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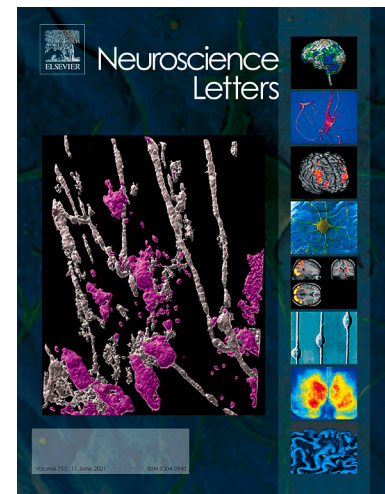
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TITLE PAGE

Title

Circadian functioning of Locus Cœruleus of the nocturnal rat and diurnal rodent *Arvicanthis*

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(Footnote) Present/permanent address**Highlights**

- LC *th* mRNA levels display a unimodal rhythm in the nocturnal rat
- LC *th* mRNA levels show a bimodal rhythm in the crepuscular diurnal *Arvicanthis*
- Clock genes mRNA phase is opposite in the LC of nocturnal rats vs diurnal *Arvicanthis*

Abstract

The noradrenergic Locus Cœruleus is one of the major arousal structures involved in inducing wakefulness. While brain noradrenaline (NA) amounts display 24-h variations, the origin of NA

rhythm is currently unknown. In this study, we tested the hypothesis that NA rhythm could result from its rhythmic synthesis. Therefore, we investigated the 24-h expression profile of NA rate-limiting enzyme, tyrosine hydroxylase (*th*), in the Locus Cœruleus (LC) of the nocturnal rat and the diurnal rodent *Arvicanthis*, under 12h:12h light/dark (LD) and constant darkness (DD) conditions. In both species, *th* mRNA levels vary significantly over 24-h, but in a differential manner. In nocturnal rats, *th* mRNA profiles show a unimodal rhythm, with peak values in late day in LD, and in the middle of the subjective day in DD. In contrast, *th* mRNA rhythm in *Arvicanthis* is characterized by a bimodal profile, with higher levels at the beginning of the day and of the night in LD, and in the middle of the subjective day and night in DD. The rhythmic pattern of *th* expression may be dependent on a LC clock machinery. Therefore, we investigated the mRNA expression of three clock genes, namely *bmal1*, *per1*, and *per2*, and found that their expression displays significant variations between day and nighttime points in both species, but in opposite directions. These data show that NA rhythm may be related to circadian expression of *th* gene in both species, but differs between nocturnal and diurnal rodents. Furthermore, the phase opposition of clock gene expression in the rat compared to *Arvicanthis* suggests that the clock machinery is involved in *th* rhythm.

Keywords

Circadian, Clock Genes, Diurnal, Locus Cœruleus, Nocturnal, Tyrosine Hydroxylase

Abbreviations: Bmal1, Brain and Muscle ARNT-Like 1; CT, Circadian Time; DD, Constant Darkness; DMH, Dorsomedial Hypothalamus; GRE, Glucocorticoid Response Element; LC, Locus Cœruleus; LD, Light/Dark Cycle; NA, Noradrenaline; Per1/2, Period 1/2; SCN, Suprachiasmatic Nuclei; Th, Tyrosine Hydroxylase; TTFL, Transcriptional-Translational Feedback Loops; VTA Ventral Tegmental Area; ZT, Zeitgeber Time.

1. Introduction

The circadian system, orchestrated by the central pacemaker located in the suprachiasmatic nucleus (SCN), regulates the rhythm of many behavioral and physiological functions, including the sleep/wake cycle. Besides being under the control of the SCN, the distribution of wake and rest episodes is regulated by the arousal system [1-3]. The noradrenergic Locus Cœeruleus (LC) is one of the major arousal structures involved in inducing wakefulness [4], and contributes to modulate circadian parameters of the sleep/wake cycle [5]. Evidence shows that the functioning of the LC itself is rhythmic. Circadian variations have been reported on the firing activity of LC neurons, which is higher during the active than the inactive phase [6]. Moreover, noradrenaline (NA) levels in the brain show 24-h oscillations [7, 8]. However, the origin of NA rhythm is so far unknown. In the LC, NA levels directly depend on the activity of tyrosine hydroxylase (TH), the rate-limiting enzyme for NA synthesis [9, 10].

