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Circadian System in Diurnal and Nocturnal Rodents: Role of the Arousal System

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I. INTRODUCTION

A. NOCTURNALITY VS DIURNALITY

A1. EVOLUTION FROM NOCTURNAL TO DIURNAL

Mammals can be defined as nocturnal or diurnal based on the temporal niche they occupy. According to the "nocturnal bottleneck" hypothesis, mammals would derive from a common nocturnal ancestor (Menaker, Moreira, and Tosini 1997; Heesy and Hall 2010; Gerkema et al. 2013). Among the indicators demonstrating the nocturnality of the first mammals there are the eye anatomy (size and percentage of rods vs cones), the small size that allows a better adaptation to homeothermy, and loss of UV protection mechanisms. Indeed, paleontological studies show that the first mammals appeared on the Earth 200 million years ago (MA), during the Mesozoic era, and had to compete with the dinosaurs, which because of their ectothermic nature, were diurnal. Therefore, the majority of mammal species occupied the nocturnal niche, which gives mammals a selective advantage to escape from their diurnal predators. The mammals transition from nocturnality to diurnality occurred only during the Cretaceous–Paleogene which coincides with extinction of dinosaurs, around 66 MA (Corfe 2007). Some nocturnal mammals occupied the diurnal niche and began to evolve in different lineages independently, leading to several biological modifications related to this new diurnal environment (Hut et al. 2012; Gerkema et al. 2013).

A2. NEED FOR DIURNAL MODELS IN CHRONOBIOLOGY: ARVICANTHIS ANSORGEI

For many years, the majority of biomedical researches have been conducted on nocturnal animals. While for some fields of research, it does not represent a major problem, in others it can lead to findings difficult to be directly translated to humans. This is for instance the case in the area of chronobiology, investigating the biological rhythms and the effect of timing processes on biological events. The circadian system, synchronized by environmental factors (e.g. light), determines the temporal organization of many physiological functions, including the sleep/wake cycle. However, behavioral arousal occurs at opposite phase in relation to the light/dark cycle and the effect of light is different according to whether a species is diurnal or nocturnal (Mendoza 2021). Therefore, it is fundamental to use more diurnal species in chronobiology to design effective strategies for humans, which possess a diurnal nature. *Arvicanthis ansorgei* is a diurnal rodent coming from the southern regions of Mali. *A. ansorgei* belongs to the genus *Arvicanthis*, tribe *Arvicanthini*, subfamily of *Murinae*, and to the family

of *Muridae*. According to phylogenetic analyses, the tribe *Arvicanthini* split from its sister tribe Otomyini around 9 million years ago (Mikula et al. 2021), while divergence from other *Murinae* started around 12.3 million years ago (Aghová et al. 2018). *Arvicanthis ansorgei* colony was initiated in Strasbourg in 1998. The circadian characterization of this species showed that, although being essentially diurnal, *Arvicanthis'* locomotor activity is characterized by a crepuscular profile (*Fig. 1)*, with activity peaks at dawn and dusk (Challet et al. 2002). Many physiological parameters have been characterized in the *Arvicanthis*. Although some of these parameters show a bimodal profile, their daily peak shows a phase opposition to that observed in nocturnal rodents, such as the laboratory rat.

In both diurnal and nocturnal species, the temporal organization of behavioral activity, besides being under the control of the circadian system, is regulated by the arousal system. In the following paragraphs, the circadian system as well as two structures of the arousal system, namely the serotonergic raphe nuclei and the noradrenergic locus coeruleus, will be discussed. In addition, differences between diurnal and nocturnal rodents, and in particular between *Arvicanthis* and nocturnal rats, will be considered.

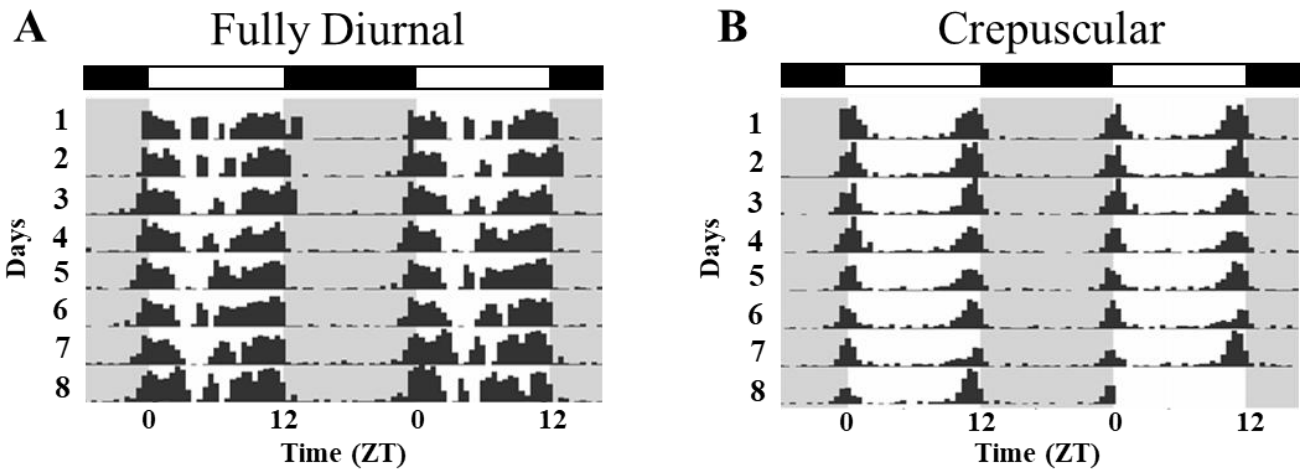

Fig. 1. Examples of two actograms in a fully diurnal (A) and a crepuscular (B) *Arvicanthis.* Locomotor activity is represented in black. White and gray areas represent the light and dark phases, respectively, of the light/dark cycle.

B. THE CIRCADIAN SYSTEM

The rhythmic variations of the Earth, due to the rotation around its axis and around the sun,

influence the organisms' internal clock, producing diurnal (light/dark alternation) and seasonal (photoperiod changes) oscillations. Virtually all living organisms possess an endogenous time-keeping system allowing them to integrate the timing information coming from the environment and to predict the external changes. The existence of internal rhythms have been first described in 1729 by the French astronomer Jean-Jacques Dortous de Mairan, who observed that the opening and closing of the leaves of the plant *Mimosa pudica* under the light/dark (LD) cycle, persisted in constant darkness (DD). Years later, in the 1950s, the acceptance of self-sustained oscillators, or biological clocks, led to the discovery of the central pacemaker. In 1972, two research groups showed that in mammals the master clock of the body is located in a region of the hypothalamus, namely the Suprachiasmatic nucleus (SCN). Lesions of the SCN lead to loss of corticosterone rhythm (Moore and Eichler 1972), as well as locomotor activity and drinking behavior rhythms (Stephan and Zucker 1972). The hypothesis that led to the discovery of the SCN was that the internal clock should have received information from light. Therefore, candidates to be the master clock were retino-recipient areas. The confirmation of the SCN role as central pacemaker came from grafted experiments transplanting the SCN of hamster having a short period in arrhythmic animals, which acquired the free-running rhythm of the donor (Ralph et al. 1990).

The SCN receives timing information from external cues and entrains the rhythm of the whole body, through neuronal, humoral and behavioral signals. Secondary clocks are present in virtually every structure of the periphery and of the brain. The oscillation of these secondary clocks is modulated by external cues as well, and by the signals coming from the SCN. On the other end, extra SCN clocks send feedback to the SCN.

B1. THE SCN AND THE CLOCK MACHINERY

Several features allow us to define the SCN as a biological clock. First, the SCN is able to produce circadian (approximately "circa", a day "diem") endogenous rhythms independently from the presence of external information (Reppert and Weaver 2002; Saper 2013; Moore 1997). For example, in absence of light-dark daily rhythms (LD alternation), the rhythms of clock gene expression in the SCN persist (Ono, Honma, and Honma 2015). Second, the SCN is able to synchronize to the environmental rhythms after receiving information coming from external cues, called Zeitgebers (ZT, from German "time givers"). The SCN may entrain to external cycling periods (*T*-cycles) within a certain range, which is species specific. In rats, this range is 22-28h, and may slightly vary based on the Zeitgeber (Madrid et al. 1998; Stephan

1983). At the same time the SCN integrates the information coming from internal signals in its own rhythm. Third, the SCN sends timing information to the other structures in the brain and in the periphery, synchronizing the whole body. Fourth, the SCN is temperature-compensated, so it is not dependent of temperature modifications that may accelerate or slow its rhythms. Finally, the SCN determines the rhythms of the whole body, since they are desynchronized in the absence of the SCN.

The molecular basis of SCN rhythmicity relies on a clock machinery characterized by transcriptional-translational feedback loops. The discovery of molecular mechanisms controlling the circadian rhythm was made in Drosophila by Jeffrey C. Hall, Michael Rosbash and Michael W. Young (2017 Nobel Prize in Physiology or Medicine). A positive and a negative loop can be distinguished. Positive loop factors drive the transcription of clock genes

that after their translation act as negative factors, determining the transcription repression of the positive clock genes (Takahashi 2017). More details will be provided in following paragraphs.

a. Anatomy and neurochemical composition of the SCN

The SCN is a bilateral nucleus composed of around 20.000 neurons. The SCN is localized in the ventral hypothalamus, above the optic chiasm (OC) (Abrahamson and Moore 2001; Patton and Hastings 2018). Based on the neurochemical distribution of its peptides, two main areas can be distinguished in the SCN (*Fig. 2*): the ventral (or ventrolateral) SCN, also called core, and the dorsal (or dorsomedial) SCN, also called shell (Moore, Speh, and Leak 2002; Antle and Silver 2005). The core is composed mainly of neurons expressing the vasoactive intestinal polypeptide (VIP) and the gastrin-releasing peptide (GRP) (Inouye and Shibata 1994; Drouyer et al. 2010). The VIP cells receive the light information from the retina and transmit it to the dorsal part (Abrahamson and Moore 2001; Jones et al. 2018; Patton et al. 2020). VIP plays a central role also in coupling and synchronizing the clock in individual cells (Patton et al. 2020; Maywood et al. 2006). The shell, rich in neurons expressing the arginine vasopressin (AVP), integrate the information from the core and sends feedback to other brain areas as well as back to the core, to stabilize the SCN network (Leak and Moore 2001; Mieda et al. 2015). Other neurotransmitters are expressed in the SCN, including the inhibitory gamma-Aminobutyric acid (GABA), expressed in almost every SCN cell and involved in cell coupling (Ono et al. 2018), and the excitatory glutamate (Ebling 1996). Although the SCN is usually divided in these two regions, their actual composition and cell distribution is far more complex (Morin et al. 2006; Antle and Silver 2005).

In the SCN the mRNA expression of neuropeptides, namely AVP, VIP and GRP, show 24-h variations. Some differences have been observed between diurnal and nocturnal rodents. Dardente and colleagues found that AVP expression in the nocturnal mouse and in the diurnal *Arvicanthis* was similarly phased both in LD and DD condition, GRP and VIP showed some differences. GRP and VIP peak was phase-advanced in DD compared to LD in *Arvicanthis,* in contrast to mice where VIP profiled were similarly phased and GRP in LD was not rhythmic (Dardente et al. 2004). However, it is worth noting that differences in SCN neuropeptides have been shown also between the mouse and the rat, two nocturnal species. Therefore, some differences may be species-specific (Dardente et al. 2002; Shinohara et al. 1993).

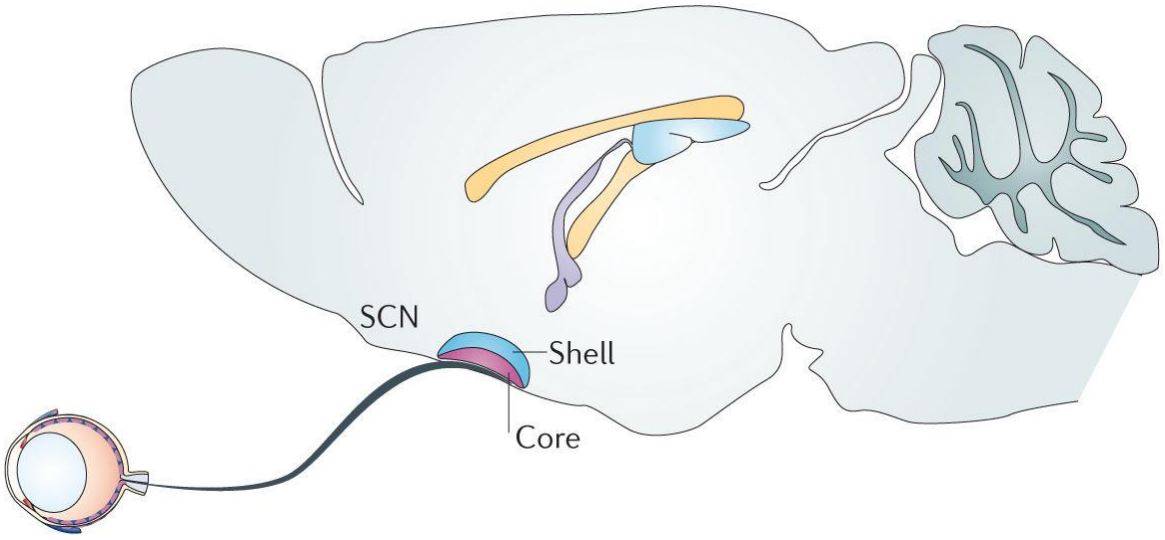

Fig. 2. Schematic representation of the suprachiasmatic nucleus (SCN), located in the hypothalamus close to the third ventricle (3V) dorsal to the optic chiasm (OC). The SCN is oft al 'shell'. The core receives the input from the retina (RHT).

(Modified from Colwell 2011)

b. The molecular clock machinery

The rhythmic functioning of the circadian clock is responsible for the oscillation of many physiological and behavioral functions. The origin of this rhythmicity relays in a self-sustained molecular machinery, consisting of a transcriptional translational feedback loop (TTFL)(Welsh, Takahashi, and Kay 2010; Takahashi 2017) (*Fig. 3*). The TTFL is composed of clock genes, whose products are mainly transcription factors. In this complex rhythmic machinery, positive and negative loops can be identified. The principal positive loop is composed of CLOCK

(Circadian Locomotor Output Cycles Kaput) and BMAL1 (Brain and Muscle Arnt Like protein 1), two transcription factors containing a protein-protein binding domain namely PAS (Per-Arnt-Sim) and a bHLH (Basic Helix Loop Helix) domains (Bunger et al. 2000; Kinget al. 1997). Thanks to the PAS domain, CLOCK and BMAL1 can form a heterodimer and linkthe E-Box sequence (CACGTG) via the bHLH domain. The E-Box sequence is present on thepromoter of the clock genes belonging to the negative loop, such as PER (Period) 1, 2 and 3 and CRY (Cryptochrome) 1 and 2 (Zylka et al. 1998; van der Horst et al. 1999; Zheng et al.

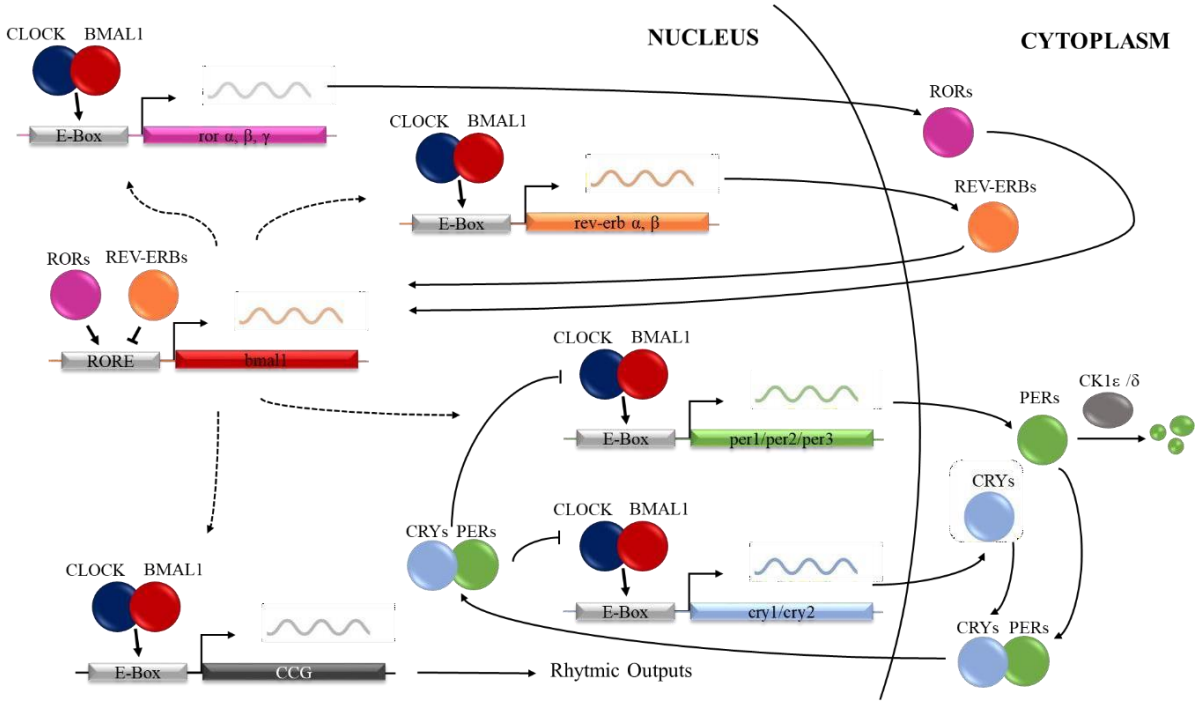

Fig. 3. Transcriptional translational feedback loop of the molecular clock machinery. Positive loop is composed by CLOCK:BMAL1 heterodimers, which bind to E-Boxes and activate the transcription of 1) elements of the negative feedback loop PERs and CRYs; 2) elements of the secondary loop REV-ERBs and RORs, which modulate *Bmal1* transcription; 3) clock-controlled genes (CCGs). PER and CRY, dimerize and inhibit their own transcription. Degradation of clock core proteins is regulated by (CK1ε/δ).

(Modified from Ko and Takahashi 2006)

Other central factors of the clock machinery are ROR (Retinoic acid-related Orphan receptor Response Element) α, β, and γ and orphan receptors REV-ERB α and β (or NR1D1 and NR1D2) (Preitner et al., 2002; Ueda et al., 2002, Sato et al., 2004). RORs positively regulate *bmal1* expression while REV-ERBs have a negative effect on the gene transcription. These two factors compete to bind the retinoic acid-related orphan receptor response elements (RORE), present on *Bmal1* promoter (Guillaumond et al. 2005; Ko and Takahashi 2006; Mongrain et al. 2008). On the other hand, CLOCK:BMAL1 induce the transcription of *Rev-erb α* by binding the E-box present on its promoter. Casein kinase 1 epsilon and Casein kinase 1 delta (CK1ε and CK1δ) regulate transcription repression and degradation of core circadian proteins (Ko and Takahashi 2006).

Many other factors intervene to regulate and to confer robustness to the clock machinery. For instance, the glycogen synthase kinase 3 β (GSK3β), a serine/threonine kinase, phosphorylates many clock genes leading to either their degradation (CRY2, CLOCK, BMAL1), their nuclear translocation (PER2), or stabilization (REV-ERBα) (Harada et al. 2005; Iitaka et al. 2005; Sahar et al. 2010; Spengler et al. 2009). Robustness to the clock machinery is among others conveyed by the neuronal PAS domain 2 protein (NPAS2), the homolog of CLOCK, which may bind to BMAL1 and induce the transcription of CCGs in absence of CLOCK (DeBruyne, Weaver, and Reppert 2007; Reick et al. 2001). In addition, other elements may drive the expression of clock genes. For example, *Per1* transcription may be activated by cAMP-response element binding protein (CREB), independently of CLOCK/BMAL1 (Travnickova-Bendova et al. 2002; Yamaguchi et al. 2000). Other factors may interfere with clock gene transcription, such as the tumor necrosis factor α (TNF-α) that inhibits E-box-dependent gene expression (Cavadini et al. 2007). Hence, clock machinery represents a complicated molecular system that auto-regulates and integrates several signals.

Of note, the phase of clock genes expression in the SCN is similar in astronomical times between nocturnal and diurnal species (Yan et al. 1999; Caldelas et al. 2003; Bae et al. 2001; Mrosovsky et al. 2001). For instance, in rats *per1* peak occurs at CT8 and *per2* at CT12 (Yan et al. 1999). in *Arvicanthis*, *per1* mRNA levels peak at CT4 and *per2* at CT8 (Caldelas et al. 2003) (*Fig. 4*). Although the peak time occur earlier in *Arvicanthis* than in rats, in both species higher *per1* and *per2* levels are observed during the subjective day.

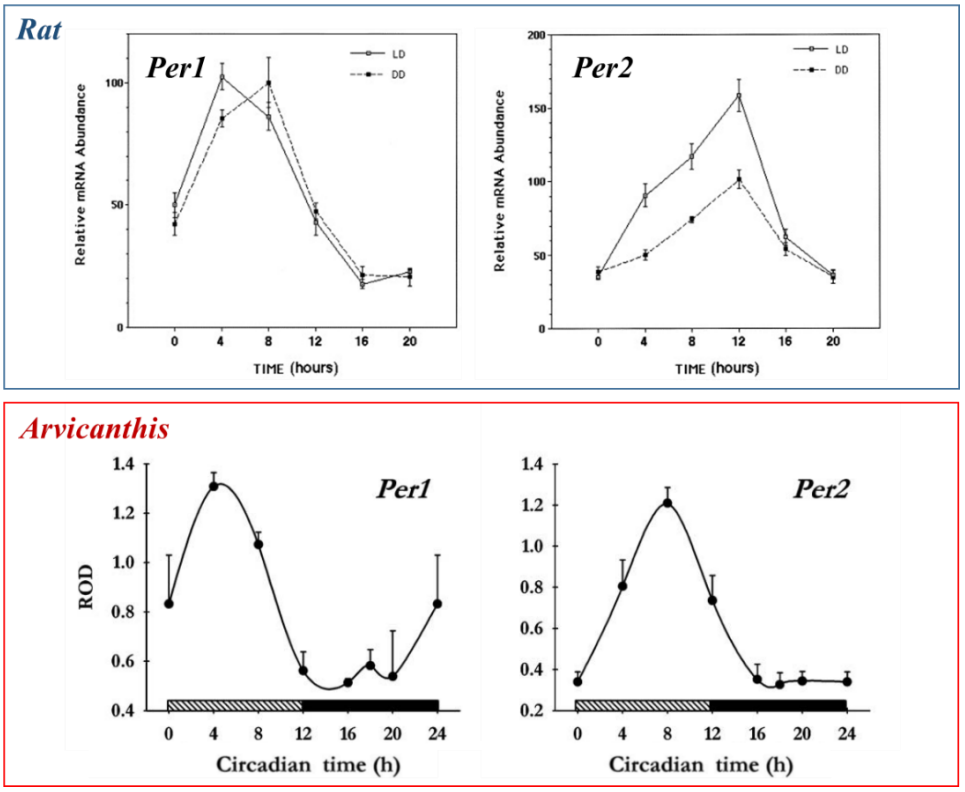

Fig. 4. Clock gene expression in the SCN of the nocturnal rat and the diurnal *Arvicanthis.* Per1 and Per2 expression within the rat (blue panel) SCN in LD and DD conditions, and *Arvicanthis* (red panel) SCN in DD condition.

(Modified from (Yan et al. 1999) and (Caldelas et al. 2003))

c. Electrical properties of the SCN

The molecular rhythm generated by the clock machinery results in electrical activity rhythm within the SCN (Colwell 2011). SCN neurons are heterogeneous at both the neurochemical and electrical level. The ventral neurons expressing mainly VIP, GRP and GABA show a low-amplitude clock gene expression, contrary to the dorsal neurons expressing mainly AVP and GABA, showing robust clock gene expression (Nakamura et al. 2005; Yan and Okamura 2002; Hamada, Antle, and Silver 2004). This is reflected in different electrical properties. Isolated SCN cells maintain the electrical circadian rhythmicity, while their endogenous period varies from 22 to 28h. The synchronization of the single cells that act as autonomous oscillators generates the rhythm of the SCN ensemble structure (Ramkisoensing and Meijer 2015). Different electrical properties characterize different subpopulations of neurons. Only 1/3 of the SCN cells are responsive to light and modify their electrical firing rate (Kim and Dudek 1993; Meijer, Groos, and Rusak 1986; Cui and Dyball 1996; Aggelopoulos and Meissl 2000).

Furthermore, it has been shown that after 6h shift, the dorsal and ventral SCN neurons desynchronize because of their different responsivity to the new light-dark cycle. While the ventral part quickly shifts to adapt to the new light cycle, the dorsal part slowly adapts (Albus et al. 2005). This difference may be due to the different innervation from the retina (Meijer et al. 1997). Furthermore, the lower amplitude of clock genes in the ventral SCN may also explain the ability of the substructure to adapt more quickly to light inputs (Dardente et al. 2004; Guido et al. 1999; Karatsoreos et al. 2004). *In vivo* and *in vitro* studies have shown that the SCN possesses characteristic electrical features, both at the level of single neurons and the multi-unit activity (MUA) levels (Meijer et al. 1997). Both in diurnal and nocturnal mammals, SCN neurons show a circadian rhythm in electrical activity that is higher during the subjective day and lower during the subjective night (Meijer et al. 1997; Inouye and Kawamura 1979; Shibata et al. 1982; Sato and Kawamura 1984). This suggests that the electrical activity of the central pacemaker neurons is independent of the locomotor activity phase of the animals. However, the electrical activity of the SCN can be modulated by behavioral activity. Recordings in freely moving nocturnal mammals show that the MUA rhythm peaks during the resting phase, i.e. during the subjective day, and that behavioral activity decreases SCN MUA (Meijer et al. 1997; Schaap and Meijer 2001). The effect of behavioral activity on the SCN neuronal firing in diurnal species is still unknown.

B2.INPUT SIGNALS TO THE SCN: PHOTIC AND NON-PHOTIC SYNCHRONIZATION

The SCN is able to oscillate autonomously, independently of external signals. However, the SCN receives information from external and internal cues, and integrates these signals into its rhythm in order to predict and to adapt to environmental changes. Synchronizer factors may modify several parameters of the SCN rhythm, i.e. the phase and/or the period. Two types of synchronizer signals can be distinguished: photic (light) and non-photic ones.

a. Photic signal: light

i. Pathways to the SCN

The most potent synchronizer of the SCN rhythm is light. Light information reaches the retina and it is transmitted to the SCN through direct and indirect pathways (*Fig. 5*). The direct

pathway is the retino-hypothalamic tract (RHT). Light acts on the photopigments rhodopsine, opsine and melanopsin, which are present on the photoreceptors, namely rods, cones and intrinsically photosensitive retinal ganglion cells (ipRGCs), respectively. Both rods and cones, and ipRGCs transmit the photic signal to the SCN and all contribute to the circadian photo-entrainment. The information received by photoreceptors is transmitted to the ganglion cells whose axons form the RHT that reaches the ventral part of the SCN and releases glutamateand pituitary adenylate cyclase-activating peptide (PACAP). As a consequence, the action of these neurotransmitters on the SCN leads to increased intracellular calcium levels, which act onclock gene expression (Tominaga et al. 1994).

An indirect pathway conveying the light information to the SCN is the geniculo-hypothalamic tract (GHT) that involves the intergeniculate leaflet (IGL) located in the thalamus. This structure integrates photic (from the retina) and non-photic (from the raphe nuclei (RN)) signals (Harrington 1997; Meyer-Bernstein and Morin 1996), and releases neuropeptide Y (NPY) and GABA in the ventrolateralSCN (Morin and Allen 2006). The modulatory role of the IGL on SCN synchronization has been described both in diurnal and nocturnal rodents (Goel et al. 2000; Morin and Allen 2006).

In some species, including the nocturnal rat, an additional indirect pathway conveying light information to the SCN has been described. This pathway involves the median RN (MRN), which receives innervations from the retina (Shen and Semba 1994).

The composition of the retina, however, displays differences between nocturnal and diurnal rodents. Nocturnal species possess a very low amount of cones, while in the diurnal *Arvicanthis ansorgei* and *niloticus* cones represent 35% of the retina photoreceptors (Bobu et al. 2008; Gaillard et al. 2008). The retina is the first organ that seems to be involved in influencing the temporal niche of a species. Indeed, mice lacking the retinal pigment epithelium enzyme 65 (RPE65), an enzyme involved in regenerating visual chromophore 11-cis-retinal (allowing the photoreceptors to be sensitive again to light after a photic stimulation), become diurnal (Doyle et al. 2008).

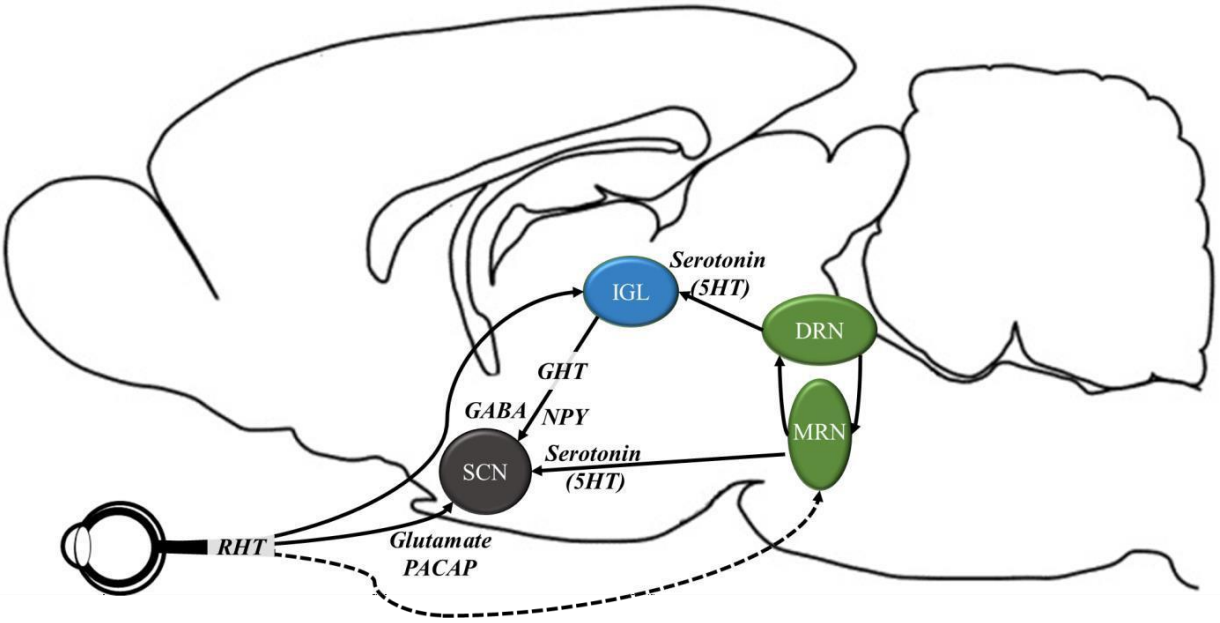

Fig. 5. Schematic representation of direct and indirect pathways from the retina to the SCN, and interconnections among the nuclei involved (IGL, MRN, DRN). The direct pathway, the RHT, results in glutamate and PACAP release into the SCN. The indirect pathway, the GHT, involves the IGL, which release GABA and NPY into the SCN. These two pathways have been described both in the nocturnal rat and in the diurnal *Arvicanthis.* An additional indirect pathway involves the MRN, which release serotonin in the SCN. This pathway has been described in rats, but not in *Arvicanthis.*

ii. Photic synchronization

Light modifies the phase of the SCN in a time-dependent way. Light has a synchronizing effect mainly during the night in both nocturnal and diurnal rodents (Slotten, Krekling, and Pévet 2005; Takahashi et al. 1984; Cohen, Smale, and Kronfeld-Schor 2009), which corresponds to their active and inactive phase, respectively. Light exposure produces circadian modifications that have an impact on different circadian parameters, such as locomotor activity. The phase modification in relation to the time of the day when a light pulse is presented, can be described by the phase-response curve (PRC). In nocturnal and diurnal rodents, the PRC to light shows a very similar profile (Slotten, Krekling, and Pévet 2005; Takahashi et al. 1984; Cohen, Smale, and Kronfeld-Schor 2009; Caldelas et al. 2003). However, some differences characterize the two PRCs. In both species, light causes a phase delay from the end of the subjective day to the middle of the subjective night and a phase advance from the middle of the subjective night to the beginning of the subjective day (*Fig. 6*). The major difference consists in the duration of the so-called dead-zone, the period of non-responsiveness to light, that is more extended in nocturnal than in diurnal rodents.

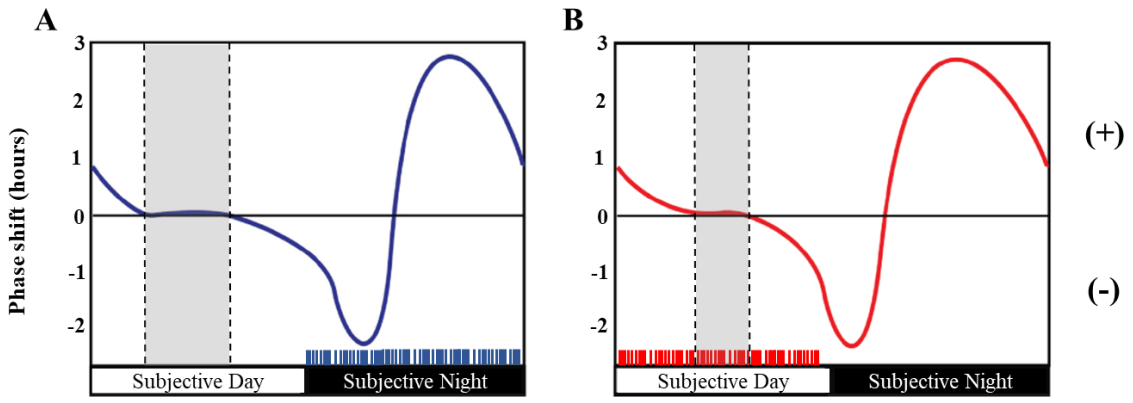

Fig. 6. Phase response curve (PRC) to light in rat (A) and *Arvicanthis* (B), showing phase advances (+) and phase delays (-) in similar times of day. Gray area represents the dead-zone (period of non-responsiveness to light), which is more extended in rat than *Arvicanthis*. The blue and the red thicks represent the episodes of activity in nocturnal and diurnal species, respectively.

(*Modified from Jha et al.2021)*

Light information synchronizes the SCN through cellular and molecular mechanisms. Light inputs result in glutamate release in the SCN that bind the NMDA and AMPA receptors, leading to an increase in intracellular Ca^{2+} levels (Golombek and Ralph 1996; Kim et al. 2005). Ca^{2+} influx activates several kinases, such as PKA, MAPK, ERK, PKCA, and PKG, which phosphorylate CREB protein (Meijer and Schwartz 2003). CREB, ones activated by phosphorylation, translocates into the nucleus and bind cAMP responsive elements (CRE) in the promoter region of genes involved in light synchronization (Ding et al. 1997; Obrietan et al. 1999).

The first clock genes to be impacted by light inputs are *Per1* and *Per2*, whose levels highly increase one hour after light exposure (Albrecht et al. 1997; Shearman et al. 1997; Shigeyoshi et al. 1997; Caldelas et al. 2003). *Per1* transcription is the result of the activation of CREB, and its binding on CRE present on *Per1* promoter (Tischkau et al. 2003; Travnickova-Bendova et al. 2002). While *Per1* transcription may be modified by light during the entire subjective night, the effect on *Per2* transcription is mainly observed at the beginning of the subjective night. Light causes slow *Per2* transcription modification in the middle of the subjective night, and has no effect at the end of the subjective night. However, some differences have been described among the SCN subregions (Albrecht et al. 1997; Caldelas et al. 2003). At the beginning of the subjective night *per1* expression is mainly induced in the ventrolateral SCN, while *per2 is* expressed both in the ventrolateral and dorsomedial SCN (Dardente et al. 2002; Yan and Silver

2002). Other clock genes may be activated by light, including *Bmal1*, *Cry2* (Abe et al. 1998; Okamura et al. 1999; Caldelas et al. 2003), however differences in light response have been observed.

Other genes responding to light are *cFos* and *Erg1*, which are activated by kinases and are important for light synchronization (Travnickova-Bendova et al. 2002; Riedel et al. 2018; Colwell and Foster 1992; Mahoney, Bult, and Smale 2001).

iii. Masking effect of light

Although the PRC to light is similar, light may have different effects between diurnal and nocturnal species acting through a mechanism called masking. Masking is mediated by melanopsinexpressing retinal ganglion cells as well, but acts in a more direct way. In nocturnal rodents, light stimuli at night lead to the suppression of locomotor activity (negative masking), while the oppositeoccurs in diurnal species, in which locomotor activity is increased (positive masking) (Shuboniet al. 2012; Shuboni et al. 2015). Dark pulses of 1h duration have less masking effects in both nocturnal and diurnal species, leading to an effect only in mice, in which dark pulse activate locomotor activity at the end of the subjective night (Shuboni et al. 2012). However, 4h dark pulses cause a different PRC in diurnal *vs* nocturnal rodents (Mendoza et al. 2007).

b. Non photic signals

A variety of stimuli, different from light may synchronize the SCN. These stimuli may be broadly categorized in two groups: the signals related to food entrainment, and the signals that results in modifying arousal state (Mistlberger and Antle 2011). Here, two non-photic signals related to arousal will be discussed: serotonin and behavioral activity.

i. Serotonin

Serotonin (5HT) is a monoamine and the principal neurotransmitter produced by the midbrain raphe nuclei (RN). The dorsal and medial raphe nuclei, which are anatomically and functionally interconnected, send direct and indirect serotonergic projections to the SCN (Peyron et al. 2018; Beliveau et al. 2015). Serotonin acts on the SCN directly, through a neural projection from the

median raphe nucleus, and indirectly, through the IGL, which receives serotonin transmission from the dorsal raphe nuclei (Meyer-Bernstein and Morin 1996; Moga and Moore 1997). Serotonin is considered as a potent synchronizer of the SCN phase.

In nocturnal rodents, 5HT injections during the middle of the subjective day cause a phase advance of the SCN, associated with decreased expression of *per1* and *per2* (Horikawa et al. 2000; Cutrera, Saboureau, and Pévet 1996) (*Fig.76*A). In *Arvicanthis*, no effect is observed when the serotonergic transmission occurs in the mid-subjective day. Phase-advances are rather observed when serotonin receptors are activated during the subjective night, but no changes in *per1* or *per2* expression within the SCN are detected (Cuesta et al. 2008) (*Fig. 7*A).

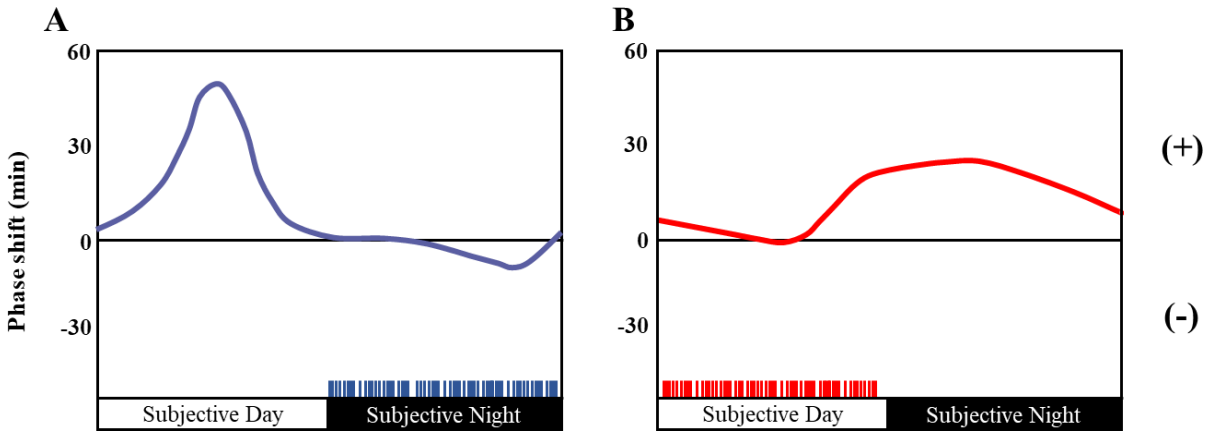

Fig. 7. Phase response curve (PRC) to serotonin in nocturnal (A) and diurnal (B) species, showingphase advance (+) and phase delay (-) at different times of day. The blue and the red thicks represent the episodes of activity in nocturnal and diurnal species, respectively.

(*Modified from Challet 2007 and Jha et al. 2021)*

Besides playing a synchronizing effect on the SCN, 5HT modulates the effect of light on the SCN, in a different way between diurnal and nocturnal rodents. In nocturnal mice, serotonin antagonizes the phase-shifting effect of light during the subjective night (Challet et al. 2001). By contrast, in the diurnal *Arvicanthis*, 5HT strengthens the phase-shifting effect of light (Cuesta et al. 2008). This effect is in accordance with a study in human, where citalopram, a 5HT reuptake inhibitor, improves circadian system sensitivity to light (McGlashan et al. 2018).

Under constant light condition, locomotor activity rhythm is phase advanced in nocturnal hamsters when dark pulses occur in the second half of the subjective day, in opposition to *Arvicanthis* in which phase advance is observed when dark pulses occur during the subjective

night (Mendoza et al. 2007). Furthermore, serotonergic activation during the subjective day potentiates the effect of dark pulses in nocturnal rodents (Bartoszewicz and Barbacka-Surowiak 2007; Mendoza et al. 2008). The effect in diurnal rodents is still unknown.

ii. Locomotor Activity

The effect of locomotor activity on the SCN synchronization has been studied using different methods, such as injection of benzodiazepines able to induce locomotor activity, or by introducing a novel wheel. In nocturnal rodents, a period of acute exercise during the second half of the subjective day, or injection of Triazolam (a benzodiazepine), each leads to phase advance the animals' activity rhythm of about 2-3h (Reebs and Mrosovsky 1989a, 1989b; Turek and Losee-Olson 1986; Turek and Losee-Olson 1987). On the other hand, induced locomotor activity produces a smaller phase-delay when it occurs at the end of the subjective night (Wickland and Turek 1991). Interestingly, locomotor activity provokes a phase-shift that is not only time-dependent, but it also changes in relation with the intensity of the motor activity (Wickland and Turek 1991; Bobrzynska and Mrosovsky 1998).

Few data are available on the phase-shifting features of locomotor activity in diurnal animals. In two diurnal rodents, the European ground squirrel and the Common marmoset, induced locomotor activity during the late subjective day determines a small phase-advance (Hut, Mrosovsky, and Daan 1999; Glass et al. 2001). However, in these animals the authors analyzed the response to a single stimulus, rather than a PRC. In humans, physical exercise in the afternoon lead to phase advance, while it causes a phase-delay when performed at night (Youngstedt, Elliott, and Kripke 2019).

As for serotonin, acute exercise in both diurnal and nocturnal animals shows a phase-dependent effect. More studies are necessary in diurnal species, to investigate whether the PRC to physical exercise is similar in all diurnal species or may be species-specific.

