Natural entrainment of circadian systems
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A circadian entrainment model for the European ground squirrel based on phase and period responses
In Chapter 2 and 3 of this thesis we reported natural activity patterns of European ground squirrels under natural light conditions. We argued that these patterns could not be explained by the generally accepted phase response entrainment model developed principally by Colin Pittendrigh (e.g. 1960; 1976; 1993). In this model daily changes in light intensity around twilight provide the crucial time cues for circadian systems to entrain. Ground squirrels virtually never in their life see the twilights and yet they are perfectly entrained. Additionally, the ground squirrels perceive large variations in light intensity due to going in and out of their burrows. Application of the phase response entrainment model to this kind of light signal yields unrealistic activity patterns. The lack of explanatory power of the phase response model for natural activity patterns of ground squirrels formed the central theme of this thesis.

Time cues other than light intensity per se were also investigated. In chapter 7 we established that circadian entrainment in European ground squirrels can not be attributed to changes in spectral composition in the course of the day. Ultraviolet light is not perceived by the ground squirrels because it is filtered from the spectrum by the lens of their eyes. Also the relative increase in blue light around the time that the sun position is just above the horizon is not perceived by the ground squirrels because they are below ground in closed burrows (Chapter 7). In Chapter 8 we established that ground squirrels are able to entrain to a nonphotic signal such as handling. However, the fact that not all animals responded to this relatively strong stimulus indicates that such nonphotic signals are at the very best weak zeitgebers and probably not important for natural entrainment in the European ground squirrel. Other nonphotic cues like daily temperature cycles and environmental sound are discussed and also rejected as potential zeitgebers for natural entrainment in Chapter 3.

Considering the findings summarized above, we re-evaluated the role of light intensity in current entrainment models. Phase responses ($\Delta\phi$) to light were combined with period responses ($\Delta\tau$) in a novel entrainment model presented in Chapter 9. Although this work was inspired by the discrepancy between theory and empirical results found in the European ground squirrel, we presented this new entrainment model more generally, describing the behaviour of a nonburrowing animal which perceives light intensity variations throughout the daily cycle. This was done because we noted that the inclusion of the $\tau$ responses improves the accuracy of entrainment in general. This discussion focusses on the question whether this general model indeed is applicable to the European ground squirrel and can provide a quantitative explanation of its activity patterns.

The problem of natural entrainment in the European ground squirrel

If the circadian system of European ground squirrels would respond to light only by shifting its phase, the natural activity rhythm of this species can not be explained (Chapter 2, 3). Phase response models would predict an offset of activity very close to civil twilight at dusk ($\psi_{\text{offset}} = 0$ h) due to the fact that the typical endogenous rhythm of these animals is longer than 24 h (average $\tau = 24.31$ h, Chapter 8). However, European ground squirrels retreat below ground about 3 h before civil twilight at dusk ($\psi_{\text{offset}} = 3$ h) and they perceive only a $\sim 1$ log unit light intensity decrease at the end of their activity period. Since this small change in light intensity is masked enormously by weather conditions and shades, including retreats in burrows, it is insufficient to correct for the difference between $\tau$ and the daily light cycle.
(T = 24 h). Hence, other types of responses to light must be considered for the explanation of the late daily appearance above ground and early daily retreat below ground in European ground squirrels.

The application of light pulses (~1h) to animals under continuous dim light conditions is a useful tool for studying the responses of the circadian system to light. The circadian system of diurnal species seems to react to a light pulse not only by shifting its phase, but also by persistently changing its period length $\tau$ (chapter 9). Both effects vary with the phase of the circadian system. When plotted against the phase of the circadian system the magnitude and direction (advancing or delaying) of phase shifts form a phase response curve (PRC) and the lengthening or shortening of $\tau$ form a $\tau$-response curve ($\tau$RC). Although there is large variability in period response as well as in phase response, the general pattern shows that shortening of $\tau$ (acceleration) by a light pulse occurs predominantly at the same phases where light advances the circadian rhythm, and lengthening of $\tau$ (deceleration) occurs where light delays the circadian rhythm (chapter 9). Thus, $\tau$ responses and phase responses work in the same direction, but a single phase shift is completed within one cycle whereas the $\tau$ response persists up to several weeks (e.g. Gerkema et al. 1993).

