9

GENERAL DISCUSSION

LIGHT SIGNALING TO THE SCN

The suprachiasmatic nucleus (SCN) functions as a major circadian pacemaker and is of great importance for regulating rhythmicity in the brain and many peripheral organs. Light is the most important signal for the SCN through which it synchronizes to the external day and night cycle. In this thesis the light signaling pathway from ocular photoreceptors in the retina to the neurons within the SCN was investigated.

In the first part of this thesis the contribution of the various mouse photoreceptors and the effects of a broad range of wavelengths of light on the SCN was investigated (chapters 3, 4 and 5). A small population of specialized retinal ganglion cells that expresses the photopigment melanopsin plays a major role in regulating the effects of light [1, 2]. The melanopsin containing retinal ganglion cells are directly light responsive and mediate photic input to the SCN [3, 4]. These photosensitive retinal ganglion cells (pRGCs) are also innervated extrinsically by the indirect synaptic input from visual rod and cone photoreceptors of the outer retina [5-8]. This provides a retinal circuitry through which the visual photoreceptors can modulate the SCN [1, 2]. The role of melanopsin in circadian photoreception [9, 10] does not preclude a role for the classical rod and cone photoreceptors in regulating this response. There is in fact increasing evidence that rods and cones contribute to irradiance encoding tasks [11-16].

Light has excitatory effects on SCN electrical activity. Typically, light pulses produce transients corresponding to light "on" and "off" with a sustained response in between that depends on the level of illumination. These different components have been linked to differential rod/cone and melanopsin inputs, with sustained responses thought to arise from melanopsin and the transients from the rods/cones [17-19]. Collectively the data from both behavioral studies and SCN recordings have falsified this hypothesis and show a mixed and complex arrangement of photoreceptor inputs regulating the circadian system. Chapter 2 discusses the role of the various photoreceptors in the response characteristics of SCN neurons to light, as well as in photoentrainment.

Chapters 3, 4 and 5 of this thesis focus on the effects of monochromatic light on the circadian system. In contrast to humans, due to a lack of UV-absorbing pigments in the crystalline lens, the eye of several other mammalian species allows transmission of UV radiation to the retina including wavelengths as low as 300nm [20]. Further, UV light elicits phase shifts in locomotor activity [21-24], suppresses pineal melatonin production [25, 26, 27], induces c-fos expression in the SCN and enables entrainment of circadian rhythms to an light-dark (LD) cycle [28, 29]. The effect of UV light on the circadian system was investigated in chapter 3. The UV wavelength was chosen to maximally stimulate the ultraviolet sensitive cones while it is outside the maximum sensitivity range of the other mouse retinal photoreceptors. UV light exposure induced similar response

characteristics in SCN impulse frequency as white light. It was long thought that melanopsin is especially important for irradiance detection. To exclude a crucial role for melanopsin in mediating the UV-light response, the UV light-induced increase in SCN neuronal activity was also tested in mice lacking melanopsin, from here on called melanopsin-deficient mice. The response kinetics to UV light in the SCN of melanopsin-deficient mice were indistinguishable from the light-induced changes in impulse frequency in wild type mice. These findings indicate that irradiance encoding and tonic light-induced changes in impulse frequencies of SCN neurons are also possible in the absence of melanopsin, indicating a role for classical photoreceptors.

The fast initial increase in SCN neuronal activity upon UV light exposure is in agreement with cone-activation. Cones generally show a short response latency (30-60 ms)[5, 7] compared to rod-mediated responses (150ms)[7] or melanopsin-mediated responses (>300ms to minutes)[3, 4, 7]. This finding suggests a role for cones in the UV-light induced increases in SCN neuronal activity. The effects of UV light could also be mediated by a low level rod or mid-wavelength sensitive cone response, because these photoreceptors can also be excited by UV light outside their maximal sensitivity range. To explore whether the UV light response at the level of the SCN originates from UVS cones, the effect of UV light on SCN electrical activity was tested when it was superimposed on bright broad spectrum white background light. The white background light did not contain any UV and was used to saturate all classes of photoreceptors, including rods and mid-wavelength sensitive cones, except the UVS cones. Blue light in addition to the white background light failed to induce any further increment in SCN neuronal activity. Interestingly, UV light application led to higher levels of SCN neuronal impulse frequency and was sustained throughout light exposure. These findings are consistent with the view that the effects of UV light responses are mediated by UVS cones.

