UNIVERSIDAD DE MURCIA
Departamento de Fisiología
Unidad de Fisiología Animal

Encarrilamiento y *masking* en el nocturnalismo de un roedor diurno (*Octodon degus*)

Memoria de la Tesis Doctoral presentada por
D. Pablo Vivanco Jodar para optar al grado de
Doctor en Biología por la Universidad de Murcia

UNIVERSITY OF MURCIA
Department of Physiology
Animal Physiology Unity

Role of entrainment and *masking* on the nocturnalism of a diurnal rodent (*Octodon degus*)

Thesis submitted by D. Pablo Vivanco Jodar to obtain the PhD. degree by the University of Murcia

UNIVERSIDAD DE MURCIA
Facultad de Biología

D. Juan Antonio Madrid Pérez, Catedrático del Departamento de Fisiología, en la Unidad Docente de Fisiología Animal, de la Facultad de Biología de la Universidad de Murcia, AUTORIZA:

La presentación de la Tesis Doctoral titulada "**Encarrilamiento y *masking* en el nocturnalismo de un roedor diurno (*Octodon degus*)**" realizada por D. Pablo Vivanco Jodar, bajo mi inmediata dirección y supervisión, en el Departamento de Fisiología, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

En Murcia a 8 de Marzo de 2010

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Dña. María de los Ángeles Rol de Lama, Profesora Titular del Departamento de Fisiología, en la Unidad Docente de Fisiología Animal, de la Facultad de Biología de la Universidad de Murcia, AUTORIZA:

La presentación de la Tesis Doctoral titulada "**Encarrilamiento y *masking* en el nocturnalismo de un roedor diurno (*Octodon degus*)**" realizada por D. Pablo Vivanco Jodar, bajo mi inmediata dirección y supervisión, en el Departamento de Fisiología, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

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UNIVERSIDAD DE MURCIA
Facultad de Biología

D. Jorge de Costa Ruiz, Profesor Titular y Director del Departamento de Fisiología de la Facultad de Biología en la Universidad de Murcia, INFORMA:

Que la Tesis Doctoral titulada "**Encarrilamiento y *masking* en el nocturnalismo de un roedor diurno (*Octodon degus*)**" ha sido realizada por D. Pablo Vivanco Jodar, bajo la inmediata dirección y supervisión de D. Juan Antonio Madrid Pérez y de Dña. María de los Ángeles Rol de Lama, y que el Departamento ha dado su conformidad para que sea presentada ante la comisión de Doctorado.

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Our reference: mjhk/pv
Date: March 1, 2010

To whom it can be concern,

I am very much delighted to write this letter of support of the PhD candidate Pablo Vivanco Jodar in view of submitting and defending his PhD thesis, entitled, "Role of entrainment and masking on the nocturnalism of a diurnal rodent (Octodon degus)" as a European thesis.

The PhD thesis consists of 7 major experimental chapters in which determinants of diurnal and nocturnal behavior in this rodent species are systematically investigated. Four of those 7 chapters, of which the PhD candidate is the first author, are accepted for publication in internationally peer-reviewed journals. This work is highly relevant in the field of circadian regulation, since it addresses the question what biological factors do contribute to the determination of diurnal or nocturnal behavior in animal species.

Please feel free to contact me when further questions may arise.

Yours Sincerely,

Martien Kas, PhD

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Date: March 1, 2010

A quien le pueda interesar,

Tengo mucho el gusto de escribir esta carta de apoyo al candidato Pablo Vivanco Jodar para que deposite y defienda su tesis doctoral, titulada “Encarrilamiento y masking en el nocturnalismo de un roedor diurno (*Octodon degus*)”, como tesis Europea.

La tesis doctoral consiste en 7 capítulos experimentales en los que se han investigado los causantes del comportamiento diurno y nocturno en esta especie de roedores. Cuatro de los 7 capítulos, de los cuales aparece el candidato a tesis como primer autor, han sido aceptados para su publicación en revistas de prestigio internacional. Este trabajo es muy relevante en el campo de la regulación circadiana ya que responde a qué factores biológicos contribuyen a la determinación del comportamiento diurno o nocturno de las especies animales.

Por favor, siéntase libre para contactar conmigo si necesita de cualquier otra información.

Sinceramente,

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P. VIVANCO THESIS EVALUATION

(University of Murcia)

Referee: Prof. Yvan Touitou

The dissertation of Pablo Vivanco, entitled "Role of entrainment and masking on the nocturnalism of a diurnal rodent (Octodon degus)" is made up of the presentation of 7 papers of the author, either already accepted (n=4) or submitted (n=2) or in the way to be submitted (n=1).

The thesis consists of 4 chapters:

- The background in the theme of this study is presented in an interesting introduction of 28 pages followed by the objectives of the research;
- 7 research papers (accepted or submitted) form the major part of the dissertation;
- The last two parts are the discussion and conclusions.

This thesis is a very interesting piece of work dealing with important matters in the research on biological rhythms since it is related to the effects of environmental factors on the temporal structure of a diurnal rodent model. Four papers have been published in high-impact scientific journals namely The Journal of Pineal Research (1 paper) and Chronobiology International (3 papers). Three papers are under submission. In all of these papers P. Vivanco is the first author.

My opinion is that this work adds very significantly to the literature on biological rhythms. I consider this research as an outstanding one, well exposed and worth presenting as a thesis of the University of Murcia for the obtention of a PhD degree by Pablo Vivanco.

Professeur Yvan Touitou

March 6th, 2010

EVALUACIÓN DE LA TESIS DE P.VIVANCO

(Universidad de Murcia)

Referencia : Prof. Yvan Touitou

La tesis de Pablo Vivanco titulada, "Encarrilamiento y masking en el nocturnalismo de un roedor diurno (Octodon degus)" está formada por la presentación de 7 artículos, algunos ya aceptados (n=4), o enviados (n=2) o en vías de ser enviado (n=1).
La tesis consiste en 4 bloques:

-El background del tema de este estudio se ha presentado en una interesante introducción de 28 hojas seguidas por los objetivos de la investigación;
-7 artículos de investigación (aceptados o enviados) forman la mayor parte de la tesis;
-Las dos últimas partes son la discusión y las conclusiones.

Esta tesis es una trabajo muy interesante que trata sobre importantes cuestiones en la investigación de los ritmos biológicos ya que se relaciona a los efectos de los factores medio ambientales en la estructura temporal de un roedor diurno modelo. Cuatro artículos han sido publicados en revistas científicas de alto impacto, llamadas Journal of Pineal Reseach (1 artículo) y Chronobiology International (3 artículos). Tres artículos han sido también enviados. En todos estos artículos, P. Vivanco es el primer autor.

Mi opinión es que este trabajo contribuye de manera muy significativa a la literatura de ritmos biológicos. Considero esta investigación extraordinaria, bien expuesta y que merece ser presentada como tesis en la Universidad de Murcia para la obtención del grado de doctor por Pablo Vivanco.

Profesor Yvan Touitou

6 de Marzo de 2010

JULIET *Wilt thou be gone? It is not yet near day:*
It was the nightingale, and not the lark,
That pierced the fearful hollow of thine ear;
Nightly she sings on yon pomegranate-tree
Believe me, love, it was the nightingale.

ROMEO *It was the lark, the herald of the morn,*
No nightingale: look, love, what envious streaks
Do lace the severing clouds in yonder east:
Night's candles are burnt out, and jocund day
Stands tiptoe on the misty mountain tops.
I must be gone and live, or stay and die.

William Shakespeare (1597) *Romeo and Juliet*, Act III, Scene V

JULIETA ¿Tan rápido te marchas? Todavía falta mucho para que amanezca:
Es el canto del ruiseñor, y no el de la alondra,
el que penetró el fondo temeroso de tu oído;
Cada noche se posa a cantar en aquel granado
Créeme, amado mío, es el ruiseñor.

ROMEO Es la alondra, heraldo de la mañana,
no es el ruiseñor. Observa, amada mía, cómo la luz envidiosa
enhebra las nubes deshechas del Oriente:
Ya se extinguen los luceros de la noche, ya el día
avanza de puntillas por las brumosas cumbres de los montes.
Debo irme y vivir o quedarme aquí y morir.

A mi familia

Index

Introduction

1- ORIGINS OF THE CLOCKS

From the beginning, Earth has been rotating on its own axis producing a cycle of around 24h alternating day and night. Several hundred millions ago, the primitive Earth atmosphere caused that the differences between the life during the solar day and the night were huge. Primitive life forms had to deal with enormous amount of damaging gamma, *X*, ultraviolet and infrared radiations due to the direct effect of the solar light (Hrushesky et al., 2009). Some of these life forms acquired biochemical strategies, as confining light-sensitive processes to the night, to cope with the environmental disadvantage of the solar light (Paranjpe & Sharma, 2005). The temporal control and regulation of the biochemical machinery into more suitable times was only possible after the appearance of clock-controlling mechanisms. These biological clocks have been severely subjected to selective pressure through the evolution, they have evolved, and as consequence nowadays almost all the life organisms, from prokaryotes to eukaryotes, are provided with clocks (Hrushesky et al., 2009).

2- DISCOVERING THE BIOLOGICAL CLOCK

The human has been always aware of the existence of rhythms in the nature. Although this knowledge was surely earlier, first written comes from the Ancient Greek. Archilochus, a Greek poet natural from the Paros' island, wrote in 650 B.C. about *the rhythms which rule the human beings* (Harris, 2003). In the fourth century B.C. Hippocrates of Cos, considered the Father of the Medicine, based his method in the observation and the experimentation. On his work *Aphorisms* related the appearance of certain diseases with the season of the year. Around the same century, Androsthenes of Thasus, scribe and naturalist of the expedition of Alexander the Great, when he was in Tyros island, on the actual Persian Gulf, noticed that the daily movement of the leaves of the tree *Tamarindus indicus*, which opened during the day and closed at night (Bretzl, 1903).

It passed two thousand of years when, in 1729, a French astronomer, called Jean-Jacques d'Ortous de Mairan, performed casually an experiment that evidence the endogenous nature of the biological clocks. He moved a sensitive plant, probably *Mimosa pudica*, which opens its leaves during the day and closes at night, into a dark place (Fig. 1).

He noticed that the opening and the closing of the sensitive leaves happened daily, even when the light of the sun was no present in the room (de Mairan, 1729). This fact was totally against the extended believe in that time, in which the rhythms in the nature were generated by the direct action of the environmental agents (exogenous nature of the rhythms).

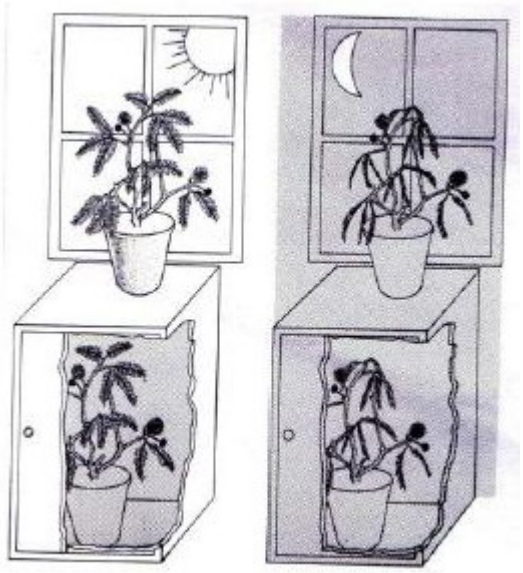


Figure 1. Representation of the Mairan's experiment. Original picture from Moore-Ede et al., 1982.

After thirty years, three independent researchers, John Hill (1757), Henri-Louis Duhamel du Monceau (1758) and Johann G. Zinn (1759), reported similar results in other plant species but they also could invert the rhythms by inverting the light-dark and the temperature cycle.

In 1814, Julien-Joseph Virey, a French pharmacist of a military hospital, completed his doctoral thesis in medicine. His work, titled *Éphémérides de la vie humaine, ou recherches sur la révolution journalière et la périodicité de ses phénomènes dans la santé et les maladies (*Ephemerides of the human life or research on the daily revolution and the periodicity of its phenomena in health and diseases), is considered the first published scientific work directly related to biological rhythmicities (Virey, 1814). For this reason, some investigators point out to 1814 as the year of the birth of the Chronobiology (Reinberg et al., 2001).

In fact, Virey textually wrote:

> *"Cette rotation successive de nos fonctions chaque jour, de la veille, du sommeil, de la réparation nourricière, des excrétions et sécrétions, ¿n'établit-elle pas une périodicité habituelle et comme innée dans tout le jeu de nos organes? N'est-ce pas comme un sytème de rouages engrenés l'un dans l'autre,* ***une sorte d'horloge vivante****, montée par la nature,* ***entraînée*** *par le mouvement rapide du soleil et de notre sphére." (Original)*
>
> *"This successive rotation of our functions each day, of the wake, of the sleep, of the nutritional reparation, of excretions and secretions, is it not establish by a daily periodicity, as like innate in all the activity of our organs? Does it not look like a system of gear-wheels meshed each other,* ***a kind of living clock****, mounted by the nature, and* ***entrained*** *by the rapid movement of the sun and of our sphere?" (Personal translation, modified from* Reinberg et al., 2001*)*

One important advance to accept the endogenous nature of biological rhythms was made, in 1832, by the Swiss botanist Augustin Pyramus de Candolle. He placed the sensitive plant, *Mimosa pudica*, in conditions of constant dark (DD) or constant light (LL). Under DD, he discovered that the sensitive leaf movements do not present a periodicity of 24h, as expectable, but of 22-23h (de Candolle, 1832). This was the first evidence of a free-running rhythm under constant conditions. De Candolle resumed that *"plants present an inherent tendency to show periodic movements*"; therefore, he indirectly pointed out an endogenous origin of the rhythms.

The German plant physiologist Wilhelm Pfeffer, critical with the results obtained previously, argued that the leaf movements persisted due to little leaks of light that penetrated in the dark rooms where were performed these studies. It is not known if Pfeffer knew the existence of Duhamel's studies, which were performed in wine caves or in compartments closed and covered by dark blankets (Kung & Yang, 1995); however, following his idea, he repeated the experiments by himself (Pfeffer, 1873). After several studies, he convinced, reported the veracity of the results and provided much more information about the leaf movement and the periodicities different from 24h in a large amount of plants (Pfeffer, 1915).

In the same way, Charles Darwin, together with his son Francis, published a book titled "T*he power of movement in plants*" (Darwin & Darwin, 1880) where they suggested that the movement of the leaf in some plants is inherent to the plant, and due to "some special purpose". The reason of the movements they proposed consists

in the protection of leaves against the cold temperatures of the night, i.e. the movement of the leaves toward vertical positions (up or down) ensures a heat radiation to each other, and not to the cold sky, as in horizontal position (Kung & Yang, 1995).

Through the XIX and early XX centuries, several authors had repeated and expanded the observations from the leave movements in some plants; however, it was the only daily rhythm perceived from the nature known to that date.

An interesting appreciation was made by the Swiss naturalist August Forel some day while having breakfast with his family in their terrace. In that day, some bees, attracted by the delicious marmalade, flied around the breakfast. This fact disturbed the family, in such a way that the next day they decided to have breakfast inside their house. However, Forel noticed that the bees continued visiting the terrace during some days later. This fact was no misperceived by him, and after several experiments he concluded that bees should have an internal mechanism to measure the time (Forel, 1910). The German Biologist, Hugo Berthold Von Buttel-Reepen, coming to the same conclusion, coined the term *Zeitgedächtnis or time-sense* to define the phenomenon observed in bees. More studies were performed by Karl von Frisch and his pupil Ingeborg Beling demonstrating that bees could be trained to visit the flowers at one specific time of the day (Beling, 1929).

Two important advances in the knowledge of circadian rhythms were due to Erwin Bünning. This German biologist demonstrated the natural heritability of the endogenous periodicity when worked with bean plants of different period in their leaf-movement rhythm (Bünning, 1935). Furthermore, he developed the concept of photoperiodism in plants, in which plants have circadian rhythms of light and dark sensitivity, permitting them to measure the duration of these phases in both short- and long- days, i.e. the photoperiod (Bünning, 1936). He also coined the term biological clock in 1935.

Another important discovery was done by two German biologists Gustav Kramer and Klaus Hoffmann interested in the navigation of migratory birds. They concluded that birds are orientated with respect the movements of the sun through the day (a sun compass), thanks to the existence of something similar to a time-keeping system inside the animals that permit them knowing the external time (Kramer, 1950; Hoffman 1954). This was a definitive evidence of the existence of biological clocks.

Franz Halberg, one of the founders of the modern Chronobiology, coined the term circadian (from the Latin `*circa´* meaning `around´; and `*dian´* meaning `day´), to refer the rhythmicity of periodicity around a day. He is considered the father of the Chronopharmacology due to his intensive studies on the application of drugs at different times of the day (Mathews et al., 1964). **Jürgen Aschoff** and **Colin Pittendrigh** are considered the fathers of the Chronobiology. They developed the two basic models for explaining the process of entrainment (the parametric model by Aschoff and the non-parametric one by Pittendrigh), which are the angular stone of the circadian rhythms. Aschoff is also famous by his experiments of isolation of humans in a bunker, to study their free running rhythms (Aschoff, 1965). At the same time, Pittendrigh, with his studies of the circadian rhythms of the pupal eclosion of *Drosophila pseudoobscura*, he established the basic properties of the circadian rhythms, as the endogenous nature, the temperature independence, and its ability to be entrained by external cycles (Pittendrigh, 1960). The Chronobiology, as a scientific discipline, is considered that it was born after the International meeting celebrated in 1960, in Cold Spring Harbor, New York.

3- PROPERTIES OF BIOLOGICAL CLOCKS

Thanks to the initial experimentations, it has been possible to understand certain properties of the circadian rhythms and, therefore, of the biological clocks that drives them (Moore-Ede, 1982):

- Persisting of the rhythmicity when isolated from environmental time cues, i.e. the appearance of free running rhythms under constant conditions that demonstrates the endogenous nature of circadian rhythms;
- Temperature independence, i.e. the circadian clock oscillates with a period that does not change significantly when the environmental temperature changes;
- The ability of circadian rhythms to be entrained by environmental cycles, i.e. the circadian rhythms can adopt a specific period and phase relationship with respect the environmental cycle.

Therefore, to date it is known that almost all the living forms own a biological clock. A central clock that is self-sustained and oscillates with a periodicity, or ***tau*** ***(τ)***, which is species-specific, for example 23.6h for a mouse or 24.5h for the human

(Refinetti, 2006b). This central clock is really a pacemaker, since its oscillation phase controls the rhythmicity of other peripheral clocks, spreading the temporal signal through the whole organism. However, this internal time generated by the pacemaker is daily reset by the action of the environmental agents, mainly by the light-dark cycle, but also temperature or food availability. The environmental agent that can reset the pacemaker is called ***zeitgeber***, a German word coined in 1954 by Jürgen Aschoff and means `time-giver´. By means of resetting the pacemaker, it generates a process of modulation, or entrainment, until achieved a specific period and phase relationship with the *zeitgeber* cycle.

In order to determine whether an environmental agent acts as a *zeitgeber* on the circadian system (Fig. 2), some criteria were proposed (Moore-Ede, 1982):

1) After removal other possible time cues, *tau* of the free running rhythm when the *zeitgeber* is not present must be different from when it is.
2) When the *zeitgeber* is present, the period of the circadian rhythm must be equal to that of the environmental cycle (Period Control).
3) A stable relationship between the phase of the rhythm and the *zeitgeber.*
4) When the *zeitgeber* is removed, the rhythm must start to free run according to the phase when the *zeitgeber* was present (Phase Control).

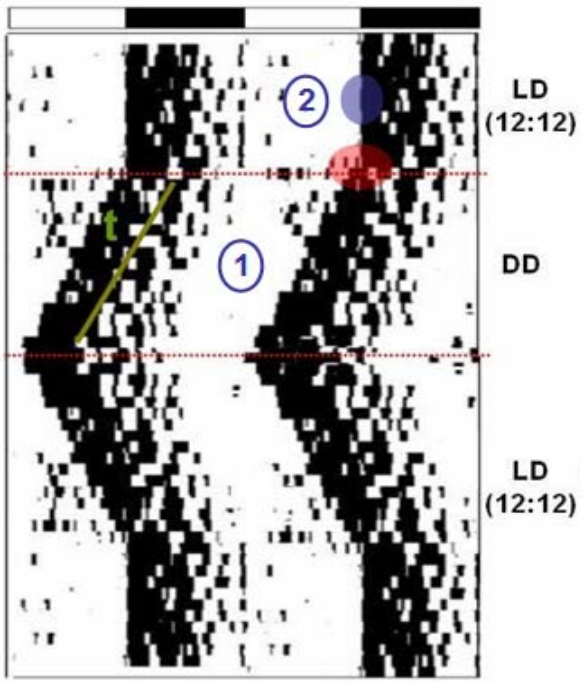


Figure 2. Criteria for entrainment to LD cycle

1) Under DD, t≠T
2) Under LD, t=T
3) Under LD, when light offs, the animal becomes active always at the same time (blue circle)
4) Under DD, free running in accordance with the previous phase under LD, with no abrupt shifts (red circle)

Zeitgeber: LD cycle 12:12
Periodicity of the *zeitgeber*: **T**=24h

Circadian rhythm studied: Locomotor activity of a rat

Endogenous periodicity of the rhythm without the *zeitgeber* (DD): **t**=23.5h (green line)

3.1- Entrainment *versus* Masking

Frequently, the same natural agents that entrain the pacemaker can at the same time mask the overt rhythms, overriding the information of the pacemaker (Fig. 3).

Therefore, the circadian overt rhythms result from the interaction between, at least, two different mechanisms. One involves the central pacemaker, and the second implies the direct action of external or internal agents, such as light or temperature (Daan, 2000; Mrosovsky, 1999). Thus, while the first one implies that the rhythm slowly adjusts its periodicity depending on a predictable external environment, the second one produces a fast change of biological rhythms to unpredictable or sporadic events (Johnson et al., 2003).

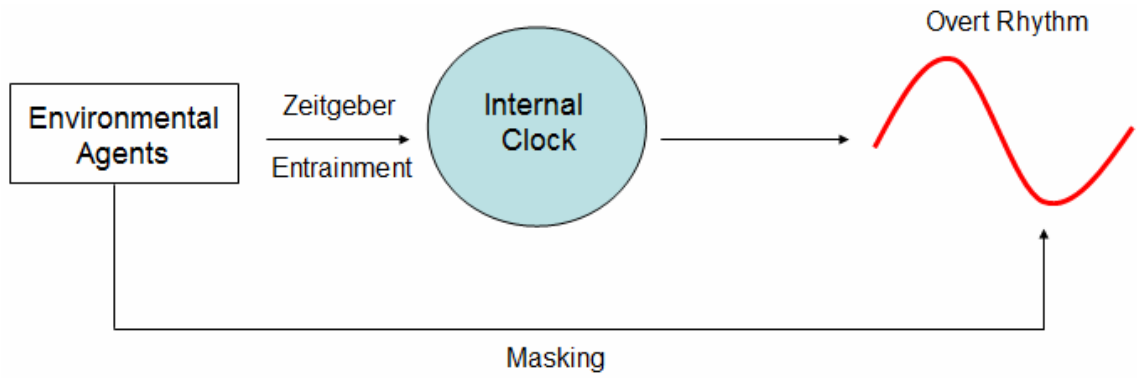


Figure 3. Simplified representation of the differential pathways for entrainment and masking processes.

Traditionally, masking has been classified according to the response of the overt rhythm: if an increase in a biological variable, for example wheel running activity, occurs, masking is defined as positive, while a decrease is defined as negative (Mrosovsky, 1999).

The type of response has also been classified depending on the masking stimulus and the animal's chronotype. For example, a diurnal animal usually shows positive masking when light is present or when light luminance is increased, but negative masking when darkness or a decrease in light luminance occurs. Alternatively, a nocturnal animal displays positive masking in response to darkness and negative masking after light exposure. Paradoxical masking effects, i.e. light inhibiting

the activity of diurnal animals and stimulating it in nocturnal ones, have also been described (Erkert & Gröber, 1986).

3.2- Entrainment *versus* Synchronization

In the modern Chronobiology literature, these two concepts are usually used indistinctly; however both present profound differences (Johnson et al., 2003). Synchronization implies that the waveform of the driving time cue, as the LD cycle, coincides with the waveform of the driven studied rhythm. This is achieved merely by masking effects, without the participation of the pacemaker. However, entrainment implies a period and phase control by the *zeitgeber*, thus the pacemaker participation is a must (Johnson et al., 2003).

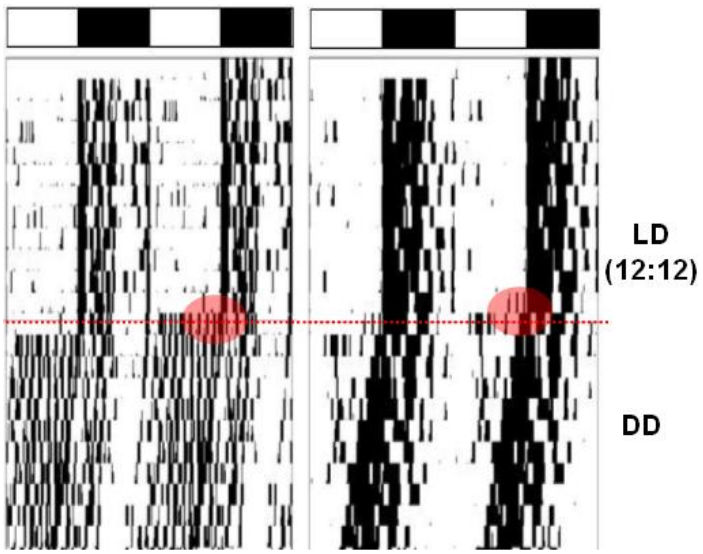


Figure 4. Differences between a synchronized (left) and an entrained (right) locomotor activity rhythm in two animals subjected to LD and DD cycles. Notice the transition showed by the red circle.

To distinguish if a rhythm under study is entrained or masked, the best way is to remove the *zeitgeber* signal and prove that phase control was present (fourth criteria of entrainment, Fig. 2). In this sense, when the animal is subjected to a environment without time cues, as DD, the phase of the rhythm will indicate whether the rhythm was entrained, if it stars to free run from the previously phase in LD (Fig. 4, right), or masked, if it starts to free run from another different phase (Fig. 4, left), and thus an abrupt shift appears.

4- THE CIRCADIAN SYSTEM

From a theoretical point of view, the circadian system is composed by the following elements (Fig. 5):

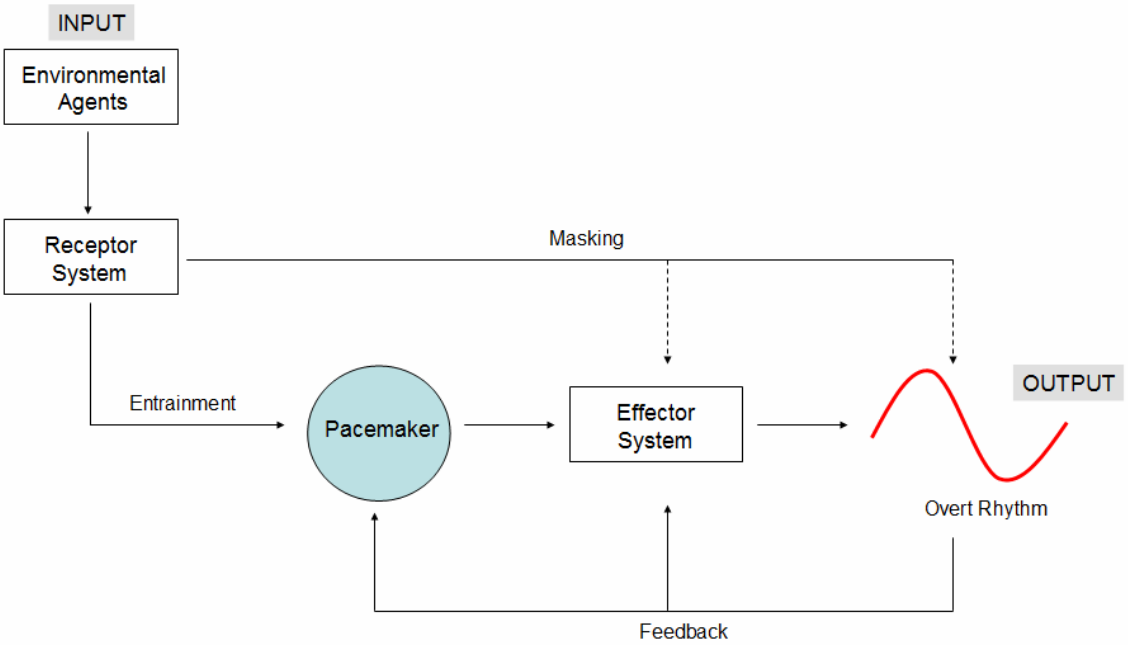


Figure 5. Scheme of the elements and mechanisms operating in the circadian system.

The sequence Input-Pacemaker-Output is the basic scheme that resumes the system (Madrid & Rol de Lama, 2006). The input signal is the environmental time cue, for example the light-dark cycle that is perceived by a specialized receptor system (in this case, the photoreceptor system). This temporal signal will be transmitted to the pacemaker, modifying its activity (entrainment). The temporal signal generated by the pacemaker will be then transmitted, via effectors, until generating the overt rhythm. The rhythm by itself also produces a feedback on the pacemaker, or in some point of the effector system, modifying their activities. In addition, the environmental agents can mask directly the overt rhythm, or act in some point of the effector system.

4.1- The mammalian circadian system structure

In 1972 two researching groups located independently in mammals the main biological clock which controls the circadian rhythmicity of the whole organism in a specific zone of the hypothalamus (Moore & Eichler, 1972; Stephan & Zucker, 1972). This zone was the suprachiasmatic nucleus (SCN). The SCN is located just above the

optic chiasm, i.e. where the innervations of the optic nerve cross (Fig. 6). In fact, the SCN is composed by two nuclei situated at both sides of the third brain ventricle (Moore, 1983).

4.1.1- Input pathways

In 2002, David M. Berson and colleagues discovered a previously unknown function for retinal ganglion cells containing melanopsin whose axons directly innervate to the SCN. This retinohypothalamic tract (RHT) has been demonstrated to be the main light input pathway to the circadian system. Together with the melanopsin-containing ganglionar neurons, the classical visual system of cones and rods also contribute to the circadian rhythmicity. The light is the main *zeitgeber* on the circadian system; however, other environmental signals, as temperature or feeding cycles, scheduled physical exercise, or social contacts, act on the pacemaker. In these cases, the temporal information reaches the SCN through other indirect pathways (Harrington, 1997; Morin, 1999).

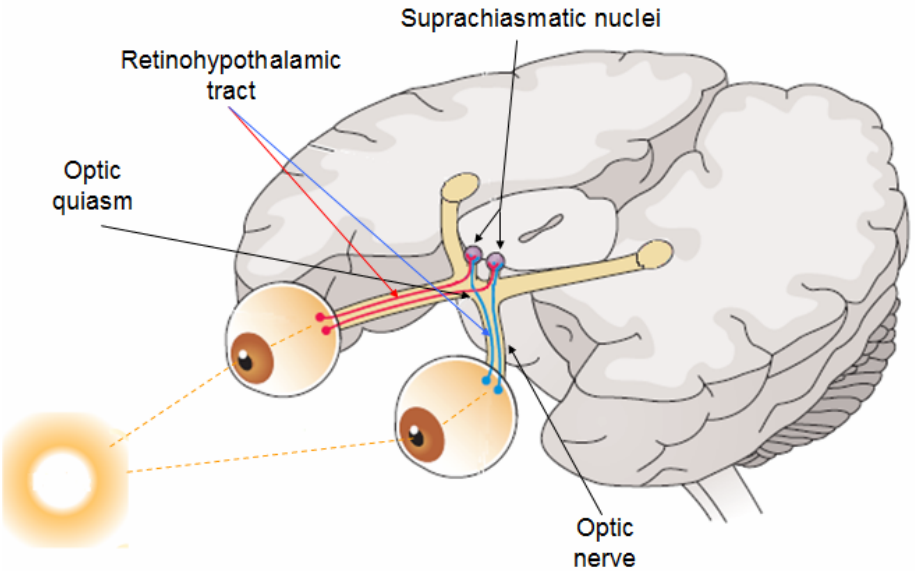


Figure 6. The retinohypothalamic pathway in mammals. Modified from Rosenzweig et al. 2004.

4.1.2- The central pacemaker

From a neuroanatomical and functional point of view, the SCN can be differentiated into two main regions, core and shell. The dorsomedial region, or **shell**, is

characterized by the majority presence of arginine-vasopressin (**AVP**) expressing neurons; and the ventrolateral, or **core**, mainly by vasoactive-intestinal peptide (**VIP**) expressing neurons (Sofroniew & Weindl, 1980; Stopa et al., 1988). The dorsomedial region receives projections from the ventrolateral SCN and communicates this information to the rest of the brain areas. While AVP expression in the shell is directly related to the clock machinery operation (Jin et al., 1999), neuropeptides in the core are more dependent on light conditions (Shinohara & Inouye, 1995).

