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CHAPTER 5

Caffeine increases light responsiveness of the circadian pacemaker

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Abstract

Caffeine is the most commonly used psychoactive stimulant worldwide. It reduces sleep and sleepiness by blocking access to the adenosine receptor. The level of 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 have shown that sleep deprivation reduces circadian clock phase-shifting capacity and decreases SCN neuronal activity. In addition, application of adenosine agonists and antagonists mimics and blocks, respectively, the effect of sleep deprivation on light-induced phase shifts in behavior, suggesting a role for adenosine. In the present study, we examined the role of sleep deprivation in 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 of SCN neuronal activity was attenuated after 6 hrs of sleep deprivation prior to light exposure. Subsequent intraperitoneal application of caffeine was able to restore the response to light. Finally, we performed behavioral recordings in constant conditions, and found enhanced period lengthening during chronic treatment with caffeine 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 behavioral data together provide evidence that caffeine enhances clock sensitivity to light.

Introduction

Caffeine is the psychoactive stimulant that is most commonly used 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.⁴⁻⁶ These results suggest that circadian clock functioning may be modified by an increase in homeostatic sleep drive and thus by changes in sleep pressure. Light is the most important environmental cue for entrainment of the biological clock. Photoc information reaches the SCN via the eyes through the retinohypothalamic tract. Retinal ganglion cells project via this monosynaptic pathway to the SCN. Following activation by light, glutamate is released at the nerve terminals,^{7,8} leading to an increase in SCN neuronal activity.⁹⁻¹⁶ Application of glutamate mimics the effect of light on the SCN.⁸ Light exposure at the beginning of the night delays the circadian rest–activity rhythm, and sleep deprivation attenuates this phaseshifting effect.^{17,18} Interestingly, administration of a selective A1 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 A1 adenosine receptor antagonist.^{20,21} Administration of an A1 adenosine receptor agonist decreased light-induced expression of c-Fos. 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 focused on the effects of disturbed sleep and caffeine on the 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 (LL) and constant darkness (DD).



Materials and methods

Animals

All animal experiments were approved by the Animal Experiments Ethical Committee of the Leiden University Medical Centre (The Netherlands). All experiments were carried out in accordance with 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 behavioral experiments and for *in vivo* electrophysiological recordings of the SCN. Mice were housed individually, and food and water were available ad libitum. The 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 approximately three cups of coffee in humans.¹

Behavioral activity

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

Sleep deprivation

Sleep deprivation was performed during behavioral experiment 1 and during the electrophysiological recordings. During sleep deprivation, mice were kept awake by the researcher. Every time that the mice appeared drowsy, they were mildly disturbed by noise, movement of bedding, or the introduction of new nesting material or food into the cage.^{25,26} As a consequence, the mice showed low levels of behavioral activity such as grooming and low levels of locomotor activity throughout the sleep deprivation procedure.

Behavioral experiment 1

Mice ($n = 8$) were housed with running wheels in a light–dark (LD) cycle of 12 hrs of light and 12 hrs of darkness. After at least 10 days in LD, mice were placed in DD. On the second day in DD, the mice were either exposed to a 15-min light pulse at circadian time (CT) 14, were sleep-deprived between CT8 and CT14, or were sleepdeprived between CT8 and CT14 and exposed to a 15-min light pulse at CT14. The three conditions were applied in a randomized cross-over design. CT14 is 2 hrs after the start of the active period and the time point at which the largest phase delay can be expected.²⁷ Running wheel activity was recorded throughout the entire experiment.

Behavioral experiment 2

Mice ($n = 17$) were housed with PIR detectors in an LD cycle of 12 hrs of light and 12 hrs of darkness. After at least 10 days in LD, mice were placed in either DD ($n = 9$) or LL ($n = 8$). Between the 10th day 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, USA) was implanted, aimed at the SCN of a mouse by the use of 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 at a 5° angle with the following coordinates: 0.61 mm lateral from Bregma and 5.38 mm ventral to the dura. After at least a week of recovery, the mice were placed in a custom-designed recording chamber for measurement of SCN electrical activity. In the recording chamber, the mice 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 into digital pulses, which were stored for offline analysis in 2-s or 10-s epochs (CIRCAV1.9 custom-made software). During the recording, the mouse was able to move freely. The movement of the mouse was recorded by PIR detectors. All data were stored for offline analysis.

