Behavioral activity increases neuronal activity in the circadian clock of diurnal *Arvicanthis ansorgei*

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Abstract

The central circadian clock, located in the suprachiasmatic nucleus (SCN) within the brain, regulates daily patterns of activity and physiology. Many studies indicate that exercise at certain times of the day is beneficial for maintaining circadian health; however, the underlying mechanisms in diurnal species such as humans are poorly understood. We therefore investigated the effect of behavioral activity on SCN neuronal firing rate in the diurnal rodent *Arvicanthis ansorgei* by performing *in vivo* electrophysiological recordings in freely moving animals. We found that SCN firing was acutely increased at the onset of behavioral activity; the firing rate remained elevated during episodes of behavioral activity ranging from seconds to hours, and then returned to baseline levels after behavioral activity ceased. We also found that diurnal behavioral input increases SCN rhythm amplitude, while crepuscular activity results in a bimodal rhythm in the SCN. To confirm that behavioral activity affects the SCN via activation of extra-SCN areas, we performed *ex vivo* recordings and found that SCN firing in both fully diurnal and crepuscular animals has a unimodal rhythm, with a single peak occurring at midday. Together, these data indicate that exercise increases the SCN's rhythm in diurnal *Arvicanthis* and does so via an excitatory effect on the SCN's neuronal firing rate. Thus the electrical activity rhythm that is generated at a molecular level is increased by behavioral activity.

Keywords: Diurnality, suprachiasmatic nucleus, exercise, *in vivo* electrophysiology, Sudanian grass rat.

Introduction

Nearly all life on Earth developed an internal clock that can entrain the timing of physiology processes and behaviors to daily changes in the environment (Hastings et al., 2018; Takahashi, 2017). Diurnal and nocturnal species are active primarily during the day or night, respectively, while some species are crepuscular (i.e., active primarily during dawn and/or dusk) (Challet, 2007). In mammals, the timing of physical activity is regulated by the central circadian pacemaker known as the suprachiasmatic nucleus (SCN), a bilateral structure located in the hypothalamus. The SCN generates a rhythm with a period of approximately 24 hours and then conveys this rhythm to the brain and the periphery, thereby establishing synchronized rhythmicity at the systemic level (Abrahamson & Moore, 2001; Hastings et al., 2018; Pauls et al., 2016). Several pathways that convey this functional output from the SCN have been identified and include the glucocorticoid regulatory pathway, melatonin regulation, and the timing of behavioral activity (Kalsbeek et al., 2006).

Interestingly, in addition to driving clock output, behavioral activity in turn affects our central timing (Hughes & Piggins, 2012; Jha et al., 2021). Thus, in humans exercise can induce a phase shift in the circadian clock's rhythm (Baehr et al., 2003; Buxton et al., 1997; Buxton et al., 2003; Van Reeth et al., 1994; Youngstedt et al., 2019), facilitate entrainment to non-24-hour cycles (Miyazaki et al., 2001), and accelerate realignment to a shifted light-dark cycle (Barger et al., 2004; Eastman et al., 1995; Yamanaka et al., 2010). The acute effects of exercise on the circadian system have been reported in nocturnal rodents such as rats, mice, and hamsters, showing that behavioral activity in these species acutely suppresses the SCN's electrical activity (Meijer et al., 1997; Schaap & Meijer, 2001; van Oosterhout et al., 2012; Yamazaki et al., 1998).

To measure the effects of behavioral input in the SCN of diurnal species, we recorded electrical activity in the SCN of freely moving diurnal *Arvicanthis ansorgei* using implanted electrodes. In addition, we recorded *ex vivo* brain slices in order to measure the SCN's intrinsic rhythm in the absence of behavioral input. We found that unlike nocturnal species—in which exercise inhibits SCN activity—the SCN in *Arvicanthis* is excited by behavioral activity. We also found that the excitatory effect of behavioral activity in crepuscular animals leads to a bimodal pattern of SCN activity. Finally, our *ex vivo* recordings of SCN slices prepared from both fully diurnal and crepuscular animals revealed a unimodal SCN rhythm, indicating that the behavioral input measured *in vivo* likely originates from brain regions located outside of the SCN.

Results

Behavioral activity increases the electrical activity of SCN neurons

We first performed SCN recordings in freely moving *Arvicanthis* (n=11 animals) while simultaneously recording the animals' behavioral activity. We found that the SCN was more active during the day, when the animals were physically active. At an ultradian (i.e., minutes-hours) timescale, upward deflections occurred at the times in which the animal was active (Figure 1A). We then fit a curve to all inactive timepoints, revealing a daily rhythmic profile; a curve fitted to all active timepoints showed a similar profile, albeit at a higher MUA frequency (Figure 1A). On average, the timepoints at which the animals were active corresponded to higher multiunit activity (MUA) (Figure 1B). At the onset of behavioral activity, we observed an acute increase in SCN neuronal firing (Figure 1C); these increases

in firing rate were present in both the light and dark periods (Figure 1D), and their magnitude was similar between day and night (Figure 1E).

