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Research Report

Effects of chronic caffeine on patterns of brain blood flow and behavior throughout the sleep–wake cycle in freely behaving mice

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Abstract

Caffeine has significant effects on neurovascular activity and behavior throughout the sleep–wake cycle. We used a minimally invasive microchip/video system to continuously record effects of caffeine in the drinking water of freely behaving mice. Chronic caffeine shifted both rest and active phases by up to 2 h relative to the light–dark cycle in a dose-dependent fashion. There was a particular delay in the onset of rapid eye movement (REM) sleep as compared with non-REM sleep during the rest phase. Chronic caffeine increased wakefulness during the active phase and consolidated sleep during the rest phase; overall, there was no net change in the amount of time spent in the wake, sleep, or REM sleep states during caffeine administration. Despite these effects on wakefulness and sleep, chronic caffeine *decreased* mean cerebral blood volume (CBV) during the active phase and *increased* mean CBV during the rest phase. Chronic caffeine also increased heart rate variability in both the sleep and wake states. These results provide new insight into the effects of caffeine on the biology of the sleep–wake cycle. Increased blood flow during sleep caused by chronic caffeine may have implications for its potential neuroprotective effects through vascular mechanisms of brain waste clearance.

Keywords: caffeine, sleep, rapid eye movement, brain blood flow, optical intrinsic signal

Significance Statement

Caffeine is the most widely used psychoactive substance worldwide, but the understanding of its physiological and behavioral effects remains incomplete. This study uses a novel minimally invasive monitoring system in freely behaving mice over extended time periods to show complex effects of chronic caffeine consumption on brain blood flow, movement, and heart rate throughout the entire sleep–wake cycle with specific actions on the active and rest phases as well as rapid eye movement (REM) sleep. These effects have implications for the effects of caffeine in both physiological and pathological settings. Increased brain blood flow during sleep due to chronic caffeine could play a role in its neuroprotective effects via enhanced waste clearance.

Introduction

Caffeine is commonly consumed by the general population and is well known for its effects on physical and mental function (1, 2). It also modulates a variety of disease states. One example is migraine, for which acute administration of caffeine may be therapeutic, whereas withdrawal from chronic caffeine use can be a migraine trigger (3). Another example is neurodegenerative diseases, particularly Parkinson's disease, for which caffeine intake may have protective effects (4). The basic mechanisms underlying the effects of caffeine on normal daily function and pathological states remain poorly understood.

Caffeine is a nonselective adenosine receptor antagonist with similar affinity for different adenosine receptor subtypes (A_1, A_{2A}, A_{2B}, and A_3 receptors) (5, 6). The different functions and locations of these receptors on neural and nonneural cells may be involved in the heterogeneous effects of caffeine in the central and peripheral nervous systems. Studies using adenosine receptor knockout mice suggest that the arousal effects of caffeine are due to effects on A_{2A} receptors and not A_1 receptors (7). Adenosine receptors mediate distinct direct and indirect effects on both neural and vascular cells. Acute administration of caffeine has been reported to cause reductions in global brain blood flow in humans (8, 9). This decrease in blood flow, which might seem paradoxical in the context of enhanced cognitive activity, has been reported to be offset by increased oxygen extraction such that overall metabolism remains stable (9). Caffeine may

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also have marked effects on the circadian cycle (10–13) and associated fluctuations in neurovascular activity (2).

One of the challenges of assessing the effects of caffeine on nervous system function is that these effects may vary substantially depending on the day–night cycle and on whether caffeine administration is acute or chronic. It has been proposed that adenosine levels increase in the brain throughout the awake period and with sleep deprivation, thereby contributing to the metabolic drive to sleep (14–17). Given its primary action as an adenosine receptor antagonist, the effects of caffeine might therefore be expected to differ depending on brain region-specific variations in levels of adenosine that occur during the circadian cycle. Most studies of the effects of caffeine in animals investigate responses to acute administration of caffeine. In this study, we quantify the effects of caffeine administration in the drinking water as a noninvasive approach that enables investigation of chronic caffeine consumption in a manner that is similar to the oral caffeine consumption during the awake period in humans.

A variety of approaches have been used to monitor movement, behavior, and neurovascular activity in rodents. While movement and behavior can be measured noninvasively via video-based methods, electroencephalography (EEG) has typically been the approach used to monitor brain activity, particularly changes in brain activity associated with different phases of sleep (18–20). However, continuous EEG recordings in rodents are invasive, typically requiring breaching of the skull for fixation of electrodes. We have developed a novel minimally invasive microchip and video-recording method that enables continuous recording of multiple physiological parameters in addition to movement and behavior over many sleep–wake cycles (21, 22). The microchip system measures cerebral blood volume (CBV) (which indicates cerebral blood flow), heart rate (HR), respiration, and head movement, while the video system tracks the location of the animal and measures overall movement in the enclosure. This approach allows for comprehensive characterization of neurovascular activity and behavior throughout the light–dark cycle, including rapid eye movement (REM) sleep and non-REM (NREM) sleep. We used this approach to investigate physiological and behavioral features of the circadian cycle before, during, and after chronic administration of caffeine.

Results

Baseline sleep and wake states

Sleep was defined by minimal head movement for 2.5 min. This designation of sleep was confirmed by the absence of body movement based on video recording and by a corresponding decrease in CBV which occurred consistently (except during REM sleep). REM sleep was detected within the sleep blocks based upon characteristic marked increases in CBV in the absence of any head or body movement (Fig. 1). EEG changes consistent with REM sleep preceded CBV changes, such that there could be a slight difference in the calculation of time spent in REM sleep based on the CBV changes as compared with EEG (18, 21).

At baseline, mice showed consistent patterns of sleep and wakefulness relative to the light–dark periods. Mice were predominantly awake during the dark periods and predominantly asleep during the light periods, but wakefulness was commonly interrupted by sleep and vice versa. (Fig. 2a). We refer to times during which mice were continuously asleep or awake for at least 2.5 min as "sleep blocks" or "awake blocks." We refer to the phases (typically ~12 h each) during which animals were predominantly asleep or awake as the rest phase or active phase. Consistent with previous reports (23), we found that C57Bl6 mice had a biphasic pattern of activity during the dark period, with a transient epoch of increased sleep blocks with decreased CBV and movement occurring at ~7–8 h after lights were turned off. This "siesta" during the active phase typically lasted for 2–3 h and commonly included periods of REM sleep.