In chapter 3, we also explored the effects of UV light on sleep induction. No differences in initial sleep induction were reported between UV light and white light. More importantly, UV light was shown to be equally effective in inducing sleep in wild type mice and melanopsin-deficient mice. Interestingly, a recent study also describes normal sleep induction in melanopsin-deficient mice using white light. However, sleep in these mice is not maintained after the first hour [30, 31]. Additionally, melanopsin-deficient mice show reduced light-induced phase shifting responses, reduced masking and reduced period lengthening in constant light [10, 32]. These findings indicate a deficit in melanopsin-deficient mice to carry information on sustained illumination levels to non-image forming brain areas and suggest a role for melanopsin in mediating prolonged light information.

The functional relevance of the ability of the murine circadian system to detect UV light remains unclear. A possible role may arise from the spectral composition

of daylight. The ratio of UV light over longer wavelengths of light which enters the atmosphere is much higher during dawn and dusk compared to its value in daytime [29]. Mice could use UV light detection as a cue to detect time of day, or distinguish between dawn and dusk.

To determine the response of the SCN to longer wavelengths of light, similar *in vivo* electrophysiological SCN recordings were performed in response to blue (467nm) and green (505 nm) light and are discussed in chapter 4. In wild type mice blue and green light exposure led to increments in SCN neuronal activity that are indistinguishable from responses to white light, with fast transient and sustained components. To investigate whether melanopsin is required for this response, similar experiments were performed in melanopsin-deficient mice. In these mice we detected light responses in SCN neuronal discharge rates indistinguishable from those observed in wild-type mice, indicating an additional role for classical photoreceptors in mediating responses to longer wavelengths of light.

Recent studies emphasize the differences in response kinetics following photic stimulation of the outer versus inner retinal photoreceptors at the level of the retina: the melanopsin-based pRGC responses are typically sluggish and last for the duration of light exposure [3, 5, 7]. Rod/cone-mediated responses are fast and retain the capability to signal a tonic increase in firing rate at the level of the pRGCs during light exposure [8, 33]. These response characteristics have all been observed in light-evoked in vitro recordings obtained from pRGCs in wild-type mice and melanopsin-deficient mice [33], demonstrating the integration of rod/cone and melanopsin phototransduction pathways at the level of the pRGCs. The extracellular recordings of SCN neurons as well as the recordings at the level of the pRGCs show that input from rods and cones is sufficient to maintain irradiance detection across a broad range of wavelengths of light.

To unambiguously determine the response characteristics of melanopsin at the level of the SCN, *in vivo* electrophysiological recordings in rodless coneless mice were performed. These mice lack all rod and cone photoreceptors and have melanopsin containing pRGCs as the only functional photoreceptor in their retina. SCN neurons of these mice typically respond sluggish and we failed to detect light responses at lower light intensities. These mice are able to entrain to a light-dark cycle and show normal phase shifting responses to light.

To test the ability of cone photoreceptors to drive tonic light responses in the SCN we performed bleaching experiments in melanopsin-deficient mice. Rod photoreceptors were desensitized by 15-minute exposure to bright broad spectrum white light, while bright light exposure does desensitize cone photoreceptors to a lower extent [34]. After desensitization of rod photoreceptors, sustained increments in SCN neuronal activity in response to long wavelengths of light were not altered (Figure 1, van Diepen, unpublished). These findings support the hypothesis that cone photoreceptors are capable of driving steady light responses in the SCN.