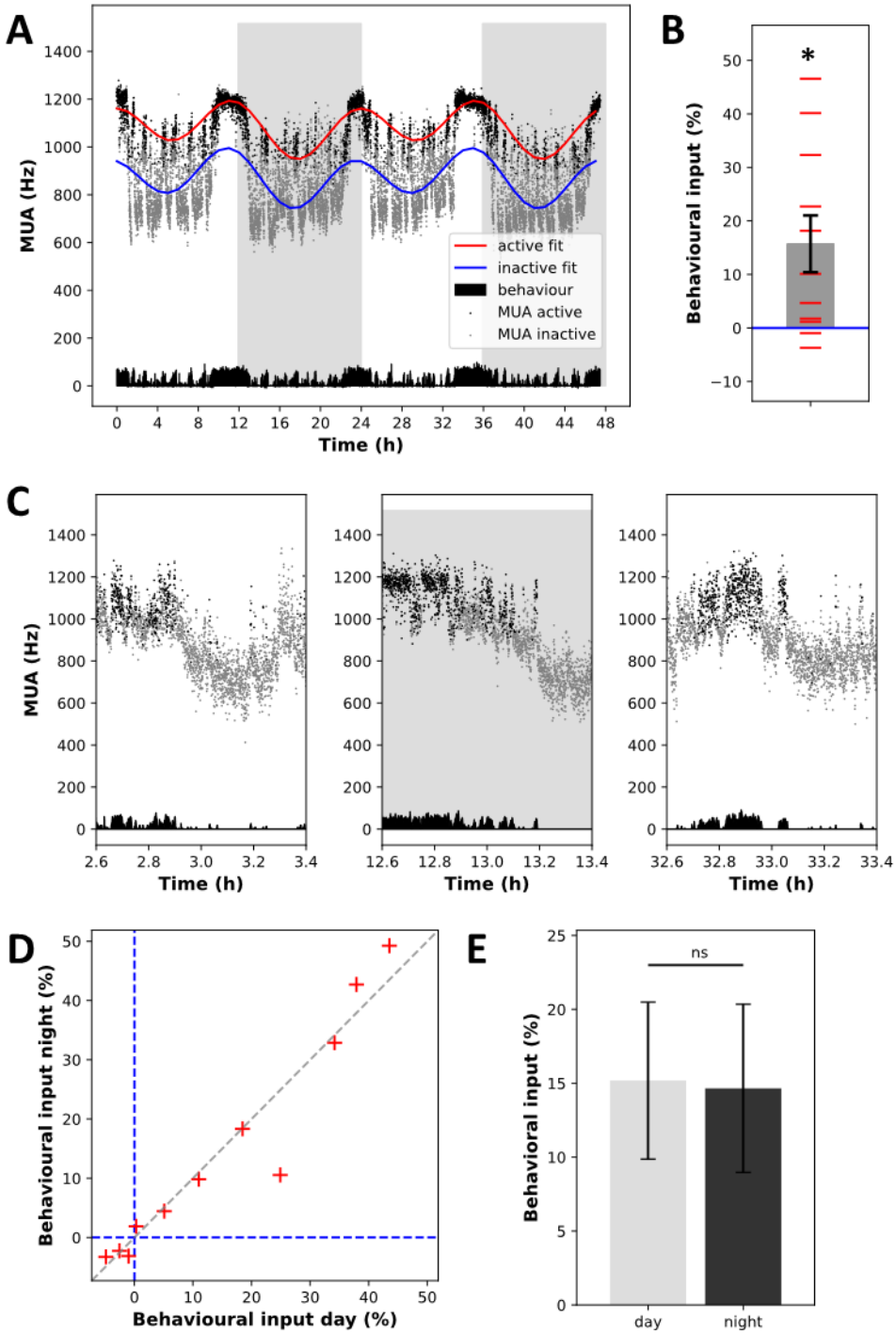


Figure 1: Physical activity increases SCN electrical activity measured *in vivo*. (A) Representative 48-hour recording of multiunit activity (MUA) measured at active timepoints (black symbols) and inactive timepoints (light gray symbols). The gray shaded background indicates darkness, and the animal's physical activity is indicated in black at the bottom. The red and blue curves were fitted to the active and inactive timepoints, respectively. (B) Each horizontal red line represents the magnitude of the behavioral input for an individual animal. (C) Representative magnified time courses of MUA measured during bouts of physical activity. (D) The magnitude of behavioral input during the night plotted against the magnitude of behavioral input measured during the day for each animal; the dashed gray line represents the 1:1 correlation ($y=x$). (E) Summary of the magnitude of behavioral input measured during the day and during the night (n=11 animals); ns, not significant.