The core is considered as an integration centre for input information so it receives photic information directly from the retina thought the retinohypothalamic tract (Johnson et al., 1988) and indirectly from the intergeniculate leaflet (Harrington, 1997). This last pathway is also involved in relaying non-photic information to the SCN (Mrosovsky, 1996). Moreover, a third important afferent pathway of information to the SCN comes from the serotoninergic neurons from the raphe nuclei of the brainstem, that has been related to photic and non-photic entrainment (Morin, 1999).

4.1.2.1- The machinery of the clock

To date, it has been identified the most of the molecular basis of the biological clock. Each neuron of the SCN is a circadian oscillator itself, driven by a molecular clock (Fig. 7). Its rhythm is generated by a transcriptional-translational feed back loop between two groups of clock genes: positive and negative elements (Ko & Takahashi, 2006).

Clock and *Bmal1* genes act as positive elements and are responsible for the synthesis of two transcriptional factors which, after heterodimerization, induce the expression of negative components of the molecular circadian clock such as Periods (*Per 1,2,3)*, Cryptochromes (*Cry1* and *Cry2*) and nuclear receptor subfamily 1 (*Rev-Erbα*). These negative elements, after dimerization (PER-CRY) undergo a nuclear translocation and act as suppressors of *Clock* and *Bmal1* expression. Thus, the levels of positive and negative elements oscillate in antiphase with a period of approximately 24 h.

Some components of the molecular clock, such as REV-ERBα, BMAL1 and CLOCK also induce a number of extra-clock genes, called clock controlled genes which are not directly involved in the clock machinery but are able to induce the expression of many target genes (Bozek et al., 2009).

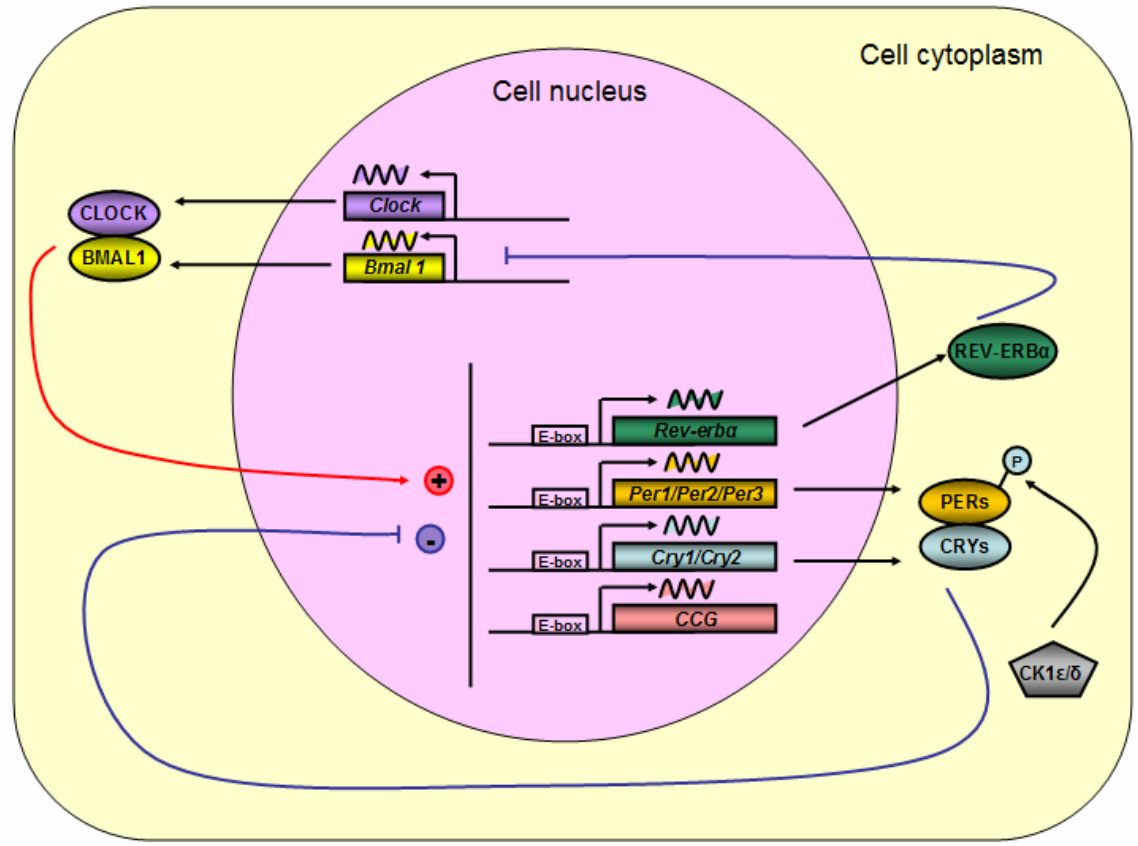


Figure 7. The molecular machinery of the clock. CCG, clock-controlled genes.

4.1.3- Outputs from the pacemaker

The temporal signal generated by the SCN is spread through the whole organism to coordinate the different biological circadian rhythms. The SCN controls the output targets by two main ways: nerve innervation of neural structures, modulated by, for example, the rhythmic change in the parasympathetic/sympathetic balance; and humoral transmission of substances such as melatonin, prokineticine-2, TGFα, and nocturnin (Reghunandanan & Reghunandanan, 2006; Reiter, 1991; Cheng et al., 2002; Kramer et al., 2001; Wang et al., 2001).

Regarding the neural communication of the SCN with output targets, the main efference from the pacemaker is found in nearby structures in the hypothalamus and in the thalamus (Fig. 8), as the medial preoptic nucleus, the medial part of the paraventricular and dorsomedial nucleus of the hypothalamus, the anterior part of the paraventricular nucleus of the thalamus, and principally the subparaventricular zone (Reghunandanan & Reghunandanan, 2006). On the other hand, an interesting study

demonstrated that tissues of SCN inside semipermeable capsules and transplanted into ablated-SCN hamsters could restore their lost circadian locomotor rhythms (Silver et al., 1996).

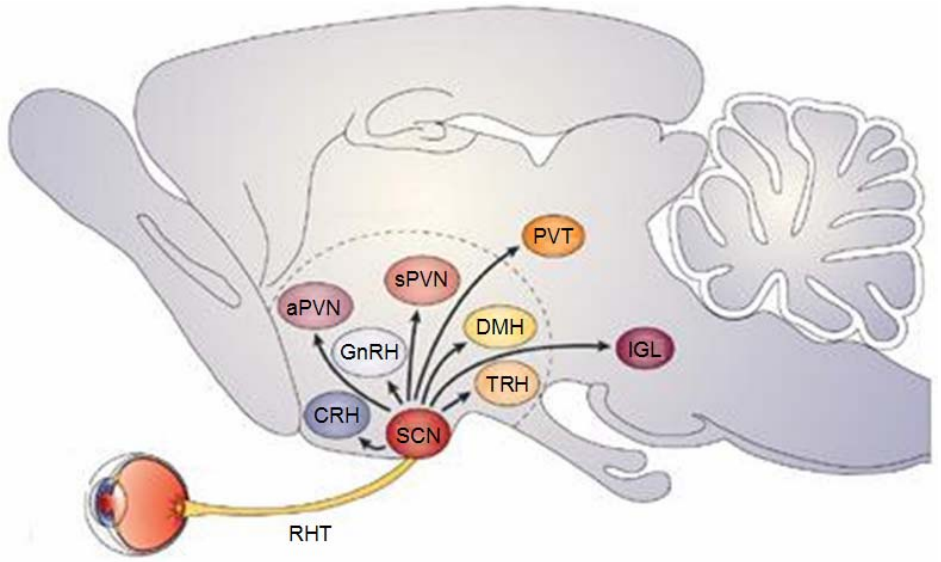


Figure 8. Neural outputs of the SCN. Endocrine neurones that synthesize corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH) and gonadotropin-releasing hormone (GnRH). aPVN, autonomic paraventricular neurons. IGL, intergeniculate leaflet. DMH, dorsomedial nucleus of the hypothalamus. sPVN, subparaventricular nucleus of the hypothalamus. PVT, paraventricular nucleus of the thalamus. Modified from Fu & Lee, 2003.

4.1.3.1- The melatonin

One essential output component for the circadian system function is the hormone melatonin which is daily secreted by the pineal gland (Reiter, 1991). The SCN controls the melatonin secretion rhythm due to the release of noradrenaline on the pineal gland, via sympathetic nerve fibres. This communication SCN-pineal is not direct but the temporal information travels down into the superior cervical ganglion in the spinal cord and come back up into the pineal gland (Fig. 9). Therefore, the pineal gland converts the electric signals from the SCN into a humoral response by the secretion of melatonin that it is released via bloodstream. In all vertebrate species, plasma melatonin peaks during the night, regardless of whether the animal shows nocturnal or diurnal behavior, thus this hormone has been considered the “chemical expression of darkness” (Reiter, 1991).

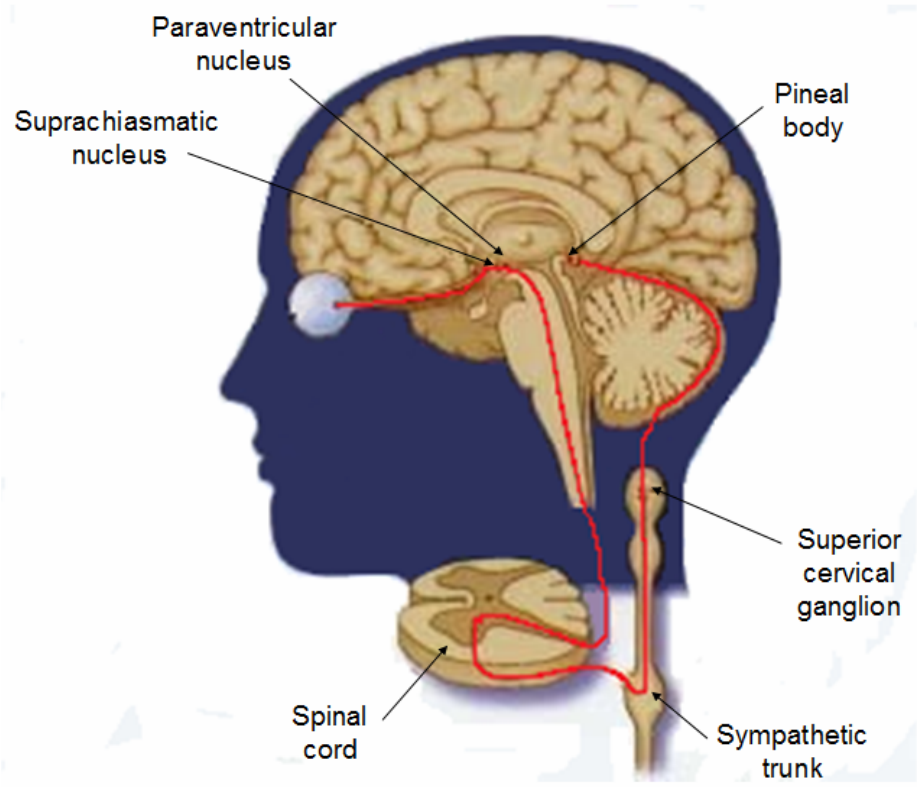


Figure 9. Neural pathway for temporal information from the retina to the pineal gland.

In diurnal species, the nocturnal rise in melatonin coincides with the physiological rest period, i.e. the increased sleepiness, decreased body temperature and locomotor activity, and increase in the immune responses (Van den Heuvel et al., 2005). However, in nocturnal species, melatonin is associated with the active period, i.e. increased locomotor activity and body temperature, and decreased sleepiness. According to Mendelson et al. (1980), endogenous melatonin should promote those behaviors associated with night, in other words, resting in diurnal and activity in nocturnal species.

The circadian rhythmicity of plasma melatonin is significantly impaired by aging in several species, including humans. In most species that have been studied, it is specifically the nocturnal melatonin that decreases as the animal ages (Reiter, 1995). These findings, together with its documented antioxidant, immunostimulant, antitumoral and hypnotic effects (Van den Heuvel et al., 2005), lead to propose the use of melatonin as an anti-aging therapy for human beings.

Most studies of exogenous administration of melatonin have been performed with nocturnal rodents to demonstrate the role of melatonin as a *zeitgeber* for the circadian system (Bothorel et al., 2002). In these species, free radicals should be produced in phase with their melatonin rhythm, a significant difference with respect to diurnal animals, in which melatonin and oxidative stress are in phase opposition. In recent years, considerable effort has been made to study diurnal animal models, such as the Nile grass rat or the mole rat as alternatives to nocturnal rodents (Slotten et al., 2002).

4.1.4- Extra-SCN circadian oscillators

The classical conception of the circadian system such as a unidirectional, (from the SCN to the whole organism) and uniclock model, (the SCN is the only circadian oscillator), has been subjected to a dramatic challenge in the last few years by two sets of new findings.

One important advance has been the discovery of peripheral oscillators distributed through the organism, i.e. structures with the ability to show circadian oscillations for few days when are studied isolated (Kowalska & Brown, 2007). Peripheral oscillators have been described in many tissues and organs, such as heart, lung, liver, intestine, adrenal and adipose tissue. These peripheral oscillators must receive periodical inputs from the SCN in order to prevent the spontaneous dampening of their rhythmical activity with time. However, they are also sensitive to their own synchronizers such as feeding time, local temperature, glucocorticoids, etc (Fig. 10). Other brain areas have also been proposed as self-sustained circadian pacemakers. Among these, retina and olfactory bulb are master oscillators which are able of self-sustained circadian output under isolation (Guilding & Piggins, 2007).

On the other hand, it has been demonstrated the existence of a bidirectional link between SCN and the physiology of the organism (Fig. 10). Recent findings show that experimental manipulation of an organism's physiology, as for example by allowing wheel running exercise or by fat induced obesity, can modify the function of the circadian pacemaker itself (Lax et al., 1998; Dibner et al., 2010).

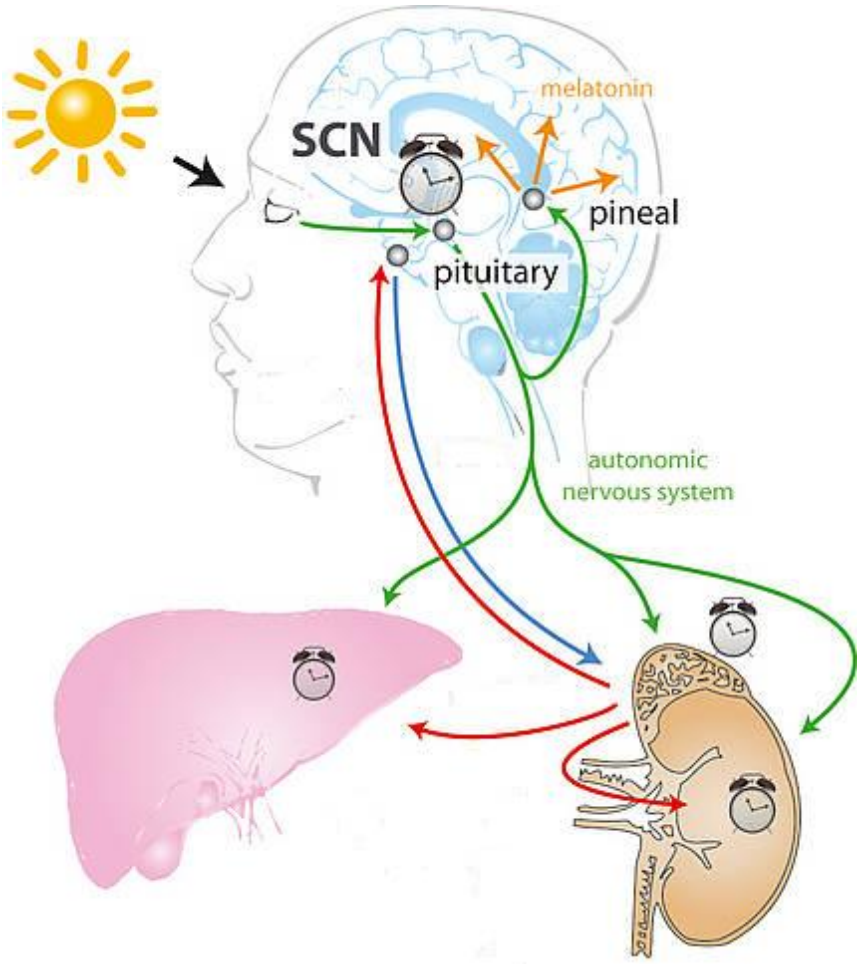


Figure 10. Peripheral oscillators controlled by the SCN. From Dr. Henrik Oster.

4.2- Effects of light on the circadian system

The cycle light darkness (LD) is the most important *zeitgeber* and, at the same time, the most important masking agent for the circadian system. Almost all circadian rhythms can be entrained to LD cycles (Johnson et al., 2003).

Jürgen Aschoff demonstrated that light exerts a continuous effect on the animals´ biological clock (Aschoff, 1981). Indeed, it was observed that increasing or decreasing of light intensity provoked variations in the endogenous periodicity, *tau*, according with the diurnal or nocturnal chronotype of the animal. These observations were called the ***Aschoff´s rules***. In diurnal animals, Aschoff found that when increased the light intensity, *tau* was shorten. However, in nocturnal animals, the same increase

provoked a *tau* lengthening. Although these rules are generally validated to nocturnal animals, in the case of diurnal ones there are a lot of exceptions.

4.2.1- Light entrainment

Based on these continuous effects of the light, Aschoff proposed the *parametric*, or tonic, model to explain the process of entrainment of the circadian systems. This model consisted in that the light exerts a continuous action on the clock to entrain it to the LD cycle. Moreover, it was suggested that the acceleration or deceleration of *tau*, as an angular velocity, generated by the changes in light intensities through the solar day would allow adjusting continuously the pacemaker to the external time (Aschoff, 1981).

On the other hand, Colin Pittendrigh proposed an alternative explanation that was called the *non-parametric*, or phasic, model of entrainment of the circadian systems. In this case, the model was inspired on the effects of discrete pulses of light on the circadian system (Pittendrigh & Daan, 1976a). Different phase variations produced in a free running rhythm by the exposition to a stimulus in specific circadian times can be drawn in a phase response curve (PRC).

Theoretically, the entrainment status in animals is achieved by the adjustment of the endogenous period, *tau* (τ), with respect the period of the external cycle (T), by means of phase variations ($\Delta\varphi$) at specific times of the day, in the form $\boldsymbol{\tau}$**- T=** $\Delta\varphi$. This model has been very successful in predicting the entrainment status of circadian rhythms mainly in nocturnal rodents. However, both parametric and non-parametric entrainment models are not mutually exclusive.

4.2.2- Neural basis for masking by light

Of the two processes involved in the control of overt rhythmicity, entrainment and masking, the latter has received much less attention over the decades. Nowadays, the mechanisms, the anatomical location and the molecular biology implicated in masking processes remain poorly understood.

Based on retina mutant mice (*rd/rd*) studies, it has been suggested that negative masking by light relies on luminance sensitive melanopsin ganglionar cells, which send information to the SCN via the retinohypothalamic tract (Mrosovsky, 1994; Mrosovsky & Hattar, 2003). Positive masking by light, however, seems to be mediated by the classic visual system (Edelstein & Mrosovsky, 2001).

Several studies have been conducted to answer the question regarding whether masking is located in or nearby the SCN and the role of the clock in masking. These studies have mainly involved SCN lesions (Redlin & Mrosovsky, 1999) and/or subjecting the animals to light-dark ultradian cycles (Borbély & Huston, 1974). Unfortunately, the results found so far are not consistent. While masking by light disappeared after SCN lesion in some experiments (Ibuka et al., 1977), it failed to do so in others (Fuller et al., 1981). This discrepancy was explained by the existence of possible collateral damage to the optic quiasm during the procedure to perform the SCN lesion (Mistlberger, 1994b).

4.2.3 Internal dissociation of circadian system by light

The light exerts profound effects on the circadian system. One very impressive is the phenomenon of *splitting* of the circadian rhythms. Splitting consists in the fragmentation of a unique and compacted rhythm into two independent components due to the manipulation of the lighting conditions. This phenomenon has been reported, when an animal, as the hamster, is subjected to LL cycles (Fig. 11). According to this observation, Pittendrigh & Daan (1976b) proposed the famous model of two oscillators, **M**-**E**.

This model explained that the circadian system is composed by two coupled oscillators, the morning component (M) and the evening (E) one, to generate the circadian rhythmicity. Furthermore, it was suggested that while the oscillator M entrains to dawn transition, the oscillator E does to dusk; and also that a phase relationship should exist between them. In this way, the circadian system would have the capacity to measure the daylength due to the phase relationship between both oscillators.

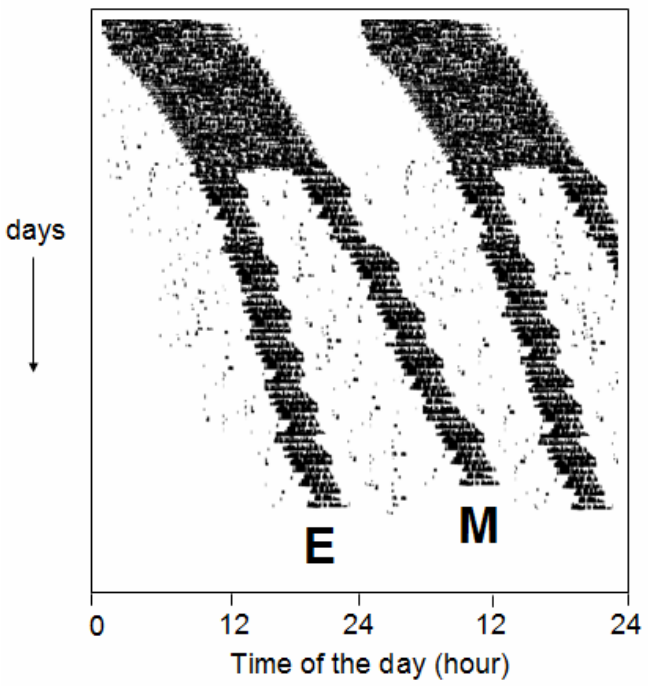


Figure 11. Actogram showing the free-running locomotor activity rhythm of a golden mouse subjected to LL conditions. Note that suddenly, the compacted free running rhythm is fragmented into two independent oscillation components, the evening component (E) and the morning component (M).

On the other hand, another fragmentation of the circadian system is obtained after subjecting animals into cycles with a period different from 24h, called T-cycles or ahemeral cycles. This dissociation has been demonstrated in rats and mice under shorter (de la Iglesia et al., 2004) and longer (Scannapieco et al., 2009) than 24h LD cycles. In all the cases, the whole output signal was dissociated into two rhythmical components: a Light Dependent Component (**LDC**), influenced by light, and a Non-Light Dependent Component (**NLDC**), presented under free running with a different periodicity from that of the T-cycle (Campuzano et al., 1998). While the NLDC has been related with the pacemaker control, the LDC has been associated to both, pacemaker entrainment and masking by light (Fig. 12).

From a neuroanatomical point of view, the dissociation process has not been explained yet; however several experiments have demonstrated that the desynchronization of areas inside the pacemaker can be involved, as the dorsomedial (*shell*) and ventrolateral (*core*) zones. In fact, it was possible to dissociate both pacemaker areas by 22h T-cycles in rats (de la Iglesia et al., 2004). On the other hand, another set of experiments demonstrated the possibility of a differential involvement of the right and left side of the SCN, as occurred in hamsters subjected under LL conditions (de la Iglesia et al., 2000).

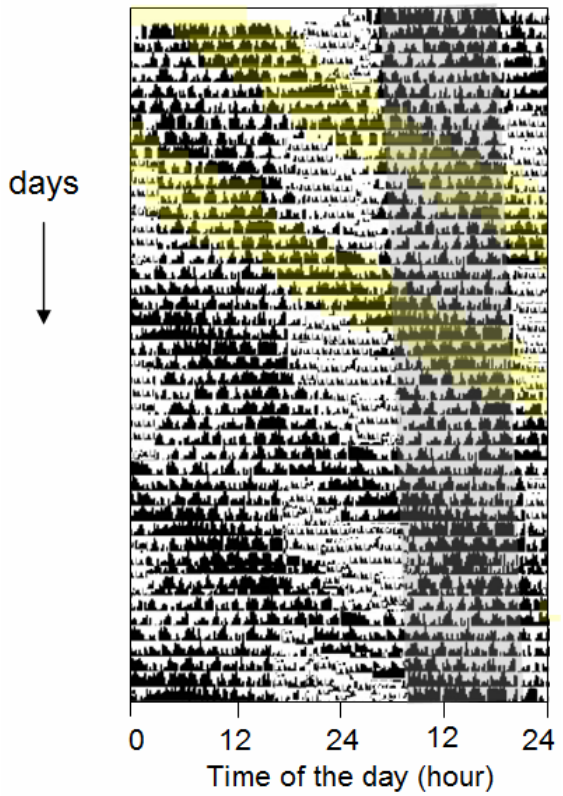


Figure 12. Actogram of locomotor activity (24 h module) of a ground squirrel subjected to a cycle of 28h (T28).
The grey box indicates the NLDC and the oblique yellow area, the time of light on, and the LDC component.

4.3- Effects of temperature on the circadian system

Temperature is a critical factor in the survival of organisms, so they have developed different mechanisms to cope with environmental temperature challenges. In fact, homeothermy, poikilothermy and heterothermy are examples of different physiological strategies adopted to withstand temperature changes.

Although temperature also displays daily cycles that are closely related to LD cycles, few studies have been conducted on the ability of daily thermal cycles to function as *zeitgeber* in homeotherms. From the 1960s until today, a number of papers have described the entrainment properties of temperature cycles upon free-running activity rhythms in birds, such as house finches (Enright, 1966), in heterothermic mammals, such as some species of mice and bats (Pohl, 1998; Erkert & Rothmund, 1981), and in homeothermic mammals like rats, mice, squirrels, macaques and monkeys (Francis & Coleman, 1990; DeCoursey, 1960; Tokura & Aschoff, 1983; Aschoff & Tokura, 1986). Entrainment was not achieved by all individuals. For this reason, temperature cycles are considered a weak *zeitgeber* for mammals. However, a phase response curve as a function of temperature pulses in rats has been drawn by Francis & Coleman in 1997.

4.4- Effects of the food on the circadian system

In the same way that temperature, food availability is an important factor in the active life of animals; and therefore, it acts upon its circadian system. It has been demonstrated that food availability is able to entrain the circadian rhythms of fish, birds and mammals (Sánchez-Vázquez et al., 1997; Hau & Gwinner, 1996; Kaur et al., 2008). However, food availability is considered a *zeitgeber* weaker than light, since this last exerts a more precise control on the circadian rhythms than food availability (Refinetti, 2006b).

To understand the implication of feeding on the circadian system, animals have been studied in laboratory conditions under different feeding situations, such as *ad libitum*, short or long food restrictions, or fasting. Initially, it was generally believed that scheduled feeding alone, without any caloric restriction, cannot entrain the SCN pacemaker (Mistlberger, 1994a); however, some recent studies in mice have demonstrated that the circadian clock can be reset by just a signal associated with feeding time (Castillo et al., 2004).

On the other hand, when animals are subjected to restricted feeding schedules, it has been described an increase in the locomotor activity and in the secretion of adrenal corticosterone and digestive enzymes two or three hours prior to the food administration (Mistlberger, 1994a). This food-anticipatory activity (FAA) has been documented in many animals. It has been hypothesized that FAA is controlled by a circadian pacemaker different from the suprachiasmatic nuclei, as it does not disappear after SCN bilateral lesions (Mistlberger, 1994a). In this sense, it has been proposed that, apart from the central light-entrainable oscillator (**LEO**), i.e. the central pacemaker in the SCN; another main circadian oscillator might exist, a food-entrainable oscillator (**FEO**) [Stephan, 2002].

Many studies have tried to locate the physical substrate of FEO; however, a clear anatomical localization has not been discovered to date (Mistlberger & Rusak, 1998; Davidson et al., 2003). In this sense, some studies point out to the dorsomedial hypothalamic (DMH) nucleus as a key component of FEO (Fig. 13) and in the regulation of the circadian rhythms by feeding cues (Mieda et al., 2006).

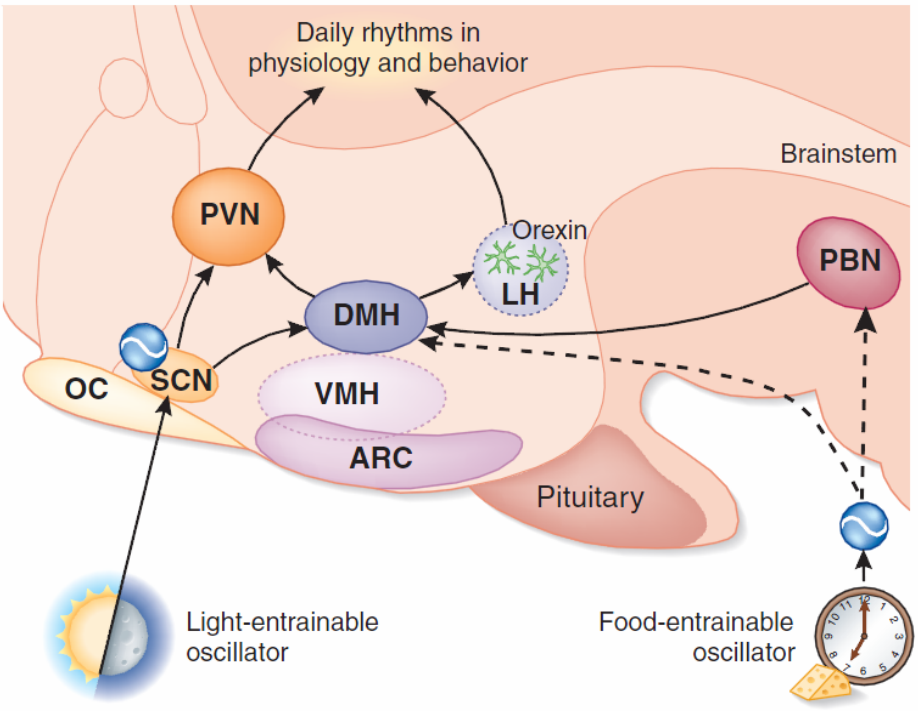


Figure 13. Control of the circadian rhythms by the two main entrainable oscillators, LEO and FEO. While the light cue acts through the suprachiasmatic nucleus (SCN), food cue can act: directly through the dorsomedial hypothalamus (DMH) or indirectly through the Parabrachial nucleus (PBN). The information from both, SCN and DMH, converge on the Paraventricular nucleus (PVN) and the orexigenic cells of the lateral hypothalamus (LH), to generate the output rhythmicity. Ventromedial hypothalamic nucleus (VMH); Arcuate nucleus (ARC). From Herzog & Muglia, 2006.

5- CIRCADIAN RHYTHMICITY IN ANIMAL'S ECOLOGY

Circadian rhythms can be considered as a strategy to deal with predictable environmental events with a 24h periodicity. The mammalian endogenous central pacemaker generates an internal timed signal that travels throughout the entire organism. The signal is used for assigning a precise temporal niche to physiological and behavioral activities (Paranjpe & Sharma, 2005). In nature, animals that are active and display daily biological events, as foraging, feeding, social or reproductive activities, at the right time of the day increase their chances of survival (Sharma, 2003).

Animals rarely are continuously active, but they are active either during the day (diurnal), during the night (nocturnal), or around dawn and dusk (crepuscular). Species

have adopted these behavioral patterns as a result of long periods of evolution in which adaptation and natural selection have operated jointly. In this sense, nocturnal and diurnal animals exhibit differences in their visual sensitivity and acuity, as well as in the chemo- or mechano-sensory detection (Bowmaker & Hunt, 2006). To these broad chronotypes may be added two more types: species with activity distributed during the day, night, and twilight (cathemeral), and species with the ability to shift from diurnal to nocturnal and *vice versa* (dual).

Dual phasing behavior have been found in different vertebrates, from fish such as the European sea bass, *Dicentrarchus labrax (*Sánchez-Vázquez et al., 1995); migratory birds as the Passeriforme *Silvia borin* (Gwinner, 1996); to mammals as the mole rat, *Spalax ehrenbergi* (Oster et al., 2002); the Nile grass rat*, Arvicanthis niloticus* (Blanchong et al., 1999); the Mongolian gerbil, *Meriones unguiculatus (*Weinert et al., 2007*)*; the golden spiny mice, *Acomys russatus* (Cohen et al., 2010); or the degu, *Octodon degus* (García-Allegue et al., 1999).

5.1- Temporal niche switches in nature

Although it is not a common feature in all animals, phase inversions occur in nature (Mrosovsky, 2003). These behavioral changes can be triggered by one apparent causal factor; however, it is likely that an interrelationship between several of these factors occurs, as is the case in the following examples (Kronfeld-Schor & Dayan, 2008).

The Atlantic salmon, *Salmo salar*, and the brown trout, *Salmo trutta*, are fishes mainly diurnal that switch to a nocturnal pattern when the water temperature reduces in winter (Bremset, 2000). Several ecological hypotheses for explaining this inversion have been raised. One of them consists in the fact that these poikilothermic fishes reduce its metabolism activity and swimming capacity; therefore, they are more vulnerable for homeothermic predators, as minks or herons, during the daytime. Other example of inversion by a temperature restraint occurs to the bateared fox, *Otocyon megalotis*. This animal forages in winter during the day, avoiding the lower temperatures of the night; however, in summer forages during the night, avoiding in this case the high sun temperatures of the day (Lourens & Nel, 1990). An interesting phase switch is found in the blind mole rat, *Spalax ehrenbergi*, that live underground, in their burrows, during all the year. This mole presents a clear diurnal activity pattern in winter

and a mainly nocturnal one in summer. It has been suggested that changes in the environmental temperature can be involved (Kushnirov et al., 1998).

