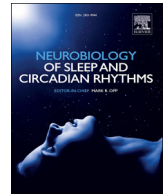




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Research paper

## Electrophysiological characterization of sleep/wake, activity and the response to caffeine in adult cynomolgus macaques<sup>☆</sup>

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## ABSTRACT

Most preclinical sleep studies are conducted in nocturnal rodents that have fragmented sleep in comparison to humans who are primarily diurnal, typically with a consolidated sleep period. Consequently, we sought to define basal sleep characteristics, sleep/wake architecture and electroencephalographic (EEG) activity in a diurnal non-human primate (NHP) to evaluate the utility of this species for pharmacological manipulation of the sleep/wake cycle. Adult, 9–11 y.o. male cynomolgus macaques ( $n = 6$ ) were implanted with telemetry transmitters to record EEG and electromyogram (EMG) activity and Acticals to assess locomotor activity under baseline conditions and following injections either with vehicle or the caffeine (CAF; 10 mg/kg, i.m.) prior to the 12 h dark phase. EEG/EMG recordings (12–36 h in duration) were analyzed for sleep/wake states and EEG spectral composition. Macaques exhibited a sleep state distribution and architecture similar to previous NHP and human sleep studies. Acute administration of CAF prior to light offset enhanced wakefulness nearly 4-fold during the dark phase with consequent reductions in both NREM and REM sleep, decreased slow wave activity during wakefulness, and increased higher EEG frequency activity during NREM sleep. Despite the large increase in wakefulness and profound reduction in sleep during the dark phase, no sleep rebound was observed during the 24 h light and dark phases following caffeine administration. Cynomolgus macaques show sleep characteristics, EEG spectral structure, and respond to CAF in a similar manner to humans. Consequently, monitoring EEG/EMG by telemetry in this species may be useful both for basic sleep/wake studies and for pre-clinical assessments of drug-induced effects on sleep/wake.

### 1. Introduction

The cyclic alternation between wakefulness and sleep appears to be ubiquitous among mammals (Siegel, 2005; Lesku et al., 2008) and, over the last several decades, has also been recognized to occur in other vertebrates (Beckers and Rattenborg, 2015; Shein-Idelson et al., 2016; Oikonomou and Prober, 2017) as well as invertebrates including molluscs (Vorster et al., 2014), flies (Artiushin and Sehgal, 2017), nematodes (Trojanowski and Raizen, 2016) and ctenophores (Nath et al., 2017). Among mammals, the sleep/wake patterns of non-human primates (NHPs) more closely resemble the consolidated periods of sleep and wakefulness of humans than do the polyphasic sleep/wake cycles of

nocturnal rodents. Among NHPs, sleep/wake patterns have previously been studied in squirrel monkeys (Edgar et al., 1993; Klerman et al., 1999), rhesus macaques (Daley et al., 2006; Hsieh et al., 2008; Darbin et al., 2009; Tannenbaum et al., 2016), juvenile cynomolgus macaques (Authier et al., 2014; Rachalski et al., 2014) and other species (Balzamo et al., 1977). The similar sleep/wake architecture of NHPs and humans has resulted in testing of preclinical compounds in NHPs for efficacy as well as safety prior to initiation of clinical studies (Uslaner et al., 2013; Tannenbaum et al., 2016).

The adenosine receptor antagonist caffeine (CAF) is undoubtedly the most widely-consumed, exogenous wake-promoting substance. Although CAF was initially thought to promote wakefulness by

**Abbreviations:** A1, Adenosine sub-type 1 receptor; A2a, Adenosine sub-type 2 receptor; CAF, Caffeine; EEG, Electroencephalogram; EMG, Electromyogram; i.m., Intramuscular; LMA, Locomotor activity; NHP, Non-human primate; NREM, Non-rapid eye movement; N1, NREM Stage 1; N2, NREM Stage 2; N3, NREM Stage 3; REM, Rapid eye movement; ROL, REM onset latency; SOL, Sleep onset latency; SEM, Standard error of mean; TST, Total sleep time; WASO, Wake after sleep onset

<sup>☆</sup> The work was performed at SRI International. No clinical trials were performed.

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antagonizing the adenosine A1 receptor (Virus et al., 1990; Benington et al., 1995), more recent studies have implicated the adenosine A2a receptor (Huang et al., 2005). However, at doses generally consumed by humans, CAF produces its arousing effects by partial nonselective blockade of adenosine A1 and adenosine A2a receptors (Einöther and Giesbrecht, 2013; Clark and Landolt, 2017). Adenosine levels in the brain are positively correlated with the previous amount of waking (Porkka-Heiskanen et al., 1997; 2003) and adenosine administration induces NREM sleep with high amplitude slow-waves (Schwierin et al., 1996; Benington et al., 1995). Hence, adenosine is one of the substances thought to be involved in homeostatic sleep regulation (Landolt, 2008). CAF is known to reduce or disturb sleep in a dose-dependent manner (Karacan et al., 1976; Hindmarch et al., 2000; Rosenthal et al., 1991; Yanik et al., 1987) and to reduce EEG slow wave activity in subsequent NREM sleep in humans and rodents (Carrier et al., 2009; Drapeau et al., 2006; Landolt et al., 1995a, 1995b, 2004; Schwierin et al., 1996). In squirrel monkeys, an adenosine receptor-mediated alerting effect of CAF is suggested by CAF attenuation of the dose-dependent decrease in lever-pressing under fixed interval schedules of food presentation induced by both selective as well as non-selective A1 and A2 adenosine receptor agonists (Howell and Byrd, 1993). In juvenile (2.5–3 y.o.) cynomolgus macaques, CAF (10–30 mg/kg, p.o.) has been reported to decrease the duration of NREM and REM sleep, reduce low frequency EEG activity (1–8 Hz) during wakefulness, and to increase high frequency EEG activity (20–50 Hz) during NREM sleep (Authier et al., 2014).

We have recently identified EEG correlates for correct responses during a sustained attention task in adult cynomolgus macaques (Goonawardena et al., 2016). Although NMDA receptor antagonists were used in that particular study, we have also assessed the EEG in conjunction with the improved performance produced by CAF on sustained attention tasks, which has led us to examine the effects on CAF on sleep/wake and EEG characteristics in greater detail. In the present study, we report the sleep/wake architecture of middle-aged adult (9–11 y.o.) cynomolgus macaques across the night and assess the acute effects of CAF on nocturnal sleep. At the dose tested, CAF caused elevated wakefulness throughout the 12 h dark phase which led us to conduct a follow up 36 h recording after CAF administration. To our surprise, we found no evidence of sleep homeostasis despite a prolonged period of CAF-induced wakefulness during the 12 h dark phase.

## 2. Materials and methods

### 2.1. Animals

Male cynomolgus macaques (*Macaca fascicularis*; 9–11 years old; 7–10 kg) were maintained in constant environmental conditions (temperature  $21 \pm 3^\circ\text{C}$ ; humidity 30–70%; 12:12 h light:dark cycle). All animals received a full daily regimen of standard certified commercial chow (Purina Animal Nutrition, Gray Summit, MO) supplemented with fresh fruit and vegetables and had access to water *ad libitum* in their home cage. Animals were individually housed but had visual, auditory and olfactory contact with other monkeys throughout the study, as well as access to chew toys. Videos and music were played in the housing room to provide environmental enrichment during the day. In accordance with Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and SRI guidelines, behavioral (e.g., activity level, locomotion, coordination) and clinical (e.g., appetite) signs were evaluated daily by a research scientist, particularly before and after surgery, and during the sleep studies. All efforts were made to minimize animal suffering and reduce the number of animals used. All procedures were reviewed and approved by the SRI International Animal Care and Use Committee (IACUC) and were in compliance with National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. SRI International is an AAALAC-accredited institution.

### 2.2. Surgical procedures

Animals were fully anesthetized [telazolone (4 mg/kg, i.m.) and 1% isoflurane] and placed on the surgical table in a dorsal recumbent position. Using aseptic surgical procedures, a longitudinal incision was made approximately 2 cm lateral to the *linea alba* by trained surgeons and a disk-shaped telemetry transmitter (D70-EEE; DSI, St-Paul, MN) was placed between the internal oblique muscle and the aponeurosis of the *transverse abdominis* muscle. The aponeurosis was sutured to hold the transmitter in place, the tips of EEG electrodes were individually sutured to prevent fluid from migrating up the leads, and the electrodes then were tunneled subcutaneously towards the back of the animal. The deeper layer of the abdominal incision was closed with a running subcutaneous pattern and, subsequently, the skin was closed with a running subcuticular pattern using absorbable sutures for both. Finally, several horizontal mattress stitches (3-0 non-absorbable Prolene; Ethicon US, LLC) were placed to remove tension from the incision.

Thereafter, the animal was placed into a stereotaxic frame and its head was secured with ear bars and a mouth/eye adaptor. The back and head/neck regions were aseptically prepared and a small skin incision was made on the back along the dorsal midline just below the scapular region. The fascia was bluntly dissected down the animal's flank to retrieve the telemetry transmitter electrodes. A final skin incision was made on the head along the midline from the orbital ridge posteriorly to the occipital notch. Using a long hemostat, the biopotential leads were tunneled from the back incision to the head incision. The underlying connective tissue and muscles were incised to expose the skull, and retractors were used to hold the skin and muscle in place. Utilizing a stereotaxic approach, EEG electrodes were placed in the left and right frontal cortices according to the 10–20 system in humans and referenced to electrodes placed over the occipital cortex (left Fp1: AP +18.0 mm, ML +15.0 mm; left Oz reference: AP –22.0 mm, ML +3.0 mm from bregma; right Fp2: AP +18.0 mm, ML –15.0 mm; right Oz reference: AP –22.0 mm, ML: –3.0 mm from bregma). Coordinates were marked with a sterile pen and 4 burr holes were drilled through the skull in which stainless steel screws were inserted until the tips were estimated to touch the *dura mater*. The exposed wire tips were then wrapped around the 4 screws and silver epoxy was applied over the wire/screws to ensure good electrical conductivity. Once the epoxy was fully dried (approx. 15–20 min), all wires/screws were insulated with dental cement (FuliCEM resin modified glass ionomer cement; Net 32 Inc., Cary, NC). The EMG electrodes were implanted bilaterally into the superficial neck musculature (*trapezius*) and secured in place with non-absorbable (3-0) sutures. Once the dental cement was fully dried, the muscle, connective tissue, intra-dermal layer and skin were sutured separately (simple interrupted, 3-0 absorbable). All animals received perioperative anesthetic monitoring, postoperative analgesia for  $\geq 72$  h and antibiotics for 10–14 d, and were allowed a minimum of 21 d recovery period before experiments.

### 2.3. Electrophysiological and locomotor activity (LMA) recording

Telemetry receiver boards (RPC-1; DSI, St. Paul, MN) were mounted individually to each home cage to facilitate real-time EEG/EMG recording. Continuous acquisition of the EEG and EMG telemetry signals occurred at a sampling rate of 500 Hz using DataQuest A.R.T. (version 4.36; DSI); the frequency range of the D70-EEE telemetry transmitters was 0.3–100 Hz. LMA (counts per 0.25 min) was collected through the use of Actical accelerometers (Philips Respironics, NV) that were attached to each animal's collar. The Acticals have a processor with a 32 Hz sampling rate that enables recording of the frequency and intensity of the subject's movements in the vertical, horizontal and diagonal planes (counts/s). Following each experiment, Acticals were removed from the collars and the data were downloaded using the Actical software (version 3.1; Philips Respironics, Murrysville, PA) for offline analysis. The counts/s were then averaged across 15 s intervals

(counts/0.25 min). All data are presented according to Zeitgeber Time (ZT) in which ZT0 = onset of lights and ZT12 = lights off.

