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CIRCADIAN RHYTHMS IN THE AUDITORY SYSTEM

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Circadian rhythms in the auditory system

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To the most precious person in my life

In memory of the old and embracing the new

PREFACE

With a luminous star portrayed on the left side and a lunar crescent on the right side, Van Gogh's Road with Cypress and Star mediates between the two words of light and darkness. The periodic alterations of day into night and their effects on health and disease have been an intriguing scientific inquiry since early human history. As long as 3000 years ago, Hippocrates had noticed: ''Whoever wishes to investigate medicine properly, should proceed thus: firstly to consider time and the effects of seasons…for knowing the changes of the seasons, the risings and settings of the stars, one should be able to know beforehand what sort of a year is going to ensue''. Current scientific knowledge illustrates that the day-night cycles are influencing the genetic setting in our bodies, in order to optimize our physiological response to daily challenges. Hence, investigating the mechanisms of this regulation will increase our understanding of physiological functions. I hope that the studies presented in this thesis will illustrate the importance of circadian rhythms for the auditory function and help develop new scientific ideas and hypotheses.

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ABSTRACT

Circadian clocks have been found in numerous cell types and tissues of the body, allowing organisms to coordinate their daily biological functions. They generate cycles of behavioral and physiological processes with a period length of ~24h, which are synchronized to the rotation of the Earth and the subsequent daily changes in illumination, temperature and humidity. The circadian system evolved in order to ensure anticipation and adaptation to these predictable environmental changes, thereby optimizing physiological functions. The current view is that all the cellular clocks of the body are organized in a hierarchical system, which consists of a central master clock and a network of peripheral clocks found in different organs. The circadian organization facilitates temporal control of physiological functions and ensures tissue homeostasis. Prior to this thesis, the consequences of circadian regulation in the auditory system had not been deeply explored. The studies presented here are aiming to investigate the role of the circadian rhythmicity in regulating auditory function. We focused on mechanisms underlying noise sensitivity, involving neurotrophic factors and glucocorticoid hormones.

Our results demonstrate that circadian rhythms play a significant role in modulating auditory sensitivity to noise trauma. Mice were found to be more vulnerable to night noise exposure, which triggered permanent hearing loss, whereas day-exposed mice recovered to normal hearing thresholds. This diurnal variation was associated with the presence of a circadian cochlear clock and the circadian control of the brain-derived neurotrophic factor (BDNF). We next found a molecular clock machinery in a central auditory structure, the inferior colliculus (IC), which demonstrated a differential response to day or night noise trauma, and was independent from that of the cochlear clock. Focusing on the cochlear clock, we next identified self-sustained single-cell oscillators originating from sensory and neuronal populations. Cellular clocks were tonotopically arranged, suggesting that networks of individual oscillators may organize circadian rhythms along the length of the cochlea. Finally, we examined the interaction between glucocorticoids and the cochlear clock in regulating the diurnal sensitivity to noise. We found that the absence of circadian glucocorticoid rhythms abolished the greater vulnerability to noise trauma at night, as hearing thresholds recovered completely. This response was linked to glucocorticoid-dependent control of inflammatory cochlear genes. Finally, treatment with the synthetic glucocorticoid dexamethasone at day time, but not at night, protected against noise damage, highlighting the importance of endogenous glucocorticoid rhythms on the effects of otoprotective drugs.

In summary, sensitivity to noise insults is greater at specific phases of the circadian cycle, both at behavioral and molecular level and is mediated through complex interaction between circadian clocks, BDNF and glucocorticoids. Overall this thesis is describing a novel feature of the auditory system that would likely have major clinical implications.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following publications that are referred in the text to by their Roman numerals:

I.   Meltser, I. Cederroth, C.R., **Basinou, V.**, Savelyev, S., Lundkvist, G.S, Canlon, B. TrkB-Mediated Protection against Circadian Sensitivity to Noise Trauma in the Murine Cochlea. *Current Biology, 2014, 24: 658-63*

II. Park, J.S., Cederroth, C.R., **Basinou, V**., Meltser, I., Lundkvist, G., Canlon, B. Identification of a Circadian Clock in the Inferior Colliculus and Its Dysregulation by Noise Exposure**.** *The Journal of Neuroscience, 2016, 36, 5509 –5519*

III. **Basinou, V**., Buijink, R., Lundqvist, G., Cederroth, C.R., Michel, S., Canlon, B. Tonotopic Arrangement of Cellular Clocks in the Cochlea. *Manuscript*

IV. **Basinou, V**., Cederroth, C.R., Wenger, B., Gachon, F., Canlon, B. Glucocorticoids regulate the circadian response to noise damage in the cochlea. *Manuscript*

PUBLICATIONS NOT INCLUDED IN THIS THESIS

I.   **Basinou, V.,** Park, J.S., Cederroth, C.R., Canlon, B. Circadian regulation of auditory function. *Hearing Research, 2016, In press*

CONTENTS

LIST OF ABBREVIATIONS

ABR	Auditory brainstem response
ADX	Adrenalectomy
ASR	Acoustic startle response
CCG	Clock controlled genes
CLOCK	Circadian locomotor output cycle kaput
CRY	Cryptochrome
CT	Circadian time
DEX	Dexamethasone
DHF	7, 8 dihydroxyflavone
GC	Glucocorticoid
GR	Glucocorticoid receptor
IHC	Inner hair cell
IL	Interleukin
i.p	Intraperitoneal
MR	Mineralocorticoid receptor
NIHL	Noise-induced hearing loss
OHC	Outer hair cell
PER	Period
PMT	Photon multiplier tube
PTS	Permanent threshold shift
ROR	Retinoid-related orphan receptors
SCN	Suprachiasmatic nucleus
SGN	Spiral ganglion neuron
SPL	Sound pressure level
TrkB	Tyrosine receptor kinase B
TTFL	Transcriptional/translational feedback loop
TTS	Temporary threshold shift
ZT	Zeitgeber time

1 INTRODUCTION

1.1 CIRCADIAN CLOCKS

The rotation of the earth around its axis and the sun subjects our world to a daily and a seasonal periodicity. This generates cycles of illumination, temperature and humidity that are exquisitely predictable. The ability to anticipate predictable periodic changes in the external environment (e.g illumination, temperature, food availability and the presence of predators) provides evolutionary advantages to organisms, as it allows them to optimize their physiological and behavioural responses to future challenges. For instance, migratory birds depend on precise tracking of the time of day in order to navigate their flyways (Gwinner et al., 2012; Yamazaki et al., 2000), nocturnal rodents need to hide in their burrows before dawn in order to avoid predators, and even prokaryotic organisms, such as cyanobacteria, require an accurate sense of periodic time, in order to perform photophobic processes at night and photophilic processes at day time (Paranjpe and Sharma, 2005). Thus, optimal timing of an activity can be of vital importance for an animal's survival in a constantly changing environment (DeCoursey PJ, 1997).

Biological clocks evolved under the influence of rhythmic environmental cues in order to provide an internal representation of time and allow organisms to exploit temporal niches (light/dark, cold/warm, wet/dry, etc) with all the predictable consequences. The environmental factors that influence the function of these biological clocks are called Zeitgebers (German for ''time-giver'') and include the light-dark cycle, temperature, food intake and social interactions (Davidson and Menaker, 2003; Lowrey and Takahashi, 2004). The light-dark cycle is considered a major Zeitgeber and probably the most significant in evolution, because it is an explicit predictor of the daily cycle as well as the seasonal cycle (length of day fluctuations). However, the clock being an endogenous timing system, is capable of generating biological rhythms even in the absence of environmental cues. This ability ensures that the physiological functions of an organism will continue even in "temporal isolation".

When Zeitgebers are not present, the clock sustains a rhythm of about 24h which is called circadian rhythm (from the Latin words ''circa'' and ''diem'', meaning approximately one day), with a corresponding circadian time (CT). By convention, CT12 is defined as the onset of activity in nocturnal animals. In order to produce an accurate 24h period, the clock adjusts its rhythm on a daily basis. This adjustment is mediated mainly through entrainment to the daily light-dark cycle, meaning synchronization of the circadian time to the external time. Consequently, the clock time conforms to that of the Zeitgeber and is referred as a Zeitgeber time (ZT).

1.1.1 Molecular properties of circadian clocks

Circadian rhythms are innate and are governed by genetically programmed mechanisms. The discovery of genes encoding circadian behavioural rhythms in *Drosophila melanogaster* (Konopka and Benzer, 1971) initiated an intense scientific effort to identify genes that regulate the clock machinery, known as clock genes, and led to the hypothesis that rhythms are generated at the molecular level. Hardin, Hall and Rosbash in 1990 proposed an autoregulatory mechanism that represents the basic principle of the molecular clock (Hardin et al., 1990). Briefly, clock gene mRNA is translated into clock protein that translocates into the nucleus and suppresses its own transcription. Reduced levels of mRNA and consequently clock protein will then kick-start the transcription, thereby restarting the cycle.

In mammals, the generation of circadian rhythms is a cellular process that involves interlocked auto-regulatory transcriptional/translational feedback loops (TTFL) (Albrecht, 2002; Kolbe et al., 2015; Kondratov et al., 2007). At the core loop, the positive elements CLOCK and BMAL1 form heterodimers and induce the transcription of the negative-feedback elements *Period* (*Per1* and *Per2*) and *Cryptochrome* (*Cry1* and *Cry2*), by binding to E-box elements at the promoter and enhancer regions of these genes. Their protein products, CRY and PER, form heterodimers that translocate to the nucleus where they inhibit the CLOCK/BMAL1 action by removing this complex from the E-box motifs of *Cry* and *Per*, and thus repress their own transcription (**Figure 1A**). In an interlocking loop, the CLOCK/BMAL1 complex activates the ROR (RORα, RORβ and RORγ) and REV-ERB (REV-ERBα and REV-ERBβ) proteins. These proteins bind to ROR response elements (RREs) within the *Bmal1* and *Clock genes* and activate or repress their expression respectively (**Figure 1B**).

Tight coordination of the positive and negative elements of transcription, as well as post-transcriptional and post- translational modifications impose time delays that produce an accurate and robust cellular oscillator with a 24h periodicity (Reppert and Weaver, 2002). Furthermore, the transcription of the *Per* genes can be modified by a variety of immediate early transcriptional factors that are activated by hormones, temperature, neurotransmitters and second messengers (Dibner and Schibler, 2015). For instance, glucocorticoids bind to glucocorticoid responsive elements (GREs) within the *Per* genes (**Figure 1A**). In a similar way, Ca2+/cAMP responsive elements (CRE) and heat-shock elements (HSEs) bind to CRE-binding proteins (CREB) and the heat-shock factor HSF1 respectively (**Figure 1A**). These mechanisms allow the adjustment of *Per* rhythms and resetting of the molecular clock by systemic cues.

Circadian clocks are ubiquitously present throughout the body (Mohawk et al., 2012). Gene expression rhythms at cellular level result in a coherent rhythmic output at tissue level and subsequently a rhythmic behavior or organ function (**Figure 2**). In that perspective, the accurate integration of timing information within the molecular clock assists one major function: to impose temporal organization of key physiological processes. This is carried out by specific clock output genes, called clock-controlled genes (CCGs), whose rhythmic patterns
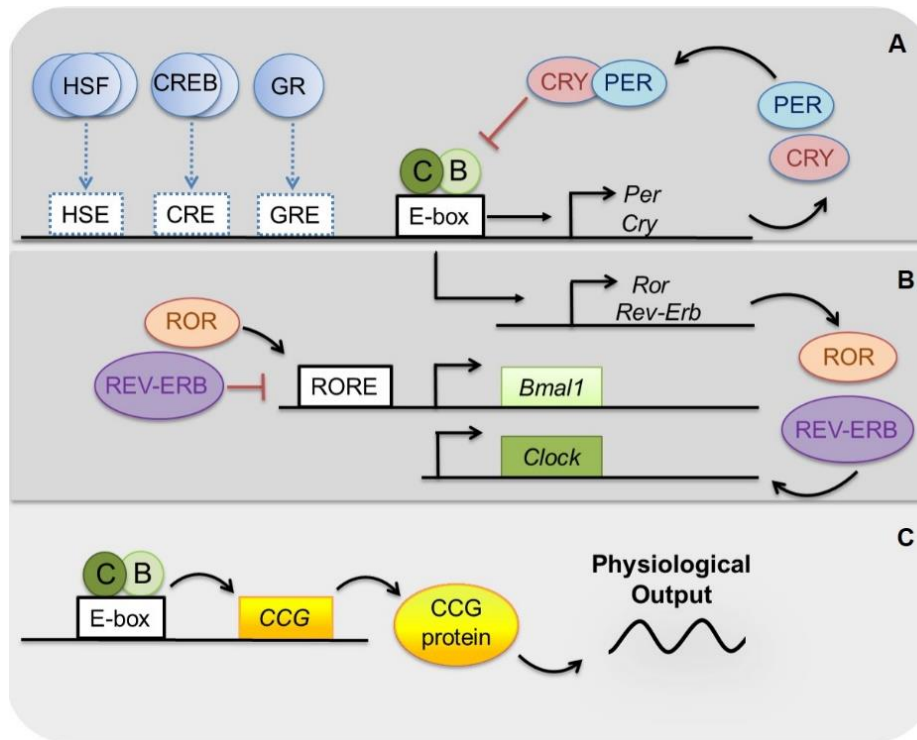
Figure 1. **The molecular clock**. The mammalian molecular is composed of two interlocked negative feedback loops of gene expression. *A) Core loop*: CLOCK (C) and BMAL1 (B) bind to E-box DNA motifs and induce the transcription of the *Per* and *Cry* genes (*Per1, Per2, Cry1* and *Cry2*). Accumulation of PER/CRY complexes inhibit the transactivation potential of C and B, thereby repressing their own transcription. As a consequence, PER and CRY levels decline, allowing C and B to initiate a new cycle of gene expression. *Per1* and *Per2* transcription can be additionally induced by systemically regulated transcriptional factors: Heat shock factor (HSF) binding to heat shock elements (HSE), cAMP responsive element (CRE)-binding protein (CREB) and glucocorticoid receptor (GR) binding to glucocorticoid responsive elements (GRE). *B) Interlocking loop*: CLOCK and BMAL1 activate the orphan nuclear receptor genes *Ror* (*Rorα*, *Rorβ* and *Rorγ*) and *Rev-Erb* (*Rev-Erbα* and *Rev-Erbβ*). The transcription of *Bmal1* and *Clock* is then regulated through competition between REV-ERB repressors and ROR activators, acting on retinoid-related orphan receptor response elements (RORE). *C) Regulation of clock-controlled genes (CCGs):* C and B can regulate the transcription of CCGs by binding to E-box elements on their promoter area. These genes then are translated into CCG protein products and regulate physiological processes in a temporal way. Printed with permission form Elsevier (Basinou et al., 2016).

