Cover Page

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CAFFEINE INCREASES LIGHT RESPONSIVENESS OF THE MOUSE CIRCADIAN PACEMAKER

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ABSTRACT

Caffeine is the most used psychoactive stimulant worldwide. It reduces sleep and sleepiness by blocking access to the adenosine receptor. Adenosine increases during sleep deprivation and is thought to induce sleepiness and initiate sleep.

Light-induced phase shifts of the rest-activity circadian rhythms are mediated by light responsive neurons of the suprachiasmatic nucleus (SCN) of the hypothalamus, where the circadian clock of mammals resides. Previous studies showed that sleep deprivation reduces circadian clock phase shifting capacity and decreases SCN neuronal activity. In addition, application of adenosine agonists and antagonists mimic and block the effect of sleep deprivation on light-induced phase shifts in behaviour, suggesting a role for adenosine.

In the present study we examined the role of sleep deprivation and the effect of caffeine on light responsiveness of the SCN. We performed *in vivo* electrical activity recordings of the SCN in freely moving mice and showed that the sustained response to light in SCN neuronal activity was attenuated after a 6-hour sleep deprivation prior to light exposure. Subsequent i.p. application of caffeine was able to restore the response to light. Finally, we undertook behavioural recordings in constant conditions and found an enhanced period lengthening during chronic caffeine treatment in drinking water in constant light conditions.

The data suggest that increased homeostatic sleep pressure changes circadian pacemaker functioning by reducing SCN neuronal responsiveness to light. The electrophysiological and behavioural data together provide evidence that caffeine enhances clock sensitivity to light.

6

INTRODUCTION

Caffeine is the most used psychoactive stimulant to reduce sleepiness worldwide. It mainly acts as an adenosine receptor antagonist and disrupts sleep and increases alertness in many mammals [1]. Adenosine is one of the substances thought to be involved in the homeostatic regulation of sleep [2]. Its levels in the brain increase in the course of sleep deprivation and decrease during recovery sleep [3]. Recent evidence indicates that vigilance state changes affect neuronal activity in the suprachiasmatic nucleus (SCN), the location of the mammalian circadian clock. SCN neuronal activity is dependent on the arousal state of the animal and decreases in response to an increase in homeostatic sleep drive induced by sleep deprivation [4] [5, 6]. These results suggest that circadian clock functioning may be modified by an increase in homeostatic sleep drive and thus with changes in sleep pressure.

Light is the most important environmental cue for entrainment of the biological clock. Photic information reaches the SCN via the eyes through the retinohypothalamic tract (RHT). Retinal ganglion cells project via this monosynaptic pathway to the SCN. Following activation by light, glutamate is released at the nerve terminals [7, 8], which leads to an increase in SCN neuronal activity [9-16]. Application of glutamate mimics the effect of light on the SCN [7].

Light exposure at the beginning of the night delays the circadian rest-activity rhythm, and sleep deprivation attenuates this phase-shifting effect [17, 18]. Interestingly, administration of a selective A_1 adenosine receptor subtype agonist reduces the size of the phase shift induced by light similarly to sleep deprivation [19-21]. This effect is blocked by administration of an A_1 adenosine receptor antagonist [20, 21]. Administration of an A_1 adenosine receptor agonist decreased light-induced expression of c-Fos [19]. These findings suggest a role for adenosine in modulating the effect of sleep deprivation on the phase-shifting capacity of the circadian clock in response to light.

The current study focuses on the effects of disturbed sleep and caffeine on light responsiveness of the circadian system. We recorded SCN electrical activity in freely moving mice and examined the effect of sleep deprivation on the response of SCN neuronal activity to light pulses. To modulate this activity pharmacologically, we studied the effect of the non-selective adenosine receptor antagonist caffeine on the light-induced changes in SCN neuronal discharge after sleep deprivation. Finally we determined the effect of caffeine in the drinking water on changes in the endogenous period of the rest-activity rhythm in constant light and constant darkness.

METHODS

Animals

All animal experiments were approved by the Animal Experiments Ethical Committee of the Leiden University Medical Centre (the Netherlands). All

6

experiments were carried out in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes. Male C57/Bl6 mice (Harlan, Horst, the Netherlands, age 3-12 months) were used for behavioural experiments and to perform *in vivo* electrophysiological recordings of the SCN. Mice were housed individually and food and water were available *ad libitum*. Ambient room temperature was maintained at 20 ± 2 ˚C.