Behavioral input increases the amplitude of the SCN rhythm

Given the excitatory effects of behavioral activity on MUA measured in the SCN of *Arvicanthis*, we hypothesized that behavioral input would affect the amplitude of MUA rhythms in the SCN of diurnal species. Thus, in diurnal animals activity during the day would likely increase MUA activity to higher levels, while inactivity during the night would decrease MUA to lower levels (shown schematically in Figure 2A). In contrast, in crepuscular animals activity at dawn and dusk would lead to a bimodal MUA rhythm in which two distinct peaks occur at the transitions between light and dark (shown schematically in Figure 2B).

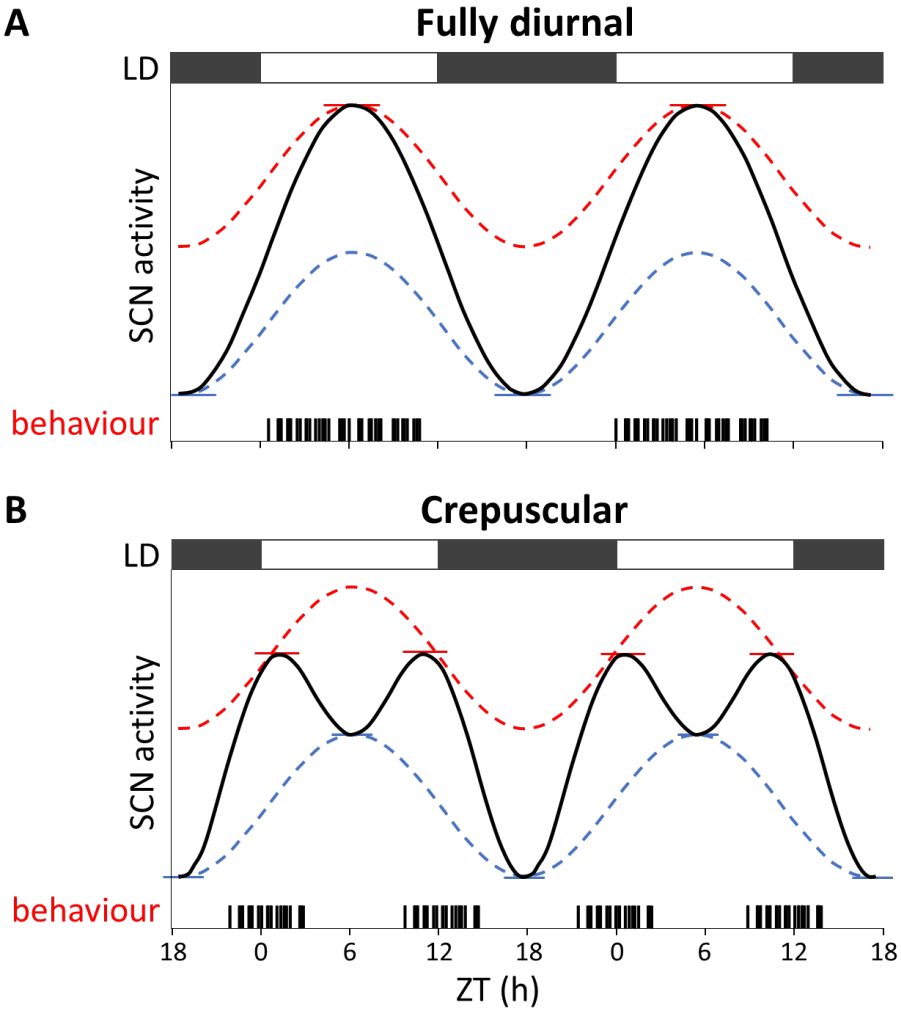


Figure 2: Schematic depiction of the MUA pattern predicted in diurnal and crepuscular animals. (A) In fully diurnal animals, activity during the day increases the amplitude of the total MUA rhythm (black line). The red and blue dashed lines represent the fit to the idealized MUA levels measured during activity and inactivity, respectively. (B) In crepuscular individuals, activity at dawn (the transition from dark to light) and dusk (the transition from light to dark) leads to a bimodal MUA rhythm. In both panels, the light-dark cycle (LD) is shown above the graph, and behavioral activity is shown at the bottom. ZT, Zeitgeber time, with ZT0 and ZT12 corresponding to lights on and lights off, respectively.

Crepuscular activity drives a bimodal pattern of SCN neuronal firing

Next, we determined the shape of the 24-hour rhythm by fitting the curve to all datapoints. We found that some of our recordings had a unimodal MUA rhythm (Figure 3A), while other recordings had a more bimodal MUA rhythm reflecting the animal's behavior (Figure 3B). We therefore quantified the

bimodality of the MUA rhythm and the crepuscularity of the behavioral activity using an "MUA crepuscularity index" and a "behavioral crepuscularity index", respectively; each index ranged from 0 to 1, corresponding to the lowest and highest degree of crepuscularity, as shown in Figure 3C. Plotting the MUA crepuscularity index against the behavioral crepuscularity index for each animal revealed a significant correlation between the two (Figure 3D).