On the other hand, migratory birds, as the garden warblers, *Silvia borin*, present diurnal patterns during the seasons of summer and winter; however, during the migratory seasons, autumn and spring, they become more nocturnal. This nocturnalism has been termed as ***Zugunruhe*** or migratory restlessness (Gwinner, 1996). It has been hypothesized that this nocturnal activity is an adaptation to cover prolonged journeys.

With the exception of the blind mole rat, the photoperiod is a causal factor involved in all the cases. The daylength affects directly the pattern of activity in voles, such as *Microtus montanus*, which become more diurnal with short days of winter (Rowsemitt et al., 1982).

5.2- Phase inversions in the laboratory

All these phase shifts found in nature also occur when the animals are kept under controlled conditions in the laboratory. In fact, these inversions usually are more drastic and faster.

One example is found in the individuals of golden, *Acomys rusattus*, and common, *Acomys cahirinus*, spiny mice. Both species coexist in the same rocky desert areas of Israel, being the golden spiny mouse typically diurnal and the common one nocturnal. In a very interesting field study with both species sharing the same habitat, when most of common spiny mice were trapped and removed, golden spiny mice became nocturnal (Shkolnik, 1971). This temporal partitioning has been proposed to be a mechanism of coexistence between related species (Kronfeld-Schor & Dayan, 2003). Moreover, when individuals of both species were introduced into the controlled conditions of a laboratory, most of golden spiny mice immediately shifted the activity to the nocturnal phase (Kronfeld-Schor et al., 2001).

The Nile grass rat, *Arvicanthis niloticus*, is another example of phase inversion in laboratory conditions. These rodents are mainly diurnal in the wild and in laboratory conditions; however, when a wheel running is available on their cage, some individuals invert their activity to the nocturnal phase in just one day (Redlin & Mrosovsky, 2004).

The mechanisms involved in the nocturnalism of the Nile grass rat and, in general, in all the phase inversions observed in the laboratory, are considered only related to masking by light.

6- *OCTODON DEGUS*

The degus is an endemic rodent from Central Chile which is mainly distributed between Vallenar and Curico (from 28 º to 35º latitude South) on the west slope of the Andean mountains, up to 1200m of elevation (Woods & Boraker, 1975). The first description of the animal was as *Sciurus degus* in 1782 by Juan Ignacio Molina. Subsequently, in 1832, Edward Turner Bennett classified it in the genus *Octodon*. Finally, Waterhouse in 1848 defined the final scientific name, used to date, as *Octodon degus* (Fig. 14).

Figure 14. A degus draw by Waterhouse, 1848

The Family name, *Octodontidae*, and the Genus name, *Octodon*, are based on their dentition, since their cheekteeth are folded resembling a figure of eight, as can be observed in Fig. 15.

Figure 15. Degus cheekteeh and its caraceristic eight-like shape

In the literature, degus is considered sometimes as a hystricomorph or a caviomorph rodent. Apart from that, there are discrepancies to validate the term hystricomorph, as it implies to accept the unity of the South American hystricomorphs together with the African ones; some authors prefer the term caviomorph due to cultural reasons (Bustos et al., 1977). The reference to a degus as a caviomorph (‘*Cavia´* and `*morfos´* = literally means ‘with form of a cavia’) is more realistic than hystricomorph (`*hystrico´* that derives from *Hystrix*, the noun of the genus of the porcupines in the Old Word and `*morfos´* = `with form of a porcupine´).

Octodon degus is becoming an increasingly popular laboratory animal model for chronobiology and aging studies, as it develops degenerative diseases such as diabetes, cataracts and Alzheimer-like diseases common in the human as age increases (Inestrosa et al., 2005; van Groen et al., 2010).

6.1- The nocturnalism in degus

The degu has been traditionally characterized as diurnal, with two major activity bouts at dusk and dawn. However, a great intra- e inter-individual variability of chronotypes has been described in laboratory conditions.

An impressive paper of Kas & Edgar (1999) titled “A nonphotic stimulus inverts the diurnal-nocturnal phase in *Octodon degus*” demonstrated that the wheel running activity triggered the nocturnal chronotype in some a previously diurnal animals (Fig. 16). They concluded that the nocturnalism status achieved in degus was produced by masking effects of light upon the overt rhythm.

Several years later, Kenagy et al. (2002) demonstrated in a wild population of degus, a seasonal variation of the activity and foraging rhythms according to the environmental temperature. In this sense, degus tended to increase their diurnal activity during winter days, and shifted their activity to dawn and dusk during the hot, dry days of summer.

In the same way, Theresa Lee (2004) reported that the relative proportion of chronotypes may be dependent on environmental temperature, with few nocturnal degus at low ambient temperatures (18ºC) and relatively high number of nocturnal ones at higher temperatures.

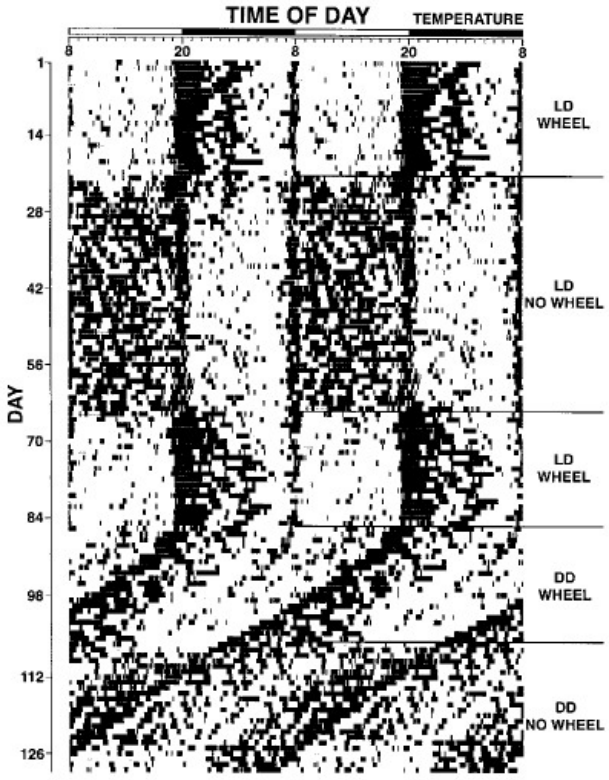
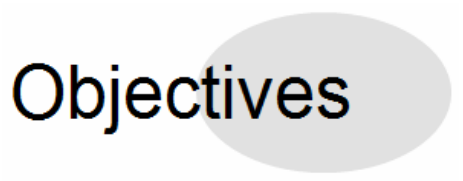


Figure 16. Representative actogram from the paper of Kas & Edgar (1999), showing the body temperature rhythm through several experimental phases: LD with wheel running, LD without wheel, again LD with wheel, DD with wheel, and finally DD without wheel.


Objectives

Considering the previous knowledge on *Octodon degus*' rhythmicity, the aim of this thesis is to understand the mechanisms involved in the nocturnalism of this diurnal species. To this, it was necessary to answer the following questions:

Causal

What external agents trigger nocturnalism?

- Is wheel running activity? (Chapter 1)
- Is feeding availability? (Chapter 2)
- Is environmental temperature? (Chapter 3)

Mechanicist

Who drives the inversion to the nocturnal phase?

- Is the pacemaker phase control or masking by light? (Chapter 4)
- Can the pacemaker output be dissociated from masking by light? (Chapter 5)

Functional

What makes different a nocturnal individual from a diurnal one?

- Does melatonin secretion generate a differential response? (Chapter 6)
- Is the suprachiasmatic nucleus different to each other? (Chapter 7)

The specific objectives of this thesis project are:

1- To verify whether wheel running activity inverts the diurnal phase preference of degus to a nocturnal one.

2- To verify whether degus' nocturnalism is exclusively related to masking effects by light.

3- To test whether the degus´ nocturnalism can be induced by a nocturnal feeding schedule.

4- To probe whether ambient temperature is the main key for the nocturnal activity of degus.

5- To characterize the masking by light response and its stability with respect to wheel running availability and ambient temperature in diurnal and nocturnal individuals subjected to ultradian cycles of one hour of light and one hour of darkness.

6- Evoke circadian dissociation between the pacemaker and the component masked by light using T-cycles (28 and 21 hours).

7- To test whether endogenous and/or exogenous melatonin is a physiological factor involved in defining the chronotype on a dual species.

8- To evaluate the possible differences in the expression of the main neuropeptides (arginine vasopressin and vasoactive-intestinal polypeptide) and Fos protein on the suprachiasmatic nucleus in diurnal and nocturnal degus.

Experimental Chapters

- Experimental Chapter 1

"Two steady-entrainment phases and graded masking effects by light generate different circadian chronotypes in *Octodon degus*"

- Experimental Chapter 2

"Nocturnalism induced by scheduled feeding in diurnal *Octodon degus*"

- Experimental Chapter 3

"Temperature cycles trigger nocturnalism in the diurnal homeotherm *Octodon degus*"

- Experimental Chapter 4

"Pacemaker phase control vs. masking by light: setting the circadian chronotype in dual *Octodon degus*"

- Experimental Chapter 5

"Dissociation of the circadian system in *Octodon degus* induced by T28 and T21 LD cycles"

- Experimental Chapter 6

"Looking for the keys to diurnality downstream from the circadian clock: role of melatonin in a dual-phasing rodent, *Octodon degus"*

- Experimental Chapter 7

"Temporal expression of arginine vasopressin, vasoactive intestinal polypeptide and Fos protein in the hypothalamus of diurnal and nocturnal *Octodon degus*"

Experimental Chapter 1

Title: "Two steady-entrainment phases and graded masking effects by light generate different circadian chronotypes in *Octodon degus*"

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TWO STEADY-ENTRAINMENT PHASES AND GRADED MASKING EFFECTS BY LIGHT GENERATE DIFFERENT CIRCADIAN CHRONOTYPES IN *OCTODON DEGUS*

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Processes involved in the operation of the circadian pacemaker are well characterized; however; little is known about what mechanisms drive the overt diurnal, nocturnal, or crepuscular behavior in a species. In this context, dual-phasing rodents, such as *Octodon degus*, emerge as a useful model to decipher these keys. Two main chronotypes, nocturnal and diurnal, have been traditionally described in laboratory-housed degus based on the percentage of activity displayed by the animals during the scotophase or photophase. However, if one considers also the entrainment phase angle during the first days following a change from LD to DD conditions, a third chronotype (intermediate)—or more properly, a continuous grading of circadian expressions between diurnal and nocturnal chronotype—can be observed. Our experiments suggest the pacemaker of the diurnal animal is entrained to the photophase, and light does not exert a negative masking effect. The pacemaker of the nocturnal degus, on the other hand, is entrained to the scotophase, and light exerts a strong negative masking effect. Finally, the intermediate chronotype is characterized by variable negative masking effect of light overlapping a pacemaker entrained to the photophase. The phase shift inversion from diurnal to nocturnal chronotype is related to the availability of a wheel in the cage, and the effect may be located downstream from the clock. However, body temperature rhythm recordings, less affected by masking effects, point to an involvement of the circadian pacemaker in chronotype differentiation, as transient entrainment cycles, and not an abrupt phase shift, were detected after providing access to the wheel. The diurnality of degus seems to be the result of a variety of mechanisms, which may explain how different processes can lead to similar chronotypes. (Author correspondence: jamadrid@um.es)

Keywords *Octodon degus*, phase inversion, transient, masking, diurnalism, chronotype

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INTRODUCTION

Temporal niche selection reflects circadian system adaptation, flexibility, and specialization. Animals do not exhibit continuous activity throughout the 24 h; rather, they concentrate their activity in the photophase (light phase), if they are diurnal; in the scotophase (dark phase), if nocturnal; in both the light and dark phases, if cathemeral (Curtis & Rasmussen, 2006); or around dawn and dusk, if crepuscular.

Temporal niche selection results from the interaction between at least two different mechanisms. One involves the action of a central pacemaker, the suprachiasmatic nucleus (SCN) of the hypothalamus in the case of mammals. The second mechanism involves the direct action of internal or external agents that mask the overt rhythm without affecting the pacemaker (Daan, 2000; Mrosovsky, 1999). Thus, while the SCN is an endogenous self-sustaining oscillatory system that slowly adjusts its periodicity depending on a predictable external environment (primarily the ambient light [L]:dark [D] cycle), the masking effects allow a fast adaptation of biological rhythms to unpredictable or sporadic events (Johnson et al., 2003).

The interactions between the pacemaker and masking effects yield an overt behavioral rhythmic expression in nature. The temporal behavioral pattern is reinforced by physiological adaptations to environmental lighting conditions. In this way, nocturnal and diurnal animals exhibit differences in visual sensitivity and acuity, as well as chemo- or mechanosensory detection, depending on the particular characteristics of their diurnal or nocturnal environment (Bowmaker & Hunt, 2006).

Although the operation of the circadian pacemaker has been well characterized, little is known about the mechanisms that determine nocturnalism or diurnalism. While neuronal activity and glucose utilization circadian rhythms within the SCN are similar (Schwartz et al., 1983), differences in c-Fos expression in the SCN (Krajnak et al., 1997) and in the neuronal electrical response within and outside the SCN from light or optic nerve stimulation (Jiao & Rusak, 2003) have been observed between some diurnal and nocturnal mammalian species (van der Veen et al., 2008). However, SCN function of some diurnal rodents differ but little from their nocturnal relatives (Smale et al., 2008).

These data suggest that some phase-control mechanisms are likely located outside of the central pacemaker (Smale et al., 2008). Output humoral molecules, like prokineticin-2, transforming growth factor alpha, or even melatonin (Vivanco et al., 2007), and also other neural substrates, such as the subparaventricular area of the hypothalamus (Schwartz et al., 2004), seem to be involved.

To date, most studies on the mechanisms that differentiate nocturnal and diurnal traits have compared different species with different

adaptations. However, it remains uncertain whether the findings of these studies reflect differences in the temporal niche or in species-related characteristics. For this reason, dual phasing species emerge as a useful model to better understand the chronobiological basis of diurnalism. Examples of dualistic behavior have been found in different vertebrates, from fish such as *Dicentrarchus labrax (*Sánchez-Vázquez et al., 1995) to mammals like *Spalax ehrenbergi* (Oster et al., 2002), *Arvicanthis niloticus* (Blanchong et al., 1999), *Meriones unguiculatus* (Weinert et al., 2007), *Acomys russatus* (Levy et al., 2007), *Microtus socialis* (Zubidat et al., 2007), and *Octodon degus* (García-Allegue et al., 1999).

The degus is a caviomorph rodent from Central Chile with a striking phase inversion capacity in the laboratory. Field studies indicate that the degu is a predominantly diurnal rodent (Fulk, 1976), which becomes crepuscular as a function of environmental temperature (Kenagy et al., 2002). Under laboratory conditions, some individuals shift from diurnal to nocturnal phasing in just one or two days after being subjected to a non-photic stimulus, namely, the availability of wheel running in its cage (Kas & Edgar, 1999). The rapidity of these changes support the current view that the differences of temporal phasing in the 24 h activity patterns between nocturnal and diurnal animals are not due to the central pacemaker, but rather to some downstream mechanism. However, the possibility that the intrinsic features of oscillators differ between nocturnal and diurnal species has not been excluded (Mrosovsky & Hattar, 2005; Smale et al., 2008).

The aim of this study was to test whether varying degrees of masking and phase angle entrainment changes of the circadian pacemaker might account for the highly variable chronotype features of degus in response to wheel availability. To this end, we analyzed locomotor activity and body temperature rhythms in degus with and without free wheel access, both under LD and DD conditions.

MATERIALS AND METHODS

Animals and Housing Conditions

Thirty-two *Octodon degus* between 11 and 26 months of age were obtained from a colony maintained at the Animal Service Unit of the University of Alicante (Spain). The animals were individually housed in plexiglas cages (52 × 15 × 27 cm, L × H × W) equipped with wheels in an isolated room with controlled humidity (60%), temperature (23 ± 1°C), and photoperiod (LD 12:12). The animals had mutual visual and acoustical contact during the experiment. Light was provided by two lateral fluorescent lamps controlled by an electronic timer (Data Micro, Orbis) and having an intensity of 350–400 lux at cage level. The degus were fed ad

libitum with commercial rat chow (A04 rat-mouse maintenance Panlab). All experimental procedures were performed in accordance with the Principles of Animal Care (Portaluppi et al., 2008) and Spanish laws.

Data Recording

Wheel running activity (WRA) was recorded as wheel turns per 10 min interval using a data acquisition system (Electronic Service at the University of Murcia, Spain). Body temperature (T_b) was measured at 60 min intervals using a miniature data logger (ThermoChron®, Data loggers iButton, Maxim Integrated Products, Sunnyvale, California, USA) having an accuracy of 0.1°C. For this purpose, sterilized data loggers were implanted intraperitoneally, under aseptic conditions, using fluothane as anesthesia (Forane®, Abbot Laboratories S.A., Madrid, Spain). Reabsorbable suture material (2/0, Safil®Quick B/Braun, Barcelona) was used to close the abdominal layers, and non-reabsorbable silk was used to suture the skin. No mortality or morbidity was observed after the surgery. The experiment began following a two-week recovery period. At the end of the experiment, the data logger was removed under the same conditions in which it was implanted. iButton readout hardware was used to transfer temperature data to a computer.

In order to quantify ambulatory activity (AA) during the period without wheel running, infrared motion sensors (Omron, photoelectric switches, E3S-AD62, Japan) were installed on the long side of each plastic cage. Each time the degus wandered about that zone, a beam of light was interrupted that caused the generation of an output signal. Ambulatory activity was recorded as the total number of interruptions per 10 min interval, using the same data acquisition system as for WRA.

Experimental Design

Two different experiments were performed to characterize degu chronotypes after wheel running availability.

Experiment 1

The aim of this experiment was to analyze the chronotype response of naive degus animals after giving them access to the wheel. To this end, 16 male degus (11 months of age) were individually housed under LD 12:12 with the wheel blocked. After eight days, the wheels were unlocked in the middle of the light phase, allowing the animals to freely run for a span of 20 days. Subsequently, the animals were subjected to another 21 days of constant darkness (DD) in order to determine the existence of a previous

entrainment to LD cycle. Finally, the animals were once again subjected to another LD 12:12 cycle for eight days to study resynchronization.

Experiment 2

This experiment was performed to determine the stability of an individual chronotype over time. For this purpose, another 16 degus (eight males and eight females) animals 18 to 26 months of age were recorded during 376 days. The animals were held under LD 12:12 in individual cages, with free access to the wheel as well as food and water ad libitum. During the experiment, degus experienced no variation in environmental temperature, handling procedure, or any known possible stressing situation, except for changes in the L:D cycle. All degus were subjected to the following series of lighting conditions: 22 days under LD 12:12 (lights on at 03:00 h, off at 15:00 h), 21 days under DD ($<$0.05 lux of dim red light), and 178 days under the previous LD 12:12. After that, the animals were subjected to a 5 h phase shift (lights on at 08:00 h, off at 20:00) for 25 days, and then were again shifted 1 h (lights on at 09:00 h, off at 21:00) for 82 days. Following this, the animals were subjected to 16 days under DD and, finally, 32 days under LD 12:12 (lights on at 08:00 h, off at 20:00 h).

Chronotype Characterization and Data Analysis

To characterize the chronotype expression of degus as nocturnal, diurnal, or intermediate, in addition to the classical numerical criteria (such as the percentage of diurnal/total activity), we included the analysis of the entrainment phase angle observed in the first days under DD after a previous LD schedule. Thus, when an animal under LD conditions showed a diurnal/total activity ratio $>$60%, and under DD started to free run in accordance with the diurnal phase in LD, this animal was considered to be diurnal. A nocturnal animal was defined as one with a diurnal/total activity ratio $<$20%, and who started to free run in the DD period, taking into consideration the nocturnal phase observed in the previous LD cycle. Intermediate animals exhibited a wide diurnal/total activity ratio, ranging from an apparently nocturnal to a diurnal chronotype, and showing a phase advance (between 2 and 6 h) from the first day under DD conditions. Using these criteria, no degus from the initial population needed to be excluded due to unstable or unclassifiable wheel running patterns.

Average actograms, mean waveforms, chi-square periodograms, and acrophases from a cosine fit of WRA and T_b rhythms were performed using software specifically designed for chronobiological analysis (El Temps, version 1.228; © Díez-Noguera, University of Barcelona). Cosinor

analysis was used to determine a phase reference for WRA and T_b rhythms. This inferential statistical method, involving the fitting of a 24 h cosine curve to the individual data by the method of least-squares, determines the following information:

- a probability or p value that indicates the statistical significance of the fitness of the cosine curve to the data; and
- three rhythmic parameters: acrophase (either the clock time or the phase angle, calculated in reference to local midnight, of the maximum value of the fitted cosine curve), mesor (the cosinor determined overall 24 h mean), and amplitude (one-half of the total cosine excursion).

To determine the daily acrophase evolution of the WRA and T_b rhythms for each chronotype, the mean and the standard error of the mean (SEM) of the acrophases (peak times) were calculated based on the statistically significant individual circadian rhythms documented by the cosinor analysis. Statistically significant ($p < 0.05$) 24 h cosine approximation was documented for all animals of three study conditions.

Ambulatory activity values recorded by the infrared motion sensor, such as that which occurs when locking and unlocking the wheel during the experimental periods, were normalized for each animal by dividing every value of the waveform by the individual 24 h mean.

Comparisons between endogenous periodicities under free-running conditions were made using an unpaired t-test ($p < 0.05$). A one-way analysis of variance (ANOVA) was used to compare differences between chronotypes with regards to the amount of WRA and mean body temperature. A repeated-measures ANOVA was performed in order to compare locomotor activity and temperature during locked and unlocked wheel phases. The experimental variables were tested for normality of distribution and homogeneity of variances. A linear regression was performed to establish the relationship between locomotor activity and temperature using SPSS 13.0 software.

RESULTS

Experiment 1

When the diurnal/total WRA ratio was the only criteria used to classify degu chronotypes, animals subjected to an LD 12:12 cycle exhibited patterns that were either predominantly diurnal or predominantly nocturnal. About 63% of degus (10 of 16) commenced their WRA approximately 1 h before light onset, and ran during the photophase and the first few hours

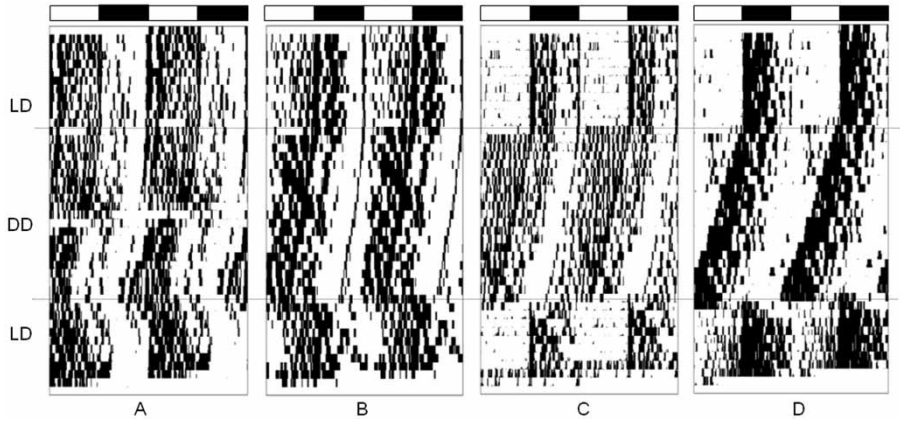

FIGURE 1 Representative double-plotted wheel running activity (WRA) actograms for: (A) diurnal behavior, (B) intermediate with diurnal behavior, (C) intermediate with nocturnal behavior, and (D) nocturnal chronotypes of *Octodon degus*. Each actogram represents 43 days of recording during which the animals were subjected to 12 days of LD 12:12 followed by 21 days of DD, and then changed back to LD 12:12 for nine days.

of the scotophase (see Figures 1A and 1B), whereas WRA for the remaining six degus was largely restricted to darkness (see Figures 1C and 1D).

However, when the phase angle of entrainment from the first days under DD was considered, three different categories emerged: diurnal, nocturnal, and intermediate chronotypes. Both extreme circadian expressions, diurnal and nocturnal, exhibited a stable phase angle of entrainment, as well as a true entrained state, as revealed by the phase control exerted by the previous LD condition (i.e., both chronotypes started to free-run in accordance to the previous circadian phase in LD; see Figures 1A and 1D).

The intermediate chronotype included degus that were predominantly diurnal, but with a significant amount of activity at night (Figure 1B), in addition to clearly nocturnal animals with very little activity during the day (Figure 1C). However, contrary to entrained nocturnal and diurnal degus, all of these individuals showed a significant phase advance the very first day they were subjected to DD conditions. Under these constant conditions, intermediate-type degus started to free-run in a similar phase as that observed in the diurnal type animals. Thus, intermediate types exhibited a negative masking effect induced by light (see Figures 1B and 1C) under entrained conditions. Using these classification criteria, we found 6/16 degus to be diurnal, 5/16 to be nocturnal, and 5/16 to be intermediate types.

The transition from LD to DD and back again to LD conditions further supported the existence of a negative masking effect of light in nocturnal and intermediate, but not diurnal, chronotypes. Intermediate animals

displayed a variation between individuals in the negative masking effect, as can be observed in Figures 1B and 1C, whereas nocturnal chronotype animals exhibited a strong negative masking effect. This negative effect of light on nocturnal degus was clearly evident when animals were subjected once again to LD conditions after DD. During this transition, all nocturnal and some intermediate-type degus switched their WRA to a nocturnal phase in a single day, which might be confused as an inversion of the activity phase preference (see Figures 1C and 1D). However, this apparent inversion did not occur in the T_b rhythm, suggesting that it is much less affected by negative masking of light. Indeed, as can be observed in Figure 2, the body temperature showed progressive transitions in all chronotypes after the change from the DD to LD condition.

Differences between chronotypes became even more evident when analyzing WRA waveforms averaged over the seven days under LD (see Figure 3). In diurnal degus, wheel running activity occurred throughout the entire day, with peaks occurring around dawn and after dusk, and with greater activity during the photophase than scotophase. The animals also exhibited a secondary bout of activity during the first part of the dark phase (see Figure 3A). In contrast, the WRA of nocturnal degus was almost entirely confined to the scotophase (see Figure 3C). Intermediate degus exhibited a pattern similar to that of nocturnal animals, although with a little more activity during the light phase and less during the dark phase (see Figure 3B). A closer inspection mean waveform of the WRA revealed a small peak of activity before lights-on and a very pronounced one after lights-off, regardless of the degu's chronotype.

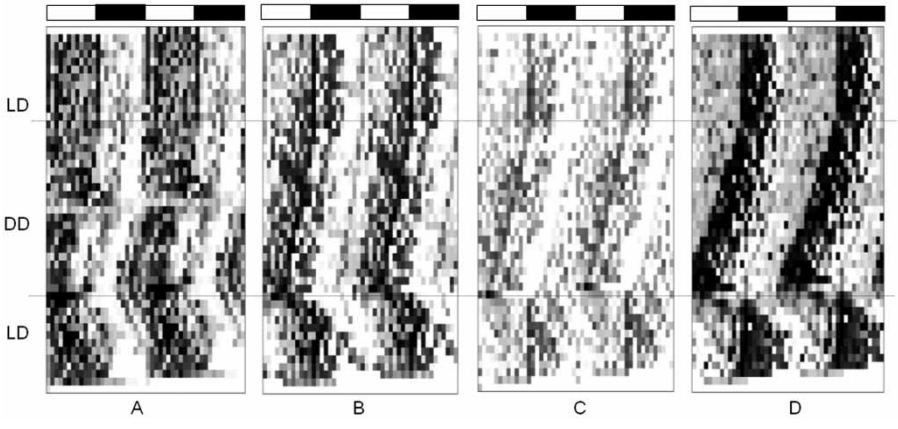

FIGURE 2 Double-plotted body temperature (T_b) actograms for the same *Octodon degus* as in Figure 1: (A) diurnal behavior, (B) intermediate with diurnal behavior, (C) intermediate with nocturnal behavior, and (D) nocturnal chronotype. See Figure 1 for details. The body temperature ranged from 35.5°C to 38°C (the darker the color, the higher the temperature).

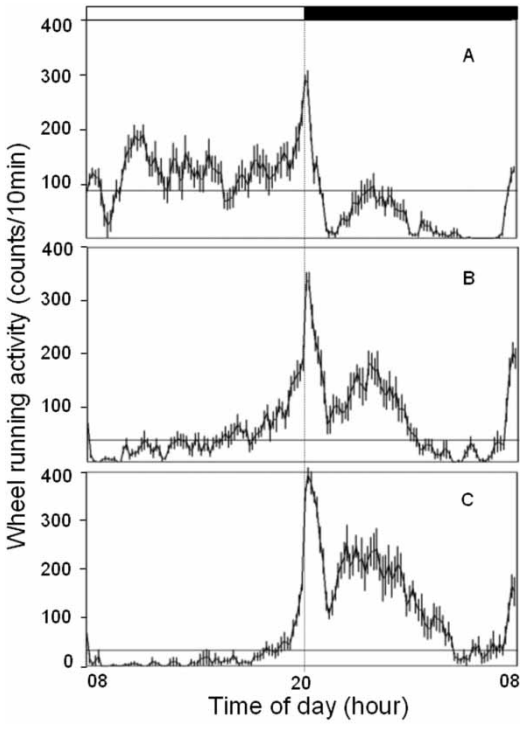

FIGURE 3 Wheel running activity (WRA) rhythm mean waveforms for the (A) diurnal, (B) intermediate, and (C) nocturnal chronotypes of *Octodon degus* subjected to LD 12:12. The horizontal line corresponds to the median for the 24 h values. Vertical lines represent the standard error of the mean (SEM) for each average value. The dotted vertical line indicates the light/dark transition for comparison purposes.

The previously reported ability of *Octodon degus* to switch their circadian phasing almost instantaneously when provided free access to a wheel (Kas & Edgar, 1999) was tested by locking and unlocking the wheel for the three circadian chronotypes (see Figure 4 and Table 1). All degus were diurnal at the start of the experiment while their wheel was locked; however, a subset of animals became nocturnal in response to the wheel availability. Thus, when naive animals that had no previous exposure to the wheel were placed in a cage with a locked wheel, they all displayed a very similar T_b rhythm with a preference for the diurnal phase (see Figure 4A), even though they would later express different activity chronotypes. Both nocturnal and diurnal chronotypes exhibited exactly the same T_b pattern, with higher temperatures during the photophase compared to the scotophase (see Table 1). Intermediate degus also displayed the same pattern, but with lower T_b values both during the day and night. With the exception of the maximum T_b value in the case of intermediate degus (repeated measures ANOVA, $p = 0.026$), no statistically significant differences were observed among the chronotypes prior

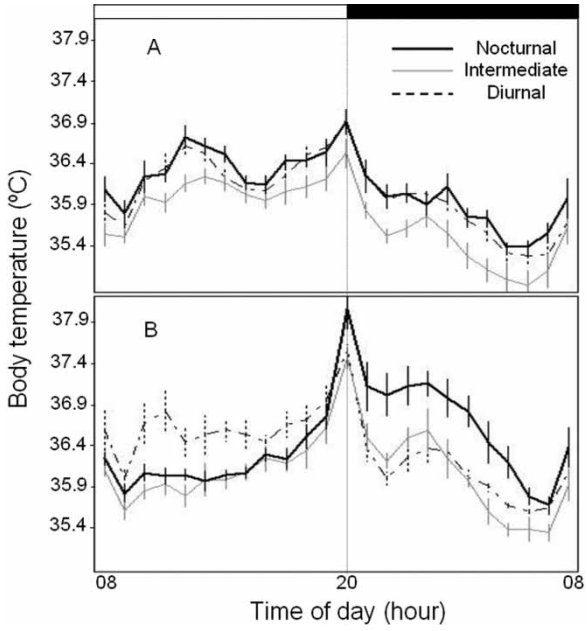

FIGURE 4 Body temperature rhythm mean waveforms for the diurnal (dotted line), intermediate (grey line), and nocturnal (black line) chronotypes of *Octodon degus*. The animals were subjected to LD 12:12 with the wheel (A) locked and (B) unlocked. Vertical lines represent the SEM of each average value. The dotted vertical line indicates the light/dark transition for comparison purposes.

to wheel access regarding their day-night distribution of locomotor activity or T_b.

When the wheels were unlocked, evidence of the three chronotypes emerged. While the pattern of the T_b rhythm in diurnal degus remained unchanged, nocturnal degus decreased their diurnal T_b and increased their nocturnal T_b (see Figure 4B). It is noteworthy that intermediate individuals behaved as nocturnal chronotype during the light phase and as diurnal chronotype during the dark phase. Wheel running availability generated statistically significant differences in mean WRA ($p = 0.002$ and $p = 0.001$ for the light and dark phase, respectively), maximum WRA ($p = 0.027$ and $p = 0.037$ for the light and dark phase, respectively), and nocturnal mean T_b ($p = 0.005$) between diurnal and nocturnal chronotypes (see Table 1). Intermediate degus showed intermediate levels in almost all WRA values, demonstrating no statistically significant differences when compared to diurnal or nocturnal animals. However, intermediate mean T_b values presented significant differences during the light phase compared to diurnal degus ($p = 0.014$) and during the dark phase compared to nocturnal ones ($p = 0.004$). Free access to wheel running generated no significant differences between the three chronotypes in ambulatory activity, minimum T_b, or maximum T_b values.