In this study, we tested the hypothesis that NA rhythm originates from its synthesis, and is therefore dependent on *th* expression. For that purpose, we investigated the 24-h profile of *th* mRNA levels. Considering the NA role in promoting wakefulness and the correlation of LC activity with the animal's arousal state [11, 12], we hypothesize that NA synthesis rhythm differs between nocturnal and diurnal animals. Hence, we compared *th* expression in the nocturnal rat and in the diurnal *Arvicanthis ansorgei*.

To go further, we raised the question about the origin of *th* rhythmicity. The circadian system may drive *th* rhythmic transcription through different mechanisms. First, the SCN may send direct/indirect neuronal projections to the LC. Accordingly, an indirect SCN projection has been described to reach the LC *via* the dorsomedial hypothalamus (DMH) [6]. Second, the SCN may convey rhythmic signals to the LC through hormonal outputs, such as glucocorticoids. In agreement with this assumption, a glucocorticoid response element (GRE) is present in *Th* promoter [13]. Third, *th* rhythmic expression may be under the control of clock genes expressed in the LC. Indeed, at a molecular level, circadian rhythms are generated by a self-sustained clock machinery, characterized by transcriptional-translational feedback loops (TTFL) [14, 15]. The core of this TTFL consists in positive and negative loops involving specific clock genes and proteins (e.g. *Bmal1*, *Clock*, *Per1/2*, *Cry1/2*) which are responsible not only for their own transcription/repression, but even for the transcription/repression of other genes. In the mouse midbrain, CLOCK, which heterodimerizes with BMAL1, represses *th* expression by directly binding *Th* promoter at sequences named E-boxes [16]. E-box sequences have been identified in *Th* gene promoter of the rat [17, 18].

In this study, we investigated whether the clock machinery may be involved in *th* rhythmic transcription in the rat and *Arvicanthis* LC. For that purpose, we measured the daily expression of three clock genes: *bmal1*, *per1* and *per2*.

2. Materials & Methods

2.1. Animals & housing conditions

All experiments were performed in accordance with the guidelines of the European Commission Directive 2010/63/EU, and the French Ministry of Higher Education and Research. Adult male Sudanian grass rats (*Arvicanthis ansorgei*) weighting 150-250g (Chronobiotron, UAR 3415, CNRS Strasbourg, France), and adult male Wistar rats weighting 200-400g (Charles River Laboratories, Saint-Germain-Nuelles, France) were used. All the animals were individually housed in Plexiglas cages, entrained to 12h:12h light/dark (LD) cycle (light: 150 - 200 lux, dark: red dim light, <5 lux, lights on and lights off defining Zeitgeber (ZT) 0 and ZT12, respectively), and provided with food and water ad libitum.

2.2. Experimental protocol and samples collection

For *th* quantification, 84 rats and 84 *Arvicanthis* were entrained to 12h:12h LD cycle. Two days before the sacrifice, 42 rats and 42 *Arvicanthis* were exposed to constant darkness (DD), while the others were maintained in LD. Animals were killed by decapitation, after CO₂ sedation, every 4 hours (seven time points), starting at ZT6 for the LD group and at circadian time (CT) 6 for the DD group for rats, starting at ZT2 and CT2 for *Arvicanthis* (Supplementary Fig. 1). For rats, the last time point was taken at ZT6 in LD and at CT6 in DD of the second experimental day, indicated as ZT6' and CT6', respectively. For *Arvicanthis*, the last time point is ZT2 in LD and CT2 in DD of the second experimental day, indicated as ZT2' and CT2', respectively. ZT12 and CT12 were defined as the beginning of dark phase under LD and subjective night in DD, respectively. Brains were quickly removed, frozen in cold isopentane (-30 °C) and stored at -80 °C. Serial coronal 20 µm-sections were cut along the extension of the LC (Interaural from -1.4 mm to -0.2 mm, Paxinos & Watson, 1986) with a cryostat (Leica Instruments GmbH, Nussloch, Germany), and collected on sterile slides. Slides were stored at -20 °C until use.

For RT-qPCR quantification of clock genes, rats and *Arvicanthis* were kept in a 12h:12h LD cycle and sacrificed at ZT2, ZT10 and ZT18 (6 animals per group). Brains were quickly removed and frozen in cold isopentane (-30 °C), and subsequently stored at -80 °C. The right and left LC were dissected from four consecutive coronal sections (300 µm thickness, 2 mm diameter), using the 4th ventricle as reference. LC tissue was stored at -80 °C until use.