#### 2.4. Experimental design and dosing procedures

All animals were acclimated to the dosing procedure for multiple sessions using 0.9% saline solution injections (i.m.) prior to start of each experiment. Three experiments were conducted:

**Experiment 1. Characterization of basal activity and sleep/wake states.** EEG, EMG and LMA were recorded from 6 adult male cynomolgus macaques during the 12 h dark phase without any pharmacological treatment.

**Experiment 2. Acute effects of CAF on sleep/wake during the 12 h dark phase.** In this experiment, the acute effects of CAF (10 mg/kg, i.m.) and vehicle (0.9% saline solution) administered 15 min prior to lights-off on EEG, EMG and LMA were assessed in 4 adult male cynomolgus macaques (subset of animals used in [Experiment 1](#)) during the 12 h dark phase. This dose was chosen to be comparable to the lower dose used in a previous study ([Authier et al., 2014](#); although dosing was p.o. rather than i.m. in that study), and to complement ongoing studies of CAF effects on sustained attention. A 7 d washout period elapsed between the two treatments. Since animals were housed and recorded in the same room, all animals received the same treatments on any given night to avoid the potential for sleep disruption by different treatment conditions (e.g., interference of sleep in vehicle-treated animals by wakefulness induced in CAF-treated animals). This procedure could result in disruption of sleep/wake patterns of the entire group caused by a single animal with a high sensitivity to caffeine.

**Experiment 3. : Effects of CAF on sleep/wake for 36 h post-injection.** [Experiment 2](#) revealed wake-promoting effects of CAF that persisted throughout the 12 h dark phase. Consequently, in [Experiment 3](#), the effects of CAF (10 mg/kg, i.m.) and vehicle (0.9% saline solution) administered 15 min prior to lights-off on EEG, EMG and LMA were assessed in 4 adult male cynomolgus macaques for 36 h post-treatment. A 7 d washout period occurred between the two treatments and, as in [Experiment 2](#), all animals received the same treatments on any given night. None of the animals had prior exposure to caffeine or any other stimulant; [Experiments 2 and 3](#) were the first pharmacological studies that were conducted following their recovery from EEG/EMG surgery. Approximately 3 months elapsed between [Experiments 2 and 3](#).

#### 2.5. Vigilance state analysis

Vigilance states were determined by visual inspection of EEG and EMG signals using Profusion software (Compumedics USA, Charlotte, NC) according to the American Academy of Sleep Medicine (AASM) guidelines for human sleep scoring ([Iber et al., 2007](#); [Silber et al., 2007](#)) and adapted for cynomolgus macaques. Trained investigators classified each 30 s epoch as one of five states: WAKE, non-rapid eye movement (NREM) sleep stages N1, N2, or N3, or rapid eye movement (REM) sleep. WAKE was scored when > 50% of the epoch consisted of alpha (8–13 Hz) activity or low amplitude, mixed frequency (2–7 Hz) activity and active EMG. N1 (Stage 1) was scored when 50% of the epoch consisted of relatively low amplitude, mixed frequency (2–7 Hz) activity and < 50% of the epoch contained alpha (8–13 Hz) activity accompanied with lower EMG activity. N2 (Stage 2) was scored when K-complexes (1–2 Hz isolated waves) and/or sleep spindles (regular 12–16 Hz EEG sequences) were observed and < 20% of the epoch contained high amplitude (> 75  $\mu$ V), low frequency (1–4 Hz) activity. N3 (Stages 3 + 4) was scored when  $\geq$  20% of the epoch consisted of high amplitude (> 75  $\mu$ V), low frequency (1–4 Hz) EEG waves (i.e., slow wave activity). REM was scored when the epoch contained relatively low voltage, mixed frequency activity with predominant theta

activity (4–9 Hz) accompanied with low EMG activity (i.e., atonia). Epochs with prominent muscular artifacts were identified and excluded from subsequent EEG spectral analysis as described below.

#### 2.6. EEG spectral analysis

Spectral analysis of EEG data was calculated offline using a Fast Fourier transform algorithm (MATLAB, Natick, MA) on all epochs without artifacts. Fourier analysis was limited to frequencies < 100 Hz due to the frequency bandwidth of the D70-EEE transmitters. Contiguous frequencies were binned into standard frequency bands (delta: 0.3–4 Hz; theta: 4–8 Hz; alpha: 8–12 Hz; sigma: 12–16 Hz; beta: 16–30 Hz; low gamma: 30–50 and high gamma: 50–100 Hz).

The following artifact removal steps were applied. Each 30 s epoch was divided in five 6 s sub-epochs. The median values of the total EEG power across all 6 s WAKE, NREM and REM sub-epochs for the 12 h dark phase following vehicle treatment were calculated. Median values were used since mean values can be disproportionately and asymmetrically affected by large artifact values. To detect non-physiological values in the EEG power both above the median (e.g., movement artifacts) and close to zero (e.g., EEG signal dropouts), separate indices of variation above and below the median were calculated for each state (WAKE, NREM and REM). For artifact removal above the median, the formula was:

$$F > F_{50} + [3*(F_{75} - F_{50})]$$

For artifact removal below the median, the formula was:

$$F < F_{50} + [3*(F_{25} - F_{50})]$$

where  $F$  = total EEG power of a 6 s subepoch and  $F_{25}$ ,  $F_{50}$  and  $F_{75}$  refer to the 25th, 50th and 75th percentiles of the total distribution of EEG power values. These values were calculated for each animal for the entire baseline for [Experiment 1](#). For [Experiments 2 and 3](#), these values were calculated for each animal during the 12 h dark phase following vehicle treatment and then applied to all 6 s sub-epochs for both vehicle and CAF conditions. Overall,  $7.3 \pm 2.5\%$  and  $9.2 \pm 1.5\%$  of epochs were removed as artifact from the recordings of the vehicle- and caffeine-treated animals, respectively, in [Experiments 2 and 3](#).

Spectral data for each 30 s epoch was comprised of the average of all artifact-free 6 s sub-epochs. For [Experiment 1](#), group mean absolute power for each frequency band (delta, theta, alpha, sigma, beta, low and high gamma) and relative power were calculated following the artifact removal process described above. In [Experiments 2 and 3](#), artifact-free absolute power following CAF administration was normalized to each animal's own 12 h light/dark average power following vehicle. Thereafter, the mean normalized power for each frequency band (delta, theta, alpha, sigma, beta, low gamma and high gamma) within each vigilance state (WAKE, NREM and REM) was calculated for each animal before group analyses occurred. Furthermore, the power spectra for the entire frequency range over time were performed for all vigilance states and conditions in all three experiments that were visualized as time-frequency spectrograms. In addition, for [Experiment 3](#), both delta power/delta energy and cumulative delta power/delta energy during NREM sleep were calculated across the 36 h recording period. Delta energy was calculated by multiplying NREM time and NREM delta power ([Franken et al., 1999](#); [Naylor et al., 2000](#); [Gerashchenko et al., 2008](#); [Morairty et al., 2013](#)).

#### 2.7. Data analysis

The duration of each vigilance state was calculated separately across the 12 h dark and light phases, both cumulatively and on an hourly basis (percent time) for each experiment. Sleep efficiency (%) was calculated as total sleep time/total time in darkness\*100. Sleep onset was defined as the occurrence of the first 30 s epoch of N2 sleep; thus, sleep onset latency (SOL) was assessed for each animal by

calculating the elapsed time from lights-off to the first 30 s epoch of N2 sleep. REM Onset Latency (ROL) was calculated as the duration from SOL to the first 30 s epoch of REM sleep. Wake after sleep onset (WASO) was assessed by summing the total time awake to the end of the 12 h dark or light phase subsequent to sleep onset. Similarly, the number of awakenings was calculated as the number of transitions from any sleep stage to a WAKE stage across the 12 h dark or light phase following sleep onset. The number of arousals was quantified by calculating the total number of wake-like arousals ( $> 3$  s and  $\leq 15$  s) with active EMG within any sleep stage after the sleep onset. REM/NREM sleep ratios and the number of NREM/REM cycles (i.e.,  $\geq 30$  s of REM sleep preceded by  $\geq 1$  min of N2 or N3 sleep was considered a NREM/REM cycle) for the 12 h dark and light phases were also determined. For the 36 h study, hourly cumulative change from vehicle was assessed for each vigilance state following treatment. Hourly LMA averages (counts/0.25 min) were assessed for each condition across all experiments. The mean actograms for each light/dark phase were calculated by summing all the counts per 15 s sample and dividing by the number of 15 s samples within that 12 h or 24 h period.

### 2.8. Statistical analysis

Data were analyzed using SigmaPlot (Systat Software Inc., San Jose, CA) and all animals underwent all treatment conditions. Tests for normality of the data were conducted for all experiments. All data are represented as mean  $\pm$  SEM. Statistical significance on the group means for all sleep/wake parameters during baseline (Experiment 1) or following either vehicle or CAF treatment (Experiments 2 and 3) were assessed for the respective 12 h dark/light phases using individual paired *t*-tests. In Experiments 2 and 3, the hourly percent time spent in WAKE, NREM and REM was compared using two-way repeated measures ANOVAs (factor 1: treatment, factor 2: time) followed by paired *post hoc t*-tests. For LMA, paired *t*-tests between vehicle and CAF were conducted for the 12 h dark phase (18:30 to 06:30) in Experiment 2 and for the two 12 h dark phases and intervening 12 h light phase (18:30 to 06:30) in Experiment 3. For spectral analysis, individual paired *t*-tests between vehicle and CAF were conducted independently for each frequency band for absolute and normalized power spectra. In all cases, significance was defined as  $p \leq 0.05$ . For Experiments 1 and 2, the vigilance state and spectral analyses described above were performed on the 12 h dark phases. In Experiment 3, the two 12 h dark phases and the intervening 12 h light phase were separately analyzed.

## 3. Results

### 3.1. Experiment 1: Characterization of basal activity and sleep/wake states

The average LMA collected from 4 male cynomolgus macaques recorded in their home cages over a 7 d period indicated a clear diurnal pattern with the highest activity levels occurring during the light phase whereas activity was essentially non-existent during the dark phase (Fig. 1). The tendency toward higher activity evident during the first half of the light phase relative to the second half of the light phase is likely due, in part, to the daily housekeeping and cage cleaning that occurred during the early part of the light phase while animals were in their home cages.