In vivo electrophysiology experiment 1

After connection to the recording chamber, the mice ($n = 11$) were placed in DD. Sleep deprivation was performed during the *in vivo* electrophysiological recording between CT8 and CT14.5, on the third and seventh days in DD. During sleep deprivation, mice were kept awake by the researcher. Every time that the mice appeared drowsy, they were mildly disturbed by noise, movement of bedding, or the introduction of new nesting material or food into the cage. Between CT13.5 and CT14.5, the mice were exposed six times to a 5-min light pulse (light source, incandescent light; light intensity, 76 lW/cm²) with intervals of 10 min. It is well known that light exposure induces sleep,²⁸⁻³² so we verified that the mice were all active and kept their eyes open during light exposure. 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 mice were closely observed, and were kept awake by mild disturbance when necessary to ensure that, in each condition, the mice were exposed to the same amount of light.



In vivo electrophysiology experiment 2

After connection to the recording chamber, mice ($n = 6$) were placed in DD. Sleep deprivation was performed between CT8 and CT14.5, on the third and seventh days in DD. At the end of the sleep deprivation, the mice were exposed to three blocks of three 5-min light pulses. We verified that the mice were active and kept their eyes open during and between light exposures. After the first block of light pulses, the mice received a control intraperitoneal injection of 0.9% saline. Twenty to thirty minutes after the saline injection, the mice were exposed to the second set of three 5-min light pulses with intervals of 10 min between the light pulses. After the last light pulse, the mice received an intraperitoneal injection of caffeine (15 mg/kg), and 20–30 min later the mice were exposed to the third block of three 5-min light pulses. The injection volume was 0.2 mL in both conditions. **Histology** After the recording, the mice were killed in a CO₂ chamber, and a small electrolytic current was passed through the two twisted electrodes to mark their positions. The brains were collected and kept in a 4% paraformaldehyde solution containing ferrocyanide to fix 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 performed with 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 of < 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 mouse to decrease the variation.

Results

Sleep deprivation and behavior

The effect of sleep deprivation on phase-shifting capacity was determined from wheel-running activity recordings (**Fig. 1A-B**). Light exposure for 15 min at CT14 induced a delay in behavioral activity of 1.7 ± 0.2 hrs. When the mice were sleep-deprived for 6 hrs between CT8 and CT14 prior to light exposure, the size of the phase delay was significantly reduced, by 0.6 ± 0.2 hrs ($P = 0.04$, $t = 2.584$, degrees of freedom (d.f.) = 7, paired t -test, **Fig. 1C**). Sleep deprivation alone induced a phase shift of 0.71 ± 0.2 hrs.