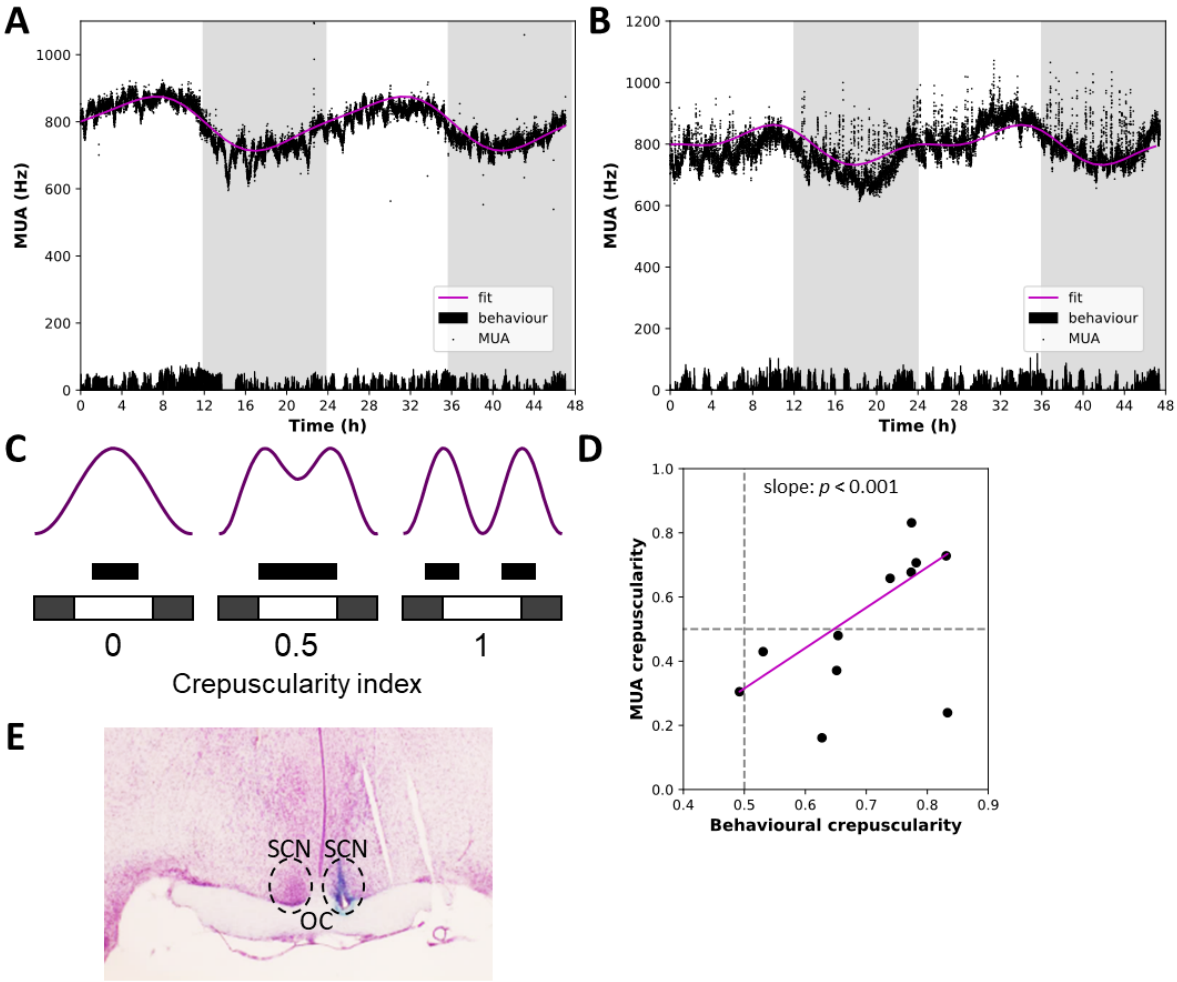
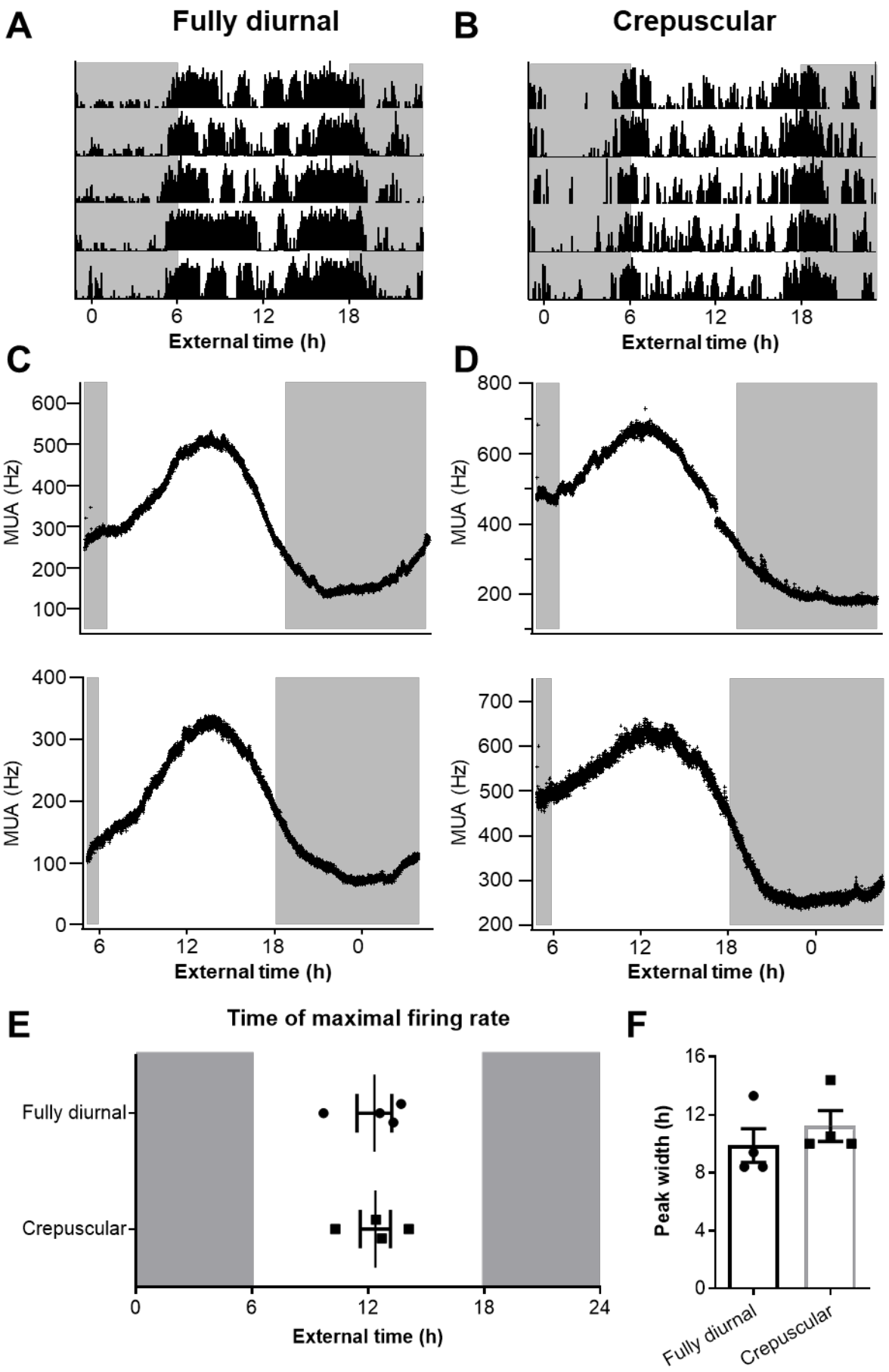


