Chapter 11
Information Transfer in the Mammalian Circadian Clock

Adrián E. Granada, Hanspeter Herzel, Achim Kramer and Ute Abraham

Abstract Most species evolved a circadian clock to adapt to the 24 h period of the solar day. In mammals, these clocks generate endogenous rhythms by regulatory gene networks in almost every cell. A pacemaker, the suprachiasmatic nucleus (SCN) as the master clock, receives environmental input and orchestrates peripheral organs via sympathetic enervation, temperature and humoral factors. However, the mechanisms by which this synchronization is achieved are largely unknown. In order to elucidate paradigms of environmental information transfer within the circadian network, we address the following questions: How is environmental information perceived by different circadian networks? Do different circadian networks vary in their responses to a given signal, and, if so, do the differences depend on inherent circadian properties? Which part of the signal (onset, offset, duration, strength) is relevant for the responses? To address these questions, we combine experimental data from cultured single cells and organotypic slices with mathematical models of circadian oscillators and find that temperature signals have a strong impact on circadian rhythms, depending on the specific circadian properties of the clock cells.

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Number of publications

Journal publications	8

11.1 Conclusion

11.1.1 Summary of the Essential Results

Combining experimental work and theoretical approaches, we quantified the information transfer from clock inputs (light-dark cycles, temperature cycles, and temperature pulses) to clock outputs, like for example activity and, clock gene expression in primary tissues and cell cultures. The response properties allowed for the classification of weak and strong circadian oscillators, depending on the coupling strength. This insight lead to a theoretical framework connecting phase response curves, entrainment and coupling strength.

11.1.2 Possible Future Work

Using bioinformatics methods, we found initial predictions of combinatorial gene regulation in liver. These results are preliminary and have to be extended by studying other tissues and by incorporating more recent high-throughput data.

11.2 Working and Result Report

11.2.1 Introduction

The mammalian circadian clock controls multiple physiological and behavioural rhythms with a period of about 24 h. These intrinsic rhythms need to be synchronized with the external environment, ensuring that fluctuating events occur at the proper times of day or night. This synchronization is achieved by periodically recurring stimuli, called zeitgebers, and requires information transfer from the environment to the clock. The circadian system is hierarchically organized and consists of a central circadian pacemaker, the hypothalamic suprachiasmatic nuclei (SCN), which relay environmental information to circadian oscillators in the rest of the body, referred to as peripheral oscillators (for a review see Albrecht 2012). The most prominent zeitgeber is the daily light-dark cycle, followed by food, temperature, social events, odours and noise. While direct synchronization by light is restricted to light-sensitive clock cells (e.g. in the eye), temperature cycles can be perceived by the majority of body cells, rendering it an elegant means to study environmental information transfer in mammalian clock cells. Our aim was to study the role of temperature oscillations as a potential zeitgeber for peripheral tissues. We combined information theory and theory of coupled oscillators to generate a set of theoretical predictions and tested them experimentally. Our predictions are:

1. When the period of the external temperature cycle (T) is close to the intrinsic period (τ), we will observe an expansion of the intrinsic amplitude (amplitude resonance effect).
2. The phase relationship between the intrinsic oscillation and the external zeitgeber is dependent on the intrinsic period. For example, relative short intrinsic periods will lead to early entrainment phases.
3. The phase relationship between the intrinsic oscillation and the external zeitgeber is dependent on the zeitgeber strength relative to the intrinsic amplitude of the oscillation.
4. Temperature pulse information is encoded in phase shifts of the circadian clock.

Mathematical derivations of our theoretical predictions can be found in previous publications (Abraham et al. 2010; Granada et al. 2013; Bordyugov et al. 2015). To experimentally test predictions 1–3, we decided to explant multiple peripheral tissues from mice and monitor their intrinsic oscillations in *vitro*. As representative

peripheral tissues, we used lung, cornea, and olfactory epithelium (OE) explanted from PER2::LUC knockin mice, an extensively used mouse model to study circadian rhythms (Yoo et al. 2004). These mice express luciferase fused to the endogenous clock protein PERIOD2. When cultured in the presence of luciferin, these tissues emit clock-gene-driven bioluminescence, which can be monitored in real time. To test prediction 4, we cultured human osteosarcoma cells (U2OS cells), transduced with a *Bmal1*-luc reporter (luciferase expressed under the control of the clock gene promoter *Bmal1*).