Chronic caffeine alters temporal patterns of active and rest phases relative to the light–dark cycle

Chronic caffeine had a dose-dependent effect on the timing and architecture of the active and rest phases. Overall, the time spent in the awake vs. sleep states was unchanged by caffeine (Fig. 2 and Table 1). Interestingly, however, chronic caffeine administration caused reduced awake blocks during the rest phase, in effect "consolidating" the rest phase (Table 1). Chronic caffeine also shifted the rest and active phases relative to the light–dark cycle in a dose-dependent fashion. During caffeine administration, the rest phase was shifted by up to 2 h later relative to the light period and extended longer into the dark period, with equivalent changes in the active phase relative to the dark period (Fig. 2 and Table 2). The temporal patterns of the active and rest phase all recovered toward baseline with discontinuation of caffeine administration (Fig. 2 and Table 2). In experiments in which a single dose of caffeine (0.6 mg/ml) was administered, the shifts described above were similar to those observed with the same dose in the ascending dose experimental design. Average vectors for NREM awake and NREM times were shifted ($n = 9$, for awake from 5:27 ± 1:38 to 6:23 ± 1:19, $P = 0.019$; for NREM sleep from 17:16 ± 1:24 to 18:04 ± 1:16, $P = 0.069$). Vectors for onset of active and rest phase shifted slightly but not significantly (for active phase from 23:49 ± 1:36 to 0:17 ± 1:21, $P = 0.94$; for rest phase from 12:43 ± 0:45 to 13:02 ± 1:03, $P = 0.57$).

Effects of chronic caffeine on REM sleep

Chronic caffeine reduced REM sleep during the active phase in a dose-dependent fashion; in some animals, it was completely abolished at high caffeine concentrations (Fig. 2a and Table 1). Chronic caffeine also shifted REM sleep relative to the rest phase, significantly delaying REM sleep following the onset of the light period (Fig. 2e and f and Table 2). This shift in the timing of REM sleep was greater than the shift in NREM sleep (Fig. 2g and Table 2), such that sleep blocks with no REM sleep were observed at the start of the rest phase (Fig. 2a). Despite this shift, there was no significant change in the total amount of REM sleep per 24-h period (Table 1), indicating that with caffeine, there was a clustering of REM sleep in the later parts of the rest phase. All values recovered toward control values in the washout period. In experiments in which a single dose of caffeine (0.6 mg/ml) was administered, the shifts in the timing of REM sleep were similar to those observed with the same dose in the ascending dose experimental design. With 0.6 mg/ml caffeine, the average vector for REM sleep was shifted significantly ($n = 9$, from 18:15 ± 1:15 to 19:28 ± 1:21, $P < 0.0001$), as was the difference between the REM sleep and NREM vector ($n = 9$, from 0:57 ± 0:32 to 1:23 ± 0:28, $P = 0.044$).

Changes in CBV associated with the awake and sleep states

Optical intrinsic signal (OIS) was measured continuously throughout the experiments as an indicator of CBV (see Methods). Mice showed stereotyped patterns of changes in CBV, head movement, body movement, and HR, which were correlated with the sleep

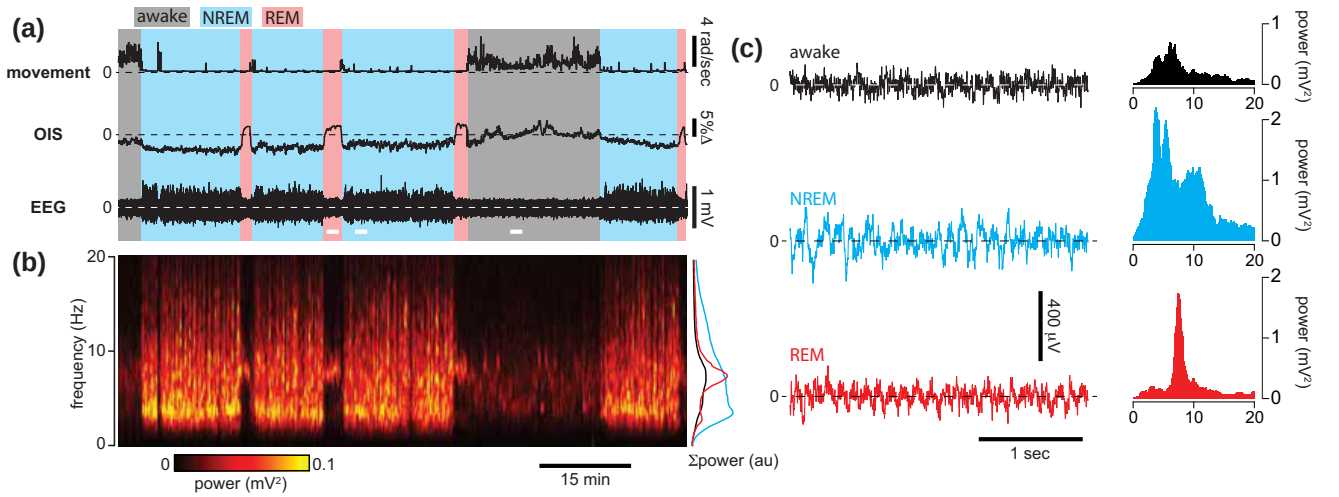

Fig. 1. REM sleep episodes shown with simultaneous EEG and OIS recording. Representative traces of a single mouse with simultaneous movement, OIS, and EEG recording. a) Recordings show periods of awake state (gray), REM sleep (red), and NREM sleep (blue) with black traces showing movement, OIS, and EEG. Each period of REM sleep identified based on a significant increase in CBV as indicated by OIS, in the absence of any movement OIS and movement is associated with expected changes in the EEG spectrum shown in b and c. b) Fast Fourier Transform (FFT) spectral analysis of EEG over time demonstrates clear differences in the power spectrum between the awake, NREM sleep, and REM sleep states. Note the high-energy band at lower frequencies (2–4 Hz frequency band) indicative of NREM sleep and a high-energy band at higher frequencies (5–8 Hz) indicative of REM sleep, whereas during the awake state, no clear bands are recognized. Power spectra were calculated over 5-s windows. c) Representative EEG segments during the awake (black), NREM (blue) sleep, and REM (red) sleep states with graphs to the right showing power spectra (0–20 Hz) for each state. Spectra show shift frequency power as expected for the different states. FFT power spectra were calculated over 2-min-long representative sections (areas indicated as white bars in Fig. 1a; segments shown are part of these sections).

and awake states (Fig. 3). At any point during the light–dark cycle, the awake state was consistently associated with an acute increase in CBV that accompanied increases in head and body movement. Conversely, the sleep state was associated with an acute decrease in CBV correlated with decreases in head and body movement. During sleep, there were transient large increases in CBV that were associated with REM sleep (from 98.6 ± 0.2% to 102.2 ± 0.2%, $P = 0.018$; Figs. 1 and 3b and c), with no accompanying movement. Consistent with previous reports (18, 24), the increases in CBV during REM commonly exceeded the CBV increases associated with the awake state.