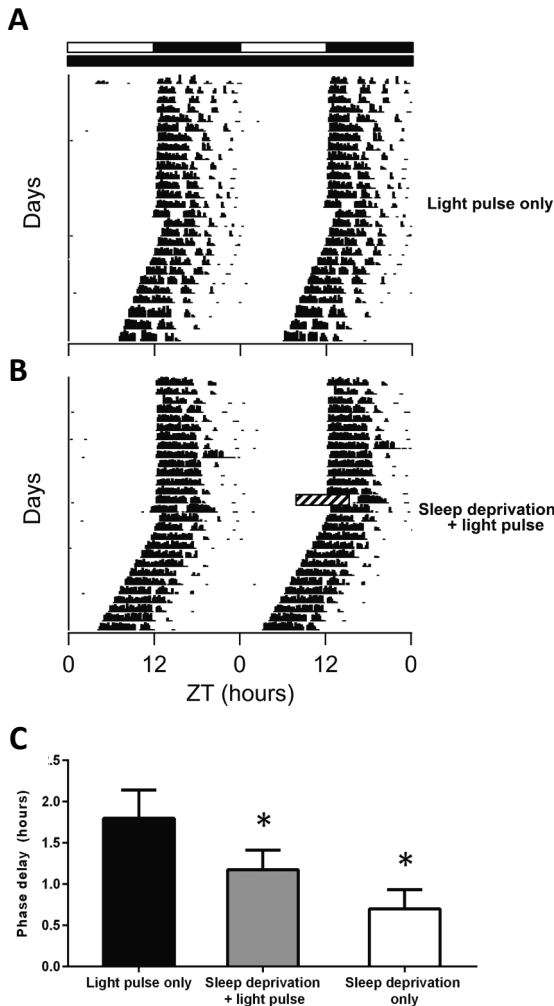


Fig. 1. (A-B) Two representative double-plotted actograms of mice demonstrating the phase-shifting response of running-wheel activity to a 15-min light pulse applied at CT14 on the second day in DD. Mice were either kept in DD (A) or sleep-deprived in DD for 6 hrs (B) prior to light exposure. The light 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 \pm standard error of the mean of wheel-running activity of mice ($n = 6$) in response to a light pulse with (grey bar) and without (black bar) prior sleep deprivation; the white bar shows the magnitude of the phase shift in response to sleep deprivation alone, without light exposure. * $P < 0.05$, paired t -test. Light exposure preceded by sleep deprivation resulted in a significantly smaller phase shift. ZT, zeitgeber time.



Sleep deprivation and SCN electrophysiology

We assessed the effect of sleep deprivation on SCN neuronal activity from *in vivo* electrophysiological multi-unit 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-D). The difference between baseline MUA frequency and MUA frequency during light exposure at CT14 was 408 ± 119 Hz (Fig. 2D). After the mice had been sleep-deprived between CT8 and CT14, the light-induced increase in SCN electrical discharge rate was significantly reduced, to 208 ± 118 Hz ($P = 0.03$, $t = 2.456$, d.f. = 10, paired t -test) (Fig. 2C and D).