Representative EEG and EMG traces from an animal recorded during the 12 h dark phase with 30 s epochs classified as either WAKE, NREM (stages N2 and N3) or REM sleep are presented in Fig. 2A. Table 1 presents the overall means  $\pm$  SEMs for various sleep-wake architecture parameters recorded from the macaques during the 12 h dark phase under baseline conditions. On average, macaques had a sleep efficiency of  $83.0 \pm 1.7\%$  during the dark phase with a SOL of  $14.0 \pm 1.9$  min and ROL of  $43.0 \pm 13.7$  min. Of the  $597.8 \pm 12.0$  min of total sleep time (TST), REM sleep was approximately 14% of TST whereas NREM sleep was 86%. Of the  $515.5 \pm 25.7$  min of NREM sleep, N1 comprised 5.2%,

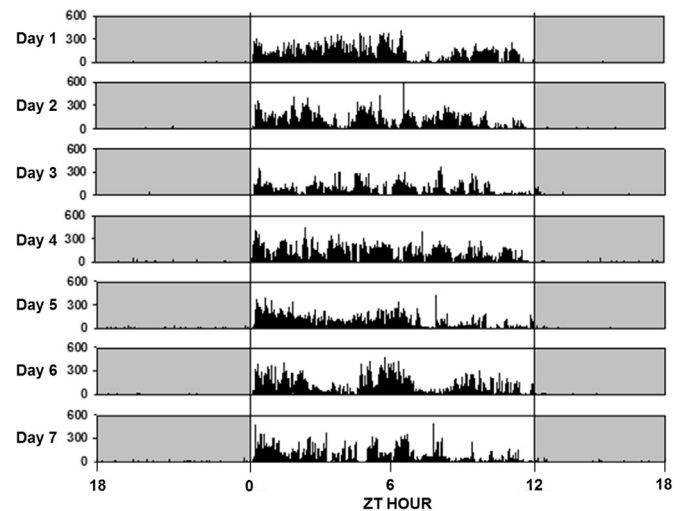


Fig. 1. Mean locomotor activity (LMA) measured by Acticals attached to collars of cynomolgus macaques. Mean activity data were collected over a 7 d period ( $n = 4$ ). Macaques show consistently high activity during the 12 h 'lights on' period and low activity during the 12 h 'lights off' period. LMA is measured in counts/0.25 min.

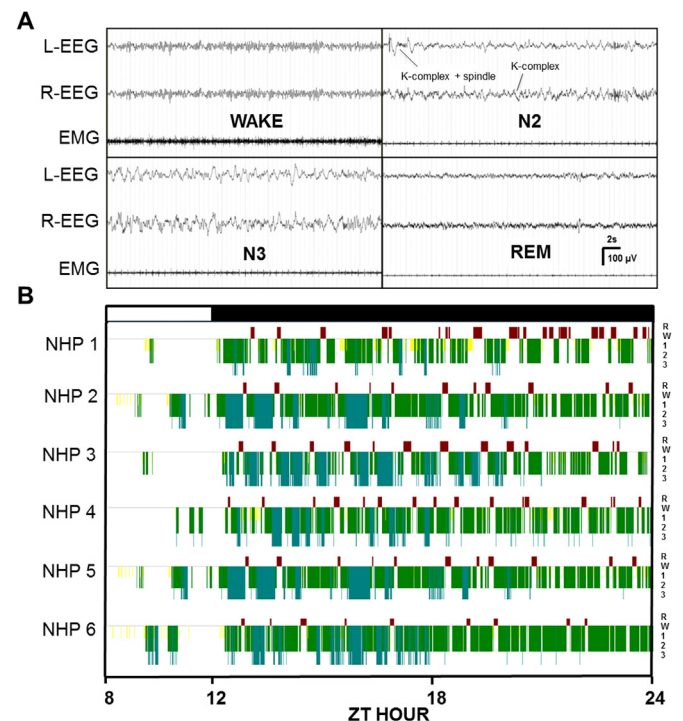


Fig. 2. Sleep-wake stages in cynomolgus macaques. (A) Representative EEG (Left- and Right-frontal cortices) and EMG (neck muscle) bipolar recordings during 30 s epochs scored as WAKE, showing characteristic low amplitude alpha (8–13 Hz) waves and high EMG activity (top left); NREM stage 2 (N2), characterized by the occurrence of K-complexes, sleep spindles (12–16 Hz) and lower EMG activity (top right); NREM stage 3 (N3), with high voltage, low frequency synchronous delta (1–4 Hz) waves (bottom left); and REM sleep, with low voltage, mixed frequencies and complete muscle atonia (bottom right). (B) Hypnograms from 6 macaques recorded for  $\sim 14$  h, including a 12 h dark period (ZT12 to ZT24). R = REM (red); W = WAKE (flat line); 1 = stage N1 (yellow); 2 = stage N2 (green); 3 = stage N3 (blue).

N2 (with K-complexes and sleep spindles similar to that observed in humans) was 72.3%, and N3 was 22.5%. On average, animals were awake for  $87.9 \pm 12.0$  min after sleep onset with  $51.7 \pm 5.0$  awakenings (i.e., transitions from any sleep stage to WAKE after sleep onset)

**Table 1**  
Sleep/wake characteristics of cynomolgus macaques during a 12 h recording in the dark phase ( $n = 6$ ).

Sleep-wake parameters	Mean $\pm$ SEM
Time in darkness (min)	720
Total Sleep Time (TST; min)	597.8 $\pm$ 12.0
Sleep Efficiency (%)	83.0 $\pm$ 1.7
Sleep Onset Latency (SOL; min)	14.0 $\pm$ 1.9
REM Onset Latency (ROL; min)	43.0 $\pm$ 13.7
Wake After Sleep Onset (WASO; min)	87.9 $\pm$ 12.0
REM Sleep Duration (min)	82.3 $\pm$ 15.4
Stage N1 NREM Duration (min)	26.8 $\pm$ 8.2
Stage N2 NREM Duration (min)	372.8 $\pm$ 28.8
Stage N3 NREM Duration (min)	116.0 $\pm$ 20.3
Cumulative NREM Sleep Duration (min)	515.5 $\pm$ 25.7
REM / NREM Ratio	0.169 $\pm$ 0.041
Awakenings (#)	51.7 $\pm$ 5.0
Arousals (#)	69.0 $\pm$ 5.4
NREM / REM Cycles (#)	11.5 $\pm$ 0.8

and 69.0  $\pm$  5.4 arousals (i.e., wake > 3 s and  $\leq$  15 s) during the 12 h dark phase. The mean number of NREM/REM cycles per night was 11.5  $\pm$  0.8.

Fig. 2B presents hypnograms for each of the 6 NHPs and illustrates that Stage 3/4 (N3) sleep primarily occurred in the first half of the dark phase whereas Stage 2 (N2) occurred throughout the night and was more prominent in the latter half of the recording. These observations are consistent with a recent study that also used telemetry to investigate sleep-wake characteristics in juvenile cynomolgus macaques (Rachalski et al., 2014).

Spectral analysis for each of the vigilance states was calculated over the 12 h dark phase and is shown in Fig. 3. Fig. 3A presents the absolute (log) power spectral profile (0.3–100 Hz) of artifact-free epochs for WAKE, NREM and REM sleep; Fig. 3B presents this information binned by frequency band within each vigilance state. As expected, relative delta power (Fig. 3C) predominated during NREM sleep (75.0  $\pm$  3.2%) in comparison to REM sleep (60.4  $\pm$  3.8%) and WAKE (68.6  $\pm$  5.0%). Similarly, the relative theta power was predominant during REM (17.7  $\pm$  1.6%) in comparison to NREM sleep (14.8  $\pm$  1.6%) and WAKE (11.3  $\pm$  1.7%). Furthermore, alpha, beta and gamma power during WAKE were all greater than NREM sleep (alpha: 11.1  $\pm$  3.4% [WAKE] vs 6.1  $\pm$  1.2% [NREM]; beta: 2.8  $\pm$  0.3 [WAKE] vs 1.1  $\pm$  0.2% [NREM] and gamma: 1.0  $\pm$  0.5% [WAKE] vs 0.15  $\pm$  0.05% [NREM]; Fig. 3C).

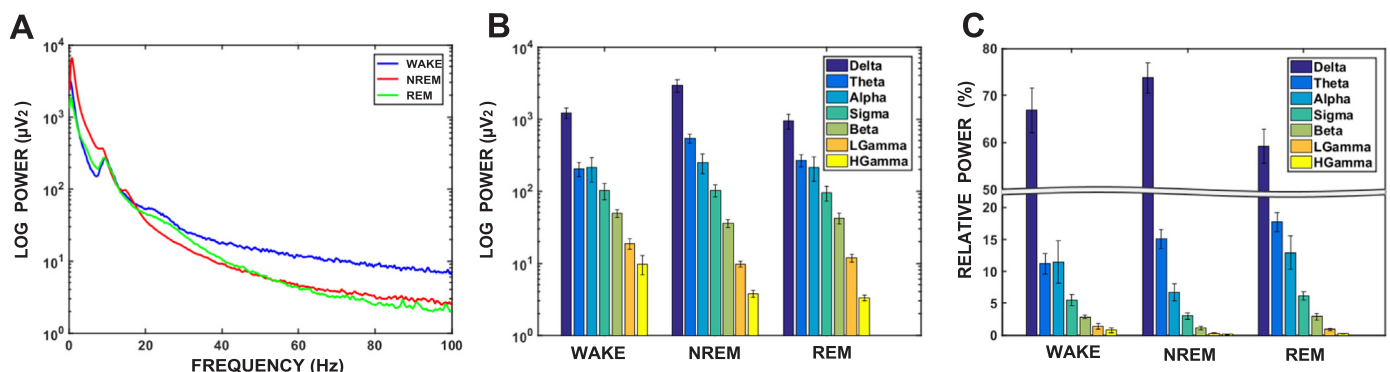
### 3.2. Experiment 2: Acute effects of CAF on sleep/wake during the 12 h dark period