Drug preparation
Caffeine (LUMC pharmacy) was dissolved in drinking water at a concentration of 0.8 mg/ml (0.08%). This concentration was found to have significant effects on circadian activity [22] and is equivalent to the caffeine concentration in ordinary drip coffee. During *in vivo* electrophysiological experiments, caffeine was injected at a concentration of 15 mg/kg. This concentration was shown to significantly increase waking in the following hours [23, 24] and is equivalent to ± 3 cups of coffee in humans [25].

Behavioural activity
Mice were individually housed in cages containing either a plexiglass running wheel (diameter 24 cm) or a passive infrared motion detector (PIR) (Hugrosens Instruments, Löffingen, Germany). PIRs and sensors on the running wheels were connected to a ClockLab data collection system (Actimetrics, IL, USA). The amount of wheel rotations were measured and stored on a computer in 1-minute bins. Animal care and experimental procedures were carried out under dim red light conditions.

Sleep deprivation
Sleep deprivation was performed during behaviour experiment 1 and during the electrophysiological recordings. During sleep deprivation, animals were kept awake by the researcher. Every time the animals appeared drowsy they were mildly disturbed by noise, movement of bedding or introducing new nesting material or food into the cage [26, 27]. As a consequence the animals showed low levels of behavioural activity such as grooming and low levels of locomotor activity throughout the sleep deprivation procedure.

Behaviour experiment 1
Mice (n=8) were housed with running wheels in a light-dark cycle of 12 hours light and 12 hours darkness (LD). After at least 10 days in LD, mice were released in constant darkness (DD). On the second day in DD the mice were either exposed a 15-min light pulse at Circadian Time (CT) 14, were sleep deprived between CT8 and CT14 or were sleep deprived between CT8 and CT 14 and exposed to a 15-min light pulse at CT14. The three conditions were applied in a randomized cross-over design. CT14 is two hours after the start of the active period and the time point

where the largest phase delay can be expected [28]. Running wheel activity was recorded throughout the entire experiment.

Behaviour experiment 2

Mice (n=17) were housed with PIRs in a LD cycle of 12 hours light and 12 hours darkness. After at least 10 days in LD, mice were released in either DD (n=9) or constant light (LL, n=8). Between the 10th and the 20th day in these conditions the mice received caffeine (0.08%) in their drinking water.

In vivo electrophysiology

Under deep anaesthesia (ketamine, 100 mg/kg; xylazine, 20 mg/kg; atropine 1 mg/kg) a tripolar stainless steel electrode (PlasticsOne, US) was implanted aimed at the SCN of a mouse using stereotaxic equipment with a digital readout (Stoelting). Two twisted polyimide-insulated electrodes were aimed at the SCN for differential recording of neurons and a third reference electrode was placed in the cortex. The electrodes were implanted under a 5 degree angle using the following coordinates; 0.61 mm lateral from Bregma and 5.38 mm ventral to the dura. After surgery, the mice were allowed to recover for a week. The mice were then placed in a custom-designed recording chamber to measure SCN electrical activity. In the recording chamber the animals were connected to a flexible cable and a counterbalanced swivel system. The electrical signal was amplified and bandwidth filtered (0.5-5 kHz). Window discriminators were used to convert action potentials in digital pulses, which were stored for off-line analysis in 2-s or 10-s epochs (CircaV1.9 custom-made software). During the recording the animal was able to move freely. The movement of the animal was recorded by PIR detectors. All data was stored for offline analysis.

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In vivo electrophysiology experiment 1

After connection to the recording chamber, the mice (n=11) were released in DD. Sleep deprivation was performed during the in vivo electrophysiological recording within the 3rd and the 7th day in DD between CT8 and CT14.5. During sleep deprivation, animals were kept awake by the researcher. Every time the animals appeared drowsy they were mildly disturbed by noise, movement of bedding or introducing new nesting material or food into the cage. Between CT13.5 and CT14.5 the animals were exposed six times to 5-min light pulses (light source; incandescent light, light intensity; 76 μW/cm^2) with intervals of 10 minutes. In the control condition, the mice were exposed to similar light pulses between CT13.5 and CT14.5 without prior sleep deprivation. The conditions were applied in a randomized cross-over design. During light exposure in both conditions, the animals were closely observed and were kept awake by mild disturbance when necessary to ensure that in each condition the animals were exposed to the same amount of light.