2.3. Radioactive *in situ* hybridization

Sense (GGGGAGCTGAAGGCTTATGGTGCAGGGCTGCTGTCTTCCTACGGAG) and antisense (CTCCGTAGGAAGACAGCAGCCCTGCACCATAAGCCTTCAGCTCCCC) oligoprobes (Sigma Aldrich) for *th* were designed based on the rat *th* mRNA sequence. The sequence identity of the probes with the *Arvicanthis th* sequence were analyzed on the *Arvicanthis niloticus* (taxid:61156) genome using Nucleotide BLAST (<https://blast.ncbi.nlm.nih.gov/scd-rproxy.u-strasbg.fr/Blast.cgi>). Probes (46-nucleotides) were 3' end-labelled with [³⁵S]-dATP (46.25 GBq/mol, PerkinElmer, Waltham MA) using the Terminal Deoxynucleotidyl Transferase (40U, Thermo Scientific). Hybridization with oligoprobes on brain slices was performed as described by Caputo et al. 2022 [19], with the following modifications. Hybridization was carried out by depositing 80 µl of oligoprobes (0.1 pMol) in a solution containing 50% deionized formamide, 4X sodium saline citrate (SSC), 1X Denhardt's solution, 0.25 mg/ml yeast totalRNA, 10 mg/ml salmon sperm DNA, 10% dextran sulfate, and 300 mM dithiothreitol. After hybridization, the sections were washed in 1X SSC for 5 min at room temperature (RT) and stringency washes were performed in 0.1X SSC for 30 min at 50 °C and then in 0.05X SSC for 30 min at 52 °C. Sections were finally dehydrated, air dried at RT and then exposed to an autoradiographic film (Kodak BioMax; Kodak, Rochester, NY), with a ¹⁴C standard.

2.4. Quantitative analysis of *th* mRNA

Quantitative analyses of the autoradiograms were performed by using NIH ImageJ software. Six 20 µm coronal sections (distance between two sections= 80 µm) along the caudo-rostral extension of the rat and *Arvicanthis* LC (see Fig. 2B and 2D), were analyzed. For each section, surface of hybridization and total optical density (OD) were measured in the right and left LC and the specific signal intensity was calculated by subtracting the non-specific OD, measured in surrounding area, where the signal was not specific. OD was normalized to relative levels of mRNA, using a ¹⁴C radioactive scale (KBq/g).

2.5. Real-time quantitative polymerase chain reaction

Total RNA was extracted from rat and *Arvicanthis* LC samples using RNeasy Lipid Tissue Mini Kit (74804, QIAGEN) following the manufacturer's recommendations. RNA concentration and purity were measured using a NanoDrop ND-1000 V 3.5 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Integrity of the RNA was assessed by using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Synthesis of cDNA was performed using, 300 ng of rat total RNA and 400 ng of *Arvicanthis* total RNA, using Thermo Scientific™ Maxima™ H Minus cDNA Synthesis Master Mix, with dsDNase (15696019, Thermo Fisher

Scientific) following the manufacturer's recommendations. RT-qPCR was performed using PowerUp™ SYBR™ Green Master Mix (A25776, Thermo Fisher Scientific) and run on the Applied Biosystems Real-Time PCR Instruments and on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Real-time PCR amplification was performed after 120 s of denature at 95 °C, and then run for 40 cycles at 95 °C for 3 s, 60 °C for 30 s. The amplified clock genes were *bmal1*, *per1*, *per2* (Supplementary Table 1). Data were analyzed by using the $2^{-\Delta\Delta CT}$ method [20], and normalized to that of two housekeeping genes: *gapdh* and *36b4* for rat data, two primers pairs for *gapdh*, amplifying different segments of the gene, for *Arvicanthis* data. Changes in clock gene expression were referenced to the mean expression level of the three ZTs.