TABLE 1 Locomotor activity and body temperature values of the three circadian chronotypes of *Octodon degus* (diurnal n = 6, intermediate n = 5, and nocturnal n = 5) subjected to LD 12:12 with the wheel locked and unlocked

		WR Locked		WR Unlocked	
		□	■	□	■
WRA Mean per l h (counts)	Diurnal			722 ± 93 a	291 ± 76 a
	Intermediate			272 ± 102 b	553 ± 83 ab
	Nocturnal			98 ± 102 b	867 ± 83 b
WRA Max. In 1h (counts)	Diurnal			1417 ± 211 a	1278 ± 183 a
	Intermediate			837 ± 231 ab	1583 ± 200 ab
	Nocturnal			453 ± 231 b	2065 ± 200 b
AA Mean per 1h (counts)	Diurnal	802 ± 51	398 ± 51	704 ± 81	495 ± 81
	Intermediate	741 ± 56	459 ± 56	502 ± 89	698 ± 89
	Nocturnal	642 ± 56	558 ± 56	487 ± 89	713 ± 89
AA Max. In 1h (counts)	Diurnal	1983 ± 265	1148 ± 130	1604 ± 243	1193 ± 170
	Intermediate	1558 ± 290	1407 ± 143	1388 ± 267	1866 ± 187
	Nocturnal	1027 ± 290	1226 ± 143	1291 ± 267	1371 ± 187
Tb Mean (°C)	Diurnal	36.29 ± 0.09	35.88 ± 0.11	36.59 ± 0.10 a	36.16 ± 0.10 a
	Intermediate	36.05 ± 0.10	35.54 ± 0.12	36.08 ± 0.11 b	36.11 ± 0.11 a
	Nocturnal	36.38 ± 0.10	35.97 ± 0.12	36.19 ± 0.11 ab	36.74 ± 0.11 b
Tb Min. (°C)	Diurnal	35.65 ± 0.11	35.23 ± 0.13	35.90 ± 0.10	35.57 ± 0.10
	Intermediate	35.50 ± 0.12	34.97 ± 0.15	35.59 ± 0.11	35.31 ± 0.11
	Nocturnal	35.75 ± 0.12	35.38 ± 0.15	35.82 ± 0.11	35.65 ± 0.11
Tb Max. (°C)	Diurnal	36.87 ± 0.09 a	36.94 ± 0.13	37.32 ± 0.16	37.55 ± 0.15
	Intermediate	36.44 ± 0.10 b	36.57 ± 0.15	36.67 ± 0.18	37.46 ± 0.16
	Nocturnal	36.89 ± 0.10 a	36.95 ± 0.15	36.81 ± 0.18	38.09 ± 0.16

Values are expressed as mean ± SEM.

Different letters in the same column indicate statistically significant differences among the circadian chronotypes (ANOVA, $p < 0.05$).

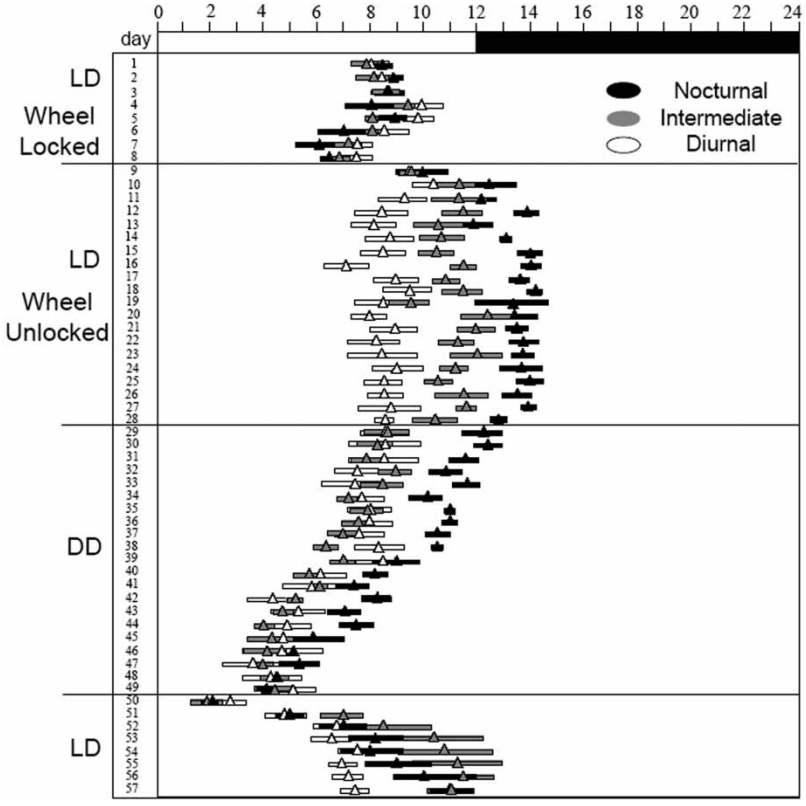

FIGURE 5 Daily evolution of chronotype differentiation in body temperature rhythm acrophases (peak times) for diurnal (white triangles, n = 6), intermediate (grey triangles, n = 5), and nocturnal (black triangles, n = 5) degus subjected to LD 12:12 and DD conditions with the wheel locked and unlocked. The triangles represent the acrophase mean for each chronotype, whereas the horizontal rectangles represent the SEM.

The day-to-day evolution of the differentiation into chronotypes induced by wheel unlocking can be observed in Figures 5 and 6. These figures show the evolution of T_b and WRA acrophases under LD and DD conditions. While the wheels were locked, all degus displayed a diurnal phase preference as shown by their T_b (see Figure 5), with no differences being evident among the chronotypes with regards to the phase angle of entrainment. However, when the wheel was unlocked at ZT8 (ZT is a standard of time based on the period of a zeitgeber; ZT0 = the time of lights on), each animal started to differentiate progressively into its particular circadian chronotype.

Both WRA and T_b exhibited a progressive phase shift until a steady phase angle of entrainment was achieved in the nocturnal and diurnal chronotypes, with the phase angle (measured using the acrophase) difference between diurnal and nocturnal animals being 6 and 7 h for T_b and WRA, respectively. Intermediate animals showed more unstable WRA

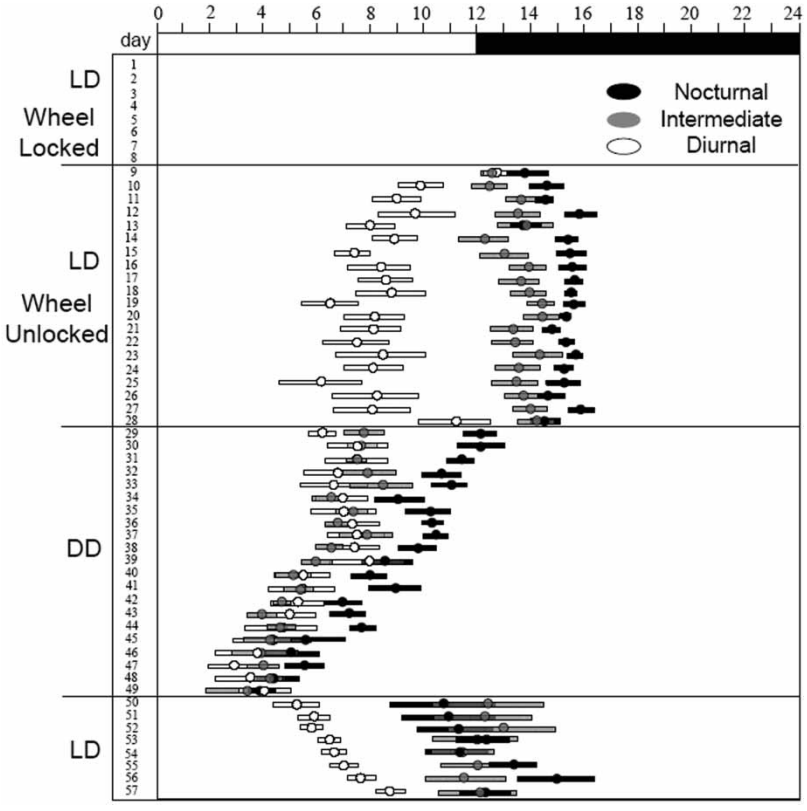

FIGURE 6 Daily evolution of chronotype differentiation in wheel running activity rhythm acrophases (peak times) for diurnal (white circles, n = 6), intermediate (grey circles, n = 5), and nocturnal (black circles, n = 5) degus subjected to LD 12:12 and DD conditions with the wheel locked and unlocked. The circles represent the acrophase mean for each chronotype, whereas the horizontal rectangles represent the SEM.

and T_b phases than nocturnal and diurnal ones, and a slight WRA and T_b phase advance with respect to nocturnal degus.

In order to further investigate the differences between chronotypes, the degus were then subjected to DD conditions, and entrainment by the previous LD conditions was evaluated. A closer inspection of the T_b acrophases (see Figure 5) revealed the T_b rhythm of diurnal and nocturnal degus started to free-run from the previous phase under LD conditions. However, intermediate degus showed a significant phase advance during the first day under DD, starting to free-run with the same phase angle as diurnal degus for both T_b and WRA (see Figures 5 and 6). No statistically significant differences in the WRA circadian period were detected among the chronotypes under DD (1423 $\pm$ 3, 1421 $\pm$ 2, and 1411 $\pm$ 5 min for diurnal, intermediate, and nocturnal degus, respectively). However, a slight trend toward a shorter tau value was observed in nocturnal than diurnal and intermediate chronotypes.

In order to determine the resynchronization dynamics, and to test the masking influence of light, the light cycle was changed from DD to LD 12:12 (see Figures 5 and 6). An abrupt change in the activity rhythm phase, indicating a negative masking effect by light, was observed from the first day under LD in both nocturnal and intermediate chronotypes, but not in diurnal animals. In the latter case, several transient cycles appeared before reaching a stable phase angle of entrainment (see Figure 6). The large phase shift observed in nocturnal and intermediate degus might be considered an inversion of the locomotor activity rhythm. However, this hypothesis may be discarded, as re-entrainment of T_b acrophases (see Figure 5) did not correlate with WRA in nocturnal or intermediate degus, and no abrupt change in the T_b acrophase phase was observed. The negative masking effect induced by light was the factor responsible for these abrupt phase shifts in WRA.

It has been suggested that the switch from diurnal to nocturnal chronotype is dependent on environmental temperature and humidity. To evaluate whether differences in degu thermoregulatory capabilities are potentially responsible for chronotype differentiation, locomotor activity was correlated with T_b (see Tables 2A and 2B) during the light and dark phases. Under locked wheel conditions, no significant differences were found in the slope, in the extrapolation of the T_b regression line to 0 counts of locomotor activity (T° 0), or in the predicted temperature at 1000 counts/1 h (T° 1000, which may be considered a standardized index of the temperature reached during high locomotor activity), among the chronotypes during either the light or dark phase.

The differentiation of chronotypes under the unlocked wheel condition paralleled the appearance of significant differences between diurnal and nocturnal degus in the slope (repeated measures ANOVA, $p = 0.001$) and T° 1000 ($p = 0.006$) during the dark phase, and in T° 0 ($p = 0.007$) during the light phase (Table 2B). The lowest slope during the dark phase appeared in nocturnal degus. It is also interesting to note that nocturnal individuals were able to run at 1000 counts/1 h with only a minor increase in T_b ($36.05 \pm 0.28^\circ$C as compared to $35.51 \pm 0.14^\circ$C at T° 0), while diurnal degus exhibited higher temperature levels when running on the wheel at night ($36.99 \pm 0.27^\circ$C as compared to $35.83 \pm 0.09^\circ$C at T° 0). No statistically significant differences were found in the 24 h WRA mean (12160 ± 1545, 9905 ± 1692, and 11589 ± 1692 counts for diurnal, intermediate, and nocturnal degus, respectively).

Experiment 2

In order to determine the occurrence of spontaneous inversions (by non-controlled factors) in the activity pattern, the WRA for a group of degus was studied for an entire year. Figures 7A and 7B show

TABLE 2 Linear correlation between locomotor activity (counts/1 h) and body temperature (°C) with the wheel locked and unlocked for diurnal (n = 6), intermediate (n = 5), and nocturnal (n = 5) degus subjected to a LD 12:12

A AA/ Temperature	WR Locked			B WRA/ Temperature	WR Unlocked		
Slope (10^{-4})	Diurnal Intermediate Nocturnal	3.1 ± 2.1 1.1 ± 2.1 4.3 ± 2.0	9.3 ± 2.0; 5.7 ± 3.9 8.0 ± 2.1	Slope (10^{-4})	Diurnal Intermediate Nocturnal	8.2 ± 1.4 10.5 ± 1.5 1.7 ± 5.6	11.6 ± 1.8 a 12.5 ± 1.6 ab 5.4 ± 1.3 b
T° 0 (°C)	Diurnal Intermediate Nocturnal	36.05 ± 0.18 35.96 ± 0.18 36.07 ± 0.16	35.47 ± 0.13 35.27 ± 0.24 35.58 ± 0.14	T° 0 (°C)	Diurnal Intermediate Nocturnal	35.99 ± 0.12 a 35.79 ± 0.07 a 36.36 ± 0.11 b	35.83 ± 0.09 a 35.44 ± 0.11 b 35.51 ± 0.14 ab
T° 1000 (°C)	Diurnal Intermediate Nocturnal	36.36 ± 0.39 36.08 ± 0.37 36.50 ± 0.36	36.41 + 0.33 35.84 ± 0.63 36.38 + 0.35	T° 0 (°C)	Diurnal Intermediate Nocturnal	36.81 ± 0.26 36.84 ± 0.22 36.53 ± 0.67	36.99 ± 0.27 a 36.65 ± 0.27 ab 36.05 ± 0.28 b

Values are expressed as mean ± 95% confidence intervals.
T° 0 is the temperature extrapolated for a locomotor activity count of 0/h. T° 1000 is the temperature extrapolated for a locomotor activity count of 1000/h.
Different letters in the same column indicate significant differences among circadian chronotypes (ANOVA, $p < 0.05$).

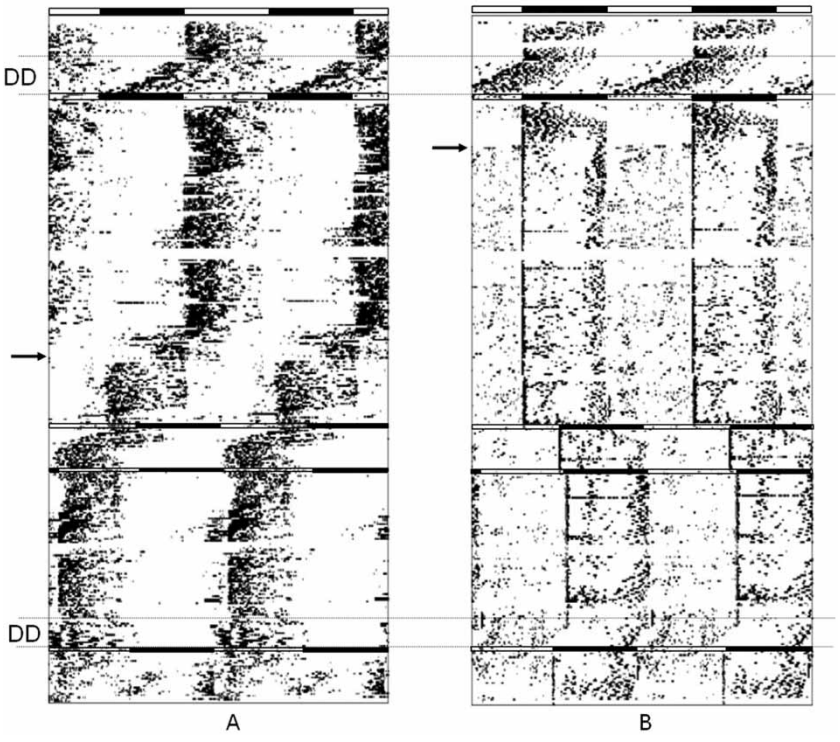

FIGURE 7 Double-plotted actograms of wheel running activity for a representative (A) 45-month-old male and (B) 18-month-old female degus recorded for 376 days and subjected to different lighting conditions: the initial 22 days under LD 12:12 (lights on at 03:00 h, off at 15:00 h), 21 days under DD, 178 days under LD 12:12 (lights on at 03:00 h, off at 15:00 h), 25 days under LD 12:12 (lights on at 08:00 h, off at 20:00 h), 82 days under LD 12:12 (lights on at 09:00 h, off at 21:00 h), 16 days under DD, and finally 32 days under LD 12:12 (lights on at 08:00 h, off at 20:00 h). The black arrow indicates spontaneous rhythm inversion.

two representative examples of the different possibilities for chronotype expressions throughout an animal's life. The WRA actogram in Figure 7A shows an example of a sudden rhythmicity change (black arrow). In this case, a diurnal degu (a 45-month-old male), after having displayed a stable WRA rhythm for several months, changed to a nocturnal pattern and, after two LD shifts, returned to its previous diurnal phase preference. In Figure 7B, a female degu (18 months of age) initially exhibited a clearly entrained nocturnal pattern, with no phase shifting when subjected to DD conditions. After resynchronization to the LD cycle, the animal experienced a spontaneous change in rhythmicity (black arrow), shifting to a more crepuscular pattern. This behavioral change was maintained during the remainder of the WRA recording, with the animal displaying a clear phase advance, characteristic of intermediate individuals, when subjected to the second DD period.

DISCUSSION

Our experiments suggest that *Octodon degus* may be considered as a primarily diurnal animal in the field and when held in cages without wheel availability; however, WR induces an almost immediate differentiation into three different chronotypes: *diurnal*, with its pacemaker entrained to the photophase and no negative masking effect by light; *nocturnal*, characterized by a pacemaker entrained to the scotophase and a strong negative masking effect by light; and finally *intermediate*, a chronotype characterized by a gradual negative masking effect by light, overlapping a pacemaker entrained to the photophase as in the diurnal chronotype. Once the chronotypes emerged, the thermoregulatory response to vigorous exercise during the photophase and the scotophase was significantly different among the circadian chronotypes. We hypothesize that the switch to nocturnal activity shown by a subset of animals is a mechanism that allows high activity levels in the wheel while avoiding overheating.

Animals are rarely continuously active, but they are active either during the day (diurnals), during the night (nocturnals), or around dawn and dusk (crepuscular). Species have adopted these behavioral patterns as a result of long periods of evolution in which adaption and natural selection have operated jointly. In such species, night or daytime behavior is fixed genetically and is frequently conditioned not only by its circadian system but also by the existence of sensory specialization. To these broad categories may be added two more types: species with activity distributed during the day, night, and twilight (cathemeral); and species with the ability to shift from diurnal to nocturnal and vice versa (dual). Dual phasing behavior has been described in fish, birds, and some mammals, such as the unstriped Nile grass rat, the blind mole rat, and the degus (Blanchong et al., 1999; García-Allegue et al., 1999; Oster et al., 2002).

From the first description of degus as *Sciurus degus* by Giovanni Ignazio Molina (1782), and subsequently as the genus *Octodon* by Edward Turner Bennett (1832), characterization of their circadian rhythmicity has been an arduous task due to a very high intra- and inter-individual behavioral variability. Early studies based on observations both in nature and in captivity considered degus to be a diurnal species, with morning and evening activity peaks (Fulk, 1976), or as a crepuscular-diurnal or crepuscular-nocturnal rodent (Iriarte et al., 1989). However, laboratory studies where the animals were able to exercise on a wheel further increased the complexity of the issue. As a result, morning, evening, intermediate- (García-Allegue et al., 1999; Labyak et al., 1997), as well as a predominantly nocturnal chronotype (Kas & Edgar, 1999) were additionally described. However, exogenous melatonin induced both a reduction in core body temperature in all chronotypes (Vivanco et al., 2007) and a PRC as expected for a diurnal mammal (Morris & Tate, 2007).

Perhaps the discrepancies between laboratories can be explained by differences in the environmental housing conditions and by the different criteria used to define degu chronotypes when attempting to systematically classify individual behavioral patterns. When one only considers the WRA ratio between the photophase and scotophase, two relatively distinct wheel running activity and T_b patterns emerge: diurnal and nocturnal. The simultaneous presence of nocturnal and diurnal animals when free access to a wheel is provided was also seen by Refinetti (2006) and Kas and Edgar (1999), who obtained percentages similar to ours. However, classification of the animals as diurnal or nocturnal using the percentage of WRA as the only criterion can be equivocal. Two criteria should be considered: the activity percentage during the scotophase and the entrainment phase angle to the LD cycle (verified by submitting degus to a DD cycle). Based on these two criteria, three chronotypes (diurnal, intermediate, and nocturnal) could be differentiated, which may be equivalent to those previously described in our laboratory (García-Allegue et al., 1999) and by others (Labyak et al., 1997), who reported the existence of morning, evening, and intermediate types. Still, it would be more correct to speak of a continuous gradient of chronotype expression, as was previously considered by Refinetti (2006), depending on masking by light and degree of entrainment. Why the entrainment phase angle changes in different ways, depending on the animal's chronotype, is still not known, but it could be hypothesized that arousal induced by wheel running is involved.

The existence of nocturnal degus held in cages without wheel availability has occasionally been described (Refinetti, 2006, and unpublished observations by our laboratory). In the current study, all degus displayed a T_b diurnal pattern when the wheel was locked, regardless of the chronotype they were later assigned after unlocking the wheel. One possible explanation for this discrepancy is that our animals had not been previously exposed to a wheel, contrary to what occurred in Refinetti's study, where some animals remained nocturnal after the wheel had been removed.

As in mammals, in general, field studies have demonstrated that temporal and spatial shifts in the activity patterns of degus can be related to thermal tolerance, food quality, and predation risks (Kronfeld-Schor & Dayan, 2003; Lagos et al., 1995). Degus also change their daily activity pattern throughout the year in response to the photoperiod and temperature (Kenagy et al., 2002). High environmental temperatures inhibit their activity and induce degus to seek shady places external to their burrows, and as temperatures rise above their thermoneutral zone, they retreat into the burrow in midday. Thus, under field conditions, environmental temperature can induce dramatic changes in activity and in phase preference.

In our experiment, the thermoregulatory response to wheel exercise differed among the chronotypes. Compared to diurnal animals, nocturnal

degus displayed a less steep correlation between T_b and WRA at night, higher daytime basal temperatures (T° 0), and greater capacity to perform intense exercise at lower temperatures during the night. Intermediate animals showed no consistent responses to exercise. All of these differences seem to be induced after WR availability. It has been suggested that some degus select the dark phase to compensate for the heat produced during exercise on the wheel (Kas & Edgar, 1999). Moreover, Lee (2004) reported that the relative proportion of chronotypes may be dependent on environmental temperature, with the proportion of nocturnal degus being very low at low ambient temperatures (18°C) and relatively high at higher temperatures. Accordingly, once the wheel has been unlocked, the degus can be defined as nocturnal or diurnal based on their particular thermal response to exercise and on the T_b threshold that makes them switch their phase preference.

Although the T_b could be determinant for phasing behavior, other factors such as the existence of an endogenous circannual rhythm in body temperature (Mustonen et al., 2007), or even in thermal sensitivity, and hence in chronotype preference, cannot be excluded. A seasonal rhythmicity with changes in the temporal niche has been previously described in some mammalian species, such as voles, squirrels, and even primates (Curtis & Rasmussen, 2006; Everts et al., 2004; Haim et al., 2005).

As it has been suggested that the lineage of octodontids (order *Rodentia*, family *Octodontidae*) is composed of nocturnal species, such as *Octodon bridgesi* and *Spalacopus cyanus*, the crepuscular-diurnal pattern of *Octodon degus* is a new, and perhaps incomplete, evolutionary acquisition (Ocampo-Garcés et al., 2006). The evolutionary inheritance of nocturnal steady-entrainment may still coexist with the recently acquired diurnalism.

Our results show that diurnal and intermediate degus have slightly, but not statistically significant, longer circadian periods under DD conditions than nocturnal degus. Previous studies by Kas and Edgar (1999) suggested the phase preference inversion occurred almost immediately—within two circadian cycles—with no evidence of phase transitions, even in the absence of light masking (i.e., under DD conditions). However, our results clearly demonstrate the phase preference inversion of activity did occur after several phase transitions once the wheel was unlocked. This is particularly evident for the T_b rhythm, which gradually transitions to altered acrophase in nocturnal animals. Thus, we conclude the mechanism that determines the overt rhythm in most degus is related to phase-control mechanisms within the circadian pacemaker. However, as a rare exception, we also detected (unpublished observation) an abrupt phase shift without phase transitions in the T_b rhythm of one degu, which would indicate that such inversions can be produced by different mechanisms.

To explain the existence of entrainment in nocturnal degus, one may hypothesize that once this initial phasing of the WRA has been changed by

masking, subsequent feedback to the pacemaker by vigorous running activity may induce a change in the pacemaker's phase angle of entrainment (Chiesa et al., 2007; Gorman et al., 2006). However, there are two arguments against this hypothesis. First, intermediate and nocturnal animals with the same apparent nocturnal WRA phasing under LD exhibited different phase angles of entrainment after being transferred to DD conditions. Second, the feedback by WRA in degus does not seem to be strong enough to entrain the pacemaker (Kas & Edgar, 2001).

On the other hand, the existence of T_b phasing changes under DD conditions after making the wheel available, as described by Kas and Edgar (1999), as well as the progressive WRA and T_b phasing change that we have observed after unlocking the wheel, would seem to support the role of the circadian pacemaker in chronotype differentiation. To date, most authors have suggested the wheel running-induced switch to a diurnal phase preference probably occurs downstream from the circadian pacemaker. According to this hypothesis, the output signal from the circadian pacemaker would be integrated with environmental and homeostatic inputs to produce a particular phasing of the observed rhythm. Wheel availability would not affect the central pacemaker itself, but rather the coupling between the pacemaker and other brain areas, such as the subparaventricular zone (Schwartz et al., 2004). However, the gradual transition of T_b and WRA phasing after wheel unlocking is difficult to explain without involvement of the pacemaker itself.

Although we cannot exclude a neural mechanism downstream from the SCN influencing activity phase preference, our results suggest, in the gradual phasing of the circadian output, that the circadian pacemaker and its interaction with masking effects are involved. One might argue that diurnal animals have a SCN that overrides the masking effects induced by light, or that the circadian mechanism of diurnal degus is less sensitive to wheel running-induced hyperactivity. Alternatively, the nocturnal degus might be more sensitive to the masking effects of light, but only under specific environmental conditions (i.e., when higher body temperatures are induced). These differences may be to some extent genetically conditioned as reported for *Arvicanthis niloticus* (Blanchong et al., 1999) and/or a consequence of early environmental influences during gestation and lactation. These periods have been demonstrated to be critical in the coupling and development of the circadian system (Anglés-Pujolrás et al., 2007).

In conclusion, two steady-entrainment phases and graded masking effects by light might explain the wide phenotypic expression of degu chronotypes. Moreover, although circadian chronotype in degus is not pre-determined, as they can phase shift throughout their entire lifespan, some individual degus have a greater predisposition to invert their phase preference than others. This predisposition may be due to

differences at any or multiple levels of the circadian controls discussed above. Thus, the diurnality of degus most likely results from a variety of mechanisms, which may explain how different processes can lead to a similar chronotype. Switching between diurnal and nocturnal patterns is not an uncommon phenomenon among some diurnal rodents; however, the biological significance of this switching mechanism in their natural habitat remains unclear. It could be hypothesized that these dual species represents a transition from nocturnalism to diurnalism in the evolutive history of mammals.

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Experimental Chapter 2

Title: "Nocturnalism induced by scheduled feeding in diurnal *Octodon degus*"

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ABSTRACT

Octodon degus, a mainly diurnal rodent characterized by its ability to shift to a nocturnal locomotor pattern under laboratory conditions, was studied to determine whether restricted food access could induce nocturnalism when available during the scotophase.

To address this question, wheel running activity, feeding and body temperature rhythms were analyzed for diurnal degus housed with a wheel and subjected to either long (12h) or short (2h) food availability periods, in the latter case, with random or scheduled food access times. The results show that allowing nocturnal feeding for two hours, but not twelve, can shift a previous diurnal phase preference for wheel running activity and body temperature to the scotophase, with random feeding being more effective than scheduled food availability for inducing nocturnalism in degus. However, this behavioral inversion proved to be unstable, as the degus returned to the diurnal phase within only a few days after the restricted feeding was discontinued. In addition, the negative masking effect induced by light, which is characteristic of the degu's nocturnal chronotype, was not observed when the animals were forced to feed at night. Thus, neither long nor short, random or scheduled, food availability during the scotophase was able to induce all the characteristics of the nocturnal chronotype in *Octodon degus*.

KEYWORDS: *Octodon degus*, nocturnalism, feeding restriction, masking, LEO, FEO, dissociation, chronotype.

INTRODUCTION

Circadian rhythms can be considered as a strategy to cope with predictable environmental events with a 24h periodicity (Sharma, 2003). Most animals display a well-defined phase relationship between their activities and the light-dark cycle, and therefore can be classified as diurnal, nocturnal or crepuscular. In most species, nighttime or daytime behavior is genetically determined, thus conditioning their circadian systems. This logically implies special sensory requirements, e.g. vision is necessary in diurnal animals in order to catch prey (Heesy, 2008). However, a small number of animal species, among which there are fish (Sánchez-Vázquez et al., 1996), migratory birds (Gwinner, 1996), and mammals (Mrosovsky, 2003; Zubidat et al., 2007), display a particular ability to shift their activity phase from diurnal to nocturnal, or *vice versa*, in a few days' time. It has been suggested that phase inversion can be triggered by: a) seasonal variations in the photoperiod (Gwinner, 1996), b) temperature (Aranda et al., 1999), c) the introduction of a non-photic stimulus, such as the availability of wheel running (Kas & Edgar, 1999), and d) food availability restrictions (Saper et al., 2005).

Circadian rhythmicity under scheduled feeding (SF) conditions has been widely studied in nocturnal rodents (Kaur et al., 2008) and other vertebrates (Sánchez-Vázquez et al., 1997). Restricted feeding increases locomotor activity (food-anticipatory activity, FAA), adrenal cortiscoterone and digestive enzyme secretion two to three hours before feeding time (Mistlberger, 1994). This FAA seems to be controlled by a circadian pacemaker that is different from the suprachiasmatic nuclei (SCN), since it is not affected by SCN bilateral lesions. In addition, it is generally believed that SF alone, without caloric restriction, cannot entrain the SCN pacemaker (Mistlberger, 1994). However, in a recent study it has reported that SCN clock genes in mice (CS and house strains) can be entrained to SF under constant dark conditions, indicating that in these strains of mice, the circadian clock can be reset by a signal associated with feeding time (Abe et al., 2007; Castillo et al., 2004).

Octodon degus, a Chilean hystricomorph rodent, is becoming an increasingly popular animal model for chronobiological and aging studies. This rodent has been traditionally characterized as diurnal, with two major activity bouts at dusk and dawn; however, diurnal, nocturnal and intermediate chronotypes have been also observed in a laboratory setting, when they have unrestricted access to a wheel (Labyak et al., 1997; García-Allegue et al., 1999). As demonstrated, both masking and the circadian

pacemaker seem to be involved in the degu's chronotypical expressions (Vivanco et al., 2009). Although it has been suggested that the degus' activity phase preference is related to constraints imposed by their limited thermal tolerance (Lagos et al., 1995), the keys to diurnalism in degus and their biological meaning remain unknown.

Natural environmental factors (i.e. light, temperature, humidity, food) act upon the circadian system in a two-fold manner, synchronizing the central pacemaker and masking overt rhythms. Masking is characterized by an activation (positive masking) or inhibition (negative) of the rhythms, modulating the pacemaker output (Mrososky, 1999). Although it has been hypothesized that masking acts at a level different from the pacemaker, to date, the precise location remains unknown. Light, as the major *zeitgeber* in nature, is also the main masking factor. Positive, negative and even paradoxical effects of light have been largely described in mammals (Mrosovsky, 1999). However, other masking factors, such as feeding, temperature and humidity, have not yet been studied to the same extent.

Stable inversion of demand-feeding behavior, from diurnal to nocturnal, has been reported in another dual phasing model, the European sea bass, when subjected to scheduled feeding (Sánchez-Vázquez et al., 1995). To date, no studies on feeding restriction have been performed on degus, therefore, it remains to be determined whether food availability could alter the phase preference in this species. To address this question, we investigated wheel running activity, feeding and body temperature rhythms in degus housed with free access to a wheel and subjected to either a long or short food availability period.

MATERIAL AND METHODS

Animals and housing conditions

Twenty-seven male *Octodon degus* between thirteen and seventeen months of age were obtained from the Animal Facilities at the University of Alicante (Spain). The animals were individually housed in polycarbonate cages (Panlab, S.L.) equipped with wheels (52 x 15 x 27 cm, L x H x W) in an isolated room ("Chronolab") with controlled humidity (60%), temperature (23 ± 1ºC) and photoperiod (LD 12:12). Light was provided by two lateral fluorescent lamps controlled by an electronic timer (Data Micro, Orbis), with an intensity of 350-400 lux at cage level. The degus were fed commercial

rat chow (A04 rat-mouse maintenance Panlab). All experimental procedures were performed in accordance with the "Principles of Animal Care" (Portaluppi et al., 2008; NIH publication No. 86-23, revised 1985) and Spanish laws.