B3. SCN OUTPUT SIGNALS

The SCN is responsible for the entrainment the rhythms of the entire body. The SCN synchronizes clocks in the brain and in the periphery through neuronal, hormonal and behavioral signals (Kalsbeeket al. 2006). Here, two hormonal outputs will be described, namely melatonin and glucocorticoids. In addition, the role of locomotor activity both as output factor and feedback signal to the SCN will be discussed.

a. Melatonin

Melatonin is one of the principal hormones conveying the circadian signal to the body. Melatonin is mainly produced by the pineal gland from the amino acid tryptophan through two principal steps, the first one involving N-acetylation by the enzyme arylalkylamine-N-acetyltransferase (AA-NAT), and the second one the enzyme hydroxyindole O-methyltransferase (HIOMT) (Klein et al. 1997). In mammals, melatonin synthesis is under the control of the SCN (Teclemariam-Mesbah et al. 1999) and is therefore considered as a strong indicator of the circadian phase. After being synthesized, melatonin is directly released in the circulation (Luboshizsky and Lavie 1998). Melatonin exerts its action binding to the high affinity MT1 and MT2 receptors, expressed both in peripheral organs and in brain structures, including the SCN. Therefore, besides being an output signal, melatonin may be defined as a non-photic factor, since it feedbacks to the SCN and controls its phase and amplitude (Armstrong et al. 1986; Hardeland et al. 2008; Pévet et al. 2002). Independently of diurnal or nocturnal nature of an animal, the 24h melatonin profile is similar in all the species studied so far. Melatonin levels are low during the day and increase, reaching a peak, at night. The LD cycle is the principal melatonin modulator: light suppresses melatonin synthesis and light duration influences melatonin peak (Lewy et al. 1980; Pévet 2002; Farhadi, Gharghani, and Farhadi 2016). Although many studies have tried to elucidate the message conveyed by melatonin, its role in sleep has not been fully elucidated yet. In diurnal species, including humans, sleep occurs around two hours after the melatonin onset and exogenous melatonin promotes sleep (Lavie 1997; Zisapel 2007). However, in nocturnal animals, melatonin peak coincides with the animals' active phase: therefore, it cannot be considered as a sleep promoting hormone. On the other hand, melatonin is rather a signal conveying information about the light/dark duration, both to the SCN and to other brain structures involved in sleep regulation (Pevet, Challet, and Felder-Schmittbuhl 2021).

b. Glucocorticoids

Glucocorticoids (GC) are steroid hormones produced by the adrenal gland that control many biological functions in mammals. Glucocorticoids are involved in the regulation of metabolism (Vegiopoulos and Herzig 2007), immune functions (Cruz-Topete and Cidlowski 2015; De Bosscher and Haegeman 2009), stress (Sapolsky, Romero, and Munck 2000), and circadian rhythms (Dickmeis, Weger, and Weger 2013). Glucocorticoids are among the strongest internal synchronizers of our body rhythms (Balsalobre et al. 2000). The 24h GC plasma levels are

under the control of the SCN (Kalsbeek, van der Vliet, and Buijs 1996; Kalsbeek et al. 1996), so that GC rhythm is considered one of the principal indicators of the SCN phase, in no-stress conditions. The SCN regulates the GC synthesis of the hypothalamus-pituitary-adrenal (HPA) axis, through the release of AVP in the hypothalamic paraventricular nucleus (PVN) and in the dorsomedial hypothalamus (DMH). As a consequence, the PVN neurons release the corticotropin-releasing hormone (CRH) which stimulates the synthesis and the release of the adrenocorticotropin hormone (ACTH) within the pituitary gland (Kalsbeek et al. 2012). ACTH finally acts on the adrenal gland to stimulate the production and the release of corticosterone in rats and *Arvicanthis*, or cortisol in humans. Although the SCN regulates the GC rhythm principally through the HPA axis, it also modulates the autonomic nervous system (ANS), which affects the adrenal sensitivity to ACTH through the splanchnic nerve (Ulrich-Lai and Herman 2009). GCs exert their action on target tissues by binding to glucocorticoid receptors (GR) or mineralocorticoid receptors (MR) (Nicolaides et al. 2010; Revollo and Cidlowski 2009; Huyet et al. 2012; Yang and Fuller 2012). GRs are usually found in the cytoplasm in a complex with chaperon proteins. After GC binding, GRs change conformation and translocate into the nucleus, where they act as transcription factors. GRs regulate gene transcription through several mechanisms of action (Grbesa and Hakim 2017) (*Fig. 8*). GRs may act as homodimers and bind to promoter sequences called glucocorticoid response elements (GRE), which are identified in the majority of glucocorticoid binding sites (GBSs). GRE sequences have been described in many genes, including clock genes such as *per1* (Yamamoto et al. 2005), or in a gene involved in catecholamine synthesis, namely tyrosine hydroxylase (*th*) (Fung, Yoon, and Chikaraishi 1992; Yoon and Chikaraishi 1994). Among other mechanisms, GRs may also heterodimerize with or bind to other transcription factors in order to enhance or repress gene transcription (Grbesa and Hakim 2017). Chromatin accessibility is another factor playing a role on GR binding (Grbesa and Hakim 2017). Furthermore, GCs may also act through non-genomic mechanisms, for example by binding to membrane-associate GRs (Panettieri et al. 2019).

GCs rhythm in the plasma, as well as in the brain, have been studied in many nocturnal and diurnal species. In both, GCs levels show a peak at the onset of the active phase, corresponding to the night in nocturnal and to the day in diurnal organisms (Allen-Rowlands et al. 1980; Dalm et al. 2005; Albers et al. 1985; Weitzman et al. 1971; De Boer and Van der Gugten 1987). However, in the crepuscular *Arvicanthis,* GC rhythm display a bimodal profile with a peak before the day and a peak before the night (Verhagen et al. 2004). Kalsbeek and colleagues showed that one of the differences in corticosterone rhythm between *Arvicanthis* and nocturnal

rats is due to the effect of AVP, produced by the SCN, on corticosterone release. AVP has a stimulatory effect on corticosterone release in *Arvicanthis*, contrary to the inhibitory effect observed in nocturnal rats (Kalsbeek et al. 1992; Kalsbeek et al. 2008).

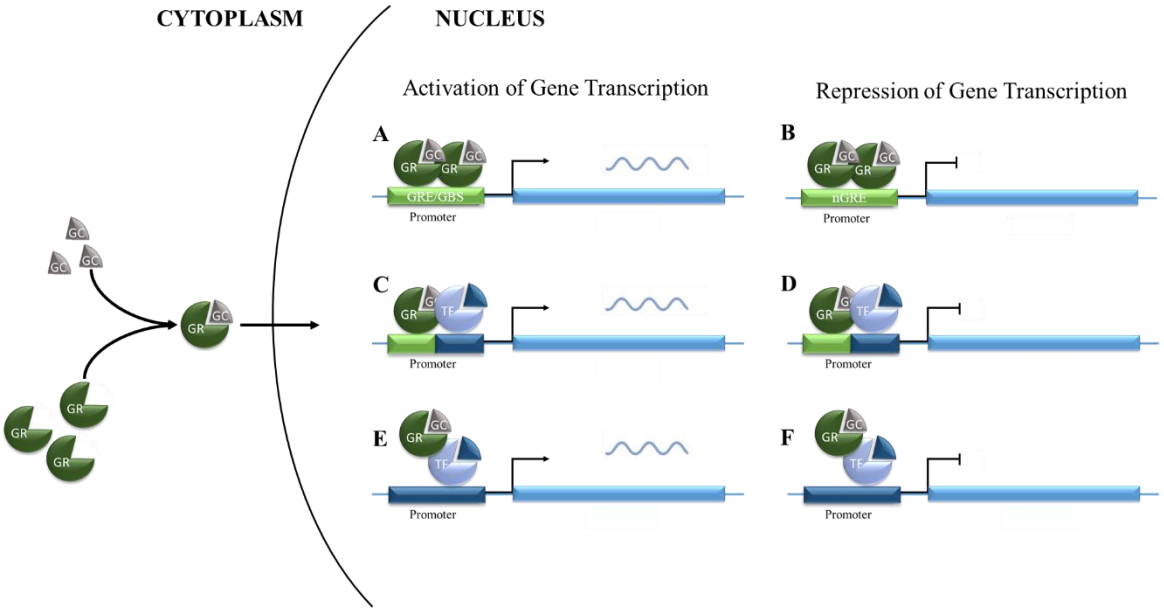

Fig. 8. Representation of the principle glucocorticoid (GC) genomic mechanisms regulatinggene transcription. GCs bind the glucocorticoid receptors (GR) in the cytoplasm, and the GC-GR complex translocates into the nucleus where it can act as 1) homodimer (A and B).binding either to positive (GRE/GBS) or negative (nGRE) glucocorticoid response elements; 2) heterodimer (C and D), with other transcription factors (TF), to induce or repress the transcription; 3) enhancer (E) or silencer (F), tethering other transcription factors.

The authors suggested that the opposite response to AVP would be due to the different phenotype of the neurons where the SCN project (i.e., glutamatergic instead of GABAergic ones). In addition,they demonstrate that in *Arvicanthis* both peaks are dependent on the SCN AVP, since AVP antagonists blocked the morning and evening corticosterone surge (Kalsbeek et al. 2008). Although the mechanisms leading to the corticosterone bimodal rhythm in *Arvicanthis* are notfully understood, the GC profile reflects the crepuscular activity of the animal. GCs may contribute to the opposite temporal organization between diurnal and nocturnal rodents, since they act on structures involved in the regulation of the sleep/wake cycle, such as the serotonergic raphe nuclei. Corticosterone modulates the rhythm of tryptophan hydroxylase 2 (*tph2*), the limiting enzyme of serotonin synthesis, in rats (Malek et al. 2007), and hamsters (Nexon et al. 2011).

c. Locomotor Activity

The SCN, synchronized by photic and non-photic input signals, controls the phase of several physiological and behavioral rhythms, including locomotor activity. Therefore, the dailyrhythm in locomotor activity reflects the state of the SCN, and may act on many nuclei in the brain, such as the IGL (Janik, Mikkelsen, and Mrosovsky 1995), the serotonergic RN (Greenwood et al. 2005; Malek et al. 2007), or the orexinergic lateral hypothalamus (Webb et al. 2008).

However, besides being a result of SCN outputs, locomotor activity provides feedback to the SCN itself. Indeed, as previously discussed, locomotor activity acts as a non-photic cue to synchronize the phase of the SCN. In addition to PRC studies to locomotor activity pulses, acute exercise has been shown to modulate SCN electrical activity (multi-unit activity – MUA)(van Oosterhout et al. 2012; Schaap and Meijer 2001). In nocturnal rodents, behavioral activity decreases the firing rate of the SCN (Schaap and Meijer 2001). Furthermore, the magnitude of the behavioral activity effect on MUA depends on the nature of the behavior (voluntary/non-voluntary walking, grooming, eating, etc) and on its intensity (van Oosterhout et al. 2012).

The mechanisms involved in conveying the behavioral activity feedback to the SCN are not fully understood. Evidence suggests the involvement of the serotonergic raphe nuclei (Hughes and Piggins 2012; Jha et al. 2021). Indeed, RN are both affected by locomotor activity and involved in synchronizing the phase of the SCN. Locomotor activity is involved in the modulation of many genes expressions of the serotonergic system in the RN (Greenwood et al. 2005; Malek et al. 2007). In addition, serotonin levels highly correlate with the behavioral state of both nocturnal and diurnal rodents (Poncet, Denoroy, and Jouvet 1993; Cuesta et al. 2008; Cuesta et al. 2009a; Shioiri et al. 1991). For example, higher serotonin levels in the SCN are measured during the night in the nocturnal rat and during the day in the diurnal *Arvicanthis* (Poncet, Denoroy, and Jouvet 1993; Cuesta et al. 2008). Furthermore, stimulation of locomotor activity leads to release of serotonin in the SCN of hamsters (Dudley, DiNardo, and Glass 1998).

The application of 5HT1a/7 receptor agonist (8-OH-DPAT) reduces SCN neuronal firing rate (Prosser, Miller, and Heller 1990; Shibata et al. 1992), showing an effect similar to that caused by locomotor activity. In addition, lesions of serotonin terminals around the SCN impair the locomotor activity entrainment in mice (Edgar and Dement 1991). The temporal organization of activity and resting episodes between the light and dark phases isnot only under the control of the circadian system, but is also regulated by the arousal system.

C. THE AROUSAL SYSTEM: THE 5HTergic RAPHE NUCLEI AND THE NAergic LOCUS COERULEUS

The arousal system is a complex neuronal network whose role is to induce and maintain wakefulness. Arousal, or wake-promoting, structures interact with sleep-promoting networks to allow the state transition in the sleep/wake cycle. The timing of sleep/wake state transitions is further regulated by the circadian system. The arousal system is composed by ascending projections reaching the cerebral cortex and descending networks to the spinal cord, stimulating sensory-motor functions (for review see (Saper et al. 2010)). Several brain areas contribute to arousal including the serotonergic (5HTergic) raphe nuclei, the noradrenergic (NAergic) locus coeruleus, the dopaminergic ventral tegmental area, and histaminergic neurons of the tuberomammillary nucleus. Furthermore, the cholinergic neurons in the basal forebrain, in the pedunculopontine and laterodorsal tegmental nuclei, as well as glutamatergic neurons in the parabrachial nucleus and the orexinergic neurons in the lateral hypothalamus are also involved in regulating wakefulness (Jones 2003; Saper et al. 2010; Scammell, Arrigoni, and Lipton 2017). The attention of this thesis will focus on the 5HTergic raphe nuclei and the NAergic locus coeruleus, considering their role in inducing and maintaining wakefulness, as well as their interactions with the circadian system.

C1. THE RAPHE NUCLEI

The raphe nuclei (RN) are principally composed of serotonergic neurons (Dahlstroem and Fuxe 1964). The serotonergic system is involved in numerous functions including mood, appetite, cognition, thermoregulation, energy expenditure, but also arousal and circadian rhythms (Lucki 1998; Yannielli and Harrington 2004).

The serotonergic neurons are located at the midline in the brainstem and can be divided in two principal groups, the inferior and the superior groups (Jacobs and Azmitia 1992). In the inferior group, three nuclei can be distinguished: the raphe pallidus, the raphe magnus and the raphe obscurus. The superior group contains four nuclei: the caudal linear nucleus, the medianraphe nucleus (MRN), the lateral (B9) neurons, and the dorsal raphe nucleus (DRN).

The midbrain MRN and DRN contain the highest percentage of 5HT neurons (around 30-50%) (Fu et al. 2010), and are both interconnected and connected to the circadian system.

a. Anatomy of 5HT neurons – interaction with the SCN

The architectural organisation of the midbrain RN has been previously described in many species, including rats (Malek et al. 2007; Lowry et al. 2008; Hale and Lowry 2011; Donner et al. 2012; Peyron et al. 2018), mice (Fu et al. 2010), and hamsters (Nexon et al. 2009). Besides distinguishing the DRN from the MRN, additional subgroups may be identified in the DRN (Peyron et al. 2018). First, the caudo-rostral extention of the DRN can be divided in three parts, namely caudal, medial and rostral DRN. Furthermore, the medial part of the DRN presents three subgroups: the dorsomedial RN, the ventromedial RN, and two lateral parts. These subdivisions may be considered as independent nuclei in accordance with receptor expression, electrophysiological properties, molecular organization and different afferent and efferent projections (Lowry et al. 2008; Huang et al. 2019; Clark, McDevitt, and Neumaier 2006; Hensler et al. 1994; Jacobs and Azmitia 1992; Kazakov et al. 1993; Urbain, Creamer, and Debonnel 2006).

Many projections have been described to reach the RN. Wake-active structures including the orexinergic lateral hypothalamus (LH), locus coeruleus (LC), and the lateral parabrachial nucleus, project to the DRN (Peyron et al. 1998; Kalén, Karlson, and Wiklund 1985; Peyron et al. 2018). While some structures, such as the lateral parabrachial nucleus, send diffuse projections to the whole DRN, others are specifically connected with subregions of the DRN. For example, the substantia nigra (SN) or the ventral tegmental area (VTA) send projections to the dorsomedial part of the DRN (Peyron et al. 2018). Many other structures send afferent projections to the RN, including the prefrontal cortex (PFC), ventral posterior complex of the thalamus (VP), zona incerta (ZI), amygdala, motor-related superior colliculus (SCm), and inferior colliculus external (ICe)(Pollak Dorocic et al. 2014). Reciprocal interconnections exist between the DRN and the MRN (Mosko, Haubrich, and Jacobs 1977; Peyron et al. 2018). In particular, the MRN innervates the lateral part of the DRN (Peyron et al. 2018). The MRN and the DRN innervate many areas of the brain, underling their roles in many functions. Among these areas ,there are the basolateral amygdala BLA, bed nucleus of the striaterminalis (BNST), habenula (Hb), medial prefrontal cortex (mPFC), nucleus accumbens (NAcb), and periaqueductal gray (PAG) (Müller and Jacobs 2010). Furthermore, important connections for the regulation of circadian rhythms are the direct projection from the Lat DRN to the intergeniculate leaflet (IGL), and the MRN projection to the SCN (Meyer-Bernstein andMorin 1996; Moga and Moore 1997).

b. Serotonin synthesis and catabolism

Serotonin (5HT) is a biogenic monoamine, classified as indolamine. In the brain, the major source of 5HT is mainly produced in the serotonergic neurons of the RN. Serotonin is synthesized from the essential amino acid L-tryptophan (L-Trp), obtained from the diet, in a 2-step process (*Fig. 9*). The first step involves the enzyme tryptophan hydroxylase (TPH) that produces 5-hydroxytryptophan (5-HTP) from the hydroxylation of L-Trp. In the second step, 5-HTP is decarboxylated to form 5-hydroxytryptamine (serotonin, 5HT) by the enzyme aromatic amino acid decarboxylase (AADC). In presence of L-Trp, the two steps of 5HT biosynthesis occur almost instantaneously. The 5HT synthesis-limiting step is represented by TPH since it has low affinity for other amino acids, while AADC has affinity for many L-aminoacids and its enzymatic activity is higher than TPH. In the brain, once synthesized, 5HT is storedin vesicles *via* the vesicular monoamine transporter 2 (VMAT2) (Peter et al. 1995). Serotonin vesicles can be found not only in presynaptic terminals, but also in the soma and in dendrites (Chazal and Ralston 1987; Descarries and Mechawar 2000). Once released, serotonin can bindserotonin receptors (5HTRs) in postsynaptic neurons, or presynaptic autoreceptors (5HT1a), involved in inhibiting 5HT release in the synaptic cleft (Cerrito and Raiteri 1979). Removal ofserotonin from the synaptic cleft occurs by the selective serotonin transporter (SERT). SERT isthe target of many drugs used in psychiatric disorders, including fluoxetine, a selectiveserotoninreuptake inhibitor (SSRI) (Wong, Bymaster, and Engleman 1995; Perez-Caballero etal. 2014). After being transported in presynaptic terminals, 5HT may be either recycled in vesicles or metabolized by monoamine oxidase (MAO). The principal 5HT metabolic product is 5-hydroxyindole acetic acid (5HIAA), obtained by the action of two enzymes, MAO-A and aldehyde dehydrogenase (Ma et al. 2004; Beck et al. 1995).

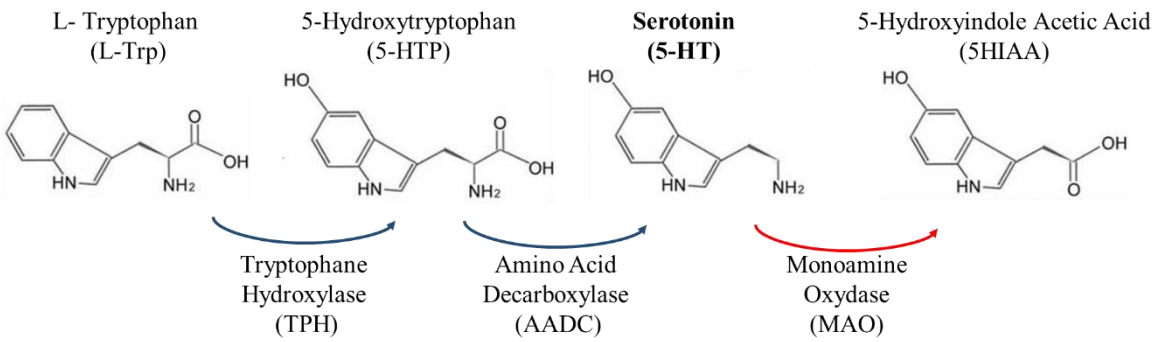

Fig. 9. Serotonin synthesis (blue arrows) and catabolism (red arrow).

c. Tryptophan Hydroxylase 2

Tryptophan Hydroxylase (TPH) is the first and rate-limiting enzyme for the biosynthesis of serotonin (5HT). Two forms of TPH have been described in mammals, TPH1 and TPH2, encoded by two different genes (Côté et al. 2003; Walther and Bader 2003; Walther et al. 2003). TPH1 is mostly expressed in the peripheral organs and in the pineal gland, where it is responsible for the synthesis of melatonin, while TPH2 is the main isoform in neurons (Malek et al. 2005; Walther and Bader 2003; Côté et al. 2003).

In the raphe nuclei, TPH2 is the most expressed form (Malek et al. 2005). Regulation of Tph2 expression is quite complex and involves a control at different levels, including at the level of its transcription (Chen and Miller 2012). In the human TPH2 gene, the core promoter region has been identified between nt -107 and 7 (Chen, Vallender, and Miller 2008), with a basal promoter located between nt -88 and 1 (Remes Lenicov et al. 2007). Among the factors involved in *tph2* mRNA regulation, calcium mobilization has been shown to induce *tph2* transcription. Indeed, in the basal promoter, a calcium-responsive element has been identified (RemesLenicov et al. 2007). Another transcription factor playing a role in transcriptional repression of*tph2* expression is the neuron-restrictive silencer factor (NRSF), that binds to the segment between nt 9-35.

Furthermore, TPH2 shows a circadian regulation both at the levels of mRNA and protein. In the rat, TPH2 shows high levels of protein in the middle of the subjective night both in the dorsal and in median raphe nuclei (Malek et al. 2004). The tph2-mRNA levels, both in LD and DD conditions, display high levels at the end of the subjective day, in anticipation to the night (Malek et al. 2005). In the hamster as well, *tph2* expression shows dailyrhythms at the levels of mRNA and protein (Nexon et al. 2009).

Although the molecular mechanisms responsible for the regulation of *tph2* rhythmic expression have not been fully elucidated yet, a major role seems to be played by the daily variations in plasma glucocorticoids. Accordingly, in rats, abolition of corticosterone rhythm results in loss of *tph2* oscillation, while *tph2* rhythm is restored, once glucocorticoid rhythm is reinstated (Malek et al. 2007). Evidence suggests that glucocorticoids may regulate *tph2* rhythmic expression at the transcription level. First, the raphe nuclei express the glucocorticoid receptor (GR) (Härfstrand et al. 1986), as well as the mineralocorticoid receptor (MR) (Arriza et al. 1988). Furthermore, GR gene deletion in the DRN in GR-floxed mice leads to increased *tph2* expression at the beginning of the day (Vincent et al. 2018). However, until now, no glucocorticoid response element (GRE) in *tph2* promoter has been described.

d. Rhythmic functioning of 5HT system

Besides being involved in the regulation of circadian rhythms, the serotonergic system itself displays several rhythmic features. Serotonin levels present a daily oscillation in target areas of serotonergic neurons including the LC (Semba, Toru, and Mataga 1984) and the SCN (Poncet, Denoroy, and Jouvet 1993; Cuesta et al. 2009b). In the SCN, not only 5HT content but also 5HT metabolite, 5HIAA, are rhythmic (Poncet, Denoroy, and Jouvet 1993; Cuesta et al. 2009b),as well as 5HT release, which shows a peak at the beginning of the subjective night in nocturnalrodents (Dudley, DiNardo, and Glass 1998; Barassin et al. 2002). Interestingly, rhythmic serotonin profiles havebeen described both in nocturnal and diurnal species. Indeed, Cuesta andcolleagues found highserotonin levels and serotonin activity during the light phase in *Arvicanthis*(Cuesta et al. 2008), in contrast to the high levels during the dark phase observed in nocturnal species (Cuesta et al.2009b). In nocturnal species, 5HT rhythm has been associated to its rhythmic synthesis, since both Tph2 protein and mRNA levels are rhythmic. In the DRN and MRN, peak of TPH2 proteinlevels occurs in the middle of the subjective night (Malek et al. 2004). Rhythmicity of TPH2 protein levels has been observed as well in the SCN (Barassin et al. 2002) and in the IGL (Malek et al. 2004), with a peak at the end ofthe subjective day. As stated in the previous paragraph, *tph2* mRNA levels in the RN oscillate as well (Malek et al. 2005), and their rhythmic transcription is driven by glucocorticoid daily surge and modulated by locomotor activity (Malek et al. 2007). Furthermore, rhythmic expression of 5HT transporter SERT in mice is rhythmic, as well as its activity, being higher in the dark phase (Ushijima et al. 2005).

Finally, 5HT neurons firing activity is high during wakefulness, decreases during NREM sleep and is almost silent during REM sleep (Sakai 2011; Jacobs and Azmitia 1992).

e. Serotonin receptors within the circadian system

The involvement of serotonin in many different biological functions is in part due to its action on several receptors. Seven classes of serotonin receptors have been identified, each grouping several subtypes (Pytliak et al. 2011):

1) $5HT_1$ receptors

Five receptor subtypes 1 have been identified, namely $5HT_{1A,}$ $5HT_{1B,}$ $5HT_{1D,}$ $5HT_{1E}$ and $5HT_{1F}$. The $5HT_{1C}$ has been reclassified as $5HT_{2C}$. These receptors are metabotropic, coupled with $G\alpha_i/G\alpha_0$ protein. They have an inhibitory role, by decreasing intracellular cAMP concentration.

The $5HT_{1A}$ receptors are expressed in the whole nervous system (Lanfumey and Hamon 2004). In the RN, principally localized in the cell body and dendrites, they act as presynaptic autoreceptors (Sotelo et al. 1990; Riad et al. 2000) and inhibit the electrical activity of serotonergic neurons (Sprouse and Aghajanian 1986), as well as 5HT synthesis and release (Hillegaart 1990; Hjorth and Sharp 1991). $5HT_{1A}$ receptors are expressed also in the SCN, where they are involved in the SCN synchronization (Bockaert et al. 2006). The most used $5HT_{1A}$ receptor agonist is the 8-OH-DPAT or (+) 8-OH-DPAT, although they have a lower affinity for the $5HT_7$ receptors as well (see below).

The $5HT_{1B}$ receptors are highly expressed in many brain structures, including the SCN, while $5HT_{1D}$ receptors are weakly expressed in the CNS, including the DRN. Both these receptor subtypes are localized in presynaptic terminals where they inhibit the release of neurotransmitters. In mice, $5HT_{1B}$ receptors are involved in regulating SCN synchronizationby inhibiting GABA release from SCN neurons.

2) $5HT_2$ receptors

Under this class of receptors three subtypes have been included, namely $5HT_{2A,}$ $5HT_{2B}$ and $5HT_{2C}$. These metabotropic receptors are coupled with $G\alpha_q$ protein and mainly show an excitatory action. $5HT_2$ receptor activation results in increasing intracellular concentration of inositol trisphosphate (IP3), Ca^{2+}, and diacylglycerol (DAG). Although the presence of 5HT2A receptors has been described in the SCN (Moyer and Kennaway 1999), their effect on circadian rhythms is still unknown. On the contrary, studies on 5HT2C receptors in regulating SCN synchronization support the its role on the phase shifting effects of light on the circadian system (Moyer and Kennaway 1999). These receptor subtypes may be both pre and postsynaptic. Specific agonist and specific antagonist have been identified, namely WAY-161503 and SB 242084, respectively.

3) $5HT_3$ receptors

Five receptor subtypes have been described in the 5HT3 receptor family, namely $5HT_{3A}$, $5HT_{3B,}$ $5HT_{3C,}$ $5HT_{3D}$ and $5HT_{3E}$. The 5HT3 receptors, unlike all the other 5HT receptors, are ionotropic. Serotonin binds the 5HT3 channel receptors, permeable to sodium, potassium and calcium ions, and causes an excitatory response in neurons. 5HT3 receptors are mainlylocalized in presynaptic terminals and are involved in modulating neurotransmitters release. Inorder to be

fully functional, heterodimeric combination of two subtypes ($5HT_{3A}$ and $5HT_{3B}$) isnecessary.The 5HT3 receptors have been suggested to be involved in the photic-like response of the circadian clock (Graff et al. 2007).

4) $5HT_4$ receptors

Seven receptor variants, which differ for their C-terminal sequence, are grouped under the $5HT_4$ class. These receptors are metabotropic, coupled with Gαs protein. They have an excitatory role, since their activation increases intracellular cAMP concentration. Among other structures, they are localized in the DRN where they modulate neurons activity and long-term potentiation. When they are localized at the presynaptic level, their activation leads to increase in neurotransmitter release. No role in circadian rhythms has been described at present.

5) $5HT_5$

In rodents, two receptor subtypes have been identified for this family, $5HT_{5A}$ and $5HT_{5B}$. As the $5HT_1$ receptors, $5HT_5$ receptors are coupled with $G\alpha_i/G\alpha_0$ protein and exert an inhibitory action on neurons, by decreasing intracellular cAMP concentration. They are expressed in the hypothalamus, including the SCN. However, their effect on the SCN synchronization has not been elucidated yet. According to the structures, 5HT receptors may be localized in neurons or in astrocytes.

6) $5HT_6$ receptors

Two variants, $5HT_{6A}$ and $5HT_{6B}$, have been described for this receptor family. As the $5HT_4$ receptors, $5HT_6$ receptors are coupled with Gαs protein action and increase intracellular cAMP concentration (Kohen et al. 1996). It has been suggested that $5HT_6$ receptors may be involved in psychiatric disorders (Bourson et al. 1995). For this class of receptor it exists a specific antagonist, the SB 271046.

7) $5HT_7$ receptors

Four 5HT7 receptor subtypes have been identified, namely $5HT_{7A}$, $5HT_{7B}$, $5HT_{7C}$ and $5HT_{7D}$. $5HT_7$ receptors are excitatory. They are coupled with Gαs protein action and increase intracellular cAMP concentration by activating adenylyl cyclase. These receptors activate MAP

kinases as well (Hedlund and Sutcliffe 2004). They are localized mainly postsynaptically and are found in the SCN (Clemett et al. 1999). $5HT_7$ are involved both in the regulation of REM sleep and synchronization of the SCN. It exists a specific agonist (AS19) and a specific antagonist (SB 269970). However, many studies have used the 8-OH-DPAT or (+) 8-OH-DPAT, which have low affinity for these receptors. 5HT7 antagonists show an effect similar to SSRIs (Mnie-Filali et al. 2007).

C2. THE LOCUS COERULEUS

Another arousal structure is the Locus Coeruleus which is a small bilateral nucleus located in the ponto-mesencephalic junction in the brainstem. In rodents, the LC is composed of around 1600 cells, all with the same embryonic origin and all producing the catecholamine noradrenaline (NA) (Robertson et al. 2013). The LC is the major source of NA in the brain (Von Euler 1946; Dahlstroem and Fuxe 1964). The LC is involved in regulating many functions including stress response, attention, memory, and the sleep/wake cycle. The NAergic LC neurons present high levels of discharge during wake, decrease their discharge rate during slow waves sleep (NREM) and show no discharge during REM sleep (Aston-Jones and Bloom 1981).

a. Anatomy of the LC– interaction with the RN and the circadian system

The involvement of the LC in a variety of function is in part due to its widespread connections. The LC sends projections to many areas of the brain, such as the cerebral cortex, the spinal cord, the thalamus, the hypothalamus and the basal forebrain (Jones and Yang 1985; Jones and Moore 1977; Berridge and Foote 1991; Olson and Fuxe 1971). Tracing studies showed that certain LC neurons are organized according to their efferent projections. Neurons projecting to the basal forebrain and to the spinal cord are located in the dorsal LC, while neurons projecting to the spinal cord are located in ventral LC. The neurons projecting to the hypothalamus and to the thalamus are located in the anterior and posterior LC respectively (Loughlin, Foote, and Grzanna 1986; Mason and Fibiger 1979; Schwarz et al. 2015). On the other hand, the LC receives multiple direct projections from different areas, including several arousal structures, the brainstem and the prefrontal cortex (Luppi et al, 1990). It has been shown that many brain areas receiving projections from the LC send back inputs to the nucleus (Schwarz et al. 2015). Among these areas, the LC is interconnected with the 5HTergic raphe nuclei (Morgane and Jacobs 1979; Olson and Fuxe 1971). Between these two structures, there are functional relationships, as wellas high correlation between the 24h levels of noradrenaline and serotonin

(Agren et al. 1986; Pujol et al. 1981). The interaction of the LC with other brain areas, including the RN, is important in the regulation of the sleep/wake cycle. Furthermore, indirect interconnections have been described, including one with the SCN, through the DMH (Aston-Jones et al. 2001).

b. Noradrenaline synthesis and catabolism

Noradrenaline (NA) is synthesized starting from the non-essential amino acid tyrosine, which is obtained either from the diet or from the essential amino acid phenylalanine (*Fig. 10*). The firststep of NA synthesis consists in converting tyrosine into L-dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase (TH). The enzyme DOPA decarboxylase (or aromatic L- amino acid decarboxylase (AADC)) is responsible for the conversion of DOPA into the neurotransmitter dopamine. These first two steps of NA biosynthesis occur in the cytoplasm. The final step takes place in vesicles, where dopamine is converted in NA by the enzyme dopamine β-monooxygenase (or dopamine β-hydroxylase). NA synthesis depends on TH activity, NA rate-limiting enzyme (Levitt et al. 1965). Degradation of NA occurs through the enzymes monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT), which lead to the metabolites 3-methoxy-4-hydroxymandelic acid (Vanillylmandelic acid (VMA)) and 3-methoxy-4-hydroxyphenylglycol (MHPG).

After its synthesis, NA is stored in synaptic vesicles until its release in the synaptic cleft, usually in response to an action potential. NA exerts its action on the adrenergic receptors and it is then recaptured by the noradrenaline transporters (NAT). Once in the cytosol, NA can be degraded or re-transported in the synaptic vesicles by vesicular monoamine transporters (VMAT), for further release.

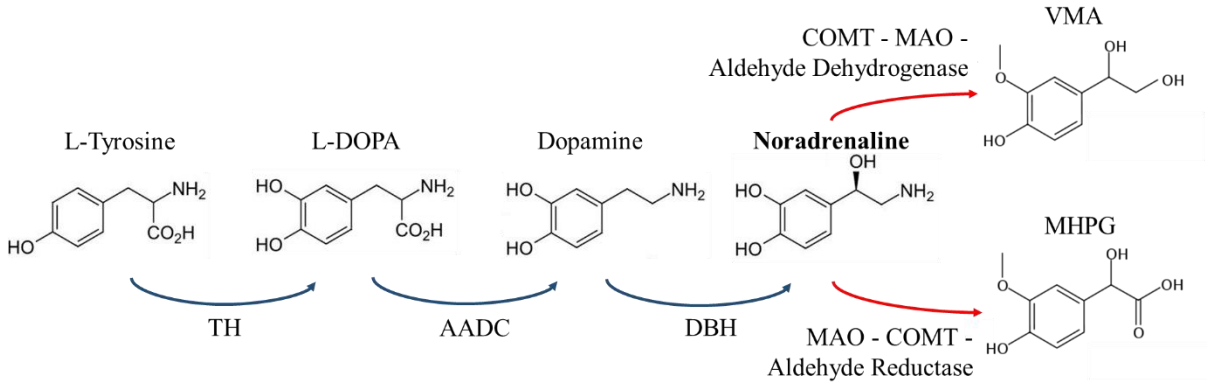

Fig. 10. Noradrenaline synthesis (blue arrows) and catabolism (red arrow).

c. Tyrosine Hydroxylase

Tyrosine hydroxylase (TH) is an enzyme belonging to the family of the aromatic amino acid hydroxylases (Grenett et al. 1987). TH protein acts as a tetramer, whose monomers are composed of 498 amino acids in the rat (Grima et al. 1985). TH catalyzes the first and limiting step of catecholamine biosynthesis (Nagatsu, Levitt, and Udenfriend 1964; Levitt et al. 1965), including noradrenaline synthesis in the LC. Tyrosine hydroxylase is controlled at multiple levels by several mechanisms. TH activity is primarily regulated by end-product feedback inhibition. Furthermore, its activity and stability are controlled by phosphorylation at four different sites. However, before being controlled at a post-translational level, tyrosine hydroxylase synthesis is regulated at the translational and transcriptional levels (Kumer and Vrana 1996; Tekin et al. 2014). Th gene expression is under the control of many transcription factors that regulate *th* transcription binding to regulatory sequences located on the *th* promoter (Tekin et al. 2014). Glucocorticoids, Ca^{2+} and stress are among the factors that play a modulatory role on *th* transcription. Indeed, *th* promoter presents, among other regulatory elements, a glucocorticoid response element (GRE), activator protein-1 (AP1), and a cAMP response element (Kumer and Vrana 1996). Furthermore, the presence of E-box sequences, that may be the target of clock gene regulation, have also been described on the rat *th* promoter (Fung, Yoon, and Chikaraishi 1992; Yoon and Chikaraishi 1994). However, the role of clock proteins on *th* transcription remains to be investigated.

d. Rhythmic functioning of NA system

The NAergic LC presents many rhythmic features. The firing activity of LC neurons shows a circadian variation, with higher firing rate during the active phase and lower discharge during the inactive phase (Aston-Jones et al. 2001; Gervasoni et al. 1998). Rhythmic variations have also been described for NA, whose levels reach a peak before the dark phase in the nocturnal rat (Semba, Toru, and Mataga 1984; Agren et al. 1986). In the diurnal rhesus monkey, the cerebrospinal fluid concentration of NA is high during daytime and minimal during the dark phase, in accordance with locomotor activity pattern (Perlow et al. 1978). The origin of the NA-LC system rhythmicity is at present not clear. However, it can be partly due to the SCN, which indirectly projects to the LC through the dorsomedial hypothalamus (DMH). Indeed, the orexinergic neurons of the DMH project to the LC and their daily activity correlate with LC firing rate. Furthermore, the orexin antagonist SB-334867-A decreases the LC firing rate during

the animals' active period (Gompf and Aston-Jones 2008). Whether other mechanisms, such as rhythmic hormonal signals or clock genes expression in LC, contribute to NA-LC system rhythmicity still remains unknown. Furthermore, the NA-LC contributes to the circadian rhythm of the sleep/wake cycle, since LC lesioned animals show decreased amplitude of the sleep/wake cycle in LD condition (González and Aston-Jones 2006).

e. Noradrenaline receptors within the circadian system

Noradrenaline can bind to three main classes of receptors, namely alpha-1 adrenergic receptors, alpha-2 adrenergic receptors, and beta adrenergic receptors, which show different affinities to NA. All these receptors are metabotropic and may have excitatory inhibitory effects. It has been shown that alpha and beta adrenergic receptors in the rat brain show both circadian and seasonal rhythms (Kafka, Wirz-Justice, and Naber 1981).

1) Alpha-1 adrenergic receptors

Three variants have been identified in this family: alpha-1a, alpha-1b, and alpha-1d receptors. Alpha-1 receptors have intermediate binding affinity to NA and are coupled with $G\alpha_q$ protein, therefore producing excitatory cellular effects.

2) Alpha-2 adrenergic receptors

Three subtypes, alpha-2a, alpha-2b, and alpha-2c receptors, are under this class. Alpha-2 receptors have the highest affinity to NA and are coupled with $G\alpha_i/G\alpha_0$ protein. Their activation results in inhibitory cellular effects. Alpha-2 receptors may be found both pre and postsynaptically. In presynaptic noradrenergic terminals, their role as autoreceptors is to inhibit further NA release.

3) Beta adrenergic receptors

Three variants of beta adrenergic receptors have been identified, namely beta-1, beta-2, and beta-3 receptors. In the brain, these receptors are found in the cerebral cortex. Beta receptors show the lowest affinity to NA and are coupled to the Gαs protein, resulting in excitatory effects.

II. OBJECTIVES

Depending on whether a mammal is nocturnal or diurnal, behavioral rhythms (such as the sleep-wake rhythm) occur in opposite phases to the light-dark (LD) cycle, while the SCN is always active at the same astronomical time. The main hypothesis of my thesis project is that structures downstream from the SCN, i.e. which receive information from the SCN or send feedback signals to the SCN, can contribute to the different temporal organization between nocturnal and diurnal animals. Among these structures, we have focused our attention on the Raphe Nuclei (RN) and Locus Coeruleus (LC), two structures which belong to the arousal system and which are also involved in circadian rhythms. Within the circadian system, serotonin (5-HT), produced in RN, is capable of phase shifting the SCN in nocturnal rats and in *Arvicanthis*. In addition, at the level of SCN, 5-HT exhibits a rhythmic variation over 24 hours, with an opposite phase in diurnal rodents compared to nocturnal rodents, and this rhythm is correlated with the arousal phase of the animals. Concerning the Locus Coeruleus, which synthesizes noradrenaline, electrophysiological studies, in particular, have demonstrated its rhythmic functioning in connection with the states of vigilance of animals.

The main objectives of this thesis are:

A. The locus coeruleus is involved in the control and maintenance of wakefulness in mammals, as well as in the circadian control of the sleep/wake cycle. To date, we do not know if the synthesis of noradreline (NA) is rhythmic. Thus, the first objective of my project is to study the expression over 24 hours of tyrosine hydroxylase (*Th*), the enzyme limiting the synthesis of norepinephrine in the Locus Coeruleus, in nocturnal rats and in diurnal *Arvicanthis*. In addition, we plan to investigate the expression of clock genes in the LC, which may be the cause of *th* rhythmicity.

B. In nocturnal rats, it has been shown that the rhythmic levels of serotonin are linked to the rate of its synthesis. Indeed, tryptophan hydroxylase 2 (*Tph2)*, serotonin rate-limiting enzyme) displays a rhythmic pattern of mRNA expression. It is not known whether serotonin synthesis is rhythmic in diurnal animals as well, and whether this rhythm is out of phase compared to the rhythm described in nocturnal rats. Thus, the second objective of my thesis project is to study the existence of a circadian rhythm of serotonin synthesis, by quantifying the expression of *tph2* in the raphe nuclei of the diurnal rodent, *Arvicanthis*. In addition, we aim at investigating the presence of a clock machinery within the RN, as potential rhythmic modulator of serotonin synthesis.

C. The mechanisms involved in controlling the rate of *tph2* are not well known, but data obtained in rats show that glucocorticoids are involved. The third objective of my project is therefore to determine whether the action of glucocorticoids in the raphe is carried out *via* a regulation mechanism of *Tph2* gene transcription.

D. Independently of an animal's behavioral phenotype (diurnal or nocturnal), the electrical activity of the SCN is higher during the day than during the night. Behavioral activity in nocturnal rodents inhibits the SCN firing rate. The effect of behavioral activity in the diurnal animals is unknown, as well as the mechanisms involved in the behavioral activity feedback to the SCN. On the other hand, many studies have shown the existence of reciprocal interactions between locomotor activity and serotonin. Thus, the fourth objective of my project is to investigate the effect of behavioral activity on the SCN of the diurnal *Arvicanthis* and to assess whether the behavioral activity feedback to SCN is mediated by serotonin.

Selon qu'un mammifère soit nocturne ou diurne, les rythmes comportementaux (tels que le rythme veille/sommeil) se produisent en opposition de phase du cycle lumière/obscurité (LD), tandis que les SCN sont toujours actifs à la même heure astronomique. L'hypothèse principale de mon projet de thèse est que les structures en aval des SCN, c'est-à-dire qui reçoivent des informations des SCN ou envoient des signaux de rétroaction aux SCN, pourraient contribuer à l'organisation temporelle différente entre les animaux nocturnes et diurnes. Parmi ces structures, nous avons porté notre attention sur les noyaux du Raphé (RN) et le Locus Cœruleus (LC), deux structures qui appartiennent au système de l'éveil et qui sont également impliquées dans les rythmes circadiens. Au sein du système circadien, la sérotonine (5-HT), produite par les RN, est capable de déphaser les SCN chez les rats nocturnes et comme chez les *Arvicanthis*. De plus, au niveau des SCN, la 5-HT présente une variation rythmique sur 24 heures, en oppositionde phase entre les rongeurs diurnes et les rongeurs nocturnes; ce rythme étant corrélé à la phased'éveil des animaux. Concernant le Locus Cœruleus qui synthétise la noradrénaline, des étudesélectrophysiologiques ont notamment mis en évidence son fonctionnement rythmique en lien avec les états de vigilance des animaux.

Les objectifs principaux de cette thèse sont :

A. Le LC est impliqué dans le contrôle et le maintien de l'éveil chez les mammifères, ainsi que dans le contrôle circadien du cycle veille/sommeil. A ce jour, on ne sait pas si la synthèse de noradrénaline (NA) est rythmique. Ainsi, le premier objectif de mon projet estd'étudier l'expression sur 24 heures de la tyrosine hydroxylase (*th*), l'enzyme limitant lasynthèse de noradrénaline dans le Locus Cœruleus, chez le rat nocturne et chez l'*Arvicanthis* diurne. De plus, nous avons quantifié l'expression des gènes horloge dans le LC, qui pourraient être impliqués dans la rythmicité de la *th*.

B. Chez le rat nocturne, il a été démontré que les niveaux rythmiques de sérotonine sont liés au rythme de sa synthèse. En effet, la tryptophane hydroxylase 2 (*tph2)*, enzyme qui détermine le taux de sérotonine, présente un profil rythmique d'expression de son ARNm. Par contre, on ne sait pas si la synthèse de la sérotonine est également rythmique chez les animaux diurnes et si ce rythme présente une phase identique ou opposée par rapport au rythme décrit chez les rats nocturnes. Ainsi, le deuxième objectif de mon projet de thèse est d'étudier l'existence d'un rythme circadien de synthèse de la sérotonine, en quantifiant l'expression de la *tph2* dans les noyaux du raphé

d’*Arvicanthis*. De plus, nous avons étudié l’expression de gènes horloge dans le RN, en tant que modulateurs rythmiques potentiels de la synthèse de sérotonine.