Effects of chronic caffeine on CBV

During chronic administration of caffeine, the acute changes in CBV that occur with the transitions between the different states (awake, sleep, and REM) were unchanged compared to control (Fig. 3b). However, chronic caffeine changed mean CBV (i.e. the baseline CBV around which these acute changes occur) significantly in a dose-dependent fashion (Figs. 3a and 4). Under control conditions, mean CBV changed little over the full 24 period (Fig. 3a). During caffeine administration, a clear cycle emerged: mean CBV increased during the latter part of the rest phase to levels that were significantly higher than during control conditions and remained elevated for beginning of the active phase (Fig. 3b). Then during the latter part of the active phase, mean CBV decreased to levels that were significantly lower than those observed in the awake phase under control conditions. The amplitude of this oscillatory change in mean CBV was increased with increasing doses of caffeine (Figs. 3a and 4). These changes in CBV recovered toward baseline after discontinuation of caffeine (Fig. 4a3 and 4d1–d6). In experiments in which a single dose of caffeine (0.6 mg/ml) was administered, the changes in CBV were similar to those observed with the same dose in the ascending dose experimental design and similarly recovered following washout (Fig. 3d).

Effects of chronic caffeine on HR and HRV

HR can be quantified based on a low-amplitude, high-frequency component of the OIS. As previously reported (21), under control conditions, average HR was higher in the awake state (678 ± 12 bpm) compared to the sleep state (606 ± 42 bpm, $P = 0.029$). During REM sleep, the average HR was comparable to that in NREM sleep (618 ± 36 bpm, $P = 0.19$; Fig. 5). Chronic caffeine caused a slight dose-dependent increase in average HR in the awake state, and a more pronounced increase during NREM sleep and REM sleep. Chronic caffeine also caused a dose-dependent increase in HR variability (HRV) during the awake, NREM sleep, and REM sleep states (Fig. 5).

Discussion

Our minimally invasive microchip/video-recording system enables continuous recording of multiple physiological and behavioral parameters over weeks to months (21, 22). This system is ideal for examining the effects of chronic experimental conditions during both the sleep and awake states over extended time periods. The multiple data streams obtained using this approach can be combined and analyzed in distinctive ways, including vector-based circular data analysis which provides unique insight into patterns of change during the circadian cycle. Daily caffeine administration in the drinking water had significant effects on cyclical changes in CBV (an indicator of blood flow) and motor activity in freely behaving mice. This approach to caffeine administration, while potentially less precise than other methods of administration, has significant advantages including reduced handling of animals and a dosing pattern that approximates that which occurs in humans who consume caffeine during their awake phase.

In this context, it is important to consider that typical diurnal variations in caffeine consumption may play a significant role in its physiological and behavioral effects. The half-life of caffeine