Data recording

Wheel running activity (WRA) was recorded as wheel turns in 10-min intervals, using a data acquisition system (Electronic Service at the University of Murcia, Spain). Body temperature (T_b) was measured at 60-min intervals, using a miniature data logger (ThermoChron® iButton Data loggers, Maxim Integrated Products, Sunnyvale, California), with an accuracy of 0.1ºC. For this purpose, sterilized data loggers were implanted intraperitoneally, under aseptic conditions, using fluothane as anesthesia (Forane®, Abbot Laboratories S.A., Madrid). Reabsorbable silk (2/0, Safil®Quick B/Braun, Barcelona) was used to suture the abdominal layers, and non-reabsorbable silk to suture the skin. No mortality or morbidity was observed after the surgery. The experiment began following a two-week recovery period. At the end of the experiment, the data logger was removed under the same conditions in which it was implanted. iButton readout hardware was used to transfer temperature data to a computer.

Feeding activity (FA) was recorded as food approaches, using a contact eatometer described elsewhere (Madrid et al., 1995). Briefly, a stainless steel rolling grid was mounted on the wire lid of each cage that the animals had to push in order to reach the food in the eating area. A microswitch fitted on the rolling grid was activated each time the grid was pushed. The system permitted continuous recording of feeding activity (food approaches), even during the period of imposed fasting. FA was recorded as microswitch contacts in 10-min intervals, using a data acquisition system (Electronic Service at the University of Murcia, Spain).

Experimental design

Three experiments were performed to characterize whether different periods of food availability can induce stable phase inversion in the degus' circadian activity and temperature patterns.

Experiment 1. Long-term food availability (LFA)

The aim of this experiment was to analyze the influence of a long period of food availability, restricted to either the photophase or the scotophase, on the degu chronotypes. To this end, fourteen degus (thirteen months of age) were held individually under LD 12:12 conditions, with free access to wheel running and *ad libitum* feeding. After eight days, feeding was restricted to 12h, during either the photophase (four animals) or the scotophase (six animals). Ten days later, food availability was inverted, so that animals that had been previously feeding during the photophase were now allowed to feed only during the dark phase, and *vice versa*. After nine days, the degus were subjected to three days of fasting. Finally, all degus resumed *ad libitum* feeding for six days. Food pellets were manually distributed or removed immediately before dark onset and after lights on.

Experiment 2. Short-term food availability (SFA)

This experiment was carried out to determine the influence of a random or scheduled 2h period of food availability per day on chronotype differentiation.
To this aim, thirteen degus (seventeen months of age) were held under LD 12:12 conditions in individual cages, with free access to wheel running and food and water *ad libitum*. After 7 days, the degus were subjected to a 2h period of food availability at random times during the scotophase (between ZT 14-20) over thirteen days. They were then allowed to feed *ad libitum* for thirty-four days, and this was followed by 2 hours a day of food availability at a scheduled time (ZT 16-18) for two weeks. Finally, the animals resumed an *ad libitum* feeding schedule for seven days.

Chronotype characterization and data analysis

Before starting the experiments, to characterize the chronotypical expression of degus, a numerical criterion based on the percentage of diurnal versus total activity was used. Thus, when an animal under LD conditions showed a diurnal/total activity ratio above 60%, it was considered diurnal. Due to the existence of intermediate chronotypes, with intermediate locomotor activity values, a nocturnal animal was defined as one whose diurnal/total activity ratio was less than 20% (Vivanco et al., 2009). Since our aim was to induce nocturnalism by feeding schedules on this dual species, only those individuals with a clear diurnal pattern were selected. Then, four

and five degus were excluded from LFA and SFA experiments, respectively, due to an unstable activity pattern.

Average actograms, mean waveforms and acrophases from a cosine fit of WRA and T_b rhythms were obtained using software specifically designed for chronobiological analysis (El Temps, version 1.228; © Díez-Noguera, University of Barcelona). To determine the daily acrophase evolution of wheel running activity and body temperature rhythms for each chronotype, the mean and standard error of the mean (SEM) of individual daily acrophases were calculated. Food anticipatory activity was determined by comparing the percentage of total daily WRA in the two hours previous to meal time during each feeding restriction phase (12h at night, 12h during the day and 2h scheduled at night) with that obtained for the correspondent *ab libitum* phases.
A repeated measures ANOVA was performed to compare locomotor activity and body temperature during the restricted feeding phases. The experimental circadian variables were tested for normality of distribution and homogeneity of variance. An unpaired t-test (p<0.05) was used for comparing food intakes between experimental phases. All statistical analyses were performed with SPSS 13.0 software.

RESULTS

Experiment 1. Long-term food availability (LFA)

At the beginning of the LFA experiment, once the numerical criteria for chronotypical classification had been determined, 10 out of 14 degus displayed diurnal WRA, FA and T_b patterns. Four animals exhibited an irregular or unclassifiable phenotype, and therefore were excluded from the experimental analysis.

Diurnal degus, such as the representative individual in Fig. 1, subjected to a 12:12 LD period and fed *ad libitum* (mean food intake= 12.3 ± 0.4 g/day), began their WRA approximately 1-2 hours before lights on, running during the light phase and the first few hours of the dark phase. Their daily T_b rhythm demonstrated a diurnal pattern, characterized by higher values during the photophase than during the scotophase.

Similarly, feeding activity was in phase with both WRA and T_b rhythmicities (top panels in Fig. 2). All circadian variables tended to show higher values during the photophase than the scotophase (Table 1).

When food availability was restricted to the dark phase (LFA), the FA rhythm clearly dissociated from the WRA and T_b rhythms (Figs. 1 and 2). Thus, while most FA was confined to the darkness period, WRA and T_b continued to display a predominantly diurnal pattern (second row of panels in Fig. 2). The mean WRA increased slightly during both light and dark phases (Table 1), and T_b showed a significant decrease in its diurnal mean value (repeated measures ANOVA, p=0.045), together with an increase in the nocturnal maximum value, probably due to the potentially thermogenic effect of food metabolism (p=0.003, in Table 1).

Similarly, diurnal food availability showed no signs of modifying the rhythmicity pattern observed during *ad libitum* feeding. It is interesting to note that when the food was available at nighttime, the degus interacted with the eatometer even during the light hours, while only diurnal activation was observed under daytime feeding conditions (Figs. 1 and 2). Statistically significant differences (repeated measures ANOVA, p<0.01) were found in almost all the circadian variables, except for maximum WRA, nocturnal mean WRA, diurnal maximum T_b and minimum T_b phase, when comparing nighttime and daytime food availability schedules (Table 1). However, no significant differences were found when comparing diurnal with *ad libitum* feeding.

Mean food intake (11.1 ± 0.4 and 11.5 ± 0.4 g/day for nighttime and daytime food availability, respectively) remained unchanged during both feeding restrictions with respect to the *ad libitum* phase (12.3 ± 0.4 g/day, paired t-test, p<0.05).

During the three-day fasting period, the degus exhibited a significant increase in their nocturnal mean and maximum WRA (repeated measures ANOVA, p=0.002, and p= 0.048, respectively) and a decrease in their minimum nocturnal T_b (p=0.001) as compared to the *ad libitum* phase. However, WRA and T_b waveforms matched a diurnal pattern (Fig. 2).

Figure 3 summarizes the relationship between WRA, T_b and FA acrophases in the LFA experiment when a stable state was achieved. A high degree of coincidence among the three variables was evident in all experimental phases; however, nocturnal food availability induced a phase shift towards the night only in FA, while WRA and T_b continued to exhibit their previous diurnal phase patterns.

Experiment 2. Short-term food availability (SFA)

When the experiment started, 8 out of 13 total degus displayed predominantly diurnal WRA, FA and T_b rhythms (Figs. 4 and 5). Two hours of food availability at random times during the scotophase (between ZT 14-20) shifted the diurnal WRA and T_b phase into a nocturnal phase in only a few days' time (Figs. 4 and 5). An increase in nocturnal mean WRA and T_b (repeated measures ANOVA, p=0.027 and p=0.012, respectively) was observed, together with a significant decrease in the diurnal mean WRA and T_b (p=0.027 and p<0.001, respectively), as well as in the maximum (p=0.002) and minimum T_b (p=0.002, in Table 2), as compared to the *ab libitum* period. Nocturnal shifting induced by random SFA was progressively achieved in WRA and T_b (Fig. 6).

WRA and T_b acrophases stabilized after four or five days following the onset of random nocturnal feeding, while FA shifted on the first day. When *ad libitum* food availability resumed, both WRA and T_b acrophases switched to a diurnal phase preference within only one day. It is striking to note that the FA acrophase resynchronized relatively slowly once the random phase ended, taking around six days to establish a stable diurnal phase (Fig. 6).

In the second part of the experiment, two hours of food availability scheduled between ZT16-18 induced a nocturnal phase inversion in WRA and T_b rhythmicities (Fig. 4). Both WRA and T_b waveforms presented a pattern similar to that observed in the previous random phase (Fig. 5, third row of panels). A significant decrease was observed for the diurnal mean WRA (repeated measures ANOVA, p=0.030) and the diurnal mean (p=0.003), maximum (p=0.021) and minimum (p=0.001) T_b values as compared to the *ad libitum* period, similar to that which occurred during the random feeding period (Table 2). Likewise, a significant increase was evident in the nocturnal mean T_b (repeated measures ANOVA, p=0.013). Interestingly, no differences in nocturnal mean WRA and minimum T_b phase were found between the scheduled food availability phase and the *ad libitum* phase. However, statistically significant differences in mean nocturnal WRA (repeated measures ANOVA, p=0.003) and diurnal T_b (p=0.009, in Table 2), but not in minimum T_b phase, were detected between the random and scheduled experimental phases.

From the beginning, both WRA and T_b acrophases showed a progressive resynchronization until reaching a stable acrophase in 6-7 days (Fig. 6). Once the

degus returned to *ad libitum* feeding, they progressively shifted their rhythmicities once again towards a diurnal phase.

A high degree of coincidence between WRA, T_b and FA acrophases was observed under both random and scheduled SFA phases (Fig. 7), unlike what was observed in the LFA experiment. It is interesting to observe that the acrophases of the three circadian variables are closer during the random feeding period than during the scheduled feeding period.

Two hours of food availability, either at scheduled or randomly assigned times (10.1 ± 0.3 and 10.0 ± 0.4 g/day, respectively), failed to result in a significant caloric reduction (paired t-test, $p<0.05$), as compared to the *ad libitum* phase (11.1 ± 0.6 g/day).

Food anticipatory activity, WRA during the 2h period prior to food availability, is shown in Figure 8. A significant increase in the percentage of total activity during those 2 hours, compared with *ad libitum* periods, were only found in the 12h availability at night (repeated measures ANOVA, $p=0.027$) and 2h scheduled at night ($p=0.020$) experimental phases.

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DISCUSSION

The present data show that two, but not twelve, hours of nocturnal food availability were capable of shifting a previous diurnal phase of WRA and T_b to the scotophase, with random feeding being more effective than scheduled food availability for inducing nocturnalism in degus. However, this behavioral inversion was not stable, as it reverted back to the previous diurnal pattern in a matter of only a few days after the restricted feeding was ended. Furthermore, the negative masking effect induced by light, a characteristic of the degu's nocturnal chronotype, was not observed when individuals were compelled to feed at night. Thus, neither long nor short (random or scheduled) food availability during the scotophase was capable of inducing all the characteristics of the nocturnal chronotype in *Octodon degus*.

This incomplete nocturnalism could be explained by feeding entrainable oscillator (FEO) involvement. The output of the light entrainable oscillator (LEO) most likely did not shift by food availability schedules here performed since: a) the animals still showed a high level of activity during the light hours when food was available

during the night, and b) the phase of minimum body temperature was maintained throughout the experimental procedures.

Under natural environmental conditions, degus exhibit a mainly diurnal or crepuscular behavior. According to field studies, a seasonal rhythmicity pattern in foraging time has been observed throughout the year (Kenagy et al., 2002). In the summer, when the weather is hot and dry, some degus forage during the darkness, probably to avoid the higher daytime temperatures (Saper et al., 2005). In nature, this thermoregulatory constraint observed for degus may be accompanied by a parallel rhythmicity in the predator-prey relationship, as pointed out by Lagos (1995) and as occurs with other mammals (Kronfeld-Schor, 2003). In fact, different degu predators have been found in both diurnal (hawks and foxes) and nocturnal (owls) temporal niches (Meserve et al., 1993).

Under laboratory conditions, the degus' ability to change their circadian phasing might be reminiscent of this adaptive ability, allowing degus to forage during either night or day with equal ease. However, our results do not entirely support this hypothesis. When degus were permitted to access food during the 12 hours of the scotophase (LFA), no WRA and T_b pattern shift occurred, although a concentration of FA before light onset and around light offset was observed. Neither changes in phasing nor masking effects on WRA or T_b were observed in response to 12 hours of nighttime food availability, thus it could not trigger a 180º phase shift of the diurnal pacemaker in degus.

Moreover, the slight influence of 12h of food availability upon the overt rhythmicity indicates that this window is not a *zeitgeber* strong enough to uncouple light and food synchronizing effects on biological rhythms. On the contrary, when food availability was restricted to 2 hours during the scotophase (SFA), important changes occurred in the degus' phasing, with differences being observed between random and scheduled feeding times. Two hours of food availability at a scheduled time induced a progressive WRA and T_b phase shift to the dark phase within six days; however, a significant WRA still occurred during the photophase. Two hours of random feeding produced a more pronounced phase shift to nocturnalism in both WRA and T_b rhythms than was observed with scheduled feeding, but again, a complete phase inversion was not achieved.

Moreover, the slow Tb resynchronization that occurs after scheduled feeding points out to a different mechanism from that responsible for the instantaneous phase shift seen with random feeding. Specially considering that, unlike scheduled feeding, random feeding does not produce FEO anticipation.

As the results show, nocturnal food availability merely induced a nocturnal phase shift in degus, but was not able to induce a stable circadian inversion or generate the most characteristic feature of the nocturnal chronotype: masking by light.

As Castillo et al. (2004) showed in mice, the entrainment of the SCN to scheduled feeding takes weeks to months to be completed, so it can be argued that our 10-13 days of scheduled feeding is not enough to allow a complete entrainment and, then a phase inversion. However, in our previous experiments we showed that a complete nocturnal/diurnal inversion occurs in very few days (4 to 5) after wheel running availability (Vivanco et al., 2009).

The nocturnal degu chronotype is characterized by the appearance of a strong negative masking effect by light, together with a nocturnal phase angle of entrainment for locomotor and body temperature rhythms controlled by the central pacemaker (LEO), although not in all cases (Vivanco et al., 2009). In our study, since feeding restriction was not able to generate masking by light in diurnal individuals, it could be hypothesized that this masking occurs only when the LEO is involved. Indeed, if wheel running is able to generate a masking response by light, it may be by the direct effect of locomotor activity upon the circadian pacemaker (Chiesa et al., 2007), therefore, food restriction should generates another kind of masking (no light dependent), acting on a different relay stage of the circadian pathway.

In spite of the existence of a continuous gradient of circadian chronotypical expression from diurnal to nocturnal degus in WRA and Tb rhythms when a wheel is available (Vivanco et al., 2009), most authors have adopted a category criteria, classifying chronotypes (Nocturnal/ Diurnal; Morning/ Intermediate/ Evening and Diurnal/ Intermediate/ Nocturnal) either by temperature (Kas & Edgar, 1999) or locomotor activity (García-Allegue et al., 1999; Ocampo-Garcés et al., 2006; Vivanco et al., 2009) or both of them (Labyak et al., 1997). However, and since diurnal pacemaker is found in most individuals (Morris & Tate, 2007), *Octodon degus* is considered mainly as a diurnal animal. Thus, the percentage of nocturnalism induced by wheel running varies greatly depending on the laboratory, experimental conditions and chronotype

classification criteria. It can range from 100% nocturnalism, as described by Kas & Edgar (1999), to 37-40% nocturnalism, as observed in our case (García-Allegue et al., 1999; Vivanco et al., 2009). In fact, this inversion may reoccur over time; for example, 30% nocturnalism had been observed in previous recordings for the same degus group that displayed a clear diurnal pattern in these experiments, despite the presence of a wheel. The existence of some seasonality in their chronotipical trend and/or the fact that these animals were exposed to the wheel for a long period of time before these experiments began could explain the low rates of nocturnalism obtained here.

Although a large number of studies have been conducted in nocturnal animals, as far as we know, this is the first time that the relationship of food availability on the entrainment status of a diurnal mammal has been studied. Moreover, in spite of the fact that feeding time is considered to be one of the most important *zeitgebers* found in nature, no studies have been conducted to clarify the influence of food availability on the duality of circadian patterns in mammals. However, this is not the case in fish.

In *Dicentrarchus labrax*, for example, a teleost fish characterized by a dualism in its locomotor and demand-feeding behavior, restricting food availability to a 12-hour period opposite to the previous spontaneous feeding phase induced a stable shift from a diurnal to a nocturnal pattern or *vice versa* (Sánchez-Vázquez et al., 1995). Furthermore, this phase inversion persisted once the feeding schedule ended. These results support the hypothesis that food availability may be one of the switching factors for the fish circadian system, determining whether they behave as nocturnal or diurnal.

Circadian rhythms have been demonstrated to be controlled by two groups of oscillators: the main circadian oscillator (LEO), based on light-information input through the retinal pathway to the SCN, and the oscillator entrained by feeding, FEO, probably located in the dorsomedial hypothalamus (DMH), and which is based on the food information input generated directly in the feeding-related peripheral organs (Mistlberger, 1994; Gooley et al., 2006). Each of the oscillator groups has an influence upon the other. In an animal entrained to a LD condition and feeding *ad libitum*, the central pacemaker (LEO) spreads a temporal signal, through the whole organism. However, when an animal is subjected to restriction feeding times, then FEO uncouples from LEO information and some rhythms (such as activity and temperature) anticipate food intake (Damiola et al., 2000; Escobar et al., 2007). In this sense, the incomplete nocturnalism in degus induced by scheduled short food availability, without any negative masking by light or stable phase shift, together with slow transients after

feeding restriction, high level of diurnal activity and minimun temperature phase maintainance could be explained only by FEO involvement.

In conclusion, two, but not twelve, hours of restricted food availability during the night can induce an incomplete and transient nocturnalism for WRA and T_b rhythms in *Octodon degus,* with random feeding being more effective than scheduled food availability. However, this phase inversion is not stable, and does not generate any negative masking effects by light, as occurs in our nocturnal degu chronotype (Vivanco et al. 2009). These results would indicate that changes induced by food availability must occur in a different location than those produced when wheel running is available. The spontaneous nocturnal phase preference shown by some degus in response to wheel running may be the evolutionary remains of an incomplete process of recent diurnalism acquisition, as suggested by Ocampo-Garcés et al. (2006), rather than a strategy to cope with the variable food supplies found in nature.

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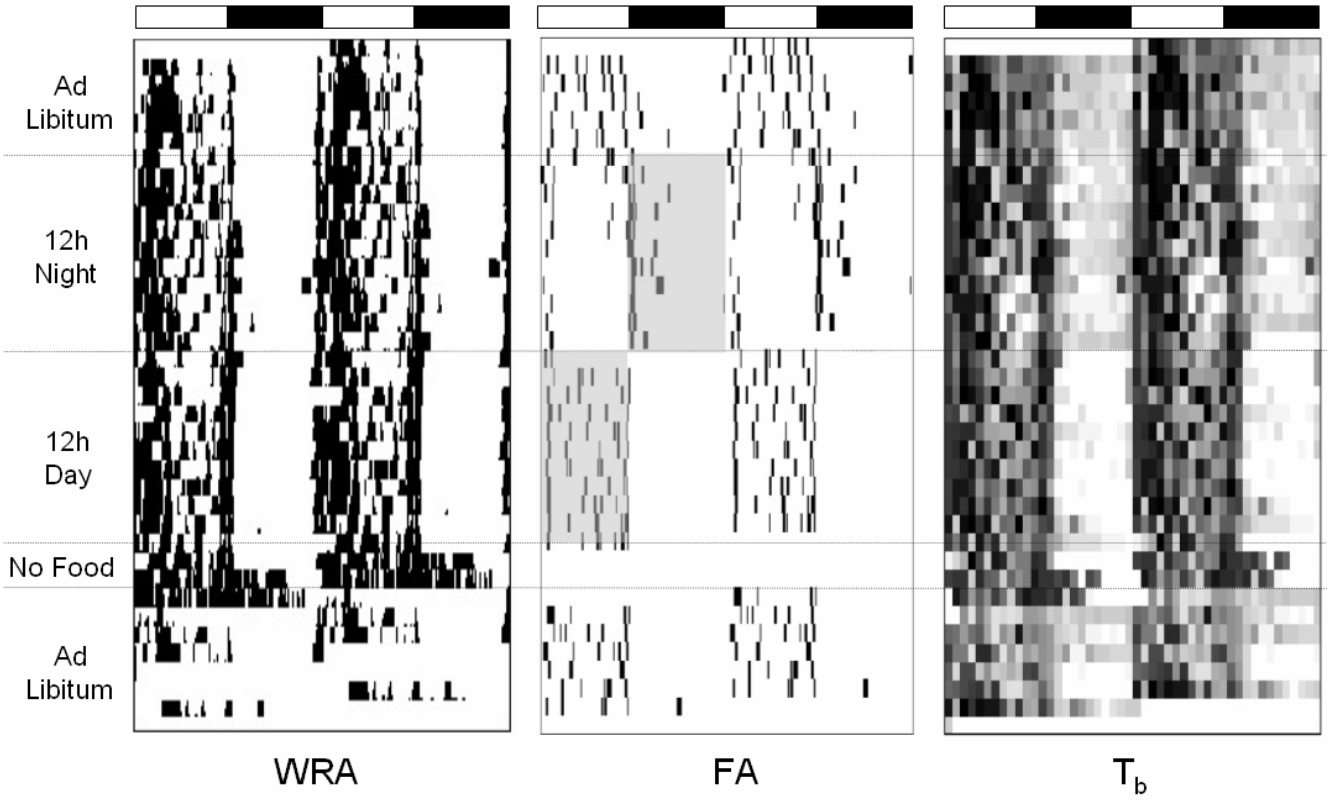

Figure 1. Actograms of wheel running activity (WRA), feeding activity (FA) and body temperature (T_b) of a representative diurnal degus during the long-term food availability (LFA) experiment. Each actogram represents 36 days of recordings and four experimental feeding phases: *ad libitum*; 12h availability during the scotophase (12h Night), 12h availability during the photophase (12h Day), a fasting period (No food) and, finally, *ad libitum* feeding once again. White and black bars at the top of the graphic represent the lighting schedule. Grey area in the FA panel indicates food availability in the 12h night and 12h day experimental stages. Data have been double-plotted for convenient visualization.

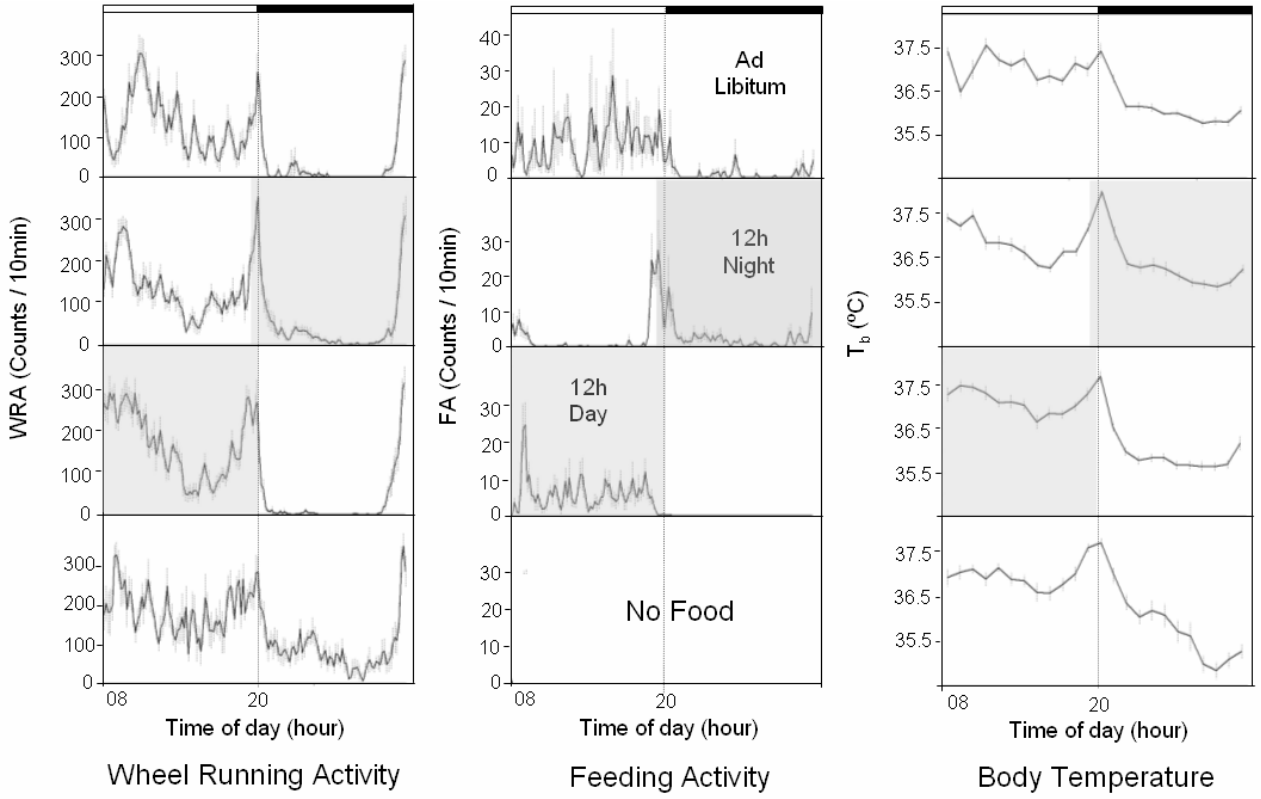

Figure 2. Wheel running activity, feeding activity and body temperature average mean waveforms corresponding to the four feeding experimental stages during the long-term food availability (LFA) experiment: *ad libitum* (top panels), 12h availability during the dark period (12h Night, shaded area in second row of panels), 12h availability during the light period (12h Day, shaded area in third row of panels) and, finally, a fasting period (No food, fourth row of panels) in diurnal degus. Vertical lines in the waveform represent the standard error of the mean (SEM) for each average value (n=10). White and black bars at the top of the graphs represent the lighting schedule. The dotted vertical line indicates the light/dark transition for purposes of comparison.

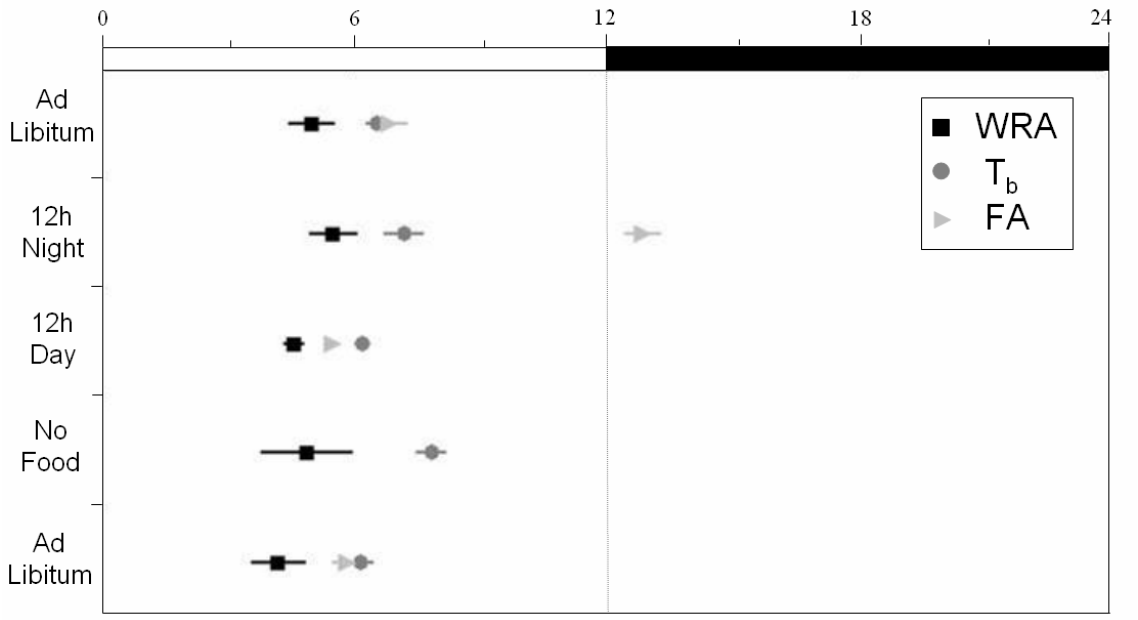

Figure 3. Mean acrophases for wheel running activity (WRA), feeding activity (FA) and body temperature (T_b) during the four experimental feeding stages during the long-term food availability (LFA) experiment: *ad libitum*, 12h availability during the dark period (12h Night), 12h availability during the light period (12h Day) and, finally, a fasting period (No food) in diurnal degus. Values represent the mean acrophase (n=10) and its standard error (SEM), as calculated by cosinor analysis.

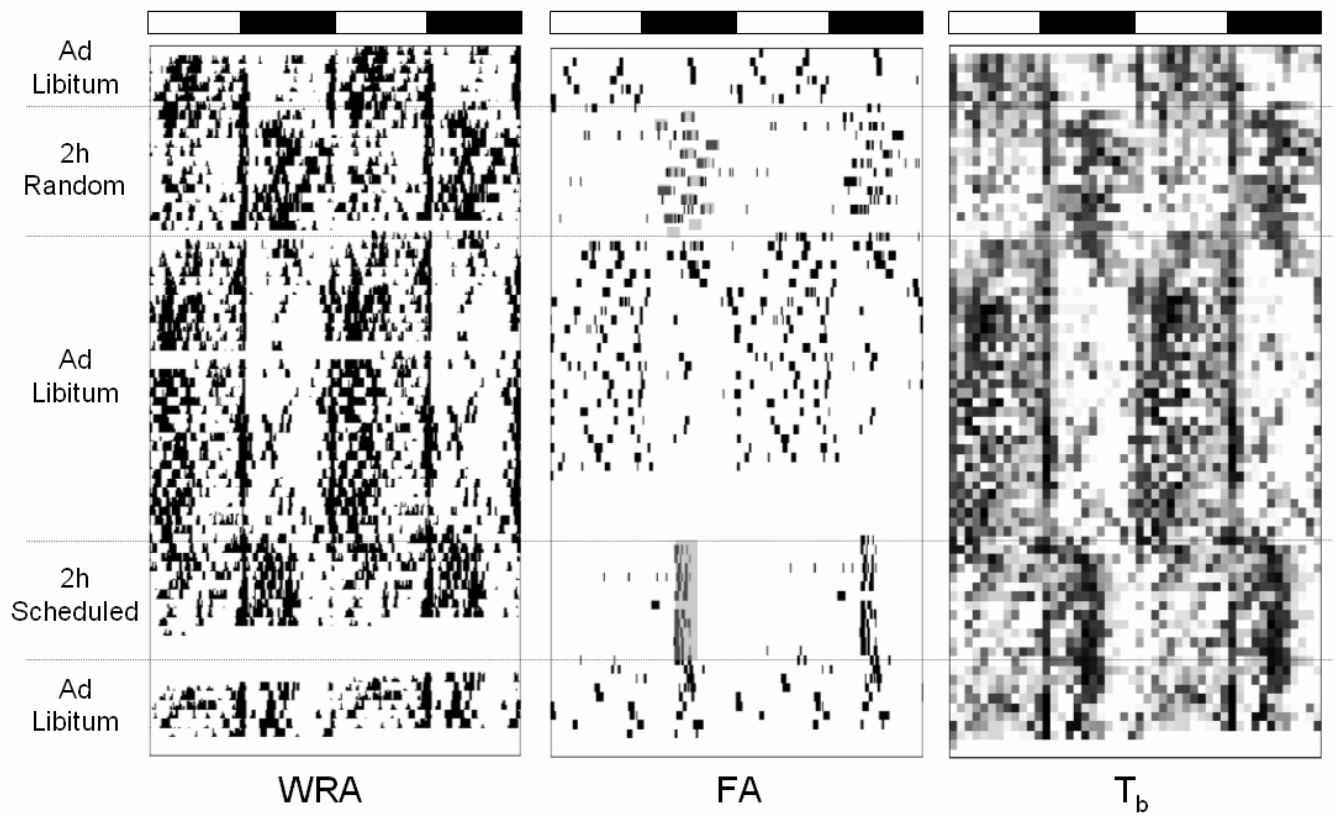

Figure 4. Actograms of wheel running activity (WRA), feeding activity (FA) and body temperature (T_b) for a representative diurnal degus during the short-term food availability (SFA) experiment. Each actogram represents a 75-day recording period and five experimental feeding stages: *ad libitum*, 2h of random availability during the night (2h Random), *ad libitum*, 2h of scheduled availability during the night (2h Scheduled) and, finally, an *ad libitum* period. White and black bars at the top of the graph represent the lighting schedule. Gray boxes at random and scheduled feeding indicate the time of feeding availability. Data have been double-plotted for convenient visualization.