C. Les mécanismes impliqués dans le contrôle du rythme de *tph2* ne sont pas bienidentifiés, mais des données obtenues chez le rat montrent, que les glucocorticoïdes seraient impliqués. Le troisième objectif de mon projet est donc de déterminer si l'actiondes glucocorticoïdes dans le raphé s'effectue *via* un mécanisme de régulation de la transcription du gène *tph2*.

D. Indépendamment du phénotype comportemental d'un animal (diurne ou nocturne), l'activité électrique des SCN est plus élevée le jour que la nuit. L'activité comportementale chez les rongeurs nocturnes diminue la fréquence de décharge des SCN, mais cet effet n'est pas connu chez les animaux diurnes. De plus, de nombreuses études ont montré l'existence d'interactions réciproques entre l'activité locomotrice et la sérotonine. Ainsi, le quatrième objectif de mon projet est d'étudier l'effet de l'activité comportementale sur les SCN de l'*Arvicanthis* et d'évaluer si cette rétroaction est médiée par la sérotonine.

III. RESULTS

A. NORADRENALINE SYNTHESIS AND CLOCK GENE EXPRESSION IN ARVICANTHIS AND RAT LOCUS COERULEUS

The noradrenergic Locus Coeruleus (LC) is an arousal structure involved in the control of the sleep/wake cycle and in the maintenance of wakefulness. The interest in investigating the functioning of the LC in diurnal *vs* nocturnal rodents is double. First, the noradrenergic LC is involved in regulating circadian rhythm (noradrenaline content in the brain is rhythmic and modulate the amplitude of the sleep/wake cycle rhythm), but the data on its rhythmic functioning are scares and only came from nocturnal rodents. Second, the LC activity in nocturnal species highly correlate with other structures of the arousal system, including the raphe nuclei. Again, no data are available on diurnal animals. In this study, we investigated the noradrenaline synthesis rhythmicity as reflected by *th* expression, in the LC of the nocturnal rat and diurnal *Arvicanthis.* Furthermore, the hypothesis of an opposite rhythm in the LC of rat *vs* *Arvicanthis* was also assessed looking at clock genes expression.

Le Locus Cœruleus (LC) noradrénergique est une structure de l'éveil impliquée dans le contrôle du cycle veille/sommeil et dans le maintien de l'éveil. L'intérêt d'étudier le fonctionnement du LCchez les rongeurs diurnes, par rapport aux rongeurs nocturnes, est double. Premièrement, le LCest impliqué dans la régulation du rythme circadien (le contenu en noradrénaline dans le cerveauest rythmique et module l'amplitude du rythme du cycle veille/sommeil), mais les données sur son fonctionnement rythmique sont peu nombreuses et ne proviennent que de rongeurs nocturnes. Deuxièmement, l'activité du LC chez les espèces nocturnes est fortement corrélée avec d'autres structures du système de l'éveil, y compris les noyaux du raphé. Aucune donnée n'est disponible sur l'interaction entre LC et RN chez les animaux diurnes. Dans cette étude, nous avons évalué la rythmicité de la synthèse de la noradrénaline, en mesurant l'expression dela *th* dans le LC du rat et de l'*Arvicanthis*. De plus, l'hypothèse de l'existence d'un rythme opposé dans le LC de l'*Arvicanthis* par rapport au rat a également été évaluée en étudiant l'expression des gènes horloge.

A1. CIRCADIAN AND DAYLY FUNCTIONING IN THE LOCUS COERULEUS OF THE NOCTURNAL RAT AND THE DIURNAL RODENT *ARVICANTHIS*

Version edited in form of article, but needs to be completed before submission.

Title

Circadian and daily functioning in the locus coeruleus of the nocturnal rat and the diurnal rodent *Arvicanthis*

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Abstract

The noradrenergic Locus Coeruleus (NA-LC) is one of the major arousal structures involved in inducing wakefulness and 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 may result from its rhythmic synthesis. Therefore, we investigated the 24-h expressionprofile of NA rate-limiting enzyme, tyrosine hydroxylase (*th*), in the Locus Coeruleus (LC) of the nocturnal rat and the diurnal rodent *Arvicanthis*, under both 12:12 light/dark (LD) and constant darkness (DD) conditions. In both species, *th* mRNA levels vary significantly over 24-h. In nocturnal rats, *th* mRNA profiles show a unimodal rhythm, with peak values preceding theactive phase, i.e. subjective night. On the contrary, *th* rhythm in *Arvicanthis* is characterized bya bimodal profile, with two higher levels in the middle of the subjective day and night. The rhythmic pattern of *th* expression may be dependent on LC clock genes machinery. Therefore,we investigated the mRNA expression of three clock genes, namely *bmal1*, *per1*, and *per2* andfound that their expression display significant variations between day and night time points in both species, but in opposite directions. These data show that NA rhythm may be related to *th*gene expression, which is circadian 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 may be involved in *th* rhythm.

Keywords

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

Abbreviations: NA, noradrenaline; LC, Locus Coeruleus; SCN, suprachiasmatic nuclei; LD, light/dark cycle; DD, constant darkness; Bmal1, Brain and Muscle ARNT-Like 1; Per1/2, period 1/2.

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. The distribution of wake and rest episodes, besides being under the control of the SCN, is regulated by the arousal system (Weaver 1998; Saper et al. 2010; Dijk and Archer 2009). The noradrenergic Locus Coeruleus (NA-LC) is one of the principal arousalstructures involved in inducing wakefulness (Jones 2003). Furthermore, previous studies haveshown that the NA-LC contributes to modulate circadian parameters of the sleep/wake cycle (González and Aston-Jones 2006). Evidence shows that the functioning of the NA-LC itself isrhythmic. Circadian variations have been reported in the firing activity of LC neurons, which ishigher during the active period than the inactive phase (Aston-Jones et al. 2001). Moreover, noradrenaline (NA) levels in the brain show 24-h oscillations (Semba, Toru, and Mataga 1984; Agren et al. 1986). However, the origin of NA rhythm is so far unknown. In the LC, NA levels strictly depend on tyrosine hydroxylase (*th*), the rate limiting enzyme for NA synthesis(Nagatsu, Levitt, and Udenfriend 1964; Levitt et al. 1965).

In this study, we tested the hypothesis that NA rhythm originates from its synthesis, and is therefore dependent on *th* expression rhythm. For that purpose, we have investigated the 24-h profile of *th* mRNA levels in 12:12 LD condition and in constant darkness (DD). Considering the role of noradrenaline in promoting wakefulness and the correlation of NA-LC activity with an animal arousal state (Aston-Jones and Bloom 1981; Rajkowski et al. 1998), we hypothesize that NA synthesis rhythm differs between nocturnal and diurnal animals. Hence, we measured*th* expression in the nocturnal rat and in the diurnal rodent *Arvicanthis ansorgei*.

To go further, we raised the question about the source 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. An indirect SCN projection has been described to reach the LC *via* the dorsomedial hypothalamus (DMH) (Aston-Jones et al. 2001). Second, the SCN may convey a rhythmic signal to the LC through hormonal outputs, such as glucocorticoids. Indeed, a glucocorticoid response element (GRE) has been described in th promoter (Hagerty et al. 2001). Third, *th* rhythmic expression may be under the control of a circadian clock in the LC. Indeed, at a molecular level, circadian rhythms are produced by self-sustained clock machinery, characterized by a transcriptional-translational feedback loop (TTFL) (Dunlap 1999; Ko and Takahashi 2006; Mohawk, Green, and Takahashi 2012). The core of this TTFL consists in positive (e.g. BMAL1) and negative (e.g. PER1/2, CRY1/2) clock

genes and protein, which are responsible not only for their own transcription/repression, but also for the transcription of other genes by binding to a promoter sequence namely E-box. Of note, E-box sequences are found in the promoter of the rat *th* gene (Fung, Yoon, and Chikaraishi 1992; Yoon and Chikaraishi 1994).

In this study, we investigate whether the clock machinery may be involved in *th* rhythmic transcription. Therefore, we measured the 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.Experiments were performed on adult male Sudanian grass rats (*Arvicanthis ansorgei*) weighting 150-250g (Chronobiotron, UMS 3415, CNRS Strasbourg, France) and adult male Wistar rats weighting 200g (Charles River Laboratories, Saint-Germain-Nuelles, France) wereused. All the animals were individually housed in Plexiglas cages, entrained to 12h light-12h dark (LD 12:12) cycle (light: 150 - 200 lux, dark: red dim light, <5 lux) and provided with food and water ad libitum.

2.2. Experimental protocol and samples collection

For *th* quantification, 84 rats and 84 *Arvicanthis* were housed in LD 12:12 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 CO_2 sedation, every 4 hours (seven time points of 6 animals) starting at *Zeitgeber* time (ZT) 6 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 is taken at ZT6 in LD and at CT6 in DD of the second experimental day, indicated as ZT6' and CT6', respectively. For the *Arvicanthis*, the last time point is ZT2 inLD 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 inDD, 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 (G. Paxinos 1986)) with a cryostat (Leica

Instruments GmbH, Nussloch, Germany) and collected on sterile slides, stored at -20°C until use. For qRT-PCR quantification of clock genes, rats and *Arvicanthis* maintained in LD were 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 4^{th} ventricle as landmark. LC tissue was stored at -80 °C until use.

2.3. Radioactive in situ hybridization

Sense (GGGGAGCTGAAGGCTTATGGTGCAGGGCTGCTGTCTTCCTACGGAG) and antisense (CTCCGTAGGAAGACAGCAGCCCTGCACCATAAGCCTTCAGCTCCCC) oligoprobes for *th* were designed based on the rat *th* mRNA sequence (Sigma Aldrich). The sequence identity of the probes with *Arvicanthis th* sequence were analyzed on *Arvicanthis niloticus* (taxid:61156) genome using Nucleotide BLAST (https://blast-ncbi-nlm-nih-gov.scd-rproxy.u-strasbg.fr/Blast.cgi), since *Arvicanthis ansorgei* genome sequence is not available. Probes (46-nucleotides) were 3' end-labelled with [^{35}S]-dATP (46.25 GBq/mol, PerkinElmer, Waltham MA) using the Terminal Deoxynucleotidyl Transferase (40U, Thermo Scientific). Hybridization was performed as follows: sections were postfixed in 4% formaldehyde for 15 min, rinsed for 2 min in 0.1 M phosphate buffer saline (PBS), acetylated twice for 5 min in 0.4% acetic anhydride in 0.1 M triethanolamine (pH 8.0), rinsed for 2 min in 0.1M PBS, dehydrated in graded ethanol series, and air dried. Hybridization was carried out by deposing 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. Sections were placed in humid boxes containing 2X SSC / 50% formamide at 37°C overnight. After hybridization, the sections were washed in 1X SSC for 5 min at room temperature (RT) and stringency washes were performedin 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 in graded ethanol series, air dried at RT and then exposed to an autoradiographic film (Kodak BioMax; Kodak, Rochester, NY), with ^{14}C standard.

2.4. Quantitative analysis of th mRNA

Quantitative analysis 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 (*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 ^{14}C 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 concentrationand 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. Real-time PCR was performed using PowerUp™ SYBR™ Green Master Mix (A25776, Thermo Fisher Scientific) and run on the Applied Biosystems Real-Time PCR Instruments and on Biorad CFX96. 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 (Livak and Schmittgen 2001), 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 level expression of the three ZTs.

2.6. Statistical analyses

Possible differences of 24h *th* mRNA labelling between the right and left LC were tested. Since no differences were found between the right and left LC, 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 interactions. 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*) weretested 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). These analyses were performed using the following equation: $y = A + (B * \cos(2\pi(\times - C)/24) + (D * \cos(2\pi(\times - E)/12))$; where A is the mean level

(mesor), B and D the amplitude and, C and E the acrophases of the rhythm. For RT-qPCR data, differences among time points were analyzed using one-way analysis of variance (ANOVA I). 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 $p<0.05$. All data are presented as mean±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. Since the *Arvicanthis ansorgei* genome is not available, *th* similarity was tested on the *Arvicanthis niloticus* sequence. BLAST analysis revealed 97.83% identity with *Arvicanthis niloticus* predicted *th* mRNA sequence. Specificity of the antisense probe was tested by a saturation test and by hybridization in the rat and *Arvicanthis* LC, and in the *Arvicanthis* Substantia Nigra (SN) as shown in Fig. 1. *In situ* hybridization of *th* antisense oligoprobe clearlylabeled the rat and *Arvicanthis* LC (Fig. 1A and 1C). *Arvicanthis* coronal sections of SNwere used as positive control (Fig. 1E). Hybridization of sense *th* riboprobe showed no specificsignalneither in the rat and *Arvicanthis LC* nor in the *Arvicanthis* SN (Fig. 1B, 1D and 1F), norin the *Arvicanthis* LC (Fig. 1F). Significant OD variations were measured in the LC, while non-specificOD measured in the surrounding area was constant.

3.2. Anatomical extension of the rat and Arvicanthis Locus Coeruleus

Hybridization with *th* antisense oligoprobe allowed clear anatomical identification of left and right LC in rat (Fig. 2A) and *Arvicanthis* (Fig. 2C). For both species, sections of 20 µm, 80 µmapart, 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, the left and right LC nuclei show a trapezoidal morphology and are positioned at the edges of the fourth ventricle. In the *Arvicanthis* LC, *th* oligoprobe hybridized on 10 anatomicallevels. As for the rat, the left and right LC nuclei are located around the fourth ventricle. Unlikein the rat, *Arvicanthis* LC

presents a crescent-shaped morphology. For both species, six anatomical levels of the LC were considered for *th* quantification (Fig. 2B and 2D).

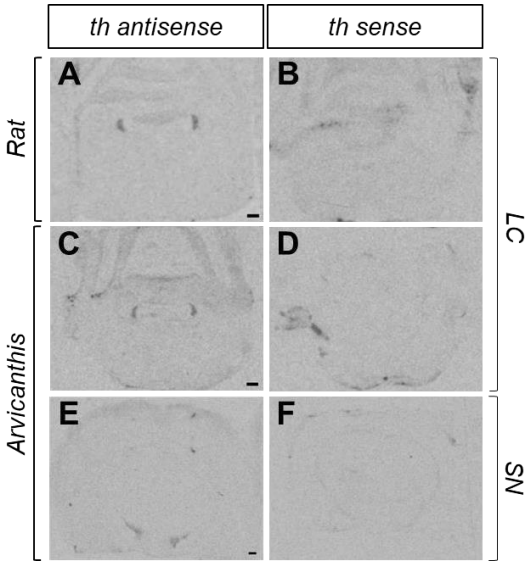
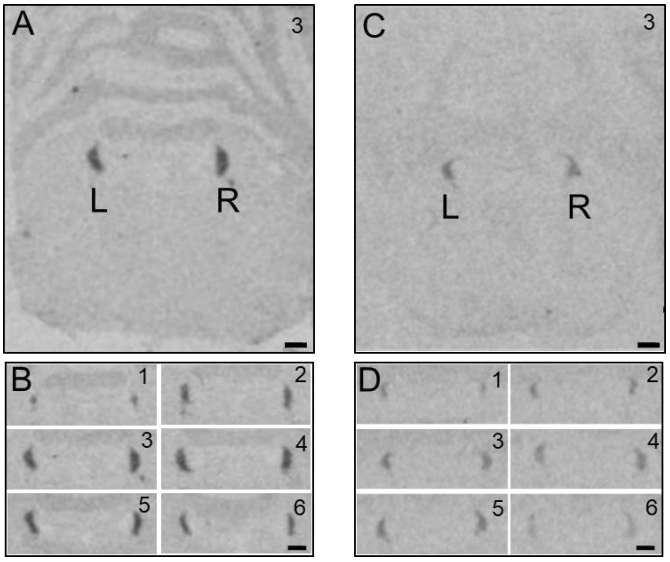


Fig. 1. Th oligoprobe quality control. In situ hybridization performed on coronal sections of the rat Locus Coeruleus (LC) (A and B), and of the *Arvicanthis* LC (C and D) and substantia nigra (SN) (E and F), using th oligoprobes antisense (A, C and E) and sense (B, D and F). Scale bar: 500 µm.

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



3.3. th mRNA profile within the rat and Arvicanthis Locus Coeruleus 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 correspond to ZT2 and CT2 for rat, and ZT6 and CT6 for *Arvicanthis*. The expression of *th* mRNA was significantly different among the levels of LC both in the rat (LD $p<0.001$, DD $p<0.001$) and in *Arvicanthis* (LD $p<0.001$, DD $p<0.001$).

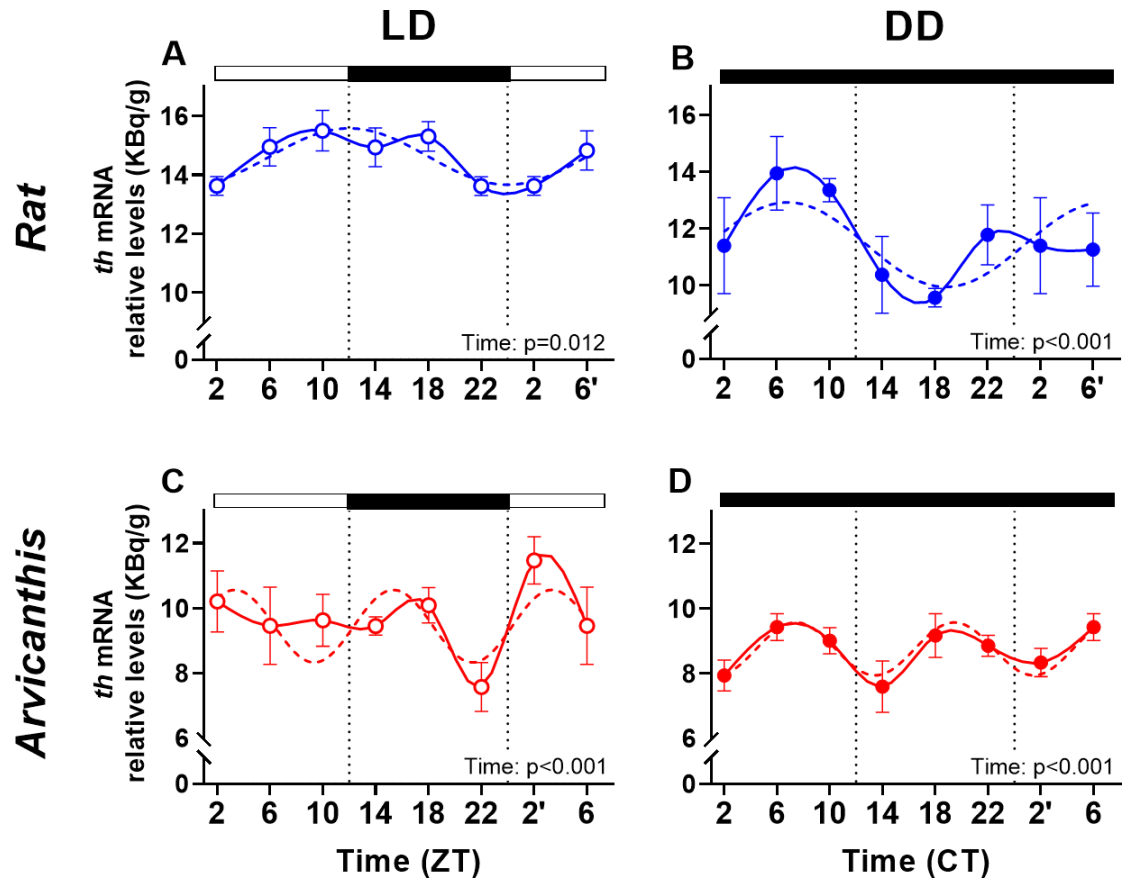


Fig. 3. In situ hybridization measurements of *th* mRNA relative levels within the rat (A and B) and *Arvicanthis* (C and D) Locus coeruleus (LC) housed in 12:12 light-dark (LD, A and C) andconstant darkness (DD, B and D) condition. Dotted lines represent the significant Cosinor fit. Points are connected by a smoothing line. White and black horizontal bars represent light anddark phases, respectively. Time is expressed as Zeitgeber Time (ZT) in LD and Circadian Time(CT) in DD. For each animal, the mean *th* levels measured within the right and left LC is plotted. Time points ZT2', CT2', ZT6' and CT6' are obtained using an experimental groups different from ZT2, CT2, ZT6 and CT6, respectively. For rats, experimental groups were all n≥5 exceptfor ZT18 (n=4), ZT6' (n=3) and CT6' (n=4). For *Arvicanthis*, experimental groups were all n≥5except 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±SEM.

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 24h 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 vsCT14 (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 (interactions "time" * "light condition"p<0.001). Post-hoc analyses show that the LD and DD profiles significantly differ at ZT2 vs CT2 (p=0.002), 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 (interactions "time" * "species" p<0.001).

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

In order to investigate whether rhythms in LC are different in nocturnal and diurnal mammals, we investigated 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 are 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 (*post-hoc* test: p=0.001) and to ZT18 (*post-hoc* test; p<0.001). Coherently, *per1* and *per2* expressions significantly increase from ZT2 to ZT18 (*post-hoc* test; both p<0.001), from ZT2 to ZT10 (*post-hoc* test; *per1* p=0.002, *per2* p=0.021), and from ZT10 to ZT18 only for *per2* (*post-hoc* test; p=0.003). The clock gene mRNA variation over time was significantly different between rats and *Arvicanthis,* for all three genes (ANOVA II, interaction "time" and "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.*

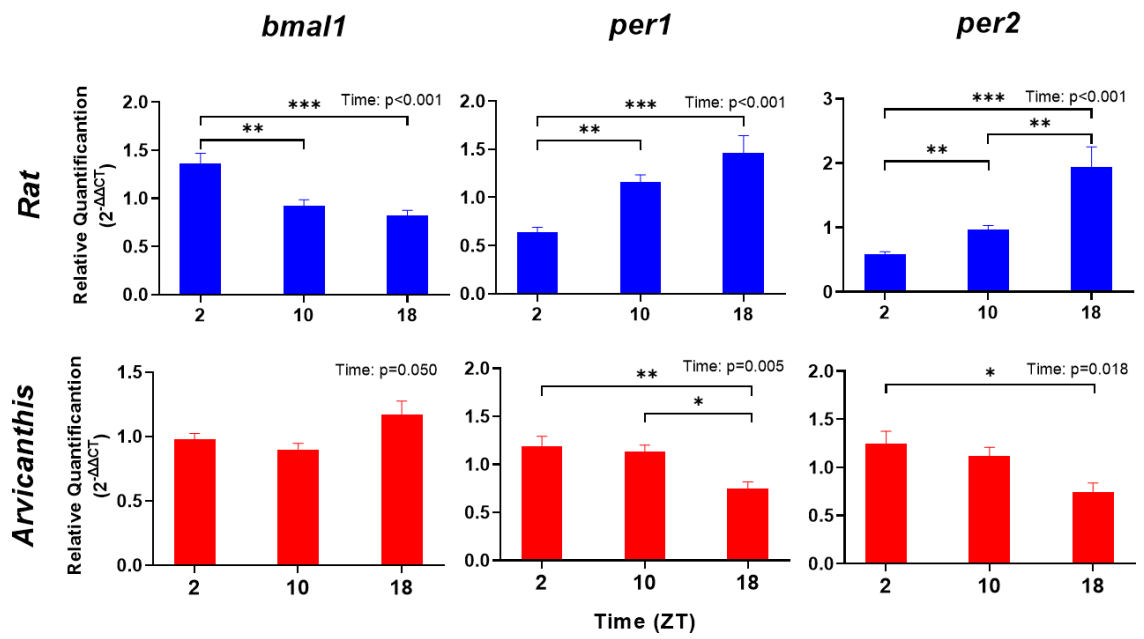


Fig. 4. qRT-PCR analyses of *bmal1*, *per1* and *per2* mRNA levels within the rat (blue bars) and *Arvicanthis* (red bars) Locus Coeruleus (LC) housed in 12:12 light-dark condition. Time is expressed as Zeitgeber Time (ZT). Experimental rat and *Arvicanthis* groups were all n≥5. Theeffect of time is significant for all genes. Post-hoc tests show differences among the time points: p<0.05 (*), p<0.01 (**), p<0.001 (***). Interaction between time and species is significant for allgenes (ANOVAII; p>0.05). Data are presented as mean±SEM

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 (Grzanna and Molliver 1980; Schwarz and Luo 2015). Though its neurotransmitter identity is quite complex, all neurons in the LC contain noradrenaline (Schwarz and Luo 2015). Therefore, theuse 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 *Arvicanthis*. Differences between the two species mainly relate to the LC shape, which is more half-moon curved in the medial part of the Arvicanthis LC, and extension, which is shorter in *Arvicanthis* than in rats of about 200 µm.

4.2. Rhythmic th mRNA expression in the rat and Arvicanthis LC

In this study, we have shown that tyrosine hydroxylase is rhythmic at the mRNA level in the LC. Rhythmicity of *th* has been observed both in a diurnal species, *Arvicanthis*, and a nocturnal species, the rat. The LC is a wake-promoting structure that regulates the circadian rhythm of the sleep/wake cycle. It is reasonable to hypothesize that temporal differences may exist between nocturnal and diurnal species, since they are awake in opposite temporal windows. Indeed, in this study we showed that the rhythm of *th* expression reflects the animals' behavioral activity pattern. In the rat, *th* levels show a unimodal rhythm with a peak occurring at the end of the day in LD condition. By contrast, in *Arvicanthis*, a species characterized by acrepuscular activity pattern (Challet et al. 2002), *th* levels display a bimodal profile in LD condition, 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 *Arvicanthis*, showing that in both species *th* rhythmicity is endogenous. *Th* rhythmicity suggests that the noradrenaline rhythm, observed in several brainareas, including hypothalamic regions involved in the circadian timing system (Cagampang, Okamura, and Inouye 1994; Manshardt and Wurtman 1968; Agren et al. 1986), may be in partregulated at the level of its synthesis. NA levels are strictly dependent on TH activity. Few data are available on the rhythmicity of TH enzymatic activity. Natali and colleagues reported circadian rhythm of TH activity in the LC, with different phases according to different mouse strains (Natali et al. 1980). Cahill and Ehret reported a bimodal TH activity in the brain stem (Cahill and Ehret 1981). Another factor affecting NA levels may be TH protein amount. Indeed,*th* mRNA 24h-variation may result in TH protein rhythm. Clemens and colleagues described 24h variation of TH protein in the spinal cord of mice (Clemens, Sawchuk, and Hochman 2005). Whether TH protein levels and enzymatic activity are rhythmic in the *Arvicanthis* and rat LC requires further investigation. NA rhythm in *Arvicanthis* brain has not been described yet. Thedifferent patterns of *th* mRNA levels, suggest that even the 24h levels of NA in LC terminals may be different between the nocturnal rat and the diurnal *Arvicanthis*. Considering the role ofNA in the circadian regulation sleep wake cycle, different rhythms in NA synthesis may contribute to determine the opposite behavioral phenotype in diurnal and nocturnal animals.

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 *Arvicanthis,* 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 NA-LC system has been highlighted in rats, in which long term exposure to DD decreases NA neurotransmission in the frontal cortex, suggesting that light is essential for physiological NA transmission (González and Aston-Jones 2006; Gonzalez and Aston-Jones 2008). In *Arvicanthis*, the effects of light on the NA-LC system have been rarely investigated. It has been shown that 1h light pulse in early night increases strongly cFOS expression in the LC of *Arvicanthis*, but does not significantly reduce it in mice (Shuboni et al. 2015), suggesting that light may have a different impact on LC activity between diurnal and nocturnal rodents. How and to which extent light affects *th* rhythm requires further investigation.

4.3. Clock gene expression in the rat and Arvicanthis LC

To investigate possible mechanisms that can be involved in the rhythmic transcription of *th*, we explored the rhythmicity of clock genes in the Locus Coeruleus. Very few data are availableon clock gene expression in the LC (Shieh 2003; Mahoney, Brewer, and Bittman 2013). Herewe showed that the clock genes Bmal1, Per1 and Per2 are expressed in the LC and vary overtimein 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 gene variations in *Arvicanthis* seem to be opposite in phase to those of the rat. 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 hamsters, showing that LC *per1* expression in TH cells is higher at CT12.5 compared to CT3 (Mahoney, Brewer, and Bittman 2013). The presence of a rhythmic clock machinery leads to the possibility that *th* rhythm may be under the control of these locally expressed clock genes. Indeed, E-box sequences have been described in *th* rat promoter gene (Fung, Yoon, and Chikaraishi 1992; Yoon and Chikaraishi 1994). The presence of E-box sequences in *Arvicanthis th* gene needs to be verified.

Although clock genes may be involved in *th* rhythmic transcription, other mechanisms may contribute to *th* 24h 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 (Aston-Jones et al.

2001). It is possible that signals resulting from indirect SCN projection may also affect *th* rhythm. Furthermore, circadian signals may be relayed to the LC through SCN hormonal outputs, such as glucocorticoids or melatonin. *Th* gene promoter contains glucocorticoid response elements (GRE) (Hagerty et al. 2001), at least in rat. Therefore, glucocorticoids rhythm may be another factor involved in *th* mRNA oscillation.

In *Arvicanthis*, *th* expression is bimodal. Based on the three time points we 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* ((Verhagen et al. 2004), Caputo et al. submitted), may contribute to *th* bimodal expression.

4.4. Conclusions

In conclusion, we found endogenous rhythms of tyrosine hydroxylase mRNA in the Locus Coeruleus of both a diurnal and a nocturnal rodent, and we showed that *th* is influenced by light condition. We also showed different temporal patterns of *th* levels, as well as in clock gene expression, in the nocturnal rat compared to the diurnal *Arvicanthis*, indicating that the rhythmic functioning of the LC is opposite in these species. Furthermore, the presence of clock genes suggests that the clock machinery may be involved in mechanisms regulating rhythmic functions within the LC, including *th* transcription. These data suggest that LC rhythm differs between nocturnal and diurnal species and may contribute to their opposite temporal organization.

Declaration of interest

None

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Gene	Species	Forward	Reverse	Reference
Bmal 1	Rattus norvegicus	CAACCCATACACAG AA GCAAAC	ACAGATTCGGAGAC AA AGAGGA	(Zhang etal. 2016)
Bmal 1	Arvicanthis ansorgei	GACACTGAGAGGTG CC ACCAA	CCATCTGCTGCCCT GAGAAT	(Bobu et al. 2013)
Per1	Rattus norvegicus	GCGTAAATGTGCCTC CT CTT	GACGTATCTTTCTT CGCCCC	IDT
Per1	Arvicanthis niloticus	CCACTGAGAGCAGCA A GAGTACA	CTGCTGCAGCCACT GG TAGA	(Bobu et al. 2013)
Per2	Rattus norvegicus	CACCCTGAAAAGAA AGT GCGA	CAACGCCAAGGAGC TC AAGT	(Mattam andJagota 2014)
Per2	Arvicanthis ansorgei	TCACCGTAGGAGA TCCGGAAT	TTTCTGCAACAGGT GCTTCCT	(Bobu et al. 2013)
*Gapdh (1)**	Rattus norvegicus	GACAACTTTGGCAT CGT GGA	ATGCAGGGATGAT GTTCTGG	IDT
Gapdh(2)	Arvicanthis niloticus	ATCCACTGGTGCT GCCAAG	CCGTTCAGCTCTGG GA TGAC	(Ikeno et al. 2016)
36B4	Rattus norvegicus	GTGCCTCACTCCATC AT CAA	TCCGACTCTTCCTT TGCTTC	IDT

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

B. SEROTONIN SYNTHESIS AND CLOCK GENE EXPRESSION IN ARVICANTHIS AND RAT RAPHE NUCLEI

The serotonergic raphe nuclei are involved in the control of arousal, as well as in regulating the circadian system. Evidence suggests that the serotonergic system may oscillate in diurnal *vs* nocturnal rodents. For instance, serotonin content in the SCN is rhythmic in both the nocturnal rodent and the diurnal *Arvicanthis*. However, the phase of the 5HTcontent rhythm within the SCN, is opposite, being higher during the dark phase in the rat and during the light phase in *Arvicanthis.* Whether the opposite rhythm of serotonin content in the SCN is linked to a different functioning of the serotonergic RN between diurnal and nocturnal rodents is still unknown. To answer this question we first investigated serotonin synthesis in the *Arvicanthis* RN. We measured the rhythmic expression of serotonin rate limiting enzyme, tryptophan hydroxylase 2 (*tph2*) and we investigated SCN outputs (i.e. corticosterone, melatonin, locomotor activity) as potential modulator of *tph2* rhythm. These findings are compared to thepublished data in rats *(Section B1)*. Furthermore, we explored the hypothesis that rhythms in serotonin synthesis may be under the control of the clock machinery within the RN. Therefore, we measured the expression of three clock genes, namely *bmal1, per1* and *per2*, in the RN of the *Arvicanthis* and the rat *(Section B2).*

Les noyaux sérotoninergiques du raphé sont impliqués dans le contrôle de l'éveil, ainsi que dans la régulation du système circadien. Les données montrent que le système sérotoninergique a un fonctionnement rythmique chez les rongeurs diurnes et nocturnes. Par exemple, les niveaux de sérotonine dans les SCN sont rythmiques à la fois chez le rongeur nocturne et chez l'*Arvicanthis* diurne. Cependant, la phase du rythme de 5HT au sein des SCN est opposée, étant plus élevée pendant la phase d'obscurité chez le rat et pendant la phase de lumière chez *Arvicanthis*. A ce jour, on ne sait pas si le rythme opposé de sérotonine dans les SCN est lié à un fonctionnement différent du RN entre les rongeurs diurnes et nocturnes. Pour répondre à cette question, nous avons d'abord étudié la synthèse de la sérotonine dans le RN d'*Arvicanthis*. Nous avons mesuré l'expression rythmique de l'enzyme limitant le taux de sérotonine, la tryptophane hydroxylase 2 (*tph2*) et nous avons étudié les sorties des SCN (c'est-à-dire la corticostérone, la mélatonine, l'activité locomotrice) en tant que modulateur potentiel du rythme de *tph2*. Ces résultats sont comparés aux données publiées chez le rat (Section B1). De plus, nous avons exploré l'hypothèse selon laquelle les rythmes de la synthèse de la sérotonine pourraient être sous le contrôle de gènes horloge au sein des RN. Ainsi, nous avons mesuré l'expression de trois gènes horloge, *bmal1*, *per1* et *per2*, dans le RN de l'*Arvicanthi*s et du rat (Section B2).

B1. CIRCADIAN RHYTHM OF SEROTONIN SYNTHESIS IN THE DIURNAL *ARVICANTHIS*

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RESEARCH ARTICLE

Bimodal serotonin synthesis in the diurnal rodent, *Arvicanthis ansorgei*

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Abstract

In mammals, behavioral activity is regulated both by the circadian system, orchestrated by the suprachiasmatic nucleus (SCN), and by arousal structures, including the serotonergic system. While the SCN is active at the same astronomical time in diurnal and nocturnal species, little data are available concerning the serotonergic (5HT) system in diurnal mammals. In this study, we investigated the functioning of the 5HT system, which is involved both in regulating the sleep/wake cycle and in synchronizing the SCN, in a diurnal rodent, *Arvicanthis ansorgei*. Using in situ hybridization, we characterized the anatomical extension of the raphe nuclei and we investigated 24 h mRNA levels of the serotonin rate-limiting enzyme, tryptophan hydroxylase 2 (*tph2*). Under both 12 h:12 h light/dark (LD) and constant darkness (DD) conditions, *tph2* mRNA expression varies significantly over 24 h, displaying a bimodal profile with higher values around the (projected) light transitions. Furthermore, we considered several SCN outputs, namely melatonin, corticosterone, and locomotor activity. In both LD and DD, melatonin profiles display peak levels during the biological night. Corticosterone plasma levels show a bimodal rhythmic profile in both conditions, with higher levels preceding the two peaks of *Arvicanthis* locomotor activity, occurring at dawn and dusk. These data demonstrate that serotonin synthesis in *Arvicanthis* is rhythmic and reflects its bimodal behavioral phenotype, but differs from what has been previously described in nocturnal species.

KEYWORDS

5HT, *Arvicanthis*, circadian, raphe nuclei, rhythm, rodent, SCN, tryptophan hydroxylase 2

Abbreviations: 5HIAA, 5-hydroxyindoleacetic acid; 5HT, serotonin; ACN, acetonitrile; CT, circadian time; DD, constant darkness; DM, dorsomedial subregion of dorsal raphe nucleus; DRN, dorsal raphe nucleus; EDTA, ethylene-diamine-tetra-acetic acid; HPLC, high-pressure liquid chromatography; IGL, intergeniculate leaflet; Lat, lateral subregion of dorsal raphe nucleus; LC, locus coeruleus; LC-MS/MS, liquid chromatography tandem mass spectrometry; LD, light/dark cycle; MRM, multiple reaction monitoring; MRN, median raphe nucleus; OC, optic chiasm; OD, optical density; PBS, phosphate-buffered saline; RCF, relative centrifugal force; RIA, radioimmunoassay; RN, raphe nuclei; RT, room temperature; SCN, suprachiasmatic nucleus; SSC, sodium saline citrate; Tph2, tryptophan hydroxylase 2; VM, ventromedial subregion of dorsal raphe nucleus; VP, vasopressin; ZT, Zeitgeber time.

1 | INTRODUCTION

In mammals, many behavioral and physiological functions are regulated by the circadian system. Circadian rhythms are orchestrated by the master clock, located in the suprachiasmatic nucleus (SCN). The SCN receives timing information from several cues and entrains the rhythmic pattern of various body's physiological and behavioral functions (e.g., fasting/feeding cycle, sleep/wake cycle), by sending neural projections and humoral signals (e.g., melatonin, corticosterone).[1,2] The most potent time cue for the SCN is the light.[3] While a correct functioning of the circadian system is crucial for maintaining optimal health,[4] disruption of circadian rhythms, for example through improper or insufficient exposure to sunlight, may increase the predisposition to develop pathological conditions, including major depressive disorder and seasonal affective disorder.[5-8] Numerous daily rhythms, such as the sleep/wake cycle, hormonal and neurotransmitters' daily oscillations are clearly disrupted in depression.[9] Although the mechanisms underlying the impact of circadian disruption on mood are still unclear, evidence suggests that among the neurotransmitters involved in depression, serotonin (5HT) may play a crucial role.[10,11] Indeed, serotonin occupies a central place in the pathophysiology of depression, as evidenced by antidepressants inhibiting 5HT reuptake, such as citalopram or fluoxetine.[12-14]

Moreover, tight relationships link the 5HT system, the circadian system and many rhythmic physiological functions. Serotonin regulates the sleep/wake cycle by inducing and maintaining wakefulness.[15] Besides, the 5HT system is also involved in the modulation of mood and locomotor activity.[16-18] Furthermore, 5HT plays a role in synchronizing the circadian system, since it is able to shift the phase of the SCN and modulate its synchronization to light.[19-23] In turn, the circadian system exerts its action on many nuclei in the brain, including the serotonergic raphe nuclei (RN). Indeed, both serotonin synthesis and release in the SCN display 24 h variations.[24-29] Evidence in nocturnal rodents suggests that, among the SCN outputs, rhythmic information may be conveyed to the 5HT system via glucocorticoids and locomotor activity.[30,31]

Indeed, behavioral activity correlates with serotonin levels both in nocturnal and diurnal species.[23,32] However, the distribution of rest/activity episodes in nocturnal and diurnal animals occurs at opposite phases relative to the light/dark cycle,[33,34] while the SCN is always active at the same astronomical time.[35-37] Moreover, recent evidence suggests that the effect of serotonin on the circadian system may be different between diurnal and nocturnal species.[23,38] Serotonin antagonizes the effect of light in nocturnal animals, while it strengthens light effect in diurnal species.[39,40]

The current knowledge about the functioning of the serotonergic system and its involvement in mood disorders stems principally from nocturnal models, while little is known about the serotonergic system in diurnal mammals. Considering the differential effects of serotonin in diurnal and nocturnal animals, it is crucial to investigate the functioning of the serotonergic system in a diurnal animal in order to guide studies in humans.

In this study, to gain more insights into the 5HT system in diurnal mammals we used a diurnal rodent, the *Arvicanthis ansorgei*. This species displays a diurnal behavioral phenotype with a crepuscular (or bimodal) pattern of general locomotor activity, with activity peaks at the light/dark and dark/light transitions. We characterized the anatomical extension of the RN and we evaluated serotonin synthesis by quantifying tryptophan hydroxylase 2 (*tph2*) mRNA levels in RN under 12 h:12 h light-dark (LD) cycle and in constant darkness (DD). Furthermore, we have measured SCN contents of serotonin and its main metabolite, 5-hydroxyindoleacetic acid (5HIAA), and we have analyzed several SCN rhythmic outputs as potential modulators of *tph2* expression profile.

This study may help understanding mechanisms that contribute to the phase-inverted outputs of the circadian system between nocturnal and diurnal mammals, so that this knowledge may be more properly translated to humans.

2 | MATERIALS AND METHODS

2.1 | Animals and housing conditions

All experiments were performed in accordance with the guidelines of the European Committee Council Directive of November 24, 1986 (86/609/EEC) and the French Ministry of Higher Education and Research. Adult (5.5 to 10 months of age) male Sudanian grass rats (*A. ansorgei*), weighting 150–250 g, were obtained from our breeding colony (Chronobiotron, UMS 3415, CNRS Strasbourg, France). All the animals were individually housed in Plexiglas cages, raised in 12 h:12 h light/dark (12:12 LD) cycle (light: 150–200 lux, dark: red dim light, <5 lux), and provided with food and water ad libitum. The general locomotor activity of 17 grass rats was recorded every 5 min by a computer-based acquisition system (Circadian Activity Monitoring System; INSERM, Lyon, France) during 4 consecutive days and actograms were analyzed using Clocklab software (Actimetrics, Wilmette, IL, USA).

2.2 | Experimental protocol and samples collection

A total of 84 *Arvicanthis* were entrained to 12:12 LD cycle. Two days before the sacrifice, 42 *Arvicanthis* were exposed to constant darkness (DD), while 42 were maintained in LD. In both conditions, animals were killed by decapitation, after CO_2 sedation, every 4 h (seven time points) starting at *Zeitgeber* time (ZT) 2 for the LD group and at circadian time (CT) 2, for the DD group. The last time point is at 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. Trunk blood was collected in hemolysis tubes containing 30 μl of 4% EDTA. Brains were quickly removed and frozen in cold isopentane (−30°C) for 1 min 20 s. Plasma samples, resulting from blood centrifugation (15 min, 4000 *g*, 4°C) were stored at −20°C, while brains were stored at −80°C. Serial coronal 20 μm sections were cut along the caudo-rostral extension of the RN with a cryostat (Leica Instruments GmbH, Nussloch, Germany) and collected on sterile slides. The distance between two collected sections was 100 μm. Since no atlas is available for the *Arvicanthis*, the rat atlas was used as reference (Interaural from +1.9 mm to −0.7 mm, Paxinos & Watson[41]) to morphologically identify the DRN and the MRN. Slides were stored at −20°C until use. For 5HT and 5HIAA quantification, 12 *Arvicanthis* were kept in a 12:12 LD cycle and sacrificed at ZT2, ZT10 and ZT18, while 12 *Arvicanthis* were exposed for 48 h to DD before being sacrificed at CT2, CT10 and CT18 (four animals per group). Brains were quickly removed and frozen in cold isopentane, and subsequently stored at −80°C. SCN tissue was microdissected from four consecutive coronal sections (300 μm thickness, 2 mm diameter), using the optic chiasm (OC) as landmark. SCN tissue was stored at −80°C until tissue homogenization.