of expression are involved in the regulation of functions such as metabolism and the cell cycle. It is not surprising that 10-20% of mRNA transcripts are clock-regulated in various tissues (Akhtar et al., 2002; Hughes et al., 2009; Panda et al., 2002a; Storch et al., 2002; Ueda et al., 2002). The cyclic transcriptional regulation of CCGs involves core clock components, such as the BMAL1, and transcription factors such as DPB, TEF, HLF and E4BP4 that are clock-controlled themselves (Gachon et al., 2006; Mitsui et al., 2001; Rey et al., 2011). Binding of these transcriptional factors to *cis* regulatory elements present on the promoter areas of CCGs activates or represses their expression (**Figure 1C**). For example, in the liver, DPB, TEF and HLF bind to D-boxes in the promoter area of the constitutive aldosterone receptor gene, which then regulates the circadian expression of enzymes that control the diurnal rhythms of detoxification (Gachon et al., 2006). In order to achieve time-specific regulation of thousands of CCGs within the body, additional regulatory mechanisms are needed. They include the

HSF1 (Reinke et al., 2008) and nuclear hormone receptors (glucocorticoid receptor, thyroid receptor or estrogen receptor) (Teboul et al., 2008). Finally, circadian proteomic studies have shown that 50% of rhythmic liver proteins are encoded by non-rhythmic mRNA transcripts (Mauvoisin et al., 2015), emphasizing the importance of translational and post-translational modifications for the control of CCGS.

1.1.2 Hierarchical organization of circadian clocks

The generation of circadian rhythms is a cellular property, however the rhythmic output function is a result of a well-coordinated clock network, called circadian system. At the top of the system's hierarchy there is a master clock that coordinates all the other clocks in the body. It is located in the hypothalamic suprachiasmatic nucleus (SCN). The SCN was named the master clock after lesion and grafting studies revealed that it is both necessary and sufficient for the generation of behavioural rhythmicity (Moore and Eichler, 1972; Ralph et al., 1990; Stephan and Zucker, 1972). The SCN consists of two nuclei, located left and right from the 3rd ventricle and contains approximately 10- 20000 neurons. Within each nucleus there is a heterogeneous neuronal population characterised by the expression of different neuropeptides, such as arginine vasopressin (AVP), vasoactive intestinal polypeptide (VIP) and gastrin-releasing peptide (GRP). Based on its neurochemical identity the SCN can be subdivided into the dorsal part containing AVP neurons, and the ventral part that contains VIP and GPR neurons (Antle and Silver, 2005). Each SCN neuron possesses an autonomous circadian oscillator. When these oscillators are interacting with each other the output circadian rhythm becomes robust and synchronous, as a result of cellular synchronization (Yamaguchi et al., 2003). As such, strong inter-cellular communication within the SCN, via electrical coupling, chemical coupling and gap junctions, provides precision in circadian rhythmicity and allows the SCN to act as the pacemaker of all bodily rhythms (Aton and Herzog, 2005; Michel and Colwell, 2001).

The SCN is synchronised to the external environment by light captured from the retina, a process called photoentrainment (**Figure 2**). Melanopsin-containing photoreceptors namely intrinsically photoreceptive retinal ganglion cells (ipRGCs) are responsible for transferring photic signals through the retinohypothalamic fibers directly to the SCN (Hannibal and Fahrenkrug, 2002). There, VIP-containing neurons in the ventral SCN encode these signals through a Ca2+-dependent kinase cascade that activates CREBs within the *Per* genes, thereby inducing their transcription and initiating cycles of TTFL (Dibner et al., 2010). This time–related information is then transmitted to the dorsal SCN. Finally, strong coordination across the SCN results in a very robust output signal that is then transmitted to other clocks in the brain (e.g olfactory bulbs and hippocampus) and clocks in the periphery (e.g. including liver, muscle and adrenal glands). The communication between the SCN and the peripheral clocks is mediated through autonomic innervation and through systemic cues, such as body temperature, hormonal signalling and feeding (Mohawk et al., 2012) (**Figure 2**). In return, peripheral clocks provide feedback information to the SCN regarding the internal status of the body, likely in the form of hormones or metabolites.

The importance of the SCN in synchronizing peripheral rhythms was demonstrated when peripheral tissues from SCN-lesioned mice showed persistent circadian oscillations *in vitro* but became de-synchronized with time (Yoo et al., 2004). A hierarchical relationship between the SCN and the peripheral clocks is therefore established as the one found in an ''orchestra'': The SCN acts as the conductor and peripheral clocks being the instruments that follow the conductor's directions in order to provide a synchronous or ''well-orchestrated'' rhythmic circadian response.

1.1.3 Entrainment of peripheral clocks

The circadian system must be continuously synchronized to both external and internal signals in order to coordinate all the individual clocks and produce a coherent rhythmic physiology. In contrast to the SCN, which is almost exclusively entrained by the light/dark cycle, peripheral clocks are known to respond to a plethora of signals directly or indirectly controlled by the SCN. As mentioned above, autonomic innervation is one of the signalling pathways connecting the SCN with the periphery (**Figure 2**). Circadian firing activity is transmitted by SCN neurons to several areas of the brain. For example, SCN efferent projections to the subparaventricular zone (SPVZ) control the rest-activity rhythms (Abrahamson and Moore, 2006). Furthermore, projections to the paraventricular nucleus (PVN) control hormonal rhythms, such as glucose in the liver (Kalsbeek et al., 2004) and glucocorticoids in the adrenal gland (Ishida et al., 2005). Additional autonomic nervous connections have been described in clocks found in the heart, kidney, pancreas, lung and thyroid gland (Brown and Azzi, 2013).

Another pathway that the SCN utilizes to entrain peripheral clocks is through humoral signalling (**Figure 2**). Glucocorticoid signals are of particular interest because they are known to synchronize circadian gene expression in isolated tissues (Balsalobre et al., 2000). Factors directly secreted by the SCN are also capable of entrainment, either acting on other brain structures or peripheral tissues. For example, diffusible factors such as prokineticin 2 (PK2) can directly entrain centrally-driven locomotor activity rhythms (Cheng et al., 2002). Interestingly, VIP has been found to regulate gene expression rhythms of core clock genes in the liver and adrenal glands (Loh et al., 2011), demonstrating that the SCN is capable of reaching target tissues far out from the brain and directly influence their clock function.

In addition to the above direct pathways, peripheral clocks can be entrained by feeding and temperature rhythms (**Figure 2**). Even subtle fluctuations in body temperature of about 1-4 °C are capable of resetting the time in peripheral clocks, possibly via HSF1 binding to heat-shock elements within core clock genes (Mohawk et al., 2012). Similarly, feeding-fasting rhythms can entrain peripheral clocks and are considered dominant Zeitgebers for several organs including liver, heart and kidney (Dibner et al., 2010). Neither feeding nor temperature cues can reset the master clock, presumably because the tight neuronal coupling within the SCN generates rigid rhythms and makes them resistant to small changes in the above signals (Abraham et al., 2010).
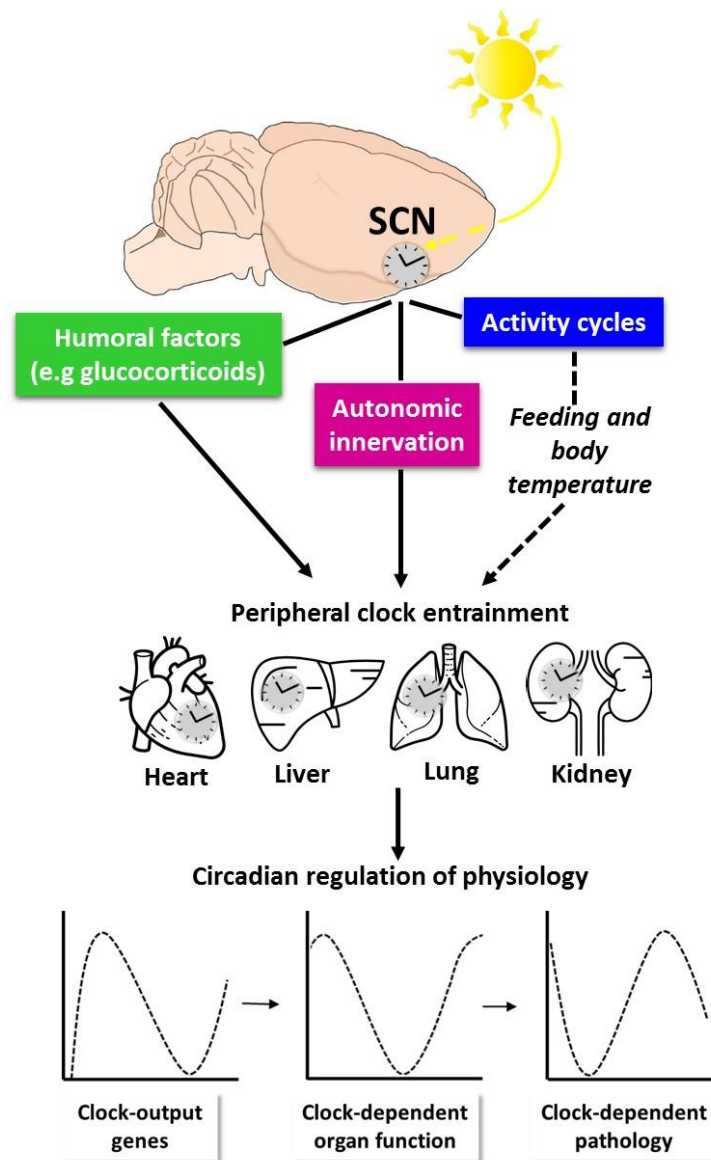
Figure 2. **The circadian system and temporal regulation of physiology**. The suprachiasmatic nuclei (SCN) is considered to be the major pacemaker of the circadian system that receives photic information directly from the retina (photoentrainment) and synchronizes peripheral oscillators found in other brain areas and peripheral tissues (entrainment). This is mediated by autonomic innervation and humoral signals (filled arrows). Indirectly, the SCN, through the regulation of rest/activity cycles, controls body temperature rhythms and feeding/fasting behavior that can entrain peripheral clocks (dashed arrow). Most peripheral tissues contain circadian oscillators that are responsible for the daily rhythmicity of their physiological functions. The rhythm generated by these individual oscillators, results in a rhythmic physiological output that creates a circadian pattern of disease susceptibility. This illustration was created by the author, with graphic components taken from freepik.com.

1.1.4 Circadian regulation of physiology

It is well established that most physiological functions including sleep and wake cycle, metabolism, feeding, immune responses, blood pressure, urine production, detoxification and hormone release are subjected to temporal regulation (Gachon et al., 2004b; Richards and Gumz, 2013). In preparation for the resting phase, anabolic processes increase, core body temperature falls and melatonin is secreted in order to induce sleep. On the contrary,

glucocorticoid hormone secretion and catabolism are increasing in anticipation of the active phase, in order to prepare animals for the activities of the day.

The discovery of peripheral clocks in fibroblasts (Balsalobre et al., 1998) and later in several peripheral tissues (Yoo et al., 2004) had a major impact in the understanding of circadian physiology, because it revealed the ability of peripheral clocks to generate self-sustained circadian oscillations, thereby suggesting a degree of autonomy in regulating tissue-specific circadian physiology. This is supported by a study showing that only 4% of cyclic transcripts found in the SCN and liver are overlapping in both tissues (Panda et al., 2002b). Most of cycling transcripts were tissue-specific and associated with principal organ functions, illustrating that peripheral clocks are to a great extent responsible for a specific circadian program matched to the tissue needs.

In order to tightly coordinate tissue-specific functions, peripheral clocks must regulate the expression of CCGs thereby creating a wide distribution of cyclic activity within the tissue. This allows distinct biological pathways to operate at particular time windows. For example, in the liver the antiphasic expression of the enzymes glycogen synthase and glycogen phosphorylase permits the separations of two chemically antagonistic processes, gluconeogenesis and glycolysis. In addition, the liver clock has been found to regulate rhythmic glucose export in a manner that counterbalances the feeding-driven rhythms of daily glucose ingestion (Lamia et al., 2008). This case demonstrates the crosstalk between SCN (through feeding rhythms) and the peripheral liver clock in order to maintain homeostasis, emphasizing the importance of peripheral clocks in regulating circadian physiology. Another example in point is the study of Marcheva et al. showing that a targeted deletion of the clock in pancreatic islets results in reduced insulin secretion and leads to diabetes (Marcheva et al., 2010).

Overall, the circadian system ensures tissue homeostasis and thus a temporal misalignment can jeopardize the optimal organ function. This situation is reflected in conditions such as jet lag, including immune dysfunction and metabolic syndrome (Brown and Azzi, 2013). Finally, the peripheral clock-dependent circadian physiology will consequently lead to a circadian variation in disease susceptibility (**Figure 2**). For example, cardiovascular events in humans, peak in the morning hours and are linked to the circadian fluctuations in blood pressure, heart rate and vasodilatation (Casetta et al., 2002; Muller, 1999) .