Figure 3: Diurnal *Arvicanthis* display both unimodal and bimodal patterns of SCN electrical activity measured *in vivo*. (A, B) Representative 48-hour recordings showing an animal with a unimodal MUA rhythm (A) and an animal with a bimodal MUA rhythm (B). The solid purple lines show the curves fitted to the datapoints. (C) Schematic depiction showing the crepuscularity index, ranging from 0 (lowest level of crepuscularity) to 1 (highest level of crepuscularity). (D) The MUA crepuscularity index was plotted against the behavioral crepuscularity index for each animal, and the linear regression was measured using the Huber loss function.

The intrinsic SCN rhythm is unimodal

Next, to determine whether the bimodal MUA rhythm in crepuscular animals is driven by behavioral activity input or arises in part due to an intrinsic bimodal pattern of SCN firing, we prepared *ex vivo* brain slices containing the SCN but lacking neuronal input from other brain structures. To compare the intrinsic activity of the SCN between fully diurnal animals and crepuscular animals, we recorded slices prepared from 4 animals that displayed fully diurnal behavioral activity (Figure 4A) and 4 animals that displayed crepuscular behavioral activity (Figure 4B) based on their actograms. Interestingly, the SCN activity in all 8 brain slices showed a unimodal rhythm, including the slices from the crepuscular animals (Figure 4C,D). Thus, no bimodal MUA pattern was observed in any slice. Moreover, neither the timing of the MUA peak (Figure 4E) nor the width of the MUA peak (Figure 4F) differed significantly

different between fully diurnal and crepuscular animals, indicating that the bimodal SCN pattern observed in our *in vivo* recordings likely originates from input derived from structures outside of the SCN and is not an intrinsic property of the SCN.