2.3 | Radioactive in situ hybridization

The sequences for sense and antisense riboprobes were designed based on the rat *tph2* mRNA sequence and were obtained as described by Malek et al.[26] The *tph2* sequence identity was analyzed on the *Arvicanthis niloticus* (taxid:61156) genome using Nucleotide BLAST (https://blast-ncbi-nlm-nih-gov.scd-rproxy.u-strasbg.fr/Blast.cgi), since *A. ansorgei* genome sequence is not available. Probes (495 bp) were transcribed from the corresponding linearized plasmids using the appropriate polymerase (MAXI script; Ambion, Austin, TX) in the presence of [^{35}S]UTP (46.25 GBq/mol, PerkinElmer, Waltham MA). Hybridization with riboprobes on *Arvicanthis* brain slices was performed as follows. Sections were postfixed in 4% formaldehyde for 15 min, rinsed for 2 min in 0.1 M phosphate buffer saline (PBS), acetylated twice for 5 min in 0.4% acetic anhydride in 0.1 M triethanolamine (pH 8.0), rinsed for 2 min in 0.1 M PBS, dehydrated in graded ethanol series, and air dried. Hybridization was carried out by deposing 80 μl of riboprobes (400 pm) in a solution containing 50% deionized formamide, 2X sodium saline citrate (SSC), 1X Denhardt's solution, 0.25 mg/ml yeast totalRNA, 1 mg/ml salmon sperm DNA, 10% dextran sulfate, and 400 mM dithiothreitol. Sections were placed in humid boxes containing 2X SSC/50% formamide at 54°C overnight. After hybridization, the sections were washed for 5 min in 2X SSC and then treated with ribonuclease A (10 μg/μl; Sigma St. Quentin Fallavier, France) for 30 min at 37°C. Stringency washes were performed in 1X SSC for 5 min at room temperature (RT) and then in 0.05X SSC for 30 min at 52°C. Sections were finally dehydrated in graded ethanol series, air dried at RT and then exposed to an autoradiographic film (Kodak BioMax; Kodak, Rochester, NY), with a ^{14}C standard.

2.4 | HPLC measurement of 5HT and 5HIAA SCN levels

SCN punches were homogenized by sonication in 0.1 mM Ascorbic Acid and centrifuged at 20.000 RCF. Twenty pmol of D4-serotonin and 30 pmol of D5-hydroxyindoleacetic acid (5HIAA) internal standards were added to all samples before sample treatment. Four volumes of ice-cold acetonitrile (ACN) were added to all samples, which were then centrifuged at 20.000 RCF for 20 min at 4°C. The resulting supernatants were placed in the speed vacuum until samples were completely dry. Samples were then suspended in 0.1 M formic acid, centrifuged at 20.000 RCF for 20 min at 4°C, and the upper phase was recovered to perform LC-MS/MS analyses. Only for serotonin and D4-serotonin, the AccQTag derivation protocol was performed before adding ACN. Analyses were performed on a Dionex Ultimate 3000 High-Pressure Liquid Chromatography (HPLC) system (Thermo Scientific) coupled with a triple-quadrupole Endura (Thermo Scientific). The system was controlled by Xcalibur Software version 2.0 (Thermo Electron, Villebon Sur Yvette, France). Five μl of each sample was loaded into an Accucore RP-MS column (reference P.N. 8636600-902, Zorbax SB/C18 micro bore rapid resolution 1.0 × 150 mm 3.5 μm, Agilent) heated at 40°C. All molecules and the deuterated compounds were eluted by applying a gradient of buffer A and buffer B. Buffer A was 1% acetonitrile (ACN)/98.9% H_2O/0.1% formic acid (v/v/v), whereas buffer B was 99.9% ACN/0.1% formic acid (v/v). The following multi-step

gradient was applied: a linear gradient of 0%–20% of solvent B at 0.09 ml/min during 6.5 min, followed by a linear gradient of 20%–98% of solvent B for 2 min, 1 min at 95% of solvent B and a linear gradient of 0.5 min of 98%–0% of buffer B. Qualitative analysis and quantification was performed in the multiple reaction monitoring (MRM) mode, using Quan Browser software (Thermo Scientific). For ionization, 3500 V of liquid junction voltage and 297°C capillary temperature was applied. The selectivity for both Q1 and Q3 was set to 0.4 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 (CE) were first determined. The transitions and the corresponding CEs used for MRM mode were the following: 347.13 charge/mass ratio (m/z) → m/z 171 (CE = 27.55 eV) for 5-HT; m/z 351.16 → m/z 171 (CE = 28.1 eV) for D4-serotonin; m/z 192.12 → m/z 118.11 (CE = 28.65 eV), m/z 91.17 (CE = 34.88 eV), and m/z 146.06 (CE = 14.55 eV) for 5-HIAA; and m/z 197.14 → m/z 122.11 (CE = 29.1 eV), m/z 150.04 (CE = 15.61 eV), and m/z 151.07 (CE = 15.91 eV) for D5–5-HIAA. Identification was based on precursor ions, daughter ions and retention times. Quantification was performed calculating the ratio of daughter ions response areas of the internal standards. Data were normalized to grams of proteins.

2.5 | HPLC measurement of corticosterone plasma levels

For corticosterone quantification, *Arvicanthis* samples were processed as follows. Fifty pmol of D4-corticosterone were added to 50 μl of each plasma sample. After adding 1 ml of ice-cold acetonitrile (ACN), samples were incubated at 4°C for 70 min and then centrifuged at 20.000 RCF for 20 min at 4°C. The upper phase was recovered, and a step of centrifugation was repeated in the same conditions. The resulting supernatants were placed in the speed vacuum until samples were completely dry. Samples were subsequently suspended in 20% ACN/0.1% formic acid (v/v), before to perform LC-MS/MS analyses. Analyses were performed on a Dionex Ultimate 3000 High-Pressure Liquid Chromatography system (Thermo Scientific) coupled with a triple-quadrupole Endura (Thermo Scientific). The system was controlled by Xcalibur Software version 2.0 (Thermo Scientific). Samples were loaded into an Accucore RP-MS column (reference P.N. 8636600-902, Zorbax SB/C18 micro bore rapid resolution 1.0 × 150 mm 3.5 μm, Agilent) heated at 40°C. Corticosterone and its deuterated compound were eluted by applying a gradient of buffer A and buffer B. Buffer A was 1% ACN/98.9% H_2O/0.1% formic acid (v/v/v), whereas buffer B was 99.9% ACN/0.1% formic acid (v/v). The following multi-step gradient was applied: a linear gradient of 0%–25% of solvent B at 0.09 ml/min during 2 min, followed by a linear gradient of 25%–30% of solvent B for 7 min, a linear gradient of 30%–98% of solvent B for 2 min, 2 min at 95% of solvent B and a linear gradient of 1 min of 98%–0% of buffer B. Qualitative analysis and quantification was performed in the MRM mode, using Quan Browser software (Thermo Scientific). For ionization, 3500 V of liquid junction voltage and 297°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 (CE) were first determined. The transitions and the corresponding CEs used for MRM mode were the following: m/z 347.11 → m/z 293.47 (CE = 17.03 eV), m/z 311.29 (CE = 15.92 eV), and m/z 329.17 (CE = 14.95 eV) for Corticosterone; and m/z 351.18 → m/z 297.10 (CE = 17.69 eV), m/z 315.18 (CE = 16.88 eV), and m/z 333.24 (CE = 15.56 eV) for D5–corticosterone. Identification was based on precursor ions, daughter ions and retention times. Quantification was performed by calculating the ratio of daughter ions response areas of the internal standards. Data were normalized to milliliters of plasma.

2.6 | Radioimmunoassay of melatonin plasma levels

Melatonin plasma concentrations were measured using a melatonin radioimmunoassay (RIA) kit (Bühlmann Laboratories AG, Schönenbuch, Switzerland) by a double-antibody based on the Kennaway G280 anti-melatonin antibody (rabbit polyclonal antibody). Reversed-phase column extracted samples and controls and reconstituted calibrators were incubated with the anti-melatonin antibody and 125I-melatonin. The sensitivity of the method is 0.3 pg/ml, the intra-assay variability less than 7.9% and inter-assay variability 8.2%. Samples were extracted on columns after conditioning with 2 × 1 ml of methanol and 2 × 1 ml of water. Sample was loaded on column and washed twice with 1 ml 10% (v/v) methanol, and 1 ml of hexane. Melatonin was eluted in 1 ml of methanol. Samples were evaporated to dryness and subsequently reconstituted in 1 ml of incubation buffer. Samples were split and run in duplicates. 100 μl of antiserum and 100 μl of tracer were added to samples (400 μl), and incubated 20 h at 4°C. After incubation, 100 μl of second antibody were added and samples were incubated 15 min at 4°C. One ml of deionized water was added and samples were centrifuged at 2000 *g* for 2 min at 4°C. The supernatant was aspirated, and radioactivity was counted in a Gamma

counter. Five standards were included: A 0.5 pg/ml, B 1.5 pg/ml, C 5.0 pg/ml, D 15.0 pg/ml, E 50.0 pg/ml.

2.7 | Quantitative analyses of *tph2* mRNA

Quantitative analyses of the autoradiograms were performed by using NIH ImageJ software. Specificity of the antisense probe was tested by hybridization of the antisense and sense probes in the *Arvicanthis* and rat raphe, and in the *Arvicanthis* locus coeruleus (LC) as shown in Figure 1. In *Arvicanthis*, *tph2* hybridization allowed accurate identification of the DRN and MRN. Furthermore, subregions of the DRN can be distinguished: ventromedial (VM), dorsomedial (DM) and two lateral (Lat) groups (Figure 2). Six 20 μm coronal sections (distance between two sections = 100 μm) along the caudo-rostral extension of the median part of the DRN and the MRN (Figure 2), were analyzed. For each section, the total optical density (OD) was measured in each subregion of the DRN and in the MRN, and the specific signal intensity was calculated by subtracting the non-specific OD, measured in the mesencephalic surrounding area, where the signal was not specific. OD was normalized to relative levels of mRNA, using a ^{14}C radioactive scale (KBq/g).

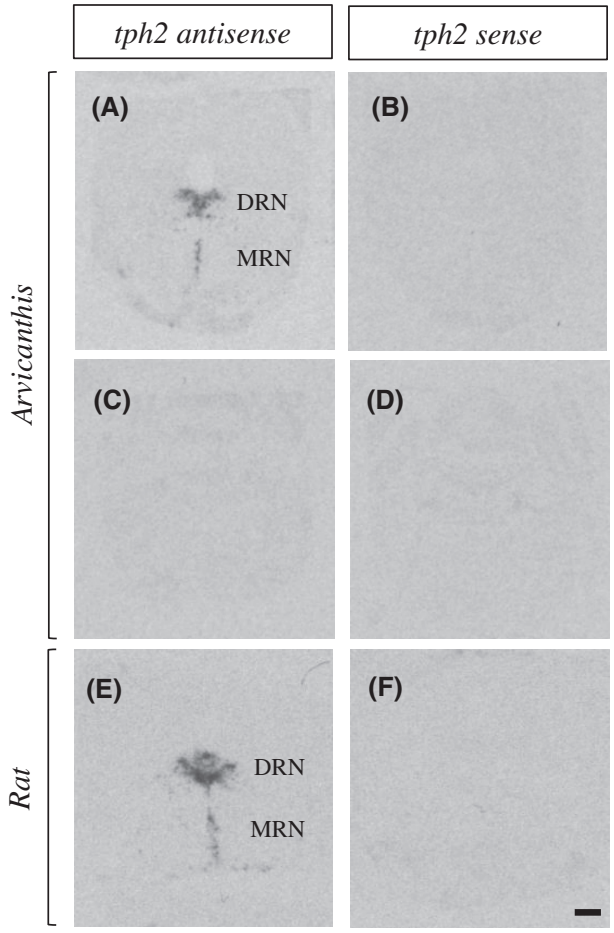

FIGURE 1 Riboprobe quality control. In situ hybridization performed on coronal sections of *Arvicanthis* (A, B, C and D) and rat (E and F) using *tph2* riboprobes antisense (A, C and E) and sense (B, D and F). *Tph2* antisense riboprobe labels the dorsal raphe nucleus (DRN) and the median raphe nucleus (MRN) in *Arvicanthis* (A) and in rat (E). No specific labeling on the *Arvicanthis* locus coeruleus (LC) using *tph2* antisense riboprobe (C) and on the *Arvicanthis* DRN and MRN (B), LC (D), or rat DRN and MRN (F) using *tph2* sense riboprobe. Scale bar: 750 μm

2.8 | Statistical analyses

To test the distribution of the mRNA expression across the caudo-rostral extension of the *Arvicanthis* raphe among the seven time points, two-way analysis of variance (ANOVA II) was performed, with factors "time," "section" and their interaction. The effect of light condition (LD vs DD) was tested by the three-way analysis of variance (ANOVA III), with factors "time," "light condition," "section" and the interaction between "time" and "light condition." *Post-hoc* analyses were performed to examine the significant difference among time points and among sections. For HPLC data of 5HT, 5HIAA and 5HT/5HIAA ratio, differences among time points within the same light condition were analyzed using ANOVA I and multiple *t*-tests. Furthermore, differences between light conditions were analyzed using an ANOVA II, with factors "time," "light condition" and their interaction. Melatonin data were analyzed using an ANOVA II, with factors "time," "light condition" and their interaction. *Arvicanthis* corticosterone data were analyzed using an ANOVA II, with factors "time," "light condition" and their interaction. Data were fitted by a non-linear regression using Cosinor analysis (SigmaPlot software, Jandel Scientific, Chicago, IL, USA). These analyses were performed using the following equation: Bimodal (12 h) rhythm: $y = A + (B * \cos(2\pi(\times - C)/12))$; where A is the mean level (mesor), B the amplitude and, C the acrophase of the rhythm. Locomotor activity was recorded for 17 *Arvicanthis*, in LD over 4 days. For each animal, the average locomotor activity counts over each hour are converted in percentage of total counts. The effect of time on mean hourly data was tested by an ANOVA I and data were fitted by non-linear regression using Cosinor analysis (as previously described). For all statistical procedures, the level of significance was set at $p < .05$. All data are presented as mean ± SEM, unless otherwise stated. The following *Post-hoc* analyses were performed: Dunn's Method for ANOVA I, Bonferroni test for ANOVA II, and Holm-Sidak test for ANOVA III. Sigma Plot (v14) and SPSS (v22) software were used for statistical analyses.

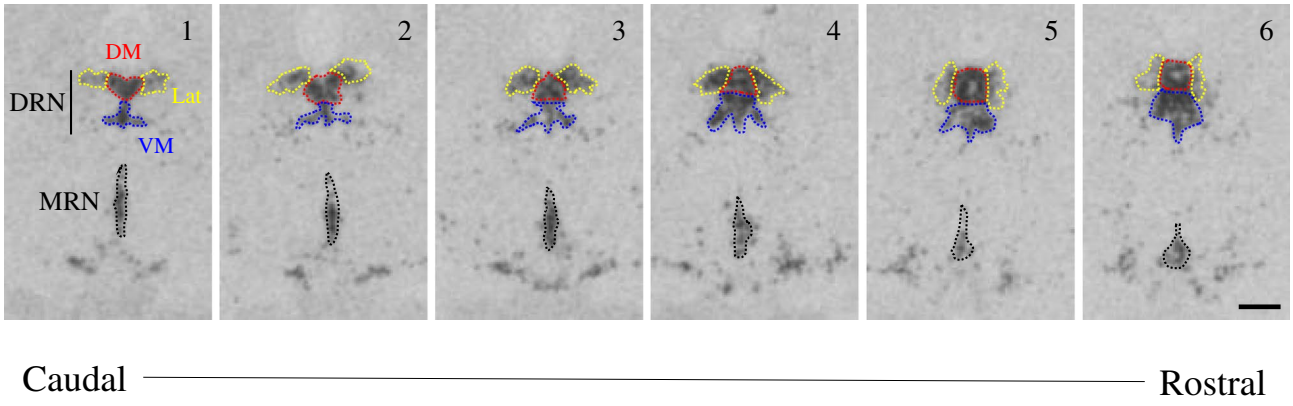

FIGURE 2 In situ hybridization of *tph2* riboprobe of *Arvicanthis* dorsal raphe nucleus (DRN) and median raphe nucleus (MRN, black dotted lines), and identification of DRN subdivisions into lateral (Lat, yellow dotted lines), dorsomedial (DM, red dotted lines) and ventromedial (VM, blue dotted lines) regions. Six successive sections are considered along the caudo-rostral raphe extension. Each section is 20 μm thick and the interval between two sections is 100 μm. Scale bars: 700 μm

3 | RESULTS

3.1 | Technical validation

The sequences for *tph2* riboprobes were designed based on the rat *tph2* mRNA sequence. Since the *A. ansorgei* genome is not available, *tph2* similarity was tested on the *A. niloticus* sequence. BLAST analysis revealed 93.94% identity with *A. niloticus* predicted *tph2* mRNA sequence. In situ hybridization of antisense riboprobe for *tph2* clearly labeled the *Arvicanthis* DRN and MRN (Figure 1A). Specificity of the probe was tested on coronal sections of the *Arvicanthis* locus coeruleus (LC), where no presence of *tph2* is expected. Indeed, no specific signal was detected on LC section (Figure 1C). Rat coronal section of DRN and MRN was used as positive control (Figure 1E). Hybridization of sense *tph2* riboprobe showed no specific signal neither in the *Arvicanthis*/rat DRN or MRN (Figure 1B,D), nor in the *Arvicanthis* LC (Figure 1F). Significant OD variations were measured in the RN, while non-specific OD measured in the mesencephalic surrounding area was constant.

3.2 | Anatomical organization of the *Arvicanthis* raphe nuclei

Raphe nuclei are characterized by different subregions, which in other species have been described as anatomically and functionally independent nuclei. It is still not known whether different raphe subgroups may be identified in *Arvicanthis*. Hybridization with *tph2* antisense riboprobe allowed an anatomical characterization of the *Arvicanthis* DRN and MRN. As shown in Figure 2, three subregions of DRN were delineated: dorsomedial (DM), ventromedial (VM) and two lateral (LAT) groups. We identified these subgroups in six successive 20 μm sections along the caudo-rostral extension of the DRN. In the same sections, the MRN was identified ventrally to the DRN.

3.3 | *tph2* mRNA profile within the *Arvicanthis* raphe nuclei

Tph2 mRNA expression was quantified in DRN subdivisions and in MRN every 4 h for seven time points, in LD and DD conditions (Figure 3). In LD, a significant effect of time on *tph2* expression is observed in all the three subregions of the DRN (ANOVA II; Lat: $p < .001$, DM: $p = .003$, VM: $p < .001$), and in the MRN (ANOVA II; $p = .049$), as shown in Figure 3A. Overall, *Post-hoc* analyses revealed two higher time points of *tph2* mRNA levels close to the light transitions at ZT10 and at ZT2, while two lower time points are observed at ZT6 and at ZT22. Indeed, significant decreases in *tph2* levels is measured at ZT6 versus ZT2 and versus ZT10 and at ZT22 versus ZT2 and versus ZT10 (Table S1). In DD as well, time had a significant effect on *tph2* mRNA levels in Lat, DM, VM and in MRN (ANOVA II; $p < .001$, $p = .003$, $p < .001$, $p < .001$, respectively), as shown in Figure 3B. Higher *tph2* expression is observed at CT22, while lower expression is measured at CT18, CT6 and CT2′ (Table S1). Cosinor analyses showed a significant bimodal fit of *tph2* mRNA expression in the Lat DRN and MRN under DD conditions (Table S2). The peak values are at CT10.9 and CT22.9 in Lat DRN, and at CT10.8 and CT22.8 in MRN. In addition, a different *tph2* expression profile is observed between the LD and DD conditions. In all the four structures, *Post-hoc* analyses show that

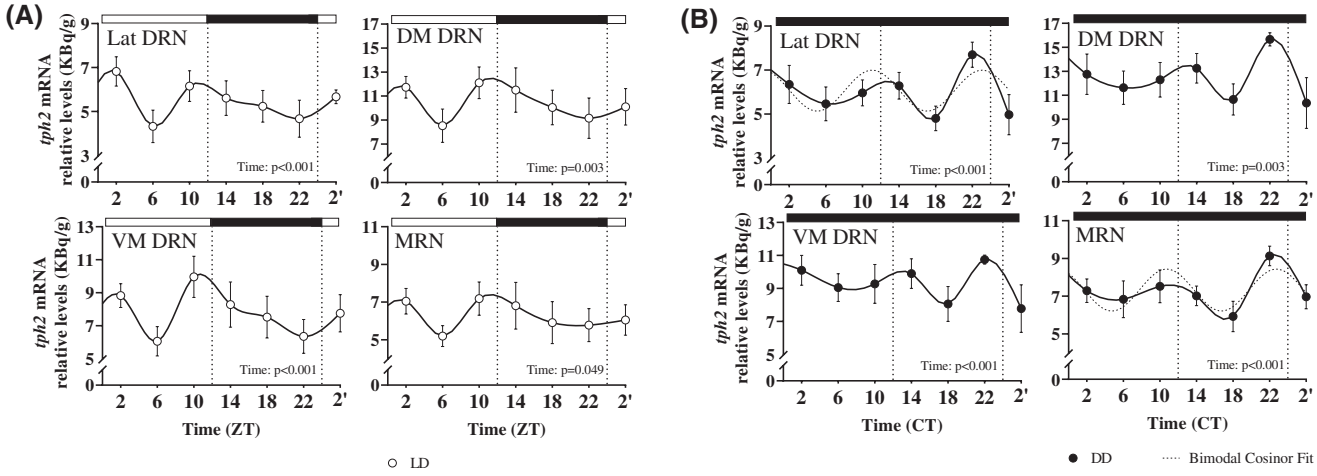

FIGURE 3 In situ hybridization measurements of *tph2* mRNA relative levels within the lateral (Lat), dorsomedial (DM), ventromedial (VM) subregions of the dorsal raphe nucleus (DRN) and in the median raphe nucleus (MRN) in *Arvicanthis* housed in 12 h:12 h light/dark (LD, open circles) (A) and constant darkness (DD, filled circles) (B) conditions. Points are connected by a smoothing line. The effect of time on *tph2* expression is significant in all conditions. Dashed line in Lat DRN and MRN (B) represents the bimodal Cosinor fit. Experimental groups were all $n \geq 5$, except for MRN at CT2′ ($n = 4$). Data are presented as mean ± SEM. Time is expressed as *Zeitgeber* time (ZT) or circadian time (CT). White and black horizontal bars represent light and dark phases, respectively

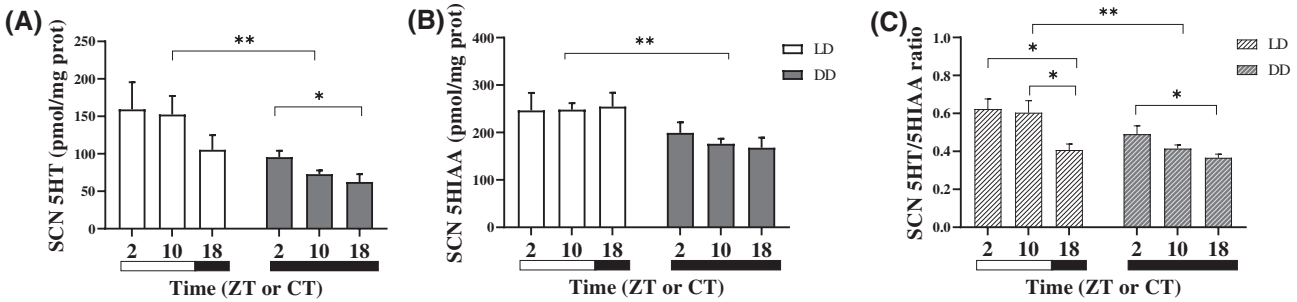

FIGURE 4 Serotonin (5HT, A) and 5-hydroxyindolacetic acid (5HIAA, B) concentration in the SCN of *Arvicanthis* measured by HPLC in LD (white bars) and DD (gray bars). 5HT/5HIAA ratio (C) under LD (white striped bars) and DD (gray striped bars) conditions. Significant effect of time and of light condition is represented with one asterisk (*) when $p < .05$ and by two asterisks (**) when $p < .01$. Data are presented as mean ± SEM. Experimental groups were all $n = 4$, except for CT2 ($n = 5$). Time is expressed as *Zeitgeber* time (ZT) or circadian time (CT). White and black horizontal bars represent light and dark phases, respectively

the differences between the two profiles is significant at the time points ZT6 versus CT6 (Holm-Sidak test; Lat: $p = .045$; DM: $p = .004$; VM: $p < .001$; MRN: $p = .015$), and ZT22 versus CT22 (Holm-Sidak test; Lat: $p < .001$; DM: $p < .001$; VM: $p < .001$; MRN: $p < .001$).

3.4 | 5HT and 5HIAA levels in the *Arvicanthis* SCN area in LD and DD

Serotonin (5HT) and its main metabolite 5-hydroxyindoleacetic acid (5HIAA) were quantified by HPLC in the SCN of *Arvicanthis* sacrificed at three time points across the LD cycle (ZT2, ZT10, ZT18), and three time points in constant darkness (CT2, CT10, CT18). We observed a significant effect of time on 5HT concentration (ANOVA I; $p = .048$) under DD cycle, with higher levels at CT2 compared to CT18 (*t*-test; $p = .042$) (Figure 4A). In addition, a significant effect of time on 5HT/5HIAA ratio in LD (Figure 4C, ANOVA I; $p = .028$) is observed, with higher ratio at ZT2 and ZT10 compared to ZT18 (*t*-test: $p = .013$ and $p = .033$, respectively). In DD, a significant higher 5HT/5HIAA ratio is measured at CT2 compared to CT18 (*t*-test; $p = .047$). Significant lower concentrations of both 5HT (Figure 4A, ANOVA II; $p = .001$) and 5HIAA (Figure 4B, ANOVA II; $p = .003$), as well as a lower 5HT/5HIAA ratio (Figure 4C, ANOVA II; $p = .003$), were measured in DD compared to LD cycle.

3.5 | Plasma melatonin levels of *Arvicanthis* housed in LD and DD

Melatonin is one of the main outputs of the SCN, and a strong indicator of the circadian phase. Plasma melatonin concentration was measured by radioimmunoassay in LD and DD conditions. In both light conditions, melatonin levels significantly changed over time (Figure 5, ANOVA II; $p < .001$), with low levels during the (subjective) day and a peak at ZT or CT22 ($p < .001$ compared with all the other time points). No significant difference is measured between the LD and DD melatonin profiles (ANOVA II; light condition: $p = .089$, interaction: $p = .989$).

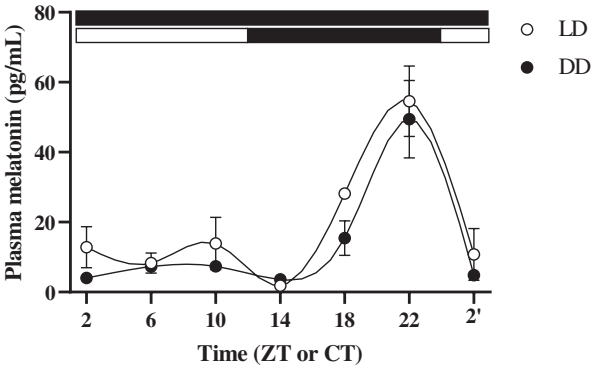

FIGURE 5 Melatonin plasma levels measured by radioimmunoassay. ANOVA II reveals a significant effect ($p < .001$) of the time in both LD (open circles) and DD (filled circles) conditions. Points are connected by a smoothing line. Data are presented as mean ± SEM. All experimental groups were $n \geq 5$, except for ZT6 ($n = 2$), ZT14 ($n = 3$) ZT18 ($n = 4$) and ZT22 ($n = 3$). Time is expressed as *Zeitgeber* time (ZT) or circadian time (CT). White and black horizontal bars represent light and dark phases, respectively

3.6 | Plasma corticosterone levels of *Arvicanthis* housed in LD and DD

As shown in Figure 6A,B, plasma levels of corticosterone significantly varied over time, both in LD (ANOVA I; $p = .017$) and DD (ANOVA I; $p = .004$) conditions. A significant increase at ZT22 was measured in LD compared to ZT18, and at CT22 in DD compared to CT18 and CT14. Cosinor analyses revealed a significant bimodal rhythm over 24 h both in LD ($p < .001$, $R^2 = .312$; Figure 6A), and in DD ($p < .001$, $R^2 = .266$, Figure 6B). The two peaks of corticosterone are observed at ZT10.5 and ZT22.5 in LD, and at CT9.5 and CT21.5 in DD. ANOVA II did not show any significant difference between LD and DD corticosterone profiles (light condition: $p = .633$, interactions: $p = .572$).

3.7 | General locomotor activity of *Arvicanthis* in LD

Locomotor activity data were analyzed for seventeen of the *Arvicanthis* housed in LD condition. All the animals were more active during the day than during the night. Indeed, 71.6% ± 1.8% of the activity occurred during the day and 28.9% ± 1.8% during the night (ANOVA I; $p < .001$, Figure 7A). Sixteen *Arvicanthis* showed a bimodal pattern of activity, with higher percentage of the activity occurring at the light/dark and dark/light transitions. One *Arvicanthis* had a unimodal locomotor activity profile, with higher activity occurring throughout the light phase (data not shown). Figure 7B shows the mean daily locomotor activity profile, expressed as hourly percentage of counts. The effect of time is significant (ANOVA I; $p < .001$), and data are significantly fitted by a bimodal Cosinor regression ($p < .0001$, $R^2 = .332$; Figure 7B). The two peaks of activity

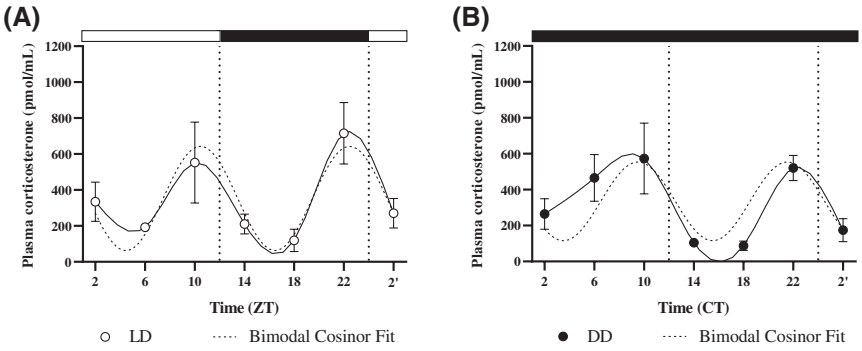

FIGURE 6 *Arvicanthis* plasma corticosterone levels measured by HPLC, in LD (A, open circles) and DD (B, filled circles). Points are connected by a smoothing line. ANOVA I analysis indicate a significant effect of time in LD ($p < .05$) and DD ($p < .01$). Dashed line represents the bimodal Cosinor fit in LD (A) and DD (B). Experimental groups were all $n = 6$, except for ZT6 ($n = 5$). Data are presented as mean ± SEM. Time is expressed as *Zeitgeber* time (ZT) or circadian time (CT). White and black horizontal bars represent light and dark phases, respectively

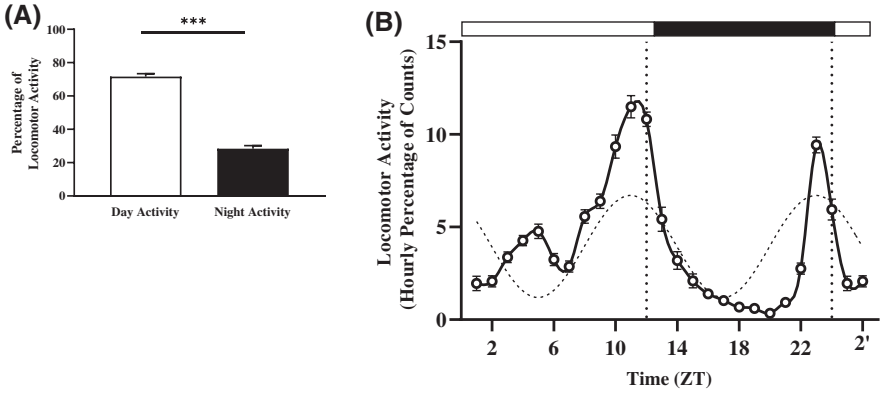

FIGURE 7 In panel A, percentage distribution of *Arvicanthis* locomotor activity between light (day activity, white bar) and dark (night activity, black bar) phases. Bars represent mean + SEM ($n = 17$, recording days = 4). Significance is expressed by three asterisks (***$p < .001$). In panel B, locomotor activity profile of *Arvicanthis* maintained in 12 h:12 h light/dark condition, plotted as hourly percentage of counts. Points are connected by a smoothing line. Points represent mean + SEM ($n = 17$, recording days = 4). The effect of time is significant ($p < .001$). Hourly data (open circles) are fitted by a bimodal Cosinor regression (dashed line). Time is expressed as *Zeitgeber* time (ZT) or circadian time (CT). White and black horizontal bars represent light and dark phases, respectively

are at ZT11.2 and ZT23.2, both occurring right after the peaks in corticosterone (ZT10.5 and ZT22.5).

4 | DISCUSSION

In the present study, we investigated serotonin synthesis in the diurnal rodent *Arvicanthis* by looking at serotonin rate-limiting enzyme, *tph2*. *Tph2* mRNA levels display a rhythmic bimodal profile over 24 h under both LD and DD conditions, showing that *tph2* rhythm persists in constant condition and is therefore endogenously regulated. Under both LD and DD cycles, higher levels of expression occurred around the (projected) light transitions. Of note, the *tph2* profiles observed in DD differed from that in LD. This finding suggests that light has an effect on serotonin synthesis, since it modifies the pattern of *tph2* endogenous rhythm. Among the SCN outputs studied, a bimodal profile was observed in corticosterone plasma levels in both LD and DD conditions, as well as in locomotor activity, which reflected *tph2* patterns. On the other hand, melatonin plasma rhythms in LD and DD were always unimodal with a peak during the night.

4.1 | Technical and anatomical consideration of *tph2* expression in *Arvicanthis*

Two isoforms of the rate-limiting enzyme of serotonin biosynthesis, TPH1 and TPH2, have been described in mammals.[42] The TPH2 isoform is predominant in the neurons and that of TPH1 in the peripheral organs, including the pineal gland.[26] In this study, we investigated *tph2* expression levels in the diurnal *Arvicanthis* using radioactive antisense riboprobe designed upon the rat *tph2* sequence to perform in situ hybridization in the RN. As *A. ansorgei* genome sequence has not been published yet, the similarity of our *tph2* riboprobe was tested on the *A. niloticus* genome, a species belonging to the same genus (https://www.ncbi.nlm.nih.gov/genome/annotation_euk/Arvicanthis_niloticus/100/). The high percentage (93.94%) of identity with the *tph2* predicted mRNA sequence demonstrated that it is suitable to detect *Arvicanthis tph2*. Specificity of the probe was confirmed by the absence of labelling in areas outside the RN and inside the RN when using the sense riboprobe. In addition, as was observed in rats,[26] hamsters[28] and mice,[43] *tph2* hybridization signals in *Arvicanthis* superimpose 5HT labelling, as previously shown by Adidharma and collegues.[44] Riboprobe hybridization allowed us to identify the anatomical extension of the RN in *Arvicanthis*. The architectural organisation of the RN has been previously described in other mammals (e.g., rats,[27] mice,[43] hamsters[28]), in which rostral, medial, and caudal parts of the DRN are delineated. In *Arvicanthis*, we distinguished the dorsal from the median raphe nuclei, and we considered the medial part of the DRN. We identified as medial DRN the portion of the DRN where we could distinguish the lateral, dorsomedial, and ventromedial subdivisions. We quantified *tph2* expression in these subgroups separately. Indeed, as previously shown in other mammals, these subregions may be considered as independent nuclei in accordance with receptor expression, electrophysiological properties, molecular organization and different afferent and efferent projections.[45-50] Furthermore, the RN subregions may be differently involved in the pathophysiology of depression, based on their sensitivity to anxiety and stress signals.[45]

4.2 | Rhythmic expression of *tph2* mRNA in the raphe nuclei of *Arvicanthis*

In *Arvicanthis*, serotonin synthesis is characterized by a bimodal rhythm. Within a given light condition (LD or DD), *tph2* expression shows similar profiles in all the RN structures, with higher levels around the light transitions. Under DD condition, *tph2* bimodality is evidenced by a 12 h-Cosinor fit in the Lat DRN and the MRN. Interestingly, among the RN subregions, those two are the structures more directly involved in the regulation of circadian rhythms. The MRN directly projects to the SCN,[51,52] while the two Lat DRN project to the IGL,[51] which in turn sends projections to the SCN.[53] Under the LD cycle, the bimodal expression of *tph2* mRNA levels persists. However, the *tph2* patterns differ between the LD and DD conditions.

The origin of *tph2* bimodality remains unclear as well as the mechanisms leading to different *tph2* profiles in LD vs DD. One hypothesis is that peaks of *tph2* expression occur at the transitions between (subjective) day/night and (subjective) night/day in both LD and DD conditions, but that in DD the whole curve is advanced. As shown in rats,[31] in *Arvicanthis tph2* expression could be driven by corticosterone (bimodal) rhythm. Although the overall phase of corticosterone rhythm does not change in DD compared to LD, Cosinor fit shows peaks in DD at CT9.5 and CT21.5, that is, 1 h in advance compared to the respective peaks in LD at ZT10.5 and ZT22.5. It is possible that, if the whole DD profile is advanced, it is partly due to corticosterone signals. Furthermore, locomotor activity may affect *tph2* levels.[31] In this study, locomotor activity data in DD are not available, but it cannot be excluded that locomotor activity peaks in DD are phase advanced as well. In an unpublished study, the period of locomotor activity in DD in *A. ansorgei* is shorter than 24 h. Therefore, it is possible that locomotor activity peaks are phase advanced after 2 days in DD. Finally, other light-dependent mechanisms may have an impact on *tph2* expression. Light influences the 5HT system not only through the circadian system (e.g. via corticosterone rhythm), but also via other pathways.[31,44,54] While in many rodent species, the DRN receives light information through direct projections from the retina,[55-57] a similar direct projection has not been observed in *Arvicanthis*.[58] Thus, in *Arvicanthis* light information may be conveyed to the 5HT system through other structures of the arousal system. For instance, orexin neurons, which are activated by light in *A. niloticus* but not in mice,[44,58,59,60] may mediate differential effects on the DRN activity between LD and DD, leading to changes in 24 h profiles of *tph2* mRNA levels. This strong difference between the LD and DD *tph2* profiles in *Arvicanthis* is not observed in nocturnal rats, in which LD and DD *tph2* profiles are similar.[26] These data suggest that light may have a stronger impact on serotonin synthesis in diurnal than in nocturnal rodents, and underscore the importance of using diurnal models for many research questions involving arousal structures like the serotonergic system.

4.3 | Bimodal rhythmic profiles of plasma corticosterone and locomotor activity in *Arvicanthis*

Plasma levels of corticosterone in *Arvicanthis* display a rhythmic bimodal profile in LD condition, with two peaks at the light transitions, in agreement with Verhagen et al.[61] Higher corticosterone levels were found at the end of the light and of the dark phases. Furthermore, a similar bimodal profile was detected in DD condition, highlighting that corticosterone bimodal rhythm is endogenous. Indeed, many studies have shown that glucocorticoid rhythm is under the control of the SCN.[62-64] Glucocorticoids surge has an anticipatory effect on locomotor activity.[65,66] Coherently, in our study we observed that the two peaks of corticosterone plasma levels precede the two peaks of locomotor activity observed at the light/dark transitions in LD. *Arvicanthis* corticosterone profile strongly differs from what has been observed in many species, where only one peak is measured.[67-70]

4.4 | Daily and circadian melatonin plasma levels in *Arvicanthis*

Melatonin is one of the principal outputs of the SCN and affects many structures in the brain and in the periphery.[71,72] In this study, we investigated melatonin plasma profile in *Arvicanthis*: we measured low melatonin level during the biological day and a peak at the end of the night, similarly to what has been shown in many nocturnal species and in humans.[73-79] Our data are coherent with the melatonin profile previously described in the *Arvicanthis* pineal gland in LD condition.[80] In addition, we showed a similar rhythm in DD condition, confirming that the melatonin rhythm is endogenous. Our findings agree with previous studies and with the sleep promoting effect of melatonin in diurnal but not in nocturnal species.[81-83]

4.5 | 5HT and 5HIAA levels in SCN in *Arvicanthis*

To gain more insights about serotonin synthesis in relation to *Arvicanthis* behavior, we evaluated the 5HT and 5HIAA SCN content at two time points of the biological

day and at the middle of the biological night, in LD and DD conditions. In our study, under LD condition, the effect of time was not significant. However, in DD we observed higher 5HT levels at the beginning of the subjective day compared to the middle of the subjective night. These data suggest that rhythm of 5HT in the SCN of *Arvicanthis* is endogenous. Finally, both LD and DD 5HT/5HIAA ratios, used as index of serotonin neuronal activity, were higher during the day than during the night, in relation to *Arvicanthis* activity state,[84] in accordance with Cuesta and colleagues.[40] A similar correlation with arousal has been shown in nocturnal rodents, where 5HT and 5HIAA content and release in the SCN is rhythmic and presents a peak at the beginning of the night (onset of activity).[29,85,86] Serotonin and 5HIAA rhythms are therefore in antiphase between nocturnal and diurnal rodents.

In DD condition, mean levels of 5HT, 5HIAA and 5HT/5HIAA ratio are lower than in LD condition. These findings are in accordance with a previous publication in Drosophila, showing that constant darkness decreases serotonin levels in the brain.[87] Furthermore, it corroborates the strong effect that light may have on serotonin, as previously shown on *tph2* levels.

In the nocturnal rat, a temporal sequential relationship was demonstrated between the rhythmic patterns of *tph2* mRNA in raphe cell bodies, the TPH2 protein and the 5HT release within the SCN.[26,27,29] Although 5HT/5HIAA ratio in the SCN is higher during the *Arvicanthis* active phase (Ref. [40], present study), the 5HT pattern of release in this species is still not known. Further investigation is necessary to assess whether the bimodal rhythm in serotonin synthesis is associated to a bimodal 5HT release in the SCN in *Arvicanthis*, and to elucidate the mechanisms leading from a bimodal 5HT synthesis to a unimodal rhythm in 5HT and 5HIAA content in the SCN.[40]

5 | CONCLUSION AND PERSPECTIVES

In this study, we characterized for the first time the *tph2* anatomical extension of the dorsal and median raphe nuclei of the diurnal *Arvicanthis*. We observed a bimodal rhythmic expression of *tph2* mRNA levels, and we demonstrated that the nature of this rhythm is endogenous. We correlated this bimodal *tph2* profile to the crepuscular locomotor activity rhythm of our animals, and to the bimodal corticosterone plasma levels. However, further experiments should be performed to confirm modulatory effects of locomotor activity and corticosterone on *tph2* rhythm in *Arvicanthis*. Finally, 5HT/5HIAA ratio within the SCN of *Arvicanthis* is higher during the day (active phase) compared to the night (rest period).

Questions arise about the origin of bimodality in *Arvicanthis* and whether it directly depends on the SCN. According to the SCN outputs that we measured, the SCN rhythmic activity may be either bimodal (bimodal corticosterone rhythm) or unimodal (unimodal melatonin rhythm). This question could be potentially answered by electrical recording of the SCN in vitro.

Our data show that the circadian variations of the serotonergic system and the effect of light on its rhythms differ between nocturnal and diurnal rodents. These observations suggest that the serotonergic system may participate to the distinct temporal organization underlying nocturnality and diurnality. These findings in a diurnal mammal may be helpful for studies on human mood disorders, such as depression in which alteration of numerous biological rhythms including serotonin neurotransmission and sleep/wake cycles have been described.

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DISCLOSURES

The authors declared no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

AUTHOR CONTRIBUTIONS

Rosanna Caputo was involved in study design, data collection and interpretation, statistical analyses, and writing of the manuscript; Vincent-Joseph Poirel was involved in study design; Etienne Challet contributed to study conception and data interpretation; Johanna H. Meijer was involved in data interpretation; Sylvie Raison was involved in conception, design and coordination of the study, data interpretation and writing of the manuscript. All authors critically reviewed the manuscript and have approved the publication of this final version of the manuscript.

DATA AVAILABILITY STATEMENT

All raw data (autoradiographic films, actograms, HPLC and RIA assay data) that support the findings of this study are available on request from the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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			Lat DRN		DM DRN		VM DRN		MRN	
			P value	Difference (%)	P value	Difference (%)	P value	Difference (%)	P value	Difference (%)
LD	*ANOVA II*	*Time Effect*	**<0.001**		**0.003**		**<0.001**		**0.049**	
	Post Hoc Analyses	*ZT2 vs ZT6*	**<0.001**	**57.76**	0.052		**0.021**	**45.76**	0.258	
		ZT2 vs ZT22	**0.003**	**55.97**	0.212		**0.042**	**38.96**	1	
		ZT10 vs ZT6	**0.036**	**42.33**	**0.016**	**41.88**	**<0.001**	**64.62**	0.104	
		ZT10 vs ZT22	0.158		0.07		**<0.001**	**56.94**	0.746	
DD	*ANOVA II*	*Time Effect*	**<0.001**		0.003		<0.001		<0.001	
	Post Hoc Analyses	*CT22 vs CT6*	**0.002**	**40.92**	**0.005**	**34.63**	0.503		**0.009**	**33.55**
		CT22 vs CT18	**<0.001**	**60.15**	**<0.001**	**47.06**	**0.008**	**33.42**	**<0.001**	**54.14**
		CT22 vs CT10	**0.047**	**29.17**	**0.04**	**27.47**	1		0.259	
		CT22 vs CT2'	**<0.001**	**54.90**	**<0.001**	**51.19**	**0.002**	**38.22**	0.091	
		CT2 vs CT2'	0.225		0.429		**0.027**	**29.89**	1	

Table S1. Effect of time on *tph2* levels within the Arvicanthis dorsal raphe nucleus (DRN) subdivisions, namely lateral (Lat), dorsomedial (DM), ventromedial (VM) and in the median raphe nucleus (MRN), housed under 12h:12 light/dark (LD) cycle or in costant darkness (DD). Data ara analyzed using two-way ANOVA. Post hoc analyses are provided when significance between two time points is observed at least in one structure. Significant results are highlighted in bold.

