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Sleep deprivation and caffeine treatment potentiate photic resetting of the master circadian clock in a diurnal rodent

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Abbreviation: CT-Circadian time; EEG- electroencephalogram; EMG-Electromyogram; SCN-Suprachiasmatic nucleus; ZT- Zeitgeber time

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Abstract

Circadian rhythms in nocturnal and diurnal mammals are primarily synchronized to local time by the light-dark cycle. However, non-photoc factors, such as behavioural arousal and metabolic cues, can also phase-shift the master clock in the suprachiasmatic nuclei (SCN) and/or reduce the synchronizing effects of light in nocturnal rodents. In diurnal rodents, the role of arousal or insufficient sleep in these functions is still poorly understood. In the present study, diurnal Sudanian grass rats, *Arvicanthis ansorgei*, were aroused at night by sleep deprivation (gentle handling) or caffeine treatment that both prevented sleep. Phase-shifts of locomotor activity were analyzed in grass rats transferred from a light-dark cycle to constant darkness and aroused in early night or late-night. Early, but not late night sleep deprivation induced a significant phase-shift. Caffeine on its own induced no phase-shifts. Both sleep deprivation and caffeine treatment potentiated light-induced phase-delays and phase-advances in response to a 30-min light pulse respectively. Sleep deprivation in early, but not late night potentiated light-induced c-FOS expression in the ventral SCN. Caffeine treatment in midnight triggered c-FOS expression in dorsal SCN. Both sleep deprivation and caffeine treatment potentiated light-induced c-FOS expression in calbindin-containing cells of the ventral SCN in early and late night. These findings indicate that, in contrast to nocturnal rodents, behavioural arousal induced either by sleep deprivation or caffeine during the sleeping period potentiates light resetting of the master circadian clock in diurnal rodents and activation of calbindin-containing suprachiasmatic cells may be involved in this effect.

Key words: Circadian rhythms, Behavioural arousal, Suprachiasmatic nucleus, Photoc resetting, Phase-shift

Significance statement

Arousing stimuli have the ability to regulate circadian rhythms in mammals. Behavioural arousal in the sleeping period phase-shifts the master clock in the suprachiasmatic nuclei and/or slows down the photic entrainment in nocturnal animals. How these stimuli act in diurnal species remain to be established. Our study in a diurnal rodent, the Grass rat, indicates that sleep deprivation in the early rest period induces phase-delays of circadian locomotor activity rhythm. Contrary to nocturnal rodents, both sleep deprivation and caffeine-induced arousal potentiate the photic entrainment in a diurnal rodent. Such enhanced light-induced responses could be relevant for developing chronotherapeutic strategies.

Introduction

The phasing of the cycle of sleep and wakefulness with respect to day and night is one of the key rhythmic components that divide animals into night-active (nocturnal) and day-active (diurnal) species. There has been considerable research on circadian rhythms in nocturnal rodents with regard to the synchronizing properties of light and other cues, including behavioural arousal and metabolic factors. Diurnal species, however, have been studied much less as compared to nocturnal rodents, especially for non-photic synchronizing factors. Since the temporal organization of sleep and arousal is inverted between nocturnal and diurnal animals, we asked how arousal synchronizes circadian rhythms in diurnal animals.

In addition to the sleep-wake cycle, a number of other daily rhythms, such as feeding-fasting cycle and hormonal rhythms, are oppositely phased relative to the light-dark cycle between nocturnal and diurnal mammals (Kumar Jha et al., 2015). However, the central clock in the suprachiasmatic nucleus (SCN) of the hypothalamus, which regulates rhythmicity of most behavioural and physiological parameters, is most active at the same astronomical time in these two categories of mammals. For instance, glucose utilization and firing rate in the SCN are high during the day and low at night in both diurnal and nocturnal animals (Smale et al., 2003). Genesis of circadian rhythmicity is based on the rhythmic expression of core clock genes such as *Pers* (*Per1-3*), *Crys* (*Cry1-2*), *Bmal1*, *Clock* and nuclear receptors *Rev-erba/β* and *Rora/β* (Mohawk et al., 2012). Temporal patterns of expression of most of these clock genes in the SCN are similar in nocturnal and diurnal species (Albrecht et al., 1997, Shearman et al., 2000, Mrosovsky et al., 2001, Caldelas et al., 2003).

The molecular clock machinery is reset by environmental cues. In particular, light can synchronize the SCN neurons via a cascade of transcriptional activation. Both diurnal and nocturnal species are most sensitive to the synchronizing effects of light at night. Within nocturnal and diurnal species the mechanisms underlying photic resetting are essentially similar in terms of temporal sensitivity and direction of light-induced phase-shifts (Challet, 2007). As a rule, light exposure during the early and late subjective night elicits phase-delays and advances, respectively (Daan and Pittendrigh, 1976, Slotten et al., 2005). In addition to light, numerous non-photic factors have abilities to reset the phase and period of the SCN clock and influence its photic resetting (Mrosovsky, 1996, Challet and Pevet, 2003, Challet, 2007). Some of these factors are based on involuntary arousal in the resting period. Notably, it has been found that sleep deprivation induced by gentle handling during the resting period leads to phase-advances of locomotor activity rhythm in the nocturnal Syrian hamster (Antle

and Mistlberger, 2000). Moreover, this treatment attenuates light-induced phase-delays in both hamsters and mice (Mistlberger et al., 1997, Challet et al., 2001, van Diepen et al., 2014). On the other hand, systemic administration of the adenosine antagonist caffeine during the usual resting period induces arousal without shifting the SCN clock in Syrian hamster, whereas it blocks light-induced phase-shifts (Antle et al., 2001, Vivanco et al., 2013).

Most studies concerning non-photoc entrainment have been carried out in nocturnal hamsters and mice. To study behavioral arousal as a possible non-photoc synchronizing agent in diurnal rodents, we first examined whether sleep deprivation or caffeine-induced arousal affects the phase-shifting effect of light in the Sudanian grass rat (*Arvicanthis ansorgei*). The magnitude of sleep deprivation and caffeine-induced arousal was quantified by electroencephalogram (EEG) combined with electromyogram (EMG) recording. Second, we used c-FOS as a marker of neuronal activation to investigate the molecular mechanisms of entrainment; we also examined c-FOS expression in the SCN of behaviorally aroused animals. Third, we phenotyped the neurons expressing c-FOS in the SCN. Fourth, to better understand the neurochemical basis and assess the state of arousal, we measured serotonin, adenosine and caffeine concentrations in the SCN and midbrain raphe area of animals, aroused or not by caffeine and sleep deprivation.

Materials and Methods

All the experiments were performed in accordance with the U.S National Institute Health Guide for the Care and Use of Laboratory Animals (1996), the French National Law (implementing the European Directive 2010/63/EU) and approved by the Regional Ethical Committee of Strasbourg for Animal experimentation (CREMEAS) and French Ministry of Higher Education and Research, #01050.02).

Animals and housing conditions

Male Sudanian grass rats (*Arvicanthis ansorgei*), 3-4 months old, were obtained from a local breeding colony (Chronobiotron platform, UMS3415, CNRS, University of Strasbourg) in Strasbourg, weighing 100–150 g at the beginning of the study. During baseline, animals were maintained at 23°C under a 12 h light/12 h dark cycle (LD) (light intensity during light and dark periods (red light on) was respectively 200 lux and < 3 lux). Lights went on at 07:00 AM and off at 07:00 PM defining *Zeitgeber* time (ZT) 0 and ZT12, respectively. During the actimetry experiments, animals were housed individually in Plexiglas cages (35 × 22 × 40

cm) equipped with running wheels (diameter: 34 cm) with *ad libitum* food and water in LD. Wheel-running activity was continuously recorded and stored in 5 min bins by Vital-View (Minimitter Inc., Sunriver, OR, USA). After a period of 2 weeks, animals were transferred into constant darkness (DD; red light, < 3 lux). Animals included in the present study displayed bimodal patterns of diurnal locomotor activity.

EEG and EMG surgery

A classical set of EEG and EMG electrodes was implanted according to methods previously described and adapted to *Arvicanthis ansorgei* (Hubbard et al., 2015). The electrodes were implanted under deep anaesthesia (intraperitoneal injection of ketamine 80 mg/kg and xylazine 7 mg/kg), then soldered to a connector and cemented to the skull, before the skin was sutured. Holes were drilled in the skull and animals were implanted with two epidural EEG (frontal: 2.0 mm lateral to middle, 2.0 mm anterior to bregma; parietal 2.0 mm lateral to middle, 2.0 mm anterior to lambda) and one reference electrode (Hubbard et al., 2015). In addition, two EMG electrodes were inserted in the neck muscles along the back of the skull in a manner that they did not touch each other. Thereafter, EEG electrodes were cemented to the skull and the skin was sutured where needed. Animals were allowed to recover from surgery and habituate to the recording conditions for a minimum of two weeks.

Experimental design

Experiment 1: Assessment of EEG and EMG changes in response to sleep deprivation and caffeine treatment

We assessed the actual sleep loss during early and late subjective night of sleep deprivation or caffeine treatment. A first set of animals (n=6) were transferred to DD for 2 days, and EEG and EMG were recorded using commercial Compumedics hardware (Neuvo 64-512 Channel EEG HD-LTM) and software (Profusion PSG 4 Software and Neuroscan Curry 7). For sleep deprivation, on the first day of DD, animals were left undisturbed (baseline condition used as control procedure). On the second day, they were sleep-deprived by gentle handling during 4 h in early subjective night from projected Circadian Time (CT) 12 (defining as projected time of lights off during the previous light-dark cycle) to CT16 (n=6). Animals were allowed to synchronize to LD 12:12 for a few days before repeating the same procedures in DD as above except that sleep deprivation occurred in late subjective night, from CT16 to CT20 (n=5).

Similarly, the animals were exposed for two days to DD to analyze the effect of caffeine administration. Continuous sleep recordings were performed on the first day of DD as baseline and on second day of DD to analyze the effects of intraperitoneal (i.p.) injection at CT14 of either caffeine (CAF, 30 mg/kg in 0.9% saline; C0750, Sigma-Aldrich, St. Louis, MO, USA) or vehicle (0.9% saline; VEH). Animals were allowed to synchronize to LD 12:12 for a few days before repeating the same procedures in DD in a cross-over design of caffeine and vehicle treatment at CT18 (n=4).

Experiment 2: Early night sleep deprivation

We next studied the circadian clock resetting by sleep deprivation (SD) in early night. On the first day of DD, a second set of animals (n=24) were transferred to new cages (without running wheel) at projected CT12. Twelve out of them were sleep deprived by gentle handling (Hubbard et al., 2015) from CT12 to CT16. The remaining 12 animals were kept undisturbed as controls (CTR). After sleep deprivation, half of the animals from both the sleep deprived and control group were subjected to a 30-min light pulse of fluorescent white light (200 lux at the level of animals; “Light”) at CT16, while the other animals were kept in darkness (“Dark”). Thereafter, all animals were transferred back to their home cages. Thus, there were 4 groups (each n=6): (i) CTR + Dark, (ii) SD + Dark, (iii) CTR + Light and (iv) SD + Light. Then, animals were left undisturbed in DD for 8 days before being resynchronized to LD for at least 2 wks.

Thereafter, to investigate the molecular responses, the 24 animals that were used in the above mentioned actimetry experiment were divided into four groups again (n=6 per group, as indicated above, but newly randomized) and transferred to DD where they were sleep deprived and exposed to a light pulse as described above. Animals were euthanized under a dim red light (TL-D 18W Red SLV, Philips, <3 lux at the level of animals) 1 h after the end of sleep deprivation in the “Dark” groups and 30 min after light exposure in the “Light” groups, i.e., CT17.

Experiment 3: Late night sleep deprivation

A third set of animals (n=24) were used here. The procedures for this study were the same as for Experiment 2, except that the sleep deprivation period was from CT16 to CT20, while animals from groups (iii) and (iv) were exposed to a light pulse at CT20. Then, animals were left undisturbed in DD for 8 days before being resynchronized to LD for at least 2 wks. The treatments were repeated in DD as above, except that brains were sampled at CT21 on the first day of DD.