The model presented in chapter 9 simultaneously used the PRC and the $\tau$RC curve to evaluate entrainment under fluctuating (natural) light conditions. Under entrained conditions, this model predicts that the $\tau$ response to light results in an overall intrinsic period of the circadian system indistinguishable from 24 h. When $\tau$ is close to 24 h only very small phase shifts are needed to correct for variations in light intensity and for stochastic variations in phase and period of the circadian oscillating system. The limited environmental daily light variation perceived by ground squirrels may be able to entrain their circadian system under the assumption that their $\tau$ response curve tunes the intrinsic circadian period virtually to 24 h. In order to study this assumption we measured both the phase and the period responses to light pulses in the European ground squirrel and used the resulting PRC and $\tau$RC for simulating entrainment under natural conditions using a model as presented in Chapter 9.

**Phase and $\tau$ response curves for the European ground squirrel**

25 ground squirrels under continuous dim red light conditions (0.02-0.06 lux) were treated with brief light pulses (1-h; 1700 lux; at eye-level; Osram L40W/20SA white tube lights) once every 14 days. In total 100 responses to a light pulse (1-9 per individual) were derived from those records where the animals displayed stable circadian rhythms before and after the light pulse. Phase shifts and period changes were calculated by comparing a pre-pulse and post-pulse regression line through the daily centres of gravity of activity (Kenagy 1980), which were used as a phase reference and defined as circadian time 6. The first 4 cycles after the light pulse were not included in the post-pulse regression in order to exclude transient days. Post-pulse regression lines were extrapolated to the day of the pulse to calculate the differences in pre- and post-pulse phase of the circadian system. Differences between the slopes of pre- and post-pulse regression lines were used to calculate induced changes in period. Calculated phase shifts and period changes were plotted on a circadian time axis. Data points from the same individual within each 2-h circadian time bin were averaged. A sine wave with 2 sub-harmonics was fitted through the data points using a least squares method.
The shape of the resulting PRC (Figure 10.1, upper panel) closely corresponds with the average type 1 PRC in diurnal mammals (chapter 9, figure 9.1). Advances (maximally 3 h) occur in the late subjective night and the early subjective day between ct 17.1 and ct 5.9. Delays (maximally -3.33 h) occur in the late subjective day and early subjective night between ct 5.9 and ct 17.1. There is no indication of a zone without phase shifts around ct 6 (“dead zone”). This feature may be specific for diurnal animals (4). Alternatively, it may reflect a general problem of estimating circadian phase in diurnal mammals. In general, diurnal mammals have less clear onsets and offsets of activity as compared to nocturnal mammals. This may induce errors in estimating circadian phase that, in turn, will reduce the dead zone of the PRC.

The shape of the resulting τRC (Figure 10.1, lower panel) was similar to the PRC with shortening of τ (maximally 0.22 h) occurring from ct 21.5 till ct 8.6 and lengthening (maximally 0.47 h) occurring from ct 8.6 till ct 21.5. Thus the results are consistent with the expec-

Figure 10.1 Phase response curve (PRC; top panel) and period response curve (τRC; lower panel) for the European ground squirrel. Phase shifts and period changes are indicated in hours and plotted against the phase of the circadian activity rhythm in circadian hours. The centre of gravity of activity was taken as the phase reference point for the middle of the subjective day (circadian time 6; ct 6) and the period of each cycle was set to 24 circadian hours. For both, the PRC and the τRC, a sinusoidal curve with two sub-harmonics was fitted to the data points as shown in the graphs.
tation that $\tau$ response and phase response work in the same direction for different phases of the circadian cycle. The positive correlation between phase shifts and $\tau$ changes was highly significant ($p < 0.0001$). The ratio between period and $\tau$ changes was on average $\Delta \phi / \Delta \tau = 9.96$. For the purpose of simulations (see below) we decided to fit a general acceleration / deceleration curve to all data points: the $\tau$ changes were multiplied by 9.96 and added to the phase shift data points. The combined data set was used to estimate a new PRC, again by fitting a sine wave with 2 sub-harmonics. In order to calculate the new $\tau$RC, the amplitude of the resulting PRC was scaled down by dividing it by 9.96.

**A natural entrainment model for the European ground squirrel**

In order to evaluate the importance of $\tau$ responses for natural entrainment in the European ground squirrel, we simulated the process of entrainment in a model as described in chapter 9 of this thesis. We simulated entrainment only for long days (21 June) and the daily maximal light intensity curve used in the model was based upon irradiance measurements in the enclosures in Haren around the 21st of June (chapter 3, chapter 7). Based upon these measurements a random decrease in light intensity due to overcast was set to a maximum of 1.5 log units. Random variation in period and phase was identical to the values used in the model of chapter 9.