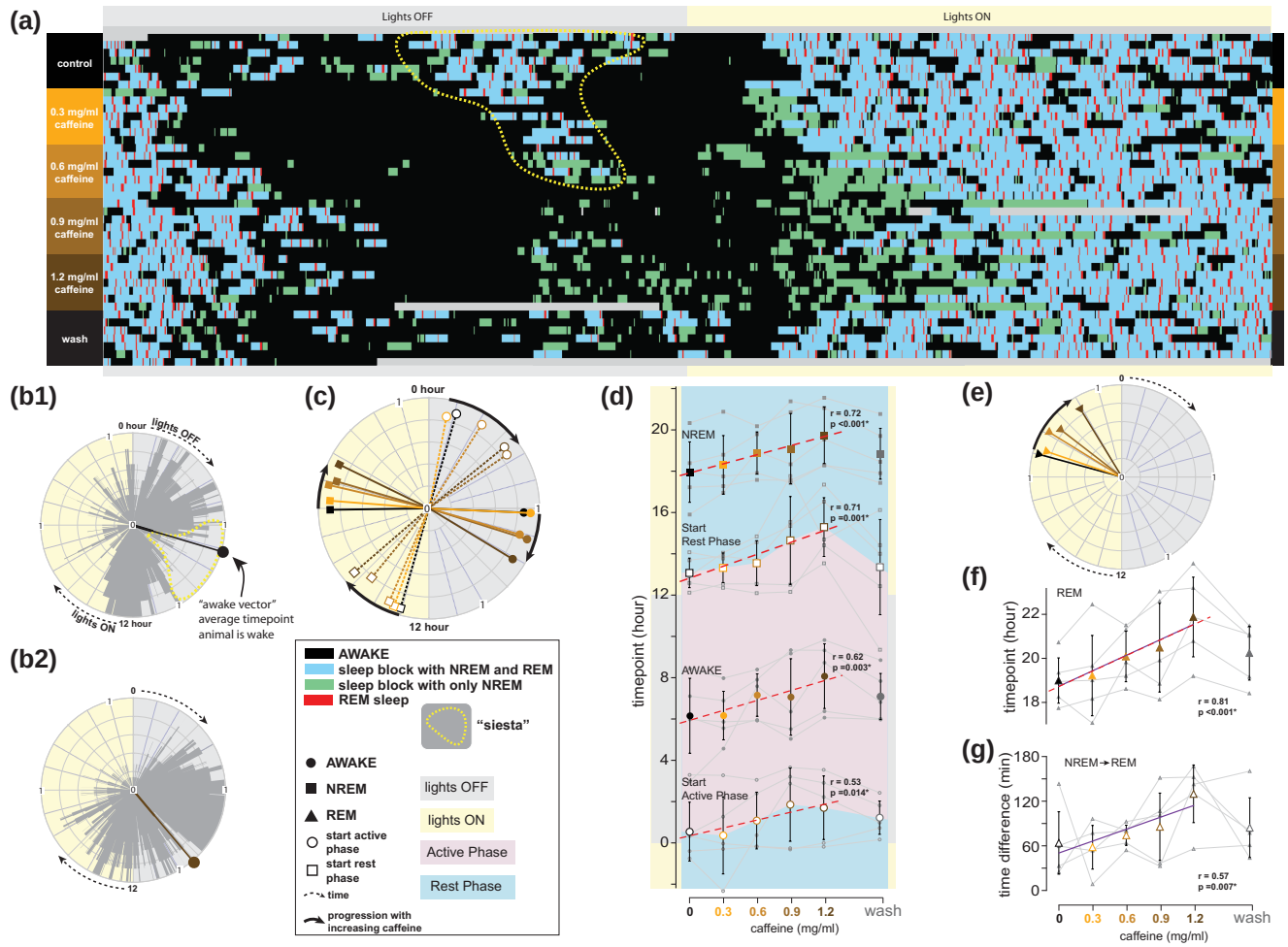

Fig. 2. Effects of chronic caffeine on the timing of awake, NREM sleep, and REM sleep states. a) Raster plot showing effects of different concentrations of caffeine in the drinking water (left bar) in a typical experiment. Each row represents a single 24-h period, with lights off and on indicated by the bars at the top and colors representing the wake, sleep, and REM sleep states determined based on movement and CBV (see Figs. 1 and 3). Gray color indicates missing data due to interruption in sensor recording. Increasing doses of caffeine are administered for 7 days after a 7-day control period and followed by a 7-day washout period. Chronic caffeine administration abolishes the "siesta" period during the active phase and shifts both NREM sleep and to a greater extent REM sleep relative to the light–dark cycle. b1, b2) Circular 24-h plots of data in a) showing the probability of being in the awake state during control period (b1) and during 1.2-mg/ml caffeine administration (b2). Dark gray areas show the probability of being awake, with radius representing the probability (from 0 to 1). Time is represented clockwise, with the start of the dark period (light gray) at 0 h at the top and the start of the light period (yellow) at 12 h at the bottom. The average vector from each distribution of awake probabilities is shown (black line with filled circle); the angle of the vector is an approximation of the mean time of the active phase. c) Solid lines are vectors calculated for awake state (closed circles) and NREM sleep state (closed squares) averaged across animals for each caffeine dose. The angle of the vectors indicates time on the 24-h "clock" where the mean of the active period and rest periods occurs. Dotted lines are the average vectors for time points at which the transitions from rest to active phase (open circles) or active to rest phase occur (open squares). d) The same time points as in c but now plotted on a linear time axis (y-axis) as a function of the caffeine concentration (x-axis). The final point in each plot shows the washout period. Traces show a dose-dependent shift in timing of phases with recovery toward baseline in the washout period. e) The vector (as described in c), for REM sleep. f) Same as d but for REM sleep shows the shift in time of the mean of the REM distribution. g) The difference in the time shift of the NREM vector vs. the time shift of the REM vector, showing that the shift for REM sleep is greater than that for NREM sleep. Error bars in d and f indicate circular SD. Small gray lines and markers in d, f, and g are individual values measured in each animal. Red dotted lines are trend lines.

Table 1. Time spent in different states (averages ± SD, $n = 5$).

Caffeine (mg/ml)	Awake (%/day)	NREM (%/day)	REM (%/day)	Time awake during rest phase (%/phase)	Time in REM during active phase (%/phase)
0 (control)	56.1 ± 10.3	40.7 ± 9.9	3.2 ± 0.6	31.2 ± 3.3	2.7 ± 1.4
0.3	62.9 ± 2.7	33.6 ± 2.5	3.5 ± 0.3	27.2 ± 6.6	1.9 ± 1.7
0.6	59.7 ± 3.9	36.8 ± 3.5	3.5 ± 0.4	25.6 ± 4.1	1.1 ± 1.0
0.9	56.8 ± 5.8	39.6 ± 5.5	3.6 ± 0.3	24.7 ± 3.9	1.5 ± 2.1
1.2	55.8 ± 7.3	41.0 ± 6.7	3.2 ± 0.9	22.6 ± 3.1	0.8 ± 0.8
R	−0.24	0.026	0.000	−0.655	−0.747
P	0.621	0.583	0.998	**0.001****	**0.00001****
Power	0.078	0.085	0.05	0.924	0.987
Wash	58.8 ± 7.0	34.4 ± 6.5	2.8 ± 0.9	25.7 ± 2.0	4.7 ± 1.9

** and bold indicate statistically significant values.

Table 2. Time points of start and average awake, NREM, and REM vectors and the difference in the shift of the NREM and REM vectors. All values are ±circular SD.

Caffeine (mg/ml)	Start active phase (hh:mm ± SD)	Start rest phase (hh:mm ± SD)	Avg. awake (hh:mm ± SD)	Avg. NREM (hh:mm ± SD)	Avg. REM (hh:mm ± SD)	Avg. NREM-Avg. REM (min ± SD)
0 (control)	0:32 ± 1:26	13:04 ± 0:43	6:10 ± 1:49	17:57 ± 1:28	19:01 ± 1:02	64 ± 42
0.3	0:21 ± 1:52	13:19 ± 0:43	6:11 ± 1:11	18:18 ± 1:25	19:15 ± 1:49	58 ± 29
0.6	1:05 ± 1:23	13:32 ± 1:05	7:10 ± 1:02	18:53 ± 1:01	20:07 ± 1:09	74 ± 13
0.9	1:51 ± 1:47	14:38 ± 2:07	7:05 ± 1:51	19:05 ± 1:42	20:31 ± 2:01	86 ± 45
1.2	1:41 ± 1:33	15:17 ± 1:25	8:05 ± 1:34	19:44 ± 1:23	21:54 ± 1:49	130 ± 39
R	0.530	0.713	0.620	0.719	0.809	0.569
P	**0.014****	**<0.001****	**0.003****	**<0.001****	**<0.001****	**0.007****
Power	0.723	0.971	0.880	0.975	0.998	0.799
Wash	1:13 ± 0:48	13:21 ± 2:18	7:06 ± 1:07	18:50 ± 1:14	20:15 ± 1:13	84 ± 40

** and bold indicate statistically significant values.

in mice has been reported to be in the range of 1–3 h (25, 26). Caffeine is metabolized to paraxanthine, which has similar effects on adenosine receptors, and may therefore extend the effects of caffeine consumption (27, 28). Even accounting for the effects of paraxanthine, variation in the consumption of caffeine during the sleep–wake cycle, will lead to significant fluctuations in adenosine receptor antagonism during any 24-h period. The overall physiological and behavioral effects of chronic caffeine consumption in humans may therefore be not only due to the effects of peak concentrations during the awake phase but also due to the effects of falling levels of caffeine during the sleep phase. Another important source of diurnal variability may be the levels of adenosine. Brain adenosine levels are believed to increase throughout wakefulness and decrease during sleep (2, 17, 29). Fluctuating levels of adenosine may therefore also contribute to different effects of caffeine in the sleep and awake states.

Chronic caffeine and temporal features of the sleep–wake cycle

Caffeine had significant effects on the temporal patterns of sleep and wakefulness relative to the light–dark cycle. At baseline, wakefulness during the dark period was commonly interrupted by brief periods of sleep, and the reverse occurred during the light period. Also, consistent with previous reports, we found that at baseline, C57Bl6 mice showed a biphasic pattern of activity during the dark (active phase), with a consistent increase in sleep blocks and decreased movement occurring ∼8 h into the dark period. Episodes of REM sleep occurred consistently during this "siesta" period. Interestingly, this "siesta" pattern has been reported to be specific to the C57Bl6 strain, suggesting that it is genetically encoded (23). Administration of caffeine reduced or abolished the "siesta" and had the overall effect of consolidating wakefulness during the active phase. Conversely, we found that chronic caffeine was associated with decreased awake blocks during the rest phase. This consolidation of sleep in response to chronic caffeine has been observed in other studies (20).