A
Fully diurnal
B
Crepuscular
0
6
12
18
External time (h)
C
D
MUA (Hz)
600
500
400
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200
100
800
600
400
200
400
300
200
100
0
700
600
500
400
300
200
6
12
18
0
External time (h)
E
Time of maximal firing rate
Fully diurnal
Crepuscular
0
6
12
18
24
External time (h)
F
Peak width (h)
16
12
8
4
0
Fully diurnal
Crepuscular

Figure 4: Intrinsic SCN electrical activity is unimodal for both fully diurnal and crepuscular *Arvicanthis*. (A, B) Representative actograms showing the daily behavioral activity of a fully diurnal animal (A) and a crepuscular animal (B). (C, D) Representative recordings of *ex vivo* brain slices containing the SCN prepared from fully diurnal (C) and crepuscular (D) animals; note that the example slices in both cases have a clear unimodal pattern. The shaded gray areas indicate the periods of darkness to which the animals were entrained prior to preparing the *ex vivo* slices. (E, F) Summary of the time at which peak firing occurred (E) and summary of the width of the peak (F) measured in slices prepared from fully diurnal and crepuscular animals (n=4 animals each).

Discussion

Although exercise has been shown to inhibit SCN neuronal activity in nocturnal species such as hamsters (Yamazaki et al., 1998), rats (Meijer et al., 1997; Schaap & Meijer, 2001), and mice (van Oosterhout et al., 2012), the effect of activity on the SCN in diurnal animals has not been investigated (Jha et al., 2021). To address this question, we measured the effect of behavioral activity on the *in vivo* firing rate of SCN neurons in freely moving diurnal *Arvicanthis ansorgei*. We found that unlike nocturnal species, behavioral activity increases neuronal activity in the SCN of *Arvicanthis*, with an incremental effect superimposed on the SCN's intrinsic circadian rhythm. In fully diurnal animals, this resulted in a higher peak in SCN activity at midday; in crepuscular *Arvicanthis*, the result was a bimodal pattern of SCN electrical activity. In contrast with our *in vivo* data, *ex vivo* brain slices containing the SCN displayed only unimodal rhythms, even when the slices were prepared from crepuscular animals. Based on these results, we conclude that: *i*) circadian behavior originates in the SCN, *ii*) crepuscular behavior originates outside the SCN, and *iii*) crepuscular behavior is integrated into the SCN's electrical activity.

The incremental effects of behavioral activity on MUA measured in diurnal *Arvicanthis* (up to 46.6%) are similar in magnitude to the inhibition reported for nocturnal species (ranging from 5-60%) (Schaap & Meijer, 2001). We found that 6 out of 11 recordings (54.5%) in our study showed behavioral input (based on an arbitrary threshold of a 10% change at active timepoints relative to inactive timepoints). Also in rats and mice, responses to behavior were not measured in all recordings. In addition, and in contrast to observations reported in nocturnal species (Schaap & Meijer, 2001), the magnitude of the behavioral input that we measured in *Arvicanthis* was similar between day and night. One possible explanation for this difference may be that in nocturnal species the relatively low firing rate of SCN neurons cannot be decreased further by the inhibitory effects of behavioral activity (i.e., a floor effect), while the firing rate in the SCN of diurnal *Arvicanthis* may not have reached a ceiling and was increased further by behavioral input.

In general, the firing rate of SCN neurons is high during the day and low during the night based on *in vivo* recordings in nocturnal rodents such as hamsters (Yamazaki et al., 1998), rats (Inouye & Kawamura, 1979; Meijer et al., 1997; Schaap & Meijer, 2001), and mice (van Oosterhout et al., 2012), as well as in diurnal chipmunks (Sato & Kawamura, 1984). These findings have been confirmed using *ex vivo* SCN recordings obtained from several nocturnal rodents (Groos & Hendriks, 1982; Meijer et al., 1997; Shibata et al., 1982) and diurnal *Rhabdomys* (Bano-Otalora et al., 2021). We also observed this pattern in fully diurnal *Arvicanthis* animals; interestingly, however, we observed a bimodal pattern in crepuscular *Arvicanthis* animals, likely due to the excitatory effects of behavior on the rate of SCN firing, which re-shaped the MUA waveform to reveal peaks at both dawn and dusk.

For an animal that is typically active during the day and inactive during the night, excitatory behavioral input can increase the amplitude of the SCN's neuronal activity, provided that the animal is active during the day and inactive during the night. This same phenomenon also occurs in nocturnal species, but with the exact opposite mechanism in which nocturnal activity and inhibitory input cause an increase in amplitude by lowering the trough of the SCN rhythm. As discussed above, all nocturnal species studied to date have a 24-hour sine rhythm in SCN electrical activity (Inouye & Kawamura, 1979; Meijer et al., 1997; Schaap & Meijer, 2001; van Oosterhout et al., 2012; Yamazaki et al., 1998), whereas many of our recordings in *Arvicanthis* had a bimodal rhythm in the SCN that can be explained by these animals' crepuscular behavioral activity (increased physical activity during dawn and dusk).

In the absence of behavioral input—for example, in *ex vivo* slices (Meijer et al., 1997)—we found that all SCN recordings had a unimodal MUA rhythm. This finding suggests that the MUA waveform is intrinsically unimodal and the brain regions that receive the behavioral input information were not contained within the hypothalamic slices.