The natural above ground activity phase of European ground squirrels in the enclosures in Haren around the 21st of June started on average from 6.96 h local time (average within individual SD = 0.95 h) and ended at 18.74 h (average within individual SD = 1.01 h). Thus, the active phase ($\alpha$) was set to 11.78 h in duration and centred around ct 6, which was also the phase reference point for the PRC and $\tau$RC measurements. Within $\alpha$, European ground squirrels are ~ 50 % of the time above ground and exposed to natural light. They face complete darkness when below ground (chapter 2, 3, 4). This behaviour introduces extreme light intensity variation, which was introduced into the model by reducing the light levels to 0 with a probability of 50% within $\alpha$. Light levels within the rest phase ($\rho$) were always set to 0.

The intrinsic $\tau$ with which the model was launched was set at 24.31 h. This value was taken from free-running European ground squirrels under continuous dim light conditions (0.02-0.06 lux; chapter 8).

**Simulation results**

Both the PRC and the $\tau$RC are measured under continuous dim red light conditions (0.02-0.06 lux). For a diurnal mammal we can assume that under natural daylight conditions the animals are light adapted during a major part of their activity period. For the circadian system this would translate to a reduction in the amplitude of the PRC and the $\tau$RC. If both effects are mediated via the same photic pathway, the reduction in light sensitivity would be similar for both phase responses and $\tau$ responses. We first analysed the effect of simultaneous reduction of the PRC and the $\tau$RC amplitude.

The average phase of activity onset (ct 0.11) and activity offset (ct 11.89) calculated over the last 100 days of 1000 days simulation runs is plotted in figure 10.2 (lower two panels). The middle panel shows the consequence of reducing the amplitude of the PRC and the $\tau$RC simultaneously (i.e. reducing the sensitivity to light). This results in shifting the active phase...
Figure 10.2 Simulation results compared to the observed activity pattern around June 21. Maximal light intensity used in the model simulations divided over 7 log units is plotted against the time of day (curve in top panel). Arrows indicate civil twilight at dawn and at dusk. Average activity onset and activity offset measured by light sensitive telemetry around June 21 (chapter 3) is indicated with dots. Error bars indicate the mean of the individual standard deviations of activity onsets and activity offsets. Dotted vertical lines indicate the 95% confidence limits around the mean activity onset and the mean activity offset. Middle panel indicates the model output for activity onset and activity offset (thick lines) and their standard deviations (thin lines) plotted against the time of day. The relative amplitude of the PRC and the τRC is varied simultaneously from 1 (as measured under continuous dim light) to 0 (no response to light). Dotted horizontal lines indicate those values where the model simulations are not significantly different from the observed activity patterns (relative PRC and τRC amplitude = 0.165).

Lower panel illustrates the relative contribution of period responses in the entrainment model. The relative PRC amplitude is fixed at 0.165 and the τRC amplitude is varied from 0 to 1. When τRC amplitude is set to 0, the model output deviates significantly from the observed activity patterns under natural conditions (outside the 95% confidence limits). When the relative amplitude of the τRC is higher than 0.9 there is no significant difference between the model simulations and the observed activity pattern.
into the light phase of the day. This can be explained as follows. When the amplitude of the PRC and τRC are reduced, higher light intensities are required at the end of the active phase to attain similar phase shifts. Hence, entrainment occurs when the end of the active phase is shifted (i.e., advanced) into the light phase of the day.

Reducing the sensitivity to light eventually results in a match between the observed activity patterns in the enclosures and the simulation results. This appears to occur when the PRC and τRC amplitude are reduced to 0.15-0.18 (~1/6) of the amplitude as measured under dark adapted conditions (relative sensitivity to light = 1). Under those conditions the standard deviation of the average onset and offset of activity also matches the standard deviation of these phase markers as measured in the field.

