Hierarchical organization of the circadian timing system

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SUMMARY AND CONCLUSION

In order to cope with and to predict 24-hour rhythms in the environment, such as light and darkness, temperature or the availability of food, most, if not all, organisms have circadian timing systems. These systems generate circadian rhythms in a wide range of behavioral and physiological processes. For most animal species, a main circadian pacemaker has been identified in an anatomically defined location in or close to the brain. During the last years, however, it has become clear that circadian systems are complex and that additional oscillators exist, both within and outside the central nervous system. For a correct functioning of the circadian timing system, these oscillators should be synchronized to one another and properly phased to the environmental cycle. Synchronization between the oscillators occurs via interactions or coupling pathways, whereas the light-dark cycle is used for synchronization to the outside world. In the first chapter of this thesis, a review discusses the presence of circadian oscillators within several animal species as well as the mechanisms of the interactions between them.

The most important mammalian circadian pacemaker is located in the suprachiasmatic nuclei (SCN) at the base of the hypothalamus in the brain. Circadian rhythmicity is generated in individual SCN cells by interlocking negative and positive molecular feedback loops. The main input of the circadian system is light, which reaches the SCN via a specialized pathway, the retinohypothalamic tract. Light causes changes in SCN neuronal activity and clock gene expression, with the magnitude of the effects depending on the timing of exposure. These changes underlie the phase shifting effects of light on the circadian system, which is the main mechanism by which entrainment to the environmental light-dark cycle is accomplished. Interestingly, the phase shifts that are induced by light in the onset of behavioral activity often show different magnitudes and kinetics than the phase shifts in activity offset. This phenomenon has been explained using a model consisting of two coupled oscillators that are differentially affected by light and control either the activity onset or offset.

Besides light, specific types of behavior as well as certain pharmacological agents can have an effect on the mammalian circadian system. Among others, they induce phase shifts in behavioral activity rhythms and therefore can be used to examine the attributes of the oscillators that are in control of the activity onset and offset. The experiments described in chapter 2 investigate the phase shifting effects of two pharmacological agents, the opioid agonist fentanyl and the benzodiazepine midazolam. The data show that administration of midazolam and fentanyl in the mid- to late subjective day induces large phase advances in the activity onset, whereas the activity offset does not phase advance. Large phase delays of the activity offset are the result of administration during the late subjective night or early subjective day, a time when the effects on the activity onset are only small. These results demonstrate

that the activity onset and offset respond in a different manner to administration of fentanyl and midazolam, corresponding with the results obtained for light pulses, and it is concluded that the systems controlling behavioral activity onset and offset are qualitatively different.

The data described in chapter 2 do not reveal whether the dissimilar phase shifting responses of activity onset and offset are generated at the pacemaker level in the SCN or whether systems outside the SCN differently affect behavioral activity onset and offset. Chapter 6 provides indications for the presence of two groups of oscillators within the SCN that react in a different manner to a photic stimulus. On the first day after a 6-hour phase delay of the light-dark cycle, the neuronal activity rhythm in a slice preparation shows bimodal patterns in the ventral and the dorsal parts of the SCN. The two peaks merge in the course of several days in constant darkness, yielding a unimodal and completely phase-delayed peak on day 6 after the shift. A surgical cut, separating the dorsal and ventral parts of the SCN, results in unimodal electrical activity peaks in both SCN regions. The rhythm in the ventral SCN shows an almost complete phase delay, whereas the rhythm in the dorsal SCN does not shift in phase. Also in response to a phase advance of the light-dark cycle, the ventral part of a cut SCN shows a somewhat larger phase shift than the dorsal part. These data suggest that within the SCN, two regions are present that respond with different kinetics to photic stimulation. The large and immediate phase shift of the ventral SCN may be explained by the dense retinal innervation of this region, whereas the minor retinal projection to the dorsal SCN may account for the slow phase shifting response of this area. The bimodal peaks in the neuronal activity of intact slices indicate that phase information is normally transmitted between the two SCN regions. Application of the $GABA_A$ antagonist bicuculline to an intact slice has the same effects as a cut between the dorsal and the ventral SCN: the occurrence of out-of-phase unimodal peaks in neuronal activity. The data indicate therefore that GABA plays an important role in signaling between the dorsal and the ventral SCN.