This unique set-up allows us to expose PER2::LUC peripheral tissues and *Bmal1*-luc U2OS cells to temperature cycles and single pulses, and simultaneously monitor changes in their circadian amplitudes, periods and phases in response to external signals in real-time live conditions.

11.2.2 Methods

Bioluminescence Monitoring and Temperature Cycles

Organotypic slices. Lung (n = 6), cornea (n = 4), and OE (n = 4) were explanted from PER2::LUC mice and cultured as described (Abraham et al. 2010; Saleh et al. 2015; Yoo et al. 2004). Immediately after culture, tissues were placed in temperature adjustable light-tight boxes (Technische Werkstaetten Charité, Berlin, Germany) equipped with photomultiplier tubes (HC135-11MOD, Hamamatsu, Japan) at 37 °C and 5% CO2. During bioluminescence recording, a daily temperature cycle with a period of 20 h was applied to each slice individually. Specifically, 10 h of cold with temperatures ranging from 31 to 36.5 °C alternated with 10 h of 37 °C. In order to simulate gradual temperature changes at dusk and dawn, each temperature step comprised a gradual temperature increase or decrease, respectively, over a course of 2 h. The first phase of the temperature entrainment cycle was always the 10-h cold phase and started at the minimum of PER2::LUC expression of the respective cycle (usually the third to fourth cycle) as determined by online registration. Temperature entrainment comprised six to seven temperature cycles and was followed by several days of constant 37 °C. Bioluminescence from all slices was recorded in 5-min bins for at least 12 days. For a visualization of the experimental procedure see Fig. 11.1.
U2OS cell culture. U2OS cells stably transfected with *Bmal1*-luc (Vollmers et al. 2008) and cultured in 96-well plates were at a density of 20.000 cells per well as described by Maier et al. (2009). Prior to bioluminescence recording, cells were synchronized by adding 1 μM dexamethasone to the culture medium for 30 min. Photon counts/30 min were measured in a 96-well plate luminometer (Topcount, Perkin Elmer, Rodgau, Germany) at 35.5 °C. 23.5 h following the start of the bioluminescence recording, one 96-well plate was removed from the topcount and left at 28.3 °C for 60 min (60 min temperature pulse), while the control plate was left untouched.

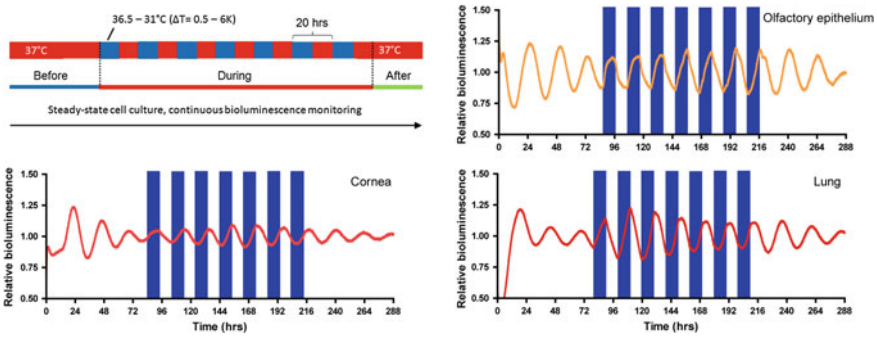

Fig. 11.1 Schematic representation of the experimental procedure (*top left*) and representative bioluminescence recordings (*top right* and *bottom*). Lung, cornea and OE carrying a clock-gene-driven bioluminescence reporter were exposed to 20-h temperature cycles of varying temperature differences during a steady-state culture. Before and after temperature cycles, a constant temperature of 37 °C was maintained. *Blue bars* represent cold phases