1.2. CIRCADIAN PATTERNS OF GLUCOCORTICOID FUNCTION

1.2.1 Glucocorticoid actions and secretion modes

Glucocorticoids (GCs) are steroid hormones produced by the adrenal cortex that help maintain body homeostasis during development and adulthood. They regulate a variety of metabolic processes, such as lipogenesis and gluconeogenesis (Wang, 2005), immune reactions (Oppong and Cato, 2015), cognitive functions and stress responses (Groeneweg et al., 2011). Activation of the hypothalamic-pituitary-adrenal (HPA) axis results in the

release of GCs in the bloodstream and target tissues, where they exert diverse effects, like mobilization of cellular energy stores and regulation of gene expression. Glucocorticoid signalling is mediated through the glucocorticoid (GR) and the mineralocorticoid (MR) receptors, which act as ligand-dependent transcription factors. Binding of GCs to the GR leads to the translocation of the GC-GR complex into the nucleus. In the nucleus, it induces the transactivation or transrepression of target genes by binding to GRE in their promoter. Furthermore, GCs can induce rapid non-genomic actions, mediated by a membrane-bound form of GR or by the MR (Groeneweg et al., 2012). The cellular actions of GCs depend on the expression levels of their receptors and the hormone bioavailability. Both types of receptors are expressed in the inner ear. They are mostly found in the primary auditory neurons (spiral ganglion neurons, or SGN) and the spiral ligament of the cochlear duct and to a lesser degree in the sensory hair cells (Yao and Rarey, 1996). Their functional role includes the regulation of ion homeostasis (Kim et al., 2009; Lee and Marcus, 2002) and modulation of auditory sensitivity to noise insults (Canlon et al., 2007).

The GC bioavailability is locally regulated by the 11-β-hydroxisteroid dehydrogenase (11βHSD) enzymes that convert the biologically inactive keto form to the biologically active hydroxyl form and vice versa, and nuclear transport molecules such as the FK506 binding proteins (FKBP51 and FKBP52) and the multidrug resistance P-glycoprotein (MDR1). Furthermore, circulating hormone availability is regulated by carrier proteins in the blood (such as the corticosteroid-binding globulin or CBG) and also by the temporal control of its secretion patterns. Corticosterone, the major endogenous glucocorticoid hormone in rodents, shows a pronounced circadian rhythmicity and an underlying ultradian rhythm (occurring with a period shorter than 24h). The ultradian pulses occur every 30-60 min and their amplitude increases towards the circadian peak time, which appears at the beginning of the active phase in early evening (Lightman et al., 2008). The circadian pattern of GC release is important for the daily regulation of physiological functions and alterations in this rhythmicity are related to many diseases, including metabolic syndrome, Cushing's syndrome and mood disorders (Chung et al., 2011).

1.2.2 Circadian regulation of GR signalling

Before binding of the circulating GC, the cytoplamatic GR is at a low affinity state due to a ''foldosome'' complex, which consists of the several heat-shock proteins (HSP), including the HSP70-HSP90 organizing proteins (HOP). Release of HOP by binding of the HSP90 and p23 sets the GR in a high affinity state, able to bind the GC ligand and translocate into the nucleus. Many HSPs show circadian regulation and as previously discussed (paragraph 1.1.1), are involved in resetting of core clock rhythms through binding to HSEs. Thus, circadian regulation of HSP/GR complexes may regulate GR output pathways (Furay et al., 2006). The GR also shows a circadian pattern of expression as reported in the brain and in adipose tissue (Dickmeis, 2009). Moreover, modulation of GR transactivation by core clock proteins has been reported in several studies (Han et al., 2014; Lamia et al., 2011) with the CRY proteins being the most prominent candidates for the repression of GR actions. These studies suggest that

peripheral clocks could indirectly influence the physiological functions of all tissues in the body, by modulating the ubiquitous and diverse actions of glucocorticoids.

1.2.3 Circadian regulation of GC secretion

Within the HPA axis, the PVN produces corticotropin-releasing hormone (CRH) and regulates the release of the adrenocorticotropin hormone (ACTH) from the anterior pituitary gland. ACTH binds to melanocortin type 2 receptor (MC2R) in the adrenal cortex and stimulates the synthesis of corticosteroids **(Figure 3)**. In parallel to GC rhythms, ACTH release follows a similar circadian pattern but with lower amplitude levels. Both these oscillatory profiles are primarily attributed to SCN control of the HPA axis, since SCN lesion leads to blunted ACTH and GC rhythms (Moore and Eichler, 1972). However, SCN grafts into lesioned animals cannot restore those endocrine rhythms, suggesting that missing neuronal connections may be vital in the generation of these rhythms. Direct SCN projections to the medioparvocellular division of the PVN (mpPVN), which contains CRH expressing neurons,
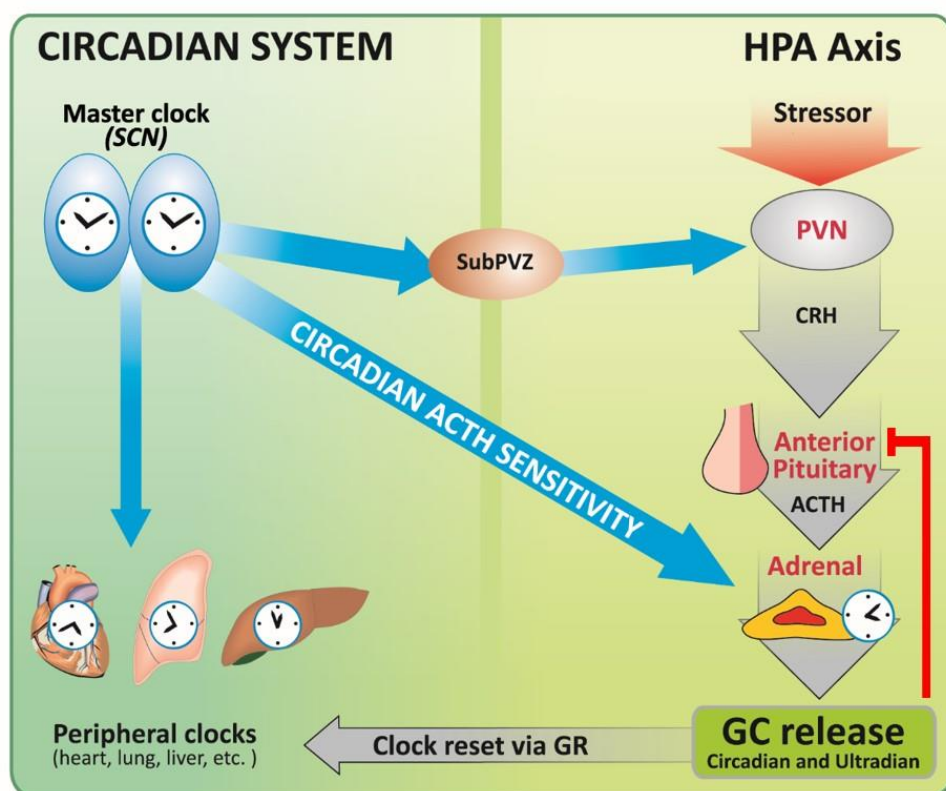
Figure 3. Interactions between the circadian system and the HPA axis. The hypothalamic PVN receives stress inputs and activates CRH-secreting neurons. CRH is directly secreted in the anterior pituitary gland. As a results, ACTH is being secreted in the circulation. In the adrenal cortex it stimulates the synthesis and secretion of GC that, in turn, provide negative feedback to the pituitary gland. Ultradian oscillations of GC secretion are sustained by a feedback loop between the adrenal cortex and the pituitary gland. GCs synchronize/ 'resets' the peripheral molecular clocks. The SCN controls the daily activity of the HPA axis via projections to the PVN and by the autonomic innervation to the adrenal cortex. *ACTH, adrenocorticotropic hormone; CRH, corticotropin-releasing hormone; GC, glucocorticoid(s); GR, glucocorticoid receptor; HPA axis, hypothalamic-pituitary-adrenal axis; PVN, paraventricular nucleus of the hypothalamus; SCN, suprachiasmatic nucleus; subPVZ, subparaventricular zone.* This illustration was created by Gabriella Lundkvist who gave the author permission for printing.

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seem to play this role (Dickmeis, 2009). In addition, indirect projections to the mpPVN, with the subparaventricular zone (subPVZ) as an intermediate relay, may also be involved (Dickmeis, 2009). SCN control of GC rhythms is also mediated independently of the HPA axis. Autonomic innervation to the adrenal gland leads to GC regulation via increased sensitivity to ACTH (Ishida et al., 2005) (**Figure 3**). Finally, the adrenal gland possesses a peripheral circadian clock that regulates the GC rhythm through adrenal clock-autonomous mechanisms, linked to the steroidogenic pathway (Son et al., 2008) (**Figure 3**).

1.2.4 Glucocorticoid regulation of circadian rhythms

In addition to metabolic control, GCs play a critical role in the regulation of circadian physiology as they are considered to be the main mediators of entrainment signals to peripheral clocks, able to reset circadian gene rhythms. The GC analog dexamethasone is a well-established agent for synchronising circadian gene expression both *in vivo* and *in vitro* (Balsalobre et al., 2000). Clock resetting stems from the transcriptional regulation of *Per1* and *Per2* via BMAL1-dependent binding of GR to GRE and E-box elements on the promoter region (Cheon et al., 2013; Yamamoto et al., 2005). Besides clock gene modification, glucocorticoid rhythms regulate a large proportion of the peripheral rhythmic transcriptome. In the liver, only 1/3 of the hepatic circadian functions is driven by the liver clock while the biggest part is controlled by glucocorticoid rhythms (Oishi et al., 2005). In fact 60% of the circadian transcriptome in the liver is controlled by glucocorticoid signalling (Reddy et al., 2007). In a similar way, 50% of rhythmic mRNA transcripts in smooth skeletal muscles are glucocorticoid regulated (Almon et al., 2008). Finally, glucocorticoid signalling in certain brain regions might be involved in providing feedback input to the SCN (Buijs and Escobar, 2007). Overall, glucocorticoid actions contribute to the temporal organization of physiology by coordinating clock output pathways and by stabilizing behavioural rhythms, in order to more efficiently adapt to sporadic environmental changes.

1.3 AUDITORY SYSTEM AND RELATION WITH CIRCADIAN RHYTHMS

1.3.1 Auditory stimulation as an entrainment signal

Several studies have revealed that the sensory system is involved in circadian entrainment. Social cues through auditory and olfactory stimuli have been reported as effective synchronisers of circadian rhythms (Davidson and Menaker, 2003). Noise has been shown to act as strong Zeitgeber for humans, as sound stimulation at night can phase shift circadian rhythms of melatonin and core body temperature (Goel, 2005), as well as synchronize activity rhythms (Davidson and Menaker, 2003). In addition, bird behavioural rhythms can be entrained by conspecific bird songs (Menaker and Eskin, 1966) and even by white noise (Reebs, 1989). Taken together, these studies point towards a connection between the circadian and the auditory systems. In support of this, studies in rodents has shown circadian patterns of acoustic startle response (Chabot and Taylor, 1992; Frankland and Ralph, 1995) and a diurnal sensitivity to audiogenic-induced convulsions (Halberg et al., 1958a). Moreover, antibiotic-mediated ototoxicity appears to be clock-regulated with more

damaging effects occurring at night time (Soulban et al., 1990; Yonovitz and Fisch, 1991). Finally, outer hair cell function in humans appears to fluctuate throughout the day (Cacace et al., 1996; Haggerty et al., 1993).

1.3.2 Auditory system and hearing loss

The inner ear is the peripheral organ of the auditory system, responsible for encoding sound waves into electrical information, which is then conveyed to the central auditory system and leads to sound perception. The cochlea is the auditory component of the inner ear, which contains the sensory organ called organ of Corti (**Figure 4A**). The function of the cochlea is to transform sound in to electrical signal that is then transmitted to the brain via the auditory nerve. In the organ of Corti, there are two types of sensory cells, the inner hair cells (IHC) and the outer hair cells (OHC). Sound waves, transmitted by the perilymph (extra-cellular cochlear fluid) cause a displacement in the basilar membrane and subsequently the tectorial membrane. This movement causes a deflection of IHC stereocilia (hair-like bundles of the sensory cells), leading to depolarization of the cell and generation of electrochemical signals (**Figure 4B**). The synapse between the IHC and afferent auditory fibers is activated through the neurotransmitter glutamate. Glutamate release initiates an action potential that propagates through spiral ganglion neurons (SGN) towards the central auditory system (**Figure 4B**). The OHC become depolarized and amplify the initial sound vibration. Through the auditory nerve, the sound information encoded by the cochlea is being transmitted to the brainstem and then the auditory cortex, where the perception of sound is realized.

According to the World Health Organization, over 5% of world's population is suffering from disabling hearing loss and this impairment highly affects life quality. Noise trauma, which is correlated with environmental and occupational noise (Ishii and Talbott, 1998; Ishii et al., 1992), consists of a high-intensity sound exposure that is damaging the auditory system and can cause severe hearing impairment (Noise-induced hearing loss or NIHL). Depending on the intensity and duration of the noise exposure, as well as the individual's sensitivity, the noise damage can lead either to permanent or temporary and reversible hearing loss (Davis et al., 2003). If the hearing impairment is reversible, it is characterized by a temporary hearing threshold shift (TTS) and recovery after 24-48h, whereas irreversible damage is accompanied by permanent hearing threshold shift (PTS) that is non-recoverable. Recent studies have revealed that even in TTS, although the hearing threshold fully recovers, there is a substantial permanent damage of neuronal synapses that result in hearing loss in older age (Kujawa and Liberman, 2009). There are potential targets for therapy against hearing loss (Muller and Barr-Gillespie, 2015), however, only synthetic glucocorticoid steroids have shown potential therapeutic effects, although with mixed success (Rauch, 2008).