Having established the basal sleep/wake characteristics during the

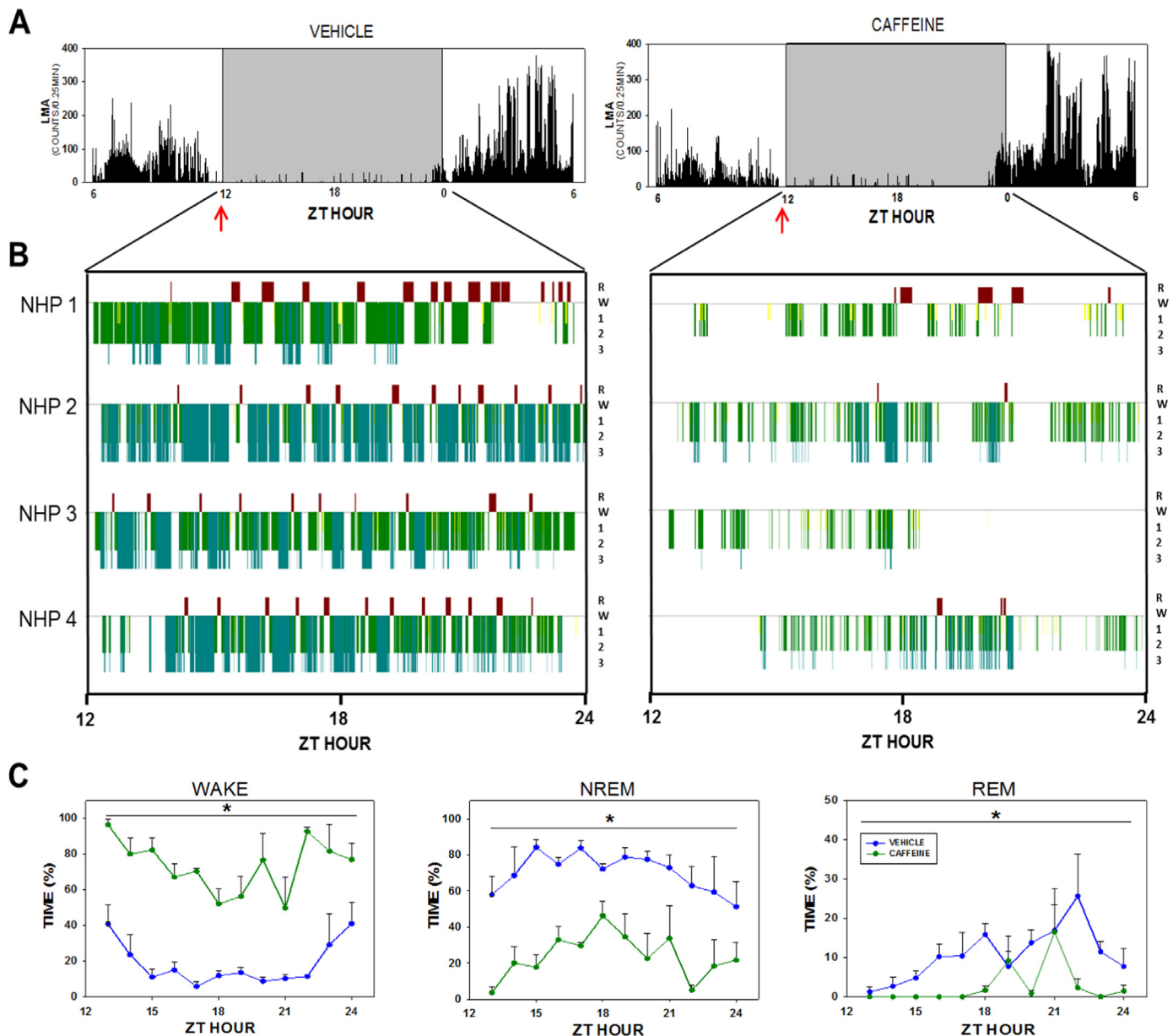
12 h dark period in Experiment 1, we next sought to determine whether the wake-promoting agent CAF was effective in prolonging wakefulness in male cynomolgus macaques when administered at a time of day when the homeostatic drive to sleep is high. Intramuscular injections of CAF (10 mg/kg;  $n = 4$ ) were administered 15 min prior to lights off. Although LMA in their home cages was unchanged during the subsequent 12 h dark period (Fig. 4A), TST was reduced to about 1/3 of the TST of vehicle-injected animals (Table 2;  $t = 9.31$ ;  $p < 0.01$ ). Following vehicle treatment, macaques showed a SOL of 12.6  $\pm$  2.8 min after light offset, 120.8  $\pm$  11.8 min of wakefulness after sleep onset, and a sleep efficiency of 81.0  $\pm$  1.9% during the 12 h dark period (Table 2). Similar to the baseline study, REM sleep was about 13% and NREM sleep was about 87% of TST in the vehicle-treated animals and there were 11.0  $\pm$  0.7 NREM/REM cycles per night. Of the 506.4  $\pm$  30.0 min of NREM sleep, N1 comprised 3.2%, N2 was 50.5%, and N3 was 46.3%. As in baseline, N3 sleep also predominated during the first half of the night while more consolidated REM sleep was evident towards the latter part of the 12 h dark period (Fig. 4B). Vehicle-injected controls showed a ROL of 92.1  $\pm$  22.2 min.

Fig. 4C presents the hourly distribution of WAKE, NREM and REM sleep during the 12 h dark period for both vehicle- and CAF-injected animals and Table 2 presents the effects of CAF on multiple sleep parameters in cynomolgus macaques. Consistent with the stimulant properties of the drug, CAF-treated animals showed significant decreases in TST ( $p < 0.01$ ), sleep efficiency ( $t = 9.31$ ;  $p < 0.01$ ), the amounts of both REM ( $t = 7.22$ ;  $p < 0.01$ ) and NREM ( $t = 7.60$ ;  $p < 0.01$ ) sleep and a converse increase in wakefulness ( $t = 9.31$ ;  $p < 0.01$ ) during the 12 h dark period. In particular, CAF-treated animals showed a reduction in N3 sleep compared to controls (Table 2;  $t = 3.44$ ;  $p < 0.05$ ). Although the difference was not statistically significant due to high variation in the effect of CAF among animals, there was a trend ( $p = 0.10$ ) for CAF to prolong SOL compared to controls (73.8  $\pm$  27.7 vs 12.6  $\pm$  2.8 min). CAF-treated animals also showed a significant increase in ROL ( $t = 13.00$ ;  $p < 0.01$ ), WASO ( $t = 5.75$ ;  $p < 0.01$ ) and fewer NREM/REM cycles ( $t = 22.05$ ;  $p < 0.001$ ) during the 12 h dark period. These results are consistent with the conclusion that sleep is disrupted by CAF in macaques.

Spectral power within the conventional frequency bands was calculated for each vigilance state during the 12 h dark period following vehicle and CAF administration (Fig. 5). Fig. 5A shows the absolute power spectral profile from 0.3–100 Hz for WAKE, NREM and REM sleep. CAF significantly reduced absolute delta ( $t = 7.45$ ;  $p < 0.05$ ), theta ( $t = 5.43$ ;  $p < 0.05$ ), and high gamma power ( $t = 9.35$ ;  $p < 0.05$ ) during wakefulness (Fig. 5B). When absolute power values for CAF treatment were normalized to the vehicle condition for each animal (Fig. 5C and D), CAF-treated animals also showed significant decreases in delta ( $t = 5.21$ ;  $p < 0.01$ ), theta ( $t = 15.2$ ;  $p < 0.01$ ), and high gamma power ( $t = 6.70$ ;  $p < 0.01$ ) during wakefulness as well as



**Fig. 3.** EEG spectral power analyses under baseline conditions in cynomolgus macaques ( $n = 6$ ). (A) Absolute (Log) power from 0.3–100 Hz for WAKE, NREM and REM sleep during the 12 h dark period. (B) The mean ( $\pm$  SEM) of absolute (Log) power and (C) Relative power for delta, theta, alpha, sigma, beta, low gamma and high gamma (left to right) for each vigilance state during the 12 h dark period.



**Fig. 4.** Effects of caffeine (10 mg/kg, i.m.) on sleep/wake during the dark period in cynomolgus macaques. (A) Mean locomotor activity (LMA) across a 24 h light/dark period measured by Acticals attached to collars of macaques ( $n = 4$ ) treated with either vehicle or caffeine. Red arrows indicate the time of injections. (B) Hypnograms of 4 macaques recorded during the 12 h dark period (ZT12 to ZT24) after vehicle (left) and caffeine (right) treatment. (C) Hourly distribution of WAKE, NREM and REM sleep during the 12 h dark period in macaques treated with either vehicle or caffeine. Values are mean  $\pm$  SEM ( $n = 4$ ). \* = caffeine is significantly different from vehicle ( $*p \leq 0.05$ ). [R=REM (red); W=WAKE (flat line); 1=stage N1 (yellow); 2=stage N2 (green); 3=stage N3 (blue)].

reductions in delta power ( $t = 8.17$ ;  $p < 0.05$ ) and increases in beta ( $t = 16.17$ ;  $p < 0.01$ ) and low gamma power ( $t = 4.69$ ;  $p < 0.05$ ) during NREM sleep. Hence, CAF has a complex state-dependent effect on EEG spectral activity whereby it reduces low frequency and very high frequency activity during wakefulness and increases moderately high frequency activity during NREM sleep. Epoch-by-epoch analysis across the entire frequency range showed that the CAF-induced effect was prominent throughout the 12 h dark phase and no transient changes across time were evident (data not shown).

### 3.3. Experiment 3: Effects of CAF on sleep/wake for 36 h post-injection

As illustrated in Fig. 4C, the wake-promoting effects of CAF persisted throughout the 12 h dark period with little indication of rebound sleep during that night. To investigate this further, the effects of an

identical dose of CAF (10 mg/kg, i.m.) were assessed in the same animals ( $n = 4$ ) across a 36 h period to determine whether the results illustrated in Fig. 4C during the first post-injection night were replicable and, secondly, to determine whether a sleep rebound occurred during the subsequent 24 h period. Both vehicle- and CAF-treated animals showed a robust activity rhythm with peak activity occurring during the light phase (Fig. 6A), as expected for this diurnal species. In contrast to Experiment 2, CAF produced a significant increase in LMA ( $t = 3.31$ ;  $p < 0.05$ ) during the initial 12 h dark period (night 1) although no significant differences in LMA occurred during the subsequent 12 h light (day 1) or dark (night 2) periods.

During night 1, the vehicle-treated group showed a total of  $525 \pm 26.5$  min of TST, which was comparable to the  $583.4 \pm 13.4$  min of TST for the vehicle-treated animals in Experiment 2 (Tables 2 and 3). The vehicle-treated group had a SOL of  $18.8 \pm 5.8$  min measured from

**Table 2**  
Sleep/wake characteristics of cynomolgus macaques during the dark phase after administration of vehicle or caffeine (10 mg/kg, i.m.) 15 min before light offset in Experiment 2 (n = 4).

Sleep-wake parameters	Vehicle (Mean ± SEM)	Caffeine (Mean ± SEM)
Time in darkness (min)	720	720
Total Sleep Time (TST; min)	583.5 ± 13.4	190.6 ± 40.2*
Sleep Efficiency (%)	81.0 ± 1.9	26.5 ± 5.6*
Sleep Onset Latency (SOL; min)	12.6 ± 2.8	73.8 ± 27.7
REM Onset Latency (ROL; min)	92.1 ± 22.2	280.2 ± 9.7*
Wake After Sleep Onset (WASO; min)	120.8 ± 11.8	455.6 ± 56.0*
REM Sleep Duration (min)	77.0 ± 20.7	19.1 ± 12.8*
Stage N1 NREM Duration (min)	16.5 ± 4.3	18.9 ± 3.3
Stage N2 NREM Duration (min)	255.5 ± 57.0	119.9 ± 25.6
Stage N3 NREM Duration (min)	234.5 ± 73.4	32.8 ± 17.8*
Cumulative NREM Sleep Duration (min)	506.4 ± 30.0	171.5 ± 43.8*
REM / NREM Ratio	0.161 ± 0.054	0.161 ± 0.131
Awakenings (#)	49.3 ± 1.9	77.5 ± 11.2
Arousals (#)	78.0 ± 11.3	58.2 ± 17.2
NREM / REM Cycles (#)	11.0 ± 0.7	2.0 ± 0.8*

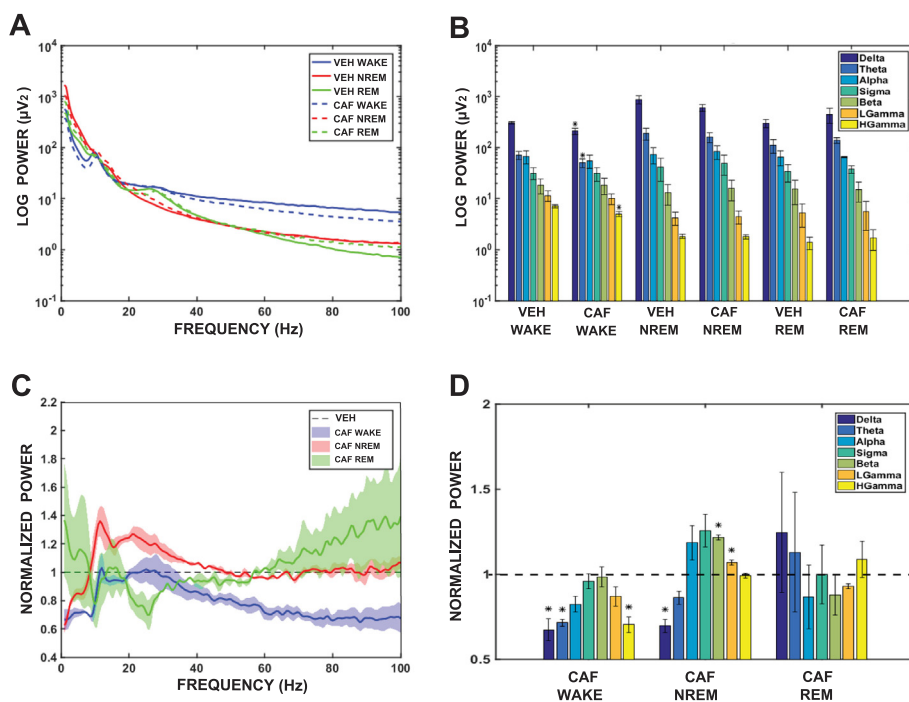
\* p ≤ 0.05 vs. vehicle

light offset and a WASO of 175.9 ± 23.2 min with an overall sleep efficiency of 72.9 ± 3.7% during the initial 12 h dark period (night 1; Table 3). REM sleep was 17.2% and NREM sleep was 82.8% of TST in the vehicle-treated animals and, similar to Experiment 2, there were 12.0 ± 1.9 NREM/REM cycles per night (Table 3). Of the 434.9 ± 26.5 min of NREM sleep during night 1, N1 comprised 2.3%, N2 was 75.0%, and N3 was 22.7%. The vehicle-treated control group showed a ROL of 83.4 ± 41.2 min, similar to that observed in Experiment 2. As in both Experiments 1 and 2, the animals had more N3 sleep during the first half of the dark period following vehicle (Fig. 6B).