Caffeine administration also significantly shifted the rest and active phases relative to the light–dark cycle by up to 2 h. Similar findings have been reported in previous studies in mice (11, 13). An increased sleep latency in response to caffeine has been reported in multiple human studies (2), although this effect may be reduced by adaptation to chronic caffeine consumption (30). Overall, there was no net change in the amount of time spent in the sleep vs. awake states during caffeine treatments. These results indicate that the effects of caffeine may mimic those of a genetically mediated delayed sleep phase shift in humans (31), rather than simply reducing overall sleep as is commonly believed. In human studies, chronic caffeine consumption has been reported to result in decreased sleep efficiency and sleep quality, although this effect may not be observed under controlled laboratory conditions (2, 30). Our results suggest that a perception of reduced sleep quality in humans could be due to a shift in the sleep phase as opposed to reduced efficiency of sleep or increased awakenings during the sleep phase. This would be particularly true if the time of awakening is not flexible relative to light–dark cycle, such that there is no ability to compensate for the sleep phase shift with delayed awakening.

Caffeine effects on REM sleep

Chronic caffeine had a dose-dependent effect on the timing of REM sleep relative to the light–dark cycle, but did not significantly change the overall amount of REM sleep in a given 24-h cycle. At higher doses of caffeine, REM sleep during the active phase was significantly decreased and in some animals completely abolished. In addition, there was a marked shift in REM to later in the rest phase, such that we observed an increased occurrence of sleep blocks without REM at the beginning of the rest phase. A similar effect of caffeine on REM sleep latency has been reported in humans (32). Despite this substantial shift in the REM sleep state, the fact that caffeine did not change the overall amount of REM sleep reflects a concentration of REM sleep in the latter part of the rest phase. One explanation for this pattern is that a falling level of caffeine leads to greater REM sleep as the rest phase progresses. These results suggest that adenosine may be specifically involved in regulating the onset and frequency of REM sleep to an even greater extent than the regulation of NREM sleep. The functional consequences of the "concentration" of REM sleep toward the latter half of the rest phase are not clear, but it is possible that this, along with a shift in sleep onset, could be involved in the perception of reduced sleep quality with caffeine, particularly if there is a fixed time of awakening.

Caffeine has paradoxical effects on the relationship between activity and CBV

In the setting of normal neurovascular coupling, increased brain activity is typically correlated with increased CBV. With our system, this correlation can be demonstrated when mice are under anesthesia during which repetitive cortical bursting activity as demonstrated by field potential recording is consistently correlated with repetitive dilation of cerebral surface arteries and increases in CBV as indicated by OIS (21). In previous studies and in the present study, we show that increases or decreases in head and body movement of the mouse in the awake state or sleep

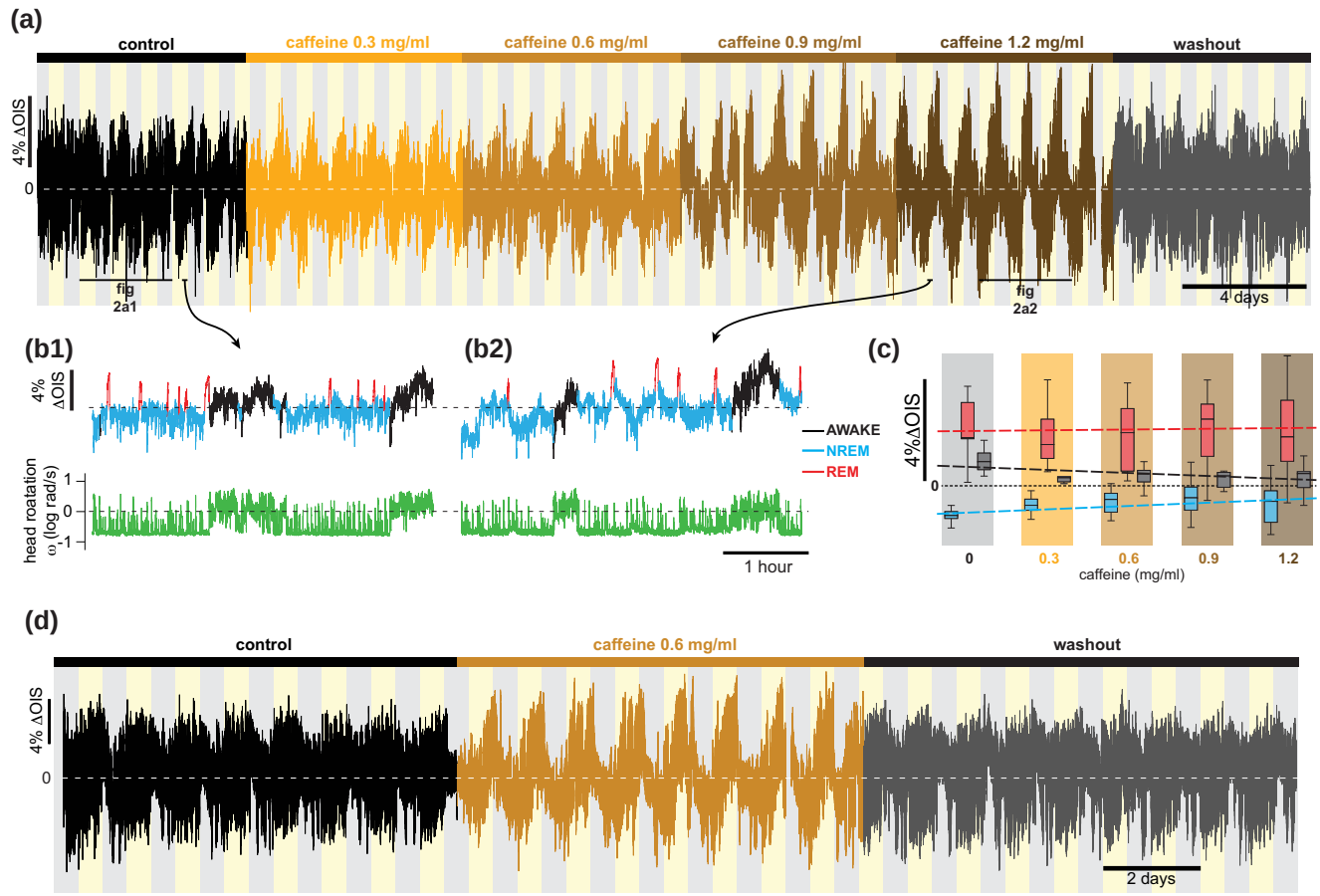

Fig. 3. Effects of chronic caffeine on CBV. a) Representative trace of OIS from a single mouse demonstrates CBV fluctuations over a 41-day period. Different caffeine doses are indicated at the top of the trace and by corresponding change in the color of the trace. Dark periods are indicated by gray bars, whereas light period by yellow bars. Increasing doses of caffeine have a distinct effect on the CBV changes associated with the light/dark cycle (also see Fig. 3). b1, b2) Zoomed in traces of CBV (top) and head movement (bottom, green) over 4-h periods at baseline and during administration of 1.2 mg/ml caffeine. In the top trace, blue represents NREM sleep, red represents REM sleep, and black represents the awake state. The acute changes associated with each state are not significantly affected by caffeine. c) Z-score normalized daily average of CBV during the NREM sleep (blue) REM sleep (red) and awake states (gray) at baseline and with increasing doses of caffeine. Box indicates quartiles; whiskers represent minimum and maximum. CBV when awake decreased with increased caffeine ($R = -0.391$, $P = 0.003$) but increased during NREM ($R = 0.253$, $P = 0.008$). CBV during REM was not significantly affected by caffeine ($R = -0.001$, $P = 0.994$). Dotted lines are trend lines. d) Representative trace of CBV in a mouse who received a single concentration of caffeine (0.6 mg/ml) in the drinking water for 7 days followed by washout. CBV changes are similar to those observed at the same concentration in the ascending dose experimental design.