Cochlear damage induced by noise exposure can lead to metabolic (excessive potassium influx, oxidative stress, etc.) or physical damage (disorganization of stereocilia, hair cell loss, neuronal loss, etc). Permanent hearing loss is mainly characterized by degeneration of auditory neurons (Pourbakht and Yamasoba, 2003) and hair cell loss (Nordmann et al., 2000). On the contrary, there is no hair cell loss or neuronal degeneration in TTS (Nordmann et al.,
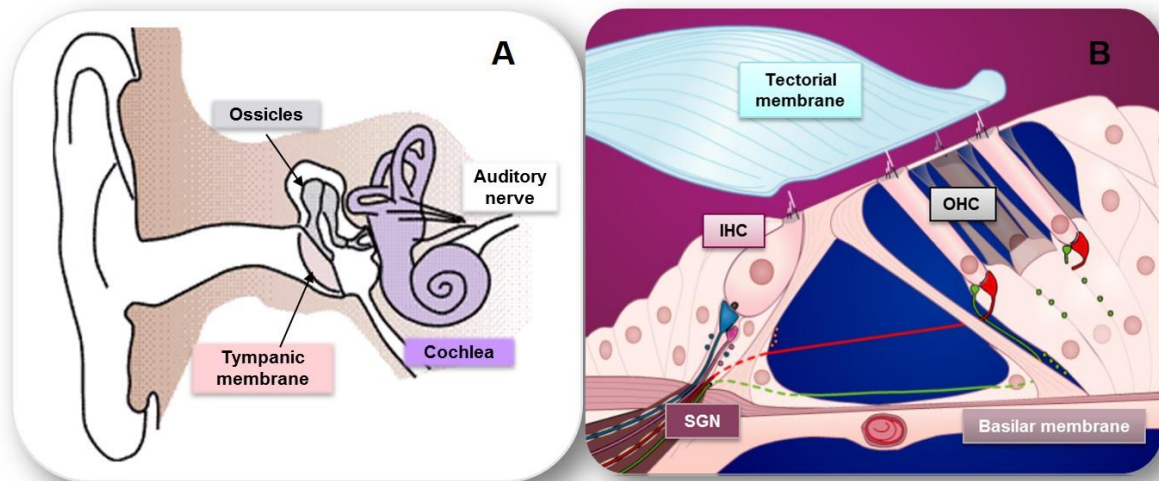
Figure 4. Peripheral auditory system. **(A)** *Schematic overview of the anatomy of the ear*. Sound waves propagate through the auditory canal and towards the tympanic membrane. Sound vibrations are then transferred through the middle ear ossicles towards the inner ear, which contains the cochlea. In the cochlea, sound waves are encoded into electrical information that reaches the brain through the auditory nerve. **(B)** *Cross section of the organ of Corti.* Sensory cells are arranged in one row of IHC and three rows of OHC along the basilar membrane and are innervated by SGN. *IHC: Inner hair cell, OHC: Outer hair cell, SGN: Spiral ganglion neurons.* Adapted with permission from Iain at the English language Wikipedia [C-BY-SA-3.0 (http://creativecommons.org/licenses/by-sa/3.0/)], via Wikimedia Commons (A) and the ''Journey into the World of Hearing" (www.cochlea.eu), by R. Pujol et al., NeurOreille, Montpellier (B).

2000) and the noise damage is mainly causing activation of reactive oxygen and nitrogen species, intracellular Ca^{2+} increase and excessive glutamate release. Glutamate excitotoxicity causes swelling of the cochlear nerve terminals and loss of the synapses between IHC and SGN dendrites (Pujol et al., 1993). The nerve terminals can recover through re-growth to restore hearing sensitivity.

Activation of the HPA axis can modulate the cochlear sensitivity and protect against NIHL (Canlon et al., 2007). One possible protective mechanism is through GC-dependent regulation of cochlear immune response following noise trauma. After noise exposure, inflammatory cells, macrophages and phagocytes migrate to the cochlea (Fredelius and Rask-Andersen, 1990; Hirose et al., 2005; Zhang et al., 2012) and activation of pro-inflammatory cytokines (IL-1, IL-6 and TNF) induces immune responses (Fujioka et al., 2014). Glucocorticoids are known to suppress these immune responses and up-regulate anti-inflammatory proteins through transrepression and transactivation mechanisms. Moreover, GR signaling can protect against hearing loss through modification of mitogen-activated protein kinase (MAPK) pathways and activation of the otoprotective neurotrophin, brain-derived neurotrophic factor (BDNF) (Meltser et al., 2009, 2010). BDNF is a predominant neurotrophin in the inner ear, strongly expressed in SGN, that has a major role in cochlear innervation during development and auditory neuron maintenance and survival in the adulthood (Ramekers et al., 2012). BDNF mutant mice display significant hearing loss and mice deprived of the BDNF-specific receptor TrkB have reduced neuronal innervation (Fritzsch et al., 2004). Treatment with BDNF is shown to improve neuronal survival (Hiltunen et al., 1996; Wise et al., 2005), demonstrating neuroprotection in the auditory system. Even though the protective role of glucocorticoids and BDNF has been demonstrated, the precise mechanisms of action are not fully explored.

2 AIMS OF THESIS

The aim of this thesis was to examine the role of circadian rhythms in regulating noise sensitivity, with a focus on neurotrophin and glucocorticoid mediated mechanisms. The specific objectives were the following:

- To determine the presence of a molecular clock machinery in the cochlea and the inferior colliculus (IC), and how it is modulated by noise exposure.

- To examine the interaction between neurotrophic signalling and the cochlear clock on protection against NIHL.

- To characterize the organization of cellular oscillators within the cochlear clock.

- To investigate the role of glucocorticoids on regulating the cochlear clock and the sensitivity to noise trauma.

3 MATERIALS AND METHODS

3.1 ETHICS STATEMENT AND ANIMAL HANDLING

All animal procedures were conducted under the guidelines of Karolinska Institutet and with approval from the ethical committee (Stockholm's Norra Djurförsöksetiska Nämnd, application numbers: N370-12 and N156-14). In Paper III, animal experiments that were performed in Leiden University Medical Center were in accordance to the Dutch law on Animal welfare and approved by the Dutch government (Ethical number: DEC 11010). The transgenic PERIOD2::LUCIFERASE (PER2::LUC) mouse strain, generated from a C57BL/6 background (Yoo et al., 2004) was used for bioluminescence recordings and bioluminescence imaging. CBA/Sca and PER2::LUC mice were used for noise exposure and audiological assessment, adrenalectomy procedures, protein and gene expression analysis. All mice were 1-4 months old. They had free access to water and food (Lactamin R34, Lantmännen) and were housed under a 12:12 h LD cycle (Lights on at 6 AM or ZT0 and off at 6PM or ZT12), in 19^0C-21^0C and 46-48% humidity. For experiments that required housing in constant darkness, mice were kept in light-sealed rooms under the same temperature and humidity conditions. Animal handling at night (ZT14-16) was performed under red light.

3.2 NOISE EXPOSURE AND AUDITORY BRAINSTEM RESPONSE (ABR) MEASUREMENTS

In order to generate hearing loss we used a noise trauma paradigm in awake mice, based on a previously described protocol (Tahera et al., 2006). Non-anesthetized mice were individually placed in wire-mesh cages and into a sound-proof chamber. There, they were treated with free-field broadband noise of 6 - 12 kHz, at 100 dB sound pressure level (SPL) for 1 h. After noise exposure, mice were transferred back to their original cages. For assessment of circadian variability, exposures were performed either in the morning (ZT3-6) or in the evening (ZT14-16).

For the evaluation of hearing function, we used ABR recordings. ABR is an evoked potential response of auditory activity in the auditory nerve and subsequent fiber tracts and nuclei within the auditory brainstem pathways. The recordings were performed in anesthetized mice (under 100 mg/kg ketamine and 8 mg/kg xylazine), using subcutaneously positioned needle electrodes. The body temperature of mice was monitored and maintained at 37^0C, using a heating pad. ABR thresholds were assessed at the frequencies of 6, 12, 16 and 24 kHz (Paper I) or 8, 12, 16 and 24 kHz (Paper IV). The stimuli were first presented at high intensity levels (75-80 dB) and then decreased in steps of 5 dB, until a positive response was not seen. Hearing threshold was defined as the lowest intensity at which a visible ABR wave was detected. ABR measurements were performed pre- and post-noise exposure at subjective day and night. Post-trauma measurements were performed 24 h and 2 weeks after exposure. The final measurements (2 weeks post-exposure), were made only during the day because i) we found no differences in basal ABR levels between day and night and ii) 2 weeks post-trauma the ABR levels are

considered stable. Data were presented as ABR threshold shifts (mean ± SEM), which constitute the threshold difference between pre-exposure and post-exposure measurements.

3.3 ASSESSMENT OF CIRCADIAN OSCILLATIONS WITH LUCIFERASE REPORTER TECHNOLOGY

In order to monitor the real-time dynamics of clock genes/proteins in cultured tissues, we employed a luciferase reporter technique, according to a previously published protocol (Savelyev et al., 2011; Yamazaki et al., 2000).

We isolated tissues from the transgenic knock-in PERIOD2::LUCIFERASE mice produced by Yoo *et al.(Yoo et al., 2004)*. The mice contain a fusion protein of PERIOD 2 (PER 2) and the firefly enzyme LUCIFERASE. In the presence of luciferin, luciferase catalyses the oxidation of luciferin and PER2 expression can be monitored as photon emission. The relative number of emitted photons positively corresponds to the relative amount of produced PER2 protein, and the bioluminescence rhythms match the endogenous PER2 rhythm (Yoo et al., 2004). Applying this principle, with the use of photonmultiplier-tube (PMT) detectors mounted inside a light-tight chamber, we were able to detect and amplify the cyclic variation in PER2 expression, allowing for continuous and long-term monitoring of PER 2 dynamics. The protocol we follow for tissue culturing and real-time bioluminescence recording is being briefly described in the following paragraphs (3.3.1-3.3.3).

3.3.1 Organotypic tissue culture

Tissues were isolated from PER2::LUC mice and cultured as previously described (Savelyev et al., 2011; Yamazaki et al., 2000). Briefly, 35 mm culture dishes were filled with 1.2 ml of DMEM-based culture medium with air-buffering capacity, containing 0.1 mM of D-luciferin sodium salt (Biothema), and a culture membrane (Millipore, PICMORG50) was placed on top of the liquid surface. After placement of the tissue, the culture dish was sealed with a 40mm cover glass and vacuum grease (Dow Corning Corp). At the final step, the culture dishes were transferred to a 37°C light-tight incubator for bioluminescence recordings. Cochleae (Papers I and IV) were dissected free of bone and stria vascularis and were kept in culture for a minimum of 6 days. For bioluminescence imaging (Paper III) dissected cochleae were cut perpendicularly at the modulus under the middle turn and the osseous spiral lamina was carefully removed, in order to generate two flat segments. In Paper II, we isolated whole IC from adult or early post-natal mice and kept them in culture for 4 days. In addition, IC sections were obtained by brain sectioning using a vibratome and a thickness of 250 μm and were kept in culture for 3 days.

3.3.2 Bioluminescence recordings and data analysis

Bioluminescence recordings were performed using the LumiCycle Luminometer (Actimetrics, Wilmette, IL, USA) or a photonmultiplier tube (PMT) detector assembly (Hamamatsu), placed in a 37°C incubator. Analysis of PER2::LUC rhythms (period,

amplitude, and phase) was performed by a fitting sine wave to a 24h-running average after subtraction of the baseline, using the Lumicycle Analysis software (Actimetrics, Wilmette, IL, USA) and the Origin software (Microcal software). The first day of measurements was excluded from the analysis. The amplitude was calculated from 3 half-cycles (1.5 cycles), as the difference between highest (peak) and lowest (trough) photon count. Phase was determined as maximum (peak) luminescence between 24 and 48 h after the start of the recording. Phase shifts were calculated by comparing the peak time before and after treatment. For period calculations we used the average period (time between two consecutive peaks) of 5 circadian cycles of recordings.

3.3.3 Bioluminescence imaging and single-cell analysis

Organotypic cochlear cultures were placed on an upright microscope (BX51WIF, Olympus) housed in a light-tight and temperature controlled chamber (Life Imaging Services). The microscope was equipped with a cooled CCD camera (ORCA –UU-BT-1024, Hamamatsu). Bioluminescence images were obtained using 29 min exposure time. Image acquisition was controlled by Image Pro Plus software (Media Cybernetics). For data analysis, the image frames were analyzed in Image Pro for tracking of bioluminescence of selected regions of interest. For single-unit analysis, cell-like regions were selected using an in-house written program for analyzing live cell recording (Stacks). For every cell-like region, the average brightness was calculated for each image frame and the subsequent time series were further analyzed in Matlab (Mathworks). Finally, the resulting single-cell luminescence traces were evaluated for sustained PER2::LUC signal and circadian properties, such as period length of 20-28 h.

3.4 PHARMACOLOGICAL TREATMENTS

Mice were treated with an intraperitoneal injection (i.p) of 4 ml/kg for the above drugs prior to noise trauma exposure: 5 mg/kg 7,8-Dihydroxyflavone hydrate (DHF, Tocris, 3826) dissolved in 18% DMSO in PBS solution (Paper I), 0.5 mg/kg Dexamethasone 21 phosphate disodium salt (DEX, Sigma Aldrich, D1159) dissolved in PBS solution (Paper IV). For *ex vivo* experiments we used: 60μM DHF dissolved in 18% DMSO in PBS solution (Paper I), 5 μM DEX dissolved in PBS (Paper IV), 25μM Mifepristone (RU-486, Sigma Aldrich, M8046) (Paper IV), 5μM Spironolactone (Sigma Aldrich, S3378) dissolved in ethanol (Paper IV) and 50 μM Forskolin (Sigma Aldrich, F3917) dissolved in PBS (Papers II and IV).

3.5 ADRENALECTOMY AND CORTICOSTERONE MEASUREMENTS

Bilateral removal of the adrenal glands (adrenalectomy) was performed in anesthetized mice (under 100 mg/kg ketamine and 8 mg/kg xylazine) with a dorsal midline incision and lateral retroperitoneal incisions. After surgery, muscle and skin incisions were closed using absorbable sutures (5-0, C-3 3/8c, Vicryl, Ethicon Vicryl, and V385H) and analgesia (0.1 mg/kg Temgesic) was provided. Drinking water was replaced with 0.9% sodium chloride solution

(saline). ABR measurements were performed at least 2 weeks after the operation. For the evaluation of plasma corticosterone levels, blood was collected in heparinized tubes and was centrifuged at 1400rpm, +4C, for 10 min. Corticosterone levels were determined with an ELISA kit according to the manufacturer's instructions (Corticosterone EIA kit, Enzo Life Sciences, Farmingdale, NY).