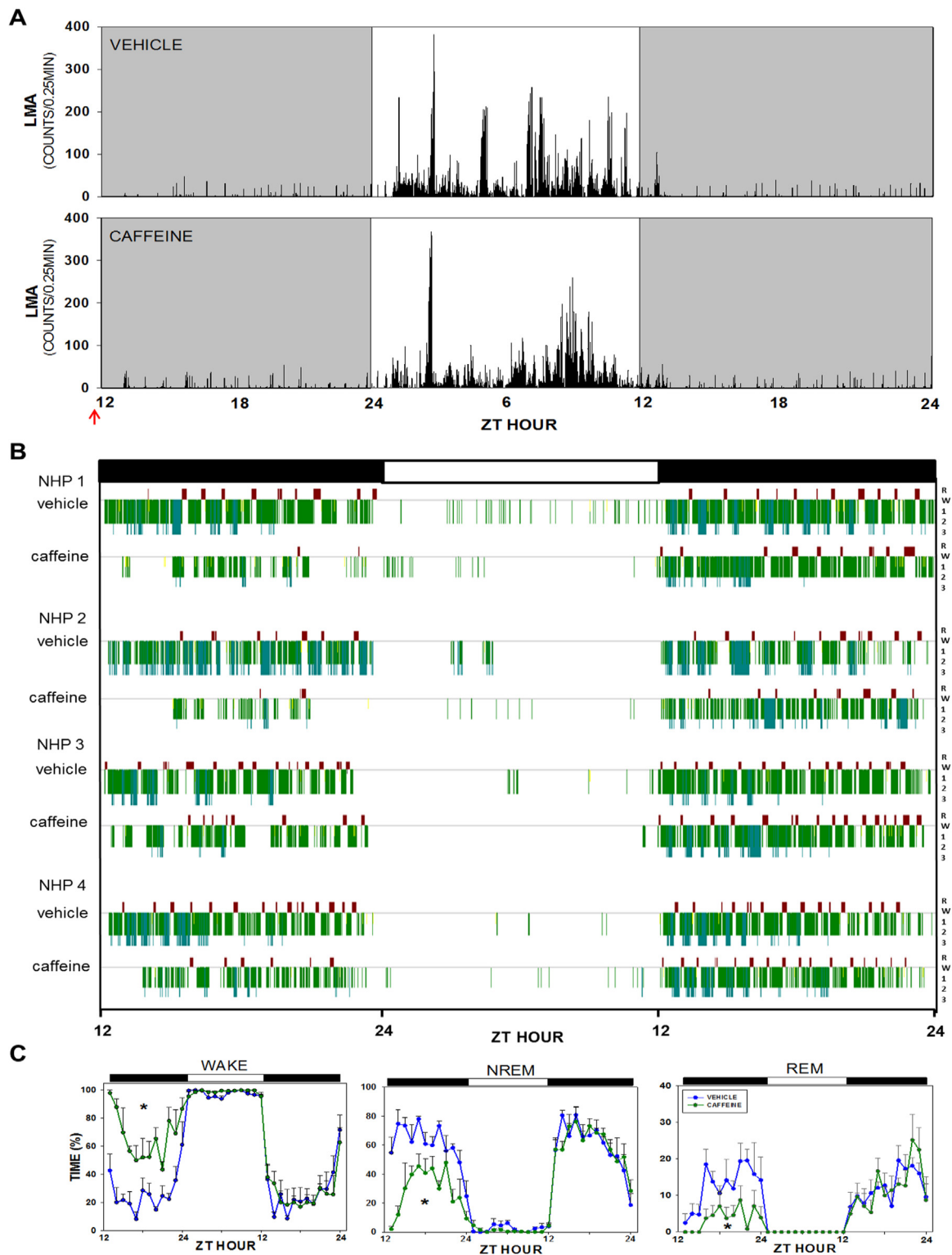
Most of the CAF-induced effects on sleep-wake parameters observed in Experiment 2 were replicated during night 1. As indicated in Table 3, CAF significantly decreased TST (t = 5.06; p < 0.05), sleep efficiency (t = 5.06; p < 0.05), the amounts of both REM (t = 7.94; p < 0.01) and NREM (t = 3.96; p < 0.05) sleep and, conversely, increased wakefulness (t = 5.06; p < 0.01) during night 1. Fig. 6C presents the hourly distributions of WAKE, NREM and REM sleep for the 36 h post-injection for both the vehicle- and CAF-injected animals. As in Experiment 2,

CAF-treated animals had reduced N3 sleep duration compared to the vehicle-treated group (Table 3; t = 2.86; p < 0.05) during night 1. Also consistent with Experiment 2, there were trends for CAF to delay SOL (100.5 ± 34.7 vs 18.8 ± 5.8 min; p = 0.072) and ROL (250.3 ± 70.7 vs 83.4 ± 41.2 min; p = 0.086) in comparison to the vehicle-treated group during night 1. The CAF-treated group also had more WASO (t = 4.01; p < 0.05) but fewer arousals (t = 4.63; p < 0.01) compared to controls, fewer NREM/REM cycles (t = 7.83; p < 0.01) and a smaller REM/NREM ratio (t = 3.46; p < 0.05) during night 1. Along with the data in Experiment 2, these results are consistent with the conclusion that CAF is disruptive to sleep in macaques.

In contrast to the acute effects on night 1, no significant differences were observed in any sleep-wake parameter between the CAF-treated and vehicle-treated groups during the subsequent 12 h light phase (Table 3). As expected, both treatment groups slept significantly less during the light phase than the previous night. TST (17.6 ± 5.7 min in vehicle-treated and 9.3 ± 3.1 min in CAF-treated animals) consisted of NREM sleep without any REM sleep during the 12 h light phase. Hence,



**Fig. 5.** Caffeine effects on EEG spectra recorded during the 12 h dark period in cynomolgus macaques. (A) Absolute (Log) power spectrum from 0.3–100 Hz and (B) power in the delta, theta, alpha, sigma, beta, low gamma and high gamma bands (left to right) for WAKE, NREM and REM sleep during the 12 h dark period following vehicle or caffeine (10 mg/kg; i.m.) treatment. (C) Normalized power from 0.3–100 Hz and (D) corresponding delta, theta, alpha, sigma, beta, low gamma and high gamma bands (left to right) for WAKE, NREM and REM sleep during the 12 h dark period following vehicle or caffeine treatment. Values are mean ± SEM (n = 3). \* = Caffeine is significantly different from vehicle (\*p ≤ 0.05).



**Fig. 6.** Effects of caffeine on sleep/wake over a 36 h period in cynomolgus macaques. **(A)** Mean locomotor activity (LMA) across a 36 h light/dark period measured by Acticals attached to collars of macaques ( $n = 4$ ) treated with either vehicle or caffeine. Red arrows indicate the time of injections. **(B)** Hypnograms for 4 macaques continuously recorded over a 36 h period following treatment with either vehicle or caffeine (10 mg/kg, i.m.). **(C)** Hourly distribution of WAKE, NREM and REM sleep during the 36 h period in macaques treated with either vehicle or caffeine. Values are mean  $\pm$  SEM ( $n = 4$ ). \* = caffeine is significantly different from vehicle during the initial 12 h dark period (\* $p \leq 0.05$ ). [R=REM (red); W=WAKE (flat line); 1=stage N1 (yellow); 2=stage N2 (green); 3=stage N3 (blue)].

sleep efficiency was low for both treatment groups:  $2.4 \pm 0.8\%$  and  $1.3 \pm 0.4\%$  for the vehicle- and CAF-treated groups, respectively. Values for SOL (vehicle-treated:  $221.5 \pm 63.8$  min; CAF-treated:  $229.0 \pm 157.6$  min) and WASO (vehicle-treated:  $480.9 \pm 58.8$  min;

CAF-treated:  $481.8 \pm 155.6$  min) were similar for both groups during day 1 (Table 3).

No significant difference in any sleep-wake parameter was observed between CAF- and vehicle-treated groups during night 2 (Table 3). Both