	LD								DD							
	Lat DRN		DM DRN		VM DRN		MRN		Lat DRN		DM DRN		VM DRN		MRN	
	Best-fit values	P value	Best-fit values	P value	Best-fit values	P value	Best-fit values	P value	Best-fit values	P value	Best-fit values	P value	Best-fit values	P value	Best-fit values	P value
Bimodal Cosinor		<0.001		<0.001		<0.001		<0.001		**<0.001**		<0.001		<0.001		**<0.001**
Mesor	5.42	<0.001	10.33	<0.001	7.74	<0.001	6.24	<0.001	**5.92**	**<0.001**	12.36	<0.001	9.25	<0.001	**7.25**	**<0.001**
Amplitude	0.75	0.0628	1.08	0.1958	0.96	0.1558	0.66	0.1958	**0.93**	**0.0352**	1.57	0.0837	0.80	0.1913	**1.09**	**0.0144**
Acrophase	1.03	<0.001	0.48	<0.001	0.15	<0.001	0.3	<0.001	**10.89**	**<0.001**	10.70	<0.001	11.02	<0.001	**10.75**	**<0.001**

Table S2. Bimodal (12h) Cosinor analysis of *tph2* levels within *the Arvicanthis dorsal raphe nucleus (DRN) subdivisions, namely lateral (Lat), dorsomedial (DM), ventromedial (VM) and in the median raphe nucleus (MRN), housed under 12h:12 light/dark (LD) cycle or in costant darkness (DD).* The equation used is $y = A + (B * \cos(2\pi(x-C)/12))$, where A is the Mesor, B is the Amplitude and C is the Acrophase of the rhythm. The Cosinor regression is considered significant when every parameter of the regression has a $p<0.05$, and is highlighted in bold.

B2. CLOCK GENE EXPRESSION IN THE RAPHE NUCLEI DIFFERS BETWEEN THE NOCTURNAL RAT THE DIURNAL *ARVICANTHIS*

Version edited in form of article, but needs to be completed before submission.

Title

Clock gene expression in the raphe nuclei differs between the nocturnal rat and the diurnal *Arvicanthis*

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Abstract

The raphe nuclei (RN) are structures of the arousal system involved in inducing and maintain wakefulness. RN principal neurotransmitter, serotonin, plays a role in the control of circadian rhythms by modulating the phase of the SCN. The functioning of the RN is rhythmic in both the diurnal rodent *Arvicanthis* and to the nocturnal rat, at least at the level of serotonin synthesis. Itis not clear whether rhythmicity within the RN is due to output signals from the SCN, or to thepresence of a clock machinery within the RN. In this study, we investigated the latter hypothesis, assessing the expression of three clock genes, *Bmal1*, *Per1* and *Per2*, in the dorsal RN (DRN) and in the median RN (MRN). Clock gene mRNA levels displayed variation over time, showing different patterns between the rat and *Arvicanthis*. These data demonstrate the presence of a rhythmic clock machinery in the RN and suggest that the opposite RN functioning between nocturnal rats and diurnal *Arvicanthis* may be due to the phase opposition of clock gene expression.

Keywords

Rhythm, *Arvicanthis*, Rat, Diurnal, Nocturnal, Rodent, Raphe Nuclei, Clock Genes

Introduction

Independently of the temporal niche (day or night) occupied, all mammals possess an internal time-keeping system, which allow them to integrate external information and to predict environmental changes (Pittendrigh 1960). The interaction between the circadian system, orchestrated by the suprachiasmatic nucleus (SCN) of the hypothalamus, and structures of the arousal system, including the serotonergic raphe nuclei (RN), is responsible for the regulation of the sleep/wake cycle (Webb, Antle, and Mistlberger 2014). The temporal distribution of rest and wake episodes between the light and dark phases is opposite in nocturnal compared to diurnal rodents. While the SCN rhythmicity is similar in nocturnal and diurnal species (Challet 2007), recent data suggest that the rhythmic functioning of the RN differs between the diurnal *Arvicanthis* and the nocturnal rat. For example, serotonin content in raphe terminals, including the SCN is rhythmic both in nocturnal and diurnal species, but peak at opposite phases (Poncet, Denoroy, and Jouvet 1993; Cuesta et al. 2008). In nocturnal rats, tryptophan hydroxylase 2 (*Tph2*), serotonin rate-limiting enzyme, show circadian rhythms both at the protein (Malek, Pévet, and Raison 2004), and mRNA levels (Malek et al. 2005). We have recently shown that *tph2* mRNA levels are rhythmic in the diurnal *Arvicanthis ansorgei* as well, but the pattern of *tph2* expression differ from the rat, while it correlates with the animal's behavioral activity. The origin of rhythmic functioning of the RN is not clear. The SCN may convey circadian information to downstream areas *via* neuronal and/or endocrine messages. A direct connection from the SCN to the RN has not been described. Therefore, circadian signals may be conveyed to the RN either through indirect projections (Deurveilher and Semba 2005), or through SCN hormonal output signals, such as the glucocorticoids (Malek et al. 2007). Furthermore, the circadian functioning of the serotonergic system may depend on the clock machinery within the RN. At a molecular level, circadian rhythms are generated by a transcriptional translational feedback loop (TTFL), involving proteins/genes forming the positive loop (e.g. Bmal1, Clock) and proteins/genes forming the negative loop (e.g. *Per1, Per2, Cry1, Cry2*) (Ko and Takahashi 2006).

In order to test whether the RN may function as an autonomous oscillator, we investigated mRNA expression of three clock genes, namely *Bmal1, Per1, Per2*. Furthermore, we compared the pattern of these clock genes between the nocturnal rat and the diurnal *Arvicanthis*, in order to investigate whether the phase of clock gene expression may be opposite between nocturnal and diurnal rodents.

Materials & Methods

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 (5.5 to 7 months of age) male Sudanian grass rats (*Arvicanthisansorgei*) weighting 150-250g were obtained from our breeding colony, and adult (2.5 to 3 months of age) male Wistar rats weighting 260-400g, were purchased from Charles River Laboratories. All the animals were individually housed in Plexiglas cages, entrained to 12h light-12h dark (LD 12:12) cycle (light: 150 - 200 lux, dark: red dim light, <5 lux), and providedwith food and water ad libitum.

Experimental protocol and samples collection

Rat and *Arvicanthis* were kept in a 12:12 LD cycle and sacrificed at three time points, namely *Zeitgeber* time (ZT) 2, ZT10 and ZT18 (6 animals per group). ZT0 was defined as the beginning of the light phase. Rats were killed by decapitation, after CO_2 sedation, and *Arvicanthis* were killed by decapitation after intraperitoneal injection of a lethal dose (182 mg/Kg) of pentobarbital (Euthasol Vet). Brains were quickly removed and frozen in cold isopentane (-30 °C), and subsequently stored at -80°C. The dorsal raphe nucleus (DRN) and the median raphe nucleus (MRN) tissues were punched from six and three consecutive coronal sections (300 µm thickness, punch 2 mm diameter), respectively, using aqueduct as reference.). DRN and MRN tissues ware stored at -80 °C until tissue homogenization.

Real-time quantitative polymerase chain reaction

Rats and *Arvicanthis* DRN and MRN samples were homogenized using a 1 ml syringe and two needles (a 23 gauge needle and a 27 gauge needle). Total RNA was extracted using RNeasy Lipid Tissue Mini Kit (74804, QIAGEN) following the manufacturer's recommendations. RNA concentration and purity (A260/A280 ratio and A260/A230 ratio) were measured using a NanoDrop ND-1000 V 3.5 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). 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. Real-time PCR was performed using PowerUp™ SYBR™ Green Master Mix (A25776, Thermo Fisher Scientific) and run on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad) for rats and on the Applied Biosystems Real-Time PCR Instruments for *Arvicanthis*. Real-time PCR amplification was performed after 120 s ofdenature 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* (primers showed in Table1). Data were analyzed by usingthe 2-ΔΔCT method (Livak and Schmittgen 2001). Rat data were normalized using two housekeeping genes, *gapdh* and *36b4*, Arvicanthis data using *gapdh*. Changes in clock gene expression were referenced to the mean level expression of the three ZTs.

Gene	Species	Forward	Reverse	Reference
Bmal1	Rattus norvegicus	CAACCCATACACAGA AGCAAAC	ACAGATTCGGAGACA AAGAGGA	(Zhang et al. 2016)
Bmal1	Arvicanthis ansorgei	GACACTGAGAGGTGC CACCAA	CCATCTGCTGCCCTGA GAAT	(Bobu et al. 2013)
Per1	Rattus norvegicus	GCGTAAATGTGCCTCC TCTT	GACGTATCTTTCTTCG CCCC	IDT
Per1	Arvicanthis niloticus	CCACTGAGAGCAGCA AGAGTACA	CTGCTGCAGCCACTGG TAGA	(Bobu et al. 2013)
Per2	Rattus norvegicus	CACCCTGAAAAGAAA GTGCGA	CAACGCCAAGGAGCT CAAGT	(Mattam and Jagota 2014)
Per2	Arvicanthis ansorgei	TCACCGTAGGAGATCC GGAAT	TTTCTGCAACAGGTGC TTCCT	(Bobu et al. 2013)
Gapdh	Rattus norvegicus	GACAACTTTGGCATCG TGGA	ATGCAGGGATGATGTT CTGG	IDT
Gapdh	Arvicanthis niloticus	ATCCACTGGTGCTGCC AAG	CCGTTCAGCTCTGGGA TGAC	(Ikeno et al. 2016)
36B4	Rattus norvegicus	GTGCCTCACTCCATCA TCAA	TCCGACTCTTCCTTTG CTTC	IDT

Table 1. Primer Sequences used for analysis of clock gene expression. IDT: primers designed using the *Integrated DNA Technologies* tools.

Statistical analyses

Differences among time points were analyzed using one-way analysis of variance (ANOVA I). Differences between species were tested using an ANOVA II, with factors "time", "species", and their interactions, followed by *Post-hoc* analyses. Non-parametric tests were used in case of non-normal distribution of the data. For all statistical procedures, the level of significance

was set at p<0.05. All data are presented as mean±SEM, unless otherwise stated. Sigma Plot (v14) software was used for statistical analyses.

Results

Clock genes mRNA expression within the rat and Arvicanthis DRN

Gene expression of three core clock genes, namely *Bmal1, Per1 and Per2* was quantified at ZT2, ZT10 and ZT18 in both rat and *Arvicanthis* dorsal raphe nuclei (*Fig.1*).

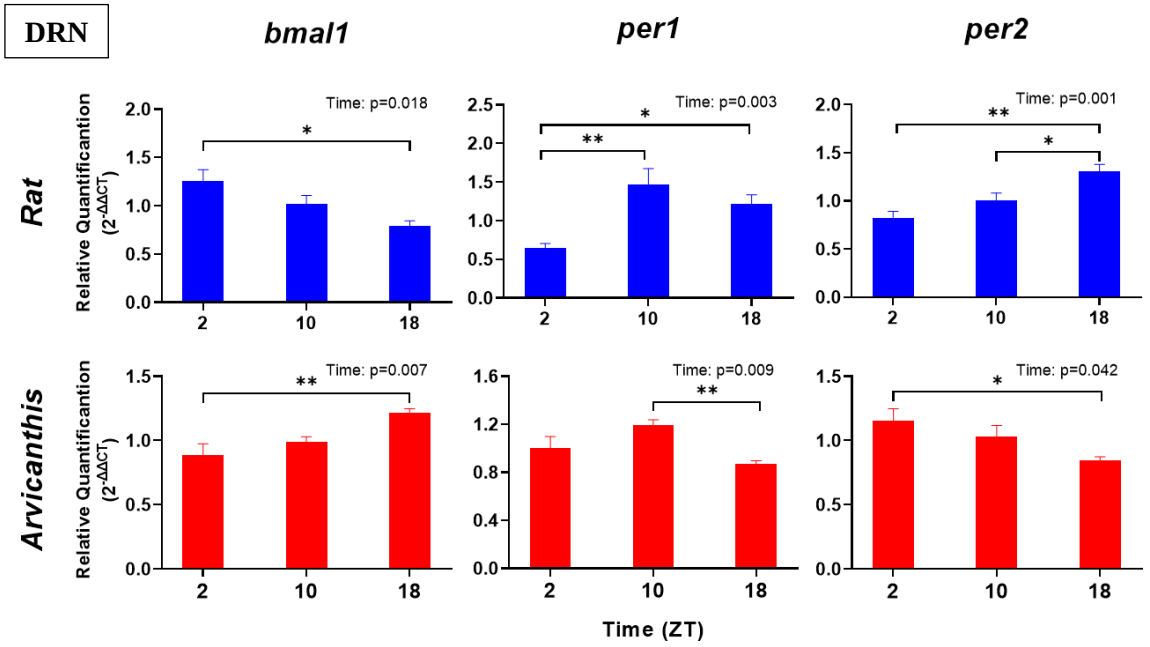

Fig. 1. qRT-PCR analyses of *bmal1*, *per1* and *per2* mRNA levels within the rat (blue bars) and *Arvicanthis* (red bars) dorsal raphe nucleus (DRN)z housed in 12:12 light-dark condition. Time is expressed as Zeitgeber Time (ZT). Experimental rat and *Arvicanthis* groups were all n≥5. The effect of time is significant for all the genes (ANOVA I; p>0.05). Post hoc tests show differences among the time points:p<0.05 (*), p<0.01 (**), p<0.001 (***). Interaction between time and species is significant for all genes(ANOVAII; p>0.05). Data are presented as mean+SEM.

The expression of all three clock genes displayed a significant variation over time both in rats (ANOVAI; *bmal1*: p=0.018; *per1*: p=0.003; *per2*: p=0.001) and in *Arvicanthis* (ANOVA I;

bmal1: p=0.007; *per1*: p=0.009; *per2*: p=0.042). In rats, higher *bmal1* mRNA levels were measured at ZT2 and lower levels at ZT18 (*post hoc* test; p=0.016), while it was the opposite in *Arvicanthis,* in which higher levels were measured at ZT18 and lower at ZT2 (*post hoc* test; p=0.008). The opposite pattern is observed for *per2,* showing higher levels at ZT18 and lower levels at ZT2 in rats (*post hoc* test; ZT18 vs ZT2: p=0.001; ZT18 vs ZT10: p=0.022), and higher levels at ZT2 and lower levels at ZT18 in *Arvicanthis* (*post hoc* test; ZT2 vs ZT18: p=0.42). The mRNA expression of *per1* was higher at ZT10 both in rats and *Arvicanthis*. However, the lower expression in rats was measured at ZT2 (*post hoc* test; ZT2 vs ZT10: p=0.003; ZT2 vs ZT18: p=0.030), while in Arvicanthis was at ZT18 (*post hoc* test; ZT10 vs ZT18: p=0.007). The clock gene mRNA variation over time was significantly different between rats and*Arvicanthis,* for all three genes (ANOVA II, interaction "time" and "species"; *bmal1*: p<0.001; *per1*: p=0.008; *per2*: p<0.001).

Clock genes mRNA expression within the rat and Arvicanthis MRN

Clock genes expression was measured at ZT2, ZT10 and ZT18 in the median raphe nucleus of rats and *Arvicanthis (Fig. 2).*

For all three genes, mRNA variation over time was significantly different between rats and *Arvicanthis* (ANOVA II, interaction "time" and "species"; *bmal1*: p=0.003; *per1*: p<0.001; *per2*: p<0.001).In the rat, the expression of all three clock genes displayed a significant variation over time (ANOVA I; *bmal1*: p=0.003; *per1*: p<0.001; *per2*: p<0.001). *Bmal1 mRNA* significantly decrease from ZT2 to ZT10 (*post hoc* test; p=0.002). The two clock genes of the negative loop, *per1* and *per2* displayed an opposite pattern to *bmal1*, with lower expression at ZT2, which significantly increase at ZT10 (*post hoc* test; *per1:* p=0.002, *per2*: p<0.01) and at ZT18 (t-test; *per1:* p=0.001, *per2*: p<0.01). In *Arvicanthis, per2* displayed significant variations over time (ANOVA I; p=0.039) higher levels at ZT10 and lower at ZT18 (*post hoc* test; p=0.040). *Bmal1* and *per2* genes expression did not varied significantly.

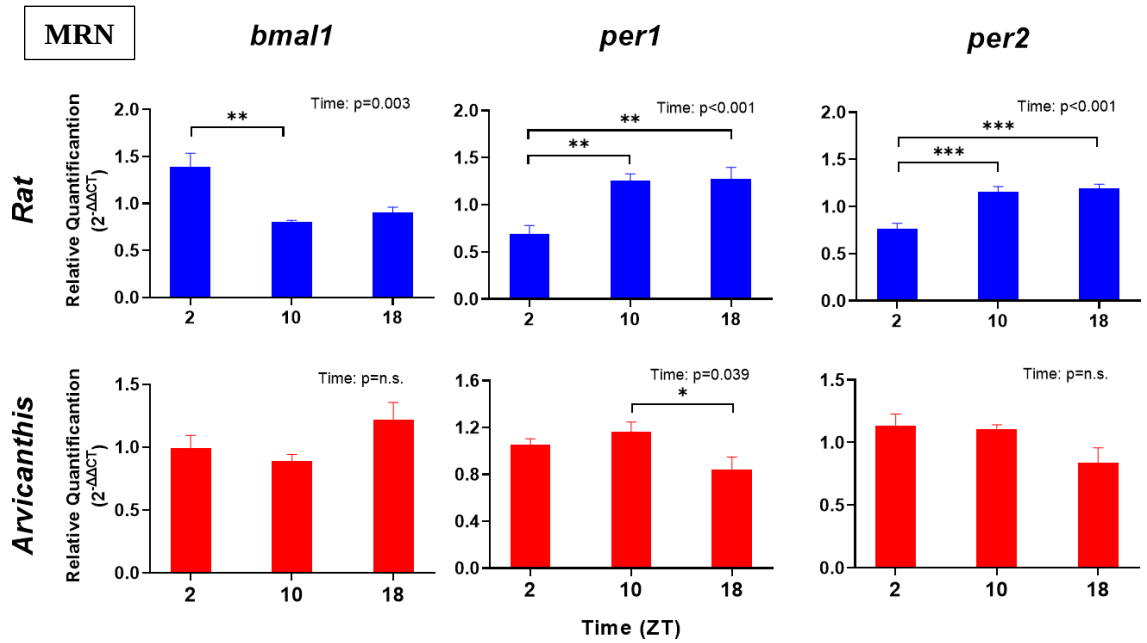

Fig. 2. qRT-PCR analyses of *bmal1*, *per1* and *per2* mRNA levels within the rat (blue bars) and *Arvicanthis* (red bars) median raphe nucleus (MRN) housed in 12:12 light-dark condition. Time is expressed asZeitgeber Time (ZT). Experimental rat and *Arvicanthis* groups were all n≥5., The effect of time is significant for all the genes (ANOVA I; p>0.05), except for *bmal1* and *per2* in *Arvicanthis*. Post hoc tests show differences among the time points: p<0.05 (*), p<0.01 (**), p<0.001 (***). Interaction between time and species is significant for all genes (ANOVAII; p>0.05). Data are presented as mean+SEM.

5. Discussion

5.1. Clock gene expression in the rat and Arvicanthis DRN and MRN

In this study, we showed that clock genes are expressed in the dorsal and median raphe nuclei in both rats and *Arvicanthis*. In the dorsal raphe nuclei of both species, gene expression of *bmal1*, a core component of the positive loop of the clock machinery, varied with opposite phase compared to *per1* and *per2*, which are part of the negative loop. Interestingly, for each gene, mRNA expression varied with phase opposition between the nocturnal rat and diurnal *Arvicanthis*. The use of qPCR performed from global tissue punches did not allow to differentiate rhythms within the subregions of the dorsal raphe nuclei (lateral, dorsomedial, ventromedial DRN), knowing that they are considered as independent nuclei based on their efferent or afferent projections, receptor expression and electrical properties (Huang et al. 2019;Clark, McDevitt, and Neumaier 2006; Hensler et al. 1994; Peyron et al. 2018). For example, the lateral part of the dorsal raphe nuclei projects to the intergeniculate leaflet (IGL), and is more directly involved in the regulation of circadian rhythms (Meyer-Bernstein and Morin 1996). However,

based on the similar rhythmic expression of other genes (such as tryptophan hydroxylase 2 (Malek et al. 2005, Caputo et al. 2022)) in the Lat, DM, and VM DRN, we do not expect phase differences in clock gene expression among the DRN subregions.

In the median raphe nucleus of rats, clock gene expressions oscillate similarly as in the dorsal raphe nuclei. On the other hand, in *Arvicanthis* only *per1* varied significantly over time, in anti-phase to *per1* expression in rats. *Bmal1* and *per2* did not show significant variations. However, this can be due to the time points analyzed, which may not correspond to the genes' peaks or troughs.

The characterization of complete daily rhythm of RN clock gene expression in these two species could be investigated by performing *in situ* hybridization with several other time points. However, the analysis of three time points was sufficient to appreciate consistent differences between rats and *Arvicanthis*. Moreover, the endogenous nature of clock machinery rhythm as well as the effect of light on clock gene expression should be studied in constant darkness as well.

Clock gene expression in the raphe nuclei may be involved in the circadian regulation of *tph2* expression, whose rhythm differs between diurnal and nocturnal rodents (Malek et al. 2005), (Caputo et al.). In rats, *tph2* rhythm seems to be under the control of corticosterone daily surge (Malek et al. 2007). It is possible, that the signal coming from glucocorticoids is integrated by the RN clock machinery, and both contribute to a produce synchronized *tph2* daily and circadian rhythms.

Furthermore, other rhythmic gene expression may be under the control of the RN clock machinery. In mice, mRNA levels of the serotonin transporter SERT, one of the main target of antidepressants, are higher during night than during the day (Ushijima et al. 2005). The clock machinery may be implicated in SERT daily variation and therefore be a possible target to improve mood disorders.

5.2. Conclusions and Perspectives

Taken together these data show that the two main raphe nuclei may function as an oscillator. It is not excluded that it may as well integrate rhythmic signals coming from the SCN. This study suggests that clock genes may contribute to the opposite rhythmic functioning of the

Arvicanthis and rat RN. The phase opposition of the RN rhythm may be one of the factor contributing to the temporal switch between nocturnal and diurnal animals.

Conflict of interest statement

The authors declared no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

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C. RHYTHMIC REGULATION OF SEROTONIN SYNTHESIS-ROLE OF GLUCOCORTICOIDS

Serotonin synthesis is rhythmic in both rats and *Arvicanthis* but the mRNA rhythm of serotonin rate limiting enzyme, tryptophan hydroxylase (*tph2*) differ between these two species. We raised the question about the mechanisms that may regulate *tph2* expression. Glucocorticoid signaling seems to be a promising candidate. Corticosterone rhythm depletion suppresses *tph2* rhythm in the rat raphe, and glucocorticoid plasma levels correlate with *tph2* pattern both in the rat (unimodal) and in *Arvicanthis* (bimodal). Since glucocorticoid receptor (GR) is expressed in the RN of both rat and *Arvicanthis,* we have hypothesized that glucocorticoids may control *tph2* mRNA expression at a transcriptional level. To test our hypothesis we used a ratserotonergic cell line, the RN46A. We treated the cells with dexamethasone, a GR agonist, to induce *tph2* expression. The final aim was to confirm glucocorticoid effect on this cell line, to subsequently perform GR chromatin immunoprecipitation in optimal condition to detect GR bound to *Tph2* gene. However, this last step was not possible due to limitations of the RN46A cell line. Details are described here, in section C1.

La synthèse de la sérotonine est rythmique chez le rat et l'*Arvicanthis*, mais le rythme de l'ARNm de l'enzyme limitant le taux de sérotonine, la tryptophane hydroxylase (*tph2*), diffère entre ces deux espèces. Nous avons recherché quels pourraient être les mécanismes impliqués dans la régulation de l'expression de la *tph2*. Les glucocorticoïdes semblent être un candidat prometteur. En effet, l'abolition du rythme de corticostérone plasmatique supprime le rythme *tph2* dans le raphé du rat. De plus, les modalités du rythme des glucocorticoïdes, i.e. unimodal chez le rat et bimodal chez *Arvicanthis* se retrouvent également sur les profils rythmiques de la *tph2*. Étant donné que les récepteurs des glucocorticoïdes (GR) sont exprimés dans le RN du rat et d'*Arvicanthis*, nous avons émis l'hypothèse que les glucocorticoïdes pourraient contrôler l'expression de l'ARNm de *tph2* au niveau de sa transcription. Pour tester cette hypothèse, nous avons utilisé une lignée cellulaire sérotoninergique de rat, la RN46A. Nous avons traité les cellules avec de la dexaméthasone, un agoniste des GRs, pour induire l'expression de *tph2*. L'objectif final était de confirmer l'effet des glucocorticoïdes sur cette lignée cellulaire, pour ensuite effectuer une immunoprécipitation de la chromatine dans des conditions optimales afin d'identifier le GR lié au promoteur du gène *tph2*. Cependant, cette dernière étape n'a pas pu être réalisée. Les détails sont décrits dans la section C1.

C1. EFFECT OF DEXAMETHASONE ON TRYPTOPHAN HYDROXYLASE 2 mRNA EXPRESSION IN RN46A CELL LINE

Version edited in form of article, but needs to be completed before submission.

Title

Effect of dexamethasone on tryptophan hydroxylase 2 mRNA expression in RN46A cell line

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Abstract

Serotonin is involved in many physiological processes, including the regulation of the sleep/wake cycle, circadian rhythms, and mood. Disruption of serotonin levels in the brain plays a central role in the pathophysiology of many mood disorders, including depression. Serotonin levels depend on tryptophan hydroxylase 2 (tph2), the rate-limiting enzyme of its synthesis. In the raphe nuclei, tph2 presents rhythmic levels of both protein and mRNA levels. The mechanisms leading to *tph2* rhythmic expressions are unknown, but evidence show that glucocorticoid signaling may be involved. In this study, by using a rat serotonergic cell line, RN46A, we investigated the effect of dexamethasone, a glucocorticoid receptor agonist on *tph2* mRNA expression. Dexamethasone (1h, 2h, 4h, 24h) treatment displayed no significant effect on *tph2* expression. Analysis of serotonergic differentiation markers, as well as the low amount of *tph2* expression measured in RN46A cells suggest that cells are not fully differentiated towards a serotonergic phenotype, and therefore is not the optimal model to study *tph2* transcription mechanisms. In order to answer our question in another way, we investigated the presence of glucocorticoid receptor element in *tph2* gene within the rat raphe nuclei. This experiment is ongoing.

Keywords

RN46A, Glucocorticoids, Dexamethasone, Tryptophan Hydroxylase 2

Introduction

The monoamine serotonin (5HT), produced in the brain by the raphe nuclei (RN), is involved in many physiological processes including the control of the sleep/wake cycle, regulation of circadian rhythms and mood (Lucki 1998; Daut and Fonken 2019). Since the 1950s, disruption of the serotonergic system has been implicated in the etiology of mood disorders, such as major depressive disorder (Loomer, Saunders, and Kline 1957), and the 5HT system is still one of the main target of antidepressant treatments (Perez-Caballero et al. 2014; Garakani et al. 2020). Serotonin levels in neurons terminals are rhythmic and the amount of 5HT synthesis is under the control of tryptophan hydroxylase 2 (TPH2), 5HT rate-limiting enzyme (Walther et al. 2003; Zhang et al. 2004). Once synthesized, serotonin is stored in presynaptic vesicles until released in the synaptic clefts. Here 5HT acts on 5HT postsynaptic receptors, and may also feedback to the RN, binding to the auto-receptor 5HTR1A (Cerrito and Raiteri 1979). 5HT is either removed from the synaptic cleft by the selective serotonin transporter (SERT) (Rudnickand Sandtner 2019), or metabolized by monoamine oxidase (MAO) (Ma et al. 2004; Beck et al.1995).

Tph2 is characterized by a rhythmic profile of both protein and mRNA levels (Malek et al. 2005; Malek, Pévet, and Raison 2004). The origin of *tph2* rhythm is unclear, but one of the hypothesis is that *tph2* rhythmicity would be under the control of output signals coming from the master clock, located in the suprachiasmatic nucleus (SCN).

The 24h temporal entrainment of the expression of several genes depends on daily 24h pattern of glucocorticoids (GCs) (Balsalobre et al. 2000), whose rhythm is under the control of the SCN (Buijs and Kalsbeek 2001). The principal mechanism of action of GCs consists in binding cytosolic glucocorticoid receptors (GR) and forming a complex that translocates into the nucleus (Revollo and Cidlowski 2009). Here, the GC-GR complex recognizes glucocorticoid response elements (GRE) or glucocorticoids binging sequences (GBS) and acts as transcription factor (Grbesa and Hakim 2017). GRE have been described in many genes, including for example the clock gene period1 (*Per1*) (Yamamoto et al. 2005).

Corticosterone plasma circadian rhythm correlates with *tph2* mRNA daily/circadian profiles both in nocturnal and diurnal rodents (Malek et al. 2005), (Caputo et al.). Indeed, Malek and colleagues (Malek et al. 2007) have shown that corticosterone plasma rhythm drives *tph2* daily variations in rats RN. Furthermore, evidence suggests that corticosterone action on *tph2* expression is GR-dependent, since ablation of GR in the mouse DRN modifies *tph2* levels

(Vincent et al. 2018). In the rat, the mechanisms involved in glucocorticoid control of *tph2* rhythm are still unclear.

The aim of this study is to investigate whether glucocorticoids may regulate *tph2* rhythm by binding to GRE on *Tph2* gene sequence, thus modulating its transcription. To test this hypothesis, we used a rat serotonergic cell line, the RN46A, which is derived from extracts of the medullary raphe nuclei of embryonic day 13 (E13) rat embryos. RN46A were treated with dexamethasone, a glucocorticoid receptor agonist, to stimulate *tph2* transcription. Part of the experiment was repeated on RN46A-B14 cell line, which expresses the brain derived neurotrophic factor (BDNF) and have been described with a more differentiated phenotype (Eaton and Whittemore 1996). The following step was to perform CUT&Tag experiment of GR, in order to investigate its binding to *Tph2* gene. However, since dexamethasone treatment did not change significantly *tph2* expression in RN46A, we performed the CUT&Tag experiment on rat RN explants. This experiment is ongoing.

Materials & Methods

Cell culture

RN46A cell line was gently obtained by Dr S. R. Whittemore and cultured as previously described (Eaton et al. 1995), with the following modifications. RN46A were derived from extracts of the medullary raphe nuclei of embryonic day 13 (E13) rat embryos and immortalized by infection with a retrovirus carrying the temperature-sensitive mutant of oncogenic simian virus-40 large T antigen (White et al. 1994). When cultured at non-permissive temperature (39°C), RN46A cells start differentiate and to express serotonergic markers such as 5HTR1, SERT, as well as low levels of *tph2* that can be stimulated by calcium treatment (Eaton et al. 1995; Remes Lenicov et al. 2007; White et al. 1994). RN46A-B14 cell line differentiates from RN46A for the expression of the brain derived neurotrophic factor (BDNF), and present a more differentiated morphology (Eaton and Whittemore 1996).

RN46A and RN46A-B14 proliferation was performed at permissive temperature (33°C) and 5% CO2 in Dulbecco′s Modified Eagle′s Medium/Nutrient Mixture F-12 Ham (DMEM:F12) medium (D8062, Sigma-Aldrich) supplemented with 10% (v/v) FBS (F2442, Sigma-Aldrich), 1% (v/v) Penicillin–Streptomycin Solution Hybri-Max (Pen/Strep) (P7539, Sigma-Aldrich), 2 mmol/L L-glutamine (G7513, Sigma-Aldrich), and 0.25mg/ml G418 (A1720, Sigma-Aldrich).

Before differentiation, cells were seeded on coated wells with collagen/fibronectin (100 μg/cm2 air-dried collagen type I solution from rat tail (C3867, Sigma-Aldrich), followed by 1 μg/cm2 fibronectin (F1141, Sigma-Aldrich)). At 75% confluence, cells were shifted to 39°C (non-permissive temperature) and the medium changed with the differentiation medium containing DMEM:F12, supplemented with 1% (w/v) bovine serum albumin (BSA), 1% (v/v) Pen/Strep (A3311, Sigma-Aldrich), 2 mmol/L L-glutamine, and 1% (v/v) N-2 supplement (17502048, Thermo Fisher). Cells were differentiated for 8 days. In addition, to improve differentiation, from day 2 to day 5 medium was supplemented with 40mM potassium chloride (KCl) (P5405, Sigma-Aldrich), and only RN46A medium was supplement for day1 to day 8 with brain derived neurotrophic factor (BDNF) (20 ng/ml) (B3795, Sigma-Aldrich).

Differentiation and dexamethasone treatment

In order to test the effect of dexamethasone on *tph2* expression, RN46A cells were differentiated for 8 days. On the eighth day, medium was changed with one of the 4 treatment media: 1- differentiation medium plus vehicle (ethanol); 2- differentiation medium plus 1000 nM dexamethasone (D1756, Sigma-Aldrich); 3- differentiation medium plus 1000 nM dexamethasone and 40 nM KCl; 4- differentiation medium plus 40 nM KCl (positive control). Cells were harvested after 1h, 2h, 4h, and 24h of treatment (n=3 wells per condition), using a RLT lysis buffer from the RNeasy Mini Kit (74104, QIAGEN). In order to test serotonergic markers of differentiation, RN46A-B14 cells were differentiated and harvested at day2, day4 and day8. In order to test the effect of different doses of dexamethasone, RN46A-B14 cells were differentiated for 8 days. On the eighth day, medium was changed with one of the 4 treatment media: 1- differentiation medium plus vehicle (ethanol); 2- differentiation medium plus 10 nM dexamethasone ; 3- differentiation medium plus 100 nM dexamethasone; 4- differentiation medium plus 40 nM KCl. Cells were harvested after 2h of treatment (n=3 wellsper condition).

Real-time quantitative polymerase chain reaction

Total RNA was extracted using RNeasy Mini Kit (74104, QIAGEN) following the manufacturer's recommendations. RNA concentration and purity (A260/A280 ratio and A260/A230 ratio) were measured using a Nano Drop ND-1000 V 3.5 Spectrophotometer

(NanoDrop Technologies, Wilmington, DE). Synthesis of cDNA was performed using, 1000 ng of total RNA, using Thermo Scientific™ Maxima™ H Minus cDNA Synthesis Master Mix, with dsDNase (15696019, Thermo Fisher Scientific) following the manufacturer's recommendations. Real-time PCR was performed using PowerUp™ SYBR™ Green Master Mix (A25776, Thermo Fisher Scientific) and run on the Applied Biosystems Real-Time PCR Instruments. 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. Primer sets for the qPCR were as follows:

Rat *Tph2* (forward 5′- GGAAGACGTCTCCATGTTTCT -3′, reverse 5′- GTGCAGTGGAATACTCTGTAGG -3′);

Rat *Tph1* (forward 5′- GCTGAACAAACTCTACCCAAC -3′, reverse 5′- TTCCCGATAGCCACAGTATT -3′);

Rat *Slc6a4* (forward 5′- GGAGATGAGGAATGAAGATGTGTCA -3′, reverse 5′- GGAAGAAGATGATGGCAAAGAACG -3′);

Rat *5htr1a* (forward 5′- CAGATGGGAGCATTGTGGAAACC -3′, reverse 5′- CAGATGGGAGCATTGTGGAAACC -3′);

Rat *Nr3c1* (forward 5′- GAAAAGCCATCGTCAAAAGGG -3′, reverse 5′- TGGAAGCAGTAGGTAAGGAGA -3′);

Rat *Th* (forward 5′- CTGTTCTCAACCTGCTCTTCTC -3′, reverse 5′- CGGGTCTCTAAGTGGTGAATTT -3′);

Rat *Per1* (forward 5′- GCGTAAATGTGCCTCCTCTT -3′, reverse 5′- GACGTATCTTTCTTCGCCCC-3′);

Rat *Gapdh* (forward 5′- GACAACTTTGGCATCGTGGA -3′, reverse 5′- GACAACTTTGGCATCGTGGA -3′);

Data were analyzed by using the 2-ΔΔCT method (Livak and Schmittgen 2001), normalized using the housekeeping gene *Gapdh*. Changes in gene expression were referenced to the level of expression of the control, set as 1.

Statistical analyses

Gene expression differences among treatments, or among differentiation days, were analyzed using one-way analysis of variance (ANOVA I), followed by *post-hoc* analyses. The effect of treatment relative to the time of treatment was tested using an ANOVA II, with factors "treatment" and "treatment time". Non-parametric tests were used in case of non-normal distribution of the data. For all statistical procedures, the level of significance was set at $p<0.05$.

All data are presented as mean±SEM, unless otherwise stated. Sigma Plot (v14) software was used for statistical analyses.

Results

Effect of dexamethasone 1h, 2h, 4h, 24h treatments on tph2 mRNA in RN46A cell line

To investigate the effect of dexamethasone, a glucocorticoid receptor agonist, on *tph2* expression, RN46A cells were treated with either dexamethasone, KCl (as a positive control), or a combination of dexamethasone and KCl, for 1h, 2h, 4h, 24h. Dexamethasone treatment did not modify significantly *tph2* mRNA levels in any of the four time conditions (ANOVA I, details in *Supp. Table 1*). However, *tph2* expression was significantly increase with KCl and dexamethasone plus KCl treatments (ANOVA I, *post-hoc* test details in *Supp. Table 1*). No significant differences were observed between KCl and dexamethasone plus KCl treatments (ANOVA I, *post-hoc* test details in *Supp. Table 1*), showing that *tph2* mRNA can be induced by KCl, but there is no interaction between KCl and dexamethasone treatments (*Fig.1)*. Although dexamethasone did not change significantly *tph2* mRNA levels compared to the control, ANOVA II analyses revealed a significant decrease of *tph2* expression after 24h of dexamethasone treatment, compared to 1h (*post-hoc* test, $p=0.008$) and 4h (*post-hoc* test, $p=0.012$) treatment (*Fig. 2)*, suggesting a minor modulatory effect of dexamethasone on *tph2* expression over time in RN46A. Dexamethasone treatment significantly increased (all $p<0.05$) the mRNA levels of *Per1*, a gene known to present a GRE sequence in its promoter (ANOVA I, *post-hoc* test details in *Supp. Table 2)* (*Fig.1).*

Dose effect of dexamethasone treatment on RN46A-B14

To investigate whether the lack of effect of dexamethasone on *tph2* was due to the dose used (1000 nM), we repeated the experiment using dexamethasone doses of 10 nM and 100 nM on RN46A (*Fig.3).* None of the two doses resulted in significant modifications (ANOVA I, *post-hoc* test; both $p>0.05$) *tph2* levels after 2h treatment, while KCl (positive control) increased *tph2* levels 9.6 fold (ANOVA I, *post-hoc* test; $p<0.001$). As positive control, *per1* expression displayed a significant increase in mRNA levels (*Fig. 3*) with 10 nM (ANOVA I, *post-hoc* test; $p<0.001$) and 100 nM (ANOVA I, *post-hoc* test; $p<0.001$) doses, but no effect of KCl (ANOVA I, *post-hoc* test; $p>0.05$).

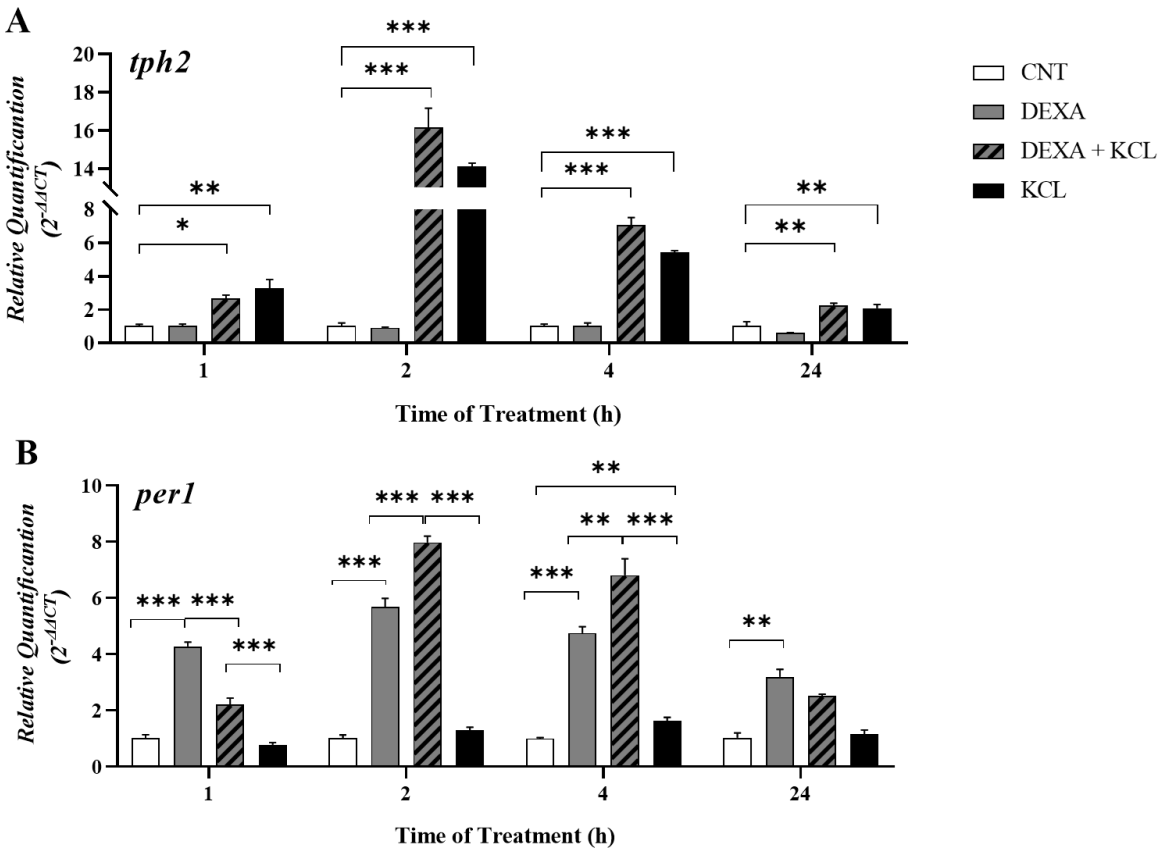

Fig.1. Dexamethasone 1h, 2h, 4h, 24h treatments on RN46A cells. *qRT-PCR analyses of tryptophan hydroxylase 2 (tph2), (A), and period 1 (per1) (B) mRNA levels after 1h, 2h, 4h, 24h treatment with control vehicle (CNT, white bars), dexamethasone 1000 nM (DEXA, gray bars), dexamethasone 1000 nM plus 40 mM potassium chloride (DEXA+ KCl, gray striped bars), and 40 mM potassium chloride (KCl, black bars). Dexamethasone treatment had no effect (ANOVA I; p>0.05) on tph2 expression, but KCl (positive control) increased (ANOVA I; p<0.05) tph2 levels, in all four time conditions (A). Dexamethasone treatment increased (ANOVA I; p<0.05) the expression of per1 (positive control), in all four time conditions (B). Post-hoc tests show differences among conditions: p<0.05 (*), p<0.01 (**), p<0.001 (***). In all conditions n=3. Data are presented as mean±SEM.*

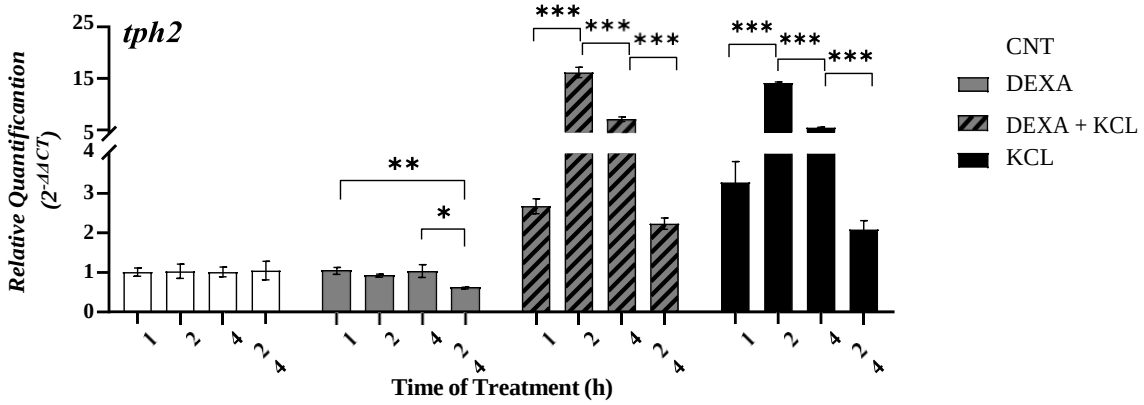

***Fig.2. Effect of* 1h, 2h, 4h, 24h treatments on RN46A cells.** qRT-PCR analyses of tryptophan hydroxylase 2 (*tph2),* mRNA levels after 1h, 2h, 4h, 24h treatment with control vehicle (CNT, white bars), dexamethasone 1000 nM (DEXA, gray bars), dexamethasone 1000 nM plus 40 mM potassium chloride (DEXA+ KCl, gray striped bars), and 40 mM potassium chloride (KCl, black bars). *Post-hoc* tests show differences among conditions: p<0.05 (*), p<0.01 (**), p<0.001 (***). Of note, dexamethasone treatment decreased on *tph2* expression after 24h of treatment compared to 1h (ANOVA II, *post-hoc test;* p=0.008) and 4h (ANOVA II, *post-hoc test;* p=0.012). In all conditions n=3. Data are presented as mean±SEM.