3.6 MOLECULAR TECHNIQUES

3.6. Immunohistochemistry

Immunohistochemical detection of proteins on cochlear and brain tissues was used for protein localization studies and synaptic ribbon quantification. Mice were transcardially perfused with 4% paraformaldehyde and cochlear and brain tissues were sampled. Cochleae were decalcified in 2% EDTA for 4 days and then cryosectioned. Cochlear and brain sections (14μm thickness) were immunostained with a rabbit-antibody against PER2 (PER21-A, Alpha Diagnostic, Texas USA; 1:100) (Papers I, II and IV) and a rabbit-antibody against c-FOS (1:250, Cell Signalling Technology) (Paper II) and secondary goat anti-rabbit antibodies (Vector Laboratories). Quantification of positive PER2 or c-FOS stained cells (Paper II) was performed with Image-Pro Plus 6.2 software (Media Cybernetics). Counterstaining with haematoxylin QS (H-3404, Vector Laboratories) was used for identification of IC sub-divisions (Paper II). In paper I, we used a mPer2^{Brdm1} mouse, carrying a mutant m*Per2* gene with a deletion in the PAS domain that produces unstable PER2 protein (Zheng et al., 1999), as a negative control for PER 2 expression in the cochlea. For the quantification of synaptic ribbons (Papers I and IV), decalcified cochlear surface preparations were immunostained with a mouse-antibody against the C-terminal binding protein 2 (1:200, 612044, BD-Biosciences) and secondary goat anti-mouse antibodies (Jackson ImmunoResearch). Confocal image stacks were analysed in ImageJ software (National Institutes of Health). Quantification was performed with an automated particle counting after converting the image to grayscale and thresholding the image.

3.6.2 Quantitative evaluation of mRNA transcripts

For assessment of mRNA transcript expression patterns at different time points around the day, we employed Quantitative real-time PCR (qRT-PCR), Nanostring nCounter assays and RNA sequence (RNAseq) techniques. A brief description of these methods is provided in the following paragraphs. Detailed information is available in the respective papers.

For assessment of gene expression changes after noise trauma (Papers I and IV), mice were kept in darkness for three consecutive days prior to noise exposure (100 dB at 6-12 kHz for 1 hour). After noise trauma tissues (cochleae, liver and IC) were collected every 4h during a 24h period. RNA extraction was performed using the Direct-zol™ RNA MiniPrep kit (ZymoResearch, Nordic Biolabs), followed by DNase I treatment (Invitrogen), as previously described (Vikhe Patil et al., 2015). In Paper I, we performed SYBR Green qRT-PCR assays. In

Paper IV, we performed NanoString nCounter assays, according to published procedures (Geiss et al., 2008). In both qRT-PCR and Nanostring analysis, 6 housekeeping genes were used for selection of the four more stable references for normalization, which was performed with the geNorm algorithm (Vandesompele et al., 2002).

For evaluation of transcriptome changes induced by glucocorticoids, we collected cochleae from ADX or sham-operated mice every 4h during a 24h period, which were previously kept in darkness for three consecutive days. Cochleae were immediately stored in RNAlater at 4°C and total RNA was extracted using the Direct-zol™ RNA MiniPrep kit, without phase-separation. DNase treatment was performed on-column as recommended by the manufacturer (ZymoResearch, Nordic Biolabs). Three replicates for each time point (CT0, CT4, CT8, CT12, CT16 and CT20) were used and in total 36 samples were subjected to RNAseq. In order to extract rhythmicity of each gene expression profile in ADX or sham operated mice, a multiple linear regression was applied to the data followed by a subsequent Bayesian information criterion (BIC) based model selection as described previously (Atger et al., 2015). Genes where the model selection did not reach a BIC weight (BICW) above 0.4 were categorized as "ambiguous". Gene ontology analysis was performed with the ontology-based PANTHER (protein annotation through evolutionary relationship) classification system (http://www.pantherdb.org/) according to published instructions (Mi et al., 2013).

3.7 STATISTICAL ANALYSIS

Data are presented as mean ± SEM. Statistical analyses were performed with GraphPad Prism 5.04 software (GraphPad software) or SigmaStat v3.5. A one-way ANOVA or an unpaired student's t-test was used. The statistical test used for each experiment is described in the figure legends.

4 RESULTS AND DISCUSSION

The circadian system imposes temporal control of organ-specific functions. The consequences of circadian regulation on hearing function had never been thoroughly studied and whether the auditory system possesses a clock machinery was unknown. In this thesis, four studies were performed aiming to investigate these fundamental questions.

In the initial study, we revealed a diurnal regulation of noise sensitivity, linked to the presence of a self-sustained molecular clock in the cochlea and the circadian control of neurotrophin activation (**Paper I**). Additionally, we provided evidence that the inferior colliculus (IC), a midbrain relay structure of the auditory pathway, also harbors a circadian clock (**Paper II**). We next examined the cellular organization of the cochlear clock and identified single-cell oscillators in sensory and neuronal populations, tonotopically arranged along the length of the cochlea (**Paper III**). In the fourth study, we investigated the role of glucocorticoid hormones, which are major synchronizers of peripheral clock rhythms, on the cochlear response to noise trauma. This study found a potential regulatory mechanism of noise sensitivity through glucocorticoid-dependent genes related to inflammation (**Paper IV**).

4.1. DIURNAL VARIATION IN AUDITORY RESPONSE TO NOISE TRAUMA

In this study, we investigated the impact of the time of the day on the capacity of mice to recover after a noise trauma. Awake mice were exposed to a noise trauma in the morning (ZT3, day group) or in the evening (ZT15, night group) and their hearing capacity was assessed by auditory brainstem responses (ABR) measurements 24 h and 2 weeks post exposure. Mice exposed to day noise recovered to normal hearing thresholds after two weeks, whereas those exposed in the evening displayed 10-20 dB hearing threshold shifts, in the absence of hair cell loss, revealing a permanent damage (**Figure 5**). This noise paradigm had been previously described to trigger a temporary threshold shift (TTS) when delivered during the day (Meltser et al., 2010). The unexpected finding that night noise exposure leads to a permanent threshold shift (PTS) illustrates that the auditory system is more susceptible to noise damage at night. Interestingly, similar shifts of hearing threshold were found 24h post exposure in both the day and the night groups (**Figure 5**), suggesting that mice had been exposed to equivalent levels of sound, thus the differential response 2 weeks post noise was not a result of noise level variability. To exclude behavioural influences on the impact of noise exposure, we examined the locomotor behaviour of mice during a day or night noise trauma (**Paper II**). We found no differences in locomotor activity, posture and the head orientation between day and night exposure, demonstrating that the phase of the sleep/awake cycle does not affect the levels of sound reaching the ear. Overall, these findings showed that the capacity of mice to recover from noise damage follows a diurnal regulation, with greater sensitivity appearing at the nocturnal phase.

This was the first report to reveal a diurnal pattern of NIHL, emphasizing the interaction between the circadian system and auditory function. This finding is of critical importance as it introduces a novel research area in the field of auditory physiology and other auditory

pathologies (e.g. sensorineural hearing loss, otitis media, autoimmune inner ear disease and tinnitus) could also follow a circadian regulation.

The clinical implications of these findings in reference to circadian variation of auditory sensitivity, are of great significance for human health, since the auditory and circadian systems in mammalian species are highly homologous. In support of this, humans studies of spontaneous otoacoustic emissions (sounds originating from the cochlea without an external stimulation) have revealed a circadian variability for different frequencies (Haggerty et al., 1993), suggesting the presence of a circadian clock in the human cochlea. In addition, circadian modulation of acoustic startle response (ASR) has been reported in rats (Chabot and Taylor, 1992; Frankland and Ralph, 1995), as well as in humans (Miller and Gronfier, 2006). We have also found that mice demonstrate a diurnal sensitivity in ASR, with greater response occurring in the morning (**Paper I**). This is important as mice are nocturnal and thus their startle response seems to be inversely correlated with activity. Similar response has been reported in humans but inverted in time (greater response in the evening), since humans are diurnal (Miller and Gronfier, 2006). It would be important to examine how the increased vulnerability of mice to night noise exposure would translate into humans. One assumption is that humans might be more vulnerable to day noise exposure, since the daily activity rhythms are opposite to that of the nocturnal mice. Nevertheless, we cannot exclude similar phasic effects of noise exposure in case that NIHL is temporally regulated by similar timing signals in both mice and humans. An example in point is melatonin rhythms that show the same pattern of release in both species (Arendt, 2006; Kennaway et al., 2002) and are known to regulate a variety of circadian physiological functions (Altun and Ugur-Altun, 2007).
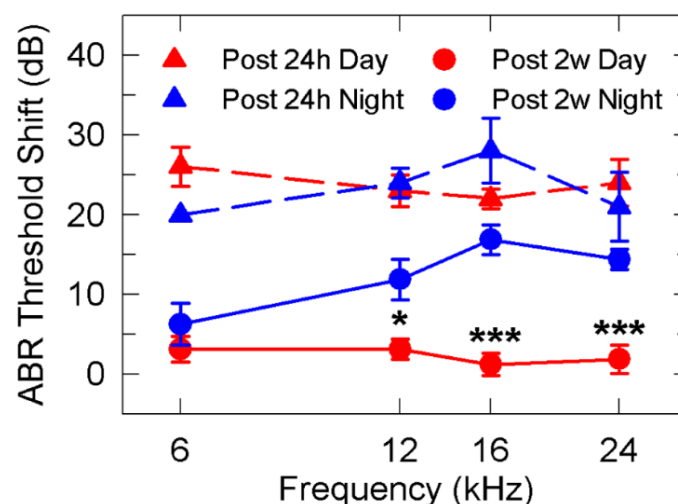
Figure 5. Diurnal sensitivity of noise-induced hearing loss. Auditory brainstem responses (ABR) in mice measured 24h (triangles and dashed lines) and 2 weeks post (filled circles) noise exposure in the morning (ZT3–5, red) or at night (ZT14–16, blue). Similar hearing threshold shifts between the two groups were observed 24h post exposure. However, 2 weeks later the morning group showed complete recovery, whereas the night group displayed high threshold shifts, thereby permanent hearing damage. Results are mean values ± SEM (n = 5–12). *p <0.05, **p < 0.001, ***p < 0.001; Two-way ANOVA with post hoc analysis. Printed with permission from Cell Press (Melster *et. al.*, 2014).

4.2. THE COCHLEA HARBOURS A CIRCADIAN CLOCK THAT IS IMPLICATED IN NOISE SENSITIVITY

The diurnal sensitivity to noise trauma suggested circadian control of the cochlear function and the idea emerged that the cochlea could contain a molecular clock machinery. To examine this hypothesis, we developed an *ex vivo* model to monitor real-time oscillations of the circadian reporter PER2::LUC (Yoo et al., 2004) in the adult mouse cochlea (**Paper I**). PER 2 was found to be expressed in the sensory cells (inner and outer hair cells) and the spiral ganglion neurons (SGN) (**Figure 6A**). Our experiments showed that the cochlea is capable of generating robust, self-sustained PER2 oscillations that persist for at least 7 days without medium change or pharmacological interventions (**Figure 6B, Paper I**). The capacity for autonomous oscillations vary among different peripheral clocks. Some of them (e.g. kidney) require entrainment signals or else their rhythms fade out rapidly, while others (e.g liver) are able to generate self-sustained oscillations in the absence of external input, suggesting organ-specific synchronization mechanisms (Yoo et al., 2004). Interestingly, the cochlear rhythm amplitude was greater compared to that of liver, a well-characterized robust peripheral clock, indicating that the cochlea is a very vigorous oscillator (**Paper I**). In addition, we examined the temporal expression of other clock genes and found circadian patterns of a variety of clock mRNA transcripts including *Per1*, *Per2*, *Bmal1* and *Rev-Erbα* (**Paper I**). The reported circadian regulation of these mRNA transcripts confirmed the reversed phase correlation between the positive (*Bmal1*) and the negative (*Per1* and *Per2*) elements of the molecular clock.

Mice deficient in the circadian transcription factor *Bmal1* [Bmal1(-/-)] display a severe phenotype characterized by loss of circadian rhythmic behaviour, reduced lifespan and additional metabolic abnormalities related to accelerated aging, namely sarcopenia, cataracts, decreased subcutaneous fat and organ shrinkage (Kondratov et al., 2006). Interestingly, their arrhythmic locomotor activity and metabolism can be rescued by overexpression of the *Bmal1* paralog *Bmal2* (Shi et al., 2010). *Per 2* deficient mice [Per 2(-/-)] have disrupted locomotor activity (Bae et al., 2001) and mice deficient in both *mPer1* and *mPer2* do not express circadian rhythms (Zheng et al., 2001). In addition, Per 2 mutant mice show glutamatergic system dysfunction (Spanagel et al., 2005), reduced innate immune responses and deficiencies in NK cell function (Liu et al., 2006). Collectively, these results highlight that core clock genes are essential for the regulation of physiology and pathogenesis and suggest that their presence in the cochlea could be important for the organ function. We believe that the cochlear clock machinery will help regulate important cochlear functions, such as the proper timing of metabolic processes in order to maintain high metabolic rates during the active phase of the day. Despite the universal presence of these clock genes in peripheral clocks, subtle variations their expression patterns may be crucial for tissue-specific functions (Panda et al., 2002a), thus understanding the specific cochlear clock machinery is important for unravelling the mechanisms of circadian physiology in the auditory system.

We next studied the effects of noise exposure on the cochlear clock machinery. Cochlear explants from night-stimulated mice cultured for a period of 6 days, show a 27% decrease in PER2::LUC rhythm amplitude compared to the sham group (**Figure 6C**). On the contrary, the

rhythm amplitude did not change after a day noise exposure (**Figure 6C**). A similar amplitude suppression was additionally found for *Per 2*, *Per 1* and *Rev-Erbα* at the mRNA level (**Figure 6D, Paper I**). More specifically, the amplitude of *Per2* mRNA transcripts decreased by 30% in cochleae from night noise-exposed mice (**Figure 6C**). Finally, the amplitude of *Bmal1* increased following night exposure, suggesting reduced negative feedback form *Rev-Erbα* (**Paper I**).