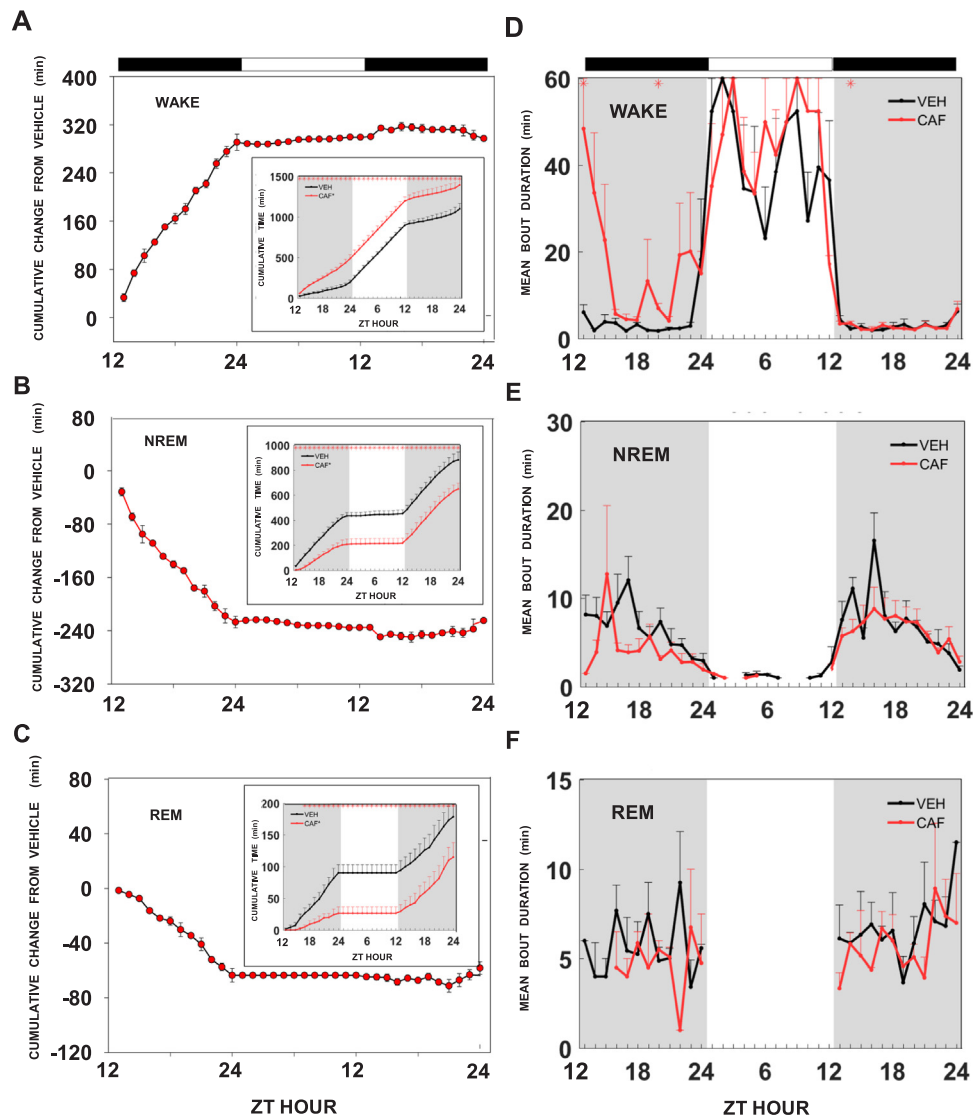


**Table 3**

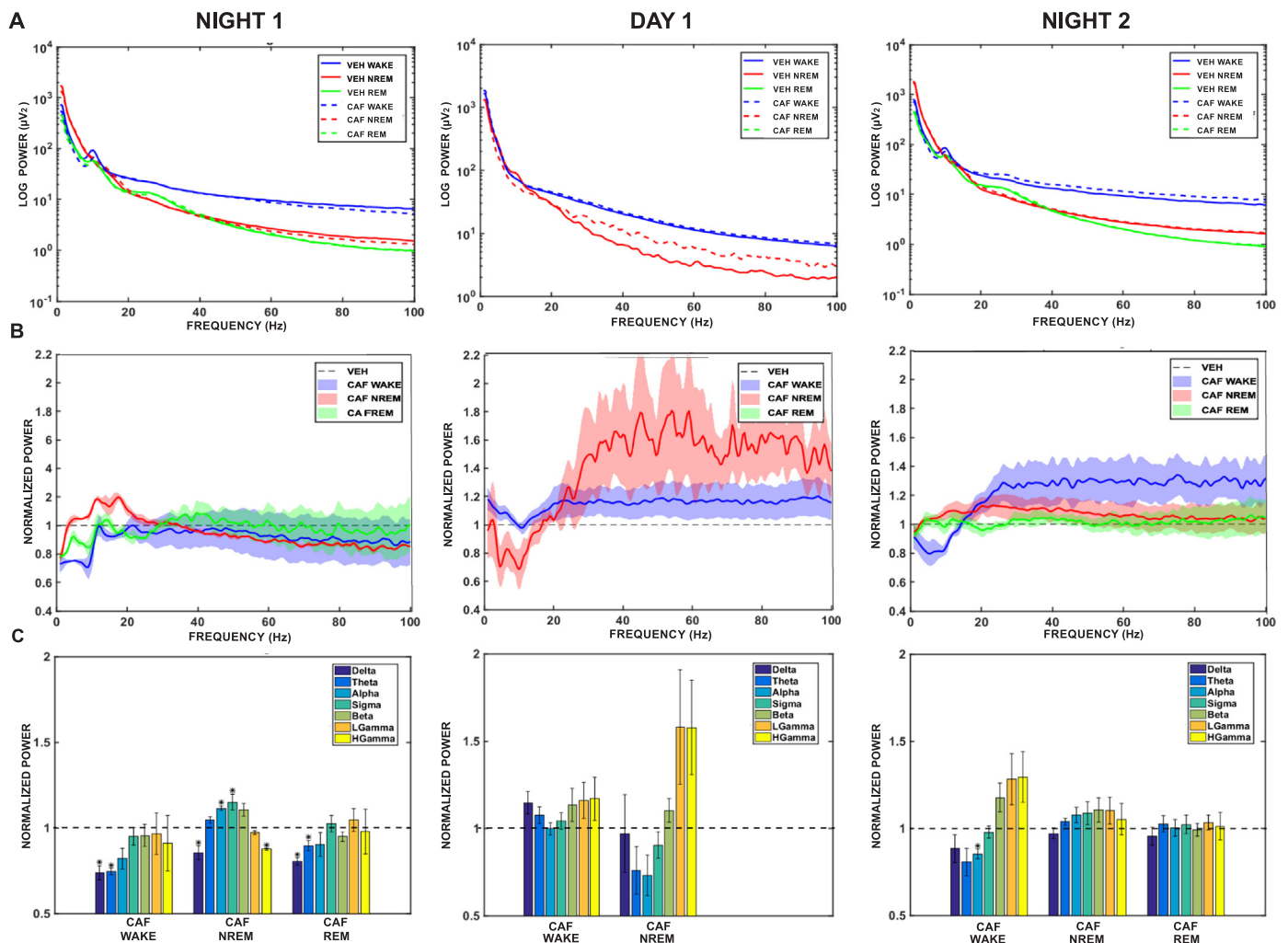
Sleep/wake characteristics of cynomolgus macaques for 36 h after administration of vehicle or caffeine (10 mg/kg, i.m.) 15 min before light offset in **Experiment 3** ( $n = 4$ ).

Sleep-wake parameters	Night 1		Day 1		Night 2	
	Vehicle	Caffeine	Vehicle	Caffeine	Vehicle	Caffeine
Time in dark or light (min)	720	720	720	720	720	720
Total Sleep Time (TST; min)	525.0 ± 26.5	234.5 ± 49.6*	17.6 ± 5.7	9.3 ± 3.1	518.1 ± 43.4	521.6 ± 34.2
Sleep Efficiency (%)	72.9 ± 3.7	32.6 ± 6.9*	2.4 ± 0.8	1.3 ± 0.4	72.0 ± 6.0	72.4 ± 4.7
Sleep Onset Latency (SOL; min)	18.8 ± 5.8	100.5 ± 34.7	221.5 ± 63.8	229.0 ± 157.6	8.8 ± 2.6	11.4 ± 5.0
REM Onset Latency (ROL; min)	83.4 ± 41.2	250.3 ± 70.7	–	–	50.4 ± 17.4	34.5 ± 29.4
Wake After Sleep Onset (WASO; min)	175.9 ± 23.2	385.0 ± 37.3*	480.9 ± 58.8	481.8 ± 155.6	193.1 ± 41.5	187.0 ± 30.2
REM Sleep Duration (min)	90.1 ± 13.1	26.6 ± 9.9†	–	–	88.6 ± 6.5	88.5 ± 14.9
Stage N1 NREM Duration (min)	9.8 ± 1.6	10.0 ± 2.8	1.0 ± 0.4	0.3 ± 0.1	8.5 ± 2.6	8.8 ± 0.5
Stage N2 NREM Duration (min)	326.3 ± 44.6	185.4 ± ± 38.3	15.1 ± 5.7	8.5 ± 3.1	335.6 ± 48.9	356.9 ± 32.7
Stage N3 NREM Duration (min)	98.9 ± 33.0	12.5 ± 2.4*	1.5 ± 1.5	0.5 ± 0.5	85.4 ± 21.5	67.5 ± 10.6
Cumulative NREM Sleep Duration (min)	434.9 ± 26.5	207.9 ± 40.5*	17.6 ± 5.7	9.3 ± 3.1	429.5 ± 41.1	433.1 ± 23.1
REM / NREM Ratio	0.211 ± 0.038	0.118 ± 0.027*	–	–	0.211 ± 0.019	0.203 ± 0.029
Awakenings (#)	94.5 ± 19.0	76.5 ± 6.1	21.8 ± 8.8	12.5 ± 4.3	84.8 ± 5.3	90.0 ± 10.6
Arousals (#)	93.8 ± 13.3	33.3 ± 3.0*	1.0 ± 0.7	1.8 ± 0.9	77.3 ± 5.6	77.0 ± 5.1
NREM / REM Cycles (#)	12.0 ± 1.9	4.5 ± 1.5*	–	–	12.8 ± 1.0	12.3 ± 1.9

\*  $p \leq 0.05$  vs. vehicle



**Fig. 7.** Cumulative change relative to vehicle for WAKE (A), NREM (B) and REM (C) sleep during the 36 h time period following caffeine administration in cynomolgus macaques. The insets in A–C show the cumulative time across the 36 h period for each vigilance state. Mean hourly bout durations for WAKE (D), NREM (E) and REM (F) sleep during the 36 h period. Values are mean ± SEM ( $n = 4$ ). Gaps in panels E and F indicate when no NREM or REM sleep occurred during a particular hour. \* = caffeine is significantly different from vehicle ( $*p \leq 0.05$ ).



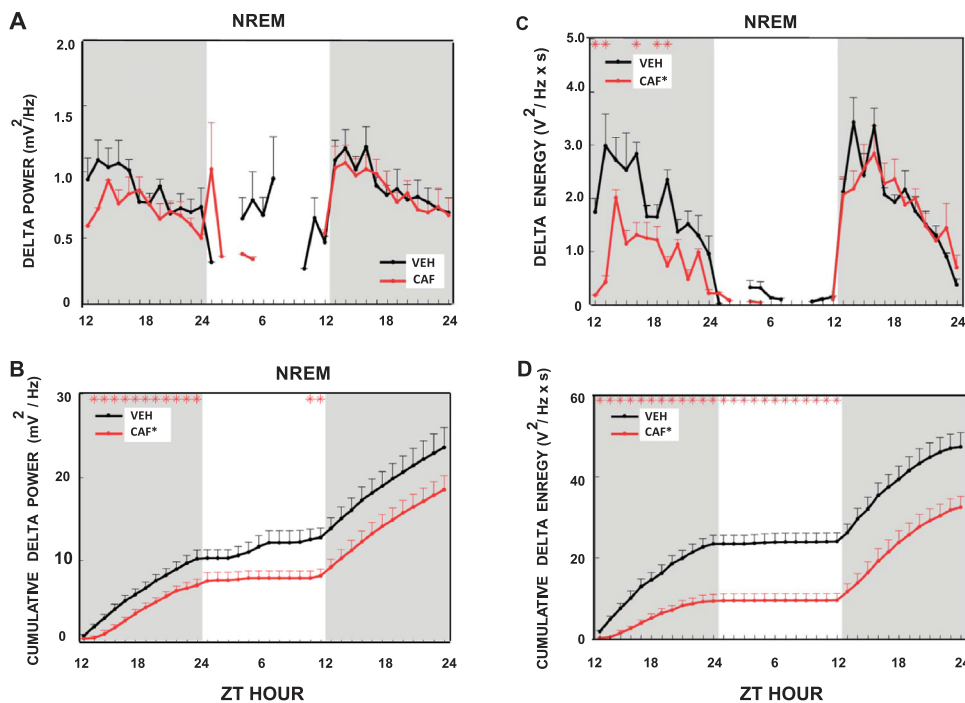
**Fig. 8.** Caffeine effects on EEG spectra recorded during the 36 h period in cynomolgus macaques. (A) Absolute (Log) power spectrum from 0.3–100 Hz for WAKE, NREM and REM sleep during night 1, day 1 and night 2 following vehicle or caffeine (10 mg/kg; i.m.) treatment. (B) Normalized power spectrum from 0.3–100 Hz and (C) corresponding delta, theta, alpha, sigma, beta, low gamma and high gamma (left to right) frequency bands for WAKE, NREM and REM sleep following caffeine treatment during night 1, day 1 and night 2. Values are mean  $\pm$  SEM ( $n = 3$ ). \* = caffeine is significantly different from vehicle (\* $p \leq 0.05$ ).