A remarkable feature of behavioral input is that the MUA increased sharply at the start of the behavior onset, but returned to baseline slowly after activity ceased. This pattern has also been observed in nocturnal rodents (Schaap & Meijer, 2001; van Oosterhout et al., 2012) and likely reflects the dynamics of a relatively slow-acting G protein–coupled receptor such as a neuropeptide receptor (Lohse et al., 2008). The most likely candidates for mediating this effect are neuropeptide Y (NPY) released from the intergeniculate leaflet (IGL) (Abrahamson & Moore, 2001; Harrington, 1997) and serotonin released from the medial raphe nucleus (Yamakawa & Antle, 2010), as both IGL lesions (Janik & Mrosovsky, 1994) and ablation of serotonergic afferent SCN pathways (Cutrera et al., 1994; Marchant et al., 1997) have been shown to reduce behavior-induced phase shifts. We therefore hypothesize that NPY and serotonin also mediate the effects of behavioral input in diurnal species, but have an opposite effect on MUA compared to nocturnal species. Injections of serotonin receptor agonists in day-active *Arvicanthis* induce a small phase advance during the subjective night (Cuesta et al., 2008), while in nocturnal rodents this phase advance occurs during the subjective midday (Horikawa & Shibata, 2004). The relationship between NPY and behavioral input is largely unknown in diurnal species; however, NPY-containing neurons in the IGL, which project to the SCN, are activated by wheel running (Smale et al., 2001).

Because behavioral input excites the SCN in diurnal species, the phase-response curve of exercise is expected to be similar to the phase-response curve induced by light pulses (Colwell, 2011). Indeed, in humans exercise advances our rhythm when performed in the early morning and is delaying in the late evening and early night (Bowman et al., 2021; Youngstedt et al., 2019). We therefore speculate that in diurnal species, behavioral activity and light can potentiate each other, as both inputs lead to the same phase response curve, in contrast to nocturnal animals in which light and behavioral activity have opposing effects on the SCN and phase response curve (Schaap & Meijer, 2001). Our hypothesis that behavioral input to the SCN is mediated by serotonergic pathways is supported by a previous report that serotonin can potentiate light-induced phase shifts in diurnal *Arvicanthis* (Cuesta et al., 2008).

In summary, we report that the SCN in diurnal *Arvicanthis ansorgei* responds acutely to behavioral input with increased firing that is sustained for the entire duration of the behavioral activity, thereby

increasing the amplitude of SCN firing in fully diurnal animals and generating a bimodal pattern of SCN firing in crepuscular animals. These results provide new insights into the basis of crepuscular activity and show that this activity and can be linked to the central clock together with the excitatory effects of behavioral activity on the clock. Finally, given the positive effects of properly timed exercise on the circadian system in humans, future studies are warranted in order to identify the neuronal pathway by which behavioral information feeds to the SCN.

Materials and Methods

Animals

Adult (3-6-month-old) Sudanian grass rats (*Arvicanthis ansorgei*) of both sexes were obtained from Chronobiotron UMS 3415 (Centre National de la Recherche Scientifique, University of Strasbourg, France) and used for *in vivo* (n=11 animals) and *ex vivo* (n=8 animals) experiments. The animals were housed in transparent plastic cages under a 12:12-hour light-dark (LD) cycle consisting of 200 lux white light <5 lux red dim light (darkness); food and water were provided *ad libitum*. Locomotor activity was recorded using a passive infrared (PIR) sensor. All experiments were performed in accordance with the guidelines established by European Commission Directive 2010/63/EU and the French Ministry of Higher Education and Research (APAFIS #6099-2016090216536741, v4).

electrophysiology

Multiunit activity (MUA) was recorded in the SCN of freely moving animals as described previously (Meijer et al., 1996). Tripolar stainless steel microelectrodes (model MS333-3-BIU-SPC; Plastics One, Roanoke, VA) were implanted in the SCN of animals anesthetized with Rompun (4 mg/kg body weight; Bayer Pharma, Puteaux, France). In brief, two polyamide-insulated twisted electrodes (bare electrode diameter: 125 µm) for differential recordings were aimed at the SCN using a stereotactic frame with an angle of 5° in the coronal plane, with the following coordinates: 0.7 mm anterior to Bregma, 0.9 mm lateral to the midline, and 7.8 mm ventral to the dura mater. A third uncoated electrode was placed in the cortex as a reference. The electrodes were fixed to the skull using screws and dental cement. After at least 5 days of recovery, the animals were connected to a custom-designed recording setup in which SCN extracellular activity was recorded while the animal was able to move freely. The electrical signal was amplified and bandwidth filtered (500-5000 Hz), and window discriminators were used to convert action potentials to digital pulses that were counted in 10-second or 1-second bins using custom-made software. The differential amplifier was based on the design reported by Yamazaki and colleagues (Yamazaki et al., 1998). The recording chamber was also equipped with a PIR sensor to measure locomotor activity.