Overall, these findings revealed the presence of a self-autonomous circadian clock in the cochlea that presumably drives the differential sensitivity to noise, as reflected in the strong suppression of circadian clock genes following a damaging night noise exposure.
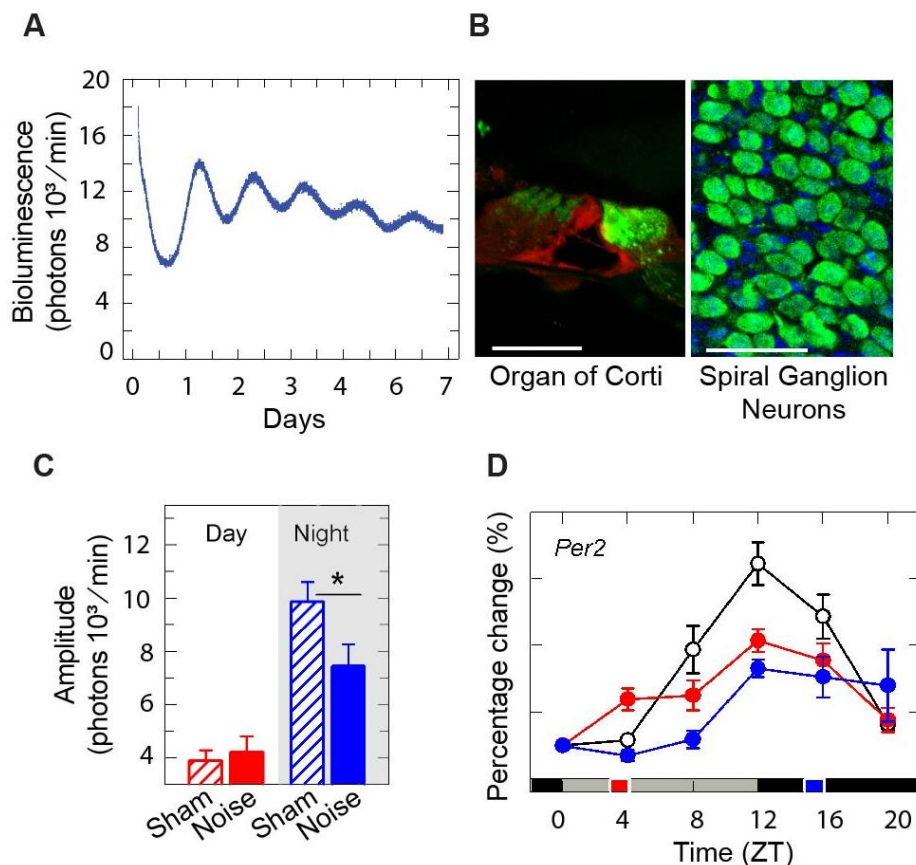
Figure 6. Cochlear clock response to noise trauma. A) Immunostaining of PER2 in the adult mouse cochlea revealed. PER2 expression was mainly found in inner hair cells, outer hair cells and in the spiral ganglion neurons. **B)** Representative bioluminescence record of circadian PER2::LUC expression in cultured mouse cochlea. **C)** Effects morning (ZT3-5, red) or night (ZT14-16, blue) noise trauma on cochlear PER2::LUC oscillations. Night noise exposure significantly reduced the PER2::LUC rhythm amplitude, whereas day exposure did not. **D)** Temporal expression of cochlear *Per2* from non-exposed mice (white circles) or mice exposed to a morning (ZT3, red circles) or night (ZT15, blue circles) noise trauma. The horizontal axis shows the sampling ZTs across a 24 h period. The vertical axis shows normalized mean values ± SEM (n = 3–4). *p <0.05, **p < 0.001, ***p < 0.001; Two-way ANOVA with post hoc analysis. Printed with permission from Cell Press (Melster *et. al.*, 2014).

4.3. CIRCADIAN REGULATION OF BDNF MEDIATES PROTECTION AGAINST NOISE TRAUMA

In order to understand what causes the increased vulnerability to night noise injury we investigated possible mechanisms of auditory damage. Permanent damage in absence of hair cell loss following a nocturnal exposure could indicate damage at the synaptic level (Kujawa

and Liberman, 2009). The presumed underlying mechanism is loss of synaptic ribbons causing a permanent uncoupling of the synapse between the inner hair cell and the afferent neuronal fibers. We examined the role of the brain-derived neurotrophic factor (BDNF), since it is an important regulator of cochlear synaptic homeostasis (Singer et al., 2014) and is known to be under circadian regulation (Jang et al., 2010a; Liang et al., 1998). We measured cochlear *Bdnf* levels after a day or night noise trauma and found a striking 35-fold increase after day noise exposure, whereas night noise exposure only triggered a 2-fold increase (**Figure 7A**). We thus hypothesized that the inability to recover for a nocturnal exposure was linked to the incapacity of the cochlea to induce *Bdnf* in response to noise.

BDNF has been shown to prevent auditory neuron degeneration and thus used against hearing loss in animal studies (Pettingill et al., 2011; Rejali et al., 2007), however clinical applicability is very challenging due to its poor pharmacokinetic properties. Mature BDNF exerts its biological effects through binding to the tropomyosin kinase type B (TrkB) receptor and recently a selective TrkB agonist, DHF, was identified with potent BDNF-like activity (Jang et al., 2010b). DHF was found to promote auditory neuron survival in an auditory neuropathy mouse model (Yu et al., 2013), illustrating that it can cross the ear-blood barrier and target the cochlear TrkB receptor. We therefore used DHF to bypass the cochlear incapacity to secrete BDNF after night noise exposure. We showed that systemic administration of DHF prior to a nocturnal exposure rescues from permanent damage, as hearing thresholds recovery was similar to those of untreated, day-exposed mice (**Figure 7B**). In addition we found that DHF could protect from loss of presynaptic ribbons (**Paper I**). Interestingly, DHF could modulate the cochlear clock function, as it was shown to increase PER2::LUC rhythm amplitude in cochlear explants, which was largely blocked by a specific TrkB antagonist (**Paper I**).

These findings demonstrate the involvement of BDNF signalling in the circadian response to noise trauma. BDNF activation is occurring at specific phases of the circadian cycle to mediate recovery of synaptic function and thus protect from the damaging effects of noise exposure. Another neurotrophin acting on the TrkC receptor, the neurotrophin-3 (NT-3), has a very significant role in synaptic plasticity and auditory recovery (Ramekers et al., 2012; Yang et al., 2011). In fact NT-3 and BDNF seem to have complementary roles in maintenance neuronal survival. Mice lacking NT-3 display 84% spiral ganglion neuronal loss and BDNF overexpression is capable of rescuing the NT-3 deficiency (Farinas et al., 2001; Fritzsch et al., 2004). Moreover, loss of TrkC function results in 50% loss of auditory neurons and knock-out of TrkB in a TrkC [-/-] background causes a complete absence of auditory neurons (Fritzsch et al., 2004). Recently, a study using mice overexpressing *Ntf3* or *Bdnf* examined differential responses to noise trauma. They found that *Ntf3* but not *Bdnf* overexpression promoted auditory recovery (Wan et al., 2014). This finding appears to be conflicting with our results on DHF rescue of hearing function. However, a possible explanation is that DHF treatment acting on the TrkB receptor, was acute (single injection) and performed at the time point that the endogenous BDNF response is poor, whereas the genetic model of *Bdnf* overexpression reflects a chronic activation of TrkB. The role of NT-3 signalling in the circadian sensitivity to noise exposure remains to be investigated. Moreover, there are several possible mechanisms of auditory

damage in absence of hair cell loss, such as impairment of the stereocilia or the tip links, generation of free radicals and increased intracellular Ca^{2+}. Investigation of these factors in the circadian response to noise would reveal additional circadian repair mechanisms.
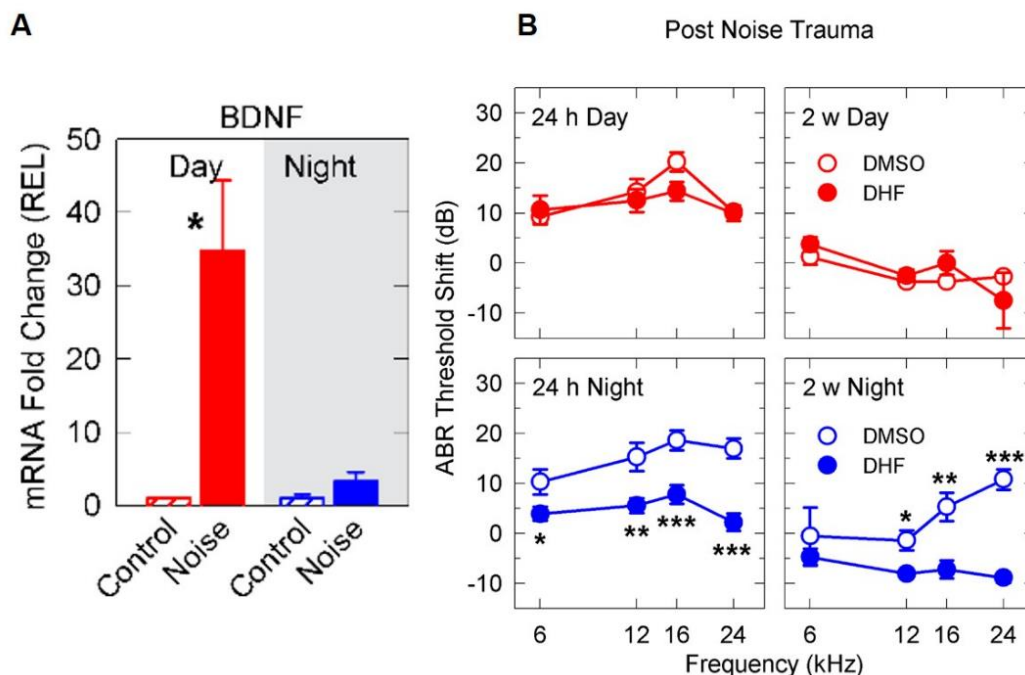
Figure 7. BDNF-dependent protection against noise damage. A) Cochlear *Bdnf* response after a day (ZT3-5, red) or a night (ZT14-16, blue) noise trauma. Day exposure induced a 35-fold increase of *Bdnf* mRNA, whereas no change occurred after night exposure. **B)** ABR threshold shifts from mice exposed to a day (ZT 3-5, red) or a night (ZT14-16, blue) noise trauma, measured 24 hr post (left panel) and 2 weeks post (right panel). DMSO-treated animals (white circles) and DHF-treated animals (filled circles). DHF administration protected from permanent hearing loss in the night exposed group, as thresholds shifts returned to normal levels 2 weeks post exposure. Results for the PER::LUC experiments are mean values ± SEM (n = 3-4). *p < 0.05, **p < 0.001, ***p < 0.001; two-tailed Student's t test (A) and two-way ANOVA with post hoc Tukey test (B). Printed with permission from Cell Press (Melster *et. al.*, 2014).

4.4. CIRCADIAN CLOCK FUNCTION IN THE CENTRAL AUDITORY SYSTEM

We examined the presence of circadian rhythms in the inferior colliculus (IC), a central auditory structure that plays an important role in noise-induced pathologies such as tinnitus, hyperacusis and audiogenic seizures (Berger et al., 2014; Salvi et al., 1990; Wang et al., 2002). Using PER2::LUC transgenic mice and real-time bioluminescence recordings we revealed a molecular circadian clock in adult IC explants (**Figure 8**). However, not all IC explants displayed oscillations and the success rate was limited to 30% (**Paper II**). Although the reasons behind this are unknown, we speculated the this could be due to culturing conditions and vulnerability of the tissue, low number of rhythmic cells or loss of synchronicity among cellular oscillators leading to a dampen tissue signal. Isolated kidney tissue has been shown to also demonstrate PER2::LUC oscillations that rapidly dampen with time, suggesting that some organs require external input in order to maintain their oscillations in culture conditions (Yoo et al., 2004). In addition, most non-SCN brain regions demonstrate weak oscillation *in vitro* or they are arrhythmic (Abe et al., 2002). These results illustrate that the brain contains multiple, damped circadian oscillators outside the SCN. In

general, the differential capacity to sustain autonomous oscillation in culture conditions creates 3 distinct groups of circadian clocks: i) the SCN that is able to sustain high-amplitude and synchronous circadian oscillations for several weeks in culture, ii) tissues, such as the liver, the pineal gland and the cochlea with circadian oscillations that persist *in vitro* for at least one week and gradually decrease in amplitude and iii) tissues, such as the IC and other brain areas that show oscillations for only a couple of days and then rapidly damp out. It is not clear why some peripheral clocks exhibit more robust oscillations than others, however the expression level of the reporter clock genes cannot solely define the oscillating capacity of the tissue. Thereby, the physiological relevance of the peripheral clock in question should be examined by functional studies in the absence of the tissue-specific clock machinery, using mouse models with core-clock gene mutations.

Next, we identified rhythmic transcripts of other clock genes (*Cry1*, *Bmal1*, *Per1*, *Per2*, *Rev-erbα*, and *Dbp*), supporting the presence of molecular clock machinery in the IC. In addition, we showed that clock genes in the IC respond to noise trauma, in an opposite manner compared to the cochlea. Night noise exposure in the cochlea caused a decrease in PER2::LUC rhythm amplitude (**Figure 6C**), whereas in the IC we found an amplitude increase (**Figure 8B**). Moreover, the amplitude of *Per2* mRNA transcripts was increased after night noise exposure (**Figure 8C**), in contrast to the cochlea that showed a decrease of *Per2* (**Figure 6D**). These findings illustrate that the cochlear and the IC clocks responds differently to noise trauma. In addition the induction of *Bdnf* mRNA transcription in the IC is similar following either a day or a night noise trauma (**Paper II**), whereas in the cochlea *Bdnf* induction is only triggered by day noise exposure, suggesting independent neurotrophic response to noise between the IC and the cochlea. Reinforcing this view, central and peripheral auditory structures trigger diverse electrophysiological responses to noise exposure (Niu et al., 2013; Salvi et al., 2000).