2.6. Statistical analyses

Possible differences of 24h *th* mRNA labelling between the right and left LC were tested. As no differences were found, the following analyses were performed on the left LC. To test the variation of *th* mRNA expression (specific OD) within the caudo-rostral extension of the rat and *Arvicanthis* LC among the seven time points, two-way analysis of variance (ANOVA II) was performed, with factors "time", "level" and their interaction. The effect of light condition (LD vs DD) was tested by ANOVA III, with factors "time", "level", "light condition" and their interactions. Differences between the two species (rat vs *Arvicanthis*) were tested by ANOVA III, with factors "time", "level", "species" and their interactions. *Post-hoc* analyses were performed to examine the significant difference among time points. Individuals' mean data were fitted by a non-linear regression using Cosinor analysis (SigmaPlot software, Jandel Scientific, Chicago, IL, USA). To fit either a unimodal (24h) or a bimodal (24h + 12h, or 12h) rhythm, the following equation was used: $y = A + (B * \cos(2\pi(x-C)/24) + (D * \cos(2\pi(x-E)/12))$; where A is the mean level (mesor), B the amplitude of the 24h rhythm, C the acrophase of the 24h rhythm, D amplitude of the 12h rhythm, and E the acrophases of the 12h rhythm.

For RT-qPCR data, differences among time points were analyzed using an ANOVA I test. Differences between species were tested using an ANOVA II, with factors "time", "species", and their interactions, followed by *Post-hoc* analyses. For all statistical procedures, the level of significance was set at $p \leq 0.05$. All data are presented as mean \pm SEM, unless otherwise stated. Sigma Plot (v14) and SPSS (v22) software were used for statistical analyses.

3. Results

3.1. Technical validation of *in situ* hybridization

Sense and antisense oligoprobes were designed on the available rat *th* mRNA sequence. As the *Arvicantis ansorgei* genome is not available, *th* similarity was tested on the *Arvicantis niloticus* sequence. BLAST analysis revealed 97.83% identity with *Arvicantis niloticus* predicted *th* mRNA sequence. Specificity of the antisense probe was tested by a saturation test and by specific signal after hybridization within the rat and *Arvicantis* LC, and within the *Arvicantis* Substantia Nigra (SN) as shown in Fig. 1. *In situ* hybridization of *th* antisense oligoprobe labeled specifically the rat and *Arvicantis* LC (Fig. 1A and 1C). *Arvicantis* coronal sections of SN were used as positive control (Fig. 1E). Hybridization of sense *th* riboprobe showed no specific signal neither in the rat and *Arvicantis* LC (Fig. 1B and 1D), nor in the *Arvicantis* SN (Fig. 1F). Significant OD variations were measured in the LC, while non-specific OD measured in the surrounding area was constant.

3.2. Anatomical extension of the rat and *Arvicantis* Locus Cœruleus

Hybridization with *th* antisense oligoprobe allowed clear anatomical identification of left and right LC in rat (Fig. 2A) and *Arvicantis* (Fig. 2C). For both species, sections of 20 μ m, 80 μ m apart, were taken along the caudo-rostral LC extension to perform *in situ* hybridization. In the rat LC, *th* oligoprobe hybridized on 12 anatomical levels. As previously described by other authors [21], the left and right LC nuclei show a trapezoidal morphology and are positioned at the edges of the fourth ventricle. In the *Arvicantis* LC, *th* oligoprobe hybridized on 10 anatomical levels. As for the rat, the left and right LC nuclei are located around the fourth ventricle. Unlike the rat, *Arvicantis* LC presents a crescent-shaped morphology. For both species, six successive anatomical levels of the LC were considered for *th* quantification (Fig. 2B and 2D).

3.3. *th* mRNA profile within the rat and *Arvicantis* Locus Cœruleus in LD and DD

In both LD and DD conditions, *th* mRNA expression was quantified every four hours for seven time points. For easier visual comparison, one time point in each graph is double-plotted in Fig. 3, which corresponds to ZT2 and CT2 for rat, and ZT6 and CT6 for *Arvicantis*. The levels of *th* expression were significantly different among the anatomical levels of LC both in the rat (LD $p < 0.001$, DD $p < 0.001$) and in *Arvicantis* (LD $p < 0.001$, DD $p < 0.001$).

In the rat, a significant effect of time on *th* expression is observed in both LD (Fig. 3A; $p = 0.012$) and DD (Fig. 3B, $p < 0.001$) conditions. Cosinor analyses showed a significant 24-h fit of *th* mRNA expression in both light conditions. The acrophases of the LD and DD rhythms are observed at ZT11.5 and CT6.4, respectively. The LD and DD *th* profiles are significantly different (interactions "time" * "light condition", $p < 0.001$). *Post-hoc* analyses show that the LD

and DD profiles significantly differ at ZT2 vs CT2 ($p < 0.001$), ZT10 vs CT10 ($p = 0.027$), ZT14 vs CT14 ($p < 0.001$), ZT18 vs CT18 ($p < 0.001$), ZT22 vs CT22 ($p = 0.019$), and at ZT6' vs CT6' ($p < 0.001$).