Experiment 4: Morning caffeine treatments

A fourth set of animals (n=24) were used for this experiment. Treatments were administered on the first day of DD. Each animal received an i.p. injection of CAF or VEH at CT0 followed 2 h after the injection by a 30-min light pulse (“Light”) or no light (“Dark”) at CT2. Thus, there were 4 groups (n=6): (i) VEH + Dark, (ii) CAF + Dark, (iii) VEH + Light and (iv) CAF + Light. Then, animals were left undisturbed in DD for 8 days before being resynchronized to LD for at least 2 wks.

Experiment 5: Midday caffeine treatments

A fifth set of animals (n=24) were used here. The procedure for this treatment was the same as for Experiment 4, except that the timing of CAF or VEH injection was at CT6 and that animals of group (iii) and (iv) were exposed to a light pulse at CT8.

Experiment 6: Early night caffeine treatments

The procedure and animals for this treatment were the same as in Experiment 4, except that the timing of the CAF and VEH injection was at CT14 and animals of groups (iii) and (iv) were exposed to light pulses at CT16. Then, animals were left undisturbed in DD for 8 days before being resynchronized to LD for at least 2 wks.

To investigate the molecular responses, the same animals used for actimetry were divided into four groups again (n=6 per group, as indicated above, but newly randomized) and transferred to DD. Animals were treated with CAF or VEH at CT14. In addition, animals in groups (iii) and (iv) were exposed to a light pulse at CT16. Animals were euthanized under a dim red light (TL-D 18W Red SLV, Philips, <3 lux at the level of animals) 3 h after CAF treatment, i.e. CT17 on the first day of DD.

Experiment 7: Midnight caffeine treatments

The procedure and animals for this treatment were the same as for Experiment 5, except that the timing of the CAF and VEH injection was at CT18 and animals of groups (iii) and (iv) were exposed to a light pulse at CT20. Then, animals were left undisturbed in DD for 8 days before being resynchronized to LD for at least 2 wks. The animals used for actimetry were then treated again as above, and euthanized at CT21 on the first day of DD.

Analysis of behavioural data

For sleep scoring, EEG and EMG signals were amplified, filtered and analog-to-digital converted to 256 Hz. Each 10-s epoch was scored by 2 independent scorers as wake or sleep by visual inspection of the EEG and EMG signals without knowledge of the recording

condition and according to criteria previously validated in a study first characterizing the sleep-wake cycle of *Arvicanthis ansorgei* (Hubbard et al., 2015). Time spent in each vigilance states was then calculated for each condition.

Wheel-running activity was continuously recorded and stored in 5 min bins by Vital-View (Minimitter Inc., Sunriver, OR, USA). All parameters for wheel-running activity were determined with Clocklab Software (Actimetrics, Evanston, IL, USA). Beginning and end of activity at ~ZT/CT0 and ~ZT/CT12 were considered as onset and offset respectively. Throughout the experiments, we selected offset points (~ZT12/CT12) for calculation of phase-shifts. The magnitude and the direction of elicited phase-shifts were calculated by linear regression (Clocklab) encompassing the 6 days before the sleep deprivation or caffeine treatments and the 6 days after it, excluding transitory cycles. Phase-shift magnitudes were calculated by considering the interval between the two fitted lines by a researcher blind to experimental procedures. Phase-shifts in response to sleep deprivation, caffeine treatment, sleep deprivation plus light or caffeine treatment plus light were assessed using the Aschoff Type II procedure (Refinetti, 2006).

Immunohistochemistry

Animals used for immunocytochemistry were deeply anesthetized with a high dose of sodium pentobarbital (i.p. 150 mg/kg) and intracardially perfused with saline (0.9% NaCl) followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4.

Each brain was rapidly removed and post-fixed in the same fixative for 24 hrs, then cryoprotected in 30% sucrose for 48 h. Brains were frozen in isopentane at -40°C and stored in -80°C. Coronal sections of 30 µm through the SCN were prepared with a cryostat (Leica CM 3000) and stored at -20°C in Watson solution.

Single labelling for FOS within the suprachiasmatic nucleus

For c-FOS immunostaining, free-floating brain sections were washed in phosphate buffer saline (PBS), pH 7.4 and incubated in a solution of 3% H₂O₂ (30% Hydrogen peroxide, Sigma) in PBS for 30 min. Brain sections were rinsed in PBS for 10 min three times and (i) incubated in 10% normal goat serum (Chemicon®, Merck-millipore, USA) in PBS with 0.3% Tween-20 (Sigma) for 1 h, followed by (ii) an incubation with a rabbit polyclonal anti-c-FOS antibody (1:4000, SC-52, Santa Cruz Biotechnology, CA, USA) for 24 h at 4°C, followed by (iii) biotinylated secondary antibody for 2 h at room temperature (1:500, goat anti-rabbit; Vector Laboratories; in PBS with 0.3% Tween-T20), followed by (iv) avidin-biotin complex

(ABC) for 1 h at room temperature (0.2% each avidin and biotin solution; ABC Vectastain Kit; Vector laboratories; in PBS with 0.05% Tween-20). Sections were then allowed to react in 3,3 diaminobenzidine tetrahydrochloride (DAB; 0.5 mg/ml, Sigma) with 0.015% H₂O₂ in tap water. In between each step after (ii), brain sections were rinsed three times for 10 min in PBS. All tissue sections were mounted onto gelatin-coated slides, dehydrated through a series of alcohol, soaked in toluene and coverslipped with Eukitt (Chem lab, Zedelgem, Belgium). We performed the staining in one run for sleep deprivation and caffeine treatment experiments.

Double labelling for FOS and calbindin within the suprachiasmatic nucleus

To phenotype the Fos immunoreactive neurons in the SCN, we performed double labelling of Fos and calbindin, based on a distinct pattern of Fos induction in mid SCN of sleep deprived and caffeine treated group in dark and their topographic semblance with calbindin expression in SCN of *Arvicanthis niloticus* (Mahoney et al., 2000). Initially brain sections were processed for c-FOS using a nickel enhanced DAB procedure as described in the single labelling for c-FOS. After reaction with ABC, tissue was reacted by mixing DAB (0.5 mg/ml), 0.015% H₂O₂ and 2.5% nickel sulphate. Following the Fos reaction, the tissue was rinsed and processed for detection of calbindin-immunoreactivity as follow: brain sections were incubated with a mouse monoclonal anti-calbindin-D-28k antibody (1:2000, C9848, Sigma-Aldrich, St. Louis, MO, USA) for 24 h at room temperature, followed by the same procedures as reported above for c-FOS single labelling (i.e., biotinylated secondary antibody, ABC, and incubation with DAB without nickel sulphate). All the tissue sections were mounted onto gelatin-coated slides, dehydrated through a series of alcohol, soaked in toluene and coverslipped with Eukitt (Chem lab, Zedelgem, Belgium)

Microscopic quantification

Randomly a single section through the central SCN per animal was selected for quantification. Cells were visualized by using a Leica DMRB microscope (Leica Microsystems, Rueil-Malmaison, France) equipped with an Olympus DP50 digital camera (Olympus France, Rungis, France). Images were taken (5X for single labelling and 20X for double labelling) standardizing all lighting parameters on the microscope and the camera software (Viewfinder Lite, Olympus) to ensure consistent stable lighting throughout the image capture. Quantifications were performed with Image J software (W. S. Rasband, US National Institutes of Health, Bethesda, Maryland). For single labelling, immunoreactive (IR)

neurons within the SCN were quantified within rectangular templates placed in dorsal (0.18 mm X 0.13 mm; 0.11 mm X 0.1 mm) and ventral (0.26 mm X 0.22 mm) regions by the observer unaware of the experimental conditions. For double labelling, the number of calbindin neurons co-localizing with c-FOS was counted by an observer blind to the experiments and only those calbindin-positive cells that presented a clear double staining of c-FOS (black nucleus) and calbindin (light brown soma) were included as co-localizing neurons.

Serotonin and adenosine content in SCN and midbrain of sleep deprived and caffeine treated animals

To conduct this experiment, a sixth set of animals (n=24) were maintained in LD 12:12 for two weeks and transferred to DD. On the first day of DD, 12 animals were transferred to new cages (without running wheel) at CT12 and 6 out of these 12 animals were sleep deprived by gentle handling (Hubbard et al., 2015) between CT12 and CT16. At the same time, the remaining 12 animals were selected for caffeine induced arousal or VEH treatment. Treatments were administered on the first day of DD. Each animal received an i.p. injection of CAF or VEH at CT14. All animals were killed at CT17 by decapitation. The brains were rapidly removed. Thereafter, SCN region and midbrain were microdissected visually and stored in -80°C.

SCN, midbrain and plasma preparation for Liquid chromatography-tandem mass spectrometry analysis

Microdissections of SCN and midbrain raphe were homogenized in Ultra pure water and centrifuged at 15,000 g. Plasma was centrifuged at 18,000 g. Ten pmoles of D3-caffeine, C5-adenosine, and D4-serotonin internal standards were added to each sample prior to the AccQTag treatment. Only serotonin (5-hydroxy-tryptamine, 5-HT) and D4-serotonin were derivatized on amine functions. The AccQTag derivation protocol was performed according to the manufacturer recommendations (Watters, Guyancourt, France). Concentrations of total soluble protein in the extracts were determined using Bradford method (Protein Assay, Bio-rad, Marnes-la-Coquette, France).

Liquid chromatography-tandem mass spectrometry instrumentation and analytical conditions

Liquid chromatography analyses were performed to quantify the presence of caffeine, adenosine and serotonin. Analyses were done on a Dionex Ultimate 3000 High-Pressure Liquid Chromatography system (Thermo Electron, Courtaboeuf, France) coupled with a

triple quadrupole Endura (Thermo Electron). The system was controlled by Xcalibur v. 2.0 software (Thermo Electron). Extracted derived samples were resuspended in 100 μ l of AccQTag derivatization kit/formic acid 0.1% and 10 μ l of the solution was loaded into an Accucore RP-MS column (ref 17626-102130; 100 x 2.1 mm 2.6 μ m, Thermo Electron) heated at 35°C. All molecules and their corresponding deuterated compounds were eluted by applying a gradient of buffers A/B. Buffer A corresponded to H₂O 99.9% / formic acid 0.1% (v/v), whereas buffer B was ACN 99.9 %/ formic acid 0.1% (v/v). A linear gradient of 20%-85% of solvent B at 400 μ L/min during 2.5 min was applied followed by a washing step (0.5 min at 85% of solvent B) and an equilibration step (1 min of 20% of buffer B).

Qualitative analysis and quantification was performed in the selected reaction monitoring mode (SRM). For ionization, 3500V of liquid junction voltage and 292°C capillary temperature was applied. The selectivity for both Q1 and Q3 was set to 0.7 Da (FWHM). The collision gas pressure of Q2 was set at 2 mTorr of argon. For all molecules, the selection of the monitored transitions and the optimization of the collision energy were first determined. The transitions and the corresponding collision energies (CE) used for SRM were the following: m/z 195.2 \rightarrow m/z 138.1 (CE = 28 eV) for caffeine, m/z 198.2 \rightarrow m/z 141.2 (CE = 29 eV) for D3-caffeine, m/z 168.2 \rightarrow m/z 136.1 (CE = 23 eV) for adenosine, m/z 173.2 \rightarrow m/z 136.1 for C5-adenosin, m/z 347.2 \rightarrow m/z 171.1 (CE = 38 eV) for 5-HT, m/z 351.2 \rightarrow m/z 171.1 (CE = 38 eV) for D4-serotonin. Identification was based on precursor ions, daughter ions and retention times. Quantification was done using the ratio of daughter ions response areas of the internal standards. Data were normalized to ml of plasma or to g of proteins.

Statistical analysis

Data are presented as mean \pm standard error of the mean (SEM). Statistical analysis was performed by SigmaPlot (version 12, SPSS Inc, Chicago, IL, USA). Significance was defined at $p < 0.05$.

Two-way analysis of variance (ANOVA) was performed to assess the effect of *Treatment* (SD versus CTR; CAF versus VEH), *Lighting condition* (Dark versus Light pulse) and the *Interaction* between these factors. Two-way ANOVA with repeated measures (for time spent in sleep and wake) was performed to assess the effect of *Treatment* (SD versus CTR; CAF versus VEH), *Time* (CTs) and the *Interaction* between these factors. When appropriate, post-hoc analysis was performed using Fisher LSD method. One way repeated measure ANOVA was performed to compare total sleep loss during treatments. To compare the 5-HT,

adenosine and caffeine contents in SCN, midbrain and plasma after sleep deprivation versus control procedure or caffeine treatment versus vehicle injection, unpaired Student's *t* test were used.