states were consistently correlated with acute increases or decreases in CBV. One exception to this correlation under baseline conditions is during REM sleep, when there were marked increases in CBV in the absence of an increase in movement. These findings are generally in accordance with previous studies (18), although one study in head-fixed mice reported an increase in CBV when mice transitioned from the awake to sleep state (24).

In addition to acute changes in CBV associated with the sleep and awake states, we have found that there are gradual fluctuations in mean CBV that occur during the light–dark cycle. Chronic caffeine had paradoxical effects on the relationship between the sleep–wake cycle and mean CBV. During chronic caffeine administration, mean CBV *decreased* during the latter part of the active phase and *increased* during the latter part of rest phase despite consolidation of sleep. The timing of these effects of chronic caffeine suggests that caffeine decreases mean CBV during the active phase, whereas the increased CBV during the rest phase could be due to the relative "withdrawal" and falling levels of caffeine during this phase. Acute caffeine administration to humans has been reported to cause a decrease in cerebral blood flow (33–36), similar to the effects that we observe in mice during their active phase. This parallels the results in mice, in which chronic administration of caffeine in the drinking water resulted in a decrease in mean CBV.

The effects of caffeine on mean CBV during the active and rest phases are not consistent with typical patterns of neurovascular coupling, in which increased brain activity is associated with increased blood flow and vice versa. The mechanisms underlying this paradoxical effect of caffeine on neural activity versus CBV are unclear but likely involve distinct effects of adenosine on neural versus vascular activity. Adenosine has predominantly inhibitory effects on cortical neurons, whereas caffeine has excitatory effects. Adenosine has predominantly vasodilatory effects, whereas caffeine inhibits this vasodilation. There is a parallel situation in the heart, where adenosine has a negative chronotropic effect based upon its inhibitory actions on the pacemaker and electrical conduction systems, but is also a vasodilator, presumably based upon direct actions on the vasculature (37). It is possible that these actions, which seem contrary to normal neurovascular coupling, are related to homeostatic and protective functions of adenosine. While in the setting of neurovascular coupling there is an increase in blood flow in the setting of

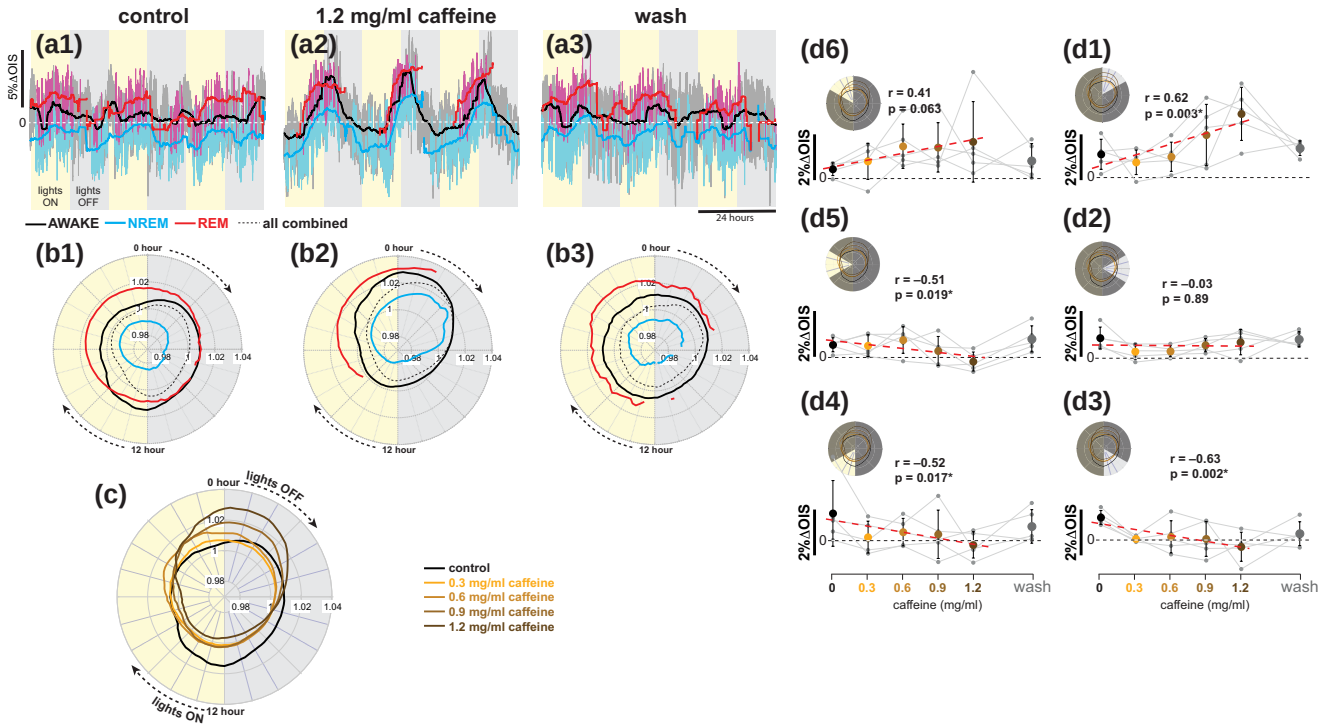

Fig. 4. Changes in CBV in response to chronic caffeine. a1–a3) Representative traces of CBV (dark gray shading) over a 72-h period with lines indicating a 4-h rolling average of CBV during NREM sleep (blue), REM sleep (red), and the awake state (black). a1 is control, whereas a2 shows CBV during 1.2 mg/ml caffeine in the drinking water. a3 shows CBV during washout period. b1–b3) Circular plots of mean CBV during NREM sleep (blue), REM sleep (red), and the awake state (black) averaged over all animals under control condition (b1) and during 1.2 mg/ml caffeine (b2) and during washout (b2). CBV is plotted clockwise over time, starting at 0 h at the top. The radius is the ΔOIS amplitude (from 0.97 at the center to 1.04 at the outer circle. CBV during REM sleep show gaps when no value could be determined for at least one animal and no proper average could be calculated. It happens at time points where in a window of ±4 h no REM sleep occurs for at least one animal. c) Same circular plot as shown in b but for the awake state only at different caffeine concentrations. With increasing doses of caffeine, the amplitude of the CBV change is larger in the upper half of the plot and smaller in the lower half of the plot and the circular CBV change rotates toward the first quadrant after the light to dark transition. d1–d6) Mean awake CBV values from 4-h time epochs from figure c (insets indicate selected window of time). CBV (ΔOIS) is plotted vs. caffeine concentrations, showing caffeine-dependent increases or decreases in CBV depending on the time of the day. The last point in each plot is the washout period, showing recovery after discontinuation of caffeine. Small gray lines and markers are individual values measured in each animal. Red dotted lines are trend lines; black upright bars indicate y-scale.