The IC has been implicated in the generation of audiogenic seizures (Faingold et al., 1992) which are reported to be more severe when induced during the night in comparison to day induction (Halberg et al., 1958b). Interestingly, mice lacking the three PAR bZip clock- genes *Tef*, *Hlf* and *Dbp* (that act as transcription factors for downstream clock-controlled genes) show increased vulnerability to audiogenic seizures (Gachon et al., 2004a). These studies are indicating a correlation between the IC clock and the sensitivity to audiogenic seizures. However, in our study we found that the IC expresses *Dpb* in a circadian manner, but we could not identify circadian expression patterns of *Tef* and *Hlf* (**Paper II**). This might be due to the sensitivity of our method to detect circadian rhythmicity patterns or it might indicate that the source of audiogenic seizures in this triple mutant might not stem from the IC, but likely from other parts of the auditory pathway. The role of the IC clock in the regulation of noise-induced pathologies is yet to be uncovered.
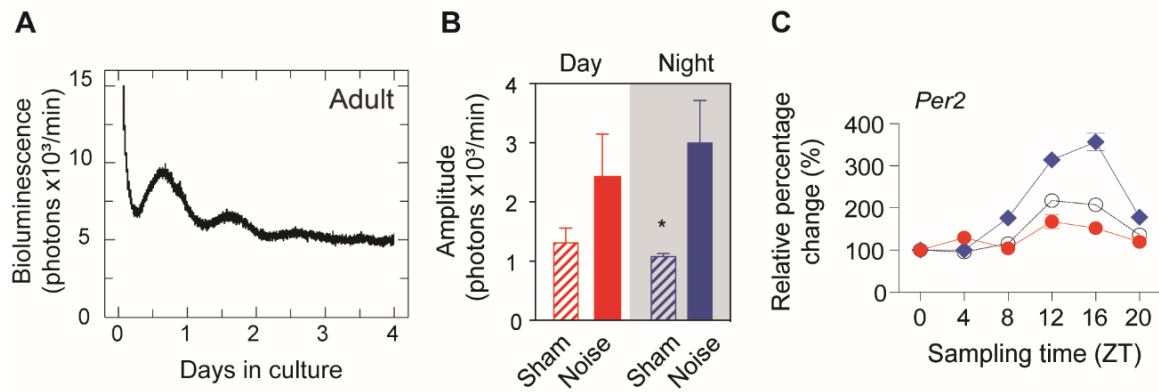
Figure 8. The IC possesses a circadian clock that responds to noise trauma A) Representative bioluminescence record of circadian PER2::LUC expression in whole IC explants. **B)** Effects of noise exposure of the IC clock. Night noise exposure (ZT15, blue) significantly increased the PER2::LUC rhythm amplitude, whereas day exposure (ZT3, red) did not. Data are presented as ± SEM (n = 3-6). **C)** Temporal expression of *Per2* in response to noise trauma. The horizontal axis shows the sampling ZTs across a 24 h period. The vertical axis shows normalized mean values ± SEM (n = 3-4). Non-exposed mice: white circles, mice exposed to a day (ZT3) noise trauma: red circles, mice exposed to a night (ZT15) noise trauma: blue circles. *$p < 0.05$, **$p < 0.001$, ***$p < 0.001$; two-tailed Student's t test (B) and two-way ANOVA with post hoc Tukey test (C) Printed from Park *et. al.,* 2016.

4.5. TONOTOPICAL GRADIENT OF CIRCADIAN RHYTHMS IN THE COCHLEA

In order to examine the cellular organization of the cochlear clock we used a bioluminescence imaging approach. We were able to detect PER2::LUC signal in cochlear preparations and identify cell-autonomous oscillators located in the sensory (inner and outer hair cells) and neuronal populations (SGN) (**Paper III**). Corroborating the whole tissue oscillations (**Figure 2B**), the average peak PER2 expression occurred in early evening and reached the trough in the morning (**Paper III**). However, we observed a peak-time variation between different cells. To further investigate the spatial distribution of these multi-phased oscillators, we examined cellular PER2::LUC rhythms originating from specific cochlear regions, the apical and the middle turns (**Figure 9**). We found a phase delay of about 3h in cells of the middle turn compared to cells in the apical turn (**Figure 9B**). The phase differences between apical and middle turns of the preparation in **Figure 9A** were apparent around the 48 h time point (**Figure 9C, D**). The apical population had an average phase of 45 hours with the majority of cells (87%) peaking between 36 and 48 hours (**Figure 9E**). On the contrary, the middle turn population had an average phase of 48 hours with most of the cells (61%) peaking between 48 and 60 hours (**Figure 9E**). Similar phase differences were also observed in isolated individual cochlear turns (apical, middle and basal) recorder under a PMT (**Paper III**). Since the PER2::LUC signals first appeared in the apical turn, we tested whether this region is required for the generation of the circadian oscillations in the whole cochlea. Removal of the apical turn did not affect PER2::LUC rhythms in the remaining tissue, suggesting that the apex is not driving the cochlear oscillations (**Paper III**). Nevertheless, when the different cochlear turns were isolated from one another they showed unique profiles of rhythmicity, indicating that inter-regional coupling is required for the generation of a coherent rhythm in the whole tissue. The phase variability in the individual cochlear turns could indicate different degrees of cellular

synchrony in each segment. This phenomenon has been studied in the SCN with bioluminescence imaging of *mPer1::luc* SCN slices. When the dorsomedial and ventrolateral portions of the SCN were isolated from each another, cellular rhythms in the dorsomedial part became de-synchronised while the ventrolateral cells continued to oscillate in phase (Yamaguchi et al., 2003). This finding suggested that the ventrolateral part is required for the synchronization of the cellular oscillators in the dorsomedial part. In order to examine whether any cochlear part would have a similar function, monitoring of cellular oscillations in each of the isolated cochlear turns would have to be investigated.
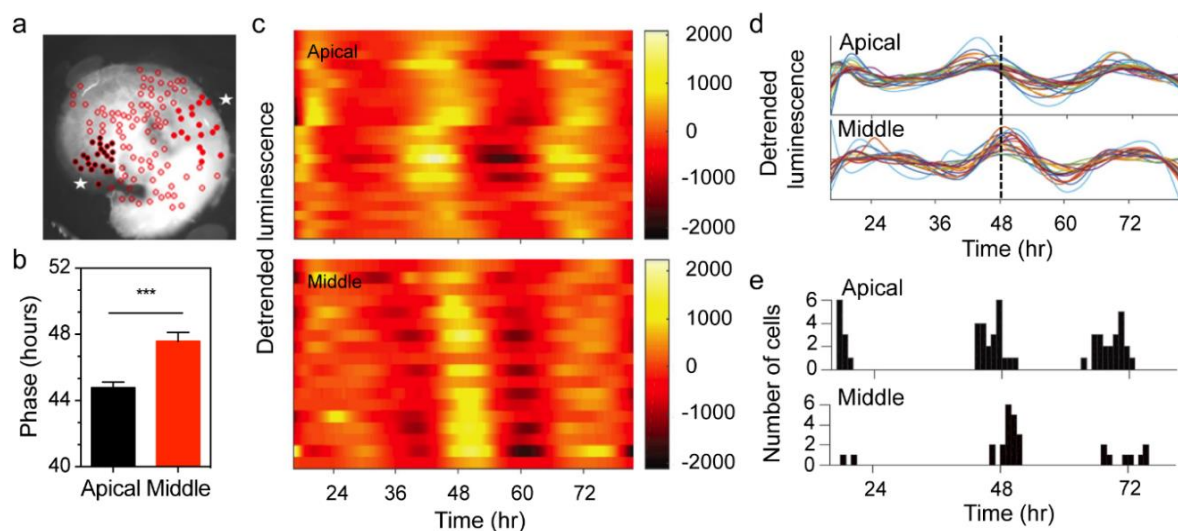
Figure 9. Longitudinal arrangement of circadian cellular oscillations in the cochlea. A) Bioluminescence image of the cochlear preparation. The asterisks indicate the selection of the cellular population in the apical turn (black filled circles) or the middle turn (red filled circles). Open circles indicate cells that were not analyzed. **B)** Comparison of PER2::LUC phase between the apical and middle turn cell populations from 3 different preparations (mean ± SEM, n = 36-56 cells, *** = $p < 0.001$, one-way ANOVA). **C)** Representative heat maps of apical and middle cell populations during 72 hours of recordings. High luminescence intensity is shown in yellow tones and low luminescence intensity is shown red tones. **D)** Bioluminescence records of individual cell oscillators in the apical and middle turns. The dashed line points to the difference in peak-time expression between the two different cell populations during the second circadian cycle of recordings (around the 48 h time point). **E)** Distribution of peak time between apical and middle turn populations, in each circadian cycle of recordings.

These findings revealed a distinct spatial and temporal distribution of cellular PER2 rhythms in the cochlea. Cellular oscillators have been described in cells such as fibroblasts (Welsh et al., 2004), however *in situ* analysis of circadian cellular clocks within peripheral tissues is more challenging, due to strong bioluminescent signal requirements. Currently, cellular oscillators have been reported from tissue cultures of bone and tendon (Lande-Diner et al., 2015) and brain (Guilding et al., 2009; Guilding et al., 2010; Landgraf et al., 2015), but there is no information about the cellular organization of these individual clocks. This study provided the first evidence of spatial arrangement of cellular clocks within a peripheral organ. A characteristic property of the cochlea is that several mechanical, electrical and cellular properties are graded along the length of the organ, resulting in sound frequency specificity along a spatial axis, referred to as tonotopy (Davis, 2003; Ricci et al., 2003; Rosenblatt et al., 1997). The identification of a longitudinal gradient of circadian rhythms is therefore

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highlighting a novel feature of the cochlear tonotopic map, with potential implications in temporal regulation of physiological functions along the length of the organ.

Cellular synchronization is essential for the generation and maintenance of coherent tissue rhythms. In the SCN, the mechanisms that mediate synchrony involve gap junctions, GABA signalling and synaptic activity (Aton and Herzog, 2005; Michel and Colwell, 2001; Shirakawa et al., 2001). The mechanisms underlying cellular synchrony in peripheral tissues are yet to be identified. Studies on non-SCN brain areas have shown that synaptic communication mediated through action potentials is not required for the generation of circadian oscillations, thereby illustrating that SCN input and other external cues are necessary for tissue synchrony (Guilding et al., 2009; Guilding et al., 2010). Further studies aiming to uncover the involvement of internal cues (in terms of inter-cellular communication) and external SCN signals in the establishment of synchrony within the cochlea, will provide important information for the mechanisms needed to sustain spatiotemporal order across the organ.

4.6. GLUCOCORTICOID-DEPENDENT REGULATION OF THE CIRCADIAN RESPONSE TO NOISE TRAUMA

We hypothesized that circadian sensitivity to noise damage might be mediated by glucocorticoids (GCs), which are tightly clock-controlled and are known to modulate auditory sensitivity to noise trauma (Canlon et al., 2007). For this purpose, we examined the interaction between GC rhythms and the cochlear clock on the circadian response to noise trauma. Depletion of GC levels through adrenalectomy (ADX), resulted in complete recovery of hearing thresholds two weeks after night noise exposure, similar to that of the day-exposed animals (**Figure 10**). The diurnal pattern of recovery (greater noise damage occurring at the nocturnal phase) was apparent in the sham-operated groups (**Figure 10**). These findings demonstrated that the loss of circadian glucocorticoid rhythmicity disrupts the circadian regulation of noise sensitivity, thereby GC rhythms play an important role in the temporal regulation of auditory function.

The effects of ADX on noise sensitivity also suggest that in order to achieve the desired effects of corticosteroid treatment against hearing loss, the endogenous hormonal rhythms should be taken under careful consideration. In support of this, we found that DEX, an anti-inflammatory drug frequently prescribed for acute hearing loss (Wei et al., 2013), protects against day noise trauma but not against night noise trauma (**Paper IV**). We thereby hypothesized that DEX treatment before a night noise trauma, when blood corticosterone levels are peaking, may interfere negatively with the endogenous glucocorticoid response to the noise stressor. In addition, it has been shown that the rhythmic action of glucocorticoids may controlled by the clock genes *Cry 1* and *Cry 2*, which form a complex with the GR to repress GR-controlled transcriptional activity at specific phases of the cycle (Lamia et al., 2011). Thus, DEX anti-inflammatory actions might reveal ineffective at the onset of the nocturnal phase, when PER2/CRY1 expression is at maximum levels in the cochlea and can inhibit GR transcriptional activity.
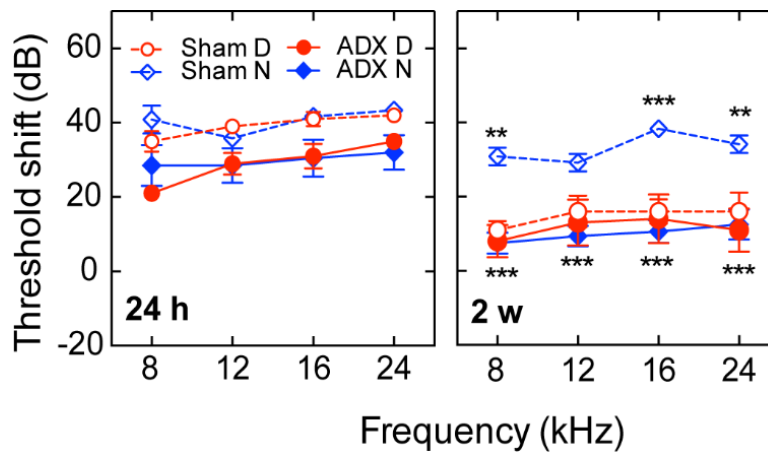
Figure 10. Depletion of circulating corticosterone promotes recovery after night noise trauma. ABR threshold shifts form ADX (full lines) or sham-operated (dashed lines) mice exposed to a day (ZT3–5, red) or night (ZT14–16, blue) noise trauma, were measured 24h (left) and 2 weeks (right) post noise exposure. Similar hearing threshold shifts were observed 24h post exposure. However, 2 weeks later the night ADX group showed recovery, whereas the night sham-operated group displayed high threshold shifts, thereby permanent hearing damage. Results are mean values ± SEM (n = 5–10). *p <0.05, **p < 0.001, ***p < 0.001; Two-way ANOVA with post hoc analysis.