In vivo electrophysiology experiment 2

After connection to the recording chamber, mice (n=6) were released in DD. Sleep deprivation was performed between CT8 and CT14.5 within the 3rd and the 7th day in DD. At the end of the sleep deprivation the animals were exposed to three blocks of three 5-min light pulses. After the first block of light pulses the animals received a control intraperitoneal (i.p.) injection of 0.9% saline. Twenty to thirty minutes after the injection with saline the animals were again exposed to three blocks of three light-pulses of 5 minutes with intervals of 10 minutes. After the last light pulse the animals received an i.p. injection of caffeine (15 mg/kg) and 20-30 minutes later the animals were exposed to the third block of three 5 minutes light pulses. Injection volume was 0.2 ml in both conditions.

Histology

After the recording the animals were culled using a CO_2 chamber and a small electrolytic current was passed through the two twisted electrodes to mark their position. The brains were collected and kept in a 4% paraformaldehyde solution containing ferrocyanide to fixate the brain tissue and to stain the recording site. After fixation the brain was sectioned coronally and stained with cresyl violet. The marked position of the electrode was verified by microscopic inspection.

Statistical analysis

Statistical analyses were carried out using SPSS (version 20) or GraphPad Prism. Repeated measurements ANOVA, paired t-tests and a linear mixed model with compound symmetry covariance structure and paired t-tests were performed to determine significant differences.

F-values, t-values and P-values are reported for each statistical test. P-values below 0.05 were considered to be significant. For the electrophysiological data the average of six (experiment 1) or three (experiment 2) light pulses was used as one datapoint per animal to decrease the variation.

RESULTS

Sleep deprivation and behaviour

The effect of sleep deprivation on phase shifting capacity was determined by wheel running activity recordings (Fig 1A and B). Light exposure of 15 minutes at CT14 induced a delay in behavioural activity of 1.7 ± 0.2 hours. When the mice were sleep deprived for 6 hours between CT8 and CT14 prior to light exposure, the size of the phase delay was significantly reduced by 0.6 ± 0.2 hours (P=0.04, t=2.584, df=7, paired t-test) (Fig 1C). Sleep deprivation alone did induce a phase shift of 0.71 ± 0.2 hours.
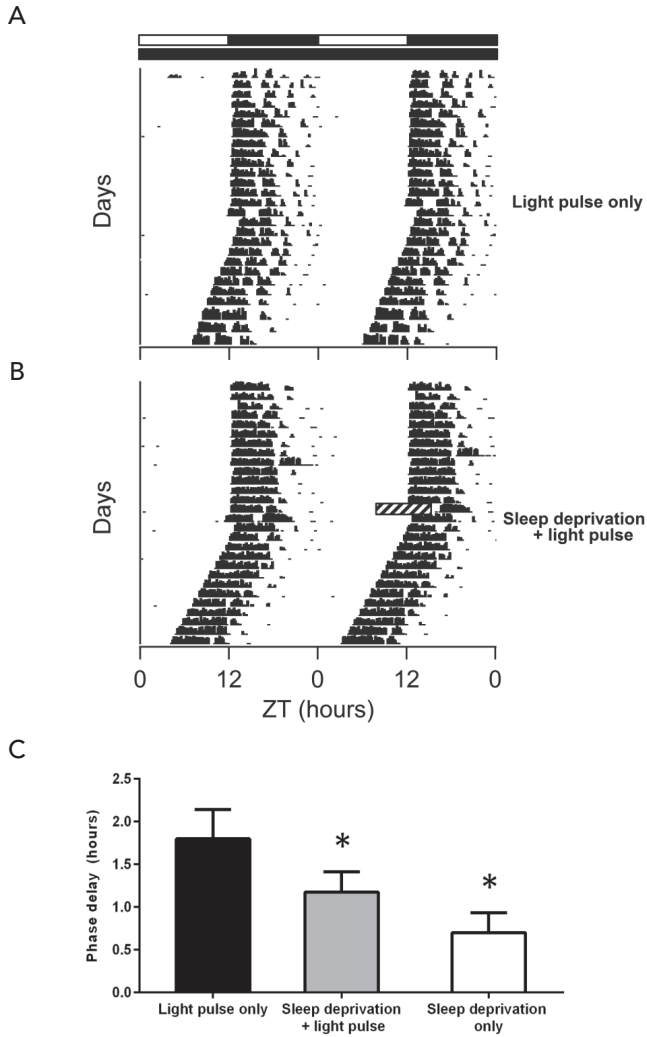
Figure 1. Two representative double plotted actograms of mice demonstrating the phase-shifting response of running wheel activity to a 15-min light pulse applied at CT14 on the second day in constant darkness (DD). Mice were either kept in DD (A) or sleep deprived in DD for 6 hours (B) prior to light exposure. The light regime is plotted as bars above the actograms. The striped area in B reflects the period of sleep deprivation (not double plotted). C. Mean phase shifts ± SEM of wheel running activity of mice (n=6) in response to a light pulse with (grey bar) and without (black bar) prior sleep deprivation. * indicates $P<0.05$, paired t-test. Light exposure preceded by sleep deprivation resulted in a significantly smaller phase shift.