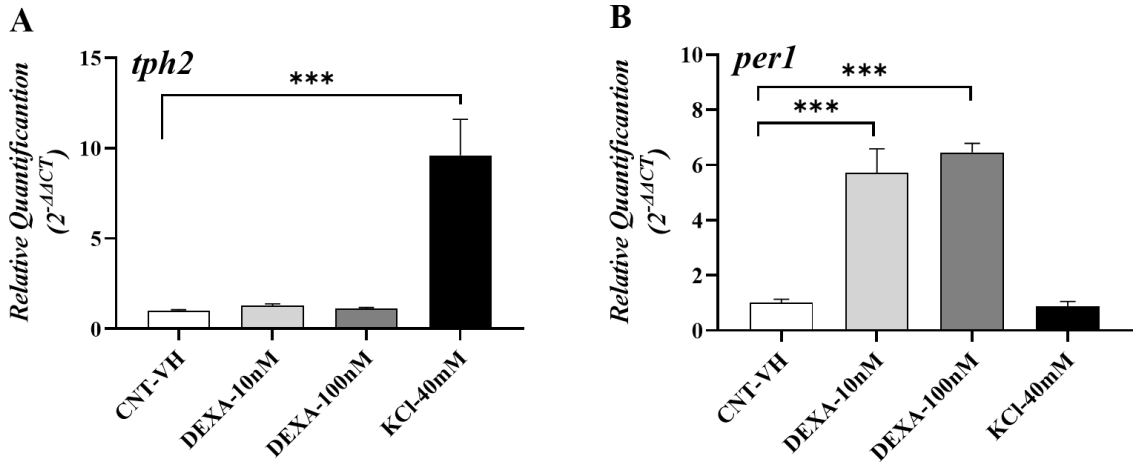

Fig.3. Dose effect of dexamethasone 2h treatment on RN46A-B14 cells. qRT-PCR analyses of tryptophan hydroxylase 2 (tph2), (A), and period 1 (per1) (B) mRNA levels after 2htreatment with control vehicle (CNT, white bars), dexamethasone (DEXA) 10 nM (light gray bars), dexamethasone (DEXA) 100 nM (dark gray bars), and 40 mM potassium chloride (KCl, black bars). Both doses of dexamethasone treatment had no effect (ANOVA I; p>0.05) on tph2 expression, but KCl (positive control) increased (ANOVA I; p<0.05) tph2 levels (A). Both doses of dexamethasone treatment increased (ANOVA; p<0.05) the expression of per1 (positive control) (B). Post-hoc tests show differences among conditions: p<0.05 (*), p<0.01 (**), p<0.001 (***). In conditions n=3. Data are presented as mean±SEM.

Serotonergic markers of differentiation in RN46A-B14

To investigate whether RN46A-B14 cell line differentiated appropriately over time, at day 2, 4 and 8 of differentiation we measured the expression of serotonergic markers (*Fig. 4A)*, namely *tph2, tph1* (expressed at low levels in serotonergic neurons), serotonin receptor 1a (*5htr1a*), and serotonin transporter (*slc6a4*), as well as tyrosine hydroxylase (*th*, expressed in catecholaminergic neurons), and glucocorticoid receptor (*nr3c1*). *Tph2* levels increased while *tph1* and *th* levels decreased at day8 compared to day2 (ANOVA I, *post-hoc* test; *tph2* $p<0.001$*; tph1* $p=0.048$*; th* $p<0.001$*)* and compare to day4 (ANOVA I, *post-hoc* test; *tph2* $p<0.001$*; th* $p<0.001$*)*, showing that RN64A-B14 differentiated towards a serotonergic phenotype. *5htr1a* expression did not change significantly (ANOVA I; $p>0.05$) and *slc6a4* levels decreased (ANOVA I, *post-hoc* test; day2 vs day4 $p=0.046$; day2 vs day8 $p=0.017$). *Nr3c1* expression did not change significantly over the days (ANOVA I, $p>0.05$).

In addition, to test whether RN46A-B14 cells expressed an exclusive serotonergic phenotype, we compared *tph2, tph1* and *th* expression at day 8 (*Fig. 4B*). Surprisingly, *Tph1* and *th* mRNA levels were 2.9 fold (ANOVA I, *post-hoc* test; $p<0.001$) and 11.3 fold (ANOVA I, *post-hoc* test; $p<0.001$) higher than *tph2,* suggesting that RN46A-B14 cells are not fully differentiated towards a serotonergic phenotype.

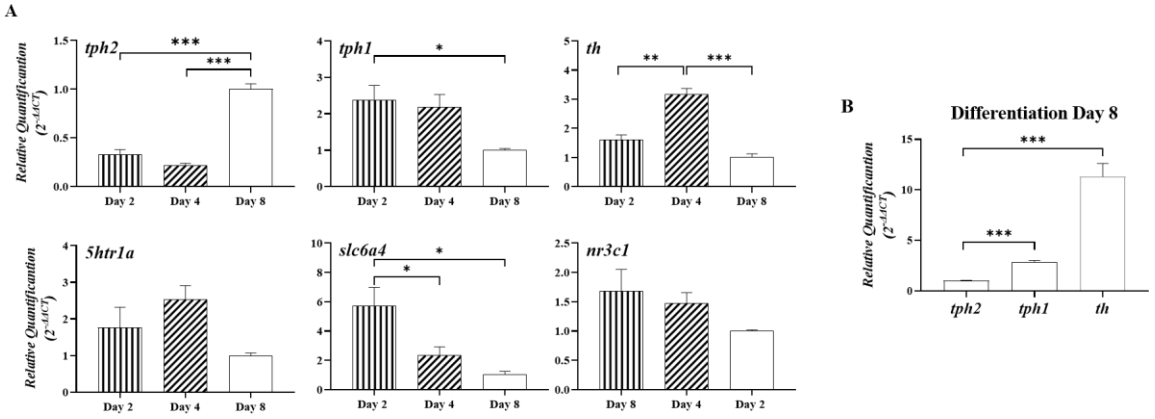

Fig.4. Serotonergic markers of differentiation in RN46A-B14 cells. qRT-PCR analyses of tryptophan hydroxylase (*tph*) 2, tph1, tyrosine hydroxylase (*th*), serotonin receptor 1a (*5htr1a*), serotonin transporter (*slc6a4*), and glucocorticoid receptor (*nr3c1*) mRNA levels at day 2 (vertical striped bars), day 4 (diagonal striped bars) and day 8 (white bars) of differentiation (A). All condition had n=3. A). qRT-PCR analyses of tryptophan hydroxylase (tph) 2, tph1, tyrosine hydroxylase (*th*) mRNA levels at day 8 of differentiation (B). Post-hoc tests show differences among conditions: $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***). In all conditions n=3. Data are presented as mean±SEM.

Discussion

Dexamethasone effect on tph2 expression in RN46A

In this study, we aimed to test the hypothesis that glucocorticoids may regulate *tph2* rhythm, by directly acting on its transcription, through GR binding to GREs (or GBS). *Tph2* mRNA levels in the rat raphe nuclei are rhythmic (Malek et al. 2005) and evidence suggests that in rats, corticosterone signaling would increase *tph2* mRNA levels at the end of the subjective day, *i.e.* when corticosterone levels increase (Malek et al. 2007). Therefore, we suppose that if corticosterone-GR complex drives *tph2* rhythmic transcription, it is time dependent. Hence, in order to confirm t*ph2*-induced transcription, we treated a serotonergic cell line, the RN46A, with dexamethasone, a glucocorticoid receptor agonist, for different time duration.

In RN46 cell line, dexamethasone had no significant effect on *tph2* expression compared to the control, in any of the four time conditions tested (1h, 2h, 4h, 24h treatment). Two positive controls were included in our experiment. First, to confirm that the dexamethasone treatment was able to induce gene transcription in this cell line we looked at the expression of *per1*, whose gene promoter contains a functional GRE (Yamamoto et al. 2005). *Per1* expression was increased by dexamethasone treatment. Second, to test that *tph2* expression could be induced in RN46A cell line, we treated cells with KCl to increase intracellular calcium levels. Indeed, it has been shown that *tph2* expression is regulated by transcription factors that are calcium-responsive such as activator protein 1 (AP-1) (Remes Lenicov et al. 2007). Expression of *tph2* was successfully induced by KCl addition. We also tested a condition in which dexamethasone and KCl treatments were provided together. Indeed, GC-GR complex may act as a transcription factor either as homodimer or as heterodimer (Grbesa and Hakim 2017), as well as *via* other factors such as AP-1 (Deblois and Giguère 2008; Reddy et al. 2009). Therefore, it is possible that glucocorticoid act in combination with other signals, such as calcium increase. However, there was no significant difference between *tph2* expression in cells treated only with KCl and cells treated with a combination of dexamethasone and KCl, suggesting that in the latter, *tph2* increase is mediated by KCl. Comparing the different time duration of dexamethasone treatment, *tph2* expression decreased after 24h of treatment compared to 1h and 4h, but not compared to the control.

As we did not obtain any effect of the dexamethasone treatment on *tph2* expression, we tested whether it was due to the high (1000 nM) dose used. We repeated the experiment using two lower doses (10 nM and 100 nM). For this experiment, we used the RN46A-B14 cell line, which

only differs from RN46A because the B14 synthesize and secrete biologically active BDNF, necessary for the cell line survival and serotonergic differentiation (Eaton and Whittemore 1996). Surprisingly, no significant effect on *tph2* levels was observed with either of the two doses.

At this stage, despite previous studies on this cell line (Chen, Vallender, and Miller 2008; Eaton et al. 1995; Nawa et al. 2017; Remes Lenicov et al. 2007; Sabir et al. 2018; White et al. 1994; Yammamoto et al. 2013), we could question the degree of differentiation and the phenotypic identity of RN46A cells. Therefore, we checked whether these cells correctly differentiated towards a serotonergic phenotype. Serotonin transporter SERT, a marker of differentiation, mRNA levels decreased unexpectedly during differentiation. *Tph2* expression increased over the days of differentiation, while *tph1* (expressed at low levels in serotonergic neurons) and *th* (expressed in dopaminergic, adrenergic and noradrenergic neurons) levels decreased, indicating that cells differentiated over time. However, *th* and *tph1* levels were much higher than that of *tph2*. The higher levels of *tph1* compared to *tph2* observed in this experiment is in agreement with what is stated in another study (Nawa et al. 2017). In other studies, either the differences between *tph1* and *tph2* levels are not expressed (Sabir et al. 2018), or no distinction is made between tph1 and tph2 (Eaton and Whittemore 1995; Eaton et al. 1995; White et al. 1994). In this cell line *tph2* is expressed a very low levels, in contrast with its expression in rat raphe neurons. This may be due either to state of the chromatin, or to the fact that, since GR may act as a heterodimer, other factors necessary to have a glucocorticoid-GR mediated *Tph2* transcription are missing. Therefore, further investigation is required before to consider RN46A an appropriate serotonergic model. Our data suggest that after 8 days of differentiation, RN46A cells are not fully differentiated in serotonergic neurons phenotype and may therefore not be the best model to study mechanisms of *tph2* transcription.

Conclusion and Perspectives

This study demonstrate that RN46A cell line is not the most appropriated model to answer to our question, i.e. if GC may act on *tph2* transcription by binding GREs (or GBS) on *Tph2* gene promoter. Therefore, our next approach is to investigate whether GR may be found on *Tph2* gene by using the CUT&Tag technique on rat RN tissue. As showed by Malek and colleagues (Malek et al. 2005), *tph2* mRNA levels gradually increase from ZT2 and reach higher levels at ZT10. Therefore, raphe samples were collected at ZT9, just before the peak of *tph2* expression. This experiment is ongoing.

Gaining insights about glucocorticoid-mediated *tph2* rhythmic expression, besides improving our understanding of molecular mechanisms, may have implication for mood disorders, such as depression, considering stress inhibits *tph2* expression and is associated with depression (Donner et al. 2012; Chen et al. 2017).

Conflict of interest statement

The authors declared no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

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	tph2															
	1h treatment				**2h treatment**				**4h treatment**				**24h treatment**			
ANOVA I	p<0.001				p<0.001				p<0.001				p<0.001			
Post-hoc test - Comparison	**Diff of Means**	**t**	**P**	**P<0.050**	**Diff of Means**	**t**	**P**	**P<0.050**	**Diff of Means**	**t**	**P**	**P<0.050**	**Diff of Means**	**t**	**P**	**P<0.050**
DEXA vs. CNT	0.0286	0.07	0.946	No	-0.0381	0.67	0.522	No	-0.44	1.754	0.221	No	0.00401	0.063	0.951	No
DEXA_KCL vs. CNT	1.669	4.087	0.014	Yes	1.207	21.22	<0.001	Yes	1.192	4.754	0.006	Yes	0.848	13.313	<0.001	Yes
KCL vs. CNT	2.265	5.548	0.003	Yes	1.149	20.206	<0.001	Yes	1.04	4.146	0.01	Yes	0.735	11.533	<0.001	Yes
DEXA_KCL vs. DEXA	1.64	4.017	0.012	Yes	1.245	21.891	<0.001	Yes	1.632	6.508	0.001	Yes	0.844	13.25	<0.001	Yes
KCL vs. DEXA	2.237	5.477	0.003	Yes	1.188	20.876	<0.001	Yes	1.479	5.9	0.002	Yes	0.73	11.47	<0.001	Yes
KCL vs. DEXA_KCL	0.597	1.461	0.331	No	-0.0577	1.014	0.565	No	-0.153	0.608	0.56	No	0.113	1.78	0.213	No

Supplementary Table 1. ANOVA I and post-hoc analysis on *tph2* mRNA expression.

	per1															
	1h treatment				**2h treatment**				**4h treatment**				**24h treatment**			
ANOVA I	p<0.001				p<0.001				p<0.001				p=0.016			
Post-hoc test - Comparison	**Diff of Means**	**t**	**P**	**P<0.050**	**Diff of Means**	**t**	**P**	**P<0.050**	**Diff of Means**	**t**	**P**	**P<0.050**	**Diff of Ranks**	**t**	**P**	**P<0.050**
DEXA vs. CNT	3.264	14.763	<0.001	Yes	4.663	15.708	<0.001	Yes	0.676	16.316	<0.001	Yes	18	2.882	0.174	No
DEXA_KCL vs. CNT	1.191	5.388	0.001	Yes	6.967	23.469	<0.001	Yes	0.83	20.019	<0.001	Yes	27	4.323	0.012	Yes
CNT vs. KCl	0.233	1.056	0.322	No	-0.286	0.963	0.364	No	-0.208	5.011	0.002	Yes	-9	1.441	0.738	Do Not Test
DEXA vs. DEXA KCl	2.073	9.374	<0.001	Yes	-2.304	7.761	<0.001	Yes	0.154	3.704	0.006	Yes	9	1.441	0.738	Do Not Test
DEXA vs. KCl	3.497	15.819	<0.001	Yes	4.377	14.745	<0.001	Yes	0.469	11.305	<0.001	Yes	18	2.882	0.174	No
DEXA KCl vs. KCl	1.425	6.444	<0.001	Yes	6.681	22.506	<0.001	Yes	0.622	15.009	<0.001	Yes	9	1.441	0.738	Do Not Test

Supplementary Table 2. ANOVA I and post-hoc analysis on per1 mRNA expression.

D. SEROTONIN EFFECT ON SCN ELECTRICAL ACTIVITY

The first parts of this thesis have shown that the functioning of arousal structures, including the serotonergic raphe nuclei, differ between the diurnal *Arvicanthis* and the nocturnal rat. It is still not clear how these differences are integrated by the SCN to produce different behavioral phenotypes (nocturnal *vs* diurnal), taking into consideration that the rhythm of the SCN is in phase in diurnal and nocturnal rodents (Challet 2007). To gain more insights about the functioning of the SCN in diurnal species we have recorded the SCN electrical activity (multi-unit activity (MUA)) rhythm in freely moving *Arvicanthis*. The MUA pattern in the SCN is well characterized in nocturnal rodents, such as rats (Meijer et al. 1997; Meijer et al. 1996; Schaap and Meijer 2001), and mice (van Oosterhout et al. 2012). In nocturnal rodents, the SCNelectrical activity has a rhythmic profile that peaks during the day and is low during the night (Inouye and Kawamura 1979; Shibata et al. 1982). Furthermore, the SCN neurons firing rate issuppressed by the animal's spontaneous behavior occurring at night (van Oosterhout et al. 2012). The magnitude of the SCN electrical suppression is dependent on the type, intensity andduration of behavioral activity (van Oosterhout et al. 2012). Thus, when the behavioral activityoccurs at the appropriate time, i.e. the biological night in nocturnal rodents, it results in increasing the amplitude of the SCN MUA rhythm, by reducing the trough level (van Oosterhout et al. 2012). In collaboration with Johanna Meijer's research group, we investigatedthe MUA rhythm in the SCN of freely moving *Arvicanthis*, during three days of LD and one day of DD. *Arvicanthis* behavioral activity and SCN MUA were recorded simultaneously in 1sbins. The *Arvicanthis* electrical firing rate was overall higher during the day and lower during the night, in phase with the animal's behavioral activity rhythm. The rhythmic SCN electrical pattern was kept in DD condition. These data in *Arvicanthis* are in coherence with the electricalactivity of the diurnal chipmunk (Sato and Kawamura 1984). In *Arvicanthis*, behavioral activityincreased the firing rate of the SCN electrical discharge. Indeed, the frequency of SCN discharge (MUA) is higher when the animal is active, compared to MUA rhythm when it is inactive (Fig. D1). The higher behavioral activity occurred mainly when the SCN electrical activity was also high, thus resulting in enhancing the SCN MUA amplitude. These data showthat behavioral activity can increase the amplitude of the SCN firing rhythm in both nocturnal and diurnal animals, by modulating SCN electrical activity in opposite directions. Moreover, the *Arvicanthis* with a crepuscular behavioral activity pattern presented a MUA bimodal profile,with peaks around the light transitions, in correspondence to locomotor activity bouts.

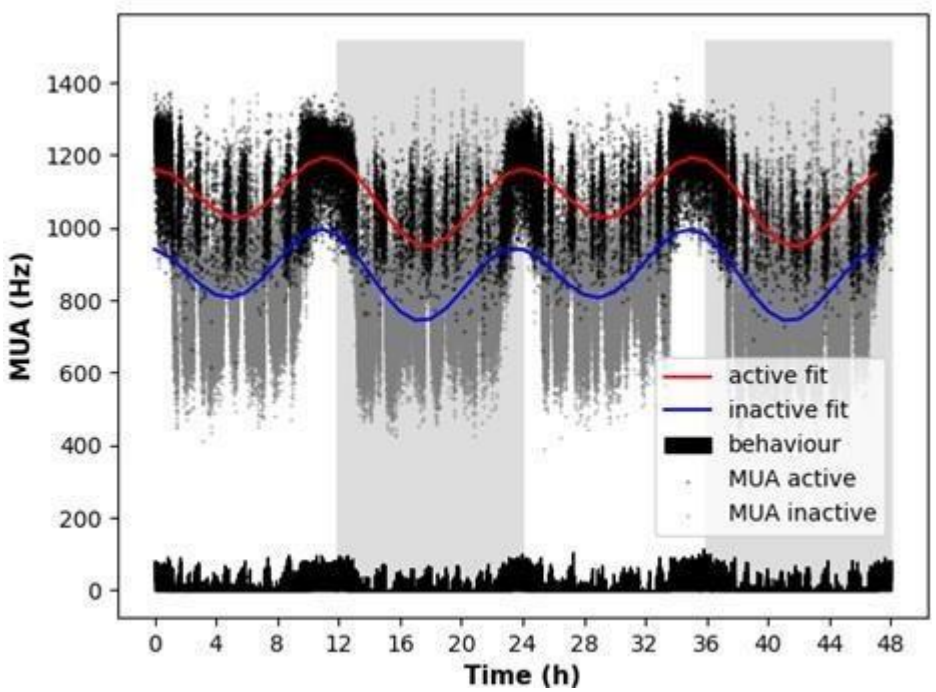

Fig. D1. Multi-unit activity within the *Arvicanthis* SCN during periods of activity (black points)and inactivity (gray points). The red line fits the MUA points during activity; the blue line fits the MUA points during inactivity. The black lines at the bottom represent behavioral activity.Grey background represents the dark phase of the light/dark cycle.

Figure provided by Schoonderwoerd, R.A

In contrast, in *Arvicanthis* with a fully diurnal activity pattern, MUA rhythm presented only one peak. *In vitro* electrophysiological recordings of the SCN from both fully diurnal and crepuscular *Arvicanthis*, showed that the SCN is endogenously unimodal, since its firing rate presents only one peak in both groups. These data suggest that the bimodality is not an intrinsic feature of the SCN, but is rather conveyed by extra-SCN structures, which may also relay the behavioral (unimodal or bimodal) feedback to the SCN (Fig. D2).

The mechanisms involved in conveying the behavioral activity feedback to the SCN are not clear. Evidence suggests that the serotonergic raphe nuclei may be involved. Serotonin levels highly correlate with the behavioral state in both nocturnal and diurnal rodents (Poncet, Denoroy, and Jouvet 1993; Cuesta et al. 2008; Cuesta et al. 2009a; Shioiri et al. 1991), and stimulation of locomotor activity leads to release serotonin in the SCN in hamsters (Dudley, DiNardo, and Glass 1998). In addition, serotonin affects the rhythm of the SCN. Serotonergic stimulation is able to shift the phase of the SCN (Bobrzynska, Godfrey, and Mrosovsky 1996;

Cutrera, Saboureau, and Pévet 1996; Tominaga et al. 1992; Horikawa et al. 2000; Challet 2007). Moreover, lesions of serotonin terminals around the SCN impair the locomotor activity entrainment in mice (Edgar and Dement 1991).

Therefore, we tested the hypothesis that serotonin may be involved in relaying the behavioral feedback signal to the SCN. For this purpose, we used fluoxetine to disrupt serotonin physiological levels (section D1). In addition, we also investigated the effect of serotonin on the *Arvicanthis* circadian system by looking at the effect of fluoxetine on the locomotor activity's endogenous period (section D2).

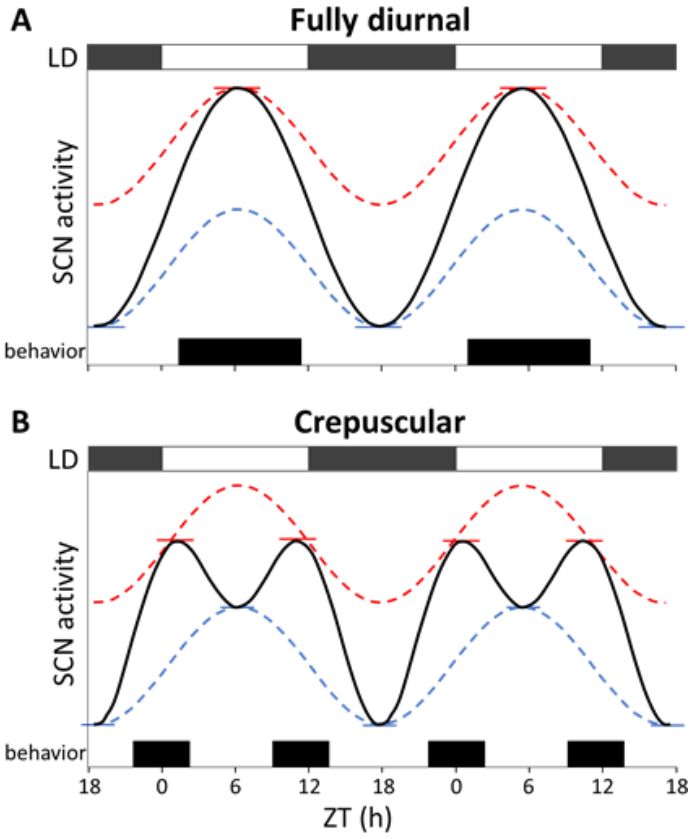

Fig. D2. Effect of fully diurnal (A), or crepuscular (B) behavioral activity pattern on SCN electrical activity. Blue line represents the SCN activity in the absence of behavioral activity; red line represents the SCN activity in the presence of behavioral activity. Black bars representbehavioral activity. The black lines represent the MUA rhythm when behavioral activity is concentrated during the day (A), or when it occurs at the light/dark or dark/light transitions (B).The horizontal bars represent the light (white bars) and dark (gray bars) phases.

Figure provided by Schoonderwoerd, R.A

Les premières parties de cette thèse ont montré que le fonctionnement des structures de l'éveil, dont les noyaux sérotoninergiques du raphé, diffère entre l'*Arvicanthis* diurne et le rat nocturne. Cependant, on ne connait pas comment ces différences sont intégrées par les SCN pour produire différents phénotypes comportementaux (nocturne *vs* diurne), notamment en considérant que le rythme des SCN est en phase chez les rongeurs diurnes et nocturnes (Challet 2007). Pour mieux comprendre le fonctionnement des SCN chez les espèces diurnes, nous avons enregistré le rythme de l'activité électrique *in vivo* des neurones des SCN (activité multi-unitaires (MUA)) d'*Arvicanthis*. Le profil MUA des SCN est bien caractérisé chez les rongeurs nocturnes, tels que le rat (Meijer et al. 1997; Meijer et al. 1996; Schaap et Meijer 2001) et la souris (van Oosterhout et al. 2012). Chez les rongeurs nocturnes, l'activité électrique des SCN présente un profil rythmique avec des niveaux élevés pendant le jour et faibles pendant la nuit (Inouye et Kawamura 1979; Shibata et al. 1982). De plus, la décharge des neurones SCN est supprimée par le comportement spontané de l'animal, se produisant principalement de nuit (van Oosterhout et al. 2012). Le degré de suppression de cette activité électrique des SCN dépend du type, de l'intensité et de la durée de l'activité comportementale considérée (van Oosterhout et al. 2012). Ainsi, lorsque l'activité comportementale se produit au moment opportun, c'est-à-dire la nuit biologique chez les rongeurs nocturnes, il en résulte une augmentation de l'amplitude du rythme MUA des SCN (van Oosterhout et al. 2012). En collaboration avec le groupe de recherche de Johanna Meijer, nous avons étudié *in vivo* le rythme MUA des SCN d'*Arvicanthis* vigiles et libres de leurs mouvements, pendant trois jours en LD et un jour en DD. L'activité comportementale des animaux et l'activité électrique des SCN ont été enregistrées simultanément. Le rythme de décharge électrique des SCN d'*Arvicanthis* est globalement plus élevée le jour et plus faible la nuit, en phase avec le rythme d'activité comportementale de l'animal. Le profil électrique rythmique des SCN est maintenu en condition DD. Ces données chez *Arvicanthis* sont en cohérence avec l'activité électrique enregistrée chez d'autres animaux diurnes (Sato et Kawamura 1984). Chez *Arvicanthis*, l'activité comportementale augmente la fréquence de décharge électrique des SCN. En effet, le rythme des SCN (MUA) est plus élevée lorsque l'animal est actif, par rapport au rythme MUA mesuré lorsqu'il est inactif (Fig.D1). L'activité comportementale est plus élevée lorsque l'activité électrique des SCN est également élevée, entraînant ainsi une augmentation de l'amplitude du rythme MUA. Ces donnéesmontrent que l'activité comportementale peut augmenter l'amplitude du rythme MUA des SCNchez les animaux nocturnes et diurnes, en modulant son activité électrique, dans des directionsopposées. De plus, les *Arvicanthis* ayant un profil d'activité comportementale crépusculaire

présentent un profil MUA bimodal, avec des pics autour des transitions lumineuses, en correspondance avec des périodes d'activité locomotrice plus importantes. En revanche, chez les *Arvicanthis* ayant un profil d'activité uniquement diurne, le rythme MUA ne présente qu'un seulpic. Des enregistrements électrophysiologiques *in vitro* ont montré que le rythme endogène desSCN est unimodal, puisqu'il ne présente qu'un seul pic à la fois chez des *Arvicanthis* diurnes et crépusculaires. Ces données suggèrent que la bimodalité n'est pas une caractéristique intrinsèque des SCN, mais est plutôt conférée par des structures extra-SCN, qui peuvent également relayer la rétroaction comportementale (unimodale ou bimodale) aux SCN (Fig. D2).

Les mécanismes impliqués dans la transmission de la rétroaction de l'activité comportementale aux SCN ne sont pas clairement identifiés. Des données expérimentales suggèrent que les noyaux sérotoninergiques du raphé pourraient être impliqués. Les niveaux de sérotonine sont fortement corrélés avec l'état de vigilance et d'activité chez les rongeurs nocturnes et diurnes (Poncet, Denoroy et Jouvet 1993 ; Cuesta et al. 2008 ; Cuesta et al. 2009a ; Shioiri et al. 1991), et que la stimulation de l'activité locomotrice entraîne la libération de sérotonine dans les SCN chez les hamsters (Dudley, DiNardo et Glass 1998). De plus, la sérotonine affecte le rythme des SCN. En effet, la stimulation sérotoninergique est capable de décaler la phase des SCN (Bobrzynska, Godfrey et Mrosovsky 1996 ; Cutrera, Saboureau et Pévet 1996 ; Tominaga et al. 1992 ; Horikawa et al. 2000 ; Challet 2007) et la lésion des terminaisons sérotoninergiques des SCN altère l'entraînement par l'activité locomotrice chez la souris (Edgar et Dement 1991).

Par conséquent, nous avons testé l'hypothèse selon laquelle la sérotonine pourrait être impliquée dans la transmission du signal de rétroaction comportementale aux SCN. Pour cela, nous avons utilisé la fluoxétine, un inhibiteur sélectif de la recapture de sérotonine, pour modifier les niveaux physiologiques de sérotonine (section D1). De plus, nous avons également étudié l'effet de la sérotonine sur le système circadien d'*Arvicanthis* en examinant l'effet de la fluoxétine sur la période endogène de leur activité locomotrice (section D2).

D1. SHORT-TERM FLUOXETINE TREATMENT DECREASES THE AMPLITUDE OF THE SCN MULTI-UNIT ACTIVITY IN ARVICANTHIS ANSORGEI

Version edited in form of article, but needs to be completed before submission.

Title

Short-term fluoxetine treatment decreases the amplitude of the SCN multi-unit activity rhythm in *Arvicanthis ansorgei*

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Abstract

In mammals, all rhythmic functions are orchestrated by the suprachiasmatic nucleus (SCN). In *Arvicanthis*, a diurnal rodent, behavioral activity enhances the SCN firing rate. Numerous studies have shown the existence of reciprocal interactions between locomotor activity and serotonin, a neurotransmitter involved both in synchronizing the SCN and in maintaining wakefulness. Thus, we evaluated the effect of serotonin on the *in vivo* electrical activity of SCN and its role in relaying the behavioral activity feedback to the SCN. SCN multi-unit activity (MUA), as well as behavioral activity, were recorded continuously on freely-moving *Arvicanthis* for three baseline days, followed by five days of fluoxetine treatment provided in drinking water (5-6 mg/kg/day). Five days-fluoxetine treatment results in decreasing the amplitude of both the SCN MUA and behavioral activity. Furthermore, fluoxetine treatment gradually decreased the slope of the correlation between behavioral activity and MUA, but did not change the behavioral feedback magnitude. This study suggests the importance of a proper 5HT rhythm for the physiological functioning of the SCN. These findings in a diurnal rodent may have implications in the treatment of depression.

Keywords: Rhythm, Circadian, *Arvicanthis*, Diurnal, Nocturnal, Noradrenaline, Tyrosine Hydroxylase, Locus Coeruleus, Clock genes

Introduction.

The temporal organization of all physiological and behavioral functions is under the control of the central clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN receives inputs from external cues and allows the body to predict and adapt to environmental changes. The electrical activity of the SCN presents a sinusoidal shape with high electrical firing rate during the day and low during the night, in both nocturnal (Meijer et al. 1997; van Oosterhout et al. 2012) and diurnal (Sato and Kawamura 1984) species. The rhythmic electrical activity of the SCN is conveyed in rhythmic outputs, which regulate many physiological functions, including behavioral activity (Houben et al. 2009; Houben, Coomans, and Meijer 2014). While robust rhythms are necessary for healthy physiological functions (Manoogian and Panda 2017), a reduced amplitude of behavioral activity rhythms is associated with increased risk to develop pathological conditions, including major depressive disorder (MDD) (Lyall et al. 2018). In both diurnal and nocturnal rodents, the behavioral activity alters the electrical activity of the SCN. In nocturnal species, behavioral activity suppress the SCN firing rate, which is already low during the dark phase (i.e. active phase of the animal), thus increasing the amplitude of the SCN rhythm (van Oosterhout et al. 2012). In the diurnal *Arvicanthis*, behavioral activity increases the amplitude of the SCN as well, but by enhancing the SCN firing rate (unpublished data), which is high during the light phase (i.e. active phase of the animal) (Sato and Kawamura 1984; Bano-Otalora et al. 2021) (unpublished data). It is still not clear how the behavioral feedback is relayed to the SCN.

The SCN receives non-photic information *via* two major inputs namely the intergeniculate leaflet (IGL) and the raphe nuclei (RN) (Meyer-Bernstein and Morin 1996), which release serotonin (5HT). Numerous evidence suggest that serotonin may be involved in conveying the behavioral activity feedback to the SCN. First, serotonin is able to shift the phase of the SCN in both diurnal and nocturnal rodents, but in opposite astronomical windows (Challet 2007). Indeed, serotonin rhythm in the SCN is opposite between diurnal (peak during the day) (Cuesta et al. 2008) and nocturnal rodents (peak at night) (Barassin et al. 2002; Garabette, Martin, and Redfern 2000), but in both species 5HT levels correlate with the animal's behavioral phenotype. Moreover, induced behavioral activity increases 5HT release in the SCN (Dudley, DiNardo,

and Glass 1998). Additional evidence comes from an *in vitro* study, showing that application of 5HT 1a receptor agonist 8-OH-DPAT applied to the rat SCN neurons *in vitro*, decreases the neurons firing rate (Shibata et al. 1992), similarly to the effect measured *in vivo*, by behavioral activity.

This study was performed in diurnal *Arvicanthis* and, in order to test whether serotonin may be involved in conveying the behavioral feedback to the SCN, we disrupted serotonin levels by treating the animals with fluoxetine, a selective serotonin reuptake inhibitor (SSRI). SCN electrical activity and the animals' behavioral activity were recorded simultaneously. We then evaluated whether fluoxetine treatment had an effect on the SCN multi-unit activity (MUA), and whether it changed the relationship between SCN MUA and the animal behavioral activity.

Materials & Methods

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 (APAFIS Nr #6099-2016090216536741 v4). Adult male*Arvicanthis ansorgei* weighting 130-200g, were obtained from our breeding colony (Chronobiotron, UMS 3415, CNRS Strasbourg, France). All the animals were individually housed in Plexiglas cages, entrained to 12h light-12h dark (LD 12:12) cycle (light: 150 - 200 lux,dark: red dim light, <5 lux), and provided with food and water ad libitum.

Microelectrode implantation

To record the multi-unit electrical activity (MUA) of SCN neurons, tripolar stainless steel microelectrodes (Plastic One MS333-3-BIU-SPC, Roanoke VA, United State) were implanted in the SCN of *Arvicanthis* at a minimum of 3 months old. The procedure has been previously described for mice (van Oosterhout et al. 2012) and rats (Meijer et al. 1996). Briefly, animals were transferred to high cages two days before the surgery, to allow them to get used to the new cage of the recording setup. Surgery was performed with the use of a stereotaxic instrument, on animals under gaseous anesthesia (4% isoflurane with O_2) injected with 4 mg/Kg of Rompun (Bayer Pharma, Puteaux). For differential recording, two twisted electrodes (Polyamide-insulated; bare electrode diameter 125 µm) were aimed to the SCN (coordinates: 0.7 mm

anterior to bregma, 0.9 mm lateral to the midline, 7.8 mm ventral to the dura mater, 5° angle in the coronal plane) and a third uncoated electrode was placed in the cortex as reference. The differential amplifierwas based on the design of Yamazaki et al. (Yamazaki et al. 1998).

In vivo SCN electrical recordings and behavioral activity recordings

After a minimum of 5 days of recovery, animals were individually housed in recording cages, and connected to the recording system. *Arvicanthis* were able to freely move during the *in vivo* recording. The electrical signal was amplified, bandwidth filtered (500 – 5000 Hz), and window discriminators were used to convert action potentials into digital pulses that were counted in 1 second bins by Circa V1.9 software (OriginLab). The recoding set-ups allowed the simultaneous recording of the SCN electrical activity and the behavioral activity, thanks to the presence of a Passive Infra-Red (PIR) motion detector positioned 30 cm above the floor of the cage. The cage was equipped with a drinking sensor to monitor the animals' drinking activity.

Fluoxetine treatment protocol

Arvicanthis behavioral and electrical activity were recorded for three days of baseline (BL) and five days of fluoxetine treatment (FT). Fluoxetine hydrochloride (F132-50MG, Sigma Aldrich) treatment was administered to the animals in the drinking water, to minimize the stress. The daily amount of water consumption was measured during the BL, and fluoxetine concentration was adjusted in order to have a consumption between 5 and 6 mg/kg/day. Bottles containing fluoxetine were protected from light to avoid degradation. Fluoxetine treatment consumption was checked daily.

Histological microelectrode verification

After the fluoxetine treatment protocol, the location of the electrode implantation was verified. Animals were sacrificed by injecting an overdose of sodium pentobarbital (Euthasol VET, 182 mg/Kg) under gaseous anesthesia (4% isoflurane with O_2). An electrical current of 40 µA was applied through the recording electrode to deposit iron at the electrode tip. The brain were then collected and immersed in a solution containing 4% of paraformaldehyde with $C_6FeK_4N_6 \cdot 3H_20$ (SigmaAldrich, Lyon, France) for a minimum of 6 hours. Brains were then cryopreserved in 30% sucrose solution and frozen in cold (-30° C) isopentane for 1 min 20 sec. Brain coronal

sectionsof 40 µm were collected and stained with cresyl violet for microscopic verification of the implantation site (*Fig.1*).

Data analysis of in vivo electrophysiology

Multi-unit activity and behavioral activity were recorded simultaneously and acquired in 1s bins for three days of BL and five days of FT. All the analyses were performed comparing meandata of the last two FT days (day 4 and day 5) to the mean data of the three BL days, in seven *Arvicanthis,* unless otherwise stated.

Data were fitted by a non-linear regression using Cosinor analysis (SigmaPlot software, Jandel Scientific, Chicago, IL, USA). These analyses were performed using the following equation: $y = A + (B * \cos(2\pi(\times - C)/24) + (D * \cos(2\pi(\times - E)/12))$; where A is the mean level (mesor), B and D the amplitude and, C and E the acrophases of the rhythm. Crepuscularity index for behavioral activity was defined as follows: behavioral activity counts at the light transitions (morning transition: ZT21-ZT3, and evening transition: ZT9-ZT15) / total daily counts. Behavioral feedback was defined as follows: delta difference between the mean MUA values during activity and the mean MUA values during inactivity ($\Delta MUA_{(Act-Inact)}$); where MUA is expressed as percentage of the baseline MUA during inactivity. The behavioral feedback per unit of behavioral activity was defined as follows: behavioral feedback ($\Delta MUA_{(Act-Inact)}$)/behavioral activity total counts (BA); where BA is expressed as percentage of behavioral activity total counts measured at BL.

Statistical analysis

Data were analyzed either using one-way or two-ways analysis of variance with repeated measures (ANOVA I RM or ANOVA II RM), or by using paired t-test. All data are presented as mean±SEM, unless otherwise stated. Sigma Plot (v14) software and GraphPad Prism (v8) software were used for statistical analyses.

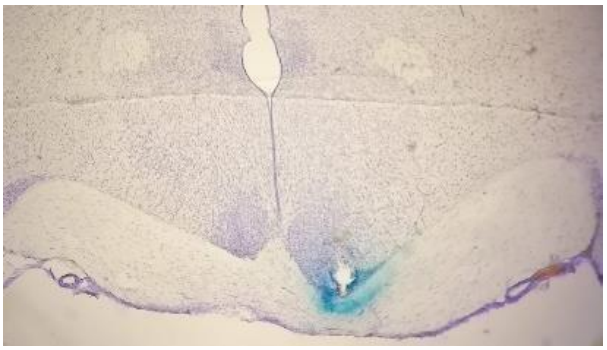

***Fig. 1*. Histological verification of electrode implantation site**. Example of a coronal section of the *Arvicanthis* SCN, located above the optic chiasm (OC) at the base of the hypothalamus. The electrode implantation site was checked using an electrolytic current and is visualized by the blue spot.

Results

Fluoxetine treatment effect on SCN multi-unit activity and behavioral activity rhythmic pattern

Fluoxetine treatment was performed successfully on seven *Arvicanthis,* in which SCN MUA and behavioral activity were simultaneously recorded (*Fig. 2*). Fluoxetine treatment had a significant effect on both MUA (treatment*time: p=0.034) and behavioral activity (treatment*time: p=0.011) pattern. Cosinor regression analysis of hourly MUA data revealed a sinusoidal rhythm of the SCN electrical activity during BL, fitted by a two-component (24h + 12h components) model, in each *Arvicanthis (*group fit shown in *Fig. 3A).* Fluoxetine treatment caused a loss of significance of the 24h component in three *Arvicanthis (*group fit shown in *Fig. 3B).* Animals' behavioral activity was characterized by a crepuscular pattern (crepuscularity index (CI); BL: 0.71±0.04, FT: 0.71±0.03), whose rhythm was fitted by a two-component (24h +12h) Cosinor regression in two *Arvicanthis* and by a one-component (12h) Cosinor regression in five *Arvicanthis,* both during BL (group fit shown in *Fig. 3C*) and FT *(*group fit shown in *Fig. 3D).* Fluoxetine treatment decreased significantly the mesor of the behavioral activity rhythm (p=0.011), but not the mesor of SCN MUA.

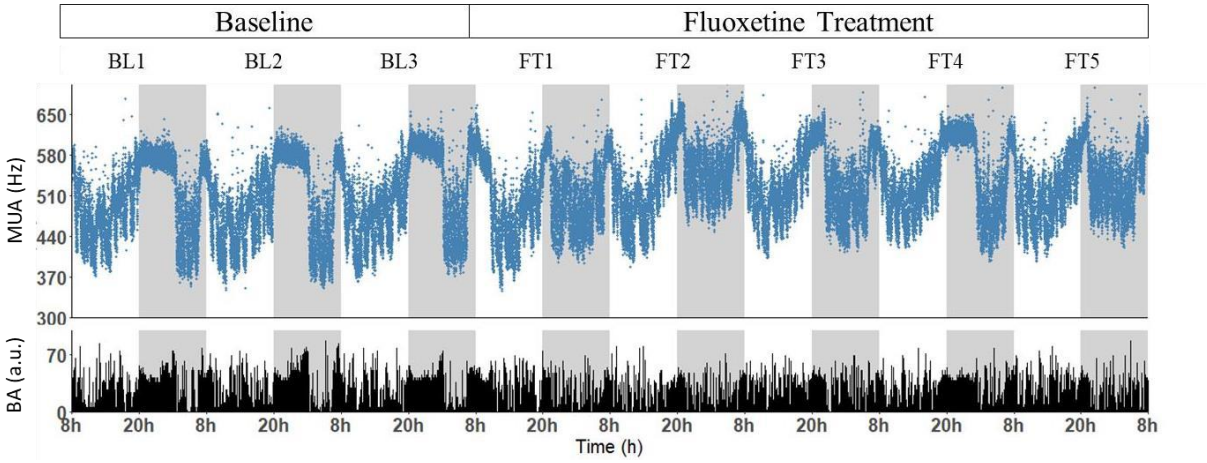

***Fig. 2. In vivo* SCN multi-unit activity and behavioral activity during baseline and fluoxetine treatment.** An example of the SCN electrical activity (blue) and behavioral activity (black) recordings in one *Arvicanthis* during three baseline days (BL1, BL2, BL3) and five days of treatment with fluoxetine (FT1 to FT5). The x axis is the time in hours, the y axis shows the discharge frequency (MUA)in Hz and the behavioral activity (BA) expressed in arbitrary units (a.u.). The gray background represents periods of darkness.