Whereas the data of chapter 6 provide evidence for dissociation within the SCN during phase resetting, chapter 5 reveals that dissociation may also occur between different levels of organization within the circadian timing system. In response to a 6-hour phase advance of the light-dark cycle, the rhythm in *Per1* clock gene expression in the SCN phase shifts immediately. Surprisingly, the behavioral activity rhythm does not show a phase shift, corresponding with *in vivo* neuronal activity in the SCN of freely moving animals. When the SCN neuronal activity is measured in a slice preparation *in vitro,* the rhythm initially shows a phase shifts of several hours, but returns to the unshifted state when the SCN remains *in situ* for several days before preparation of the slice. These data suggest that, in a slice, when extra-SCN areas are disconnected from the SCN, the neuronal activity rhythm displays the actual SCN phase. In contrast, *in vivo*, the neuronal activity of the SCN seems to be masked by the activity of extra-SCN areas that are not shifted. The eventual return of the *in vitro*

electrical activity rhythm to the unshifted phase indicates that extra-SCN areas ultimately have an entraining effect on the SCN. These findings provide new insight into problems related to shift work or jet lag and suggest that the inability of the body to adjust quickly to a shift of the light-dark cycle is not caused by the circadian clock itself, but is the result of largely unresponsive or inert extra-SCN areas having an unexpectedly strong influence on the phase regulation of the SCN. Future studies should elucidate which signaling routes within the central nervous system, or possibly also from the periphery, are involved in antagonizing the phase resetting response of the SCN.

Sleep is thought to be regulated by two processes: a sleep homeostatic process and a circadian process. These processes have long been believed to act independently, but over the last years, some indications have appeared for communication between the two. In the experiments described in chapter 4 of this thesis, this issue is addressed directly. Simultaneous recording of the SCN neuronal activity and the electroencephalogram (EEG) in freely moving rats reveals a correlation between the discharge rate of SCN neurons and vigilance state. Neuronal activity of the SCN appears high during REM sleep and low during NREM sleep. When the animal is deprived of REM sleep, SCN neuronal activity does not increase, whereas deprivation of slow wave activity during NREM sleep prohibits the decrease of neuronal activity. These data demonstrate a causal relationship between vigilance state and the discharge rate of SCN neurons. In conclusion, these studies provide first evidence for an influence of sleep centers on the circadian clock in the SCN and suggest a potential coordination of the actions of the circadian system and sleep centers in the regulation of the timing of sleep.

A major afferent pathway to the SCN originates in the intergeniculate leaflet and contains, among others, enkephalins, which are endogenous opioids. The opioid agonist fentanyl is known to induce phase shifts in wheel running activity rhythms. The experiments described in chapter 3 investigate the mechanisms by which opioids such as fentanyl affect the circadian clock in the hamster-SCN. The opioid antagonist naloxone blocks the occurrence of fentanyl-induced phase shifts, suggesting the involvement of true opioid receptors. Possibly, these opioid receptors are located within the SCN, as fentanyl is able to directly change SCN neuronal activity. Interestingly, the phase shifts induced by fentanyl can be blocked by simultaneous exposure to light. Vice versa, light-induced phase shifts and light-induced *Per1* clock gene expression are attenuated by fentanyl injection. These data indicate that light and opioids interact at the level of phase shifting mechanisms, which corresponds with the overlap of retinal and geniculate projections in the SCN. Phase shifts in behavioral activity rhythms, induced by several non-photic stimuli, have often been associated with a decrease of *Per1* and *Per2* expression levels in the SCN. Fentanyl administration does not change the expression level of *Per1* significantly, but an effect on *Per2* cannot be excluded. This indicates that for the induction of phase shifts, fentanyl affects the molecular clock at another

level than *Per1*. The data suggest that phase shifts may be induced via different intracellular pathways.

The aim of this thesis was to obtain insight in the hierarchical organization of the circadian timing system and to determine whether certain attributes of the system arise at the tissue level. Together, the data presented provide new information on the presence and significance of the interactions that exist within the circadian timing system, e.g. between components of the circadian pacemaker itself or between pacemakers and the periphery. Mechanistic insight is provided for the interaction between the dorsal and ventral regions of the mammalian SCN pacemaker, showing that the neurotransmitter GABA plays a key role in coupling these two SCN regions. The causal relationship between vigilance state and SCN neuronal activity suggests strong effects on the clock that were previously unknown. Also the opioid system is able to influence SCN neuronal activity and the phase shifting effects of fentanyl, as well as its effects on light-induced phase shifts and clock gene expression, provide additional insight in the influence of opioids on the circadian system and their interaction with the photic input of the clock. The potential strength of the influence of extra-SCN areas becomes clear from the ability of these regions to antagonize an initial phase shift of the SCN. The data show that the functioning of the circadian system as a whole is the result of communication between and within organizational levels. Another important finding of our studies is that the multiple components of the circadian system can, under certain circumstances, dissociate. For example, the phase resetting characteristics of the activity onset and offset in response to midazolam or fentanyl injection differ. Dissociation between the ventral and dorsal regions of the SCN occurs in response to a phase shift of the light-dark cycle and photic manipulation may also induce desynchrony between *Per1* expression, *in vitro* and *in vivo* SCN electrophysiology and behavior. Whereas the precise mechanisms involved in coupling remain to be elucidated, it can be assumed that they have great functional significance. Desynchronization between components of the circadian system may occur during jet lag, shift work and during adaptation to changing daylength. The hierarchical composition of the circadian timing system, the interactions within and between organizational levels, and importantly, the ability of several components to dissociate, may provide the circadian system the required plasticity to adjust to biologically relevant changes in the environment, but also keep it robust enough to remain unaffected by non-significant cues.