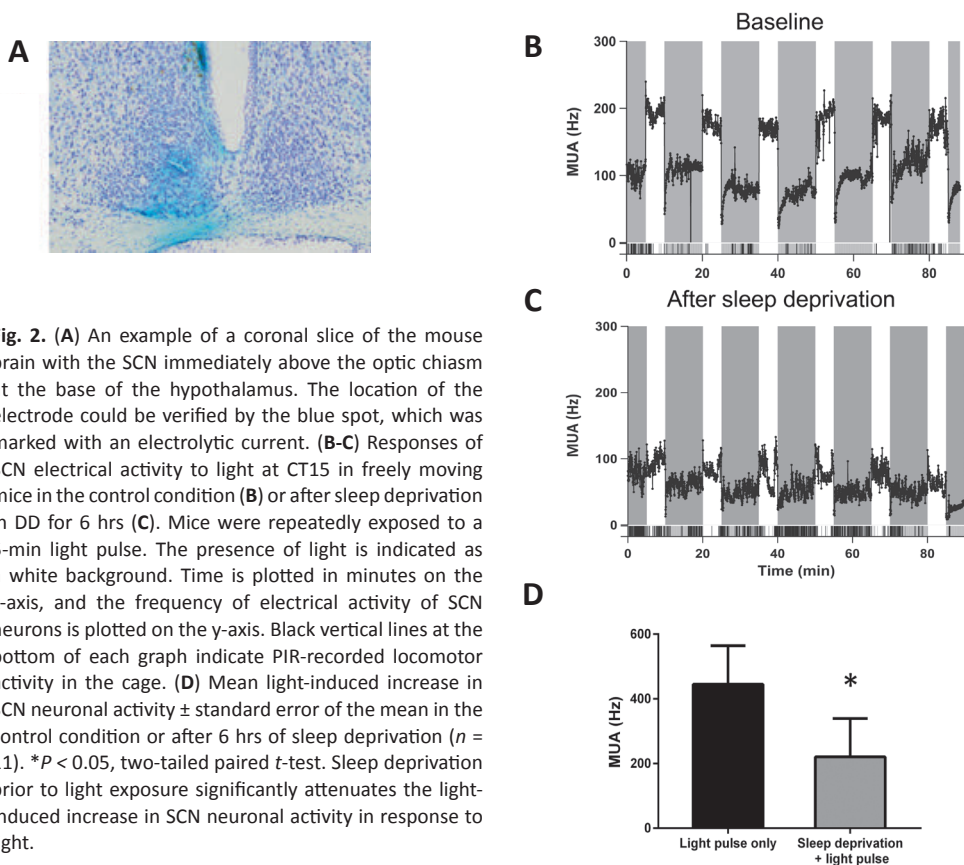


Fig. 2. (A) An example of a coronal slice of the mouse brain with the SCN immediately above the optic chiasm at the base of the hypothalamus. The location of the electrode could be verified by the blue spot, which was marked with an electrolytic current. (B-C) Responses of SCN electrical activity to light at CT15 in freely moving mice in the control condition (B) or after sleep deprivation in DD for 6 hrs (C). Mice were repeatedly exposed to a 5-min light pulse. The presence of light is indicated as a white background. 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 PIR-recorded locomotor activity in the cage. (D) Mean light-induced increase in SCN neuronal activity \pm standard error of the mean in the control condition or after 6 hrs of sleep deprivation ($n = 11$). * $P < 0.05$, 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.

Caffeine and SCN electrophysiology

To investigate the putative role of adenosine in the reduced response to light of 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 of SCN neuronal activity to light exposure ($P = 0.70$, $t = 0.412$, ratio paired t -test) (Fig. 3A-B). Administration of caffeine after sleep deprivation led to a significantly larger increase in SCN neuronal activity in response to light than the light-induced increase after saline injection ($P = 0.004$, $t = 5.097$, d.f. = 6, ratio paired t -test after significant repeated measurements ANOVA; $P = 0.046$, $F = 4.66$, d.f. = 5).

Caffeine and behavior

A linear mixed model was created with light, caffeine and the interaction between the two as predictors, and period length and behavioral activity as outcomes. Behavioral activity was decreased during LL (LD: 19,150 counts/24 hrs; LL: 13,398 counts/24 hrs; $P < 0.001$).

Caffeine did not significantly change the amount of behavioral activity (LL and caffeine: 14732 counts/24 hrs; $P = 0.07$). Overall, the mice exposed to LL had a period that was ~ 2.3 hrs longer than that of the mice in DD ($P < 0.001$). The effect of caffeine was highly significant; caffeine lengthened the period by ~ 1.6 hrs on average ($P < 0.001$). The lengthening of the period in LL by caffeine was ~ 1.3 hrs greater 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, but this was not significant ($P = 0.09$, $t = -1.970$, d.f. = 7, paired t -test). In contrast, the period length was significantly longer in LL during caffeine treatment than in the LL control ($P = 0.01$, $t = 3.236$, paired t -test) (Fig. 4).