Sleep deprivation and SCN electrophysiology

We assessed the effect of sleep deprivation on SCN neuronal activity by *in vivo* electrophysiological multiunit activity (MUA) recordings of the SCN in freely moving mice. The location of the electrode was verified by histology (Fig 2A). Successful recordings were obtained in 11 mice. In these recordings, SCN electrical discharge rates were high during the day and low during the night. All mice showed increased SCN neuronal activity in response to light (Fig 2B and D). The difference between baseline MUA frequency and the MUA frequency during light exposure at CT14 was 408 ± 119 Hz (Fig 2D). After sleep depriving the mice between CT8 and CT14, the light-induced increase in SCN electrical discharge rate was significantly reduced to 208 ± 118 Hz (*P*=0.03, *t*=2.456, *df*=10, paired *t*-test) (Fig 2C and D).

Caffeine and SCN electrophysiology

To investigate the putative role of adenosine in the reduced response to light in SCN neuronal activity after sleep deprivation, we combined sleep deprivation with administration of saline and caffeine during SCN *in vivo* electrophysiological recordings. Intraperitoneal injection of saline after sleep deprivation did not alter the response in SCN neuronal activity to light exposure (*P*=0.70, *t*=0.412, ratio paired t-test) (Fig 3A and B). Administration of caffeine after sleep deprivation led to a significant larger increase in SCN neuronal activity in response to light compared to the light-induced increase after saline injection (*P*=0.004, *t*=5.097, *df*=6, ratio paired *t*-test after significant repeated measurements ANOVA *P*=0.046, F=4.66, *df*=1,7;).
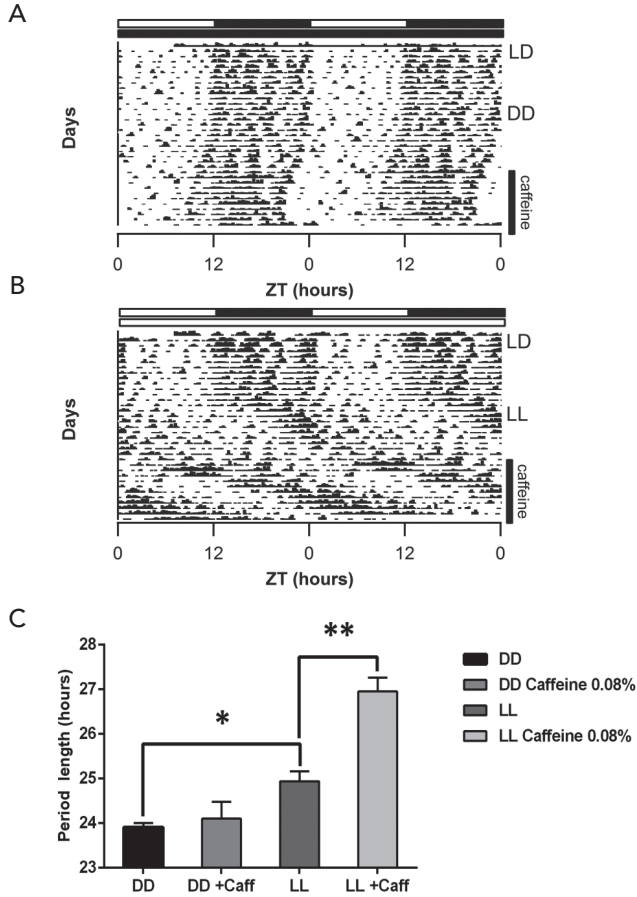
Caffeine and behaviour