**Reduction of PRC and τRC amplitude in relation to light adaptation**

Maximal similarity between the model output and behavioural measurements is found when PRC and τRC amplitude are reduced to ~1/6 of the amplitude as measured under dark adapted conditions (dim red light). This theoretically estimated light adaptation value for the circadian system can be compared to empirical measurements on light adaptation in the circadian system of the rat. Dark adapted light pulse (1 s; 510 nm) threshold responses of SCN neurons (single unit recordings) have been found to be around $10^{10}$ photons.m$^{-2}$.s$^{-1}$ in the rat (Aggelopoulos and Meissl 2000). When the animals were light adapted to $10^{18}$ photons.m$^{-2}$.s$^{-1}$ broad spectrum illumination (> 500 nm; intensity is ~ 2 log units less than solar irradiation levels, Figure 7.1), these threshold responses were found at light levels around $10^{16}$ photons.m$^{-2}$.s$^{-1}$. In order to compare these light adaptation levels in the rat to our model estimation for light adaptation in the circadian system of the ground squirrel we need to recalculate the threshold responses of the rat to the PRC and τRC amplitude axis used in our simulation model. The PRC and τRC used in our simulations was measured under dark adapted conditions (Figure 10.1) and set to 1 in our model. A dark adapted threshold response of $10^{10}$ photons.m$^{-2}$.s$^{-1}$ in the rat could therefore correspond to the dark adapted PRC and τRC amplitude of 1. In nature, green light [510 nm, as used in Aggelopoulos and Meissl (2000)] reaches a maximum intensity of ~ $10^{18.5}$ photons.m$^{-2}$.s$^{-1}$ (Chapter 7). As a result, a threshold response of $10^{18.5}$ photons.m$^{-2}$.s$^{-1}$ will not cause any phase shifts or period changes in our model simulations and would therefore correspond to a PRC and τRC amplitude of 0. We assumed a log-linear relationship between the threshold responses in the rat (ranging from $10^{18.5}$ to $10^{10}$) and the PRC and τRC amplitude used in our model simulations (ranging from 0 to 1). A reduction of the PRC and τRC amplitude to ~1/6 of its dark adapted value would thus translate to a threshold response of $10^{17}$ photons.m$^{-2}$.s$^{-1}$ under natural (light adapted) conditions (natural light adapted threshold response = $10^X$, where $X = (5/6) * (18.5 - 10) + 10 = 17.1$). This predicted natural threshold response for the ground squirrels is about 1 log unit higher than the light adapted threshold response reported for rat SCN neurons ( = $10^{16}$). Thus, the circadian system in light adapted rats seems to be 1 log unit more sensitive to light than the predicted sensitivity for the European ground squirrels under natural conditions. This difference could be explained by the duration (~30 min) and low light intensity (~2 log units less than natural daylight) used by Aggelopoulos and Meissl (2000) to adapt the rats to light.
Contribution of τ responses

The relative contribution of τ responses to the model simulations was further studied in detail around the area where the model simulations resemble field observations best (Figure 10.2, middle panel, PRC and τRC amplitude = 0.165). In the lower panel of figure 10.2, τ responses were varied by increasing the τRC amplitude from 0 (no τ response) to 1 (full τ response as measured under dark adapted conditions) while the PRC amplitude was fixed at 0.165. Without any τ response (relative τRC amplitude = 0) the system entrains to the light-dark cycle with a phase angle difference of 1.5 h between civil twilight at dusk and the offset of activity (ψoffset) and the model simulations do not resemble the field observations. At low levels of τRC amplitude ψoffset increases with increasing τ response. Similarity between natural behaviour and the model simulations emerged when the τ response was increased to ~1/10 of its dark adapted value resulting in ψonset = -3.9 h and ψoffset = 2.9 h. Further increase in the τ response primarily affected the accuracy of the rhythm (1/SD), but had only a minor effect on the average phase position. Thus only small changes in τ response to light are sufficient - but necessary - to generate the natural activity patterns of European ground squirrels. Entrainment by phase shifts only cannot explain the natural activity patterns.

Perspective

The model describes natural activity patterns of European ground squirrels reasonably well when a certain light sensitivity is assumed. This is of course no evidence that the assumptions of the model are correct. In particular the fine structure of the patterns of withdrawal behaviour of the squirrels into their dark burrows and the dynamics of light and dark adaptation, on which we have no independent empirical data, are bound to affect the outcome of the entrainment process. With these caveats in mind, I conclude that the natural pattern of activity of the European ground squirrel indeed can be generated by a system employing velocity responses to light in addition to phase responses, and not by a system employing only phase responses. Ground squirrels employ both (Figure 10.1), like most diurnal mammals do (Daan 2000). This suffices for entrainment in the absence of dawn and dusk perception. No nonphotic or spectral zeitgebers need to be involved.

This thesis is a first attempt to understand natural entrainment in a species where the animals’ own behaviour causes the major changes in perceived light intensity. Obviously these behaviourally induced light intensity changes are extreme in an exclusively diurnal animal that shows several retreats into its burrow during daytime. Nonetheless, other types of behavioural patterns such as eye closure, diurnal above ground activity in nocturnal species, moving in and out of the shade, vertical movements through a water column, or artificial lighting and curtain closing behaviour in humans will also induce variations in light intensity perceived by the circadian system. The entrainment mechanism proposed here for European ground squirrels may therefore well be applicable for natural entrainment in many other species where behaviour of the animal itself causes variation in perceived light intensity.