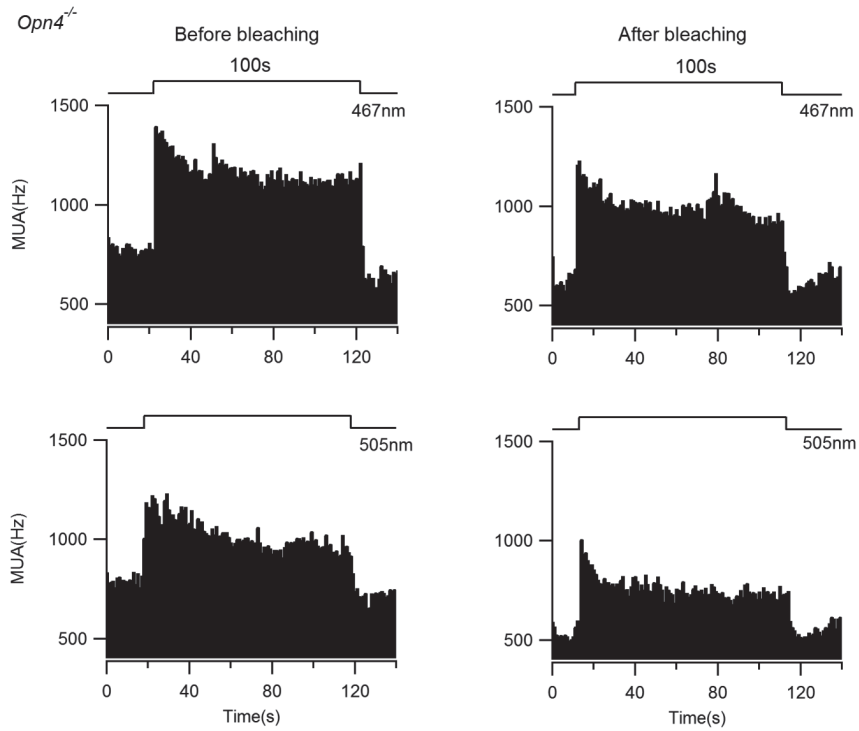

Figure 1. *In vivo* SCN electrical activity recordings of a melanopsin-deficient mouse in response to blue (λ_{max} 467 nm) and green (λ_{max} 505 nm) light. The 100s period of light exposure is plotted on top of the graph. Time is plotted in seconds on the x-axis and the frequency of SCN electrical activity on the y-axis. Left graphs show the response to light in SCN electrical activity during baseline recording. In the right graphs blue and green light exposure was preceded by desensitization of rod photoreceptors using a bleaching protocol with bright white broad spectrum light for 15 minutes.

To evaluate the contribution of cones to circadian phototransduction in more detail, behavioral and *in vivo* electrophysiological experiments were performed in mice having cones as the only photoreceptors in their retina. These transgenic mice lack melanopsin and critical elements of rod phototransduction cascades [14]. Some cone-only mice can entrain to a LD cycle to a degree that is indistinguishable as compared to wild-type mice, while others exhibit a large phase angle of entrainment or were not able to entrain to a LD cycle at all [14, 35], chapter 5). To explore the acute light input to the SCN in these mice, *in vivo* electrophysiological experiments were performed to measure light-induced increments in SCN neuronal discharge rates (chapter 5). Both transient and sustained increments in SCN neuronal activity were determined in response to 100 second light pulses of UV, blue and green light in cone-only mice. We observed an activation of SCN neuronal activity that lasted only for

about a minute in response to a 15-minute light pulse. The light-induced increases in SCN neuronal activity showed a larger sensitivity in response to UV light as compared to longer wavelengths of light. SCN neuronal discharge rates gradually returned to baseline during the 15 minute light exposure for all three wavelengths of light. These findings suggest that cones contribute to transmitting light information to the SCN at the beginning of light exposure. In line with these data Gooley and colleagues provided evidence that cones contribute to responses of the human circadian system to various wavelengths of light. In their study similar levels of melatonin suppression in response to long and short wavelength light are reported [15, 16]. It is shown that cones provide a substantial contribution to the first phase of light exposure, whereas the cone contribution decays during prolonged light exposure [15].

In the mouse retina most murine cones co-express UV-opsins and midwavelength (M)-opsins. The UV-opsins are widespread in the ventral retina, whereas the M-opsins are abundant in the dorsal retina [36, 37]. For the retina as a whole, UV-opsins outnumber the amount of M-opsins. Based on these findings the response of UV-opsins together is predicted to be larger than the response of the M-opsins. Recordings from cone photoreceptors reveal a maximum response to UV light [38]. The fact reported in chapter 5 that the light-induced increases in SCN neuronal activity in cone-only mice were lower in response to green light than to UV light, supports this finding.

Both mid-wavelength sensitive and UV sensitive cones in the outer retina induce sustained synaptic input to the pRGCs in the inner retina [39]. At the level of the cone-photoreceptor membrane, membrane currents from M-cones and UV-cones also respond in a sustained manner to steady light stimuli. The steady light stimuli range from 0.5 second up to 5 seconds [37, 38, 40]. This indicates that cone photoreceptors contribute to sustained light signaling for at least a few seconds.