A linear mixed model was made with light, caffeine and their interaction as predictors and period length and behavioural activity as outcome. Behavioural activity was decreased during LL (LD; 19150 beam breaks/24h LL; 13398 beam breaks/24h, *P*<0.001). Caffeine did not significantly change the amount of behavioural activity (LL and caffeine; 14732 beam breaks/24h, *P*=0.07). Overall, the mice exposed to LL had a period that was approximately 2.3 hours longer than the mice in DD (*P*<0.001). The effect of caffeine was highly significant, caffeine lengthened the period by about 1.6 hours on average (*P*<0.001). The lengthening of the period in LL by caffeine was about 1.3 hours more than the lengthening in DD (*P*=0.002). Paired t-tests were performed to study the effects of caffeine in LL and in DD separately. In DD, caffeine treatment induced a slight increase in period in DD, but this was not significant (*P*=0.09, *t*=-1.970, *df*=7, paired *t*-test). In contrast the period length was significantly longer in LL during caffeine treatment compared to LL control (*P*=0.01, *t*=3.236, paired *t*-test).
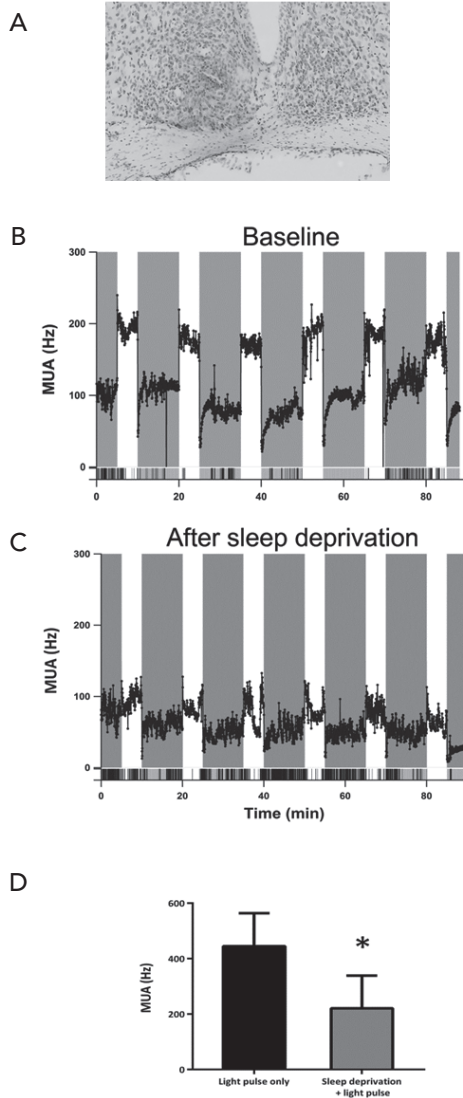
Figure 2. A. An example of a coronal slice of the mouse brain with the SCN right above the optic chiasm at the base of the hypothalamus. The location of the electrode can be verified by the blue spot which is marked using an electrolytic current. B and C. Responses in SCN electrical activity to light at CT 15 in freely moving animals in the control condition (B) or after sleep deprivation in DD for 6 hours (C). Mice were repeatedly exposed a 5-min light pulse. The presence of light is indicated as a white background in the graphs. Time is plotted in minutes on the x-axis and the frequency of electrical activity of SCN neurons is plotted on the y-axis. Black vertical lines at the bottom of each graph indicate passive infrared recorded locomotor activity in the cage. D. Mean light-induced increase in SCN neuronal activity ± SEM in control condition or after a 6-hour sleep deprivation (n=11). * indicates $P<0.05$, two-tailed paired t-test. Sleep deprivation prior to light exposure significantly attenuates the light-induced increase in SCN neuronal activity in response to light.
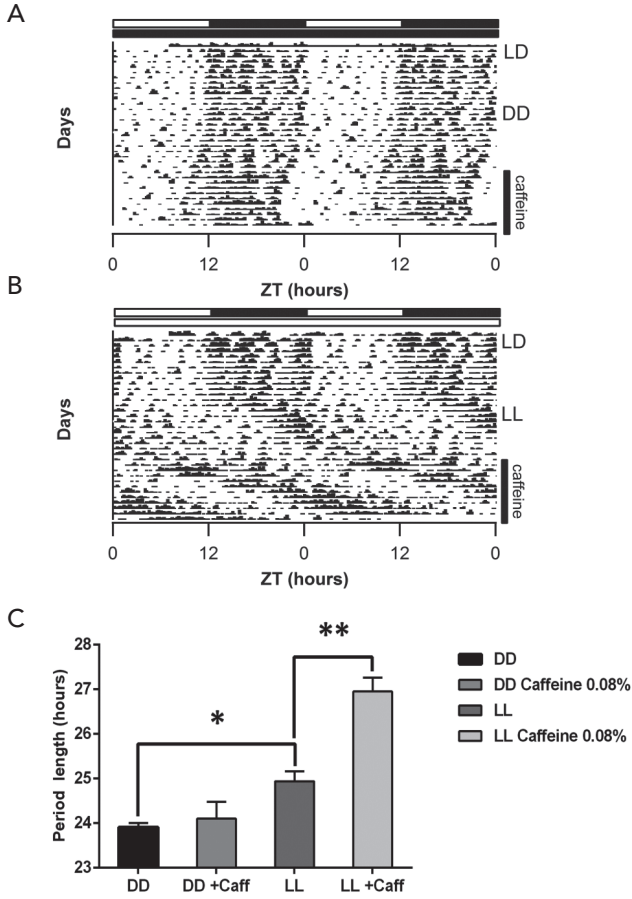
Figure 3. A. Two examples of responses to light in SCN neuronal activity after a 6-hour sleep deprivation. Mice were exposed to 5-min light pulses. In the right graphs light exposure was preceded by intraperitoneal (i.p.) injection of caffeine (15 mg/kg). Light exposure is indicated by a white background in the graphs. Time is plotted in minutes on the x-axis and the frequency of SCN electrical activity is plotted on the y-axis. Black vertical lines at the bottom of each graph indicate passive infrared recorded locomotor activity in the cage. B. Mean light-induced increases in SCN neuronal activity ± SEM after sleep deprivation are summarized (n=6). Mice received either no injection, i.p. injections of saline, or caffeine (15 mg/kg) followed by exposure to 5-min light-pulses. ** indicates $P<0.01$, ratio paired t-tests (P=0.004, t=0.4125 saline versus caffeine, P=0.697, t=5.097 sleep deprivation versus saline). Caffeine significantly enhances light-induced increases in SCN neuronal activity after sleep deprivation.