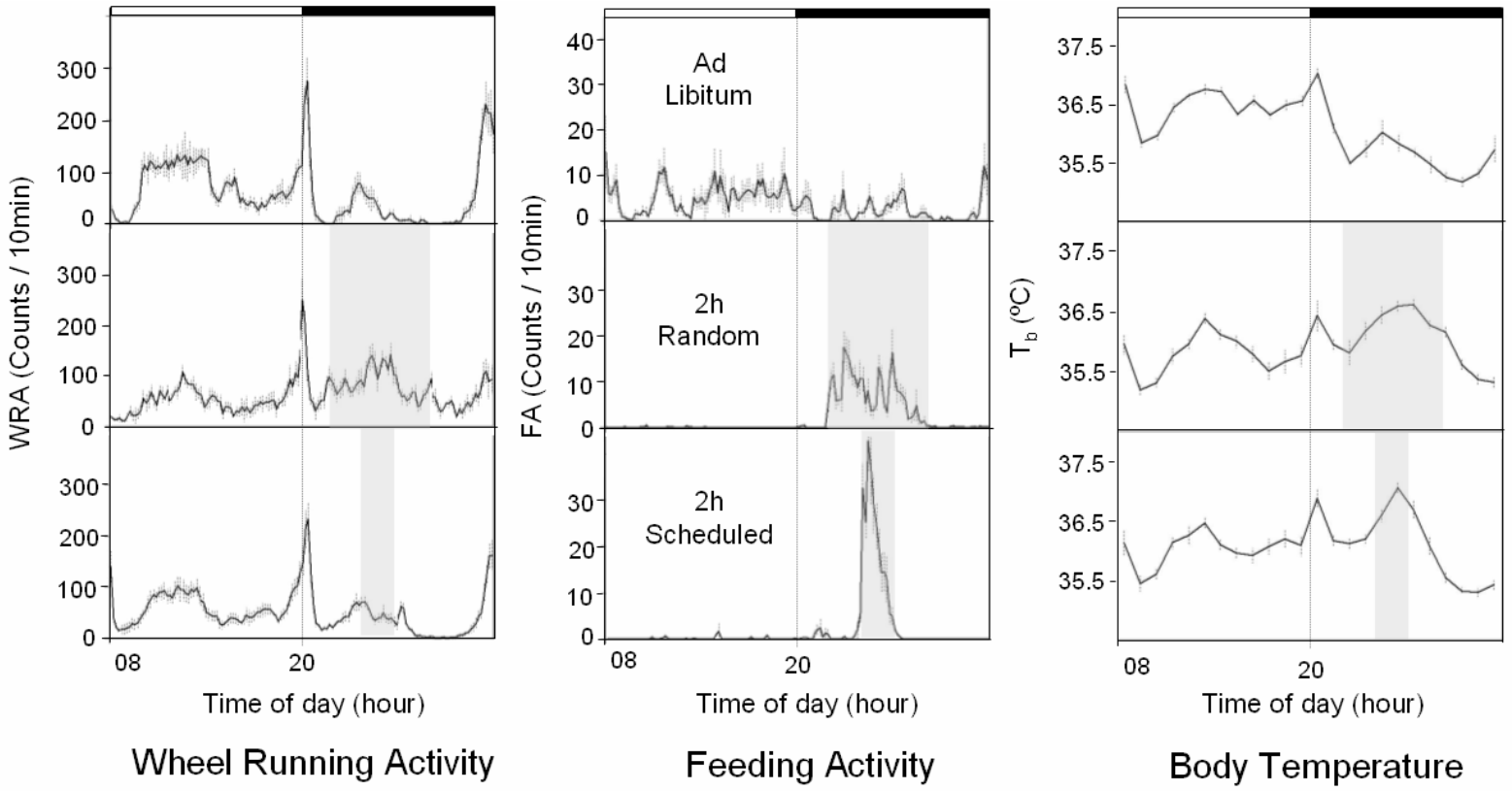

Figure 5. Wheel running activity, feeding activity and body temperature mean waveforms corresponding to the three main experimental feeding stages during the short-term food availability (SFA) experiment: *ad libitum* (first row of panels), 2h of random availability during the night (2h Random, second row of panels), and 2h of scheduled availability during the night (2h Scheduled), third row of panels) in diurnal degus. Vertical lines in the waveform represent the standard error of the mean (SEM) for each average value (n=8). Food availability is represented by a gray shaded area (second and third rows of panels). White and black bars at the top of the graph represent the lighting schedule. The dotted vertical line indicates the light/dark transition for purposes of comparison.

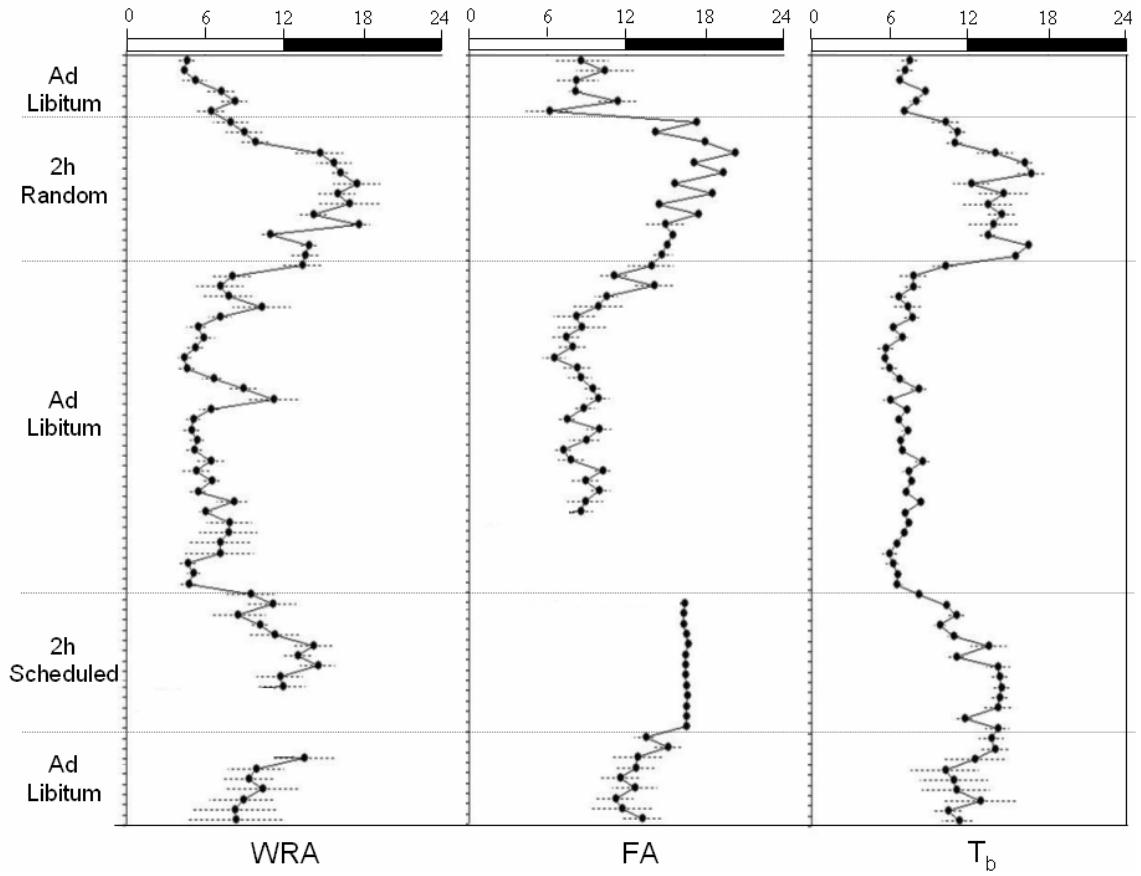

Figure 6. Daily mean acrophases of wheel running activity (WRA), feeding activity (FA) and body temperature (T_b) for the five experimental feeding stages during the short-term food availability (SFA) experiment: *ad libitum*, 2h of random availability during the night (2h Random), *ad libitum*, 2h of scheduled availability during the night (2h Scheduled) and, finally, an *ad libitum* period, for diurnal degus. The circles represent the mean acrophase (n=8) for each variable, as calculated by cosinor analysis, whereas the horizontal dotted line represents the SEM.

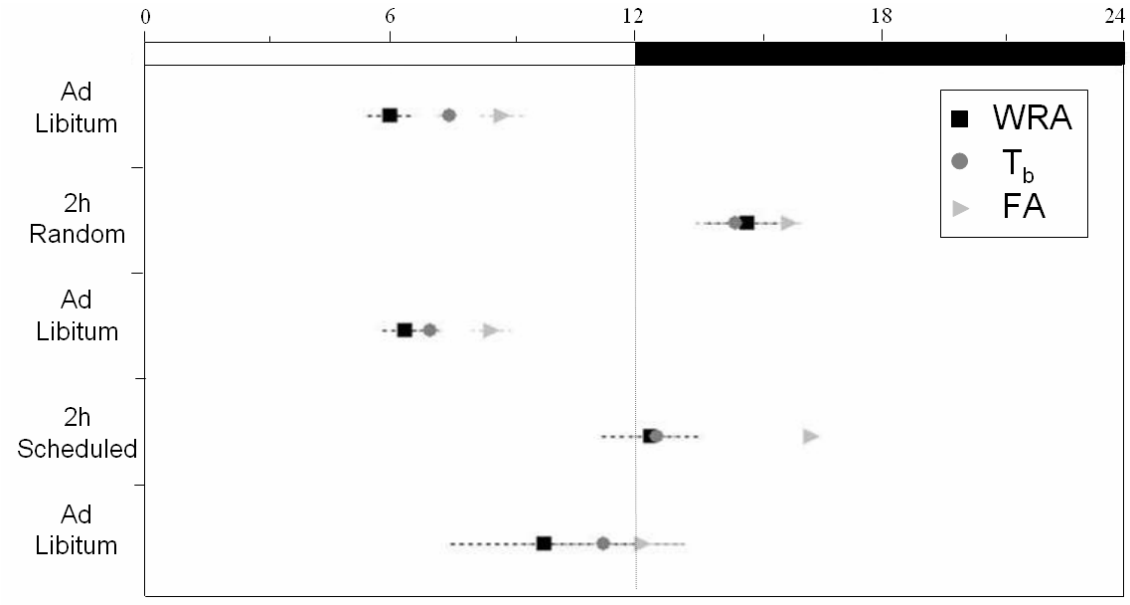

Figure 7. Mean acrophases of wheel running activity (WRA), feeding activity (FA) and body temperature (T_b) for the five experimental feeding stages during the short-term food availability (SFA) experiment: *ad libitum*, 2h of random availability during the night (2h Random), *ad libitum*, 2h of scheduled availability during the night (2h Scheduled) and, finally, *ad libitum*. Values represent the mean acrophase (n=8) and its standard error (SEM), as calculated by cosinor analysis.

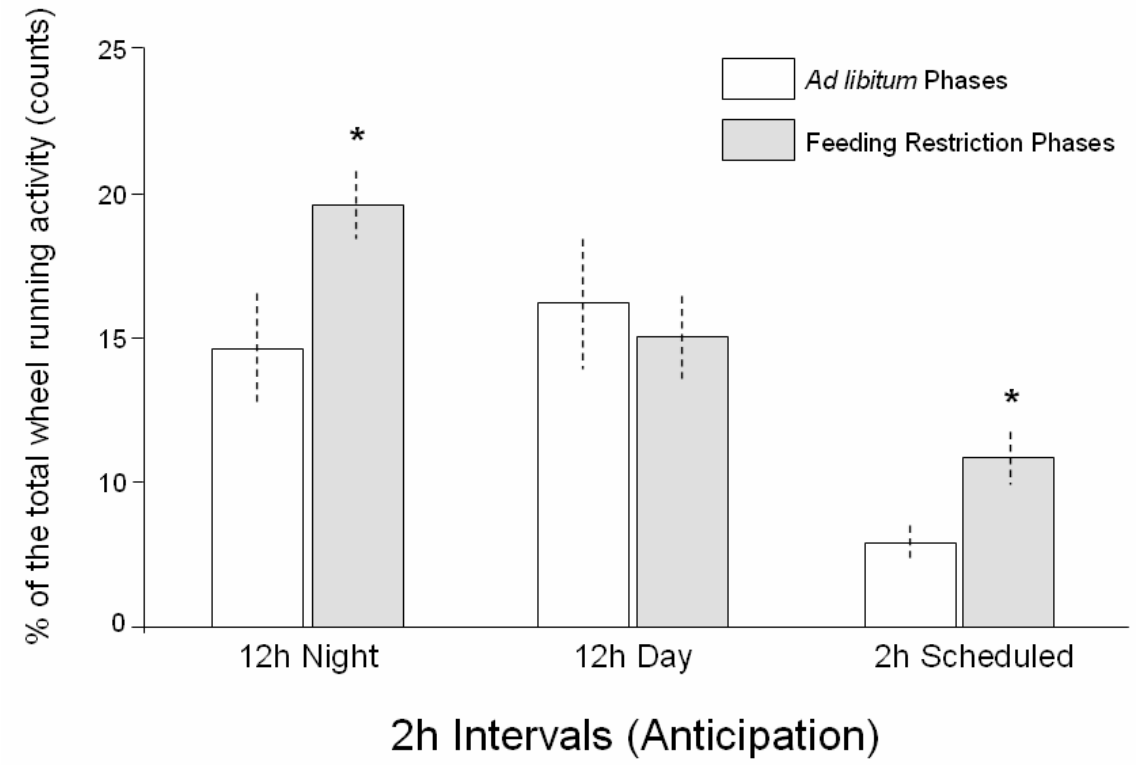

Figure 8. Food anticipatory activity expressed as percentage of wheel running activity (mean ± SEM) in the two hours previous to the meal time with respect to the total daily activity of the correspondent experimental phase (2h at night, 12h in the day, and 2h scheduled at night). Asterisks indicate significant differences between each *ad libitum* and its correspondent feeding restriction schedules ($p<0.05$).

	Ad libitum		Night		Day		Fasting	
WRA Mean per 1h (Counts)	771± 111	ab	792 ± 63	a	990 ± 61	b	1112 ± 165	ab
	190 ± 40	a	284 ± 36	a	197 ± 14	a	566 ± 50	b
WRA Max. per 1h (Counts)	1868 ± 182		1676 ± 133		1963 ± 126		1781 ± 197	
	1163 ± 97	a	1544 ± 181	ab	1300 ± 119	ab	1640 ± 121	b
T_b Mean (ºC)	37.04 ± 0.11	a	36.83 ± 0.10	b	37.10 ± 0.11	a	36.93 ± 0.11	ab
	36.16 ± 0.07	ab	36.34 ± 0.11	a	36.00 ± 0.10	b	35.89 ± 0.16	b
T_b Max. (ºC)	37.78 ± 0.12		37.55 ± 0.08		37.65 ± 0.13		37.66 ± 0.11	
	37.46 ± 0.18	a	37.97 ± 0.14	b	37.68 ± 0.13	a	37.72 ± 0.09	ab
T_b Min. (ºC)	36.29 ± 0.12	ab	36.14 ± 0.09	a	36.51 ± 0.10	b	36.18 ± 0.14	ab
	35.66 ± 0.07	ac	35.78 ± 0.09	a	35.53 ± 0.11	c	34.66 ± 0.17	b
T_b Min. phase (hh:mm)	04:24 ± 00:24		04:30 ± 00:24		04:06 ± 00:24		04:30 ± 00:18	

Table 1. Effects of long-term food availability (LFA) experiment on the circadian values of wheel running activity (WRA) and body temperature (T_b) in the four feeding experimental stages: *ad libitum*, 12h availability during the dark period (night), 12h availability during the light period (day) and, finally, a fasting period (fasting) in diurnal degus. Values are expressed as mean ± SEM. Different letters in the same row indicate statistically significant differences among the food availability schedules (ANOVA, $p<0.05$). Vertical white and black bars at the left of the graphic represent the photophase and scotophase, respectively.

		Ad libitum		Random		Scheduled	
WRA Mean per 1h (Counts)		443 ± 50	a	309 ± 43	b	357 ± 55	b
		258 ± 37	a	464 ± 36	b	257 ± 37	a
WRA Max. per 1h (Counts)		974 ± 118		716 ± 73		741 ± 103	
		1273 ± 154	a	913 ± 74	ab	853 ± 112	b
T_b Mean (ºC)		36.46 ± 0.03	a	35.79 ± 0.10	b	36.04 ± 0.09	c
		35.74 ± 0.08	a	36.07 ± 0.10	b	36.12 ± 0.08	b
T_b Max. (ºC)		37.06 ± 0.08	a	36.49 ± 0.11	b	36.65 ± 0.14	b
		37.10 ± 0.08		36.89 ± 0.12		37.19 ± 0.12	
T_b Min. (ºC)		35.73 ± 0.05	a	35.16 ± 0.11	b	35.31 ± 0.08	b
		35.13 ± 0.10		35.21 ± 0.10		35.26 ± 0.07	
T_b Min. Phase (hh:mm)		04:54 ± 00:24	a	09:00 ± 00:30	b	07:18 ± 00:36	ab

Table 2. Effects of short-term food availability (SFA) experiment on the circadian values of wheel running activity (WRA) and body temperature (T_b) in the three main feeding experimental stages: *ad libitum,* 2h of random availability during the night (random), and 2h of fixed availability during the night (scheduled) in diurnal degus. The minimum T_b phase is expressed in time (lights on at 08:00h). Values are expressed as mean ± SEM. Different letters in the same row indicate statistically significant differences among the food availability schedules (ANOVA, $p<0.05$). Vertical white and black bars at the left of the graph represent the photophase and scotophase respectively.

Experimental Chapter 3

Title: "Temperature cycles trigger nocturnalism in the diurnal homeotherm *Octodon degus*"

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ABSTRACT

Body temperature regulation within a physiological range is a critical factor for guaranteeing the survival of living organisms. The avoidance of high ambient temperatures is a behavioral mechanism used by homeothermic animals living in extreme environmental conditions. As the circadian system is involved in these thermoregulatory responses, precise phase shifts and even complete temporal niche inversion have been reported. *Octodon degus*, a mainly diurnal rodent from Chile, has the ability to switch its phase preference for locomotor activity to coincide with the availability of a running wheel. The aims of this work are twofold: to determine whether ambient temperature cycles, with high values during the day and low values at night (HLT_a), can induce nocturnal chronotypes in degus previously characterized as diurnal; and to learn whether HLT_a cycles are able to act as a *zeitgeber* in this dual-phase species.

To this end, degus were subjected to 24h HLT_a cycles, under both 12:12 LD and DD conditions. Two experimental groups were used, one with previous wheel running experience and another naïve group, in order to study the influence of the thermal cycles and previous wheel running experience on the degu's dual phasing behavior. Temperature cycles (31.3 ± 1.5 ºC during the day and 24.2 ± 1.6 ºC at night) induced a 100% nocturnalism in previously diurnal individuals. Indeed, both entrainment with nocturnal phase angle to LD and nocturnal rhythmicity induced by masking were observed. Moreover, HLT_a cycles acted by masking, confining wheel running activity rhythm to the cooler phase under DD conditions, with the naïve group being more sensitive than the experienced one.

KEYWORDS: *Octodon degus*, thermal cycles, phase inversion, nocturnalism, entrainment, masking, *zeitgeber*

INTRODUCTION

From their origins, living organisms have been subjected to rhythmic environmental conditions due to the rotation and translation movements of the Earth. As a consequence, circadian and circannual rhythmicity are coupled with two key environmental factors, light and temperature. Because of its high degree of stability, the daily fluctuation of light and darkness (LD) is used by most living organisms as the main *zeitgeber* to entrain the phase and the period of their circadian pacemaker (in mammals, the suprachiasmatic nucleus (SCN) to 24h environmental cycles.

Temperature is a critical factor in the survival of organisms, so they have developed different mechanisms in order to control it within a physiological range. Homeothermy, poikilothermy and heterothermy are examples of different physiological strategies for controlling body temperature (T_b) in animals, as well as its effect on biochemical processes. Although temperature also displays daily cycles that are closely related to LD cycles, and we have known for a long time that the pacing of circadian pacemakers is temperature-compensated (Pittendrigh, 1954), few studies have been conducted on the ability of daily thermal cycles to function as a *zeitgeber* in homeotherms. Entrainment by temperature cycles is known to occur in palm squirrels (Rajaratnam & Redman, 1998) and marsupials (Francis & Coleman, 1990), but nonetheless, temperature cycles are considered to be a weak *zeitgeber* in mammals.

Octodon degus is a primarily diurnal rodent from Central Chile that can display both diurnal and nocturnal phase preferences in locomotor activity when an exercise wheel is available in the cage (Kas & Edgar, 1999). A predator-prey relationship cycle, seasonal rhythmicity or simple food availability are some of the ecological hypotheses for explaining such an uncommon duality (Lagos et al., 1995; Kenagy et al., 2002, Vivanco et al., 2010). A physiological hypothesis raises the possibility that thermoregulation could be the key factor involved. Locomotor activity displayed during the light and warm phase could generate brain overheating that, in some individuals, might be compensated by a circadian response in the form of a 180° phase switch. In this sense, previous studies conducted at our laboratory have demonstrated that nocturnal and diurnal chronotypes differ in their thermal response to intense wheel exercise (Vivanco et al., 2009).

Behavioral strategies for thermoregulation involving the circadian system are common in homeothermic animals. These can vary from limited phase shifts in activity

(with a modification of burrow entries and exits), in order to avoid extremely hot periods, to complete temporal niche inversions, both of which have been described in birds and mammals (Goldstein DL, 1984; Fielden et al., 1992). Field studies have demonstrated that degus' locomotor activity is highly dependent on environmental temperature and humidity; they tend to increase their diurnal activity during winter days, and shift activity to dawn and dusk during the hot, dry days of summer (Kenagy et al., 2002). To date, however, no laboratory studies have been published testing the ability of ambient temperature (T_a) cycles to induce nocturnal inversion in diurnal animals or the ability of this environmental cue to act as *zeitgeber* in this species, which is characterized by a limited range of thermoregulation.

The main aim of this work is to determine whether ambient temperature cycles, with high values during the day and low at night (HLT_a), can induce nocturnal chronotypes in degus previously characterized as diurnal. To this end, we subjected male degus with and without previous wheel running experience to 24h HLT_a cycles under both 12:12 LD and DD lighting conditions.

MATERIAL AND METHODS

Thirty-nine male *Octodon degus* between 12-28 months of age were obtained from the Animal Facilities at the University of Alicante (Spain). The animals were individually housed in polycarbonate cages (Panlab, S.L.) equipped with exercise wheels (52 x 15 x 27 cm, L x H x W) in an isolated room ("Chronolab") with controlled humidity (60%), constant temperature (22.3 ± 1.5 ºC) and photoperiod (LD 12:12). Light was provided by two lateral fluorescent lamps controlled by an electronic timer (Data Micro, Orbis), with an intensity of 350-400 lux at cage level. The degus were fed commercial rat chow (A04 rat-mouse maintenance Panlab) *ad libitum*.

The HLT_a cycles had a trapezoidal profile with an increasing and a decreasing phase, each one lasting about 2h (Figure 1). High temperature levels were obtained by increasing the ambient temperature of the chamber by switching on an electric thermostatic blanket (A.E. Herga, S.L., Murcia, Spain) under each cage. All blankets were connected to an electronic timer that switched them on at 08:00h and off at 20:00h. Local environmental temperature was recorded every 10 min by a data logger iButton placed 1 cm away from the floor, outside the cage (attached to the lateral side of the cage). All experimental procedures were performed in accordance with the

"Principles of Animal Care" (Portaluppi et al., 2008; NIH publication No. 86-23, revised 1985) and Spanish laws.

Data Recording

Wheel running activity (WRA) was recorded as the number of wheel turns per 10-minute intervals using a data acquisition system (Electronic Service at the University of Murcia, Spain). Body temperature was measured at 60-minute intervals using a miniature data logger (ThermoChron®, Data loggers iButton, Maxim Integrated Products, Sunnyvale, California, USA) with an accuracy of 0.1ºC. For this purpose, sterilized data loggers were implanted intraperitoneally under aseptic conditions, using fluothane as anaesthesia (Forane®, Abbot Laboratories S.A., Madrid, Spain). Reabsorbable suture material (2/0, Safil®Quick B/Braun, Barcelona) was used to close the abdominal layers, and non-reabsorbable silk was used to suture the skin. No mortality or morbidity was observed after the surgery. The experiment began following a two-week recovery period. At the end of the experiment, the data logger was removed under the same conditions in which it was implanted. iButton readout hardware was used to transfer temperature data to a computer.

Experimental Design

The experimental designed included a group of animals with previous wheel running experience, to characterize the effect of temperature upon the duality of degus and its role as *zeitgeber*, and another group of naïve animals in order to examine the relationship between previous wheel running experience and the impact of thermal cycles on the degu's circadian system.

Experiment I. Experienced degus

Twenty-five male degus (27-28 months old) with more than one year of wheel running experience were divided in two groups, an experimental and a control group. The experimental group (n=12) was subjected to the HLT_a cycles during certain experimental phases, while the control group (n=13) was exposed to the same lighting conditions, but with a constant T_a. Control and experimental animals were individually housed in separate chambers, with wheel running access under LD 12:12 and constant temperature (22.3 ± 1.5 ºC) conditions. After 7 days, the experimental group was subjected to 24h HLT_a (31.3 ± 1.5 ºC during the day, and 24.2 ± 1.6 ºC at night; Figure

1), for a period of 17 days. The degus were then exposed to DD conditions, but maintained at the previous HLT_a (high temperatures from 08:00h to 20:00h, local time) for a period of 18 days. Afterwards, the HLT_a cycles were eliminated and the animals subjected to the constant temperature (22.3 ± 1.5 ºC), still under DD conditions, for another 10 days. Finally, both groups returned to the initial LD 12:12 and constant temperature conditions for an additional 8 days.

Experiment II. Naïve degus

A group of fourteen male degus (12-18 months old) that had never experienced wheel running (Naïve group) was divided in two subgroups: an experimental and (n=6) a control group (n=8). As in Experiment I, only experimental animals were exposed to HLT_a cycles, as described above. Control animals experienced the same lighting conditions, but with the constant temperature. On day 0, the animals were subjected to LD 12:12 and HLT_a conditions (the same as the experienced group) for 9 days; they were then exposed to DD and HLT_a for 18 days. After that, HLT_a cycles were eliminated and the temperature was kept constant (22.3 ± 1.5 ºC) under DD conditions for a period of 10 days. Finally, the animals were placed under LD 12:12 and constant temperature conditions for an additional 8 days.

Chronotype Characterization and Data Analysis

To characterize the chronotype of each degu, a numerical criterion based on the percentage of diurnal *versus* total activity was used. Accordingly, when an animal under LD conditions showed a diurnal/total activity ratio above 60%, it was considered diurnal. Due to the existence of intermediate chronotypes with intermediate locomotor activity values, a nocturnal animal was defined as one whose diurnal/total activity ratio was less than 20% (Vivanco et al., 2009). Since our aim was to test the ability of HLT_a cycles to induce nocturnalism, only those individuals with a clear diurnal pattern were selected for the experiment.

Individual actograms, mean waveforms, a chi-square periodogram and acrophases from a cosine fit of WRA and T_b rhythms were obtained using software specifically designed for chronobiological analysis (El Temps, version 1.228; © Díez-Noguera, University of Barcelona). To determine the daily acrophase evolution of wheel running activity and body temperature rhythms for each chronotype, the mean and standard error of the mean (SEM) of the individual daily acrophases were

calculated. A repeated measures ANOVA was performed to compare locomotor activity and body temperature during the different experimental phases. The experimental circadian variables were tested for normality of distribution and homogeneity of variance. All statistical analyses were performed using SPSS 13.0 software.

RESULTS

HLT_a cycles in experienced degus

From the initial twenty-five degus with wheel running experience, six individuals were removed due to their nocturnal chronotype, as determined by numerical classification, and five because of their irregular or unclassifiable phenotype. The remaining 14 degus presented clear diurnal activity and T_b patterns (8 and 6 individuals in the control and experimental group, respectively).

When subjected to 12:12 LD conditions under constant T_a (22.3 ± 1.5 ºC), diurnal degus, as the four representative animals in Figs. 2 and 3 show, ran predominantly during the photophase, but also during the one to two hours prior to or following light-dark transitions. While dusk induced a reactive response in WRA, the animals anticipated the dawn transition by 1-2 hours of activity (Fig. 2). Similarly, the T_b rhythm was characterized by higher values during the photophase than during the scotophase (Fig. 3).

When experienced diurnal degus were subjected to HLT_a cycles, 100% of the animals inverted their WRA rhythm to the nocturnal phase within just a few days while increasing nocturnal T_b (Fig. 2B, C and D and Fig. 3B, C and D), in contrast to the entire group of control degus (Figs 2A and 3A), which maintained their diurnal pattern throughout the experiment. However, changes in diurnal Tb are harder to detect considering that high environmental temperature during the day could mask the pattern (Fig. 3).

In degus under constant T_a conditions, wheel running activity occurred throughout the entire day, with peaks after dusk and around dawn, and with greater activity during the photophase than the scotophase (Fig. 4, top left panel). In contrast, the WRA of degus subjected to HLT_a and LD conditions was confined to the scotophase, with the same two peaks of activity observed in the diurnal pattern around

light-dark transitions (Fig. 4, lower left panel). Very low levels of activity were observed during the photophase.

Regarding T_b, the diurnal pattern observed under constant T_a was characterized by two peaks around light transitions and higher values during the day than at night (Fig. 4, top right panel). However, under HLT_a, an increase in T_b was observed during the first hours of the night, (Fig. 4, botton right panel). As expected, under LD, control animals displayed a diurnal pattern in WRA and T_b very similar to that observed in the experimental animals under LD and constant T_a conditions. HLT_a cycles generated a statistically significant difference in mean WRA during the day (repeated measures ANOVA, p=0.007) and at night (p=0.013), and in the maximum WRA at night (p=0.009) (Table 1). With the exception of the nocturnal mean T_b (p=0.004), no statistically significant differences were observed (i.e., maximum, minimum or the phase of the minimum T_b) between the constant T_a and HLT_a phases.

An HLT_a–induced nocturnal chronotype may be the result of two processes: entrainment and masking. Thus, once the phase angle of entrainment from the first few days under DD was taken into account, two different categories emerged: nocturnalism by masking and nocturnalism by entrainment. In the nocturnal masked animals (Figs. 2B and 3B), the degus showed a significant phase advance the very first day they were subjected to DD conditions. Under these conditions, the animals started to free-run in a phase similar to that observed in the previous LD and constant T_a treatment. In contrast, the nocturnally entrained animals started to free-run in accordance to the previous circadian phase in LD and HLT_a (Figs. 2C and 3C). Only one individual from the experienced group restricts its WRA to the cooler period of the HLT_a cycle under DD (*tau*= 1440 min) (Figs. 2D and 3D). For the remaining animals, the endogenous periodicity under DD in nocturnally entrained individuals was 1406 ± 4 min and 1401 ± 1 min for WRA and T_b, respectively. However, in nocturnal masked degus it was 1425 ± 3 min and 1422 ± 3 min for WRA and T_b, respectively.

When the HLT_a cycle was removed under DD, no modification in the endogenous rhythmicity was observed for nocturnal masked (*tau*= 1423 ± 2 min for WRA and 1416 ± 9 min for T_b) or entrained (*tau*= 1405 ± 5 min for WRA and 1402 ± 2 min for T_b) animals. However, in HLT_a-masked degus (Figs. 2D and 3D), WRA and T_b shifted abruptly to the photophase during the first day of the change, and both rhythms free-ran with a periodicity of around 1415 min. It is interesting to note that although a large phase shift appeared in the main rhythm, a closer inspection of T_b (Fig. 3D)

revealed a slow resynchronization of the previously nocturnal component, which continued until it was incorporated into the whole rhythmicity. Finally, when LD 12:12 conditions were restored, all animals resynchronized, returning to the initial diurnal temporal niche they exhibited at the beginning of the experiment.

The day-to-day evolution of WRA and T_b acrophases is represented in Fig. 5. While animals were held under constant temperature conditions, all displayed a diurnal phase preference for WRA and T_b, with the WRA acrophase preceding the T_b acrophase. However, when the HLT_a was imposed, the degus started to move progressively towards a nocturnal chronotype, reaching a stable phase within 7-8 days. Interestingly, under these conditions, the T_b acrophase anticipated the WRA acrophase by approximately 4 hours. The animals showed a significant WRA phase advance during the first day under DD and HLT_a conditions, indicating a significant contribution of negative masking by light to the nocturnalism of degus under LD and HLT_a conditions.

A closer inspection of the WRA and T_b acrophases (Fig. 5) revealed a relative coordination, with the 11 first days having stable acrophases, followed by a clear free-running period. The T_b rhythm was more resistant to change than WRA; however, a normal phase-relationship between WRA and T_b was eventually reached. Finally, diurnal acrophases were observed when the animals returned to LD and constant temperature conditions (Fig. 5).

HLT_a cycles in naïve animals

A group of 14 degus which had never experienced wheel running were divided into control (exposed to constant temperature throughout the experiment, n=8) and experimental (subjected to HLT_a cycles during certain phases of the experiment, n=6) groups.