Analysis of Time Series Data

Organotypic slices. Bioluminescence data were first trend-eliminated, and then analyzed for their period, phase and relative amplitude using Chronostar 2.0 (Stephan Lorenzen, Institute for Theoretical Biology, Humboldt-University, Germany). Time series were trend-eliminated by dividing values by a 24-h running average, thereby normalizing the magnitude to 1 to be independent of any measurement specifics, such as sensitivity/background of the photomultiplier or efficacy of the luciferase. Resulting time series oscillate around 1 with amplitudes that are relative to their mean. Periods, phases, and amplitudes before, during and after temperature cycles were estimated by fitting the cosine wave function $y = a * \exp(b * t) * \cos((2 * \pi * t * 24/c) + d)$, which includes an exponential term for damping (a = amplitude, b = damping, c = period, d = phase). The period and the relative amplitude before onset of the temperature cycle were used to determine the individual τ-T and the amplitude-adjusted zeitgeber strength (ΔT/amplitude), respectively. Phase relationships of bioluminescent oscillations to external temperature cycles were calculated as follows: The first bioluminescence peak after completion of the temperature cycles (condition referred to as After in Fig. 11.1) was picked using Chronostar 2.0. Subsequently, the difference between peak time and onset of the extrapolated cold phase (dashed blue outline in Fig. 11.3) was calculated. Statistical analyses were performed using GraphPad Prism 4 software (GraphPad software, USA).

U2OS cells. Time series were processed and analyzed with an in-house MATLAB code. Raw time series data were detrended with a moving average method and fitted to an exponentially decaying sinusoidal function (see equations in Fig. 11.4). From these fittings, phase difference values before and after the temperature pulse were extracted and statistical analysis was performed to obtain properties of the distributions.

Inclusion of Previously Published Data

In order to complement our studies on the influence of different zeitgeber strengths, we included data from lung slices (n = 4), exposed to 20-h temperature cycles with a temperature difference of 0.75 to 6 °C, that were previously published (Abraham et al. 2010). The data were analyzed as described above.

11.2.3 Results

Different Peripheral Tissues Respond Similarly to Temperature Cycles and Exhibit a Resonance Effect

As shown in Fig. 11.1, all three tissues showed self-sustained circadian clock gene expression over the course of multiple days. While intrinsic relative amplitudes of circadian oscillations were rather similar in all tissues, periods appeared to be tissue-specific, with periods close to 24 h for OE (ϕ24.3 ± 0.7 h S.D.), intermediate for cornea (ϕ24.8 ± 0.9 h S.D.) and distinctly longer for the lung (ϕ25.4 ± 0.7 h S.D.). However, the responses to temperature cycles were similar in principle: a fraction of the tissues responded with an expansion of the amplitude (see Fig. 11.1, bottom), a fraction with a reduced amplitude and the remaining tissues showed no change at all (see Fig. 11.1, top right). We plotted these responses according to the intrinsic periods of the samples, and found that when the intrinsic circadian period is close to the period of the extrinsic temperature cycle ($\tau - T \approx 0$), the majority of the tissues respond with an amplitude expansion, they resonate (Fig. 11.2). Contrastingly, when the difference between intrinsic circadian period and extrinsic temperature cycle is larger, the majority of tissues respond with amplitude reduction. Intermediate period values usually resulted in no change. This experimentally supports our theoretical prediction nr. 1 (see above), detailed in Granada et al. (2013). Interestingly, our concept of amplitude resonance can be used to explain the fact that cyanobacteria

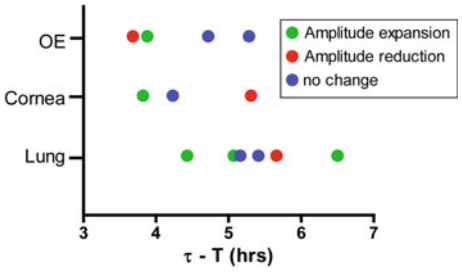

Fig. 11.2 The difference between the intrinsic period τ and the extrinsic zeitgeber cycle T determines the response of circadian oscillations in different tissues: when τ is close to T ($\tau - T \approx 0$) cornea and lung respond with amplitude expansion ("resonance"), and vice versa. They respond with no change of the amplitude for intermediate values. The response pattern of the OE was slightly different

with an unusually short intrinsic period outcompete cyanobacteria with a normal intrinsic circadian period when they are raised in a short environmental cycle, and vice versa (Ouyang et al. 1998). This would imply a resonance effect at an organism population level. Thus, our concepts seem to be applicable not only to tissues, but also to the organismal level.