Results

Characterization of sleep deprivation and caffeine-induced arousal by EEG and EMG

We characterized the sleep loss due to sleep deprivation and caffeine treatments by calculating time spent per hour in wake and sleep. Both in early and late subjective night, sleep deprivation protocol enhanced the wake duration and decreased time spent in sleep (**Fig 1**). In late, but not in early night deprivation, ANOVA revealed that time spent in wake and sleep was affected by the *Treatment* (early night: $F_{1,110} = 0.16$; $p = 0.69$; late-night: $F_{1,88} = 8.3$; $p = 0.02$). Both in early and late night, time spent in wake and sleep was affected by *Time* (early night: $F_{11,110} = 8.5$; $p < 0.001$; late-night: $F_{11,88} = 6.3$; $p < 0.001$) and *Interaction* (early night: $F_{11,110} = 2.8$; $p = 0.003$; late-night: $F_{11,88} = 3.1$; $p = 0.001$). In early night, post-hoc analysis revealed that time spent in wake was higher and sleep was lower during the last 2 h of sleep deprivation ($p < 0.05$) in sleep deprived group and this effect was near to significant at CT 14 ($p = 0.053$) (**Fig 1 A and B**). The time spent in wake was lower and sleep was higher in sleep-deprived animals to the end of the night, from CT21 to CT23 ($p < 0.05$). In late night, post-hoc analysis revealed that time spent in wake was higher and sleep was lower in sleep deprived group during the 4 h period of sleep deprivation ($p < 0.001$) (**Fig 1 C and D**). Moreover, total amount of sleep during the experimental night was reduced by sleep deprivation when it occurred in late night ($F_{1,4} = 14$; $p = 0.02$), while it was compensated by sleep rebound when animals were sleep-deprived in early night ($F_{1,5} = 0.2$; $p = 0.6$).

Caffeine treatment in both early and midnight enhanced the time spent in wake and reduced the sleep duration (**Fig 2**). In early night treatment, ANOVA revealed that the time spent in wake and sleep was not affected by the *Treatment* ($F_{1,66} = 4.7$; $p = 0.07$) but affected by *Time* ($F_{11,66} = 10.5$; $p < 0.001$) and *Interaction* ($F_{11,66} = 1.9$; $p = 0.05$). Post-hoc analysis showed that the time spent in wake was higher and sleep was lower in caffeine treated group for 5 h ($p < 0.05$) (**Fig 2 A and B**). Similarly, in midnight treatment, the time spent in wake and sleep was not affected by the *Treatment* ($F_{1,66} = 4.4$; $p = 0.08$) but affected by *Time* ($F_{11,66} = 9.03$; $p < 0.001$) and *Interaction* ($F_{11,66} = 1.97$; $p = 0.04$). Post-hoc analysis showed that the time spent in wake was higher and sleep was lower in caffeine treated group for 2 h ($p < 0.05$) (**Fig 2 C and D**). Furthermore, total amount of sleep during the experimental night was

reduced by caffeine treatment when applied in early night ($F_{1,3} = 12.8$; $p = 0.03$), while it was unchanged after caffeine injection at midnight ($F_{1,3} = 5.39$; $p = 0.1$).

Only early night sleep deprivation induces phase-delay shifts while sleep deprivation in both early and late night potentiates light-induced phase-shifts

To evaluate the possible phase-shifts of the locomotor activity rhythm in sleep-deprived animals, the offsets of the diurnal pattern of locomotor activity were analyzed by two-way ANOVA using the factors *Treatment* (SD or CTR) and the *Lighting condition* (Dark or Light pulse).

Regarding the sleep deprivation in early night (CT12-16), the behavioural shifts were significantly modified by *Treatment* ($F_{1,20} = 12.79$; $p = 0.002$) and *Lighting condition* ($F_{1,20} = 24.08$; $p < 0.001$), while *Interaction* was not significant ($F_{1,20} = 0.19$; $p < 0.66$), indicating that the effect of *Treatment* was independent of the *Lighting condition* (**Fig 3 A – D and I**). Post-hoc analysis revealed that sleep deprivation in the absence of a light pulse induced a significant phase-delay compared to undisturbed controls ($p = 0.01$) and it significantly enhanced light-induced phase-delays in the early night compared to light-exposed undisturbed control animals ($p = 0.03$). Furthermore, phase-delays in the SD + Light group were significantly higher compared to SD + Dark ($p = 0.004$). Phase-delays in the CTR + Light group was also significantly higher compared to CTR + Dark ($p = 0.001$).

With respect to sleep deprivation during late night (CT16-20), phase-shifts were modified by *Treatment* ($F_{1,20} = 6.24$; $p = 0.02$) and *Lighting condition* ($F_{1,20} = 15.31$; $p < 0.001$). The non-significant *Interaction* ($F_{1,20} = 0.18$; $p = 0.67$) indicates that the effect of treatment was independent of lighting conditions (**Fig 3 E – H and J**). Post-hoc analysis confirmed that phase-advances in the CTR + Light group was significantly higher compared to CTR + Dark ($p = 0.02$). Moreover, sleep deprivation in late night was not able to induce phase-advances larger than those of the dark control animals ($p = 0.15$). By contrast, sleep deprivation in late night significantly potentiated light-induced phase-advances compared to light-exposed undisturbed controls ($p = 0.05$). Furthermore, phase-advances in the SD + Light were significantly higher compared to the SD + Dark ($p = 0.006$).

The free-running period of wheel-running activity rhythm was close to 24 h, and not influenced by sleep deprivation at night (data not shown).

Daytime caffeine treatment does not induce a phase-shift or influence light-induced phase-shifts

Caffeine treatment (30 mg/kg) in the early subjective morning (CT0) and mid-subjective day (CT6) did not induce significant phase-shifts in the locomotor activity rhythm, nor significantly change light-induced phase-shifts. ANOVA revealed the significant effect of *Lighting condition* ($F_{1,20} = 7.4$; $p = 0.01$) in early subjective morning (**Fig 4 A – D and I**). Post-hoc analysis indicated that phase-shifts were significantly larger after VEH + Light than after VEH + Dark ($p = 0.03$). After caffeine treatment during mid-subjective day we did not observe any significant effect of *Treatment* ($F_{1,20} = 0.47$; $p = 0.49$), *Lighting condition* ($F_{1,20} = 0.39$; $p = 0.53$) or *Interaction* ($F_{1,20} = 0.1$; $p = 0.74$) (**Fig 4 E – H and J**).

The free-running period of wheel-running activity rhythm was close to 24 h and not influenced by CAF or VEH during daytime (data not shown).

Caffeine treatment in early and late night potentiates light-induced phase-shifts

Caffeine treatment in early subjective night (CT14) and mid-subjective night (CT18) potentiated light-induced phase-delays and advances, respectively. ANOVA revealed that both caffeine treatment in the early night (CAF or VEH; $F_{1,20} = 6.4$; $p = 0.02$) and light ($F_{1,20} = 40$; $p < 0.001$) induced significant phase-delays. The non-significant *Interaction* ($F_{1,20} = 1.18$; $p = 0.29$) indicates that the effect of caffeine was independent of the lighting condition (**Fig 5 A – D and I**). CAF + Light treatment induced phase-delays that were significantly greater than those after VEH + Light ($p = 0.01$) and CAF + Dark ($p < 0.001$). Phase-delays induced by VEH + Light treatment were significantly higher compared to VEH + Dark ($p = 0.001$).

Similar to early night, caffeine (CAF or VEH; $F_{1,20} = 5.4$; $p = 0.03$) and light ($F_{1,20} = 40.9$; $p < 0.001$) treatment during late night induced significant phase-advance. The non-significant *Interaction* ($F_{1,20} = 1.18$; $p = 0.29$) indicates that also during late night the effect of caffeine treatment was independent of the lighting condition (**Fig 5 E – H and J**). CAF + Light treatment induced phase-advances were significantly greater than those after VEH + Light ($p = 0.02$) and CAF + Dark ($p < 0.001$). Phase-advances induced by VEH + Light treatment were significantly higher compare to VEH + Dark ($p = 0.001$).

The free-running period of wheel-running activity rhythm was close to 24 h and not influenced by CAF or VEH during night time (data not shown).

Early night sleep deprivation potentiates light-induced c-FOS expression in ventral SCN

Light pulses at CT16 and CT20 increased c-FOS expression in the dorsal as well as ventral SCN (**Fig 6 A - D**; light effect at CT16; dorsal SCN: $F_{1,16} = 346.4$, $p < 0.001$; ventral SCN: $F_{1,16} = 126.2$, $p < 0.001$; light effect at CT20, dorsal SCN : $F_{1,16} = 94.8$, $p < 0.001$; ventral SCN: $F_{1,16} = 165.6$, $p < 0.001$). ANOVA detected effect of early night sleep deprivation on c-FOS expression in ventral SCN ($F_{1,16} = 6.38$, $p = 0.02$) and a significant interaction between sleep deprivation and light in dorsal SCN ($F_{1,16} = 4.04$, $p = 0.04$). Post hoc analysis indicated that at CT16, the light-induced c-FOS expression in the ventral SCN was significantly greater in sleep-deprived animals than in non-sleep deprived controls ($p = 0.007$).

Caffeine treatment induces c-FOS expression in the dorsal SCN and potentiates light-induced c-FOS expression in ventral SCN

We also examined the effect of caffeine-induced arousal on c-FOS expression in the master clock. During the early subjective night, c-FOS expression in the dorsal SCN was only altered by *Lighting condition* ($F_{1,20} = 30.9$; $p < 0.001$). Post-hoc analysis indicated that the number of c-FOS positive nuclei in VEH + Light-treated group was significantly higher compared to dark controls ($p = 0.004$). In the ventral SCN, c-FOS expression was altered by both *Treatment* (CAF or VEH; $F_{1,20} = 9.6$; $p = 0.005$) and *Lighting condition* ($F_{1,20} = 189.4$; $p < 0.001$). Post-hoc analysis indicated a significant increase in the number of c-FOS-positive nuclei in the ventral SCN of the CAF + Light group compared to the VEH + Light ($p = 0.003$) and CAF + Dark ($p < 0.001$) groups. The number of c-FOS positive nuclei in VEH + Light treatment group was also significantly higher than that in the dark controls ($p < 0.001$) (**Fig 7 A and C**).

During mid-subjective night, c-FOS expression in the dorsal SCN was altered by *Treatment* ($F_{1,20} = 6.5$; $p = 0.01$) and *Lighting condition* ($F_{1,20} = 96.6$; $p < 0.001$). Post-hoc analysis indicated a significant increase in the number of c-FOS positive nuclei in the dorsal SCN of CAF + Dark group compared to VEH + Dark ($p = 0.02$). The number of c-FOS positive nuclei in VEH + Light treatment group was also significantly higher compared to dark controls ($p < 0.001$). Moreover, in the ventral SCN, c-FOS expression was also altered by *Treatment* ($F_{1,20} = 6.8$; $p = 0.008$) and *Lighting condition* ($F_{1,20} = 280.2$; $p < 0.001$). A significant increase in the number of Fos-positive nuclei in the ventral SCN of CAF + Light group was detected as compared to VEH + Light ($p = 0.01$) and CAF + Dark ($p < 0.001$).

group. The number of c-FOS positive nuclei in VEH + Light group was also significantly higher compared to dark controls ($p < 0.001$) (**Fig 7 B and D**).

Sleep deprivation and caffeine treatment alone or with light pulses activate calbindin containing cells in the SCN.

To phenotype the activated cells in the SCN by arousal and/or light pulse, we performed double immunolabelling for calbindin and c-FOS. In the dorso-ventral SCN, we calculated the percentage of calbindin cells that showed c-FOS immunoreactivity (IR). Both during early night and late night sleep deprivation, ANOVA revealed that the percentage of calbindin cells co-expressing c-FOS IR were modified by *Treatment* (early night: $F_{1,16} = 37.3$; $p < 0.001$; late night: $F_{1,16} = 153.5$; $p < 0.001$) and *Lighting condition* (early night: $F_{1,16} = 46.6$; $p < 0.001$; late night: $F_{1,16} = 420.8$; $p < 0.001$) (**Fig 8 A-C**). Post-hoc analysis showed that both in early and late night, the percentages of calbindin cells showing c-FOS IR were significantly higher in SD + Dark group compared to CTR + Dark ($p < 0.001$). Moreover, these percentages were also higher in SD + Light compared to CTR + Light (early night: $p = 0.007$; late night: $p = 0.002$) and SD + Dark (early night: $p = 0.002$; late night: $p < 0.001$). In both conditions, CTR + Light group showed a higher percentage of expression than CTR + Dark ($p < 0.001$). We also analysed percentage of c-Fos IR cells that express calbindin in dark conditions. Sleep deprivation in both early and late-night significantly enhances calbindin expression in c-FOS positive cells ($p < 0.05$).