Figure 4. Double-plotted actograms of behavioural activity of mice demonstrating the effect of caffeine on free running periods in constant darkness (A) and constant light (B). The light regime is plotted as bars above the actograms. Zeitgeber Time (ZT) is plotted in hours on the x-axis and consecutive days are plotted on the y-axis. Mice were released in either DD (A) or LL (B). After ten days mice received caffeine (0.08%) via their drinking water. C. Mean period length ± SEM is shown in DD (n=8) with and without caffeine and in LL (n=9) with and without caffeine. * indicates $P<0.05$, ** indicates $P<0.01$, mixed model factor light and caffeine, unpaired t-test. Administration of caffeine resulted in period lengthening in both DD and LL. The period lengthening as a result of caffeine administration was significantly larger in LL.

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DISCUSSION

Our findings show that caffeine increases light responsiveness measured at the level of neuronal activity of the SCN as well as at the level of behavioural activity. Similar to previous studies, we report a reduction in light-induced phase delays in behavioural activity in sleep deprived mice [17, 18]. The current study elucidates a mechanism underlying this effect at the level of the SCN. Light-induced increases in SCN neuronal activity are attenuated after sleep deprivation. We demonstrate that this attenuation was restored after peripheral injection of caffeine. We undertook behavioural recordings in constant light and found an enhanced sensitivity of the circadian system to light in combination with chronic caffeine treatment. Hence, the current report provides evidence for the involvement of adenosine and adenosine receptors in modulating the light sensitivity of the circadian system.

Both in the baseline condition and after sleep deprivation the data support the notion that caffeine increases light sensitivity of the circadian system. Aschoff's rule dictates that increasing light intensity in LL conditions lengthens the circadian period of the endogenous circadian clock in nocturnal animals [29]. Chronic caffeine consumption in DD significantly lengthened the circadian period in behavioural activity in mice by approx. 25 min [22]. In the present study we found a similar lengthening, but this did not reach significance. In addition, we show that caffeine significantly increases the period of the circadian clock in LL by 95 minutes. This is more than can be expected on the basis of the effect of caffeine alone on the endogenous period in DD. Caffeine did not significantly change the amount of activity, but there was a trend towards increased activity during caffeine consumption. Increased activity can influence the period of the rest-activity rhythm but is normally associated with shorter periods [30-34]. The data suggest that the chronic caffeine consumption via the drinking water increased the sensitivity of the endogenous circadian clock to light, and in accordance lengthens the period of the clock.