The OE does not strictly follow this pattern. In particular, longer τ do not result in amplitude reduction. This might be due to tissue heterogeneity resulting in a large variation of amplitude responses to a given zeitgeber, or to the low sample size (there might be a bias: we incidentally measured OE tissues with a τ too close to T to exhibit amplitude reduction). Alternatively, the dependency of the amplitude response on τ-T might simply be different for OE.

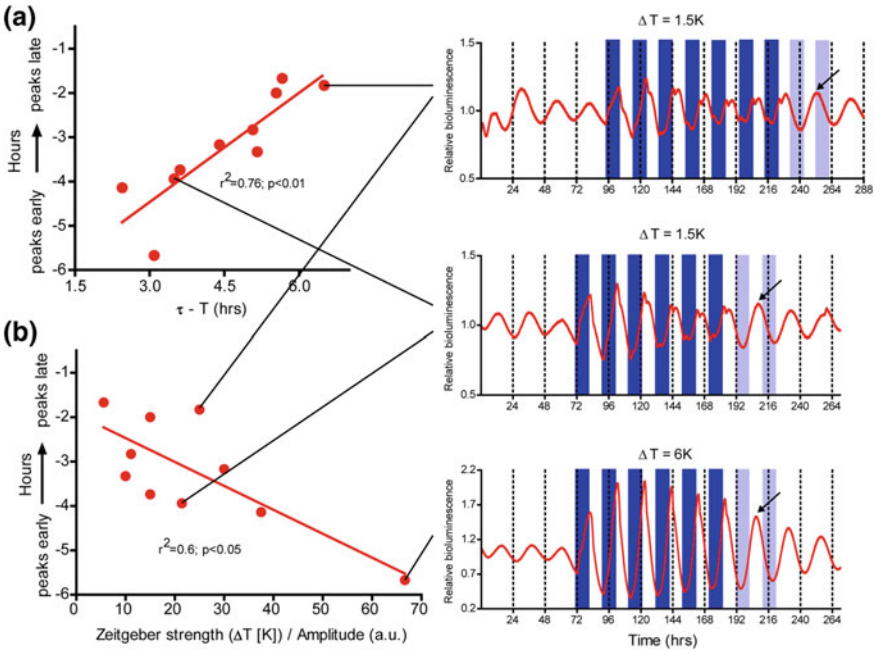

Fig. 11.3 Dependence of the phase relationship on the intrinsic period and on the zeitgeber strength in lung tissue for T = 20 h. **a** Significant positive linear correlation between τ-T and the phase relationship of the circadian oscillation with the temperature zeitgeber. In other words, the smaller the intrinsic period, the earlier the peak phase. Each *red dot* represents data of a single lung tissue. The respective bioluminescence measurements for two exemplary dots are shown on the *right*. **b** Significant negative linear correlation between the zeitgeber strength, adjusted for the intrinsic amplitude, and the phase relationship with the zeitgeber. Each *red dot* represents data of a single lung tissue. The respective bioluminescence measurements for three exemplary dots are shown on the *right*. The phase relationship with the zeitgeber was determined by calculating the difference between bioluminescence peak and extrapolated cold phase (see *black arrows*). *Blue bars* = cold phases; *light blue bars* = extrapolated cold phases