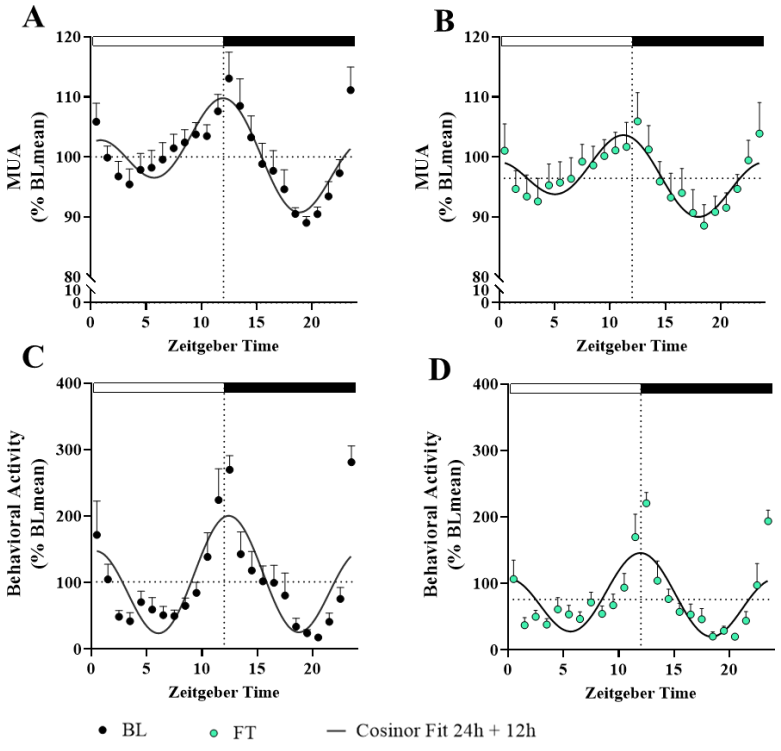

Fig. 3. Effect of Fluoxetine Treatment on MUA and behavioral activity rhythm. Cosinor regressions (24h+12h black solid line) of hourly data of electrical activity (MUA, A and B) and behavioral activity (BA, C and D) on baseline (BL) days (A and C) and on the day4 and day5 of fluoxetine treatment (FT) (B and D). Horizontal white and black horizontal bars represent light and dark phases, respectively. Time is expressed as Zeitgeber Time (ZT). Black dots in A and C represent the mean of 3 BL days in 7 *Arvicanthis*. Green dots in B and C represent the mean of day 4 and day 5 (7 *Arvicanthis*) of FT. All data are represented as percentage of the BL mean and are expressed as mean±SEM

Fluoxetine treatment effect on SCN multi-unit activity and behavioral activity rhythm amplitude and phase

Analysis of the sinusoidal electrical rhythm of the SCN revealed that fluoxetine treatment caused a significant decrease (p=0.04) of the peak-to-trough amplitude, from 24±3.6 % to 14.3±2.7 % of the mean values recorded during BL (*Fig. 4A*). The reduced amplitude of the rhythm was mainly due to the effect of the treatment on the amplitude of the 24h component, which was significantly reduced in four *Arvicanthis* (p=0.036), while was no longer significant in three *Arvicanthis (Fig. 4B).* The amplitude of the 12h rhythm did not differ significantly in FT compared to BL (*Fig. 4C*). Fluoxetine treatment reduced significantly (p=0.015) the peak-to-trough amplitude of the behavioral rhythm (*Fig. 4D*) as well, from 184.6±20.9 % to123.1±7.3 %, affecting the 12h component of the rhythm (p=0.024). Fluoxetine treatment did not cause a significant shift in the peak time of both MUA (*Fig. 5A*) and behavioral activity rhythms (*Fig. 5D*). However, the acrophase of the 12h component was significantly phase advanced both for MUA (*Fig. 5C*; p=0.016; BL ZT 11.9±0.4, FT ZT 11.1±0.4) and behavioralactivity (*Fig. 5F*; p=0.016; BL ZT 12.6±0.02, FT ZT 12.1±0.3).

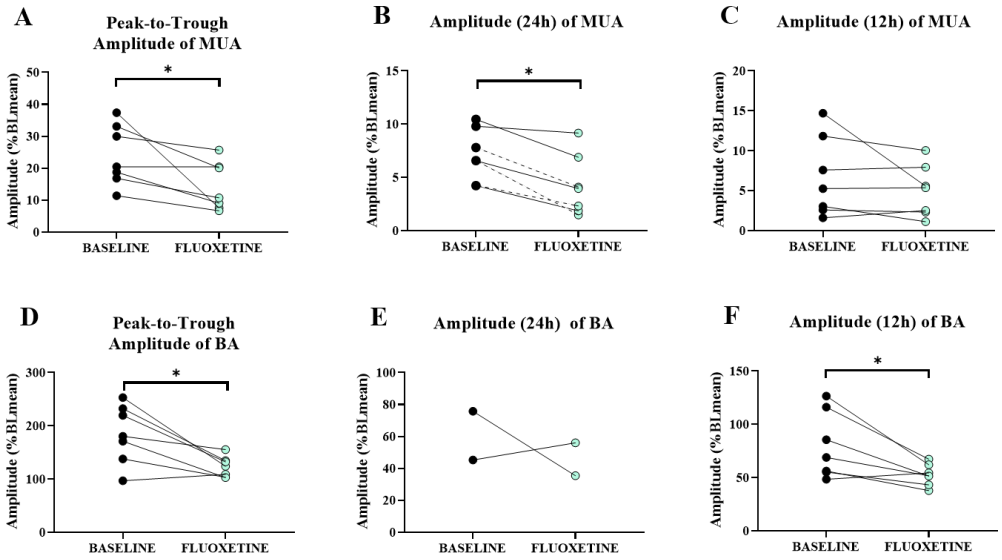

Fig. 4. Effect of Fluoxetine Treatment on MUA and behavioral activity amplitude. *Fluoxetine treatment reduced the peak-to-trough amplitude (A) of MUA sinusoidal rhythm, the amplitude of the 24h component of MUA rhythm (B), but not of the 12h component (C). Fluoxetine treatment reduced thepeak-to-trough amplitude (D) of behavioral activity (BA) sinusoidal rhythm, the amplitude of the 12h component of BA rhythm (F), but not of the 24h component (E). The difference between the baseline(black dots) and fluoxetine (green dots) data is expressed with one star (*) when p<0.05. For each animal, baseline and fluoxetine values are connected by a black line (significant Cosinor fit with fluoxetine treatment) or a black dashed line (non-significant Cosinor fit with fluoxetine treatment).*

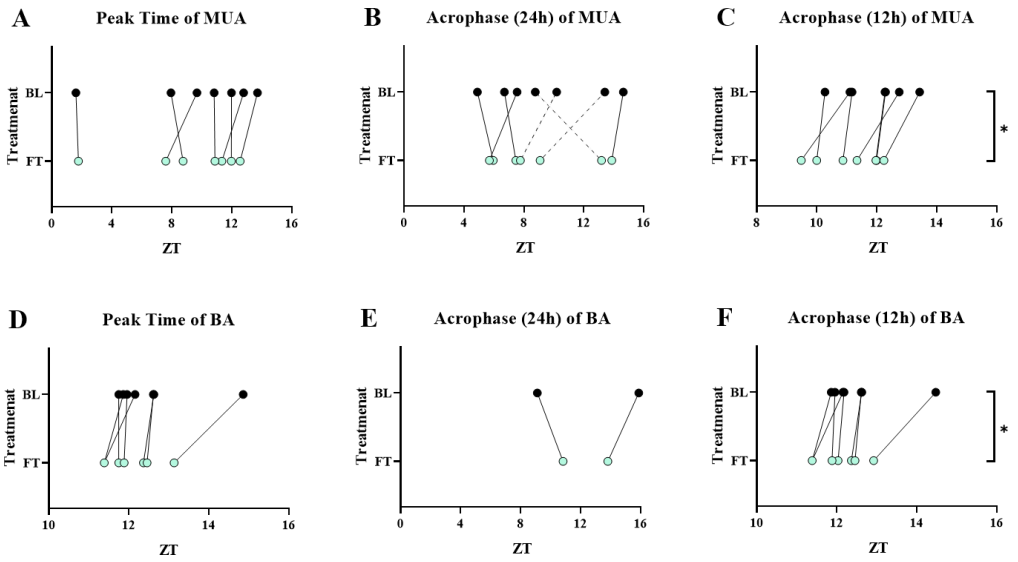

Fig. 5. Effect of Fluoxetine Treatment on MUA and behavioral activity phase. *Fluoxetine treatment did not modify the peak time (A) of MUA sinusoidal rhythm or the acrophase of the 24h component of MUA rhythm (B), but phase-advanced the 12h component (C). Fluoxetine treatment did not modify the peak time (D) of behavioral activity (BA) sinusoidal rhythm or the acrophase of the 24h component of MUA rhythm (E), but phase-advanced the 12h component (F). The difference between the baseline (black dots) and fluoxetine (green dots) data is expressed with one star (*) when p<0.05. For each animal, baseline and fluoxetine values are connected by a black line (significant Cosinor fit with fluoxetine treatment) or a black dashed line (non-significant Cosinor fit with fluoxetine treatment).*

Fluoxetine treatment effect on the behavioral feedback to the SCN multi-unit activity

To investigate whether serotonin may be involved in transmitting the behavioral feedback to the SCN, the correlation between MUA and behavioral activity was evaluated for BL and FT at day 3 (FT3) and FT at day 5 (FT5). For BL, there is a significant correlation between behavioral activity and MUA (R^2=0.416, p<0.001, slope=0.0631) *(Fig. 6A)*. The correlation is still significant for FT3 (R^2=0.152, p<0.001, slope=0.0494) *(Fig. 6B)* and for FT5 (R^2=0.152, p<0.001, slope=0.0098) *(Fig. 6C)*, but the slope of the correlation decreased gradually over the treatment.

To test whether fluoxetine treatment modified the magnitude of behavior-induced enhancement of SCN firing rate, more precisely if a unit of behavioral activity produced a similar increase in MUA at FT vs BL, the behavioral feedback per unit of behavioral activity (see materials & methods), was compared between BL and FT, showing no significant differences (*Fig. 7*).

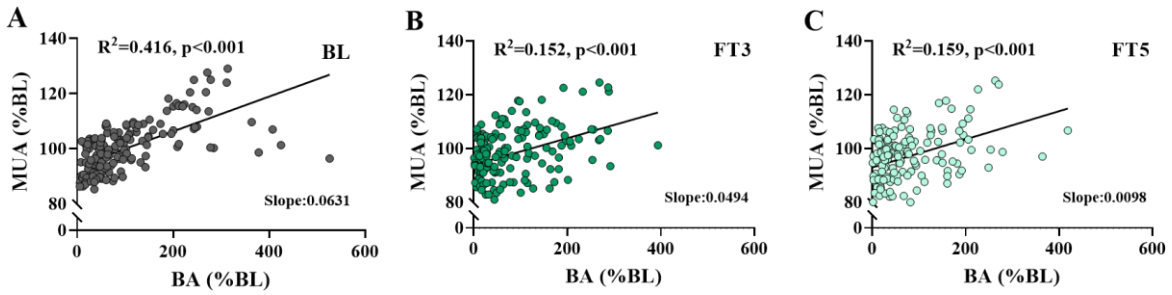

Fig. 6. Effect of Fluoxetine Treatment on the relationship between MUA-Behavioral Activity. Linear regression between MUA and behavioral activity (BA) hourly 24h data during baseline (BL, A) during day 3 of fluoxetine treatment (FT3, B), and during the day 5 of fluoxetine treatment (FT5, C). Fluoxetine treatment reduces the correlation between MUA and BA gradually throughout the treatment.

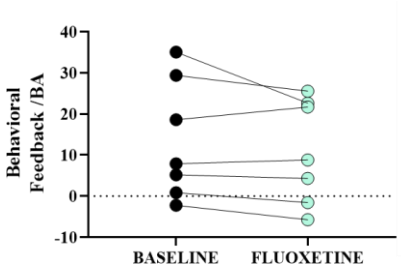

Fig. 7. Effect of Fluoxetine Treatment on the behavioral feedback. The magnitude of the behavioral feedback per unit of behavioral activity (BA) measured during baseline (black dots) was not significantly modified by the fluoxetine treatment (green dots). For each animal, baseline and fluoxetine values are connected by a black line.

Discussion

In this study, by disrupting physiological serotonin levels with fluoxetine, a serotonin selective reuptake inhibitor, we tested the hypothesis that serotonin signal may convey the behavioral activity feedback to the SCN. Fluoxetine treatment reduced the amplitude of the MUA rhythm, as well as the amplitude and the mesor of the behavioral activity rhythm. These data show the involvement of serotonin signaling in regulating the functioning of the SCN and suggest that a physiological serotonin 24h oscillation is crucial for a robust SCN firing rhythm. The magnitude of behaviorally-induced firing enhancement of the SCN was not significantly modified by fluoxetine treatment. More studies are necessary to confirm the serotonin role in relaying the behavioral feedback to the SCN.

Serotonin disruption decreases the amplitude of the SCN electrical activity and of behavioral rhythm

Fluoxetine treatment was provided to *Arvicanthis* for five days in the drinking water. Fluoxetine is the most common used antidepressant (Prozac), and it acts by blocking the serotonin reuptake, resulting in increasing serotonin levels in the synaptic cleft (Masand and Gupta 1999;Lorman 2018; Fuller and Wong 1987).

The pharmacokinetics and pharmacodynamics of fluoxetine and its active metabolite norfluoxetine, have been well documented in rodents (Caccia et al. 1990; Qu et al. 2009). In rats, after an acute oral dose of (5mg/kg) fluoxetine treatment, fluoxetine and norfluoxetine half-life in the plasma is around 7h and 15h, respectively (Caccia et al. 1990), and serotonin inthe frontal cortex is increased for 18h compared to baseline levels after an acute injection of fluoxetine or norfluoxetine (Qu et al. 2009).

Fluoxetine long-term treatment and effects differ from the short-term effects. Short-term administration of fluoxetine leads to increase extracellular serotonin levels and to the decrease the firing of raphe neurons acting to serotonin autoreceptors, such as 5HT1a and 5HT1b (Hjorth and Auerbach 1996; Gartside et al. 1995; Blier, Chaput, and de Montigny 1988). The result is a reduced 5HT release from RN (Artigas 1993). Indeed, side effects are often reported by patients during the first weeks of fluoxetine treatment (Schatzberg et al. 1987). Long-term treatment, due to chronic treatment during several weeks/months, leads to desensitization of serotonin autoreceptors, which reduces the feedback inhibition of RN firing and 5HT release, resulting in increased 5HT release and transmission (de Montigny, Chaput, and Blier 1990; Finley 1994; Blier, Chaput, and de Montigny 1988).

Therefore, in our study, with a short-term (5 days) fluoxetine treatment we expected an increase of serotonin levels in synaptic clefts, and a higher serotonin signaling in the SCN, in particular during the night, when serotonin physiological levels are low (Cuesta et al. 2008). In this study, fluoxetine treatment decreased the amplitude of the sinusoidal SCN rhythm, due to the 24h component of the rhythm. Considering that the daily levels of serotonin in the *Arvicanthis* SCN show a unimodal profile, with a peak during day (Cuesta et al. 2008), the reduced 24h amplitude of the SCN MUA rhythm is in accordance with the expected dampened amplitude of serotonin rhythm.

The SCN electrical activity is enhanced by the animal's behavioral activity (our group's unpublished data). In crepuscular *Arvicanthis*, behavioral activity increases the neuronal firing rate at the light/dark and dark/light transitions, conveying a bimodal rhythm to the SCN. In this study, the 12h component of MUA rhythm was not significantly modified by the fluoxetine treatment. However, fluoxetine treatment progressively decreased behavioral activity counts, as well as the rhythm amplitude.

The fluoxetine-dependent decrease of behavioral activity counts that we observed in this study is coherent with what has been previously described in rats (Silva, Alves, and Santarem 1999; Kennett et al. 1994), hamsters (Duncan et al. 2010), and mice strains that were not a model of depression (Dulawa et al. 2004). This effect may be explained by a 5HT feedback on the raphe, resulting in a transitory inhibition of the neuronal firing (Le Poul et al. 1995), which may affect arousal. Furthermore, it suggests that fluoxetine may cause different effects in animals with physiological 5HT rhythm, like the *Arvicanthis*, vs animal with disrupted 5HT levels, like in mice model of depression (Dulawa et al. 2004).

Altogether, these data provide evidence that fluoxetine affect the master clock and support the importance of a proper serotonin rhythm for the correct functioning of the SCN and behavioral rhythms.

Fluoxetine effect on SCN electrical activity and behavioral rhythm phase

Circadian rhythms are often altered in depression (Bunney and Bunney 2000) and improving depressive symptoms is associated with phase-advance circadian timing (Hickie et al. 2013).

The phase-advancing effect of fluoxetine on behavioral activity has been described in nocturnal and diurnal species (Cuesta et al. 2009; Cuesta et al. 2008), and fluoxetine in combination with L-tryptophan phase-advance the SCN-electrical activity *in vitro* (Sprouse, Braselton, and Reynolds 2006). In this study, we have shown that the 12h component of both behavioral activity and SCN MUA are phase-advanced with the fluoxetine treatment, but the overall sinusoidal rhythms are not. It is possible, however, that five days of fluoxetine treatment are not enough to produce a significant phase-advancing effect. The 12h rhythm is not an endogenous feature of the SCN, but is rather conveyed by areas that sends feedback signal, including the behavioral feedback. Therefore, these data suggest that exercise performed at the

appropriate time may be used as a strategy to phase advance the SCN and improve circadian rhythms.

Fluoxetine treatment and relationship between MUA and behavioral activity

Non-photic information reach the SCN through two major pathways, *via* the IGL and/or the RN (Meyer-Bernstein and Morin 1996). The serotonergic raphe nuclei is a promising candidate to convey the behavioral feedback to the SCN considering that serotonin release is increased by wheel running induced locomotor activity (Dudley, DiNardo, and Glass 1998) and serotonin shifts the phase of the SCN at opposite astronomical phases in nocturnal *vs* diurnal rodents (Challet 2007). Furthermore, serotonin levels correlate with behavior in nocturnal and diurnal rodents (Challet 2007), and behavior has opposite effects on SCN electrical activity (van Oosterhout et al. 2012) (our group unpublished data). By disrupting serotonin physiological levels with fluoxetine, we observed that the correlation between behavioral activity and MUA gradually decreased throughout the fluoxetine protocol administration. We then examined whether this decrease was due to changes in behavioral feedback. However, the magnitude of behaviorally-induced enhancement in SCN firing rate was not significantly modified by the fluoxetine treatment. We observed that there was an individual difference relative to the effect of fluoxetine on the behavioral feedback.We tried to correlate this difference in response to treatment first to the behavioral feedback at BL (some Arvicanthis show a high behavioral feedback, while in other it was close to 0), then to the position of the electrode implantation (Supplementary Fig. 1). However, no clear significant correlation was obtained before and after fluoxetine treatment. The median raphe nuclei directly project in the ventral SCN, so we expected to observe higher effect in the animals implanted ventrally (Meyer-Bernstein and Morin 1996). However, it is possible that difference may exist also based on the rostro-caudal position, as well as the medio-lateral position. A higher number of animals may help investigating whether behavioral feedback changes based on the SCN location, i.e. based on the neurons targeted, as well the effect of fluoxetine depends on that.

Conclusion

This study shows that disrupting serotonin levels affects the SCN firing amplitude and behavioral activity rhythm. It also demonstrate that fluoxetine treatment has an effect on the

master clock. These data add evidence to support the hypothesis that disturbances of the serotonergic system (as observed in depression) are accompanied by disruption of the SCN rhythm, and suggest that strategies aimed to improve circadian rhythms may be effective in depression. The role of serotonin as signal involved in transmitting behavioral feedback to the SCN requires further investigation.

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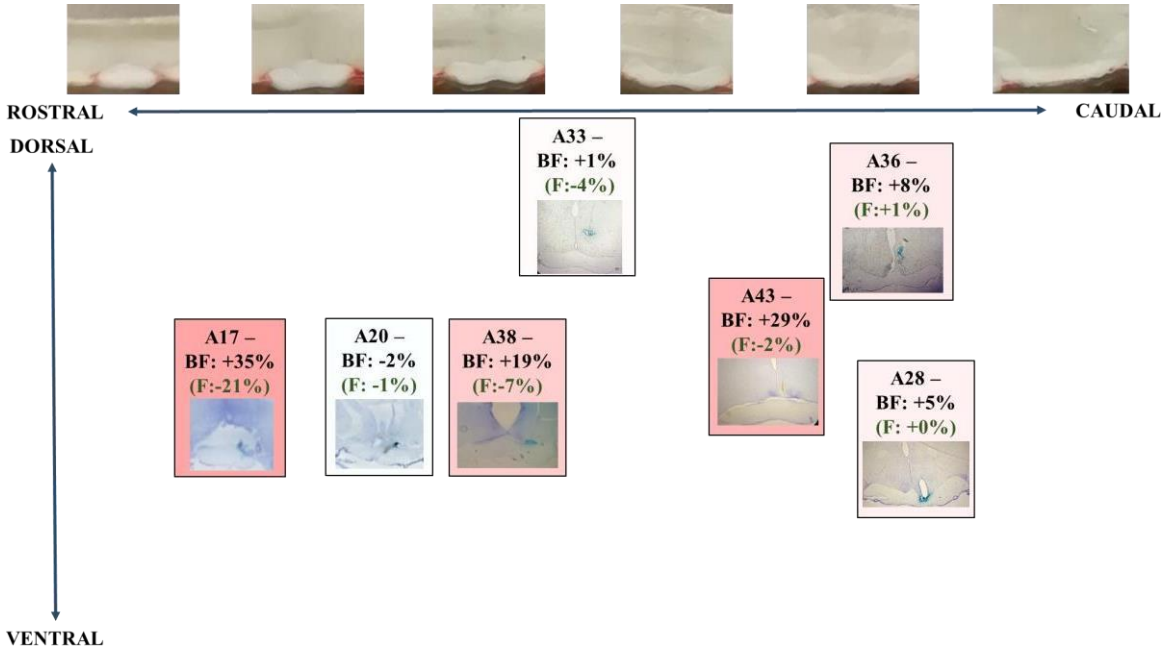

Supplementary Fig. 1. Site of implantation. Schematic representation of the site of implantation in the *Arvicanthis* SCN. Figures representing the site of implantation are positioned horizontally based on the rosto-caudal coordinate, and vertically based on the dorso-ventral coordinate within the SCN. Background color represent the magnitude of the behavioral feedback (BF) on the MUA (red: high BF, white low BF). Change in BF after fluoxetine treatment (F), is indicated as well.

D2. EFFECT OF FLUOXETINE TREATMENT ON CIRCADIAN PERIOD OF LOCOMOTOR ACTIVITY IN THE DIURNAL RODENT, IN *ARVICANTHIS ANSORGEI*

Version edited in form of article, but needs to be completed before submission.

Title

Effect of fluoxetine treatment on circadian period of locomotor activity in the diurnal rodent, *Arvicanthis ansorgei*

Authors

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Conflict of interest statement

The authors declared no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

Abstract

Fluoxetine is a serotonin selective reuptake inhibitor and is the most common treatment for major depressive disorder (MDD, depression). Circadian disturbances are often observed in depression, and chronobiotic effect of fluoxetine have been described in nocturnal rodents. Few data are available on fluoxetine in diurnal rodents. In this study, we tested the effect of fluoxetine on the circadian period of locomotor activity in the diurnal rodent *Arvicanthis.* Locomotor activity was monitored for seven days under 12:12 light/dark (LD) condition followed by fourteen days in constant darkness (DD), fourteen days of fluoxetine treatment (5-6 mg/kg/day) in DD, and fourteen days in DD post-fluoxetine treatment. Fluoxetine treatment did not change the endogenous period of locomotor activity in *Arvicanthis,* and decreased the

total activity counts. These data show that the effect of fluoxetine on circadian period in *Arvicanthis* may be different from nocturnal rodents.

Keywords: Fluoxetine, Circadian, Period *Arvicanthis*, Diurnal, Crepuscular, Serotonin

Introduction.

The serotonergic system is involved in regulating many physiological functions, including arousal, mood and circadian rhythms (Lucki 1998; Yannielli and Harrington 2004). Disruption of serotonin (5HT) levels are considered one of the principal factors involved in the etiology of mood disorders, including major depressive disorder (MDD, depression) (Germain and Kupfer 2008). Increasing data show that circadian disturbances are often reported by depressive patients. Sleep disturbances are always present in depression and are considered one of the criteria for the diagnosis of MDD (Murphy and Peterson 2015). In addition, a reduced amplitude of the locomotor activity rhythm is associated with an increased risk to develop depression (Lyall et al. 2018), and chronotherapy has been reported to alleviate depressive symptoms (Gorwood 2010). Reciprocal interactions have been described between the circadian system and the serotonergic system. Serotonin synthesis rhythm is driven by the daily surge of glucocorticoid, an output signal of the suprachiasmatic nucleus (SCN), the master clock (Malek et al. 2007). In turn, serotonin is able to shift the phase of the SCN, in opposite astronomical phases between diurnal and nocturnal rodents (Challet 2007).

Fluoxetine (Prozac), a serotonin selective reuptake inhibitor, is the first line treatment for depression. Fluoxetine block the serotonin transporter SERT, causing 5HT increase in the synaptic cleft. The effects of fluoxetine on the SCN functioning have been mainly described for acute administration. *In vitro* studies show that fluoxetine shortens the period of *Per1* in rat fibroblasts (Nomura et al. 2008) and induces phase advance of rat SCN firing, when L-tryptophan is provided (Sprouse, Braselton, and Reynolds 2006). Animal studies show that fluoxetine reduces the response to light-induced phase shift in nocturnal mice and hamsters (Challet et al. 2001; Gannon and Millan 2007), but potentiate light induced phase-shift at CT0 and CT12 in the diurnal *Arvicanthis* (Cuesta et al. 2008). These data suggest that fluoxetine in combination with light may have a different chronobiotic effect in diurnal and nocturnal species. While, administration of fluoxetine for fourteen days shortens the circadian period of wheel running activity in mice (Possidente et al. 1992), no data are available on the effect of fluoxetine on the circadian period in diurnal rodents.

In this study, we tested the effect of fourteen days of fluoxetine on the circadian period of locomotor activity in the diurnal *Arvicanthis.*

Materials & Methods

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. Eight adult (3.5 months old) male *Arvicanthis ansorgei* weighting 130-190g, were obtained from our breeding colony (Chronobiotron, UMS 3415, CNRS Strasbourg, France). All the animals were individually housed in Plexiglas cages, entrained to 12h light-12h dark (LD 12:12) cycle (light: 150 - 200 lux, dark: red dim light, <5 lux), and provided with food and water ad libitum.

Fluoxetine treatment protocol

Arvicanthis locomotor activity was recorded with infrared detectors placed above the cage and connected to an automated recording system (CAMS, Circadian Activity Monitoring System, Lyon, France). Locomotor activity was monitored for seven days under 12:12 light/dark (LD) condition followed by fourteen days in constant darkness (DD), fourteen days of fluoxetine hydrochloride (F132-50MG, Sigma Aldrich) treatment in DD, and fourteen days in DD post-fluoxetine treatment (Fig.1). Fluoxetine treatment was administered to the animals in the drinking water to minimize the stress. The daily amount of water consumption was measured during the DD preceding the treatment, and fluoxetine concentration was adjusted in order to have consumption between 5 and 6 mg/kg/day. Fluoxetine treatment was provided at day1 of treatment, replaced with fresh treatment at day 4, 7 and 11, and removed at the end of day 14. The bottle containing fluoxetine was weighted at the beginning, at the end of the treatment, and every time the treatment was replaced.

Data analysis and Statistical analysis

Data were collected in 5 min bins and analyzed with the ClockLab Software (Actimetrics, Wilmette, IL, USA) to measure circadian period (chi-square). Dara are visualized on double-plotted actograms (Fig. 2). Locomotor activity counts were analyzed using one-way analysis of variance with repeated measures (ANOVA I RM) with “treatment” as factor, followed by post-

hoc analyses. All data are presented as mean±SEM, unless otherwise stated. Sigma Plot (v14) software was used for statistical analyses.

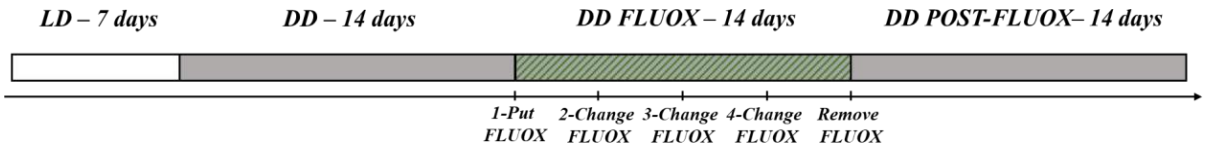

Fig. 1. Fluoxetine protocol: for each Arvicanthis locomotor activity was recorded for seven days in 12:12 light/dark (LD) condition (white bar), fourteen days in constant darkness (DD) (gray bar), fourteen days in DD with fluoxetine (FLUOX) in the drinking water (gray bar white green stripes), and fourteen days in DD after fluoxetine (POST FLUOX) (gray bar white green stripes). Fluoxetine was put on day1 of DD FLUOX, weighted and replaced four times throughout the fourteen days, and removed after fourteen days.

Results

Effect of fluoxetine treatment on locomotor activity period and locomotor activity counts

In DD condition all Arvicanthis presented a free running period shorter than 24h (*Fig 2-3*; $p<0.001$, 23.80±0.05 h). Fluoxetine treatment did not change significantly the period of locomotor activity (23.82±0.04 h). Locomotor activity period after fluoxetine treatment (23.85±0.09 h) was similar to that measured in DD and in DD plus fluoxetine.

Locomotor activity counts were similar in DD compared to LD condition. Fluoxetine treatment in DD significantly decrease activity counts of 23.5% compared to that in DD ($p=0.036$) and of 24.6% compared to LD ($p=0.024$) (*Fig. 3B*). Locomotor activity counts after fluoxetine treatment were not significantly different neither from DD, nor from fluoxetine treatment, showing that cessation of the treatment leads to re-increase locomotor activity.

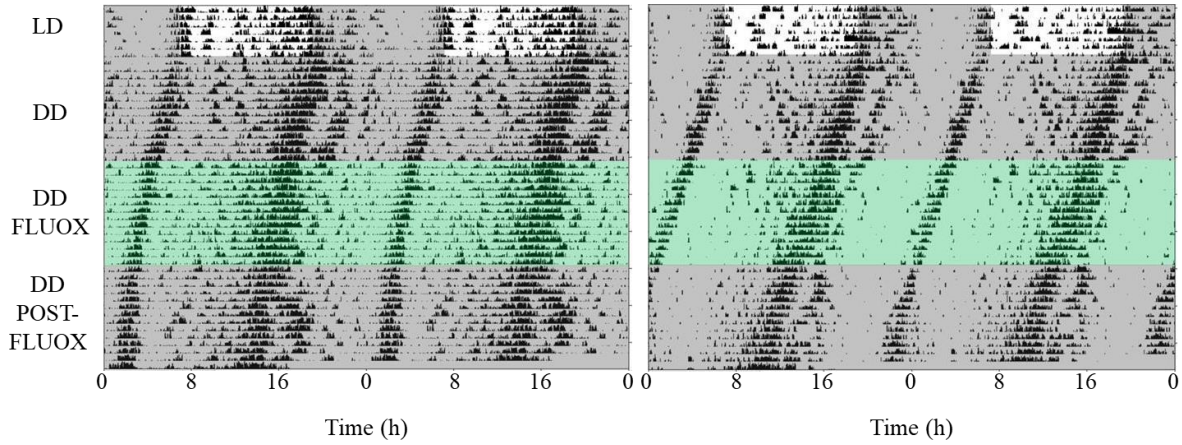

Fig. 2. Actograms of two *Arvicanthis* for seven days in 12:12 light (white background) /dark (gray background) (LD) condition, fourteen days in constant darkness (DD) (gray background), fourteen days in DD with fluoxetine (FLUOX) in the drinking water (green background), and fourteen days in DD after fluoxetine (POST FLUOX) (gray background). Time is expressed in hours (h).

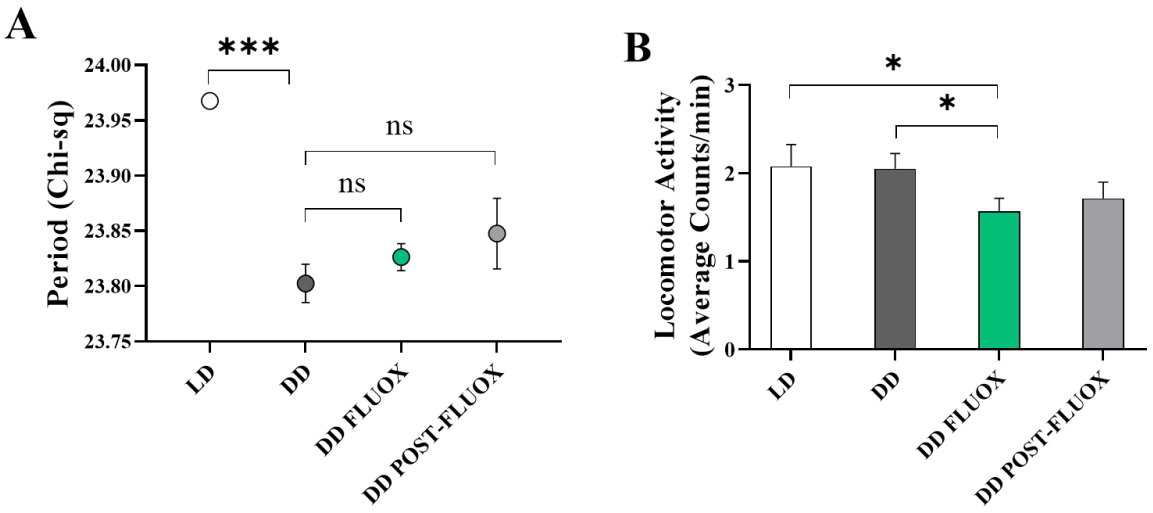

Fig. 3. Locomotor activity period in *Arvicanthis* under 12:12 light /dark (LD) condition (white circle), in constant darkness (DD) (dark grey circle), in DD with fluoxetine (FLUOX) (green circle), and in DD after fluoxetine (POST FLUOX) (light gray circle). One Way Repeated Measures Analysis of Variance (ANOVA I RM) shows that free running period in DD is significantly shorter than in LD (***$p<0.001$), but fluoxetine had no significant effect (n.s.) (A). Locomotor activity counts in *Arvicanthis* under 12:12 light /dark (LD) condition (white bar), in constant darkness (DD) (dark grey bar), in DD with fluoxetine (FLUOX) (green bar), and in DD after fluoxetine (POST FLUOX) (light gray bar). ANOVA I RM shows that locomotor activity counts are significantly reduced by fluoxetine compared to LD (*$p<0.05$), and DD (*$p<0.05$)(B). The protocol was performed on 8 *Arvicanthis.* Data are presented as mean±SEM

Discussion

In this study, we show that two weeks of fluoxetine treatment do not modify significantly the period of locomotor activity in *Arvicanthis.* The lack of effect observed in our study is in contrast with what have been shown in mice, in which fluoxetine shortens the circadian period of wheel running activity (Possidente et al. 1992). We expected to observe an increase of the circadian period, in opposition to what has been observed in mice. The lack of effect may be also due to the dose that we used (5-6 mg/kg/day), which is lower than the dose used in mice (8 mg/kg/day) (Possidente et al. 1992). However, this dose has been used by other authors andhas been shown to significantly increase serotonin levels in the brain (Van Dyke et al. 2019; Bouet et al. 2012). Hence, it is possible that in diurnal species under constant darkness the effectof fluoxetine is not sufficient to produce a significant phase shift of locomotor activity.

However, fluoxetine treatment leads to a significant decrease in the animal's locomotor activity counts, which tended to re-increase after the end of the treatment. This effect is in agreement with what have been shown in other studies. Chronic fluoxetine treatment reduces locomotor activity in non-anxious mice (C57BL/6 and 129SvEv), but not in anxious mice (BALB/c and DBA/2) (Abe et al. 1998), and two weeks fluoxetine treatment inhibits wheel running activity in hamsters (Duncan et al. 2010). The decrease in locomotor activity observed, may be due to the activation of 5HT autoreceptors in the raphe nuclei consequent to the elevated 5HT levels. This leads to transiently decrease the raphe neurons firing rate, contributing to decrease arousal (Hjorth and Auerbach 1996; Gartside et al. 1995; Blier, Chaput, and de Montigny 1988).

This study shows that in opposition to what has been observed in nocturnal mice, our treatment of fluoxetine does not modify the circadian period of locomotor activity in *Arvicanthis.* More studies in diurnal animals are required to confirm this finding. In addition, the effect of fluoxetine should be tested in constant light condition, in view of the fact that fluoxetine potentiates the light-induce phase shift in acute in *Arvicanthis* (Cuesta et al. 2008). Investigating more about fluoxetine effect on circadian rhythm, in combination with different light paradigms, on a diurnal rodent, may provide additional information to improve antidepressant strategies.

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I. DISCUSSION AND PERSPECTIVES

The main objective of this thesis was to investigate whether circadian differences characterizing structures of the arousal system may be involved in determining the different temporal organization between nocturnal and diurnal rodents. Therefore, using the diurnal crepuscular rodent *Arvicanthis ansorgei* and the nocturnal rat, two arousal structures namely the raphe nuclei and the locus coeruleus were studied, and their rhythmic functioning compared (see *Fig. 11*, which summarizes the functional and reciprocal links in rats and *Arvicanthis* between circadian system and arousal structures). The differences highlighted in these two monoaminergic structures between diurnal and nocturnal rodents show that the use of diurnal species may be fundamental in research fields analyzing pathologies linked to the sleep/wake cycle or to mood disorders, in which monoamines play a central role. Moreover, to understand the circadian regulations within the raphe nuclei and its role in connecting behavior and the circadian system, we investigated mechanisms that may be at the origin of raphe nuclei rhythmic functioning (*i.e*. clock gene expression, hormonal regulation by glucocorticoids) and we evaluated the effect of serotonin disruption on the SCN electrical activity.

A. RELATIONSHIP BETWEEN MONOAMINE AND BEHAVIORAL ACTIVITY RHYTH – DIURNAL VS NOCTURNAL RODENTS

In this thesis, we have investigated the synthesis of two monoamines, i.e. serotonin (5HT) in the raphe nuclei (RN), and noradrenaline (NA) in the locus coeruleus (LC). Serotonin and noradrenaline synthesis were investigated by considering the respective rate-limiting enzymes, tryptophan hydroxylase 2 (*tph2*) and tyrosine hydroxylase (*th*). RN and LC are two arousal structures involved in inducing and maintaining wakefulness, and based on data mainly coming from nocturnal rodents, their activity changes in accordance with the behavioral (sleep/wake) state of the animal (Sakai 2011; Gervasoni et al. 1998). At the same time, these structures are involved in the regulation of circadian rhythms. The serotonergic RN modulates the SCN directly *via* a projection originating from the MRN, and indirectly *via* the DRN-IGL-SCN pathway (Meyer-Bernstein and Morin 1996; Moga and Moore 1997; Card and Moore 1989). Indeed, serotonin modulates the phase of the SCN and its synchronization to light (Yannielli and Harrington 2004; Bobrzynska, Godfrey, and Mrosovsky 1996; Cutrera, Saboureau, and Pévet 1996; Tominaga et al. 1992; Challet 2007). The LC plays a role in regulating the amplitude of the sleep/wake cycle, as demonstrated by LC-lesioned animals, in which the sleep/wake cycle amplitude in LD is decreased (González and Aston-Jones 2006).

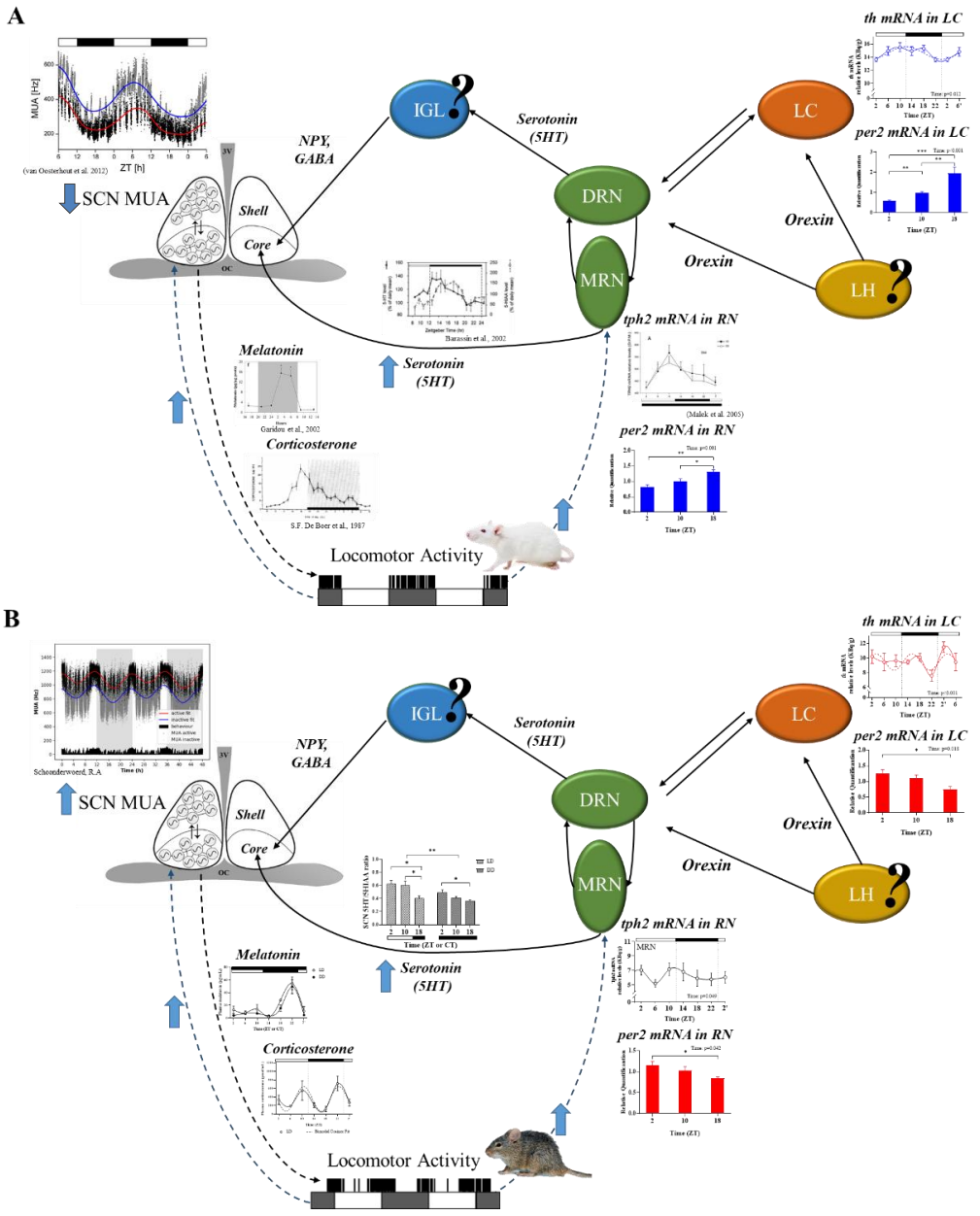

Fig. 11. Reciprocal interconnections among the suprachiasmatic nucleus (SCN), the arousal structures namely dorsal raphe nuclei (DRN), median raphe nuclei (MRN) and locus coeruleus (LC), and locomotor activity, in the diurnal *Arvicanthis* (A) and in the nocturnal rat (B). Functioning of the DRN, MRN, and LC display opposite rhythms between Arvicanthis and rats, as shown by tryptophan hydroxylase 2 *(tph2)*, tyrosine hydroxylase *(th)*, and *per2* mRNA rhythms. The dorsal raphe nucleus projects indirectly to the SCN through the intergeniculate leaflet (IGL), while the median raphe nucleus sends direct projections to the SCN, releasing serotonin (5HT). Serotonin signaling correlates with behavioral rhythm, being higher at night in rats and during the day in *Arvicanthis*. The SCN synchronizes peripheral rhythms through outputs, including melatonin (similar in rats and *Arvicanthis*), and corticosterone (unimodal rats and bimodal in *Arvicanthis*), whose peak anticipates the activity-arousal period. The SCN multi-unit activity (MUA) is higher during the day and lower during the night in diurnal and nocturnal rodents. MUA is reduce by locomotor activity in nocturnal rodent, while it is enhanced in the diurnal *Arvicanthis*. The differential rhythmic interconnections between diurnal and nocturnal rodents with the IGL and the orexinergic lateral hypothalamus (LH) are still unknown.