In *Arvicanthis*, the effect of time is significant in both LD (Fig. 3C, $p < 0.001$) and DD (Fig. 3D, $p < 0.001$) conditions. Cosinor analyses differed from the rat, and displayed a significant 12h fit with peaks at ZT3.5 and ZT15.5 in LD, and at CT7.5 and CT19.5 in DD. The *th* profiles are significantly different between the LD and DD conditions ($p < 0.001$). Post-hoc analyses show that the LD and DD profiles significantly differ at ZT2 vs CT2 ($p = 0.002$), ZT14 vs CT14 ($p = 0.002$), ZT22 vs CT22 ($p = 0.005$), and at ZT2' vs CT2' ($p < 0.001$).

Analyses comparing the rat and *Arvicanthis th* profiles show that the two patterns are significantly different both in LD (interactions "time" * "species", $p = 0.008$), and in DD ($p < 0.001$).

3.4. Clock genes mRNA expression within the rat and *Arvicanthis Locus Coeruleus* in LD

To investigate whether rhythms in LC are different in nocturnal and diurnal mammals, we measured the expression of three genes central in the functioning of the circadian clock machinery, namely *Bmal1*, *Per1* and *Per2*, at ZT2, ZT10 and ZT18. Differences among the time points were measured for all the three clock genes, both in the rat and *Arvicanthis* (Fig. 4). In the rat, the expression levels of *bmal1* significantly decrease from ZT2 to ZT10 ($p = 0.001$) and to ZT18 ($p < 0.001$). Coherently, *per1* and *per2* expressions significantly increase from ZT2 to ZT18 (both $p < 0.001$), from ZT2 to ZT10 (*per1* $p = 0.002$, *per2* $p = 0.021$), and from ZT10 to ZT18 only for *per2* ($p = 0.003$). In *Arvicanthis*, clock genes expression varied in opposite ways compared to the rat. *Bmal1* expression varied significantly over the time points ($p = 0.05$) with higher levels at ZT18, while *per1* and *per2* levels decreased from ZT2 to ZT18 ($p = 0.007$ and $p = 0.018$, respectively), and from ZT10 to ZT18 for *per1* ($p = 0.017$). The clock gene mRNA variation over time was significantly different between rats and *Arvicanthis*, for all three genes (interaction "time" * "species"; *bmal1*: $p < 0.001$; *per1*: $p < 0.001$; *per2*: $p < 0.001$). These data suggest that the LC clockwork is phased differently between nocturnal rats and diurnal *Arvicanthis*.

4. Discussion

4.1. Technical considerations and morphology of rat and *Arvicanthis* LC

In situ hybridization quantification of *th* expression in the LC was performed using radioactive antisense oligoprobe designed on the rat *th* sequence. The high percentage of similarity (97.83%) of rat *th* oligoprobe with *Arvicanthis niloticus* genome, a species belonging to the same genus, made it suitable to detect *th* in the LC of *Arvicanthis ansorgei*. The morphology

and neurochemical organization of the LC in rats have been extensively described [21, 22]. Though its neurotransmitter identity is quite complex, all neurons in the LC contain NA [22]. Therefore, the use of an antisense oligoprobe labelling *th*, noradrenaline rate-limiting enzyme, allowed an accurate morphological characterization of the LC both in the rat, and for the first time in *Arvicanthus*. Differences between the two species relate mainly to the LC shape, which is more half-moon curved in the medial part of the *Arvicanthus* LC, and extension, which is shorter in *Arvicanthus* than in rats of about 200 μm .

4.2. Rhythmic *th* mRNA expression in the rat and *Arvicanthus* LC

In this study, we have shown that *th* mRNA levels in the LC are rhythmic in both a diurnal species, the *Arvicanthus*, and a nocturnal species, the rat, as previously described in the midbrain of another nocturnal mammal, the mouse [16, 23].