Similar to sleep deprivation, in both early night and mid-night, ANOVA revealed that the percentages of the calbindin cells showing c-FOS IR were different after caffeine treatment (early night: $F_{1,20} = 27.3$; $p < 0.001$; mid-night: $F_{1,20} = 134.4$; $p < 0.001$) and light exposure (early night: $F_{1,20} = 12.18$; $p = 0.002$; mid-night: $F_{1,20} = 45.8$; $p < 0.001$) (**Fig 9 A-C**). Post-hoc analysis showed that both in early and mid-night the percentages of calbindin cells co-expressing c-FOS were significantly higher in CAF + Dark group compared to VEH + Dark ($p < 0.001$). Moreover, these percentages were also higher in CAF + Light compare to VEH + Light (early night: $p = 0.007$; mid-night: $p < 0.001$) and CAF + Dark (early night: $p = 0.005$; mid-night: $p < 0.001$). In both conditions, VEH + Light showed a higher expression percentage than that of VEH + Dark (early night: $p = 0.005$; mid night: $p < 0.001$). Remarkably, the mean effect of caffeine (CAF + Dark versus VEH + Dark) appeared even stronger than that of light (VEH + Light versus VEH + Dark), although this difference (CAF + Dark versus VEH + Light) did not reach significance. Again we analysed the percentage of

c-FOS IR cells that express calbindin in dark condition. Caffeine treatment in both early and midnight significantly enhances calbindin expression in c-FOS positive cells ($p < 0.001$).

5-HT and adenosine content in the SCN and midbrain after early night sleep deprivation or caffeine treatment

The levels of 5-HT and adenosine were assessed in SCN and midbrain after sleep deprivation and caffeine treatment during early subjective night (CT 14) (**Table 1**). The levels of 5-HT were not different in SCN and midbrain after sleep deprivation (**Fig 10 A**). Caffeine treatment enhanced midbrain levels of 5-HT significantly ($t_{12} = 3.22$, $p_{\text{two-tailed}} = 0.007$), though SCN levels of 5-HT remained unchanged after caffeine injection (**Fig 10 B**). The level of adenosine in SCN and midbrain did not change after sleep deprivation or caffeine treatment (**Fig 10 C-D**).

Caffeine content in SCN, midbrain and plasma 3 h after caffeine injection in early night

The levels of caffeine in the SCN, midbrain and plasma were quantified 3 h after caffeine or vehicle injection in the early night (**Table 2**). In addition to plasma, caffeine levels in the SCN and midbrain were markedly elevated in caffeine-treated animals ($p < 0.002$, t -test), indicating that caffeine can directly target these brain areas.

Discussion

It is widely acknowledged that behavioural arousal in the usual rest period has the ability to reset the master circadian clock (Webb et al., 2014). For instance, 3 h of sleep deprivation in the middle of the rest period induced phase-advances in Syrian hamsters (Antle and Mistlberger, 2000). Moreover, sleep deprivation and caffeine-induced arousal influence photic resetting of the SCN clock by attenuating light-induced phase-shifts in nocturnal mice and hamsters (Mistlberger et al., 1997, Challet et al., 2001, Vivanco et al., 2013, van Diepen et al., 2014). Most of the experiments supporting these statements have been carried out in laboratory rodents that are essentially night-active. It remains unresolved, however, whether these non-photic stimuli influence the SCN clock and its photic resetting of diurnal species in a similar or different manner. Using sleep deprivation and caffeine treatment as involuntary behavioural arousal, we show here that sleep deprivation in the early night induces phase-delay, whereas both nightly sleep deprivation and caffeine treatment, whatever the time at which the animals are subjected to it, enhance light-induced phase-shifts in the diurnal

Sudanian grass rat. These behavioural changes are concomitant at the cellular level with potentiation of light-induced c-FOS expression in SCN cells, in particular those containing calbindin. Noteworthy, these findings are conspicuously opposite to the results obtained earlier in nocturnal rodents, in which sleep deprivation and caffeine decrease light-induced phase-shifts.

Time-course of sleep loss during sleep deprivation and post caffeine treatments

In the present study, our sleep deprivation protocol significantly reduces sleep time and increases time spent awake in both early and late subjective night. A previous study in grass rats showed that 6 h of sleep deprivation, started at dark onset, significantly increased delta power during the next 3 h of sleep rebound (Hubbard et al., 2015). Our results indicate that following 4 h of sleep deprivation, wake duration in sleep-deprived animals went back to control levels and animals recovered sleep. We also showed that caffeine treatment (30 mg/kg) in early subjective night drastically increases wake timing during the 5 h period following the treatment. Mid-subjective night treatment induces increased wake time during the 2 h post-treatment period. These results provide a detailed monitoring of behavioural arousal during sleep deprivation and after caffeine administration.

Sleep deprivation, but not caffeine, in the early night induces phase-delays in diurnal grass rats

The phase-response curves to arousing stimuli show that both magnitude and direction of the shifts are gated by circadian phase, with maximal phase-advances during the middle of the rest period and minimal phase-delays during the latter half of the usual wake phase (Hughes and Piggins, 2012, Webb et al., 2014). Experiments aimed at mimicking sleep deprivation by intracranial or i.p. injection of an adenosine A1 agonist in the mid-sleeping period show dose-dependent shifts similar to those induced by sleep deprivation itself. In addition, the adenosine receptor antagonist caffeine attenuates the phase-shifts produced by sleep deprivation in nocturnal rodents (Antle et al., 2001).

In the present study, we found that caffeine alone does not induce significant behavioural shifts in diurnal grass rats, in keeping with an earlier study in nocturnal rodents (Vivanco et al., 2013). In humans, however, caffeine intake in the early night produces a phase-delay of the circadian melatonin phase (Burke et al., 2015). In parallel, sleep deprivation in early subjective night, without any notable hyperactivity, induces a phase-delay of the behavioural

rhythm in diurnal grass rats. Together, these data indicate that the overall shape and timing of phase-responses to sleep deprivation in nocturnal and diurnal species depend on the phase of the rest-activity cycle and are phased at opposite circadian times in both categories of species.

Sleep deprivation and caffeine treatment potentiate light-induced shifts in diurnal grass rats

Arousing stimuli such as sleep deprivation, forced activity, or injections of an adenosine A1 agonist inhibit light-induced phase-shifts (Watanabe et al., 1996, Mistlberger et al., 1997, Challet et al., 2001, Elliott et al., 2001, Sigworth and Rea, 2003). Sleep deprivation in mice attenuates the sustained response to light on SCN firing rate, while caffeine treatment normalizes the light response (van Diepen et al., 2014). Caffeine also blocks light-induced phase-delays in the mice (Vivanco et al., 2013).

We show here that 4 h of sleep deprivation in the early and late night enhances light-induced phase-delays and advances in the diurnal grass rat. Furthermore, caffeine similarly potentiates light-induced phase-shifts. These findings are opposite to the reducing effects found in nocturnal rodents (Vivanco et al., 2013). In humans, however, caffeine intake in the early night does not significantly enhance the light-induced phase-delay of the circadian melatonin phase (Burke et al., 2015). It is worth mentioning that the modulatory effects of sleep deprivation and caffeine on photic resetting in nocturnal and diurnal rodents are functionally comparable to those observed after serotonergic activation (Morin, 1999, Challet, 2007, Cuesta et al., 2008).

5-HT and adenosine levels in SCN and midbrain after sleep deprivation and caffeine treatment in the diurnal grass rats

Since the activity of the central serotonergic and adenosinergic systems is closely related with behavioural arousal, they may participate in arousal-induced resetting of the SCN. Serotonin (5-HT) concentrations in the SCN follow a daily pattern with higher levels during the active phase in both nocturnal and diurnal rodents (Poncet et al., 1993, Cuesta et al., 2008). The increased concentration of 5-HT or its main metabolite 5-HIAA in the SCN and other hypothalamic areas during sleep deprivation was also correlated to circadian phase-shifts and/or attenuated light-induced phase-shifts in nocturnal rodents (Grossman et al., 2000, Challet et al., 2001). In the current experiment, 5-HT concentrations after 4 h of sleep deprivation as measured in tissue punches, remained unchanged in the SCN and midbrain of

diurnal rodents, thus suggesting that arousal feedback would be mediated by other mediators (e.g., adenosinergic cues).

The adenosine receptor antagonist caffeine attenuates the phase-advances induced by sleep deprivation and blocks light-induced phase-delays. In addition, microinjections of an adenosine agonist in the SCN of hamsters and rats induce phase-shifts, as does sleep deprivation, and attenuate light-induced phase-shifts (Watanabe et al., 1996, Antle et al., 2001, Elliott et al., 2001). Some studies reported the presence of adenosine receptors in the SCN (Chen and van den Pol, 1997, Diaz-Munoz et al., 1999, Hallworth et al., 2002), suggesting that caffeine may act directly on SCN cells. Ryanodine receptors (RyRs) are caffeine-sensitive Ca^{2+} release channels (McPherson et al., 1991). Caffeine-mediated increases of Ca^{2+} levels in SCN neurons are dependent on the activation of RyRs (Pfeffer et al., 2009). Ryanodine and caffeine treatment applied *in vitro* during the subjective day produce phase-advances in the hamster SCN (Diaz-Munoz et al., 1999). These findings indicate that the effects of sleep deprivation and *in vivo* caffeine treatment may be achieved by activation of the adenosinergic system in the SCN. The present data do not fully support this hypothesis because adenosine levels in SCN and midbrain of grass rats, either sleep-deprived or treated with caffeine, remained essentially unchanged. On the other hand, tissue measurements do not accurately reflect release. Of note, while 5-HT levels remained unchanged in the SCN region, caffeine treatment in early night significantly increased 5-HT concentrations in the midbrain raphe. This confirms the fact that caffeine promotes serotonergic activation (Antle et al., 2001, Okada et al., 2001, Vivanco et al., 2013). Together these findings suggest that arousal feedback may also be mediated by mediators other than serotonergic and adenosinergic pathways such as orexinergic, cholinergic and glutamatergic cues). Further studies using intra-cerebral microdialysis of 5-HT, adenosine, orexin, and glutamate and acetylcholine receptor agonist are needed to solve this issue.

Cellular responses of arousal on light sensitivity of SCN

Nocturnal and diurnal species display similar temporal patterns of light-induced c-FOS expression in the SCN (Mahoney et al., 2001). Photic stimulation of c-FOS expression in the SCN has been used by many labs to study the photosensitive phase of the master clock. Previous studies in nocturnal species have shown that levels of c-FOS expression in the SCN decrease when they are aroused during their usual resting period (Mistlberger et al., 1998, Antle and Mistlberger, 2000, Dattolo et al., 2016). Activation of the adenosine A1 receptor

attenuates light-induced c-FOS and p-ERK expression in the SCN (Watanabe et al., 1996, Sigworth and Rea, 2003). Our results in the diurnal grass rat show that sleep deprivation in the early night increases light-induced c-FOS induction in the ventral SCN. Caffeine treatment in the midnight induces c-FOS expression in the dorsal SCN, albeit without inducing a subsequent phase-shift. Furthermore, caffeine treatment in both early and midnight potentiated light-induced c-FOS expression in the ventral SCN. These data show that the feedback effects of arousal on SCN cellular activity and their interaction with the light-induced expression of immediate early genes are opposite in nocturnal and diurnal rodents.

Our double immunolabelling indicates that behavioural arousal activates a large proportion of calbindin-containing cells in the SCN of diurnal rodents. Earlier studies in hamsters have already shown a role for SCN calbindin in the maintenance of circadian rhythms and photic entrainment (Hamada et al., 2003, Kriegsfeld et al., 2008, Stadler et al., 2010). Our results are the first to suggest that SCN calbindin-containing cells could be involved in the feedback effect of arousal on the master clock and its light-induced phase shifts also in diurnal species. To sum up, the present study in a diurnal rodent reveals that sleep deprivation can phase-delay the master clock and both sleep deprivation and caffeine administration potentiate light-induced phase-shifts. This work reinforces the view that modulation of the SCN clock by arousing cues may be a key function to maintain the respective temporal niche in day-active and night-active species. Moreover, the availability of a diurnal rodent model could be useful not only for comparative studies with nocturnal rodents and reciprocal interactions with human circadian research, but also for biomedical applications.