groups had similar TST amounts (vehicle-treated:  $518.1 \pm 43.4$  min; CAF-treated:  $521.6 \pm 34.2$  min) with a sleep efficiency of  $72.0 \pm 6.0\%$  and  $72.4 \pm 4.7\%$  for the vehicle- and CAF-treated groups, respectively (Table 3). Sleep efficiency for the vehicle-treated group was similar on night 2 ( $72.0 \pm 6.0\%$ ) to night 1 ( $72.9 \pm 3.7\%$ ). The absence of significant differences in the durations of NREM ( $429.5 \pm 41.1$  vs.  $433.1 \pm 23.1$ ;  $p = 0.89$ ), N2 ( $p = 0.60$ ) and N3 ( $p = 0.48$ ) sleep between vehicle- and CAF-treated groups on night 2 is particularly noteworthy. Despite the prolonged wakefulness evident in CAF-treated macaques on night 1, these results demonstrate the absence of rebound sleep during the subsequent 24 h. Fig. 7A–C presents the cumulative change from vehicle for WAKE, NREM and REM sleep for the 36 h post-treatment and demonstrates the absence of a sleep rebound as well as the unchanged NREM and REM bout durations in CAF-treated animals (Fig. 7D–F) during the 24 h subsequent to the increase in WAKE that occurred during the first night after CAF treatment.

Spectral power within the conventional frequency bands was calculated for each vigilance state during night 1, day 1 and night 2 for vehicle-treated and CAF-treated groups. Fig. 8A shows the absolute power spectral profile from 0.3–100 Hz for WAKE, NREM and REM sleep during night 1, day 1 and night 2. Similarly, the normalized power spectral profiles (0.3–100 Hz) for each vigilance state during night 1, day 1 and night 2 when absolute power values for CAF are normalized to vehicle are shown in Fig. 8B. During night 1, CAF-treated animals

showed reductions in delta and theta power during wakefulness (delta:  $t = 6.34$ ;  $p < 0.01$ ; theta:  $t = 14.08$ ;  $p < 0.001$ ) and REM sleep (delta:  $t = 9.37$ ;  $p < 0.01$ ; theta:  $t = 3.42$ ;  $p < 0.05$ ) sleep in comparison to vehicle-treated animals (Fig. 8C). In addition, CAF reduced delta ( $t = 3.74$ ;  $p < 0.05$ ) and high gamma power ( $t = 27.16$ ;  $p < 0.001$ ) and increased alpha ( $t = 8.29$ ;  $p < 0.01$ ) and sigma power ( $t = 3.22$ ;  $p < 0.05$ ) during NREM sleep compared to vehicle-treated animals (Fig. 8C). In contrast, CAF-treated animals showed no changes in normalized power across all vigilance states on day 1. However, the CAF-treated group showed a significant reduction in alpha power during wakefulness ( $t = 5.61$ ;  $p < 0.05$ ) on night 2 (Fig. 8C). Similar to Experiment 2, epoch-by-epoch analysis across the entire frequency range showed that the CAF-induced effect was prominent throughout the initial 12 h dark period with no transient changes across time (data not shown).

Since Fig. 7 indicated the absence of a homeostatic response in either NREM sleep or NREM bout duration, we assessed two other measures of sleep homeostasis: NREM delta power and NREM delta energy. Assessment of NREM delta power across the 36 h period demonstrated that CAF-treated animals showed reduced cumulative delta power during the initial 12 h dark phase (ZT13 – ZT 24; night 1) and during ZT11 – ZT12 the next day compared to vehicle controls (Fig. 9B). Furthermore, the CAF-treated group showed significant reductions in delta energy during ZT13 – ZT14, ZT17 and ZT19 – ZT20 on night 1 (Fig. 9C)



**Fig. 9.** Effects of caffeine on NREM delta (0.3–4 Hz) power and delta energy over a 36 h period in cynomolgus macaques. (A) Absolute delta power; (B) cumulative delta power; (C) absolute delta energy and (D) cumulative delta energy following vehicle or caffeine (10 mg/kg; i.m.) administration during night 1, day 1 and night 2. Values are mean  $\pm$  SEM ( $n = 3$ ). Gaps in panels A and C indicate when no NREM sleep occurred during a particular hour. \* = caffeine is significantly different from vehicle during that hour (\* $p \leq 0.05$ ).

as well as reduced cumulative delta energy during night 1 (ZT13–ZT24) and the subsequent 12 h light phase (ZT1–ZT12) compared to the vehicle-treated group (Fig. 9D).

#### 4. Discussion

In the present study, we used telemetric EEG and EMG recordings to determine the sleep/wake distribution and EEG spectral activity of the different vigilance states of adult cynomolgus macaques during the 12 h dark phase. To determine whether we could detect changes from basal sleep/wake levels, we then characterized the acute effects of CAF during the 12 h dark phase and found that CAF enhanced wakefulness throughout the 12 h dark phase even when administered late in the active phase – a time of day when sleep pressure was high. The absence of a sleep rebound during the 12 h dark phase led us to conduct a follow up study to assess the effects of CAF for 36 h following its administration to determine whether there was a homeostatic sleep response in the subsequent 24 h light and dark phases. To our surprise, we did not detect any evidence for sleep homeostasis (i.e., increases in either sleep amounts, NREM and REM bout duration, NREM delta power, or NREM delta energy) in the CAF-treated group during the subsequent 24 h light and dark phases.

##### 4.1. Telemetric recording of sleep/wake in non-human primates

When studied in their home cage, undisturbed cynomolgus macaques exhibited a clear diurnal pattern of activity, with the majority of wakefulness during the light phase and virtually no LMA during the dark phase. Although other studies have assessed activity by actigraphy (Zhdanova et al., 2002) or infrared video imaging (Masuda and Zhdanova, 2010) in macaques or sleep/wake patterns using telemetry (Hsieh et al., 2008; Authier et al., 2014; Rachalski et al., 2014), to our knowledge, this is the first study to measure LMA using Acticals in conjunction with telemetric EEG/EMG recordings for determination of sleep/wake states in cynomolgus macaques.

Most sleep studies in NHPs, including those in cynomolgus monkeys, have assessed the EEG and EMG while the primates were in restraint chairs (Bouyer et al., 1978; Holcombe et al., 1979; Almirall et al., 1999; Philip et al., 2005; Yao et al., 2013). To overcome the

limitations associated with restraint, more recent studies have used wireless telemetry methodologies in freely-moving unrestrained NHPs (Crofts et al., 2001; Hsieh et al., 2008; Authier et al., 2009; Gotter et al., 2013; Authier et al., 2014; Rachalski et al., 2014). Telemetry implants allow recording EEG and EMG in the animal's home cage, thereby minimizing potential stress. Thus, baseline sleep-wake architecture has been characterized in both freely-moving rhesus (Hsieh et al., 2008) and juvenile cynomolgus macaques (Authier et al., 2014; Rachalski et al., 2014). Our experimental subjects were housed in the same colony room for approximately two months prior to baseline sleep recordings, during which time macaques would be able to habituate to each other and stress levels should be stabilized. Consequently, the sleep/wake characteristics obtained in our 12 h baseline sleep study were very similar to those previously reported in juvenile cynomolgus macaques during the dark phase (Rachalski et al., 2014) as described below.

##### 4.2. Sleep architecture in cynomolgus macaques: comparison to previous studies

As indicated by their hypnograms (Fig. 2B), the time course of wakefulness and sleep was similar among animals in the baseline study. The sleep efficiency of 83% (Table 1) exceeds the 77.8% sleep efficiency reported in a previous study of juvenile cynomolgus macaques recorded during the 12 h dark phase (Rachalski et al., 2014), suggesting that stress was minimal under baseline conditions. TST was comprised of 14% REM and 86% NREM sleep, which is comparable to the 18.3% REM and 81.7% NREM reported previously (Rachalski et al., 2014). However, it is interesting to note that young cynomolgus macaques in that study were reported to have more REM sleep during the 12 h dark phase (~102 min) than the amount of REM sleep that we observed in adult cynomolgus macaques in the current study (77–90 min). These differences parallel the decline in the amount of REM sleep observed in humans across a comparable period of development (Van Cauter et al., 2000; Ohayon et al., 2004; Moraes et al., 2014). Within NREM sleep, comparisons between the present and the previous study of young cynomolgus macaques (Rachalski et al., 2014) are as follows: N1, 4.5% in the present study vs. 0.2%; N2, 62.4% vs. 51.1%; and N3, 19.4% vs. 30.7%. Although these numbers are generally similar, many factors can contribute to the relatively small differences. First, our monkeys were

older, ranging in age from 9–11 years vs. 2.5–3 years, with a larger body mass (7–10 kg vs. 2.6–2.8 kg). Secondly, although the telemetry transmitters, light/dark cycle, environmental temperature, diet and enrichment procedures used were identical in the two studies, the environment in which the animals were housed and recorded certainly differed. For example, in contrast to rodent studies, parameters such as the time of day during which NHPs are fed and the rooms are cleaned are the domain of veterinary staff and with limited control by an experimenter. Thirdly, although the criteria used to score epochs as N1, N2, N3 and REM were generally similar between the two studies, inter-individual scoring variability is an issue both within, as well as across, laboratories. In contrast to previous studies (Authier et al., 2014; Rachalski et al., 2014), we did not conduct electrooculogram recordings. Lastly, night-to-night variation occurs in the relative amount of sleep stages within an experimental subject that, presumably, relates to waking activity during the previous day as well as to the general physiological condition of each individual. Given these and other factors that can influence sleep stages, it is reassuring that there is relatively close agreement on many of these sleep/wake parameters as reported above.

#### 4.3. Comparison to human sleep

Overall, cynomolgus monkeys showed a more fragmented sleep pattern than humans, but our recordings and analyses occurred across the entire 12 h dark phase in comparison to the typical 7–8 h duration of a human sleep study. Macaques showed more consolidated NREM stage 3 (N3) sleep with a large amount of slow wave (delta) activity during the first half of the dark phase, while NREM stage 2 (N2) sleep, consisting of K-complexes and sleep spindles and more consolidated REM episodes, was prominent in the latter half of the dark phase. These characteristics are similar to the architecture of nocturnal sleep in humans, which suggests that sleep in cynomolgus macaques is regulated by circadian and homeostatic influences in a manner analogous to humans (Dijk and Czeisler, 1995). On average, there were about 11 NREM/REM cycles per night in macaques. Although this is considerably more than that reported in humans, the 11 NREM/REM cycles occurred over a 12 h dark phase. The proportion of NREM sleep in macaques, including N2 representing approximately half of TST, was similar to that typically recorded in human adults (Carskadon and Dement, 2010) as well as in other studies of adult NHP sleep (Breton et al., 1986; Crofts et al., 2001; Daley et al., 2006; Hsieh et al., 2008).