4.7. GLUCOCORTICOID-DEPENDENT REGULATION OF THE COCHLEAR TRANSCRIPTOME

We next found that ADX did not affect the cochlear clock response to night noise, as evaluated by recordings of PER2::LUC oscillations (**PAPER IV**). We thus assumed that the ADX-mediated protection against night noise trauma is not a result of clock de-synchronization but could involve modification of clock output pathways. To examine how glucocorticoids could regulate the cochlear transcriptome, we performed RNA sequencing (RNA-seq) analysis in cochlear samples from ADX or sham-operated mice. We were able to identify 3 distinct patterns of glucocorticoid regulation, using a clustering approach that discriminates rhythmic or constant patterns of gene expression (Atger et al., 2015). These modes of regulation are reflected in the following changes in ADX conditions: Gain of rhythmicity (model 2), unaffected rhythmicity but altered amplitude and phase rhythms (model 4), or loss of rhythmicity (model 5) (**Figure 11A**). Approximately 23% of all rhythmic genes identified were found to be under the control of GCs (**Figure 11B).**

By focusing on molecular clock components, we observed that 45% of clock genes were not affected by ADX whereas a 55% displayed only small changes in phase and amplitude (**Figure 11C, Paper IV**). For example, Per2 amplitude was reduced in ADX conditions (**Figure 11C**), corroborating with findings in liver and kidney (Sujino et al., 2012), and consistent with the notion that glucocorticoid-responsive elements (GREs) are present within the Per2 promoter region allowing for GC-regulation of PER 2 rhythms (Cheon et al., 2013). Interestingly, although glucocorticoids are known to reset clock rhythms in culture conditions (Balsalobre et al., 2000) we found that ADX had no effect on the cochlear PER2::LUC oscillations (**Paper IV**). Overall, the subtle changes in the cochlear clock machinery suggest that other entrainment cues must be of high importance for the maintenance of cochlear rhythms.

Autonomic innervation, food intake and other humoral factors may transmit timing cues, and the precise pathways are yet to be discovered.

We hypothesized that genes involved in circadian regulation of noise sensitivity would be presented in model 5, whereby the depletion of glucocorticoids leads to the loss of rhythmic gene expression in the cochlea. This pattern of regulation has been observed in the liver, where ADX abolished the circadian rhythms of glucocorticoid-dependent genes that regulate liver metabolism, although the circadian expression of clock genes was unaffected (Oishi et al., 2005). Additionally, circulating corticosterone was shown to regulate the temporal expression of the chemokine CXCR5 that drives the circadian pulmonary response, without affecting the core clock rhythms (Gibbs et al., 2014). In a similar way, glucocorticoids could modulate noise sensitivity by altering the temporal expression of glucocorticoid downstream targets involved in cochlear physiology. Model 5 was mainly composed of genes related to inflammation (**Paper IV**). For example, we found that ADX abolishes the rhythms of cluster of differentiation 109 (*Cd109*), C-C chemokine receptor type 2 and 9 (*Ccr2* and *Ccr9*), interferon gamma receptor 2 (*Ifngr2*) and interleukin- 1b (*IL 1b*) (**Figure 11D**). There are several studies highlighting the role of cochlear inflammatory responses for hearing function. For instance, in Muckle-Wells syndrome, a rare autoinflammatory disease, there is excessive IL-1 release that causes sensorineural deafness. Treatment with the specific IL-1 inhibitor anakinra has been shown to decrease serum levels of IL-1, IL-6 and IL-12 and reverse this cytokine-induced sensorineural hearing loss (Yamazaki et al., 2008). In addition, in a model of acute cochlear mitochondrial dysfunction (Hoya et al., 2004) which induces secondary inflammation responses and lead to severe hearing impairment, a 60% increase of chemokine genes including monocyte receptors such as *Ccr2* was reported (Okamoto et al., 2005). Taken together, these studies showed that increased immune responses may be detrimental for hearing recovery.

Our findings demonstrated that depletion of GCs in the ADX model leads to blunted pro-inflammatory gene expression, indicating that suppression of inflammatory responses leads to the protective effect of ADX after night noise. It is likely that the glucocorticoid regulation of the diurnal response to noise trauma is driven by changes in inflammation signalling pathways. Recently a study has demonstrated that the Toll-like receptor TLR4 which is expressed in the cochlea (Cai et al., 2014) is highly involved in inflammatory response after noise insult, leading to cell degeneration (Vethanayagam et al., 2016). TLR4 deficiency has been shown to suppress the production of pro-inflammatory cytokines and antigen-presenting molecule specifically in the organ of Corti (Vethanayagam et al., 2016). These findings suggest that cochlear immune response and pathogenesis following a noise trauma is regulated by TLR4, thereby the increased pro-inflammatory gene expression found in sham mice may be mediated through the TLR4. Investigation of the relationship between GR signalling and TLR4 will contribute to a better understanding of the inflammation-mediated cochlear damage. Finally, further studies aiming to identify GC-dependent transcriptome changes after a night noise exposure are necessary in order to reveal the specific GC-induced pathways that regulate the circadian noise sensitivity. Most importantly, this knowledge

could potentially lead to the identification of new GC-related drug targets for treatment against NIHL. This is highly clinically relevant since anti-inflammatory drugs are commonly used drugs for hearing disorders.
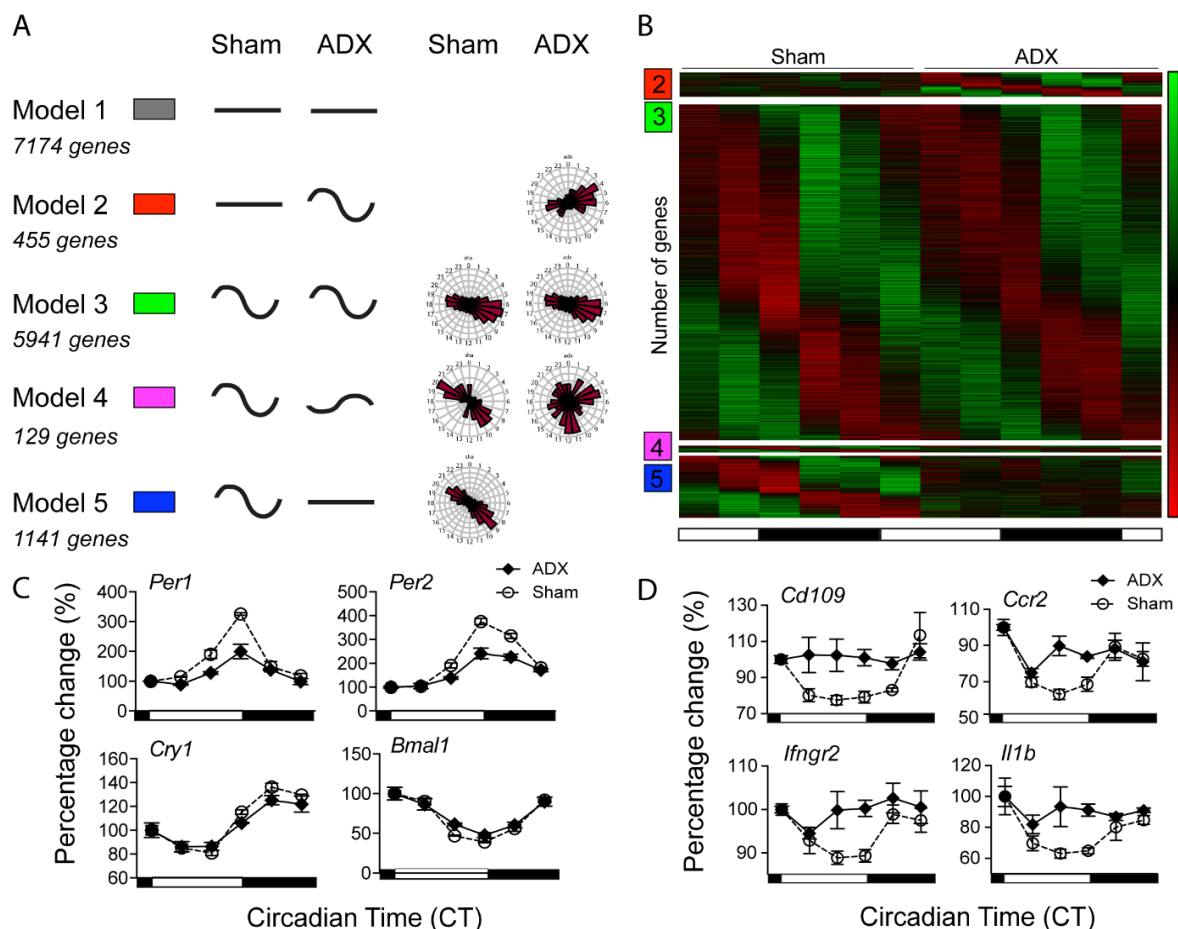
Figure 11. Glucocorticoid-dependent rhythmic regulation of cochlear transcriptome. A) RNA-seq analysis of cochlear samples form ADX or sham-operated mice revealed five models of GC-dependent transcriptome regulation, with distinct rhythmic patterns and phase specificity. Model 1: Genes that were non-rhythmic in both conditions (ADX and sham) (7174 genes); model 2: non-rhythmic genes in baseline conditions (sham-operated) that gained rhythmicity after ADX (455 genes); model 3: genes that are rhythmic and share the same phase and amplitude parameters between the two conditions (5941 genes); model 4: genes that are rhythmic but their rhythmic parameters for amplitude and phase are different (129 genes); model 5: genes that show rhythmic profiles only in sham-operated animals (1141 genes). Phase distribution for each model is shown on the right panel. **B)** Expression heat map for models 2-5, that comprise of rhythmic gene profiles in at least one of the conditions (sham operated/ADX). Mean relative expression is shown in green (low levels) and red (high levels) color tones. The white and black boxes on the bottom of the heat map indicate the light (white) and dark (black) circadian phases. **C)** Transcriptional profiles of four core clock genes in sham and ADX conditions. **D)** Transcriptional profiles of four putative glucocorticoid–dependent circadian cochlear genes. **(C-D)**The horizontal axis shows the sampling time as circadian time (CT). The vertical axis shows normalized mean values ± SEM (n = 3). All conditions were plotted as relative percentage change (RPC) using CT 0 as baseline value. Sham-operated samples: open circles/dashed lines; ADX samples: circles/full lines. The white and black boxes on the bottom of the heat map indicate the light and dark circadian phases, respectively. *Cd109*: cluster of differentiation 109; *Ccr2:* C-C chemokine receptor type 2; *Ifngr2*: interferon gamma receptor 2; *IL-1b*: interleukin-1b.

Administration of synthetic glucocorticoids is the standard pharmacological treatment for idopathic sudden sensorineural hearing loss, but the effectiveness of this treatment is controversial due to individual variability and decreased efficacy with time (Rauch, 2008). Although protective effects of GCs have repeatedly been demonstrated, inappropriate timing of GC treatment could be a reason to why most interventions fail. A therapeutic approach that consider the normal biological rhythms, namely chrono-pharmacology, will help to achieve optimal drug efficacy and minimum toxicity. Many pharmacokinetic and pharmacodynamics parameters (e.g drug absorption and distribution) as well as drug metabolism are subjected to circadian regulation, and thus drug efficacy and safety profiles would also vary with time of day (Dallmann et al., 2014). In addition, the effectiveness of the therapy may be dependent on what stage a programmed function is featured when the agent reaches the target tissue. In rheumatoid arthritis for example, there is a circadian abnormal increase in pro-inflammatory cytokines at night due to inadequate cortisol secretion, leading to increased symptoms in the morning. To target this night immune reaction, glucocorticoid administration with a night-time-release formulation has been used as a chrono-pharmacological approach and has proven more effective over the conventional treatment (Cutolo, 2016). Under these considerations, a chrono-pharmacological approach against NIHL would be of major clinical importance and it is also expected that it could have implications for other hearing disorders, such as ototoxicity and inflammation.

5 CONCLUDING REMARKS

In this thesis, I investigated the role of circadian regulation in the auditory system, focusing on implication for NIHL.

The work presented here shows a circadian regulation of hearing sensitivity and thus adds a novel feature to the complexity of the auditory system. As it was to be expected, the auditory function is not static, but it is under a continuous regulation during a 24-h cycle, with optimal function requiring tightly clock-regulated periods of intense activity, followed by rest.

The discovery of a clock machinery in the cochlea and the IC illustrates that the temporal control of auditory function is probably mediated by circadian clocks both in the peripheral and central parts of the auditory pathway.

Noise sensitivity is presumably driven by the cochlear clock, as supported by the differential clock gene expression and *Bdnf* induction after noise exposure. The specific mechanisms that underlie how changes in cochlear rhythms are coupled to the physiological response to noise insult are still unexplored, however this work provides evidence that there is a complex relationship between the cochlear clock and neurotrophins in regulating noise damage.

Furthermore, this work demonstrates that GC rhythms can regulate the diurnal feature of NIHL, presumably through mechanisms involving cochlear inflammation responses. In addition, the present results highlight the complex interaction between endogenous and exogenous GC levels in regulating the auditory response to noise damage and suggest that appropriate timing of glucocorticoid therapy may be of critical importance when treating hearing loss.

Based on the findings of this thesis, it is evident that circadian rhythms influence the effectiveness of otoprotective drugs. DHF treatment was protective against noise damage only when administered at the night, whereas DEX treatment was only effective at day-time. These results demonstrate that different drugs may require specific administration timing in order to be effective against NIHL, thereby introducing a chrono-pharmacological approach in auditory therapeutics that can help to improve current treatment regimen.

Overall, the findings presented in this thesis will have important clinical applications since there are possibly circadian auditory rhythms in humans regulating auditory function in a similar way. Noise is an environmental factor that is increasingly common in our society, and is the major contributor of auditory pathologies in humans. It would be important to examine whether people exposed to loud noise levels at certain phases of the circadian cycle (e.g shift-workers and flight crew) would be more vulnerable to NIHL.

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