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Figure legends

Figure 1. Time course of sleep and waking under DD condition and sleep deprivation experiments. (A,C) Time course of wakefulness and (B,D) sleep when sleep deprivation were done in early (upper panels) and late (lower panels) night. Data are presented as mean \pm SEM (early night: n=6; late night: n=5). Asterisks indicate a statistically significant difference (***p < 0.001, *p < 0.05) between SD and CTR groups; (*) indicates a difference close to significance threshold (p = 0.053) between the 2 groups. SD = sleep deprivation, CTR = control. Horizontal gray bars above x-axis represent timeline of sleep deprivation.

Figure 2. Time course of sleep and waking under DD condition and following vehicle and caffeine administrations. (A,C) Time course of wakefulness and (B,D) sleep after i.p. administration of VEH or CAF in early (upper panels) and midnight (lower panels). Data are presented as mean \pm SEM (n=4). Asterisks indicate a statistically significant difference (***p < 0.001, *p < 0.05) between caffeine and vehicle injected groups. CAF = caffeine, VEH = vehicle. Arrow indicates time of injection.

Figure 3. Representative double-plotted actograms of wheel-running activity during 1 week under a light-dark cycle and 1 week in constant darkness (DD), displaying phase-shifts to sleep deprivation, light pulse and the combined effects of sleep deprivation and light pulse. On the first day of DD, in early night (A-D) and late night (E-H) animals were sleep-deprived (right panels) or left undisturbed as control (left panels), in darkness (upper panels) or followed by light pulse at CT16 and CT20, respectively (lower panels). Mean phase delays (negative value) and phase advances (positive) to sleep deprivation, light pulse and combination of both in early night (I) and late night (J). Data are presented as mean \pm SEM (n=6 per group). Asterisks indicate a statistically significant difference (***p < 0.001, **p <

0.01, * $p < 0.05$). Shaded area: DD, rectangular gray and dark gray bars: time line of shifting animal out of home cage for keeping undisturbed or sleep deprived, respectively. Stars: light pulse (200 lx for 30 min.), SD = sleep deprivation, CTR = control.

Figure 4. Representative double-plotted actograms of wheel-running activity during 1 week under a light-dark cycle and 1 week in constant darkness (DD), displaying phase-shifts to caffeine treatment, light pulse and the combined effects of caffeine and light pulse. On the first day of DD, in morning at CT0 (A-D) and mid-day at CT6 (E-H) animals were administered caffeine (right panels) or vehicle (left panel) in dark (upper panels) or followed by light pulse at CT2 or CT8, respectively (lower panels). Mean phase advances (positive) to caffeine treatment, light pulses and combination of both in the morning (I) and mid-day (J). Data are presented as mean \pm SEM (n=6 per group). An asterisk indicates a statistically significant difference ($p < 0.05$). Shaded area: DD, arrows: time of injection, Stars: light pulse (200 lx for 30 min.), CAF = caffeine, VEH = vehicle.

Figure 5. Representative double-plotted actograms of wheel-running activity during 1 week under a light-dark cycle and 1 week in constant darkness (DD), displaying phase-shifts to caffeine treatment, light pulse and the combined effects of caffeine and light pulse. On the first day of DD, in early night at CT14 (A-D) and midnight CT18 (E-H) animals were administered with caffeine (right panels) or vehicle (left panels) in dark (upper panels) or followed by light pulse at CT16 or CT20, respectively (lower panel). Mean phase delays (negative value) and phase advances (positive) to caffeine treatment, light pulse and combination of both in early (I) and midnight (J). Data are presented as mean \pm SEM (n=6 per group). Asterisks indicate a statistically significant difference (*** $p < 0.001$, ** $p < 0.01$,

* $p < 0.05$). Shaded area: DD, arrows: time of injection, Stars: light pulse (200 lx for 30 min.), CAF = caffeine, VEH = vehicle.

Figure 6. c-FOS expression in the SCN after sleep deprivation. (A, B) Representative photomicrographs of c-FOS in the SCN of early and late-night sleep-deprived (right panels) and control (left panels) *Arvicanthis* in darkness (upper panels) or with exposure of light pulse for 30-min at the end of the sleep deprivation (lower panels). (C, D) c-FOS mean responses of dorsal and ventral parts of the SCN of early and late-night sleep deprived and control *Arvicanthis* in darkness or with exposure of light pulse at the end of the sleep deprivation. Data are presented as mean \pm SEM (n=6 per group). * $p < 0.05$ between sleep-deprived and control groups; # $p < 0.001$ between light and dark conditions. SD = sleep deprivation, CTR = control.

Figure 7. c-FOS expression in the SCN after caffeine treatment. (A, B) Representative photomicrographs of c-FOS in the SCN of early and midnight caffeine (right panels) or vehicle-treated (left panels) *Arvicanthis* in darkness (upper panels) or with exposure of light pulse for 30-minutes at CT16 and CT20, respectively (lower panels). (C, D) c-FOS mean responses of dorsal and ventral parts of the SCN of early and midnight vehicle and caffeine treated *Arvicanthis* in darkness or with exposure of light pulse at CT16 and CT20, respectively. Data are presented as mean \pm SEM (n=6 per group). * $p < 0.05$ between caffeine- and vehicle-injected groups; # $p < 0.001$ between light and dark conditions. CAF = caffeine, VEH = vehicle.

Figure 8. Calbindin expression in c-FOS-positive cells in the SCN after sleep deprivation. (A) Representative photomicrographs of the SCN of early night sleep-deprived (right panels)

and control (left panels) *Arvicanthis* in darkness (upper panels) or with exposure of light pulse for 30-min at CT16 (lower panels), showing c-FOS (dark black nucleus) and Calbindin (light brown soma). Arrow heads, single c-FOS immunoreactive (IR) neurons; Arrows, single calbindin IR neurons; Cross, neurons positive for both c-FOS and calbindin IR. Mean percentage of calbindin neurons with c-FOS IR in the SCN of (B) early night and (C) late night sleep-deprived and control *Arvicanthis* in darkness or with exposure of light pulse at CT16 and CT20, respectively. Data are presented as mean \pm SEM (n =6 per group). * $p < 0.001$ between sleep deprived and control; # $p < 0.001$ between light and dark conditions. SD = sleep deprivation, CTR = control.

Figure 9. Calbindin expression in c-FOS-positive cells in the SCN after caffeine treatment. (A) Representative photomicrographs of the SCN of early night caffeine (right panels) or vehicle-treated (left panels) *Arvicanthis* in darkness (upper panels) or with exposure of light pulse for 30-minutes at CT16 (lower panels), showing c-FOS (dark black nucleus) and Calbindin (light brown soma). Arrow heads, single c-FOS immunoreactive (IR) neurons; Arrows, single calbindin IR neurons; Cross, neurons positive for both c-FOS and calbindin IR. Mean percentage of calbindin neurons with c-FOS IR in the SCN of (B) early night and (C) midnight caffeine or vehicle-treated *Arvicanthis* in darkness or with exposure of light pulse at CT16 and CT20, respectively. Data are presented as mean \pm SEM (n=6 per group). * $p < 0.001$ between caffeine and vehicle injected group; # $p < 0.001$ between light and dark conditions. CAF = caffeine, VEH = vehicle.

Figure 10. SCN and midbrain contents of (A,B) 5-HT and (C,D) adenosine after early night sleep deprivation (CT12-16) and caffeine treatment (CT14) in *Arvicanthis*. Data are presented

as mean \pm SEM (n=6 per group). *p < 0.01 between caffeine and vehicle-injected groups; SD = sleep deprivation, CTR = control, CAF = caffeine, VEH = vehicle.

Tables

Table 1. 5-HT and adenosine contents in the SCN and midbrain after sleep deprivation or caffeine treatment in early subjective night.

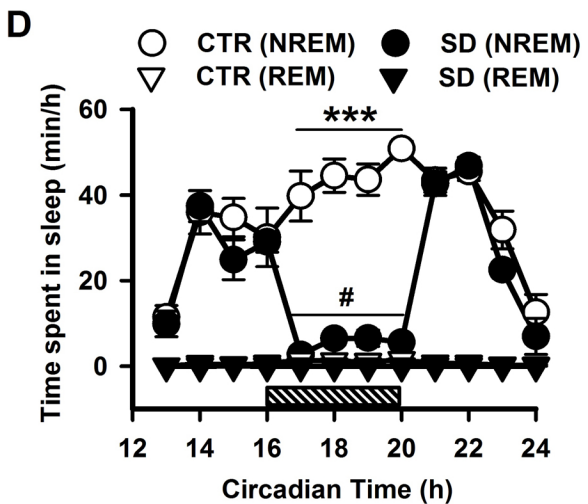
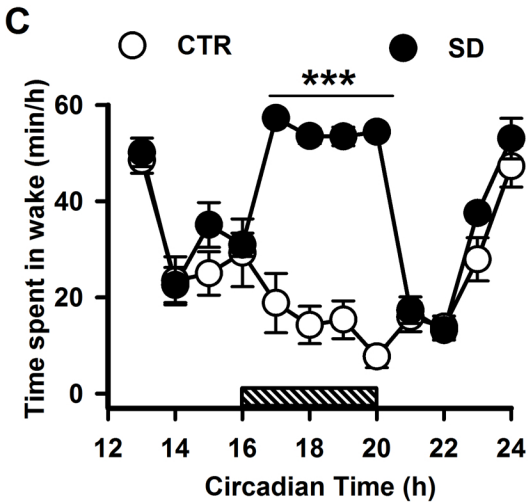
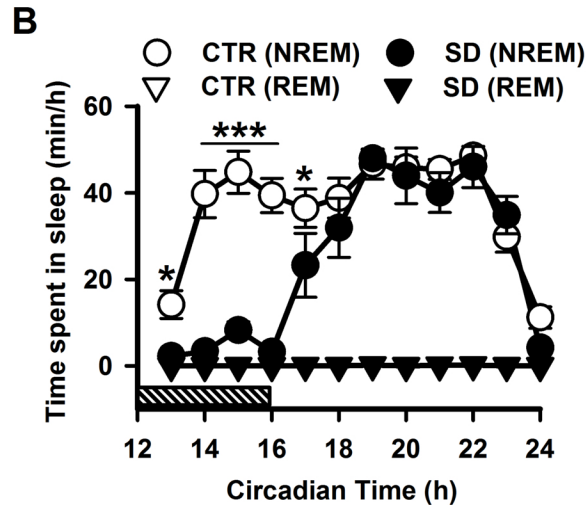
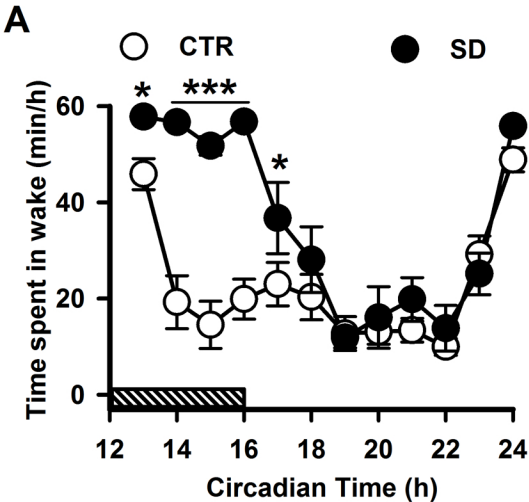
	CTR	SD	VEH	CAF
SCN 5-HT (nmol/g of tissue)	40.17±2.66	40.20±3.52	37.57±2.49	44.43±4.75
Midbrain 5-HT (nmol/g of tissue)	7.07±0.69	6.59±0.38	6.05±0.64	8.64±0.59*
SCN adenosine (µmol/g of tissue)	7.69±1.14	9.71±1.84	7.18±1.2	7.3±1.4
Midbrain adenosine (µmol/g of tissue)	22.48±1.62	23.05±1.5	23.08±1.6	22.39±1.5