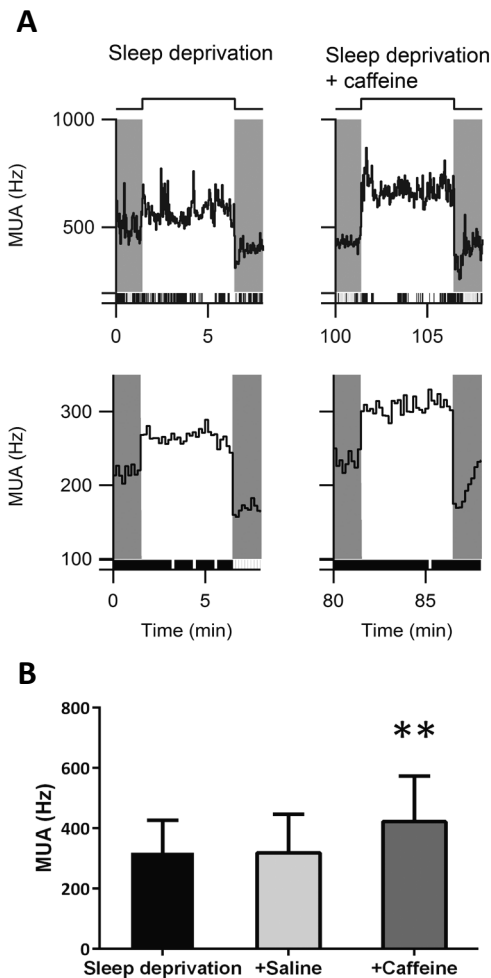


Fig. 3. (A) Two examples of responses to light of SCN neuronal activity after 6 hrs of sleep deprivation. Mice were exposed to 5-min light pulses. In the left graphs, light exposure was preceded by intraperitoneal injection of caffeine (15 mg/kg). Light exposure is indicated by a white background. Time 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 PIR-recorded locomotor activity in the cage. **(B)** Mean light-induced increases in SCN neuronal activity \pm standard error of the mean after sleep deprivation are summarized ($n = 6$). Mice received either no injection or intraperitoneal injections of saline or caffeine (15 mg/kg), and were then exposed to 5-min light-pulses. $**P < 0.01$, ratio paired t -tests (saline vs. caffeine, $P = 0.004$, $t = 0.4125$; sleep deprivation vs. saline, $P = 0.697$, $t = 5.097$). Caffeine significantly enhances light-induced increases in SCN neuronal activity after sleep deprivation.



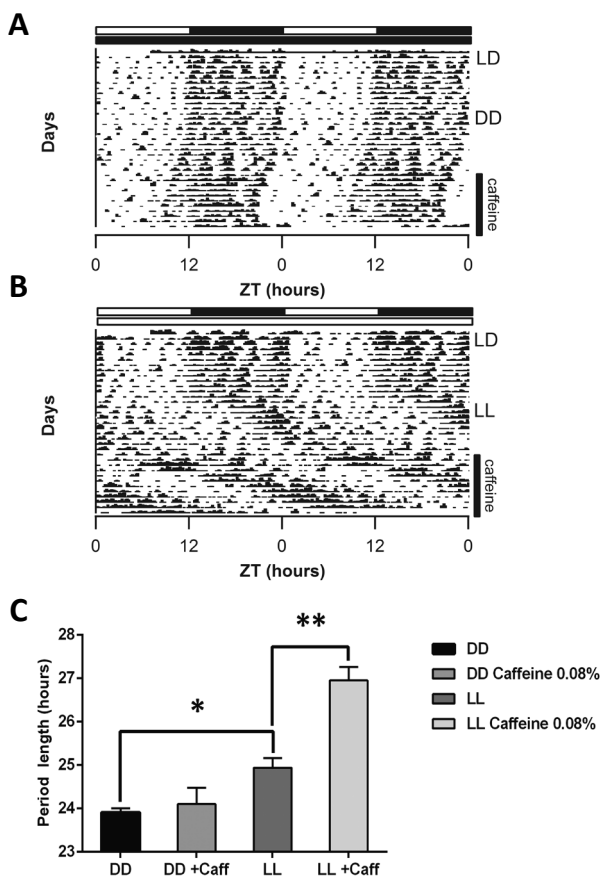


Fig. 4. (A- B) Double-plotted actograms of behavioral activity of mice demonstrating the effect of caffeine on free running periods in constant darkness (**A**) and constant light (**B**). The light regime is plotted as bars above the actograms. Zeitgeber time (ZT) is plotted in hrs on the x-axis, and consecutive days are plotted on the y-axis. Mice were released in either DD (**A**) or LL (**B**). After 10 days, mice received caffeine (0.08%) via their drinking water. (**C**) Mean period length \pm standard error of the mean in DD ($n = 8$) with and without caffeine and in LL ($n = 9$) with and without caffeine. * $P < 0.05$ and ** $P < 0.01$, mixed model with factors 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.