Dependence of the Phase Relationship on the Intrinsic Period

In order to test our predictions 2 and 3, we broadened our range of zeitgeber strength from $\Delta T = 0.5 - 1.5\,K$ to $\Delta T = 0.5 - 6\,K$. To do so, we included previously published data on lung tissue (Abraham et al. 2010). Figure 11.3a depicts the positive linear correlation ($r^2 = 0.76$, $p < 0.01$) between intrinsic period and the phase relationship with the zeitgeber, complementing our own experimental findings (Abraham et al. 2010) and those of Brown et al. (2008), who showed that information about human phases of entrainment is stored in the molecular clockwork of fibroblasts. Furthermore, our findings support theoretical predictions on the phases of entrainment (Bordyugov et al. 2015). Importantly, phase relationships are also dependent on the zeitgeber strength (Fig. 11.3b): higher zeitgeber strength (adjusted for the intrinsic amplitude) results in an earlier peak phase ($r^2 = 0.6$, $p < 0.05$), and vice versa.

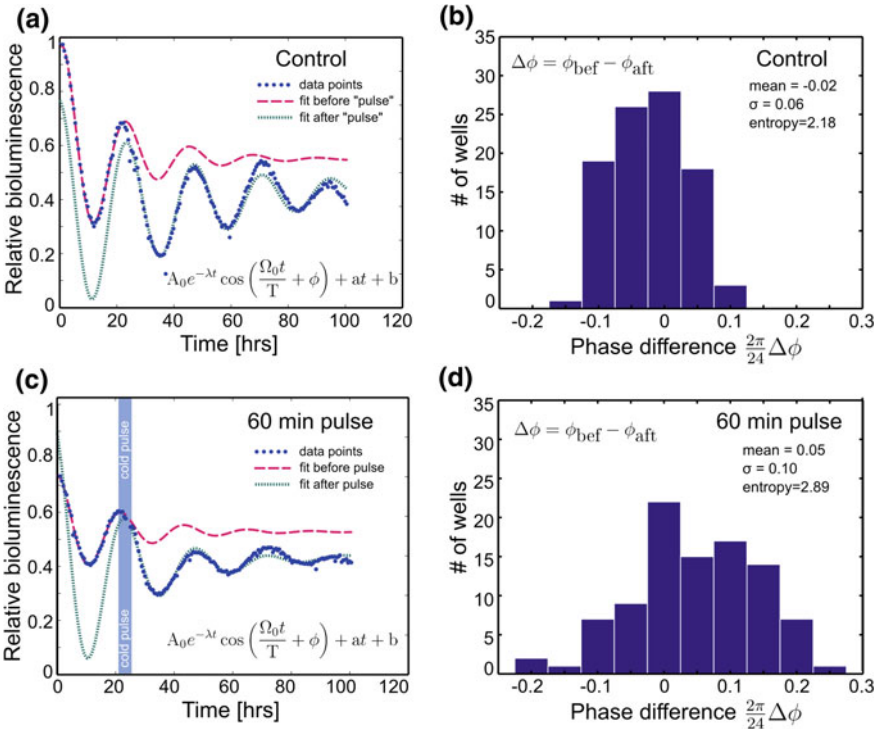

Fig. 11.4 Temperature pulses affect phases in cell culture. **a**, **c** Representative bioluminescence recordings from U2OS cells with (**c**) and without (**a**) 1-h cold exposure. **b**, **d**: The distributions of phase differences (phase before—phase after) in a 96-well plate that did (**d**) or did not (**b**) receive a 1 h cold pulse. We find a positive phase shift (0.07 ± 0.02 S.E.M.) and a broader distribution for the plate that received the pulse, i.e. the entropy is larger

Cell Culture: Temperature Pulses Increase Entropy

To expand our knowledge from tissues to dispersed circadian oscillators, we studied the effects of temperature on a human osteosarcoma cell line (U2OS cells), carrying a clock-gene-reporting bioluminescence construct. Due to the fact that circadian oscillations in U2OS cells damp rather quickly, we administered a single temperature pulse of 1h, instead of a temperature cycle. As an added benefit of the 1h-pulse setup, we were able to monitor bioluminescence from 96 cell cultures at the same time, resulting in a very high N. This allows for the quantification of distributions using entropies.