An indication for the contribution of cones is also elucidated in a mouse model in which the mid-wavelength sensitive cone was replaced by the human red-cone opsin. Phase shifts are significantly larger when these mice are exposed to discontinuous red light as compared to exposure to series of brief red light pulses, revealing a role for cones in mediating the light-induced phase shifting capacity of the circadian pacemaker to long wavelengths of light [14]. This is consistent with the presence of light-induced phase shifting responses to light in a mouse model lacking mid-wavelength sensitive cones [11]. Phase shifts are reduced in response to long wavelengths of light, while these responses are unaffected after UV light exposure. In contrast, in a mouse model lacking UVS cones phase shifting responses are decreased after UV light exposure, while the size of the phase shift is unaffected in response to long wavelength light (Watson TS, unpublished data). *In vivo* electrophysiological data are consistent with these findings showing large increases in response to blue light in UVS knockout mice and only minor increments in SCN discharge rates during UV light exposure (van Diepen, unpublished data).

The functional role of rod photoreceptors in regulation of photoentrainment is examined in transgenic mice lacking critical elements of the rod and/or cone phototransduction pathway in their retina. Rods appear capable of driving photoentrainment through bipolar cells at low light intensities and through cone photoreceptors at high light intensities [12].

Together these studies reveal that melanopsin containing retinal ganglion cells, rod photoreceptors as well as cone photoreceptors are all sufficient and capable of driving photoentrainment, although some cone-only mice show difficulties to entrain. The broad spectral tuning of the various photoreceptors provides a circuit for optimal phototransduction to the SCN. Melanopsin is capable of phototransduction to the SCN and may be important for integration of light information over longer time periods. The experiments described in this thesis suggest that rods mainly contribute to photoentrainment and phototransduction to the SCN at lower and intermediate light intensities, while cone photoreceptors play a role in phototransduction to the SCN at higher light intensities and during the beginning of light exposure.

CAFFEINE AFFECTS LIGHT SENSITIVITY OF THE CIRCADIAN SYSTEM

The amount of light information reaching the SCN is not only determined by the direct light input pathway but is also affected at the level of the SCN by adenosine and vasoactive intestinal peptide (VIP) signaling. Therefore, in chapter 6 and 7 of this thesis the role of adenosine and VIP in light processing by the SCN was investigated.

Chapter 6 discusses the effect of caffeine and increased sleep pressure on light responsiveness of the SCN. Behavioral recordings revealed that the phase-shifting capacity of the circadian system in response to light is reduced after increased sleep pressure [41, 42]. To investigate the underlying mechanism, light-induced increases in SCN neuronal activity were determined in mice when sleep pressure was high. The light response in SCN neuronal activity was diminished during increased levels of sleep pressure and restored after administration of the adenosine receptor antagonist caffeine, indicating a role for adenosine in regulating light responsiveness of the SCN.

Caffeine is the most used stimulant to reduce sleepiness. However, the effects of caffeine on light responsiveness of the SCN are not known. Adenosine receptors are present at presynaptic nerve terminals in the SCN [43, 44]. Although the abundance of adenosine receptors on these nerve terminals is low, SCN neurons do respond to adenosine application. Chapter 6 provides evidence for a functional role of adenosine in light signaling to the SCN. Light signaling to the SCN is reduced after prolonged wakefulness when adenosine levels are high. The behavioral recordings

in constant light indicate that caffeine enhances light sensitivity of the circadian system. The current findings show that sleep deprivation affects the circadian system through an adenosine dependent pathway.

Another possible route could be formed by projections from the raphe nuclei to the SCN. This pathway is mainly inhibitory, and uses serotonin, NPY and GABA as transmitters. The raphe nuclei have direct projections to the SCN and have been implicated in sleep regulation [45]. Serotonin levels increase over the course of wakefulness and prolonged wakefulness and serotonin receptors are widely expressed in the SCN. Therefore, serotonin is a possible candidate in mediating the effect of increased sleep pressure on the SCN.