Again, as shown for experienced animals, 100% nocturnalism was achieved from the first day of WR availability when the degus were exposed to LD and HLT_a cycles, concentrating most of their WRA during the scotophase (Fig. 6B). All animals in the control group entrained to the diurnal phase, presenting some activity the first hours of the dark period (Fig. 6A). In contrast to experienced animals, all naïve degus synchronized by masking their WRA to HLT_a under DD. Interestingly, the mean waveform of WRA under these conditions was very similar to that observed under LD

and HLT_a, indicating that the crepuscular peak observed before dawn in the WRA pattern of degus is not directly generated by light-dark transitions (Fig. 7).

The analysis of WRA acrophases (Fig. 8) revealed that naïve degus exhibited a nocturnal chronotype from the first day under LD and HLT_a conditions, unlike experienced degus, which needed at least 7 days to achieve a stable nocturnal WRA phase. However, both naïve and experienced degus attained the same phase angle of entrainment by the end of this phase. The diurnal chronotype of the control group progressively emerged, reaching a stable diurnal phase after the first 5 days. None of the control animals displayed a nocturnal chronotype after wheel running was made available.

When the degus were subjected to DD under HLT_a conditions, the WRA of all animals is confined to the cooler period (the subjective night), with the same phase angle that was previously observed. At the same time, in the control group, WRA free-ran with a *tau* of 1424 ± 3 min. An abrupt phase shift in the WRA acrophase was observed in the experimental group (*tau* = 1479 ± 5 min) after the change was made to constant T_a. However, it is interesting to note that a minor free-running component from the previous nocturnal phase (*tau* = 1410 ± 5 min) was also evident in all experimental animals (Fig. 6B). An abrupt change in the activity rhythm phase was observed from the first day under LD conditions in the experimental, but not the control group, which indicates a negative masking effect by light.

DISCUSSION

Temperature is presented here as a key factor involved in the nocturnalism of *Octodon degus* when a wheel is available in the cage. Environmental temperature cycles, with higher values during the day than at night, produced 100% of nocturnalism in a previously diurnal population of degus through two mechanisms: a) steady entrainment to LD; and b) negative masking effects by high temperature. This is the first report indicating that temperature can trigger nocturnalism in the degu's circadian system. Moreover, the novelty of wheel running activity potentiates both the niche inversion induced by high diurnal temperature under LD and the masking effect of the temperature cycles under DD.

Octodon degus is a Chilean rodent that has been described as mainly diurnal or crepuscular in ecological studies in its natural environment, even though a small percentage of individuals can be captured at night (Iriarte et al., 1989). In 1976, Fulk demonstrated that degus present a circannual pattern in their general activity, correlated with the photoperiod and the environmental temperature. This work was directly corroborated by Kenagy et al. (2002), who showed that in summer, when the temperature rises to 40°C by around midday, the degus' out-burrowing activity presents a crepuscular pattern, while in winter it appears unimodally diurnal, as the animal seeks open areas with sunlight.

Under controlled laboratory conditions, *Octodon degus* behaves mainly as a diurnal animal (Lee, 2004). However, when an exercise wheel is available in the cage, a nocturnal chronotypical differentiation appears in some individuals (Kas & Edgar, 1999). Steady entrainment and masking by light effects explain the great variability of circadian chronotypes in degus, from diurnal to nocturnal (Vivanco et al., 2009). Laboratory and field studies identified thermoregulation as a potential key factor involved in the circadian duality of degus.

In 1977, Rosenmann noted that degus have a very small tolerance to high temperatures (above 32 ºC), due to their limited ability for water evaporation, and concluded that a behavioral response would be necessary in order to avoid thermal stress. In fact, direct observations corroborated that high environmental temperatures inhibit degus activity and induce them to seek shady places when they are in the open, retreating into their burrow as temperatures rise above their thermoneutral zone (between 24 - 32 ºC) at midday (Kenagy et al., 2004). Similarly, as a result of their field observations, Lagos et al. (1995) stressed the importance of thermoregulatory constraints as the main cause of degus nocturnalism.

In the laboratory, previous experiments conducted by our group (Vivanco et al., 2009) have suggested that the switch to nocturnal activity in response to wheel running could be one way of attaining high levels of activity while avoiding the dangers of overheating. Indeed, compared to the diurnal chronotype, nocturnal degus displayed higher daytime basal temperatures and a greater capacity to perform intense exercise at lower temperatures at night. In addition, Lee (2004) suggested that the relative proportion of chronotypes may be dependent on ambient temperature, with nocturnalism in degus increasing as the environmental temperatures rise. However, to

our knowledge, no attempt has been made to date to test the influence of temperature cycles on nocturnalism in degus.

With regards to the duality of degus, the common point between nature and laboratory settings is thermal tolerance. Temperature may act on temporal niche preference in two interrelated ways: a behavioral response for thermoregulation (masking) and a circadian response from the pacemaker due to temperature acting as a non-photic stimulus (entrainment).

Our results suggest that in diurnal degus, vigorous running activity (such as that which occurred on the wheel) could overheat the animals during the day and generate the nocturnal phase inversion as an effective behavioral thermoregulatory response. This thermoregulatory hypothesis provides a context to understand a number of experimental observations.

First, it would explain the fact that high diurnal ambient temperature cycles generated 100% nocturnalism in the diurnal wheel runner population. Secondly, inter-individual thermal tolerance values and variable thermogenic effects of exercise would explain why circadian inversion occurred in some individuals and not in others when the exercise wheel was available under thermoneutral temperature conditions. Thirdly, the differential response of naïve and experienced wheel runner groups to the temperature cycle when the wheel was available points out the influence of acclimatization processes. Experienced animals are less sensitive to the masking effect of high temperatures and are resistant to synchronizing their WRA with the temperature cycles under DD. Acclimatization is a common physiological phenomenon in animals subjected to new environmental conditions, such as temperature, humidity or altitude (Shido et al., 1991; Frisancho, 1975).

In addition to the ability of temperature to induce nocturnal phase inversions under LD conditions, thermal cycles, under DD, are also able to keep the WRA of naïve wheel running degus confined to the cooler phase. For this to occur, temperature should act directly as a non-photic stimulus upon the circadian system. From the 1960s until today, a number of papers have described the entrainment properties of temperature cycles upon free-running activity rhythms in birds, such as house finches (Enright, 1966), in heterothermic mammals, such as some species of mice and bats (Pohl, 1998; Erkert & Rothmund, 1981), and in homeothermic mammals like rats, mice, squirrels, macaques and monkeys (Francis & Coleman, 1990; DeCoursey, 1960;

Tokura & Aschoff, 1983; Aschoff & Tokura, 1986). However, this state of entrainment was not achieved in all individuals. A phase response curve as a function of temperature pulses in rats was also drawn by Francis & Coleman in 1997.

From our results, we cannot conclude that temperature cycles function as a *zeitgeber* on the degus' pacemaker, since although all naïve animals remained synchronized to HLT_a under DD conditions, this synchronization was produced by a negative masking of the high temperature phase on WRA, rather than by pacemaker entrainment. The naïve wheel running degus confined their WRA to the cold phase (the subjective night) while running in DD, and their main rhythmicity component phase shifted to the diurnal phase when the temperature cycle was removed. In fact, Kas & Edgar (1999) found a similar switch in DD, but theirs occurred when wheel running was removed. It is remarkable that temperature cycles under DD conditions induce a WRA pattern that is very similar to that observed under LD conditions. A crepuscular peak in activity before dawn is also present when the light cycle is removed, indicating that degus can use cool-warm transition to synchronize their crepuscular peak of WRA, as they do with the dark-light transition.

The impact of temperature cycles and wheel running availability, both as regards induction of nocturnalism under LD and masking under DD, was greater in naïve animals than in the experienced group. This fact indicates the importance of acclimation to wheel running and/or to high temperatures in determining the response of the circadian system. Both temperature cycles and exercise wheel availability are non-photic stimuli for the circadian system. Based on a comparison between diurnal and nocturnal species, non-photic cues can be classified into two categories: arousal-independent and arousal dependent factors. Melatonin, which is always synthesized during the night, and brain GABA are both arousal-independent factors, whereas brain serotonin, feeding, locomotor and temperature rhythms belong to the arousal-dependent group. Since the nocturnalism induced by temperature cycles shares many of the characteristics of that induced by wheel running availability, it is possible that these two non-photic stimuli synchronize the degu's circadian system by acting on a common element, such as the activation of brain serotonin (Challet, 2007).

Based on studies indicating the lack of differences between nocturnal and diurnal master pacemakers (Schwartz et al., 1983; Koch et al., 2009, Mahoney et al. 2009), most authors have suggested that the wheel running-induced switch from a diurnal to a nocturnal phase preference probably occurs downstream of the circadian

pacemaker. According to this hypothesis, the output signal from the circadian pacemaker would be integrated with environmental and homeostatic inputs to produce a particular phasing of the observed rhythm. Wheel availability and, as our results suggest, temperature cycles, would not affect the central pacemaker itself, but rather the coupling between the pacemaker and other brain areas, such as the subparaventricular zone and the dorsomedial hypothalamus, (DMH; Schwartz et al., 2004; Saper et al., 2005). This brain center provides animals with the flexibility they need to adapt their physiology to environmental cycles, maximizing their chances of survival (Saper et al., 2005). Alternatively, recent findings point out that retinal inputs play a greater role in the diurnal-nocturnal switching than was previously suspected (Mrosovsky & Hattar, 2005).

In conclusion, temperature cycles are able to induce complete nocturnalism through two mechanisms: steady entrainment to LD, and negative masking effects by high temperature. In addition, under DD conditions, temperature cycles confine locomotor activity with the cooler phase, but only in naïve degus with no previous wheel running experience. This synchronization is mainly the result of a negative masking effect by the warm temperature. Wheel running inversion and temperature inversion share many characteristics, which suggests that a common arousal-dependent mechanism may be involved under the chronotypical duality of the degu.

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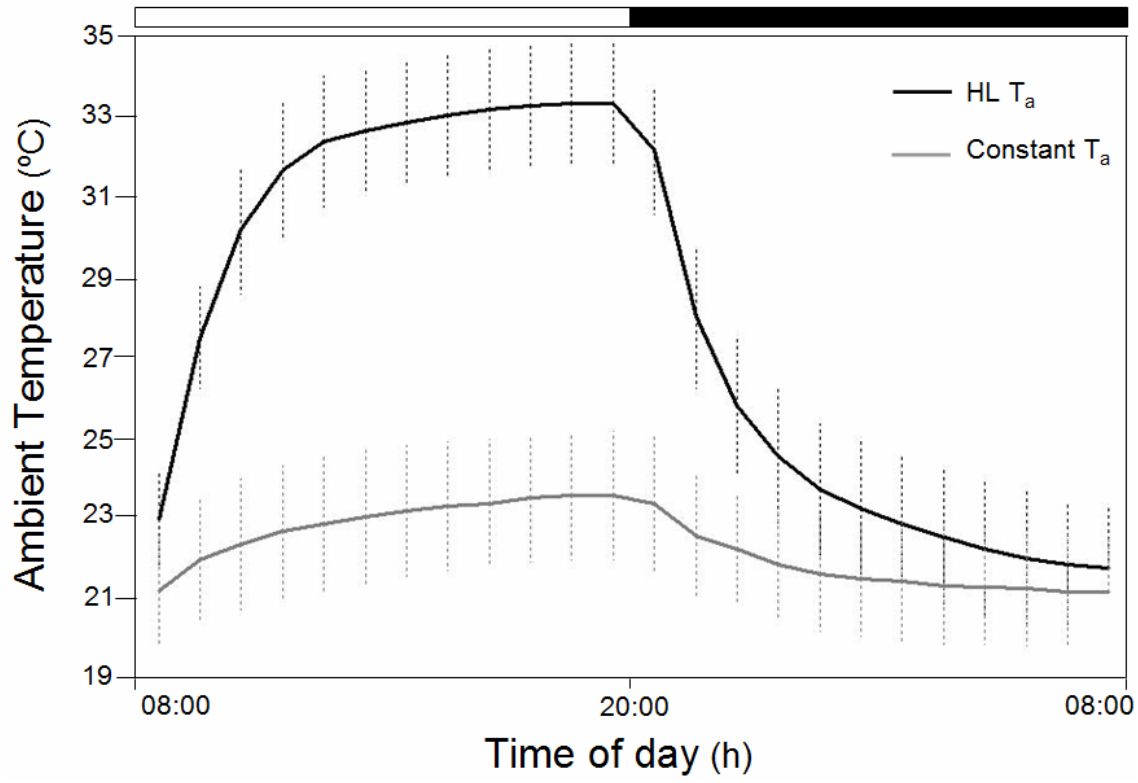

Figure 1. Mean waveform of the environmental temperature schedules used in the experiments: high-low ambient temperature (HLT_a) cycle and constant temperature. Vertical lines represent the standard error of the mean (SEM) for each average value.

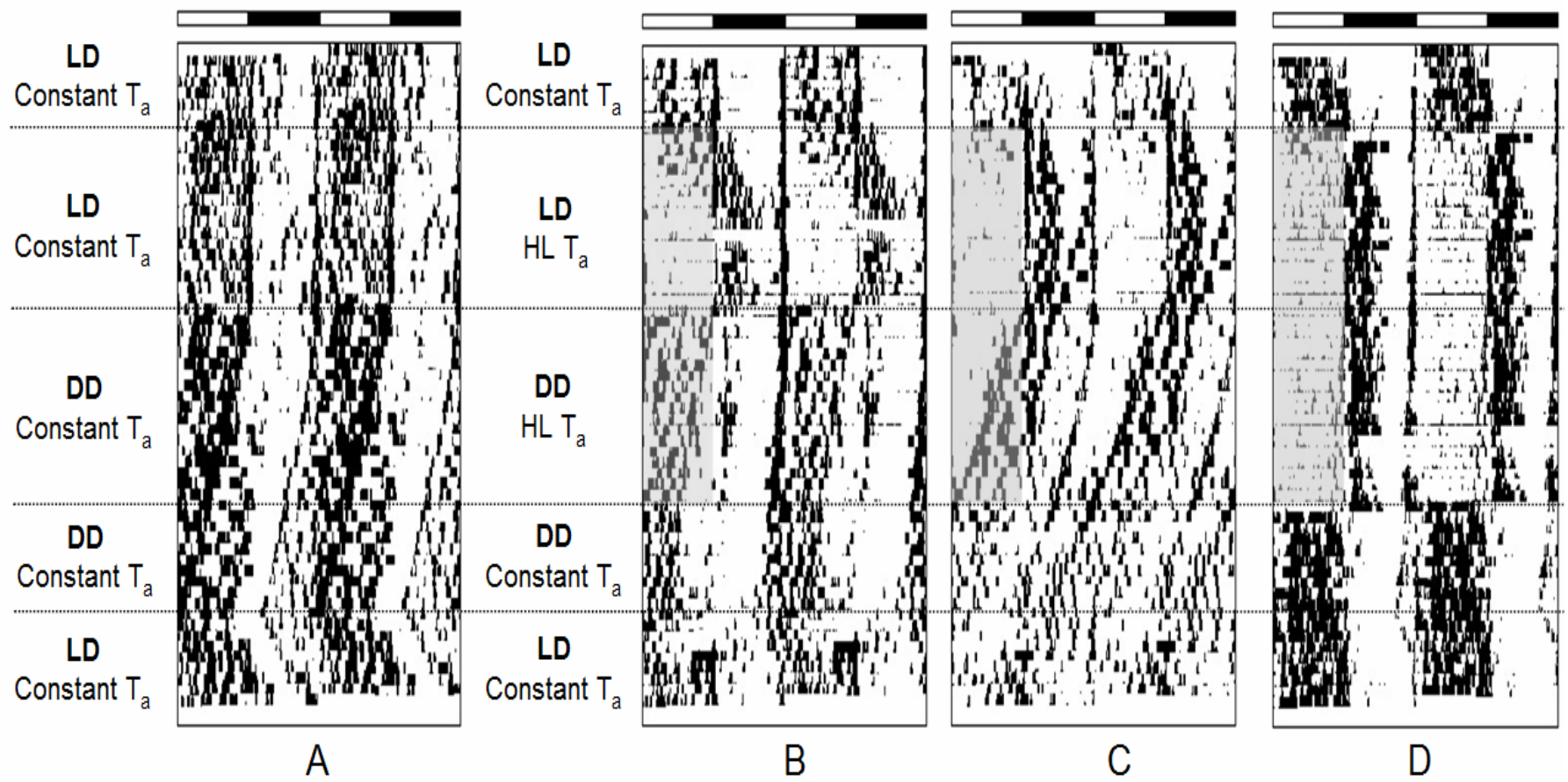

Figure 2. Double-plotted representative wheel running activity (WRA) actograms for one control (A) and three experimental degus from Experiment I (Experienced wheel running degus): (B) and (C) show nocturnalism induced by masking and entrainment with nocturnal phase angle under LD and HLT_a, respectively, and (D) exhibits synchronization to the cooler phase of the thermal cycle under DD and HLT_a. Light and temperature conditions are indicated on the left of each actogram.

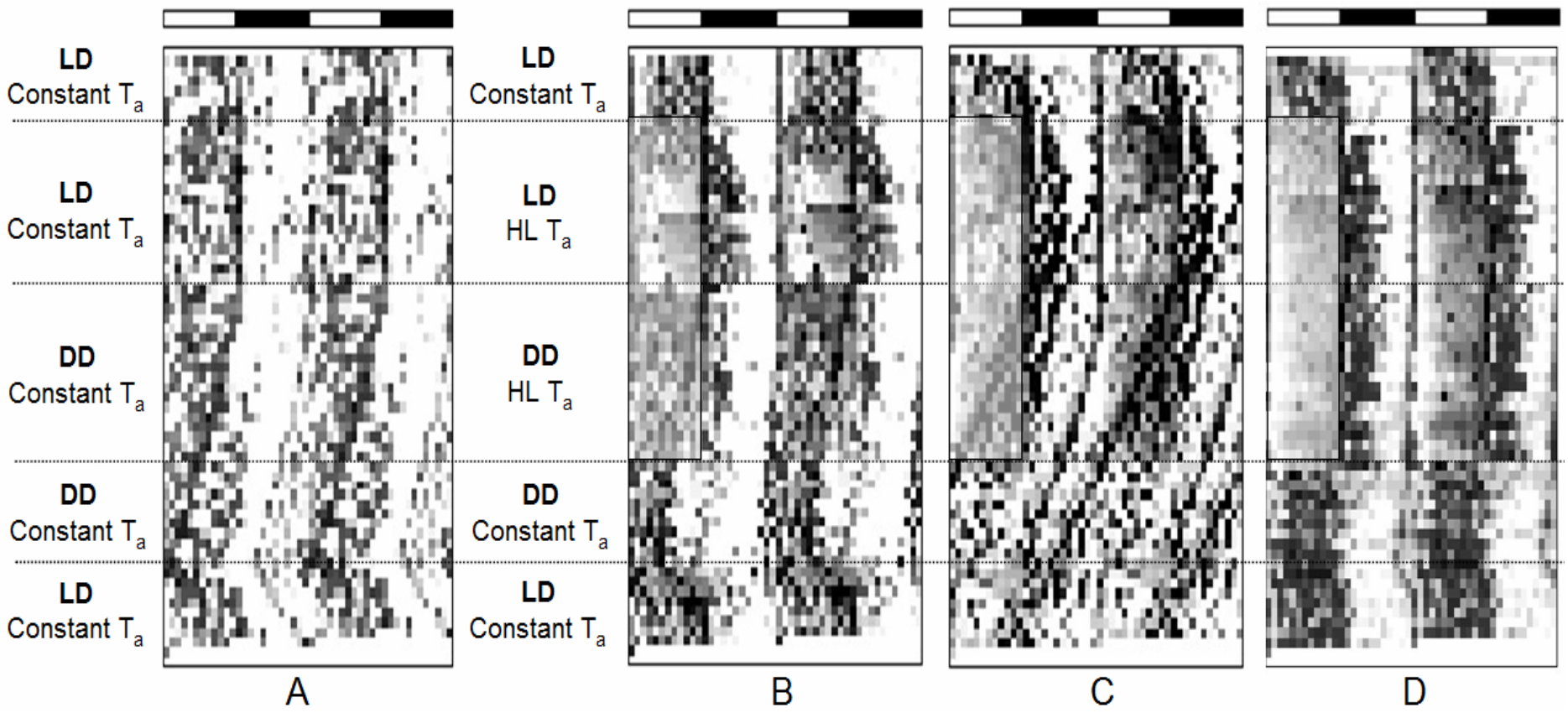

Figure 3. Double-plotted representative body temperature (T_b) actograms for the same animals represented in figure 2: one control (A) and three experimental degus from Experiment I (Experienced wheel running degus): (B) and (C) show nocturnalism induced by masking and entrainment with nocturnal phase angle under LD and HLT_a, respectively, and (D) exhibits synchronization to the cooler phase of the thermal cycle under DD and HLT_a. See Fig. 2 for details. The body temperature ranged from 36 to 38ºC (the darker the color, the higher the temperature).

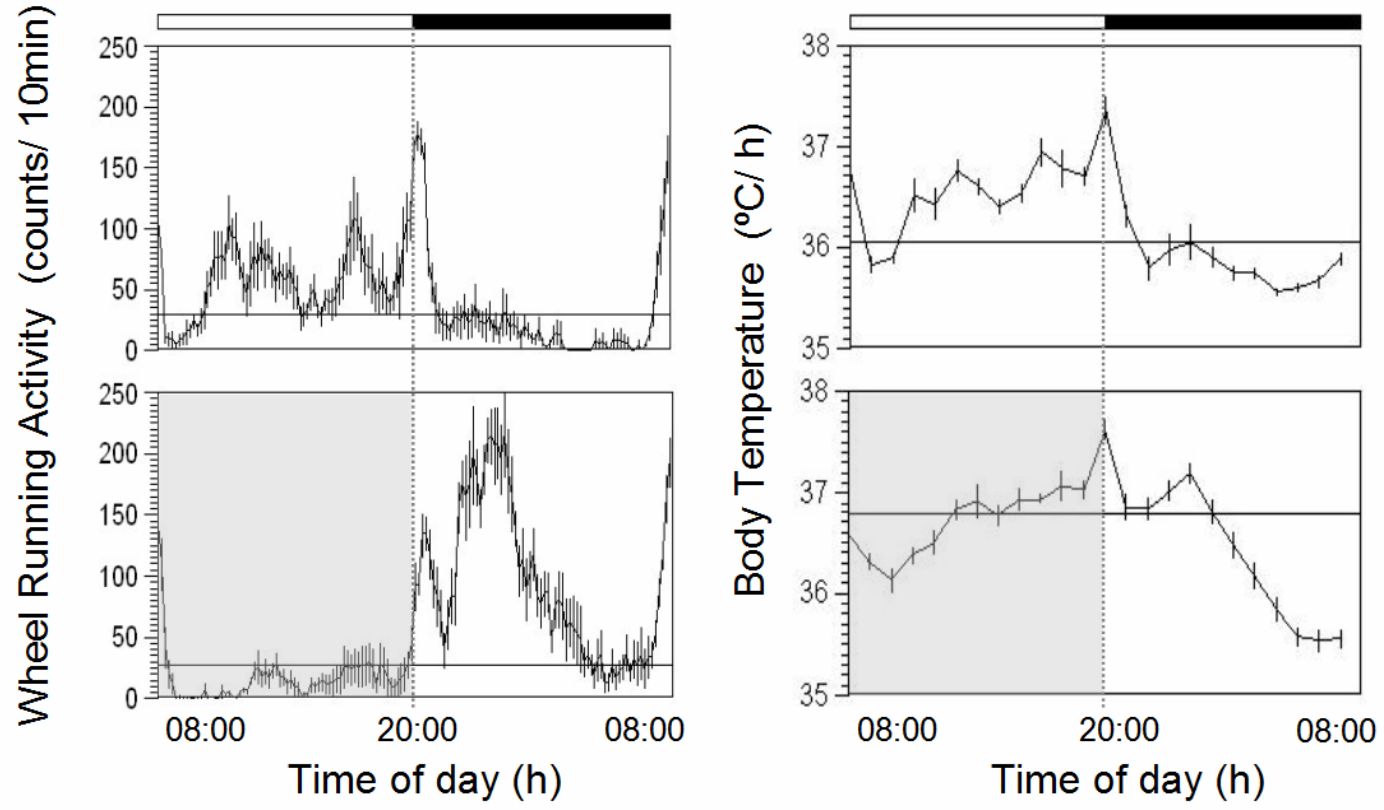

Figure 4. Mean waveforms for wheel running activity (WRA) and body temperature (T_b) rhythms for the experimental group from Experiment I (Experienced wheel running degus) subjected to a LD 12:12 cycle under constant temperature (upper panels) and LD 12:12 under HLT_a (lower panels). The horizontal line corresponds to the median for the 24h values. Vertical lines represent the standard error of the mean (SEM) for each average value. The dotted vertical line indicates the light/dark transition for comparison purposes.

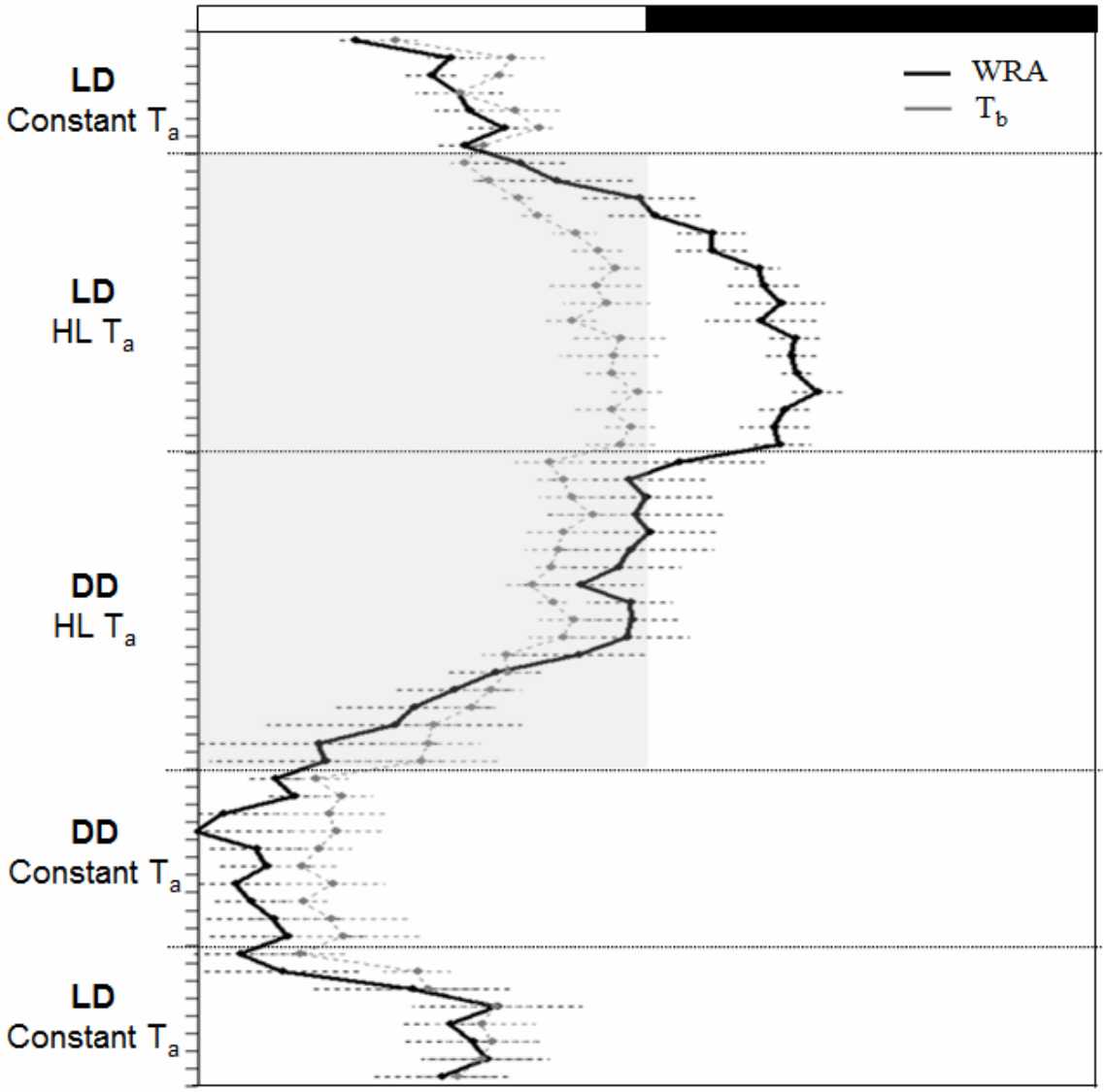

Figure 5. Daily mean acrophases for wheel running activity (WRA) and body temperature (T_b) rhythms for the experimental group from Experiment I (Experienced wheel running degus) subjected to the following sequence of conditions: LD 12:12 (7 days), LD and HLT_a (17 days), DD and HLT_a (18 days), DD and constant temperature (10 days), and back to LD 12:12 (8 days). The circles represent the mean acrophase (n=6) for each variable, as calculated by cosinor analysis, whereas the horizontal dotted line represents the SEM.

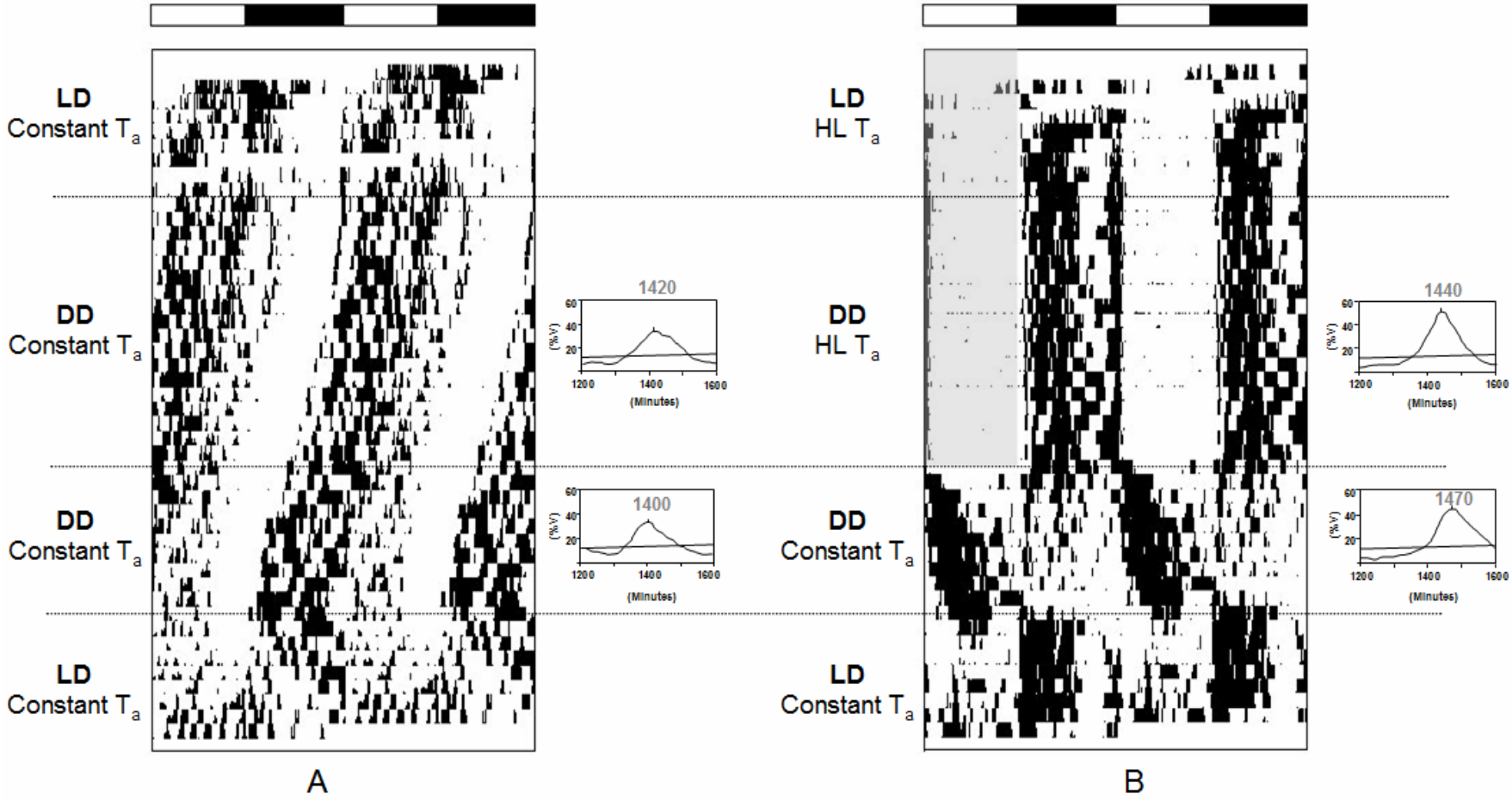

Figure 6. Double-plotted representative wheel running activity (WRA) actograms for one control (A) and one experimental degus from Experiment II (Naïve wheel running degus). Light and temperature conditions are indicated on the left of each actogram. On the right of each DD period is the individual Sokolove-Bushell periodogram with the *tau* value.