All cell cultures show some variability in their peak-to-peak phasing (Fig. 11.4a, c). However, when plotted as a frequency plot (Fig. 11.4b, d), it becomes apparent that the 1 h cold pulse resulted in a broadening of the phase difference distribution, leading to an increase in entropy.

We noticed a small trend in bioluminescence magnitude depending on the location of the cultures on the 96-well plate, with cultures situated at the edges of the plate exhibiting slightly higher bioluminescence levels. A systematic study of this trend, also known as "border-effect" (differential evaporation rates), however, revealed that the trend was not affecting the overall results.

11.2.4 Conclusions

The present findings clearly suggest that temperature serves as a universal zeitgeber. Previously, temperature entrainment has been shown for SCN and lung tissue (Abraham et al. 2010; Buhr et al. 2010). Here, we decided to further extend our experimental system to include two additional peripheral oscillators, the cornea and the OE. Although we cannot exclude that there might be some tissues that will not respond to temperature pulses at all, we assume that the majority of peripheral tissues are very sensitive, even to small changes in ambient temperature. This is functionally conceivable and mechanistically plausible since the SCN needs to communicate circadian phasing to downstream targets in order to orchestrate circadian rhythmicity among body clocks. Hence, even small changes in body temperature ($<2\,^{\circ}C$) should affect phasing in peripheral clocks. The SCN clock, however, is synchronized to the environment via light input through the eye. Consequently, it does not need to be very sensitive to temperature fluctuations (Abraham et al. 2010; Buhr et al. 2010). In fact, it might even be detrimental for the SCN to be overly reactive to temperature fluctuations, rendering the master circadian pacemaker "unstable" and "unreliable" in its phasing. Peripheral clocks presumably mainly exert local functions, therefore, it might be beneficial for them to not only respond to systemic factors transmitting zeitgeber information from the SCN, but also to local variations in temperature (e.g. skin clock). One of the key features of a zeitgeber is reliably transmitting environmental information and resetting of the endogenous clock, eventually leading to entrainment with a periodically recurring event. Although we were not able to directly test for

temperature entrainment—this would require significantly longer recordings which would compromise tissue health—we conclude from the fact that circadian oscillations assume systematic phase relationships with the temperature cycles (Fig. 11.3). For example, the association between temperature cycle and intrinsic period: similar to light-sensing organisms that grow faster in a light cycle that is close to their intrinsic period (Ouyang et al. 1998), cultured tissues will respond with an amplitude expansion when both, temperature cycle and intrinsic period, are close. Hence, while light as a zeitgeber clearly serves as a selective force in *vivo*, temperature cycles appear to be the equivalent of that in *vitro*. Furthermore, the fact that cold pulses elicit phase shifts in human fibroblasts is another indication for successful information transfer. However, undoubtedly, the responses to the temperature zeitgeber are highly variable, stressing the need for quantitative measurements, a high number of samples, and statistical analyses. This is particularly true for the OE. Whether the OE's highly variable responses are tissue-intrinsic, or whether it reflects heterogeneity in information transfer—perhaps due to a differential distribution of temperature sensitivity, or differential prevalence in signal transduction pathways—is not clear. Our data give us a first insight into temperature information transfer in peripheral tissues whose connectivity/ability of cellular communication is still controversial. We still need to investigate whether the single cell or the network serve as a unit for perception. Also, we need to identify which part of the temperature signal is most relevant (onset, offset or duration?), and its relative role with respect to other zeitgebers, i.e. do they act in a synergistic or antagonistic way when applied simultaneously? In summary, this study shows that our initial predictions/hypotheses 1 to 4 can be experimentally reproduced with temperature cycles/pulses on peripheral tissues, strongly suggesting that temperature act as a zeitgeber for peripheral clocks.

11.2.5 Educational Qualification of Researchers

Doctoral Degrees

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Bachelor's Degrees and Student Research Projects

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- Dander Bervoets, internship

- Alexander Vowinkel, internship
- Peter Larsen, internship

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Other Publications

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