One of the main aims of this study was to assess whether the rhythmic functioning of these two arousal structures correlates with behavioral rhythm in both nocturnal and diurnal species, and may therefore participate to their opposite temporal organization. Here, we show that serotonin and noradrenaline synthesis, both correlate with the animal's behavioral state, not only in the nocturnal rat, but also in the diurnal *Arvicanthis (Fig. 12).* In the rat, a unimodal circadian rhythm of *tph2* mRNA levels, with peak expression at the end of the (subjective) day has been previously described (Malek et al. 2005). In accordance, here we have shown that also *th* mRNA levels in the LC display a circadian rhythmicity, with peak values during the (subjective) day. Interestingly, in *Arvicanthis* the rhythmic expression of *th* and *tph2* mRNA is not merely opposite compared to that described in rats, but correlate with the crepuscular behavioral phenotype of *Arvicanthis*, following bimodal rhythms. In addition, the effect of light on *th* and *tph2* rhythm appears to be different between the rat and the *Arvicanthis.* As regard *th,* light phase delay the peak of *th* endogenous rhythm in rats, while it causes a phase advances of the two peaks in *Arvicanthis*. As regard *tph2,* light have no significant effect in rats, but causes a phase shift of *tph2* mRNA peak levels in *Arvicanthis*. These data show clearly that light signal have a different impact on these structures in nocturnal and diurnal species, and this may be partly due to different circuits modulating and conveying light information to the RN and the LC (Shen and Semba 1994; Adidharma et al. 2019; Adidharma, Leach, and Yan 2012; Gompf and Aston-Jones 2008). Up to now, pathway tracing studies have not shown direct connections between the retina and the RN in *Arvicanthis* (Adidharma, Leach, and Yan 2012; Gaillard, Karten, and Sauvé 2013) contrary to the rat (Shen and Semba 1994), so it is conceivable that indirect pathways may relay light information to the RN and LC. These differences are likely to participate to the switch between diurnality and nocturnality.

The peculiar bimodal shape of *tph2* and *th* rhythm in *Arvicanthis* arousal nuclei leads to raise questions about the mechanisms regulating their expression according to this bimodal rhythm. One hypothesis is that *tph2* and *th* expression rhythms are regulated at the transcription levels by bimodal hormonal signals. Considering the bimodal shape of corticosterone plasma levels, as well as evidence in nocturnal rodents showing the corticosterone involvement in *tph2* rhythm (Malek et al. 2007; Vincent et al. 2018), it is likely that glucocorticoid signaling may be involved at least in *tph2* bimodality. However, the precise molecular mechanisms involved havenot been described yet. In this project, we have tried to elucidate whether *tph2* rhythm may be dependent on GR-mediated transcription regulation. Our analyses *in silico* do not show the presence of conserved GREs (5′-AGAACAnnnTGTTCT-3) (Strähle, Klock, and Schütz 1987)

in *Tph2* gene sequence. This does not exclude the presence of GBSs with sequence variations (La Baer and Yamamoto 1994; So et al. 2008; Meijsing et al. 2009). Thus, we focused first our experiments with in vitro cell line approaches, but limitations due to the model used, the RN46A cell line, do not currently allow us to answer to this question. Other experiments on rat raphe tissue, which include the use of the CUT&Tag technique to target GRs bound to the DNA, are ongoing. The question remains open for the mechanisms involved in *Arvicanthis*.

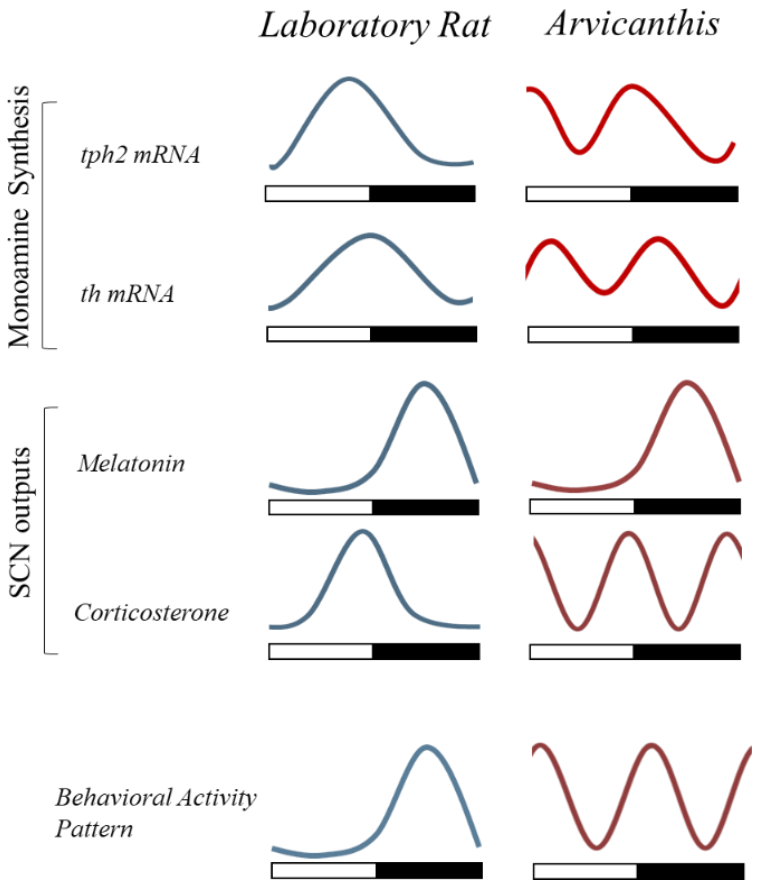

Fig. 12. Schematic representation of rhythmic expression of *tph2* and *th* mRNA expression, in relation to SCN outputs rhythm (melatonin and corticosterone), and to behavioral activity rhythm in the nocturnal rat and the diurnal *Arvicanthis*, respectively.

As regard *th* transcription regulation, GRE have been described in the promoter of the tyrosine hydroxylase gene (Hagerty et al. 2001), and *th* mRNA expression can be induced by dexamethasone treatment, as showed by us in RN46A cells and by Hagerty and colleagues in a mouse cell line (Hagerty et al. 2001). Moreover, here we show that in both RN and LC, clock genes are rhythmically expressed, and are in phase opposition in the *Arvicanthis* compared to

the rat. It is possible therefore, that *th* and *tph2* rhythms may be in part due to the clock machinery, and that modulation of these rhythms occur through output signal of the SCN, like glucocorticoids. In *Arvicanthis*, investigating the complete 24h LD and DD rhythm of clock genes could reveal two peaks and account for the bimodal rhythm of *th* and *tph2*. Hence, more studies are necessary to uncover mechanisms of *th* and *tph2* regulation in both diurnal and nocturnal species.

B. THE RAPHE NUCLEUS AS A CLOCK AND INTEGRATION OF THE GLUCOCORTICOID SIGNAL

The serotonergic raphe nuclei, thanks to its multiple neural connections and the diversity of its receptors, are involved in many physiological functions, such as appetite, mood, cognition, and arousal (Lucki 1998; Yannielli and Harrington 2004). All these functions present daily variations, suggesting that the raphe nuclei may participate to their rhythmicity. Rhythmic functions in the raphe nuclei have been previously described and include the rhythms in 5HT neurons firing activity (Sakai 2011; Jacobs and Azmitia 1992), serotonin release (Barassin et al. 2002), *tph2* protein and mRNA rhythm (Malek et al. 2005; Malek, Pévet, and Raison 2004; Nexon et al. 2009), and 5HT transporter (Ushijima et al. 2005). At a molecular level, rhythms are generated by a self-sustained clock machinery, consisting in positive and negative loops (Welsh, Takahashi, and Kay 2010; Takahashi 2017). Besides being present in the SCN, clock genes are described in many other structures of the brain and in peripheral organs, where they also show oscillating expression (Partch, Green, and Takahashi 2014). Currently, no study has shown the presence of clock genes in the raphe nuclei. Here, we have shown that rhythmic clock genes are expressed in the raphe nuclei both in the nocturnal rat and the diurnal *Arvicanthis*. The presence of rhythmic clock genes suggests that the clock machinery may coordinate rhythmic functions in the raphe nuclei. The most described mechanism to generate molecular rhythms involves CLOCK:BMAL1, which regulate gene transcription by binding toE-boxes on their promoter (Rey et al. 2011). However, other mechanisms may contribute to gene rhythmic expression, such as epigenetic modifications, which may modify the chromatin state and favor RNA polymerase II (RNAPII) recruitment (Koike et al. 2012; Le Martelot et al.2012), or circadian control on mRNA stability by regulating poly(A) tail length (Kojima, Sher-Chen, and Green 2012). These mechanisms, together with the presence of other components ofthe clock machinery, should be further investigated in the raphe nuclei.

Besides being under the control of the clock machinery, rhythmic functions within the raphe may be synchronized by timing signals coming from the master clock. It is the case of glucocorticoids, which have been shown to drive the rhythmic expression of *tph2 (Malek et al. 2007).* The mechanisms involved in glucocorticoid-dependent *tph2* mRNA rhythm are so far unknown, but evidence shows that the glucocorticoid receptor (GR) is involved (Vincent et al. 2018). Therefore, the first hypothesis is that glucocorticoid-GR complex may regulate *Tph2* transcription by binding to a glucocorticoid response element in the *Tph2* gene promoter. This hypothesis can be investigated by the use of the Cleavage Under Targets and Tagmentation (CUT&Tag) technique (Kaya-Okur et al. 2019), targeting the glucocorticoid receptor. This technique allows investigating the presence of a protein bound to the DNA by a specific antibody. It requires small amount of cells, so that even small structures may be investigated without the necessity to pool animal samples (ongoing experiment).

The second hypothesis is that clock genes may be involved in *tph2* transcription regulation. It is possible that clock genes, whose rhythms may be directly controlled by the glucocorticoid-GR complex, are responsible for *tph2* rhythmic expression. Indeed GRE have been described both on the promoter region of *Per1 (Yamamoto et al. 2005),* and an overlapping GRE with an E-box (GE*2*) in the promoter of *Per2* (Cheon et al. 2013). This hypothesis can be initially investigated by looking for the presence of E-Boxes in the *Tph2* gene promoter *in silico*, and the subsequent use of epigenetic tools, such as the Chromatin Immuno Precipitation or the CUT&Tag tecniques, targeting CLOCK or BMAL1 (Hatanaka et al. 2010).

Furthermore, to investigate whether the glucocorticoid signal is integrated by the clock machinery and is necessary for a proper rhythmic synchronization within the raphe, adrenalectomy may be performed, and clock gene daily expression may be measured. Moreover, a genome wide analysis of BMAL1 or CLOCK targets may help screening for the genes whose rhythmic transcription is regulated by clock genes. This experiment may contribute to evaluate to which extent rhythms within the raphe nuclei may be under the control of the clock machinery.

The use of both diurnal and nocturnal rodents may be helpful to understand if the integration of output signals within the raphe nuclei and clock gene-driven rhythmic mechanisms are common in both species, or if differential mechanisms may contribute to the switch between nocturnality and diurnality.

C. ORIGIN OF BIMODALITY

The use of a crepuscular rodent like the *Arvicanthis* opens an additional question, *i.e.* the origin of the animals' bimodality.

Based on the temporal niche they occupy, animals are usually categorized broadly as nocturnal or diurnal. However, mammals' rest/activity and sleep/wake patterns present a rich variety of phenotypes (McNamara et al. 2008; Siegel 2005). Indeed, the shape of mammals' activity profile ranges from unimodal with a single activity peak at day (diurnal species) or at night (nocturnal species), to bimodal, with peaks of activity at dawn and dusk (crepuscular species) (Refinetti 2008).

The temporal organization of rest and activity episodes is under the control of the SCN. However, the functioning of SCN, such as phase relationships of electrical activity and clock gene expression, in diurnal or nocturnal rodents do not present major differences (Challet 2007; Sato and Kawamura 1984; Meijer and Rietveld 1989). Therefore, it is possible that mechanisms determining the preference for a specific temporal niche involve areas outside the SCN.

Animals can regulate their activity patterns to cope with predictable environmental variations such as temperature changes and optimize periods of foraging and protection from predators. (Katandukila et al. 2013; Davimes et al. 2018; McCauley et al. 2012).

However, the mechanisms underlying and initiating bimodality are not clear. The crepuscular behavioral phenotype has been described in many diurnal rodents, such as the *Arvicanthis niloticus* (Katona and Smale 1997), *Octodon degus* (García-Allegue et al. 1999), Mongolian gerbils (Weinert, Weinandy, and Gattermann 2007), as well as in the Drosophila (Chang 2006). One of the hypothesis is that bimodality may be caused by environmental conditions, such as the temperature. Indeed, both *Arvicanthis ansorgei* and *Arvicanthis niloticus* come from Africa, in which temperatures are usually high, especially in the middle of the day. Therefore, bimodality in these rodents could be the result of a protection mechanism from elevated temperature, like in the *Arabian oryx* (Davimes et al. 2018), and act as a Zeitgeber as shown in the dromedary camel (Farsi et al. 2020). However, *Arvicanthis niloticus* has a unimodal locomotor activity in the wild (Blanchong et al. 1999) and other rodents do not come from areas where the temperature is as high as in Africa. Therefore, temperature may not fully explain the origin of bimodality.

An additional hypothesis is that a two-oscillator model would be responsible for bimodality. It has been shown that Syrian Hamsters exposed to constant light (LL) condition become bimodal. This phenomenon, called *splitting*, is characterized by two components of locomotor activity that diverge, resulting in a phase difference of 180° (12h) (Daan and Pittendrigh 1976). It has been shown that this phenomenon is caused by the SCN, in which the right and left nuclei start functioning in anti-phase. Indeed, in hamsters FOS expression shows a 24h rhythm in both SCN, but in antiphase between the right and left SCN (Butler et al. 2012).

In *Arvicanthis*, besides locomotor activity other variables are bimodal. We have shown that serotonin and noradrenalin synthesis are bimodal, as well as corticosterone plasma levels, and temperature rhythm (Cuesta et al. 2009a). However, melatonin plasma levels are unimodal. Thus, the question was to investigate whether the bimodality directly depends on endogenous features of the SCN. *In vivo* and *in vitro* recording of SCN electrical activity show that bimodality is not initiated by the SCN. Indeed, in *Arvicanthis*, the SCN electrical activity shows a bimodal pattern *in vivo*, in correlation with the crepuscularity of the animal. *In vitro,* however, the electrical activity of SCN from crepuscular animals is unimodal (unpublished data), suggesting that the bimodality recorded *in vivo* is conveyed by extra-SCN structures. These data support the hypothesis that the temporal niche of an animal may not be initiated by the SCN, but it rather shows that the SCN may integrate feedback signals, resulting in generating bimodal rhythmic activity.

The SCN electrical activity is modified by the animals' behavioral activity (van Oosterhout et al. 2012) (unpublished results). In *Arvicanthis,* the crepuscular pattern of the animals' behavioral activity correlates with the sinusoidal shape of the SCN MUA rhythm. Serotonin signaling may be involved in conveying the behavioral feedback to the SCN, contributing to the SCN bimodal rhythm. Increasing evidence supports this hypothesis, including the effect of locomotor activity to stimulate serotonin release in the SCN (Dudley, DiNardo, and Glass 1998), and the correlation between serotonin levels and behavioral state, both in diurnal and nocturnal rodents (Poncet, Denoroy, and Jouvet 1993; Cuesta et al. 2008; Cuesta et al. 2009a; Shioiri et al. 1991). In addition, lesions of serotonin terminals in the SCN, prevent a proper locomotor activity entrainment in mice (Edgar and Dement 1991). In this thesis, we showed that five-day treatment with a serotonin reuptake inhibitor, fluoxetine, affects the rhythm of the SCN electrical activity by reducing its amplitude. The treatment decreased the correlation between the MUA and behavioral activity, but no changes in the behavioral feedback magnitude were measured. This lack of significant effect may be due to the heterogeneity of behavioral

feedback observed in control condition, maybe connected with the site of electrode implantation. Therefore, more studies need to be performed in order to elucidate serotonin effect on the SCN in a diurnal rodent, and its role in conveying the behavioral activity feedback.As a next approach, the effect of serotonin receptor agonists or antagonists may be investigated*in vitro.* Among the serotonin receptors that may be involved in mediating the serotonin signaling in the SCN, the $5HTR_{1a}$ and the $5HTR_{7}$ may be tested. Indeed, Cuesta and colleaguesshowed that injection of (+)8-OH-DPAT, an agonist of the $5HTR_{1a/7}$ is able to phase advance the wheel-running activity at the end of the subjective day and during the subjective night (Cuesta et al. 2008). Application of 8-OH-DPAT to the rat SCN neurons *in vitro,* decreases theneurons firing rate (Shibata et al. 1992), similarly to the effect caused by behavioral activity *invivo*. In *Arvicanthis,* behavioral activity enhances the SCN electrical activity *in vivo.* Application of specific agonist such as the WAY-161503 for $5HTR_{2c}$ (Rosenzweig-Lipson et al. 2006), the AS19 for $5HTR_{7}$ (Liu et al. 2021), or specific antagonists like the SB 242084 for $5HTR_{2c}$ (Kennett et al. 1997), and the SB 269970 for $5HTR_{7}$ (Liu et al. 2021), may help elucidate the effect of specific 5HT receptors on the SCN electrical activity and rhythm, and lead to select a pharmacological strategy to be further tested *in vivo*.

In vivo administration of specific 5HT antagonists may provide an additional tool to test whether serotonin is involved in relaying the behavioral feedback to the SCN, as well in mechanisms contributing to the bimodal electrical SCN rhythm in crepuscular Arvicanthis.

However, the signals that feedback to the SCN may be either bimodal themselves, or unimodal but in antiphase. Serotonin and 5HIAA concentrations in the *Arvicanthis* SCN display a unimodal rhythm and peak in the second half of the day. However, the serotonergic signaling, besides being conveyed directly through the MRN projection, is also transmitted indirectly through the IGL, that release NPY and GABA (Moga and Moore 1997). It is possible that signals coming from the IGL and from the MRN may not be in phase, thus contributing to the SCN bimodality.

D. A DIURNAL MODEL: IMPLICATION FOR DEPRESSION

The understanding of the circadian system and its interaction with other systems come principally from nocturnal rodents, such as rats, hamsters or mice. However, increasing emerging evidence shows that while endogenous features of the SCN are similar between

diurnal and nocturnal rodents (Caldelas et al. 2003; Dardente et al. 2004; Sato and Kawamura 1984), signals that feedback to the SCN, as well as their integration in the SCN rhythm may strongly differ (Challet 2007; Cuesta et al. 2008). It is the case of the serotonergic system, whose difference between diurnal and nocturnal species have been described in the previous paragraphs.

Deficiency of serotonin or alteration of the serotonergic system are well recognized to be one of the principal causes of depression (Germain and Kupfer 2008), as highlighted by the widespread use of antidepressants targeting the serotonergic system, such as fluoxetine (Prozac) (Masand and Gupta 1999; Lorman 2018; Fuller and Wong 1987). As already described, reciprocal connections exist between the circadian and the serotonergic systems and perturbation of circadian rhythms have also been described in the pathophysiology of depression. Depressed individuals often report sleep disturbances, including insomnia, hypersomnia, poor sleep quality, early morning awakening (Murphy and Peterson 2015). In addition, reduction in the amplitude of activity rhythms is associated with an increased risk to develop depression (Lyall et al. 2018). In agreement, by using fluoxetine to disrupt physiological serotonin levels in *Arvicanthis*, we have shown that the amplitude of the SCN electrical rhythm is dampened, as well as the amplitude of the animal behavioral activity. These data support the hypothesis that in depression one of the causes leading to disturbances of daily rhythm is directly linked to the SCN rhythm.

Other studies, conducted mainly in nocturnal rodents suggest that fluoxetine has a chronobiotic effect. Fluoxetine shortens the period of *Per1* oscillation *in vitro* in rat fibroblasts (Nomura et al. 2008), induces phase advance of rat SCN firing *in vitro* (Sprouse, Braselton, and Reynolds 2006), normalizes disrupted locomotor activity rhythm and clock genes expression in depressive-like mice (Schaufler et al. 2016), and reduces the response to light-induced phase shift in mice and hamsters (Challet et al. 2001; Gannon and Millan 2007).

In contrast, in the diurnal *Arvicanthis* fluoxetine injections potentiate light induced phase-shift at CT0 and CT12 (Cuesta et al. 2008), showing that serotonin effect on the circadian system, as well as serotonin modulation of light, are different between diurnal and nocturnal species. In mice, fluoxetine administration in DD shortens the period of locomotor activity (Possidente et al. 1992), while we showed that it has no effect in *Arvicanthis.*

Another approach used to improve the symptomatology of depression is light therapy, which consists in exposing patients to bright light in the morning, usually for 30–90 min (Terman and

Terman 2005). Our data in *Arvicanthis* suggest that light may have a stronger impact in diurnal than nocturnal rodents. Indeed, in *Arvicanthis tph2* mRNA profiles differ between DD and LD conditions, while it is similar in nocturnal rats. In addition, overall serotonin, 5HIAA and their ratio are decreased in DD compared to LD conditions in *Arvicanthis.*

In many nocturnal animals, light reaches the raphe nuclei through a direct projection from the retina (Shen and Semba 1994; Kawano, Decker, and Reuss 1996; Fite and Janusonis 2001), while a direct projection in *Arvicanthis* has not been described (Adidharma, Leach, and Yan 2012; Gaillard, Karten, and Sauvé 2013). In diurnal animals, light signal reaching the serotonergic system may be modulated by other arousal structures, such as the orexinergic system, which is involved as well in regulating sleep and arousal (Kilduff and Peyron 2000). In *Arvicanthis niloticus* it has been shown that light activation of the dorsal raphe nucleus is mediated by the orexinergic neurons (Adidharma, Leach, and Yan 2012), which act *via* the orexin receptors OXR1s (Adidharma et al. 2019). In *Arvicanthis niloticus*, the orexinergic system project to the RN (Novak and Albers 2002). This projection has been also described in other nocturnal rodents, including rats (Peyron et al. 1998), and hamsters (Mintz et al. 2001). However, the distribution of orexin receptors *ox1r* and *ox2r* mRNA in areas involved in the regulation of the sleep/wake cycle and mood differs in diurnal and nocturnal rodents (Ikeno and Yan 2018), and may therefore convey different signals.

Considering that the orexinergic system is modified by light intensity (Lonstein et al. 2021) and that reduction of the orexin signaling is associated with increased depression-like behavior (Deats et al. 2014), future studies should investigate the interactions between the orexinergic and the serotonergic systems in diurnal rodents.

Altogether these data suggest that the effect of light, as well as the effect of antidepressant treatments, such as fluoxetine, on arousal structures and the SCN, should be investigated more in diurnal rodents, in order provide more effective strategies against mood disorders.

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Summary

In mammals, all biological rhythmic functions are orchestrated by the master clock located within the suprachiasmatic nucleus (SCN). Depending on whether an animal is nocturnal or diurnal, behavioral rhythms (such as the sleep/wake cycle) occur at opposite phases with respect to the light/dark (LD) cycle, whereas the SCN is always active at the same astronomical time. The mechanisms involved in the opposite temporal organization between nocturnal and diurnal species are mostly unknown.

The main hypothesis of this thesis is that structures of the arousal system, which receive timing information from the SCN and send it feedback signals, may be involved. Therefore, we investigated and compared the rhythmic functioning of two arousal structures, namely the serotonergic (5HT) raphe nuclei (RN) and noradrenergic (NA) locus coeruleus (LC), in the nocturnal rat and the diurnal *Arvicanthis ansorgei.*

The first aim was to investigate the rhythmic functioning of NA system in rats and *Arvicanthis* LC, quantifying the NA rate limiting enzyme, tyrosine hydroxylase (*th*) mRNA. In 12:12 LD and in constant darkness (DD) conditions, *th* mRNA levels were rhythmic in both species. However, the pattern of *th* expression was different, *i.e.* unimodal in rats and bimodal in *Arvicanthis* in accordance with their respective behavioral patterns.

The second aim was to investigate the rhythmic functioning of the serotonergic RN in *Arvicanthis*, and comparing our finding with published data in rats. The mRNA levels of tryptophan hydroxylase 2 (*tph2),* 5HT rate limiting enzyme, displayed a bimodal rhythm in LD, whose pattern was modified in DD, underlying the effect of light on the serotonergic system. This finding is in opposition to the unimodal *tph2* rhythm in rats, whose profiles are similar in LD and DD.

The bimodal *th* and *tph2* mRNA profiles in *Arvicanthis* reflected the crepuscular locomotor activity of the animals, as well as bimodal corticosterone plasma levels, but not melatonin rhythm. Finally, 5HT/5HIAA ratio within the SCN of *Arvicanthis* was higher during the day (active phase) compared to the night (rest period), in opposition to what has been shown in rats (high 5HT levels at night). The pattern of clock genes (*bmal1, per1,* and *per2*) mRNA in both LC and RN was opposite between rat and *Arvicanthis,* in relation to the astronomical phase. These data suggest that the different LC and RN rhythms between rat and *Arvicanthis* may contribute to opposite temporal organization between nocturnal and diurnal species.

The third aim was to investigate molecular mechanisms responsible for *tph2* mRNA rhythm. As in rats, corticosterone surge has been shown to drive *tph2* rhythm, the role of glucocorticoids (GC) on *Tph2* transcription was investigated. The effect of dexamethasone, a glucocorticoid receptor agonist, on *tph2* expression was assessed in a serotonergic cell line, the RN46A. Dexamethasone treatment had no effect on *tph2* expression. Analyses of differentiation markers revealed that the RN46A cell line is not fully differentiated towards a serotonergic phenotype. Further experiments using rat RN samples are ongoing to test GC-dependent rhythmic expression of *tph2*.

The fourth aim was to assess the effect of behavioral activity feedback on the SCN multi-unit activity

(MUA), knowing that the SCN MUA rhythm is higher during the day and lower during the night in both nocturnal and diurnal rodents, and that in nocturnal rodent behavioral activity decreases the SCN firing rate. *In Arvicanthis*, we showed that behavioral activity, occurring mainly during the day, enhanced the SCN firing rate. Moreover, since (i) reciprocal interconnections between the 5HT system and behavioral activity has been described, (ii) 5HT is one of the non-photic signals synchronizing the SCN, the involvement of 5HT in relaying the behavioral feedback to the SCN was investigated. To do so, we disrupted 5HT levels by treating *Arvicanthis* with fluoxetine, a 5HT reuptake inhibitor, and investigated changes in SCN MUA and behavioral activity. Fluoxetine treatment decreased the amplitude of both MUA and behavioral activity rhythms, suggesting the importance of 5HT rhythm for a robust SCN oscillation. Individual differences relative to the behavioral feedback were observed in response to the fluoxetine treatment, which did not significantly modify the magnitude of the behaviorally-induced enhancement of the SCN firing rate. Finally, we investigated the effect of fluoxetine on the circadian period of locomotor activity, in *Arvicanthis*. In contrast to the effect described in nocturnal mice, in which fluoxetine shortens the circadian period of wheel running activity, in *Arvicanthis*, fluoxetine did not modify the circadian period of locomotor activity.

Altogether, these data show that the rhythmic functioning of arousal structures, as well as their interactions with the SCN, is different between diurnal and nocturnal animals, and may participate to their different temporal organization. To provide more effective strategies against circadian-related pathologies in humans, more studies are required in diurnal rodents, which should explore additional differences relative to the interconnection between the arousal and the circadian system.

Résumé

Introduction

L’ensemble des fonctions rythmiques circadiennes est orchestré par l’horloge principale située au sein des noyaux suprachiasmatiques (SCN). La perturbation des rythmes circadiens conduit au développement de nombreux dysfonctionnements et pathologies comme par exemple la dépression. La plupart des connaissances en chronobiologie a été obtenue chez les rongeurs nocturnes (comme les rats, souris et hamster). Cependant, dans le domaine biomédical ces connaissances sont difficiles à appliquer directement à l'Homme, qui est diurne. Pendant mon projet de thèse, j'ai utilisé un modèle animal de rongeur diurne, *Arvicanthis ansorgei*, élevé au laboratoire.

Selon qu'un mammifère est nocturne ou diurne, les rythmes comportementaux (tels que le rythme veille-sommeil) se produisent dans des phases opposées par rapport au cycle lumière-obscurité (LD), alors que les SCN sont actifs au même moment astronomique chez ces deux espèces. L'hypothèse principale de mon projet de thèse est que des structures en aval des SCN, c'est-à-dire qui reçoivent des informations en provenance des SCN ou qui envoient des signaux de rétroaction aux SCN, peuvent contribuer à une organisation temporelle différente entre les nocturnes et les diurnes. Parmi ces structures, nous avons porté notre attention aux Noyaux du Raphé (RN) et au Locus Coeruleus (LC), deux structures qui appartiennent au système de l'éveil et qui sont également impliqués dans les rythmes circadiens. Au sein du système circadien, la sérotonine (5-HT), produite dans les RN, est capable de déphaser les SCN chez le rat nocturne et chez l’*Arvicanthis*. De plus, au niveau des SCN, la 5-HT présente une variation rythmique sur 24h, dont la phase est opposée chez les rongeurs diurnes par rapport aux rongeurs nocturnes et ce rythme est en corrélation avec la phase d’éveil des animaux. Concernant le Locus Coeruleus qui synthétise la noradrénaline, des études électrophysiologiques, notamment, ont mis en évidence son fonctionnement rythmique en lien avec les états de vigilance des animaux.

Objectifs

1- Chez le rat nocturne, il a été montré que le rythme de la sérotonine est lié au rythme de sa synthèse. En effet, la tryptophane hydroxylase 2 (*tph2*, enzyme qui détermine le taux de sérotonine) présente un rythme circadien de son niveau d'ARNm. On ne sait pas si la synthèse de la sérotonine chez les animaux diurnes est rythmique et en opposition de phase par rapport au rythme décrit chez le rat nocturne. Ainsi, le premier objectif de mon projet de

thèse est d'étudier l'existence d'un rythme circadien de la synthèse de la sérotonine, en quantifiant l'expression de la *tph2*, dans les noyaux du Raphé d'*Arvicanthis*.

2- Les neurones sérotoninergiques du Raphé sont étroitement interconnectés avec les neurones noradrénergiques du Locus Coeruleus et ces deux structures sont impliquées dans le contrôle et le maintien de l'éveil chez les mammifères. A ce jour, nous ne savons pas si la synthèse de noradréline (NA) est rythmique. Ainsi, le deuxième objectif de mon projet est d'étudier l'expression sur 24h de la tyrosine hydroxylase (*th*), l'enzyme limitante de la synthèse de noradrénaline, dans le Locus Coeruleus, chez le rat et chez l'*Arvicanthis*. De plus, nous avons étudié l'expression de gènes d'horloge dans le LC, qui pourraient être à l'origine d'un rythme de *th*.

3- Les mécanismes impliqués dans le contrôle du rythme de *tph2* sont peu connus, mais des résultats obtenus chez le rat montrent que les glucocorticoïdes seraient impliqués. Le troisième objectif de mon projet est donc de déterminer si l'action des glucocorticoïdes dans le raphé s'effectue via une régulation de la transcription du gène *tph2*.

4- Quel que soit le phénotype (diurne ou nocturne) d'une espèce, l'activité électrique des SCN est plus élevée pendant le jour que pendant la nuit. L'activité locomotrice modifie l'activité électrique des SCN. Chez le rongeur diurne *Arvicanthis,* l'activité locomotrice augmente la fréquence de décharge des SCN, contrairement au rat nocturne, chez lequel elle la diminue. Les mécanismes impliqués dans la rétroaction de l'activité comportementale aux SCN ne sont pas connus. D'autre part, de nombreux travaux ont montré l'existence d'interactions réciproques entre activité locomotrice et sérotonine. Ainsi, le quatrième objectif de mon projet est d'évaluer si la rétroaction de l'activité comportementale aux SCN est médiée par la sérotonine.

Résultats

1- Etude de la synthèse rythmique de la sérotonine chez *Arvicanthis*

Chez le rat nocturne, la protéine et l'ARNm tph2 sont circadiens, attestant d'une synthèse rythmique de 5-HT. Ainsi, le premier objectif était de tester si les niveaux d'ARNm-tph2 sont également rythmiques chez *Arvicanthis*, et si ce rythme est opposé par rapport aux rats nocturnes. De plus, des signaux de sortie des SCN hormonaux (corticostérone, mélatonine) et comportementaux (activité locomotrice) ont été étudiés en tant que marqueurs de la phase circadienne et en tant que modulateurs potentiels du rythme de tph2. Nous avons observé : (i) un rythme significatif des niveaux d'ARNm tph2 en conditions LD et en obscurité

constante (DD); (ii) une différence significative entre les profils d'ARNm tph2 en LD et DD alors qu'elle est similaire chez les rats nocturnes; (iii) un profil d'ARNm tph2 bimodal (2 pics/24h) alors que l'expression de tph2 est unimodale chez le rat (un pic/24h) ; (iv) un rythme bimodal dans le profil de corticostérone et l'activité locomotrice en LD et DD, alors qu'ils sont unimodaux chez les rats nocturnes ; (v) un profil de mélatonine rythmique en LD et en DD avec un pic la nuit comme chez les rats nocturnes. Ces données démontrent que chez *Arvicanthis*, le fonctionnement du système sérotoninergique est rythmique et influencé par les conditions lumineuses, mais diffère de celui du rat.

2- Etude de la synthèse rythmique de la noradrénaline chez le rat nocturne et *Arvicanthis*

Le LC est l'une des principales structures d'éveil et il est fonctionnellement interconnecté avec le Raphé. Plusieurs études ont montré que le LC noradrénergique fonctionne de manière rythmique: l'activité de décharge neuronale des neurones noradrénergiques est caractérisée par un rythme circadien (fréquence de décharge plus élevée pendant la veille) et des niveaux de noradrénaline dans le cerveau montrant un rythme sur 24h. A notre connaissance, l'existence d'une rythmicité journalière et circadienne de la synthèse de la NA dans le LC n'a pas encore été établie. Pour répondre à cette question, nous avons quantifié l'ARNm de la tyrosine hydroxylase (*th*), l'enzyme limitante la synthèse de NA, dans des conditions LD et DD, à la fois chez le rat nocturne et chez l'*Arvicanthis* diurne. Nous avons constaté que ces niveaux d'ARNm varient significativement sur 24h chez les deux espèces, à la fois en LD et en DD. Chez les rats, le profil d'ARNm th montre un rythme unimodal, avec des valeurs maximales précédant la nuit subjective, alors que chez *Arvicanthis*, ce rythme d'ARNm th est bimodal (niveaux élevés en milieu de jour et de nuit subjectives). Ce rythme circadien de *th* retrouvé dans le LC du rat et de l'*Arvicanthis* est donc soit sous le contrôle des SCN qui assurent la distribution des messages circadiens par l'intermédiaire de sorties neuronales ou endocriniennes, soit lié à l'expression de gènes horloges au sein même du LC. Nous avons évalué cette dernière possibilité en quantifiant l'expression des gènes horloge *per1, per2* et *bmal1* dans le LC du rat et de l'*Arvicanthis*. Nous avons observé que ces gènes horloges sont exprimés dans le LC des deux espèces et que leur rhtyhm est opposé.

3- Etude des mécanismes impliqués dans le contrôle du rythme *tph2* : rôle des glucocorticoïdes

Chez le rat, les glucocorticoïdes influencent la synthèse de 5HT en modulant le rythme d'ARNm *tph2*. De plus, le récepteur des glucocorticoïdes (GR) est exprimé dans les RN et la délétion du gène GR dans le RN dorsal augmente l'expression de l'ARNm *tph2* chez les souris GR floxées. Ces données suggèrent que les glucocorticoïdes pourraient réguler la

transcription de *tph2* via le GR. Par conséquent, nous avons examiné si un élément de réponse aux glucocorticoïdes (GRE) était présent dans la séquence du promoteur *tph2* et s'il contrôle la transcription de *tph2*. Pour tester cette hypothèse, nous avons utilisé une approche *in vitro* et une *ex vivo*. Tout d'abord, nous avons utilisé une lignée cellulaire issue du mésencéphale de rat, les cellules RN46A-B14, pour évaluer l'effet d'un traitement à la dexaméthasone (un agoniste de GR) à différentes concentrations, sur l'expression de *tph2*. Aucun effet significatif de la dexaméthasone n'a été mesuré sur la transcription de *tph2*. Cependant, nos vérifications quant au phénotype 5-HT de ces cellules montrent qu'elles ne sont pas entièrement et exclusivement différenciées vers un phénotype neuronal sérotoninergique. Nous avons donc par la suite évalué l'effet de la dexaméthasone sur des explants de cerveau de rat prélevés au niveau des Noyaux du Raphé. Enfin, dans une étude complémentaire menée sur des cellules du Raphé de rats, nous avons évalué la présence d'une séquence GRE en utilisant la technique CUT&Tag (Analyse en cours).

4 – Étude du rôle de la sérotonine dans la transmission de la rétroaction de l'activité comportementale aux SCN chez *Arvicanthis*

Dans cette étude menée sur *Arvicanthis*, nous avons émis l'hypothèse que la rétroaction de l'activité comportementale aux SCN (augmentation de l'activité de décharge des neurones) était médiée par la sérotonine. Pour cela, nous avons mesuré l'activité électrique des neurones des SCN in vivo (multi-unit activity) avant et après traitement à la fluoxétine. La fluoxétine, un inhibiteur sélectif de la recapture de la sérotonine, est utilisé ici pour perturber le système sérotoninergique et ainsi évaluer l'implication de la 5-HT dans cette rétroaction. L'activité électrique neuronale ainsi que l'activité locomotrice ont été enregistrées *in vivo*, en continu sur des *Arvicanthis* vigiles et libres de leurs mouvements pendant 8 jours. Les 3 premiers jours correspondent à l'enregistrement de la situation contrôle, suivis de 5 jours de traitement à la fluoxétine, administrée dans l'eau de boisson. Pendant les jours contrôles, le rythme d'activité électrique est caractérisé par un rythme bimodal et il est corrélée avec l'activité locomotrice bimodale des *Arvicanthis*. L'administration de fluoxétine entraine une diminution de l'amplitude du rythme de l'activité neuronale. De plus, on observe une diminution significative de l'activité locomotrice des animaux. Enfin, la régression linéaire positive et significative mise en évidence pendant les jours contrôles entre activité électrique et activité locomotrice, mais elle n'est pas modifiée de manière significative après fluoxétine. Ces données démontrent que la sérotonine modifie le rythme de décharge des SCN et influence l'activité locomotrice et l'activité neuronale des SCN.

Conclusions

Dans l'ensemble, les données obtenues au cours de ma thèse suggèrent que les structures du système d'éveil, en particulier les noyaux du Raphé et le Locus Coeruleus peuvent contribuer à l'organisation temporelle différente entre les espèces nocturnes et diurnes. Plus en détails :

1- La synthèse de la sérotonine est rythmique et circadienne chez l'*Arvicanthis* diurne, ainsi que chez le rat nocturne, mais le profil rythmique diffère sur 24h, en corrélation avec l'état d'éveil de l'animal. Cela montre que la 5-HT peut contribuer au changement de phénotype diurne vs nocturne.

2- La synthèse de NA est rythmique sur 24h à la fois chez le rat nocturne et chez l'Arvicanthis diurne, mais le profil d'expression diffère, suggérant que le système noradrénergique, comme le système sérotoninergique, puisse contribuer à l'organisation temporelle différente entre les espèces nocturnes et diurnes. De plus, la présence d'une machinerie d'horloge dans le LC suggère que l'expression de *th* pourrait être sous le contrôle de gènes d'horloge.

3- Chez le rat, l'expression de tph2 est circadienne et dépendante du rythme des glucocorticoïdes. La transcription de tph2 pourrait être régulée via des séquences GRE dans le promoteur *tph2*, sans exclure d'autres mécanismes et notamment, l'expression rythmique de gènes horloges dans les Raphés qui pourraient contribuer au rythme de tph2.

4- Le traitement à la fluoxétine diminue l'amplitude des rythmes de MUA et d'activité comportementale, suggérant l'importance du rythme 5HT pour une oscillation robuste des SCN. Des différences individuelles relatives à la rétroaction comportementale ont été observées en réponse au traitement à la fluoxétine, qui n'a pas modifié de manière significative l'effet de la rétroaction comportementale sur l'activité électrique des SCN.

Pour améliorer les stratégies développées contre les pathologies liées aux troubles du rythme circadien chez l'homme, d'autres études sont nécessaires chez les rongeurs diurnes, en lien notamment, avec les interconnexions existantes entre le système de l'éveil et le système circadien.

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Système Circadien chez les Rongeurs Diurnes et Nocturnes : Rôle du Système de l'Eveil

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Résumé

Chez les mammifères, les fonctions rythmiques sont orchestrées par les noyaux Suprachiasmatiques (SCN). Selon qu'un animal soit nocturne ou diurne, les rythmes d'activité/repos se produisent à des phases opposées, alors que les SCN sont actifs au même moment astronomique. Nous avons étudié le rôle de deux structures du système de l'éveil, les noyaux sérotoninergiques (5HT) du Raphé (RN) et noradrénergiques (NA) du Locus Cœruleus (LC) dans l'organisation temporelle entre les espèces nocturnes et diurnes. Nous avons montré que la synthèse de 5HT et de NA, et l'expression des gènes d'horloge dans le RN et le LC, sont opposées entre l'*Arvicanthis* diurne et le rat nocturne, et corrélées avec leurs profils comportementaux respectifs. De plus, nous avons montré que le comportement augmente l'activité électrique (MUA) des SCN chez *Arvicanthis* (contrairement au rat) et que la fluoxétine, un inhibiteur de la recapture de 5HT, diminue l'amplitude des rythmes d'activité comportementale et MUA. Ces données montrent que le fonctionnement rythmique des structures de l'éveil est différent entre les animaux diurnes et nocturnes, et peut participer à leur organisation temporelle opposée.

Mots clés : circadien, rythmes, diurne, nocturne, Arvicanthis, sérotonine, noradrénaline, noyaux du Raphé, Locus Cœruleus, noyaux Suprachiasmatiques, fluoxétine.

Summary

In mammals, all rhythmic functions are orchestrated by the Suprachiasmatic nucleus (SCN), the master clock. While behavioral activity occurs at opposite phases in diurnal and nocturnal species, the SCN is always active at the same astronomical time. We investigated whether structures of the arousal system, namely the serotonergic (5HT) Raphe nuclei (RN) and noradrenergic (NA) Locus Cœruleus (LC), are involved in the opposite temporal organization between nocturnal and diurnal species. We showed that 5HT and NA synthesis, as well as clock gene expression in the RN and LC, are opposite between the diurnal *Arvicanthis* and the nocturnal rat, and correlate with their respective behavioral patterns. Furthermore, we showed that behavior enhances the SCN multi-unit activity (MUA) in *Arvicanthis* (in opposition to the rat), and that fluoxetine, a 5HT reuptake inhibitor, dampens the amplitude of both MUA and behavioral activity rhythms. These data show that the rhythmic functioning of arousal structures and their interactions with the SCN, is different between diurnal and nocturnal animals, and may participate to their different temporal organization.

Keywords: circadian, rhythms, diurnal, nocturnal, *Arvicanthis,* serotonin, noradrenaline, Raphe nuclei, Locus Cœruleus, Suprachiasmatic nucleI, multi-unit activity, fluoxetine.