Discussion

Our findings show that caffeine increases both light responsiveness measured at the level of neuronal activity of the SCN and the level of behavioral activity. Similarly to previous studies, we found a reduction in light-induced phase delays in behavioral 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 demonstrated that this attenuation was restored after peripheral injection of caffeine. We performed behavioral recordings in LL, and found enhanced sensitivity of the circadian system to light in combination with chronic caffeine treatment. Hence, the current study 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 the 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.³³ Chronic caffeine consumption in DD significantly lengthened the circadian period in behavioral activity in mice, by ~25 min.²² In the present study, we found a similar lengthening, but this did not reach significance. In addition, we showed that caffeine significantly increased the period of the circadian clock in LL, by 95 min. 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.^{34–38} 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 lengthened the period of the clock.

In line with this, caffeine appeared to strengthen the effect of light on the circadian clock at the neuronal level. We confirmed that sleep deprivation attenuates phase delays in behavioral activity,^{17,18} and also showed that light-induced increases in SCN neuronal activity are attenuated after sleep deprivation. Previous behavioral experiments have indicated that the phase-shifting effects of repeated light pulses show saturation.³⁹ Electrophysiological experiments have shown that the response of the SCN to repeated light pulses does not diminish.^{9,15,40} These results combined suggest that the saturation in the behavioral phase response occurs downstream from the initial light response in the SCN. It has been shown previously that application of an adenosine agonist attenuates the light-induced phase delays in behavior, which are restored by an adenosine receptor antagonist.^{20,21} In contrast, a recent study showed that caffeine administration blocks light-induced phase delays; however, the dose applied was three times larger, and the activity of the animals was very much reduced under these conditions.⁴¹ 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 for boosting (re-)synchronization to a LD cycle.

The question remains how caffeine restores light responsiveness of the SCN. The choice of caffeine was reinforced by the preponderant use of caffeine in current society. Caffeine is an antagonist of the adenosine A1 and A2A 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.⁴² From the present results, we cannot determine where between the retina and the SCN caffeine influences the light sensitivity of the circadian system. In the retina, adenosine receptors and ryanodine receptors (RyRs), which are caffeine-sensitive Ca²⁺ release channels,⁴³ are present, and can be affected by caffeine.^{44–46} However, whether



adenosine levels in the retina change under the influence of sleep deprivation is unknown. Only a few studies have reported the expression of adenosine receptors in the SCN,^{47,48} 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.^{47,49} 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 A1 adenosine receptor reduces the amplitude of excitatory postsynaptic currents and depresses glutamate release.⁵⁰⁻⁵⁴ Previous studies have demonstrated that adenosine decreases intracellular Ca^{2+} levels and electrical activity via glutamatergic neurotransmission.^{47,49} The electrical activity of SCN neurons was blocked by adenosine A1 receptor agonists, and this effect was reversed by adenosine A1 receptor antagonists. Selective adenosine A2A receptor antagonists did not affect excitatory currents.⁴⁸ Therefore, transmission of light through the optic nerve to SCN neurons is probably mediated via adenosine A1 receptors present at presynaptic nerve terminals in the SCN.⁴⁸

The 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 the 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 on the SCN by blocking presynaptic A1 adenosine receptors on the retinohypothalamic tract and thereby reducing the inhibition of glutamate release to the SCN by adenosine. Another possible mechanism through which caffeine can exert its effects on the light responsiveness of SCN neurons might be via activating RyRs, which are present in the SCN.⁵⁵ Caffeine-mediated increases in Ca^{2+} levels in SCN neurons were shown to be dependent on the activation of RyRs.⁵⁶ Application of caffeine caused light-like phase shifts of the SCN in mouse brain slices, similar to the 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.⁵⁷ However, the concentrations needed to activate the RyRs *in vivo* are most likely not reached with peripheral administration of caffeine. Therefore, we hypothesize a role for adenosine receptors, in particular A1 receptors, in mediating the effects reported in our study.

Several studies in humans have reported effects of caffeine consumption on the circadian system. Melatonin release and body temperature are both used as markers of the circadian phase in humans. Caffeine ingestion suppresses melatonin release and attenuates the normal decrease in body temperature in the evening.⁵⁸ 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.^{58,59} 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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