Caffeine was also reported to lengthen period and increase amplitude of the circadian system [46, 47]. Acute application of caffeine induces a phase delay in the rhythm of peripheral clocks [47]. Together, the findings indicate that caffeine exerts an acute effect on the circadian clock in addition to the effect on the SCN in combination with light.

Caffeine consumption also affects the human circadian system. The combination of caffeine consumption and light enhances alertness, reduces melatonin release and attenuates the drop in body temperature [48, 49]. In control conditions, the release of melatonin is suppressed by light. Caffeine enhances the effects of light on melatonin suppressions in humans. The effects of caffeine in humans are therefore similar to caffeine effects in mice. Our findings reveal a putative mechanism underlying the effects of caffeine in humans.

ROLE OF VIP IN LIGHT SIGNALING TO THE SCN

VIP-receptors play a major role in mediating photic responses of the SCN. Mice lacking VIP or its receptor do not respond with a shift in their behavioral activity pattern in response to light exposure, indicating a deficit in phototransduction to the circadian system [50, 51]. Furthermore, overexpression of VIP receptors in mice leads to enhanced light sensitivity and faster re-entrainment after a shift in the light-dark cycle [52]. These findings elucidate a role for VIP in processing light information by the SCN.

Chapter 7 describes the light transduction pathway within the SCN of VIP-deficient mice. The aim of this study was to identify the mechanism by which VIP processes light information. The light-responsiveness of SCN neurons was recorded using both *in vivo* and *in vitro* electrophysiological recordings techniques and light-induced gene expression. These recordings revealed that light information was transduced from the retina to the ventral SCN indistinguishable from wild type mice. Thus, the deficit in the phase shifting capacity of VIP-deficient mice does not originate upstream of the SCN. However, light-induced increases in calcium levels were attenuated in the dorsal SCN as well as the light-induced enhancements in

the clockgene Per1 expression. These findings indicate a deficit in light-signaling within the SCN from the ventral to dorsal which may explain the absence of light-induced phase shifts in VIP-deficient mice.

EXERCISE AND THE SCN

The circadian rhythm of many species responds to the activity levels of the individual. Thus, behavioral activity which is under the control of the SCN, in turn affects the SCN, causing a feedback loop. Behavioral activity causes acute suppressions in electrical activity of SCN neurons [53-55]. The effect of behavioral activity on SCN neuronal discharge rate is dependent on the intensity of behavior [53]. Chapter 8 discusses the hypothesis that enhanced levels of behavioral activity enhance the amplitude of the SCN waveform in electrical activity by induction of suppressions in SCN neuronal activity. To test this hypothesis, *in vivo* electrophysiological recordings of SCN neurons were conducted in the absence and the presence of a running wheel. The running wheel was used to induce voluntary exercise. Voluntary exercise indeed induced a 14% increase in the amplitude of the SCN waveform. While the increase in amplitude is relatively small, it may well be within a physiological range that is functionally significant. For instance, in aged animals, a severely disrupted circadian activity pattern is associated with a 50% decrease in amplitude [56]. The enhancement of the amplitude waveform may be the underlying mechanism of the effects of exercise on circadian rhythmicity. Exercise is also known to exert beneficial effects on the sleep-wake cycles in humans [57].

CONCLUDING REMARKS

The studies described in this thesis showed that all three classes of photoreceptors are involved in signaling light information to the SCN. The experiments revealed an important contribution of classical photoreceptors in phototransduction to the SCN both in response to short- and long wavelength light. Our findings provide evidence that the different classes of photoreceptors fulfill additive functions to circadian photoreception depending on the intensity and the wavelength of light. The transmission of photic information to the SCN is not only determined at the level of the photoreceptors in the retina, but can also be affected at the level of the SCN. We showed that high levels of sleep pressure attenuate light sensitivity of the circadian system. The light sensitivity can be restored by caffeine administration. Furthermore, we found an important role for the neurotransmitter VIP in transmitting photic information within the SCN. Taken together, the studies described in this thesis show that circadian photoreception is determined by photoreceptor signaling in the retina as well as by neurotransmitter signaling at the level of the SCN. In addition to light, behavioral activity can influence SCN

function. We demonstrated a beneficial influence of exercise on the amplitude of the SCN rhythm, rendering enhanced robustness of the circadian system. Exercise may be used as a non-invasive therapeutic strategy for circadian rhythm disorders.

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