At the end of the experiment, the location of the microelectrode in the SCN was verified by histology. In brief, the animal was euthanized with an overdose of sodium pentobarbital (3 ml/kg body weight; CEVA, Libourna, France) under inhalation anesthesia with 4% isoflurane in O_2. The recording site was marked by delivering a small electrolytic current (40 µA) through the recording electrode to deposit iron at the electrode tip. The brain was then removed and fixed for 2 days in 4% paraformaldehyde containing $C_6FeK_4N_6 \cdot 3H_20$ (Sigma-Aldrich, Lyon, France). Coronal slices (40 µm thick) were then prepared, and the recording site was reconstructed.

electrophysiology

MUA was recorded in the SCN as described previously (Albus et al., 2005). The *Arvicanthis* were entrained to a 12:12-hour LD cycle for at least 30 days prior to the experiment. The animals were then sacrificed by decapitation under a dim red light at Zeitgeber time (ZT) 21±0.5 h, which corresponds to three hours before lights on. The brains were removed within one minute of decapitation, and brain slices (~450 µm thick) were prepared using a tissue chopper. The slice containing the SCN was transferred to a laminar flow chamber within six minutes of decapitation and bathed in oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 116.4, KCl 5.4, NaH_2PO_4 1.0, $MgSO_4$0.8, $CaCl_2$1.8, $NaHCO_3$ 23.8, 15.1 glucose, and 5 mg/L gentamicin (Gibco) saturated with 95% O_2 and 5% CO_2 (pH 7.2-7.4 and 290-310 mOsm). The slices were stabilized using an insulated tungsten fork and maintained for 1 hour prior to placing a 90% platinum and 10% iridium electrode (50-µm diameter) in the center of the SCN. Action potentials were amplified 10x and bandpass-filtered (0.3-3000 Hz). Action potentials that exceeded a predetermined threshold set well above noise (~5 µV) were counted in 10-second bins using custom-made software.

Data analysis

Data from the *ex vivo* electrophysiology experiments were analyzed using a custom-made program written in MATLAB. Multiunit recordings lasting at least 24 hours and expressing a clear peak in MUA were moderately smoothed using a least-squares algorithm (Eilers, 2003). The time of maximum activity was used as a marker of the phase of the SCN and was defined as the first peak in MUA. Peak width was calculated by comparing the half-max time of the rising slope to the half-max of the falling slope of the first cycle.

Data from the *in vivo* electrophysiology experiments were analyzed using Python 3.0.9 with the Pandas module version 1.3.0, and visualized using Matplotlib version 3.4.2. A 48-hour window consisting of 2 LD cycles starting with the onset of a light phase was used for further analysis; within this window, the following formula was fitted to the MUA datapoints:

$$a\ \sin\left(\frac{2\pi}{86400}x + b\right) + c\sin\left(\frac{2\pi}{43200}x + d\right) + e$$

This formula combines a sine curve with a 24-hour period (i.e., 86,400 seconds) to fit the circadian component in the MUA rhythm with a sine curve with a 12-hour period (i.e., 43,200 seconds) in order to fit the crepuscular components of the MUA rhythm. This formula was fitted separately to the timepoints coinciding with behavioral activity (indicated by the red lines in Figures 1 and 2) and to the timepoints coinciding with behavioral inactivity (indicated by blue lines in Figures 1 and 2). In the same 48-hour window, the average MUA was calculated for all timepoints coinciding with behavioral activity and for all timepoints coinciding with behavioral inactivity, and the difference between the two was used to measure the magnitude of the behavioral input. Behavioral input was calculated by dividing the average MUA of timepoints with activity by the average MUA of timepoints with inactivity, expressed as the percent change. To determine whether the magnitude of the behavioral input differed between day and night, the timepoints obtained at both phases of the cycle were analyzed separately.

To analyze crepuscular behavior, we calculated a "crepuscularity index" by dividing the level of activity in the 6-hour windows surrounding the light-dark and dark-light transitions by the total level of activity measured throughout the recordings. The MUA crepuscularity was calculated by fitting the formula

described above to all MUA datapoints and then dividing the absolute value of the 12-hour sine curve amplitude (c) by the sum of the absolute values of the 24-hour sine curve amplitude (a) and c, as shown below:

$$MUA\ crepuscularity = \frac{|a|}{|a| + |c|}$$

A crepuscularity index of 0 and 1 corresponds to no crepuscularity and complete crepuscularity, respectively.

Statistics

Data were analyzed using SPSS or the Python module SciPy version 1.7.0. To determine whether the magnitude of behavioral input differed significantly from 0, we used a one-sample Student's *t*-test. Differences in behavioral input between the day and night were analyzed using a paired Student's *t*-test. The correlation between the MUA crepuscularity index and the behavioral crepuscularity index was assessed using robust linear regression with the Huber loss function as described previously (Huber, 1964). The timing of maximal firing and the peak width measured using in *ex vivo* electrophysiology were analyzed using the Student's *t*-test. All summary data are presented as the mean ± the standard error of the mean (SEM), and differences were considered significant at $p \leq 0.05$.

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Competing interests: The authors declare no competing interests.

Data availability: Data will be provided upon reasonable request to the corresponding author.

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