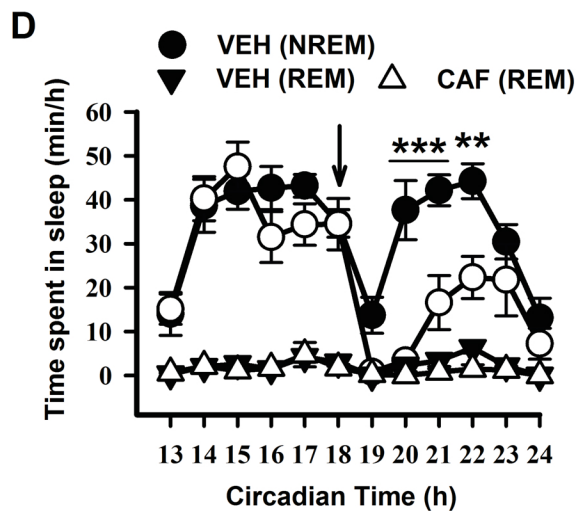
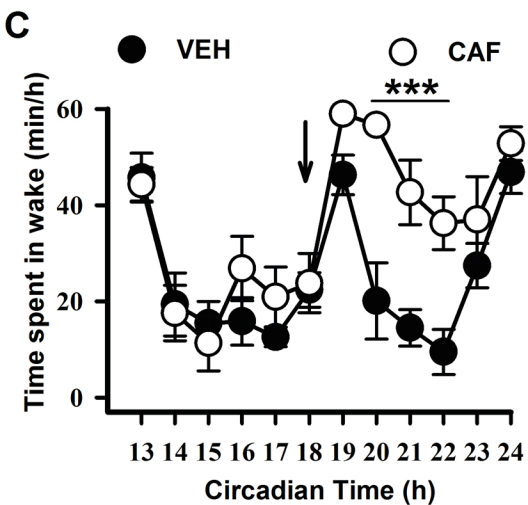
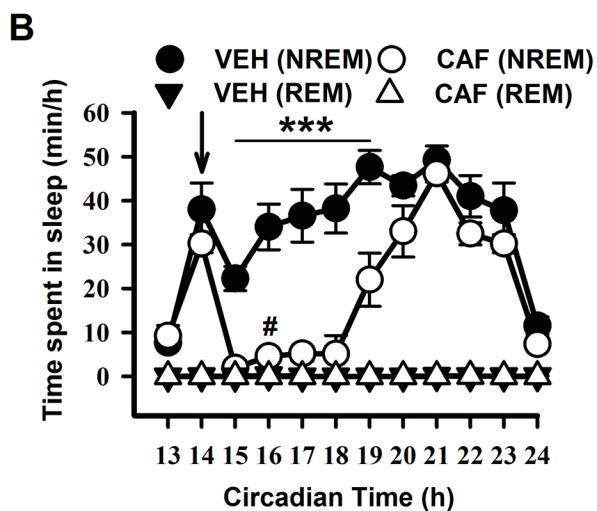
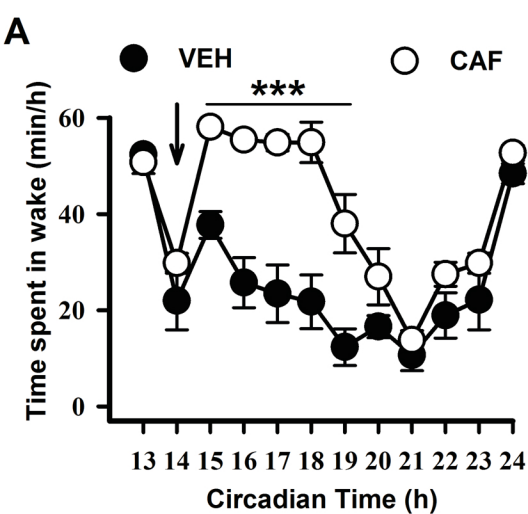
Data are presented as mean ± SEM (n=6/group) and were analyzed by Student's *t*-test (unpaired, two-tailed) between control (CTR) and sleep-deprived groups (SD), and between vehicle (VEH) and caffeine treatments (CAF); * *p* < 0.01.

Table 2. Caffeine contents in the SCN, midbrain and plasma 3 h after intraperitoneal injection of vehicle or caffeine in early night.

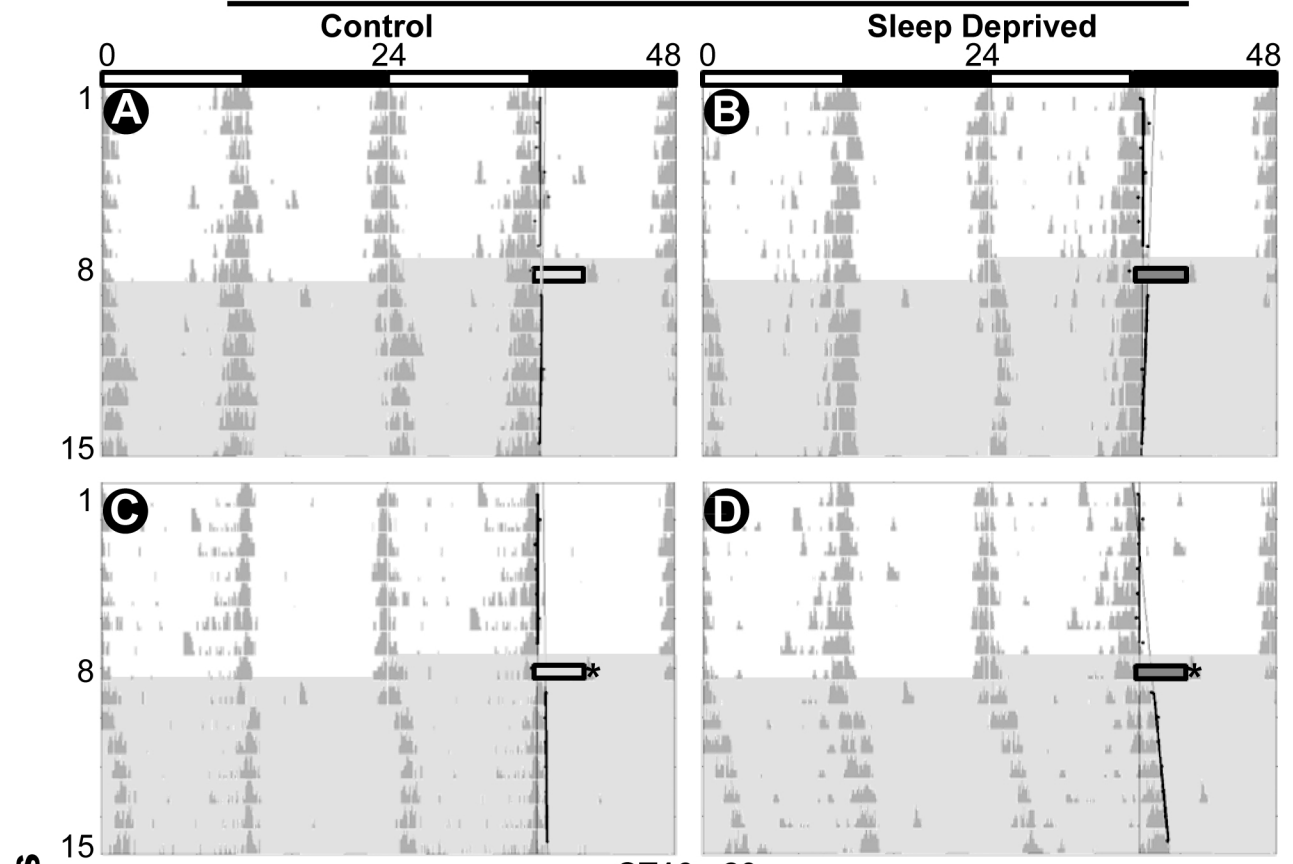
Treatments	SCN caffeine (mmol/g of tissue)	Midbrain caffeine (mmol/g of tissue)	Plasma caffeine (mmol/ml)
VEH	26.50±14.02	18.93±9.88	0.028±0.02
CAF	2691.54 ±722.35*	3180.69±212.37*	402.43±88.42*

Data are presented as mean ± SEM (n=6/group) and were analyzed by Student's *t*-test (unpaired, two-tailed) between vehicle (VEH) and caffeine treatments (CAF); * *p* < 0.002.

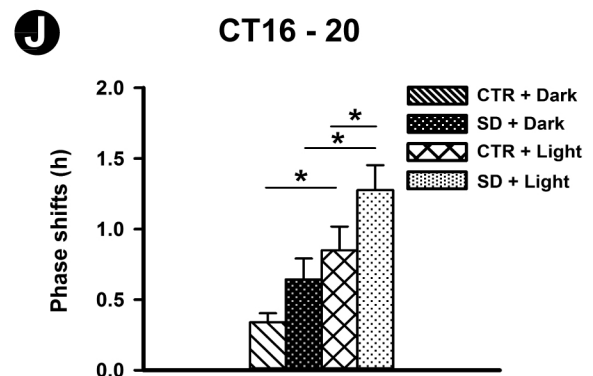
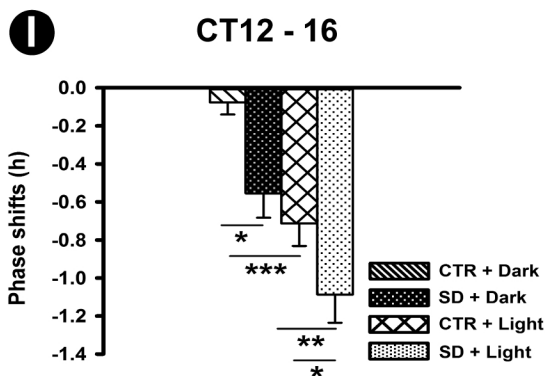
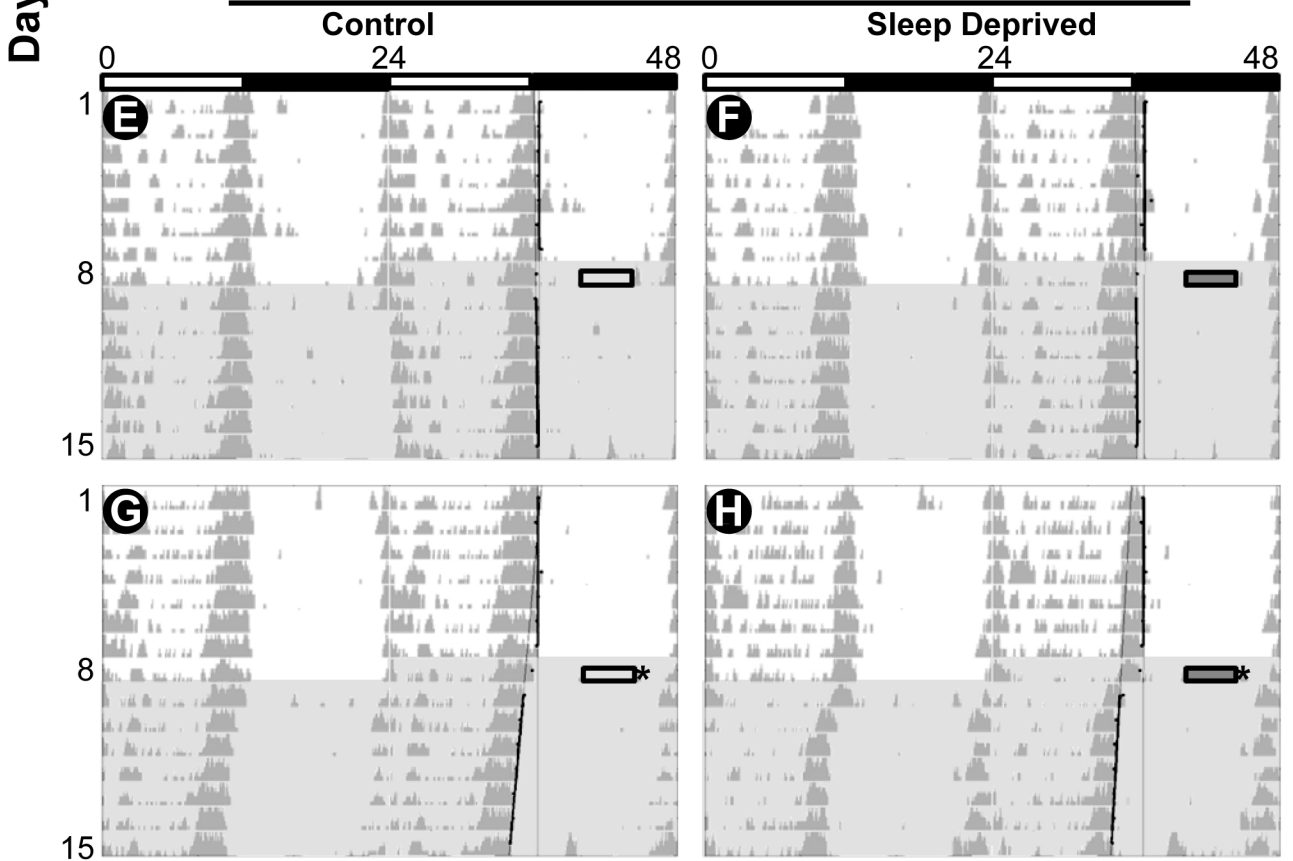