The LC is a wake-promoting structure that participates in the circadian organization of the sleep/wake cycle. It is reasonable to hypothesize that temporal differences may exist between nocturnal and diurnal species, as they are awake in opposite temporal windows. Accordingly, we showed that the rhythm of *th* expression reflects the animals' behavioral activity pattern. In LD condition, *th* levels in LC show a unimodal rhythm with a peak occurring at the end of the day in the rat. By contrast, in *Arvicanthus*, a species characterized by a crepuscular activity pattern [24], *th* levels display a bimodal profile, with a peak at the beginning of the day and a peak at the beginning of the night. Even when animals are kept in DD for two days, *th* expression maintains its unimodal profile in rats and bimodal profile in *Arvicanthus*, showing that in both species *th* rhythmicity is endogenous. Coherently, our group demonstrated that in another arousal structure, the raphe nuclei, the 24-h mRNA pattern of tryptophan hydroxylase 2, serotonin rate-limiting enzyme, is unimodal in rats [25], and bimodal in *Arvicanthus* [19].

Although *th* mRNA is rhythmic in both lighting conditions within the same species, differences are observed between *th* LD and DD profiles. In the rat, *th* mRNA peak in DD occurs in the middle of the subjective day, 5 hours before the peak observed in LD. In *Arvicanthus*, the two peaks observed in DD occur in the middle of the subjective day and in the middle of the subjective night, 4 hours delayed compared to the respective peaks in LD. Altogether, these data suggest that although *th* rhythm is endogenous, light may affect *th* expression in both nocturnal and diurnal species, though with some differences. The importance of light on the noradrenergic system has been shown in rats, in which long term exposure to DD decreases the NA neurotransmission in the frontal cortex, suggesting that light is necessary for physiological NA transmission [5, 26]. In *Arvicanthus*, the effects of light on the LC system are scarce. It has been shown that 1h light pulse applied 2h after light-off increases cFOS expression in *Arvicanthus* LC, but not in mice [27], suggesting that light may have a different

impact in diurnal and nocturnal rodents. How and to which extent light affects *th* rhythm requires further investigation.

Th rhythmicity suggests that the oscillating amount of NA observed in several brain regions [8, 28, 29], may be partly regulated at the level of its synthesis. NA levels are directly dependent on TH amount and activity. Variation of 24-h TH protein levels have been described in the ventral tegmental area (VTA) and nucleus accumbens of rats [30], and mice [23]. Natali and colleagues reported a circadian rhythm of TH activity in the mouse LC [31], while Cahill and Ehret reported a bimodal TH activity in the rat brain stem [32]. Future studies should investigate the rhythm and temporal relationship between *th* mRNA oscillation and TH protein levels/activity in the *Arvicanthis* and rat LC. Considering the role of NA in the circadian regulation of the sleep wake cycle, different rhythms in NA synthesis may contribute to determine the opposite behavioral phenotype in diurnal and nocturnal animals.

4.3. Clock gene expression in the rat and *Arvicanthis* LC

To investigate possible mechanisms leading to different rhythmic expression of *th* in rats and *Arvicanthis*, we explored the rhythmicity of clock genes in the LC. Here we showed that the clock genes *Bmal1*, *Per1* and *Per2* are expressed in the LC and vary over time in LD condition. As expected, the expression profile of *Per1* and *Per2*, that are part of the negative feedback loop of the clock machinery, are opposite to the expression of *Bmal1*, in both species. Interestingly, clock genes expression in *Arvicanthis* is opposite to that in rats. These data suggest that clock machinery and circadian rhythms of the LC may be in anti-phase in nocturnal compared to diurnal species.

Our data in rats are coherent with a study in another nocturnal species, the hamster, showing that LC *per1* expression in TH cells is higher at CT12.5 compared to CT3 [33]. The presence of a rhythmic clock machinery leads to the possibility that *th* rhythm in the LC may be regulated by clock genes. In the mouse midbrain, *Th* expression can be regulated both by CLOCK [16] and by REV-ERB α [23], another clock protein. Furthermore, the rhythmic expression of *th* in the VTA is abolished in mice with deficient clocks [34]. E-boxes sequences, recognized by CLOCK:BMAL1 heterodimer, have been described in the rat *Th* promoter [17, 18]. The presence of E-boxes in *Arvicanthis Th* gene needs to be verified.