increased neural activity, adenosine may serve to both suppress neural activity and increase blood flow as a homeostatic response. This could explain the actions of caffeine as an enhancer of neural activity in the face of decreased blood flow.

This effect of caffeine raises the question of how the nervous system could compensate for reduced cerebral blood flow in the face of increased cerebral activity. Some studies have suggested that in parallel with decreased blood flow, administration of caffeine results in increased oxygen extraction, such that overall metabolism is not compromised (9). The converse finding that was observed during sleep, where caffeine consumption during the active phase results in an increase in CBV during the rest phase, raises the possibility that this effect on cerebral blood flow during sleep could also play a role in the consequences of caffeine consumption, including its potential neuroprotective effects.

Effects of HR and HRV

Our results show that chronic caffeine caused a small but significant increase in average HR in the awake state and a more pronounced increase during NREM sleep and REM sleep, indicating that changes in HR could be involved in the increases in mean CBV during sleep. Interestingly, chronic caffeine also increased HRV, most prominently in the awake state but also in the NREM sleep state. These findings suggest that changes in autonomic regulation could be involved in the physiological and behavioral responses to chronic caffeine.

Potential neuroprotective effects of caffeine

Caffeine consumption has been reported to be associated with a reduced risk of the development of neurodegenerative diseases (38), particularly Parkinson's disease (4, 39, 40). An A_{2A} adenosine receptor-selective antagonist, istradefylline, is used for the treatment of Parkinson's disease (41). The mechanisms underlying the role of adenosine receptors in Parkinson's disease and other neurodegenerative diseases are unclear. Our results raise the possibility that one mechanism for potential neuroprotective actions of caffeine could be its effects on sleep. The clearance of metabolic waste during sleep has been hypothesized to be important in the setting of neurodegenerative diseases (42, 43). Increased blood flow during sleep and an altered pattern of the marked increases in blood flow that occur during REM sleep could enhance such waste clearance, contributing to a neuroprotective effect of caffeine consumption.

Summary

Chronic caffeine has marked effects on the timing and neurovascular features of the awake, NREM sleep, and REM sleep states, including a reversal of the normal relationship between mean brain flood flow and activity. A better understanding of these effects has

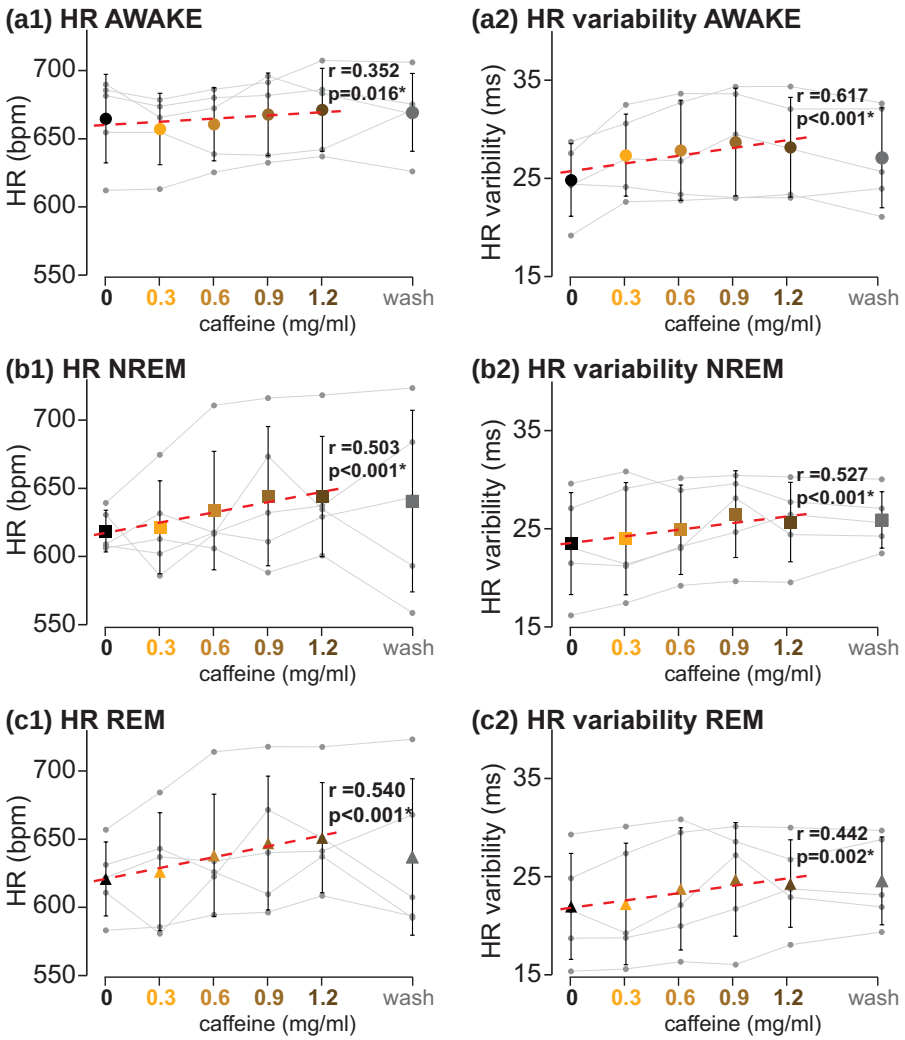

Fig. 5. Effect of chronic caffeine on HR and HRV in different states. Plots show values of average HR and HRV with different doses of caffeine. a1) Dose-dependent HR increase in awake state. a2) HRV increase awake state. b1) HR increase during NREM sleep. b2) HRV increase during NREM sleep. c1) HR increase during REM sleep. c2) HRV increase during REM sleep. Error bars indicate SD; small gray lines and markers are individual values measured in each animal. Red dotted lines are trend lines.

the potential to provide new insight into basic pathways of neurovascular coupling, as well as the mechanisms by which caffeine exerts its effects during normal daily activity as well as in the setting of neurological diseases.