The EEG spectral profiles of the vigilance states observed in cynomolgus macaques in this study were also consistent with human sleep as well as observations in other NHP sleep studies (Hsieh et al., 2008; Authier et al., 2014; Rachalski et al., 2014). As in humans, wakefulness was characterized by higher EEG activity in fast frequencies (i.e., alpha, beta and gamma bands) compared with NREM sleep. NREM was dominated by delta (1–4 Hz) slow wave activity compared to REM sleep and WAKE. In contrast, REM sleep showed predominantly theta (4–8 Hz) activity compared to NREM sleep and WAKE. However, despite the occurrence of EEG spindles during N2, a peak in the spindle frequency range was not found during NREM sleep in the present study, which is consistent with previous reports in both cynomolgus (Authier et al., 2014) and rhesus (Hsieh et al., 2008) macaques. This difference may arise from a limitation in the telemetry methodology employed, location of electrodes, or masking by elevated spectral activity in lower frequencies.

#### 4.4. Sleep characteristics in vehicle-injected animals vs. basal sleep levels

To determine whether we could detect differences in sleep/wake from basal levels, we undertook a pharmacological study in which CAF was administered prior to light offset. The sleep efficiency following i.m. vehicle injections (81%) was comparable to that observed during baseline (83%), indicating that intramuscular injections had a minimal

effect on sleep in these macaques. TST was comprised of 86.8% NREM and 13.2% REM sleep in vehicle-injected animals, which was very similar to the 86.2% NREM and 13.8% REM sleep values in the baseline study. Within NREM sleep, comparisons between the vehicle-injected and baseline studies are as follows: N1, 2.8% in the vehicle-injected vs. 4.5%; N2, 43.8% vs. 62.4%; and N3, 40.2% vs. 19.4%. In addition, the following sleep parameters were among those that were remarkably similar between the two studies: SOL,  $14 \pm 1.9$  min (vehicle-injected) vs.  $12.6 \pm 2.8$  min (baseline); NREM/REM ratios,  $0.161 \pm 0.054$  vs.  $0.169 \pm 0.041$ ; the number of NREM/REM cycles,  $11.0 \pm 0.7$  vs.  $11.5 \pm 0.8$ ; and the number of awakenings,  $49.3 \pm 1.9$  vs.  $51.7 \pm 5.0$ . These comparisons suggest that i.m. injections themselves had little effect on NHP sleep architecture, although there was less N2 and more N3 sleep in Experiment 2 relative to the baseline study (Experiment 1). Aside from the obvious difference of either the occurrence or absence of an i.m. injection, the baseline study was conducted following 4 weeks of recovery from surgery and habituation to the sleep room whereas Experiment 2 was conducted approximately 3 months later. In addition, the number of animals used for Experiment 1 ( $N=6$ ) vs. Experiment 2 ( $N=4$ ) may contribute to the greater variability in both N2 and N3 sleep that was observed in Experiment 2.

#### 4.5. Effects of caffeine on sleep/wake in cynomolgus macaques

As expected, CAF reduced TST and sleep efficiency in cynomolgus monkeys during the 12 h dark phase in both Experiments 2 and 3 as described previously (Authier et al., 2014). Although no significant changes in LMA were observed during Experiment 2, the CAF-treated group showed a subtle increase in LMA in Experiment 3. However, our data suggests that CAF increases wakefulness without producing hyperactivity in Cynomolgus monkeys. The Acticals, which are tagged to each animal's collar to measure LMA, are built with accelerometers that are more sensitive to gross vs. fine locomotor movements along the X, Y and Z axes. One factor that likely contributes to the absence of increased LMA activity during CAF-induced waking in Experiment 2 is that, during the dark period, most animals tend to sit upright on their perch while awake and show very little gross movement for the majority of the dark phase as opposed to during the light phase. In addition, the limited size of home cages (approximately  $1\text{m}^3$ ) and the small number of subjects ( $N=4$ ) may be other factors which contribute to this discrepancy. CAF also increased the SOL, ROL and total time spent awake (WASO) and decreased the overall durations of both REM sleep and NREM sleep (specifically, N3) and the number of NREM/REM cycles during the 12 h dark phase. Although CAF has previously been reported to reduce overall NREM and REM sleep durations in juvenile cynomolgus macaques (Authier et al., 2014), the authors reported that both 10 and 30 mg/kg doses of CAF reduced N2 sleep whereas the 10 mg/kg dose used in the present study only significantly decreased N3 sleep (Table 2).

In the absence of pharmacokinetic information on i.m. injections of caffeine in adult male cynomolgus macaques, it is difficult to compare this dose to oral consumption of caffeine in humans as the concentration of caffeine in coffee vs. espresso varies as much as 120-fold. The dose used here was the lower of two doses used in a previous study in which caffeine was orally-administered to juvenile macaques (Authier et al., 2014). In the present study, N2 amounts were less on average ( $255.5 \pm 57.0$  min in vehicle-treated vs.  $119.9 \pm 25.6$  min in CAF-treated;  $p = 0.199$ ). The magnitude of wake promotion following 10 mg/kg. i.m. CAF in the present study more closely resembles the 30 mg/kg p.o. dose reported previously (Authier et al., 2014). Although the reason(s) for this discrepancy between the two studies is unclear, differences in the route of administration (i.m. vs p.o.), age (9–11 years vs 2.5–3 years), weight (7–10 kg vs 2.6–3.7 kg) and/or the number of animals studied ( $n = 4$  vs.  $n = 5$ ) may be contributing factors. Acute delivery of CAF prior to sleep has also been shown to prolong sleep latency, reduce TST and increase sleep fragmentation in laboratory

rodents (Yanik et al., 1987; Virus et al., 1990; Schwierin et al., 1996; Wurts and Edgar, 2000).

CAF administration also affected EEG activity in different vigilance states by reducing low frequency activity during wakefulness and elevating high frequency activity during NREM sleep. These CAF-induced changes in sleep duration and EEG power spectral activity are consistent with observations regarding the impact of this substance on both NHP (Authier et al., 2014) and human sleep and EEG synchrony (Landolt et al., 2004; Drapeau et al., 2006; Carrier et al., 2007). Although the effects of CAF on the EEG were assessed from 0.3–100 Hz in the present study rather than from 0–50 Hz as reported previously (Authier et al., 2014), the effects on EEG spectral power were similar.

#### 4.6. Absence of a homeostatic sleep response to caffeine-induced wakefulness?

Despite a prolonged period of CAF-induced wakefulness during the initial 12 h dark phase, CAF-treated cynomolgus macaques showed no evidence for sleep homeostasis in terms of increased NREM sleep amounts, NREM and/or REM bout durations, NREM delta power or NREM delta energy in the subsequent 24 h light/dark phases. At first glance, these results suggest the possibility of “decoupling” of wake promotion by CAF and the compensatory sleep response, which would be unusual but has been suggested to occur with other pharmacological agents (Edgar and Seidel, 1997). Many studies have investigated the effects of CAF as a countermeasure to combat the effects of sleep deprivation in both humans (Landolt et al., 2004; Carrier et al., 2007) and rodents (Wurts and Edgar, 2000). In humans, sleep deprivation increased 0.75–8 Hz (delta and theta) activity and reduced spindle frequency activity in NREM sleep during the recovery nights compared to baseline conditions. In contrast, when compared to placebo, CAF (two doses of 200 mg following 11 h and 23 h of wakefulness) reduced sleepiness and EEG power in the 0.75–2.0 Hz (delta) range and enhanced power in the 11.25–20 Hz range during the recovery night (Landolt et al., 2004). Similarly, CAF (10 and 15 mg/kg, i.p.) elicited a dose-dependent increase in waking followed by a prolonged increase of slow wave (delta) activity in NREM sleep in rodents (Schwierin et al., 1996).

Although the suggestion that CAF decoupled wakefulness from sleep homeostatic mechanisms is an attractive possibility, an alternative explanation for these results is that the amount of wakefulness by CAF produced was insufficient to produce a large enough sleep debt to trigger a homeostatic response. Since CAF treatment caused a 67.4% loss of TST and 66.1% loss of NREM time in Experiment 2 and a 55.3% loss of TST and 52.2% loss of NREM time during night 1 in Experiment 3, we think this is unlikely. However, as indicated in Figs. 4C and 6C, some NREM sleep occurred beginning in the second hour after treatment indicating that CAF caused a partial, rather than total, sleep deprivation. Analogous effects have been described in rats in which wakefulness induced by 8 mg/kg CAF did not elicit sleep homeostatic responses (Seidel and Edgar, 1994) whereas 15 mg/kg CAF elevated EEG slow activity for several hours post-treatment without changing NREM duration (Schwierin et al., 1996). Consequently, despite the clear evidence for CAF-induced sleep debt illustrated in Fig. 7, it is likely that homeostatic sleep drive diminished as NREM sleep occurred beginning in the second hour post-treatment. A conclusive demonstration of the absence of a homeostatic response to CAF in cynomolgus macaques would necessitate comparison to a comparable degree of partial sleep deprivation/sleep restriction in this species, an experimental condition that was not undertaken in the present study.

Although the sleep-disrupting effects of CAF are well-documented in humans, rodents and NHPs, the mechanisms of CAF-induced wakefulness and the interaction of the drug with the homeostatic mechanisms underlying NREM and REM sleep are not fully understood. CAF is an adenosine receptor antagonist that is thought to competitively antagonize the sleep-enhancing effects of adenosine (Radulovacki et al.,

1982; Virus et al., 1990) with high affinity for A2a receptors (Bruns et al., 1986; Huang et al., 2005) and A1 receptors (Virus et al., 1990; Nehlig et al., 1992; Benington et al., 1995; Akerstedt and Ficca, 1997). The sleep-promoting effects of adenosine receptor agonists (Radulovacki et al., 1982; Virus et al., 1990) may engage neural circuits involved in sleep homeostasis within brain regions that either directly (Benington et al., 1995) or indirectly modulate cortical activation such as the preoptic area (Ticho and Radulovacki, 1991) and/or the cholinergic neurons of the mesopontine tegmentum and basal forebrain (Rainnie et al., 1994; Porkka-Heiskanen et al., 1997). Consistent with this hypothesis, CAF has been reported to decrease NREM sleep, especially the deeper stages of NREM as seen in humans and NHPs (Nicholson and Stone, 1980; Landolt et al., 2004; Drapeau et al., 2006; Carrier et al., 2007; Authier et al., 2014) and rodents (Yanik et al., 1987; Virus et al., 1990) and the associated slow-wave activity in the delta band of the EEG that serves as a marker of homeostatic sleep drive (Landolt et al., 1995a, 1995b; Schwierin et al., 1996; Landolt et al., 2004). Whether CAF promotes wakefulness by interfering with the expression of sleep or by attenuating homeostatic sleep drive is currently unclear.

In summary, we demonstrated that recording of sleep/wake and EEG spectral activity by telemetry is a useful method in adult cynomolgus macaques for translational sleep studies. Our results indicated that this species presents the proportional distribution of sleep states as well as the macro- and micro-architecture characteristic of sleep seen in humans. In addition, we showed that CAF-induced wakefulness during the 12 h dark phase was accompanied by reduced slow wave activity during wakefulness and increased higher frequencies such as alpha activity during NREM sleep without a sleep rebound in the subsequent 24 h light and dark phases. Together with previous work (Authier et al., 2014; Rachalski et al., 2014), these studies demonstrate that cynomolgus macaques can be valuable to determine the effects of pharmacological agents known to affect sleep structure and EEG activity during sleep and wakefulness in humans.

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#### Declarations of interests

The authors have no conflicts of interest or financial relationships to disclose.

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