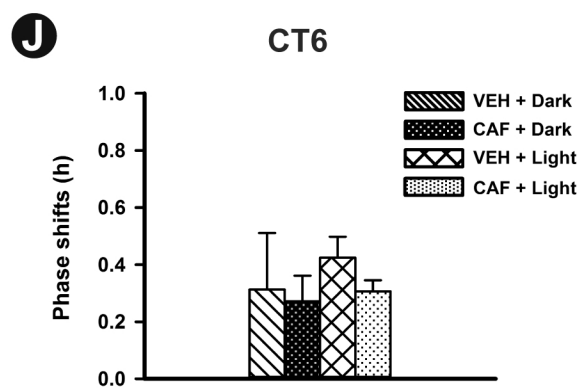
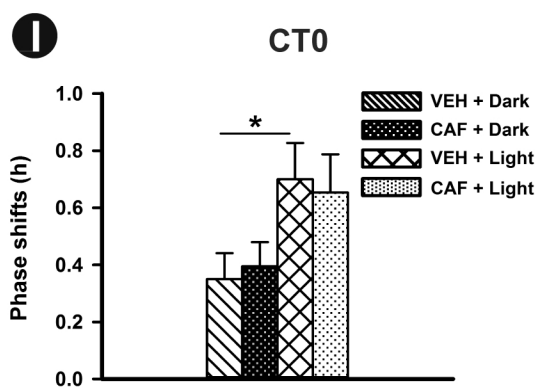
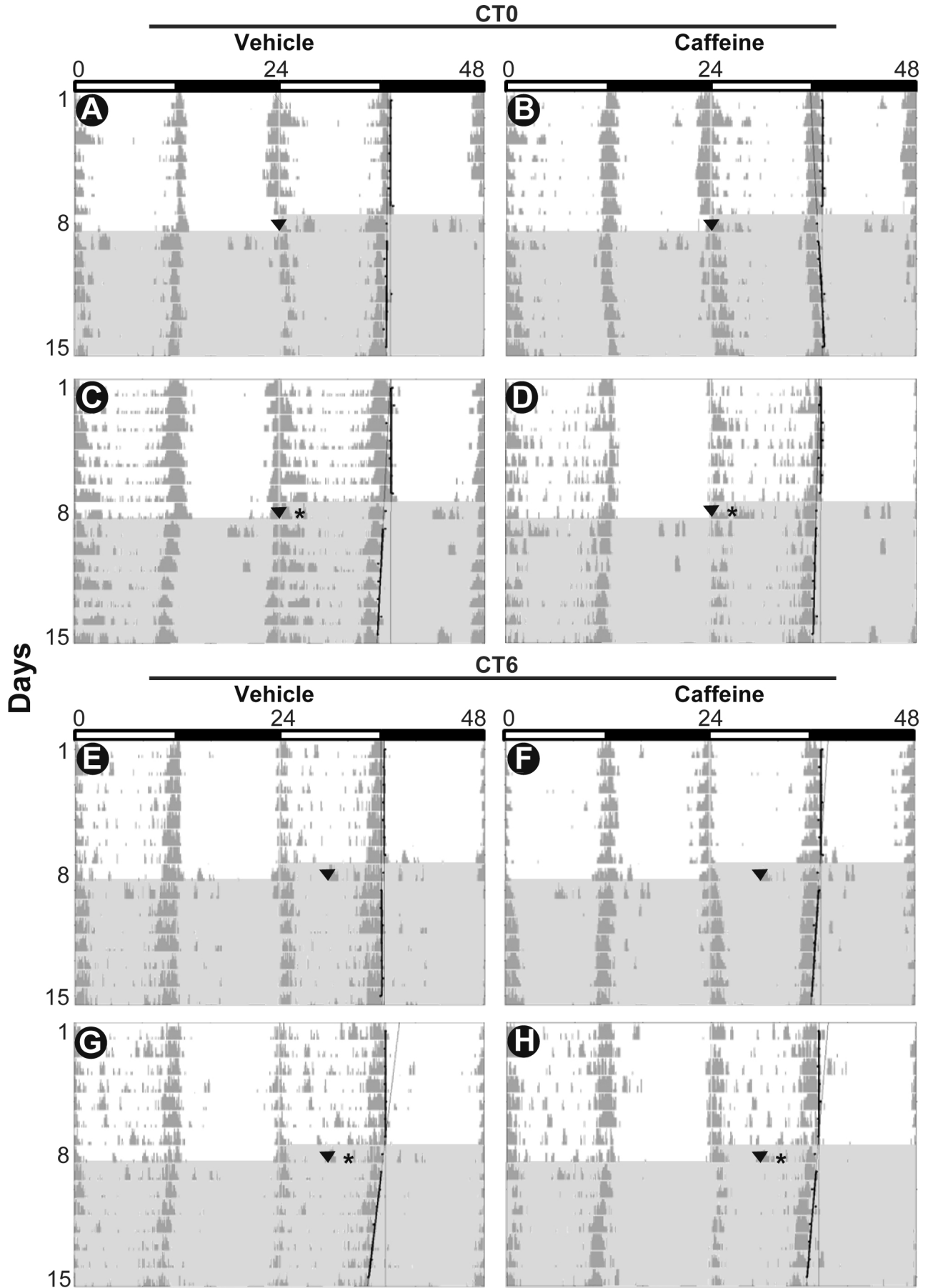


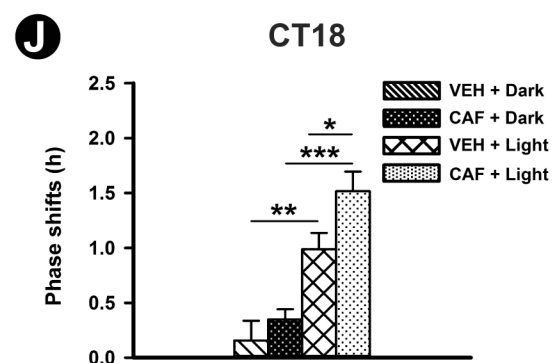
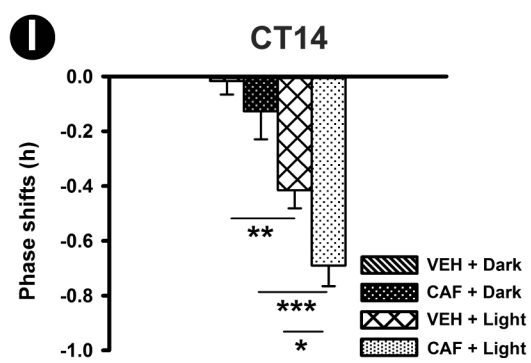
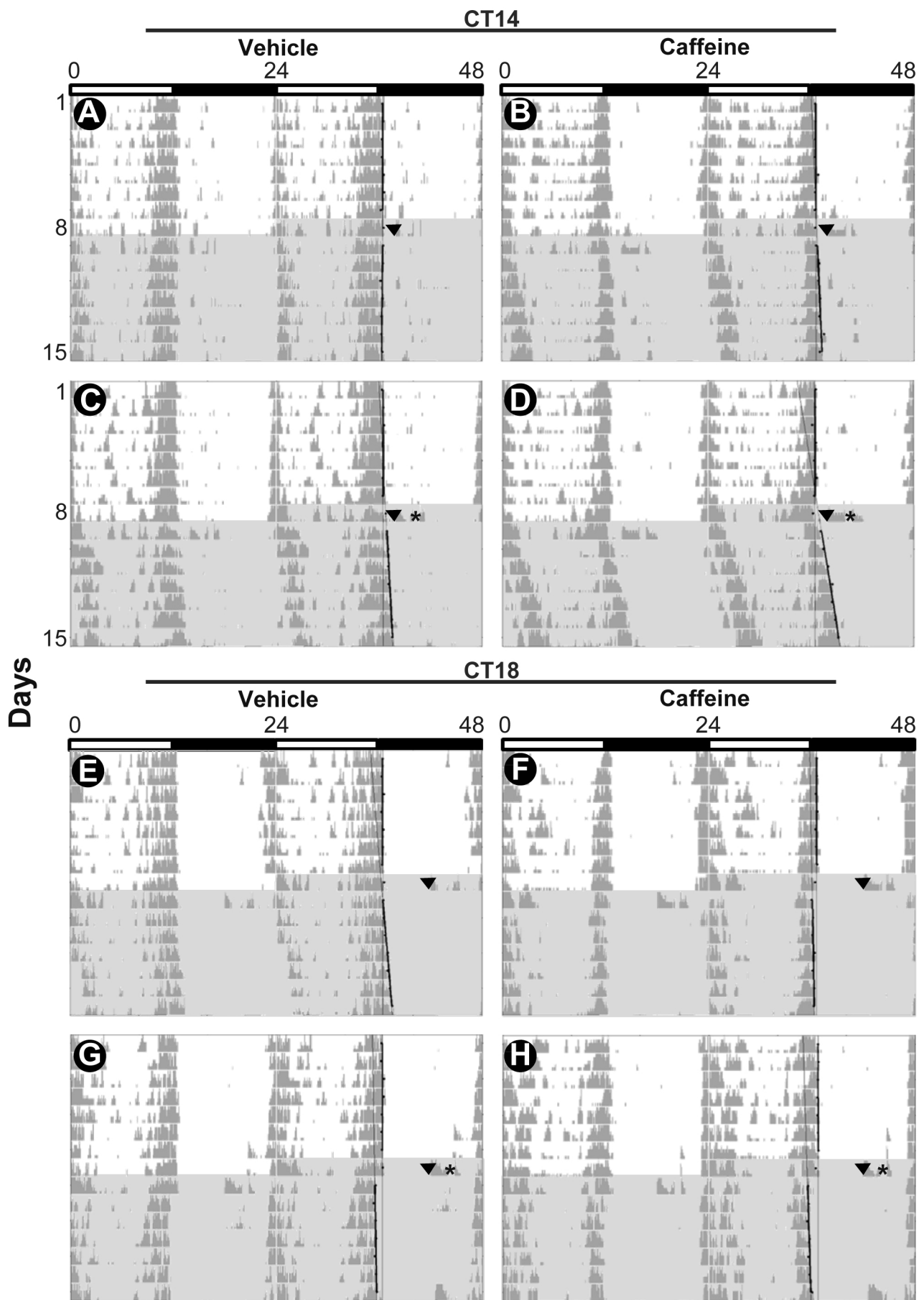
CT12 - 16