In line with this, caffeine appeared to strengthen the effect of light on the circadian clock on the neuronal level. We confirmed that sleep deprivation attenuates phase delays in behavioural activity [17, 18] and showed in addition that light-induced increases in SCN neuronal activity are attenuated after sleep deprivation. It has been shown previously that application of an adenosine agonist attenuates the light-induced phase delays in behaviour, which are restored by an adenosine receptor antagonist [20, 21]. In contrast, a recent report showed that caffeine administration blocks light-induced phase delays, however, the dose applied was three times larger and activity of the animals was very much reduced under these conditions [35]. We found that at the level of the SCN, caffeine restored light responses. These findings show that enhanced phase shifts may have been achieved by an action of the adenosine antagonist on the SCN itself and may provide a basis to boost (re-)synchronization to a light-dark cycle.

The question remains via which mechanism caffeine restores light responsiveness of the SCN. The choice for caffeine was reinforced by the preponderant use of caffeine in current society. Caffeine is an antagonist of the adenosine A_1 and A_{2A} receptors, the two major adenosine receptors in the central nervous system [1]. The density of adenosine receptors in the central nervous system is under circadian control [36]. From the present results we cannot distinguish where between the retina and the SCN caffeine influences light sensitivity of the circadian system. In the retina adenosine receptors and ryanodine receptors (RyRs), caffeine-sensitive Ca^{2+} release channels [37], are present and can be affected by caffeine [38] [39, 40]. However, whether adenosine levels in the retina change under influence of sleep deprivation is unknown. Only a few studies have reported the expression of adenosine receptors in the SCN [41, 42], which turned out to be low. Despite the modest abundance of adenosine receptors, a high percentage (70%) of the SCN neurons respond to adenosine application [42, 43]. This indicates that the low density of adenosine receptors in the SCN does not preclude a functional role for these receptors. In various brain regions, stimulation of the A_1 adenosine receptor reduces the amplitude of excitatory postsynaptic currents and depresses glutamate release [44-48]. Previous studies demonstrated that adenosine decreases intracellular Ca^{2+} levels and electrical activity via glutamatergic neurotransmission [42, 43]. Electrical activity of SCN neurons was blocked by adenosine A_1 receptor agonists and this effect was reversed by adenosine A_1 receptor antagonists. Selective adenosine A_{2A} receptor antagonists did not affect excitatory currents [41]. Therefore, transmission of light through the optic nerve to SCN neurons is likely mediated via adenosine A_1 receptors present at presynaptic nerve terminals in the SCN [41].

Aforementioned studies provide evidence for a presynaptic influence of adenosine on glutamate release to the SCN. In our study we blocked the effect of adenosine by application of caffeine and restored the light-induced increase in SCN neuronal activity. A likely mechanism underlying this effect is that caffeine increases the influence of light to the SCN by blocking presynaptic A_1 adenosine receptors on the RHT and thereby reducing the inhibition of glutamate release to the SCN by adenosine. Another possible mechanism through which caffeine can exert its effects on light responsiveness of SCN neurons might be via activating RyRs, which are present in the SCN [49]. Caffeine-mediated increases in calcium levels in SCN neurons were shown to be dependent on activation of RyRs [50]. Application of caffeine caused light-like phase shifts of the SCN in mouse brain slices, similar to phase-shifts induced by glutamate. These shifts were fully blocked by pre-incubation with RyR-blockers, which indicates a role for RyRs in regulating the light-like phase shifting effect of caffeine [51]. However, the concentrations needed to activate the RyR *in vivo* are most likely not reached by peripheral administration of caffeine. Therefore we hypothesize a role for adenosine receptors, in particular A_1 receptors, in mediating the effects reported in our study.

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Several studies in humans report effects of caffeine consumption on the circadian system. Melatonin release and body temperature are both used as markers of circadian phase in humans. Caffeine ingestion suppresses melatonin release and attenuates the normal decrease in body temperature in the evening [52]. The combination of caffeine and light was shown to be even more effective in enhancing performance, reducing melatonin release and attenuating the drop in body temperature [52, 53] suggesting that caffeine also increases light sensitivity in humans. Whether caffeine can change the endogenous period or the phase-shifting effects of light in humans remains to be determined.

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6

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6