Although clock genes may be involved in *th* rhythmic transcription, other mechanisms may contribute to *th* 24-h rhythm. In rats, a functional indirect SCN projection to the LC (SCN-DMH-LC) has been described to be responsible of LC rhythmic firing activity [6]. It is possible that signals resulting from indirect SCN projection may also affect *th* rhythm in *Arvicanthis*. Furthermore, circadian signals may be relayed indirectly to the LC through SCN hormonal

outputs, such as glucocorticoids. *Th* gene promoter contains glucocorticoid response elements (GRE) [13], at least in rats. Therefore, corticosterone rhythm may be another factor involved in *th* mRNA oscillation.

In *Arvicanthis*, *th* expression is bimodal. Based on the three time points investigated in this study, it is not possible to establish whether clock genes expression may be bimodal as well. As for rats, other mechanism may contribute to *th* rhythmic mRNA expression. For example, corticosterone rhythm, which is bimodal in *Arvicanthis* [19, 35], may contribute to *th* bimodal expression.

4.4. Conclusions

In conclusion, we found endogenous rhythms of *th* mRNA in the Locus Cœeruleus of both a diurnal and a nocturnal rodent, and we showed that *th* is influenced by light condition. Different temporal patterns of *th* levels and clock gene expression were observed in the nocturnal rat compared to the diurnal *Arvicanthis*, indicating that the rhythmic functioning of the LC is opposite in these species. Furthermore, daily changes in clock genes suggest that the clock machinery is involved in mechanisms regulating rhythmic functions within the LC, including *th* transcription. These data highlight that LC rhythms differ between nocturnal and diurnal species and may contribute to their opposite temporal organization.

Declaration of interest

Declaration of interest: none.

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Figure captions

Fig. 1. Tyrosine hydroxylase (*th*) oligoprobe quality control. *In situ* hybridization performed on coronal sections of the rat Locus Cœruleus (LC) (A and B), and of the *Arvicanthis* LC (C and D) and substantia nigra (SN) (E and F), using *th* oligoprobes antisense (A, C and E) and sense (B, D and F). Black arrows indicate *th* hybridization. Scale bar: 500 μ m.

Fig. 2. *In situ* hybridization of *th* antisense oligoprobe. Hybridization of *th* probe in the rat (A) and *Arvicanthis* (C) left (L) and right (R) Locus Cœruleus. Six successive sections are considered along the caudo-rostral extension of the rat (B) and *Arvicanthis* (D) Locus Cœruleus. Each section is 20 μ m thick and the interval between two sections is 80 μ m. Scale bars: 500 μ m.

Fig. 3. *Th* mRNA rhythm in the rat and *Arvicanthis* Locus Cœruleus. *In situ* hybridization measurements of tyrosine hydroxylase (*th*) mRNA relative levels within the rat (A and B) and *Arvicanthis* (C and D) Locus Cœruleus (LC) housed in 12:12 light/dark (LD, A and C) and constant darkness (DD, B and D) condition. Dotted lines represent the significant Cosinor fit. Points are connected by a smoothing line. White and black horizontal bars represent light and dark phases, respectively. Time is expressed as Zeitgeber Time (ZT) in LD and Circadian Time (CT) in DD. For each animal, the mean *th*-mRNA levels measured within the right and left LC is plotted. Time points ZT2', CT2', ZT6' and CT6' are obtained using an experimental group different from ZT2, CT2, ZT6 and CT6, respectively. For rats, experimental groups were all $n \geq 5$ except for ZT18 ($n=4$), ZT6' ($n=3$) and CT6' ($n=4$). For *Arvicanthis*, experimental groups were all $n \geq 5$ except for ZT10 ($n=4$) and ZT2' ($n=4$). The effect of time is significant in both species, and in both lighting conditions. Data are presented as mean \pm SEM.

Fig. 4. Clock gene mRNA levels in the rat and *Arvicanthis* Locus Cœruleus. RT-qPCR analyses of *bmal1*, *per1* and *per2* mRNA levels in the rat (blue bars) and *Arvicanthis* (red bars) Locus Cœruleus housed in 12:12 light/dark condition. Time is expressed as Zeitgeber Time (ZT). Experimental groups were all $n \geq 5$, both for rats and *Arvicanthis*. The effect of time is significant for all genes. Post-hoc tests show differences among the time points: $p \leq 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). Interaction between time and species is significant for all genes (ANOVA II; $p \leq 0.05$). Data are presented as mean \pm SEM.