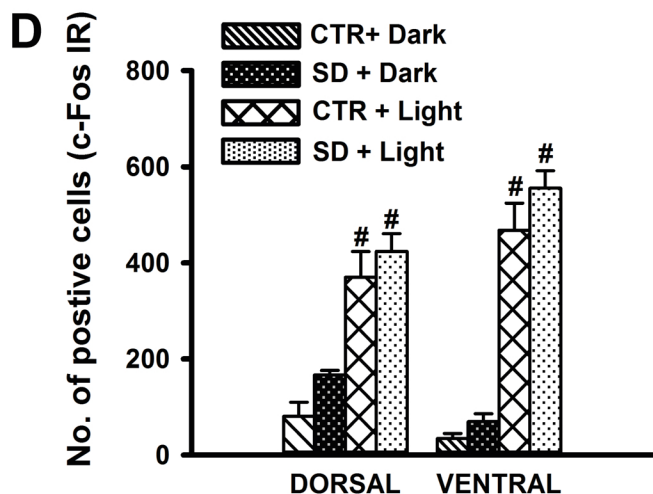
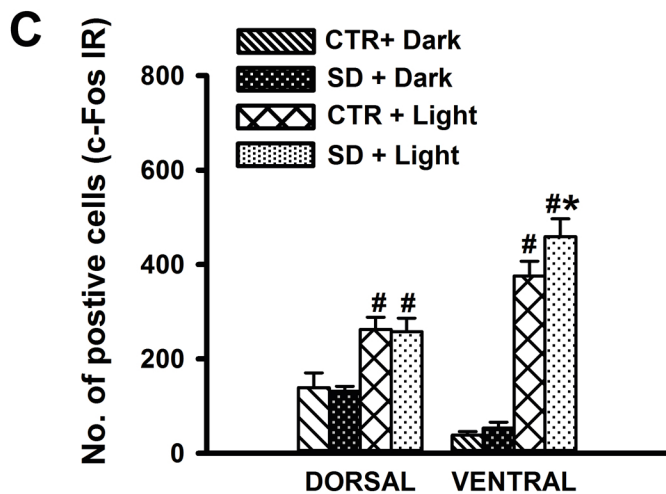
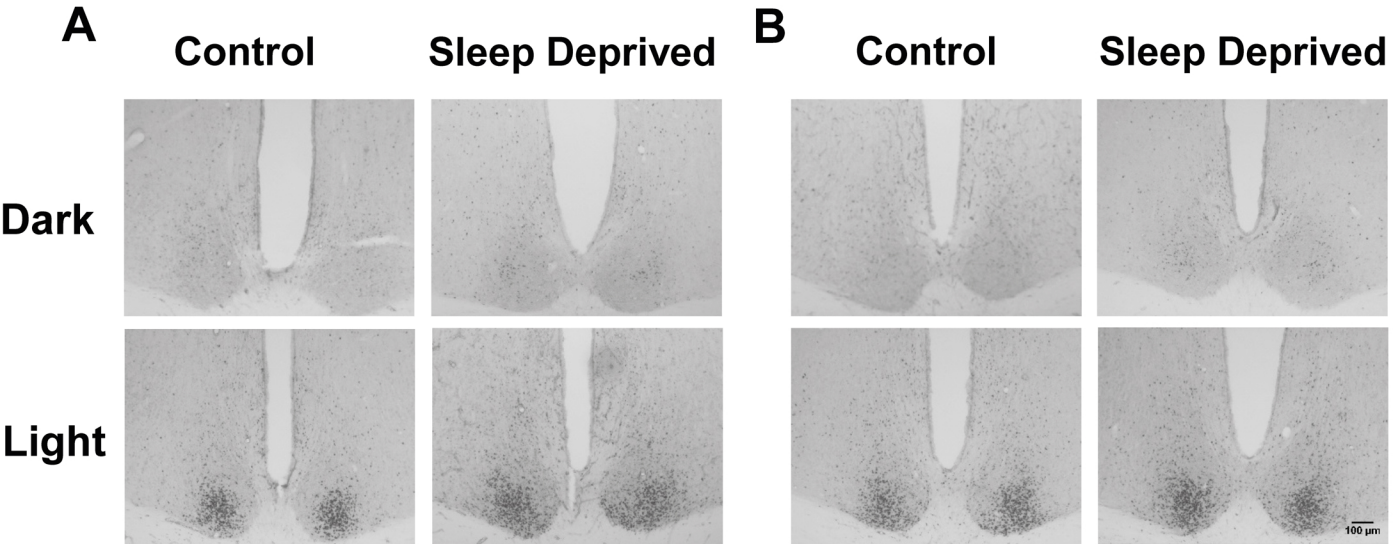


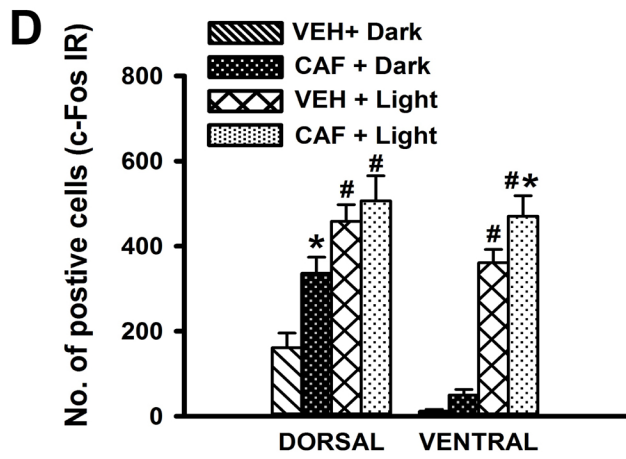
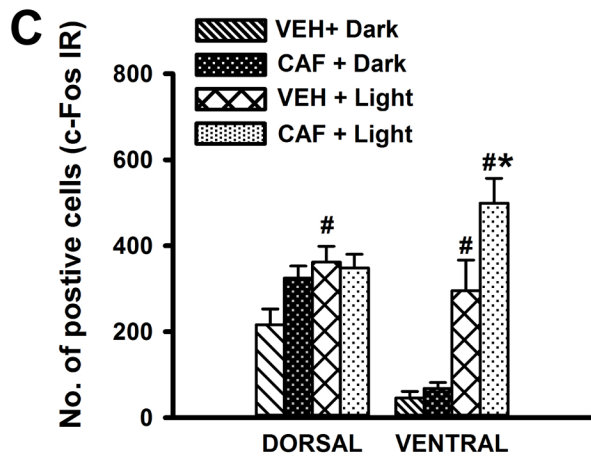
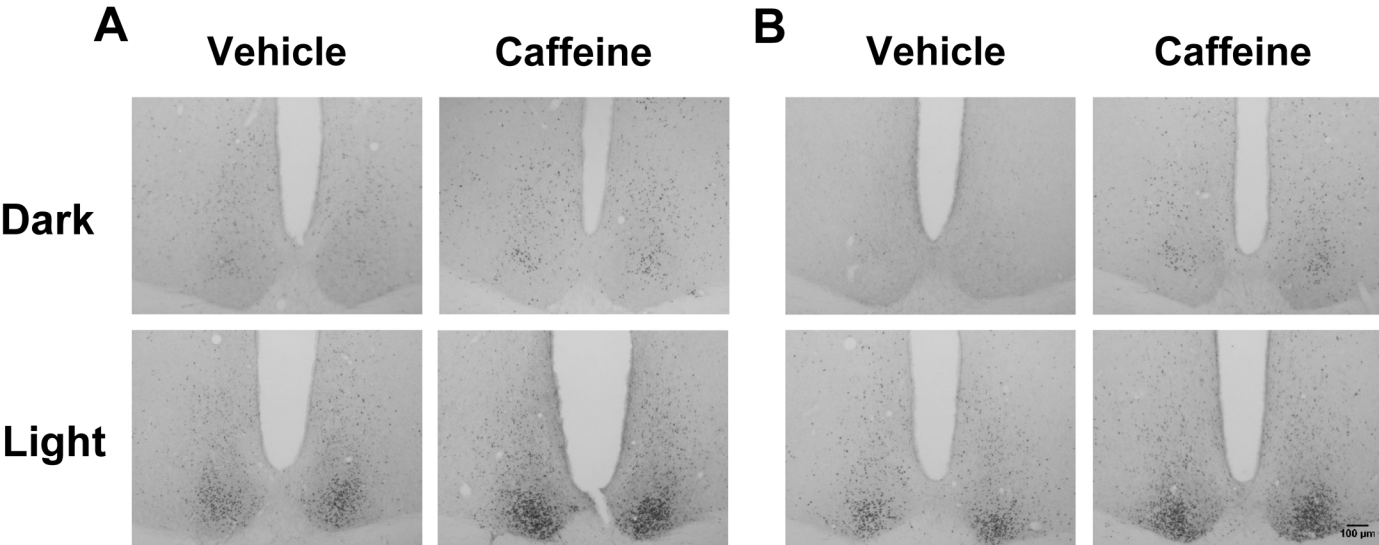
CT16 - 20









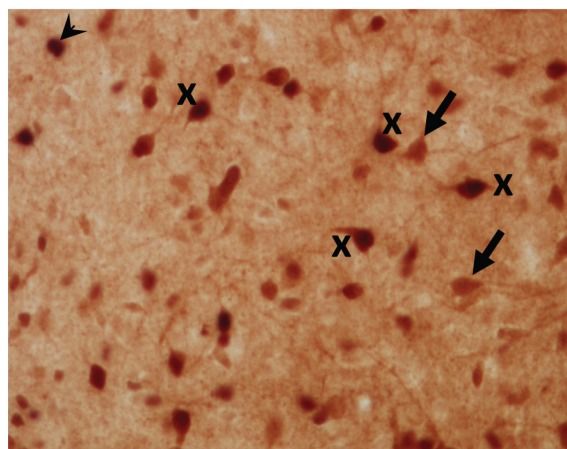
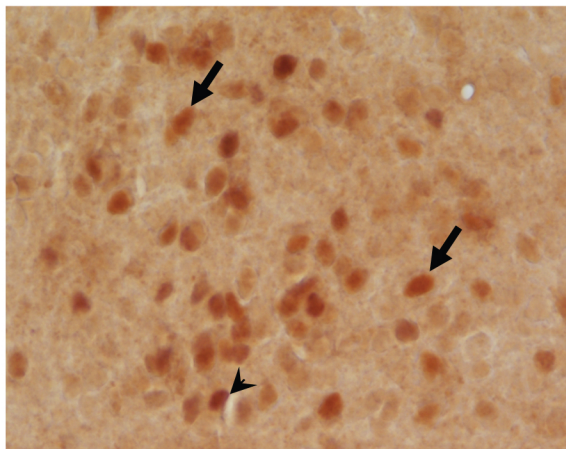


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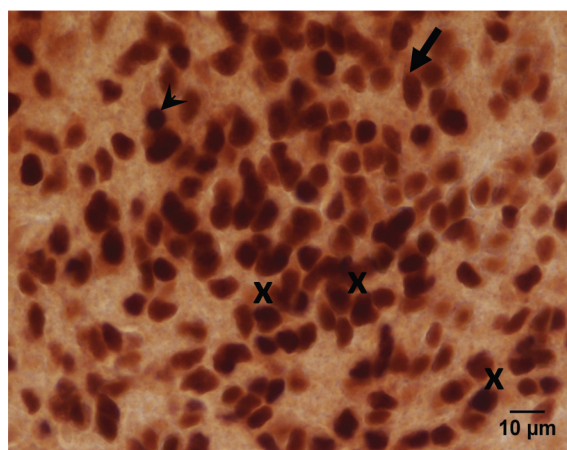
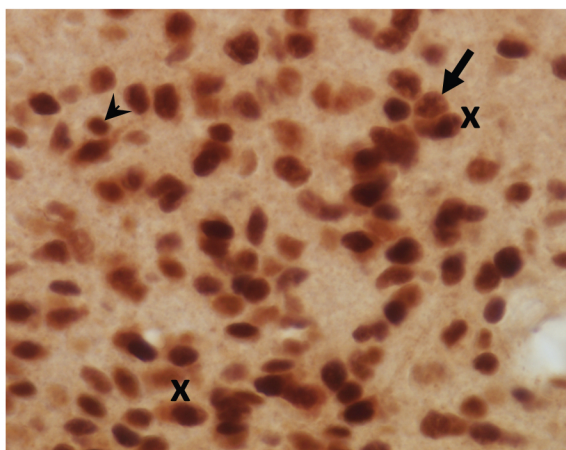
Control

Sleep Deprived

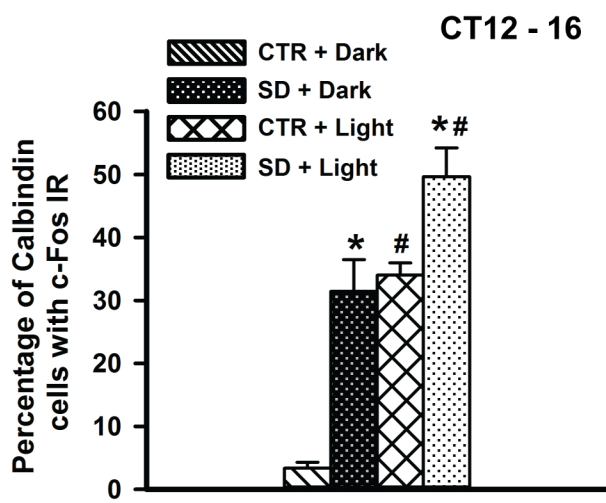
Dark



Light



B



C