Materials and methods

All studies were performed with the approval of the University of California Los Angeles Institutional Animal Care and Use Committe. Male ($n = 1$) and female ($n = 8$) wildtype C57Bl6 mice aged 12 to 25 weeks and weight 20 to 30 g were used for caffeine studies. Animals were acquired from a breeding colony and remained in standard housing conditions until the time of experimentation after which they were housed individually. Room conditions were a temperature of 20–23°C, 30–70% humidity, and a 24-h day–night cycle, with 12-h lights on and 12-h lights off. Room conditions were monitored and controlled by an automated system that was synchronized with systems involved in mouse recordings.

A microchip recording system was glued to the skull above the sensory cortex to record OIS and head movement in a brief procedure as previously described (21, 22). Carprofen (5 mg/kg subcutaneous) and bupivacaine (0.5% topical) were administered during the procedure. After recovery from the microchip attachment procedure (3–5 days), continuous recording of OIS and head movement was initiated and continued throughout the entire experiment. For the first 7 days, animals received regular drinking water. Caffeine (Sigma, St. Louis, MO, USA) was then administered in the drinking water at concentrations of 0.3, 0.6, 0.9, and 1.2 mg/ml, each dose for 7 days (the equivalent of ~5–20 cups of coffee/day in humans when accounting for differences in liver size and metabolism (44)). After caffeine administration, the animals were switched back to regular water, and an additional 7 days (washout period) were recorded. In separate experiments ($n = 4$), mice received only a single dose of caffeine (0.6 mg/ml) for 7 days followed by washout for 7 days. To verify that water intake remained the same, water bottles were weighed throughout the experiment. We found no differences in average water intake over the entire experiment (Avg 24–33 ml/week; $P = 0.59$, Mann Whitney U test).

Data were acquired with custom recording hardware and software (21, 22). Head movement and OIS were recorded from the microchip system at 256 Hz. In some experiments, mice were also

monitored with continuous video recording, using a Raspberry Pi 4b and generic IR camera with custom video software.

OIS and CBV

At the wavelengths employed in this study, OIS is primarily dependent on cerebral blood volume (CBV) (45), although the relationship between OIS and CBV is not linear and OIS may underestimate the magnitude of large increases in CBV. For analysis within one light–dark cycle, OIS was normalized with a 24-h rolling median and is expressed as ΔOIS to that median. This approach is used in preference to absolute values of OIS, because of variability of baseline absolute values from one subject to the next and very slow (weeks) drift of the OIS. The ΔOIS signal is inverted to indicate change in CBV, such that decreases in signal indicate decreases in CBV (with corresponding constriction of cortical surface arteries as indicated by simultaneous video recording (21)). To determine slow variations of the mean CBV over a full-day cycle, a rolling average of 4 h of the ΔOIS was calculated to smooth out acute changes in CBV. Mean CBV was determined for each of the different sleep states independently (awake, NREM sleep, and REM sleep), to determine the sleep state-specific baseline CBV at each time point in the day. To compare CBV over longer periods than 24 h and still account for slow drifts in OIS, we applied a rolling z-score normalization with a window of 24 h. Z-score normalization adjusts every value so that the mean is 0 and the amplitude is expressed as number of SD of the original signal. This avoids potential outliers or differences caused by skewed distributions due to other factors.

Determination of sleep states

Sleep was defined by minimal head movement for 2.5 min. This designation of sleep was confirmed by the absence of body movement based on video recording, and by a corresponding decrease in CBV which occurred consistently (except during REM sleep). REM sleep was detected within the sleep blocks based upon characteristic marked increases in CBV in the absence of any head or body movement (21). These CBV increases with REM sleep have been previously reported in mice (18), and the correlation between these increases in CBV and REM sleep was verified with simultaneous EEG recording (Fig. 1). High-frequency sampling of the CBV also allowed for extraction of HR and beat to beat detection, as previously described (21, 22).

Data and statistics

To consider shifts in timing of events relative to the 24-h light–dark cycle, some of the data are analyzed and represented in a circular fashion. For all sleep states (i.e. the awake, NREM sleep, and REM sleep states), the distribution over 24 h was highly non-uniform (for all states $P<<0.001$, Rayleigh test for unitary circular distribution) as expected. To calculate the average time point when a state (NREM sleep, REM sleep, and awake) occurs, states were considered every second, and average time points were calculated as the direction of the mean vector calculated from all occurrences of a certain state (Fig. 1b). For further calculation, this time point was represented as a unitary vector for that animal and condition. To determine average time points across animals, unitary vectors for each animal (and condition) are taken and averaged as vectors (Fig. 2). The spread on this data is expressed as circular SD (calculated from the average vector length).

To determine start/finish of the rest and active phases, the probability of being in the awake state was determined for each condition in each animal at every second of 24 h. To further smooth this distribution, a repeated (500×) rolling window (5 min) was used. The start of the active phase was defined as the time point where the probability of being in the awake state becomes more than 50% closest to switch from light to dark. The start of the rest phase was defined as the time point where the probability of being in the awake state becomes less than 50% closest to switch from dark to light.

Data analysis was performed using automated procedures, written in Python 3 (www.python.org) or Igor (Wavemetrics). For data visualization and analysis in Python 3, the numpy (www.numpy.org), SciPy (www.scipy.org), pandas (pandas.pydata.org), dask (dask.org), pingouin (pingouin-stats.org), and Holoviews (holoviews.org) modules were used. Distribution of the data was assessed using kernel density plots. To compare multiple parameters, repeated measures ANOVA and Kruskal were used depending on the distribution. Data are shown as mean ± SD, unless reported differently. Correlations were calculated using repeated measures correlation (46). This approach accounts for nonindependence in observations by using analysis of covariance (ANCOVA) to adjust for interindividual variability. Degrees of freedom were calculated using $N(k-1)-1$ where N is the number of animals and k the number of observations within each animal. Power was calculated as 1 − type II error (25). Linear trend lines are sometimes added for visualization but not used for further analysis, unless a clear linear relationship could be assumed. $P<0.05$ was considered significant. With multiple comparisons, P-values were adjusted using Benjamini/Hochberg false discovery rate correction. For circular data analysis (i.e. time points in daily cycles), averages were calculated using vector averaging and expressed ± circular SD. To compare circular data for two conditions (control vs 0.6 caffeine), the Moore test was used (paired nonparametric test for circular data).

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Author contributions

D.Y.Y, G.C.F., and A.C. conceived of experiments; K.A., D.Y.Y, and S.N. performed experiments; K.A., D.Y.Y, S.R., G.C.F., and A.C. performed data analysis, D.Y.Y, S.R., and G.C.F. developed hardware and software; all contributed to writing of the paper.

Data availability

All data generated during and/or analyzed during the current study and code used in the analysis will be available on the Dandi archive (https://dandiarchive.org/dandiset).

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