Supplementary captions

Supplementary Fig. 1. Experimental protocol: animals' sacrifice for the mRNA quantification. A total of 84 rats and 84 *Arvicanthis* were entrained to 12h:12h light/dark (LD) cycle. Forty-eight hours before the day of the sacrifice, 42 rats and 42 *Arvicanthis* were exposed to constant darkness (DD), while the others were maintained in LD. Animals were killed by decapitation, after CO₂ sedation, every 4 hours (seven time points), starting at ZT6 for the LD group and at circadian time (CT) 6 for the DD group for rats, starting at ZT2 and CT2 for *Arvicanthis*. For rats, the last time point was taken at ZT6 in LD and at CT6 in DD of the second experimental day, indicated as ZT6' and CT6', respectively. For *Arvicanthis*, the last time point is ZT2 in LD and CT2 in DD of the second experimental day, indicated as ZT2' and CT2', respectively. ZT12 and CT12 were defined as the beginning of dark phase under LD and subjective night in DD, respectively. Brains were quickly removed, frozen in cold isopentane (-30 °C) and stored at -80 °C.

Supplementary Table 1. Primer Sequences used for analysis of clock gene expression. IDT: primers designed using the *Integrated DNA Technologies* tools. * Gapdh (1) used for both rat and *Arvicanthis*.

Author contributions

Rosanna Caputo: Conceptualization, Investigation, Formal Analysis, Writing-Original Draft

Vincent-Joseph Poirel: Methodology, Validation, Writing - Review & Editing

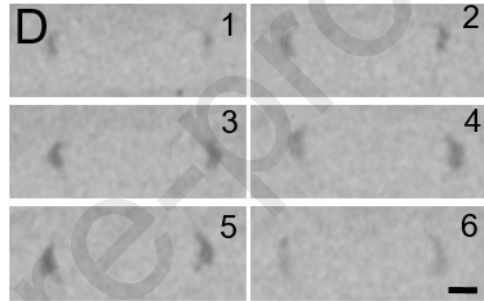
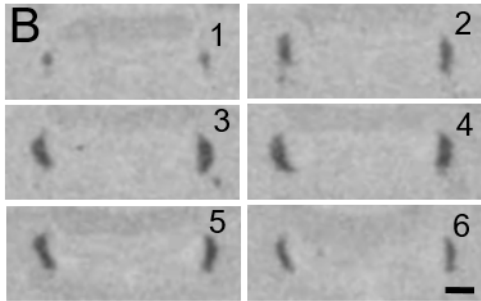
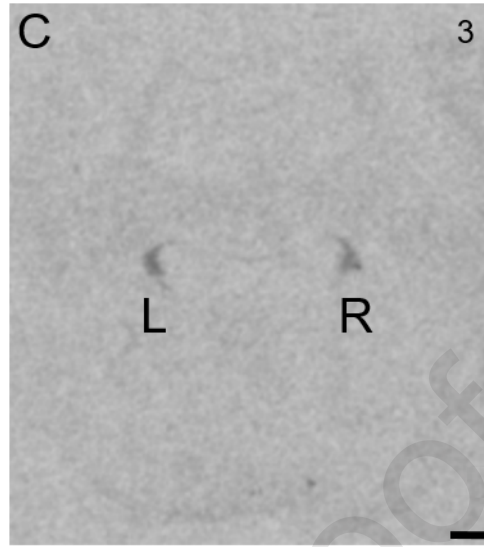
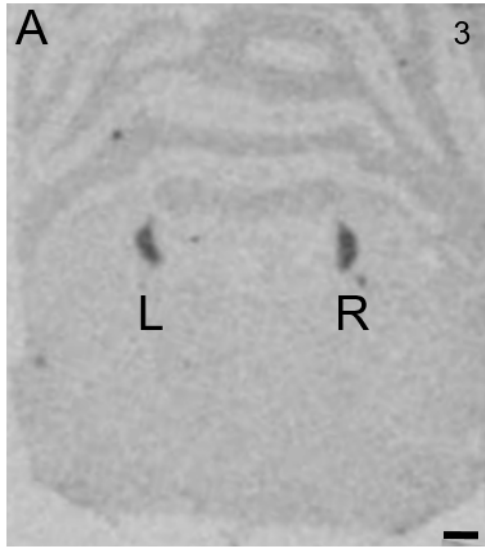
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