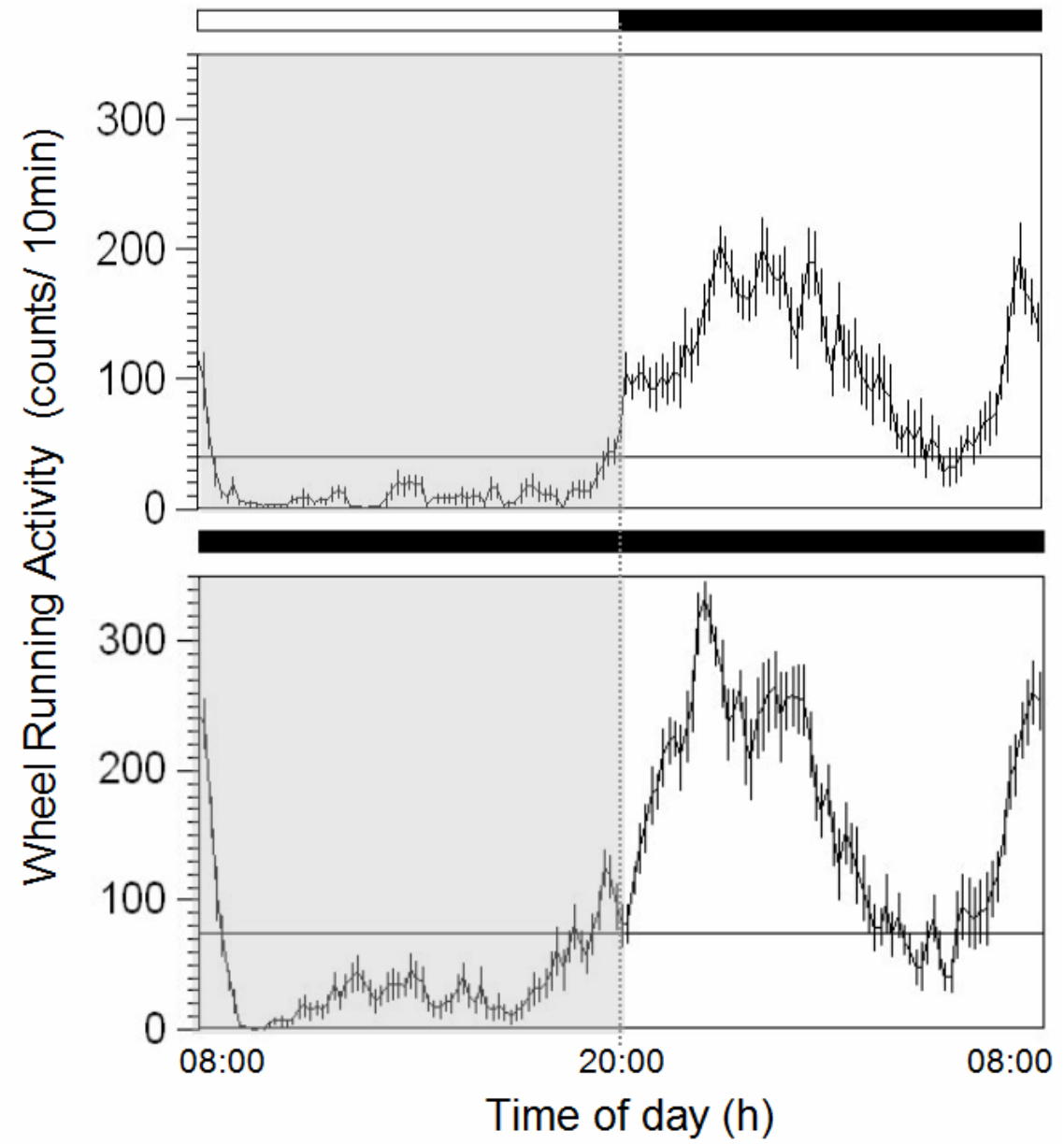

Figure 7. Wheel running activity (WRA) rhythm mean waveforms for the experimental group from Experiment II (Naïve wheel running degus) subjected to a LD 12:12 cycle with HLT_a (upper panel) and DD with HLT_a (lower panel). The horizontal line corresponds to the median for the 24h values. Vertical lines represent the standard error of the mean (SEM) for each average value. The dotted vertical line indicates the light/dark transition for comparison purposes.

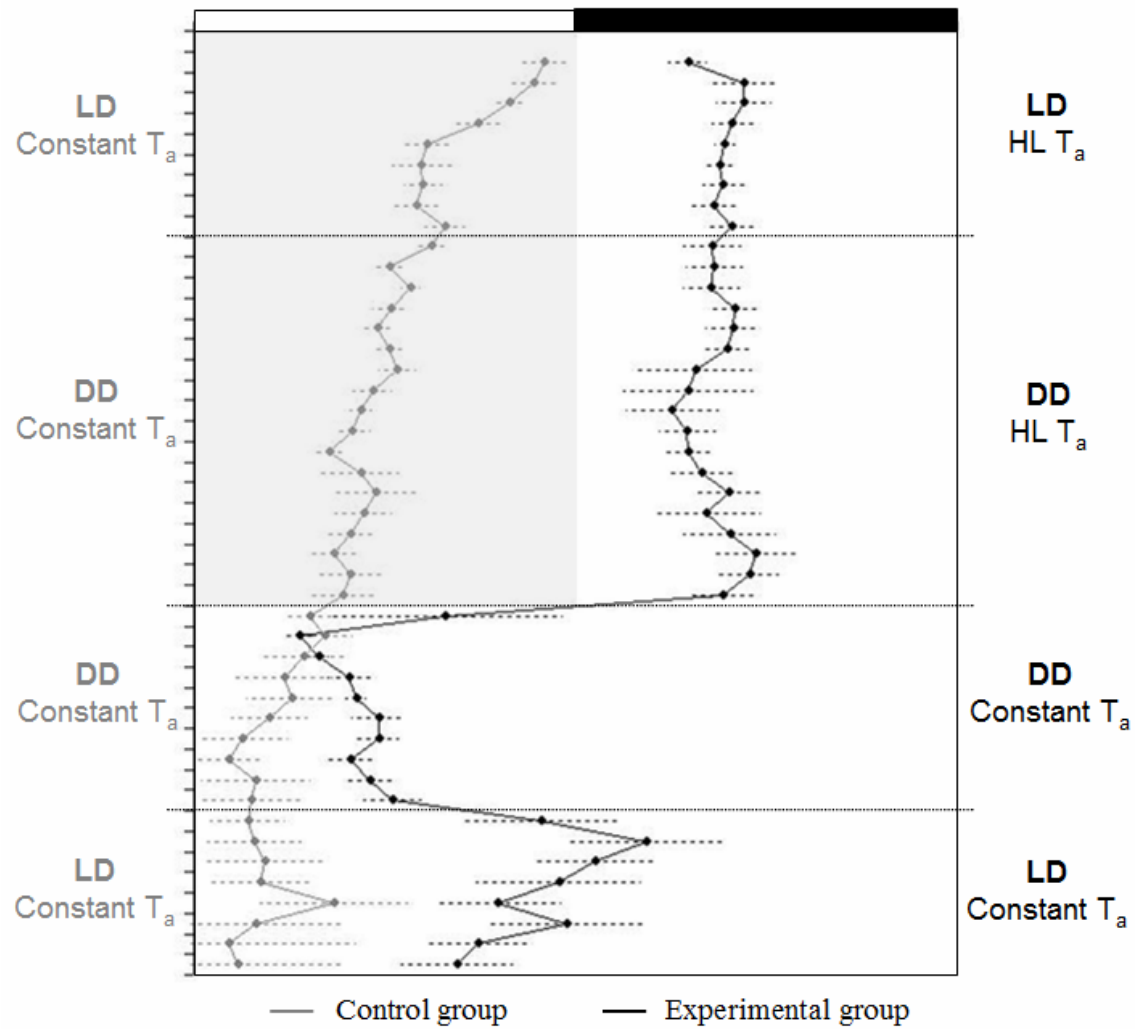

Figure 8. Daily mean acrophases for wheel running activity (WRA) rhythm for the control and the experimental groups from Experiment II (Naïve wheel running degus). The circles represent the mean acrophase (n=6 for experimental and n=8 for control) for each variable, as calculated by cosinor analysis, whereas the horizontal dotted line represents the SEM. Lighting and temperature conditions are indicated on the left and right of the graph for control and experimental animals, respectively.

			Constant T_a		HL T_a	
WRA Mean per 1h (Counts)	Experimental		394 ± 63	a	133 ± 29	b
	Control		342 ± 95		451 ± 83	
	Exp		175 ± 57	a	644 ± 116	b
	Con		197 ± 41		205 ± 47	
WRA Max. per 1h (Counts)	Exp		673 ± 94		523 ± 112	
	Con		696 ± 157		871 ± 173	
	Exp		913 ± 187	a	1455 ± 148	b
	Con		838 ± 92		879 ± 163	
T_b Mean (ºC)	Exp		36.60 ± 0.20		36.71 ± 0.03	
	Con		36.83 ± 0.16		36.73 ± 0.13	
	Exp		35.96 ± 0.16	a	36.44 ± 0.25	b
	Con		36.28 ± 0.21		36.23 ± 0.15	
T_b Max. (ºC)	Exp		37.11 ± 0.21		37.34 ± 0.10	
	Con		37.36 ± 0.21		37.28 ± 0.15	
	Exp		37.32 ± 0.28		37.68 ± 0.19	
	Con		37.48 ± 0.14		37.62 ± 0.16	
T_b Min. (ºC)	Exp		35.86 ± 0.19		35.86 ± 0.09	
	Con		35.96 ± 0.12		36.17 ± 0.05	
	Exp		35.51 ± 0.15		35.41 ± 0.25	
	Con		35.64 ± 0.26		35.71 ± 0.15	
T_b Min. Phase (hh:mm)	Exp		04:12 ± 00:36		05:48 ± 01:06	
	Con		04:42 ± 00:18		04:18 ± 00:30	

Table 1. Locomotor activity and body temperature mean values for the control and the experimental group in Experiment I (Experienced wheel running degus) subjected to LD 12:12 with constant temperature and with HLT_a. Values are expressed as mean ± SEM. Different letters in the same column indicate statistically significant differences among circadian chronotypes (ANOVA, $p<0.05$). The minimum T_b phase is expressed in *zeitgeber* time, considering ZT=0 as lights on (08:00 h).

Experimental Chapter 4

Title: "Pacemaker phase control vs. masking by light: setting the circadian chronotype in dual *Octodon degus*"

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ABSTRACT

There are two main processes involved in the expression of circadian rhythmicity: entrainment and masking. While the first operates via the central pacemaker to anticipate predictable environmental conditions, masking (mainly induced by light), functions as a direct modulator of the circadian output signal induced by non-predictable events. The Chilean rodent *Octodon degus* presents both diurnal and nocturnal phase preferences in locomotor activity when given free access to an exercise wheel. Two steady-entrainment phases and graded masking by light seem to generate the wide variability of chronotypes in this species. The aim of this study was to characterize the differential masking by light according to the individual chronotypes, their stability over time and the influence of wheel running availability and ambient temperature upon the circadian duality of degus.

To this end, diurnal and nocturnal degus were subjected to ultradian cycles (1:1h LD), with and without wheel running availability, and under both normal and high diurnal ambient temperature cycles. Our results show that diurnal and nocturnal degus present a stable masking by light, each according to their respective chronotype. Thus, while diurnal animals increased their activity with light, in nocturnal degus, light induced a sharp drop in wheel running activity. These two types of masking responses appeared not only when the animals were synchronized to the 12:12 LD cycle, but also under ultradian cycles. Both masking effects persisted when wheel running was made unavailable and when the animals shifted their circadian activity patterns in response to ultradian cycles or to diurnal exposure to high temperatures. In conclusion, our results show that the positive and negative masking effects of light on diurnal and nocturnal degus, respectively, seem to occur independently of relative phase control by the central pacemaker, wheel running availability or the negative masking induced by high environmental temperatures.

KEYWORDS: Duality, *Octodon degus*, ultradian, masking, pacemaker

INTRODUCTION

The mammalian endogenous central pacemaker, the suprachiasmatic nucleus (SCN), generates an internal timed signal that travels throughout the entire organism. This signal is used for assigning a precise temporal niche to physiological and behavioral activities (Paranjpe & Sharma, 2005). In the wild, animals that are active and display daily biological events (e.g., foraging and feeding, as well as social and reproductive activities) at the right time of the day increase their chances of survival (Sharma, 2003).

There are two main systems that drive overt behavioral rhythmicity: one can be attributed to pacemaker entrainment by *zeitgebers*, and the other is based on the direct masking effects of the *zeitgeber* itself upon overt rhythms (Aschoff, 1999). While the first mechanism is driven by a relatively well known and localized system, we know far less about the second. In nature, light acts as both the main *zeitgeber* and the most important masking agent. Masking responses can be classified as positive or negative based on whether they increase or decrease the animal's activity, respectively (Mrosovsky, 1999).

Moreover, the type of response also depends on the masking stimulus and the chronotype. For example, a diurnal animal usually shows positive masking when light is present or when light luminance is increased, but negative masking when darkness or a decrease in light luminance occurs. Alternatively, a nocturnal animal displays positive masking in response to darkness and negative masking after light exposure. Paradoxical masking effects, i.e. light inhibiting the activity of diurnal animals and stimulating it in nocturnal animals, have also been described (Erkert & Gröber, 1986).

Octodon degus is Chilean Caviomorph that exhibits a circadian duality in its activity pattern. This species is able to invert its diurnal phase preference to a nocturnal preference when an exercise wheel is available in the cage or in response to high diurnal temperatures (Kas & Edgar, 1999; Kenagy et al., 2002). Two steady-entrainment phases, combined with the masking effects of light, seem to explain the wide variability of chronotypes that can be observed in this species (Vivanco et al., 2009). However, to date, the existence of negative and positive masking effects has not been directly tested in degus under ultradian LD cycles.

The aim of this work was to characterize the masking effects of light on nocturnal and diurnal degus subjected to 1:1h LD cycles when wheel running was made unavailable by locking the wheel or when diurnal environmental temperatures were increased.

MATERIAL AND METHODS

Sixteen *Octodon degus* (7 females and 9 males) between 35 and 63 months of age were obtained from the Animal Facilities Unit at the University of Alicante (Spain). The animals were individually housed in polycarbonate cages (Panlab, S.L.) equipped with exercise wheels (52 x 15 x 27 cm, L x H x W) in an isolated room ("Chronolab") with controlled humidity (60%), temperature (24.1 ± 0.3 ºC) and photoperiod (12:12 LD), where the lights were turned on at 8:00. Light was provided by two lateral fluorescent lamps controlled by an electronic timer (Data Micro, Orbis), with an intensity of 350-400 lux at cage level. The degus were fed a commercial rat chow (A04 rat-mouse maintenance Panlab) *ad libitum*.

The high environmental temperature cycles that were applied had a trapezoidal profile with an increasing and a decreasing phase, each lasting about 2h. High temperature levels were obtained by switching on an electric thermostatic blanket (A.E. Herga, S.L., Murcia, Spain) under each cage in order to increase the ambient temperature in the chamber. All blankets were connected to an electronic timer that switched them on at 08:00 and off at 20:00. The local environmental temperature was recorded every 10 min using a data logger iButton (ThermoChron®, Data loggers iButton, Maxim Integrated Products, Sunnyvale, California, USA) attached to the side of the cage, on the outside surface, 1 cm above the floor. All experimental procedures were performed in accordance with the "Principles of Animal Care" (Portaluppi et al., 2008; NIH publication No. 86-23, revised 1985) and Spanish laws.

Data Recording

Wheel running activity (WRA) was recorded as wheel turns per 10-min interval using a data acquisition system (Electronic Service at the University of Murcia, Spain). In order to test the influence of wheel running availability on masking by light, the wheel was partially locked so that it allowed only a 45º turn to be made. In this manner, although we could record every WRA attempt, the animals were prevented from

engaging in intense locomotor activity (LA), which enabled us to determine each individual's phase preference.

Experimental Design

Initially, all animals were subjected to a 12:12 LD cycle and an ambient temperature (T_a) of 24.1 ± 0.3 ºC. After 16 days, this light cycle was changed to an ultradian cycle (1:1 LD) that was maintained until the end of the experimental period. Under the ultradian cycle, animals were subjected to two experimental situations (Fig. 1):

1) Locked exercise wheels. Following a 13 day period under ultradian cycles, the wheel was locked for 14 days. After this period, the wheel was made available once again until the end of the experiment.

2) High diurnal environmental temperature cycles. After 27 days of recuperation following the WR manipulation, the ambient temperature was increased every day at 08:00 (external time) and decreased at 20:00. This temperature cycle (28.3 ± 1.2 ºC during the day and 23.8 ± 0.6 ºC at night) was maintained for 31 days. Finally, following a 14-day period to allow the animals to recover from the temperature manipulation under ultradian LD cycles, a 12:12 LD cycle was restored and maintained for 18 days.

Chronotype Characterization and Data Analysis

To characterize the chronotype of each degu, a numerical criterion based on the percentage of diurnal versus total activity was used, as has been described elsewhere (Vivanco et al., 2009). Briefly, when an animal under LD conditions showed a diurnal/total activity ratio above 60%, it was considered diurnal. A nocturnal animal was defined as one whose diurnal/total activity ratio was less than 20%. One degu was excluded due to an unstable activity pattern. No gender differences could be established, so all animal data were pooled.

Individual actograms, mean waveforms, chi-square periodograms and acrophases from a cosine fit of the WRA rhythm were obtained using software specifically designed for chronobiological analysis (El Temps, version 1.228; © Díez-Noguera, University of Barcelona). An *alpha* period (activity) was considered to occur when WRA reached a level above the 1440 min median value and was maintained for at least 2 hours. A *rho* period (resting) was a time period in which the WRA fell below the 1440 min median value for at least 2 hours. To determine the daily acrophase

evolution of the wheel running activity rhythm for each chronotype, the mean and standard error of the mean (SEM) were calculated for the individual daily acrophases. A repeated measures ANOVA was performed to compare diurnal and nocturnal individuals. An unpaired t-test was performed to compare the endogenous periodicity between diurnal and nocturnal degus under each experimental phase. All statistical analyses were performed with SPSS 13.0 software.

RESULTS

At the beginning of the experiment, animals entrained to a 12:12 LD cycle were classified according to the numerical criteria (see the Material and Methods section) as either diurnal (n=8) or nocturnal (n=7). A representative record for both a diurnal and a nocturnal degu is presented in Fig. 2. In the case of diurnal individuals, locomotor activity (LA) began 1-2 hours before lights on and continued up through the first few hours of the dark period (Fig 2A). In nocturnal degus, LA was mainly restricted to the dark period (Fig. 2B). Both chronotypes displayed the same response to light-dark transitions: an increase when the lights were switched off, and an anticipatory peak before the lights were switched on. In both chronotypes, lights on was immediately followed by a decrease in WRA (Fig. 3).

During the first day under the ultradian cycle (1:1 LD), all nocturnal degus phase shifted to a subjective diurnal phase (Fig. 2B), as is characteristic of nocturnal masked individuals. The day-to-day WRA acrophase evolution presented in Fig. 4 shows a large phase advance in nocturnal animals (10-12 hours). During the following experimental ultradian stages, there were no phase differences between nocturnal and diurnal animals, and both groups behaved as if they were the same chronotype. However, they exhibited a differential masking effect by light, as shown in Fig. 5.

Mean waveforms calculated for 120 min (Fig. 5, left panels) and 1440 min (Fig. 5, right panels) periods show the positive masking (increase of WRA) induced by lights on in diurnal animals and by lights off in nocturnal animals. The diurnal-like masking response (Fig. 5, top panels) was more sinusoidal than the square-shaped pattern observed in nocturnal degus (Fig. 5, center panels).

Significant differences (p<0.01) were found in masking by light between diurnal and nocturnal degus, mainly during the light phase for the 120 min waveform (Fig. 5,

left panels). Negative masking by darkness in diurnal individuals was different from that induced by light in nocturnal degus, as LA in this latter group was reduced to zero. By contrast, diurnal degus did not exhibit such a marked reduction during darkness. It is interesting to note that both chronotypes presented the same positive darkness-induced masking (Fig. 5, right panel, at 1380-1440 min) as occurred when the light was switched off under 12:12 LD conditions. A small group of animals previously characterized as diurnal exhibited a masking that was not well defined, with small peaks of activity associated with light-dark and dark-light transitions (Fig. 5, bottom panels).

Under this ultradian cycle, all individuals were entrained to the same phase, and they also showed the same period, as indicated by the Sokolove-Bushell periodogram (1440 ± 3 min and 1439 ± 1 min for diurnal and nocturnal degus, respectively). Curiously enough, and although the masking response was different depending on the chronotype, the 24h WRA pattern was similar for both groups (Fig. 5); with mean values of 15-16h for activity (*alpha*) and 8-9h for resting (*rho*).

In order to study the influence of wheel running availability on masking by light and its involvement in the duality exhibited by the degus, the exercise wheel was partially locked. WRA attempts (that is, attempts made without actually performing true wheel running activity) were then used to determine the phase preference for each individual.

A 120 min waveform plot revealed a masking response for each chronotype that was similar to that previously observed when the wheel was available (Fig. 6). No abrupt phase shift was detected in the circadian rhythmicity of diurnal or nocturnal degus that could be attributed to the locking or unlocking of the exercise wheel (Fig. 4). Furthermore, when the wheel was locked, no significant differences were observed in the period for diurnal (1434 ± 5 min) *versus* nocturnal degus (1430 ± 3 min).

In order to determine the influence of high ambient temperature (an environmental factor which can induce nocturnal inversions) upon masking by light and its involvement in the circadian duality of degus, animals were subjected to an ambient temperature of 28.3 ± 1.2 ºC during the day and 23.8 ± 0.6 ºC at night. Under these conditions, all individuals synchronized their WRA to the subjective dark phase, that is, the cooler one, with an endogenous periodicity of 1441 ± 1 min and 1439 ± 1 min for

diurnal and nocturnal animals, respectively. Two representative individuals are shown in Fig. 7.

Figure 4 clearly demonstrates a slow synchronization of WRA in all animals, with no large phase shifts before achieving the final nocturnal phase. Both diurnal and nocturnal-like masking responses were repeated in the same individuals, but this time, the main WRA shifted to the cooler phase (Fig. 8). Significant differences ($p<0.05$) in the masking response were once again found between diurnal and nocturnal degus during the light phase of the 120 min waveform (Fig. 8, left panels). In this experimental stage, *alpha* was reduced to 11-12h, probably due to the negative influence on activity exerted by the high ambient temperature.

When the high diurnal temperature cycle was removed, all individuals started to free run from the previous phase with no abrupt transitions (Fig. 4), and expanded their *alpha* phase to resemble that present before the temperature cycle (Fig. 7). Finally, when the ultradian cycle was removed and the animals returned to a 12:12 LD cycle, each chronotype slowly re-synchronized until achieving its previous phase observed at the beginning of the experiment (Fig. 4).

Some diurnal animals showed clear masking responses in some experimental stages, but not in others. There were also two exceptional diurnal degus that did not present the predictable diurnal-like masking response, rather a paradoxical one. These two individuals (one of them shown in Fig. 9) were excluded from the diurnal graphical representations. However, all individuals consistently presented the same basic response, i.e., diurnal- or nocturnal-like masking. None of the degus inverted its masking response due to the locking of the exercise wheel or high diurnal temperatures.

DISCUSSION

Our results show that masking effects by light in *Octodon degus* do not involved pacemaker phase control. Diurnal and nocturnal degus displayed stable masking by light, according to their respective chronotype. Thus, while diurnal animals increased their activity in the presence of light, light induced a pronounced WRA drop in nocturnal degus. These two types of masking appeared under ultradian LD cycles and persisted when the exercise wheel was locked and when the animals were exposed to high diurnal ambient temperature cycles.

Octodon degus is a species of caviomorph rodents from Central Chile that presents both diurnal and nocturnal phase preferences in its locomotor activity. Although the degus' circadian pacemaker has been mainly described as being entrained to the diurnal phase, nocturnal entrainment also exists (Vivanco et al., 2009). This species has the ability to invert from a diurnal to a nocturnal phase preference when an exercise wheel is available in the cage, although not in all individuals (Kas & Edgar, 1999).

Both scheduled nighttime feeding and high diurnal environmental temperatures also induce nocturnalism in degus, the latter in 100% of the previously diurnal animals (Vivanco et al., 2010a,b). Previous results from our group have shown that wheel running availability induces a differentiation into three different chronotypes: diurnal, with WRA entrained to the photophase and no negative masking by light; nocturnal, characterized by WRA entrained to the scotophase and a strong negative masking by light; and finally, intermediate animals, characterized by a gradient of negative masking by light acting on a pacemaker entrained to the photophase, as in the diurnal chronotype (Vivanco et al., 2009). In the present study, all animals behaved as diurnal, with WRA entrained to the photophase and no negative masking by light, or as an intermediate chronotype, i.e. with nocturnal WRA entrained to the photophase and negative masking by light.

To date, partially based on studies that indicate no differences between the nocturnal and diurnal pacemaker (Schwartz et al., 1983), it has been generally accepted that the switch from diurnal to a nocturnal phase preference probably occurs downstream from the circadian pacemaker (Vivanco et al., 2007). This circumstance may have an adaptive value because after a switch from diurnal to nocturnal activity, the master clock may go on keeping time for a number of functions, such as synchronization with conspecifics, photoperiodic responses, anticipation to prey and predators, etc., while the timing of some specific behaviors, such as WRA, might be altered to cope with a particular need (e.g. to avoid overheating) without affecting others (Mrosovsky, 2003).

It may be possible that degu chronotypes are set without affecting the central pacemaker itself, but rather by modifying the coupling between the pacemaker and other nearby downstream areas, such as the subparaventricular zone and the dorsomedial hypothalamus (Schwartz et al., 2004; Saper et al., 2005). However, this

mechanism does not exclude a direct or indirect effect of light on some variables through masking.

Of the two processes involved in the control of overt rhythmicity, entrainment and masking, the latter has received much less attention over the decades. Nowadays, the mechanisms, the anatomical location and the molecular biology implicated in masking processes remain poorly understood. Based on retina mutant mice (rd/rd) studies, it has been suggested that negative masking by light relies on luminance sensitive melanopsin ganglionar cells, which send information to the SCN via the retinohypothalamic tract (Mrosovsky, 1994; Mrosovsky & Hattar, 2003). Positive masking by light, however, seems to be mediated by the classic visual system (Edelstein & Mrosovsky, 2001).

Several studies have been conducted in order to answer the question regarding whether masking is located in or nearby the SCN and the role of the clock in masking. These have mainly involved SCN lesions (Redlin & Mrosovsky, 1999) and/or subjecting the animals to light-dark ultradian cycles (Borbély & Huston, 1974). Unfortunately, the results found so far are not consistent. While masking by light disappeared after SCN lesion in some experiments (Ibuka et al., 1977), it failed to do so in others (Fuller et al., 1981). One explanation for this discrepancy is the existence of possible collateral damage to the optic quiasm during the procedure to perform the SCN lesion (Mistlberger, 1994).

Our results show two different types of masking, including nocturnal-like with negative masking by light and diurnal-like with negative masking by darkness. This is consistent with direct masking and entrainment effects of light being separate phenomena, as shown by previous data from our group (Vivanco et al., 2009), and also by other authors through the persistence of masking in SCN-lesioned hamsters (Redlin & Mrosovsky, 1999).

As a matter of fact, the independence of the masking effect by light and pacemaker entrainment properties influencing nocturnalism in degus is supported by the following findings: a) Nocturnal animals that shifted to a diurnal phase after being transferred from 12:12 to 1:1 LD conditions still presented a negative masking effect by light. b) The persistence of nocturnal-like masking in individuals without wheel running availability, the main stimulus that triggers the nocturnal phase inversion in degus; and c) the stability of the different types of masking responses when high diurnal ambient

temperatures forced the WRA rhythm to be confined to the cooler phase, as a result of a negative masking effect that is now prompted by warm temperature. However, we believe that the hypothesis that masking responses may be modulated by the central pacemaker, as suggested by others (Aschoff & von Goetz, 1988 and 1989), cannot be ruled out.

In conclusion, light masking is stable regardless of WRA phase preference, environmental temperature or even exercise wheel availability, and thus it seems to be independent of pacemaker entrainment, contributing significantly to the degus' temporal niche selection. However, considering the data presented in this article and in previous publications from our group (Vivanco et al., 2009, 2010a,b), it would seem that the phenotypic expression of degu chronotypes (nocturnal, diurnal or intermediate) is the result of a hierarchical interaction between the pacemaker entrainment (nocturnal or diurnal), masking exerted by light, and masking by temperature. This may explain the high variability in activity and temperature rhythm patterns observed in this species.

ACKNOWLEDGMENTS

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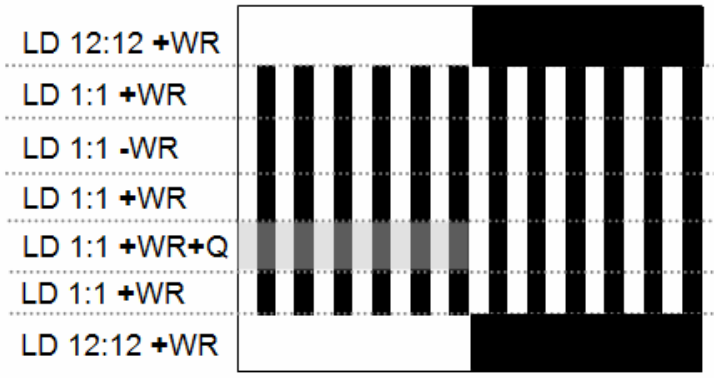

Figure 1. Schematic representation of the different experimental phases: 12:12 LD cycle with wheel running (WR) available; 1:1 LD cycle with WR available; 1:1 LD cycle with WR unavailable; 1:1 LD cycle with WR available once again; 1:1 LD, WR available and high diurnal ambient temperature cycle; 1:1 LD, WR available and constant ambient temperature; and finally 12:12 LD cycle with WR available and constant ambient temperature.

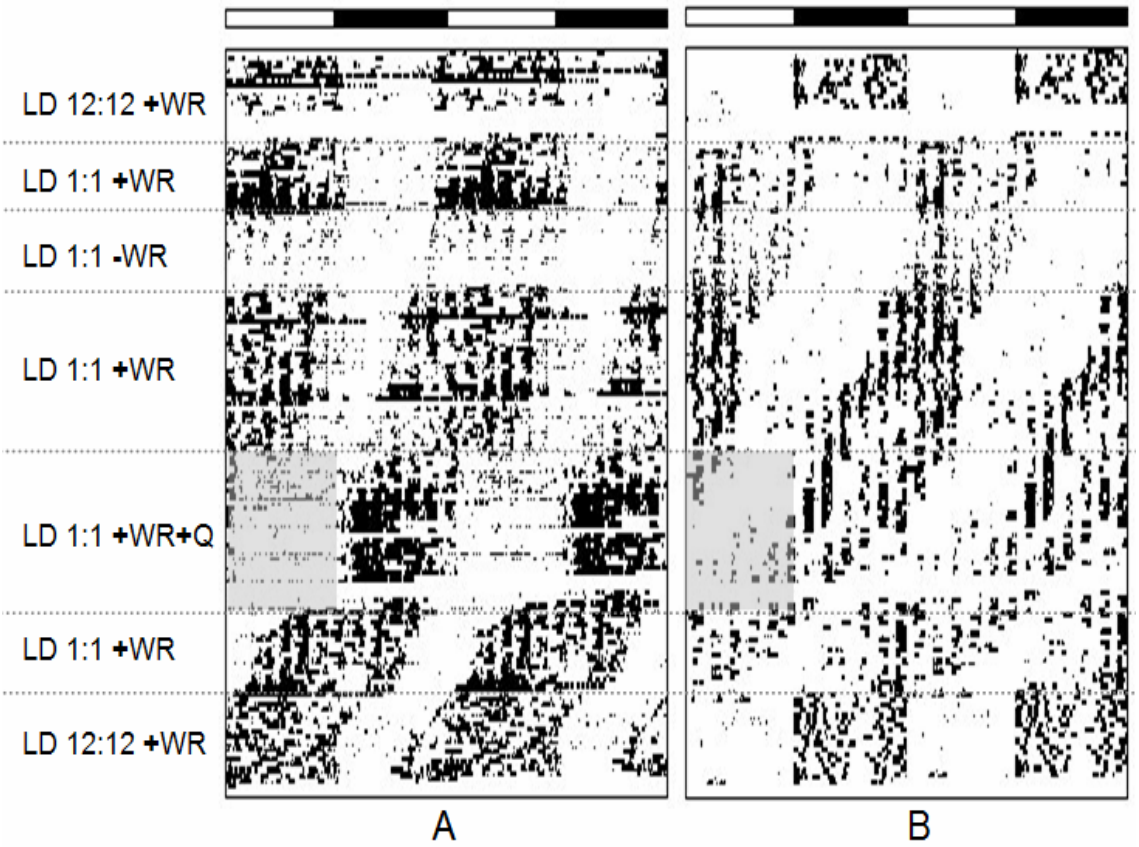

Figure 2. Double-plotted representative wheel running activity (WRA) actograms for a diurnal (A) and a nocturnal (B) degu during the different experimental phases (see figure 1 legend for details). The grey box on the actogram shows the period when the ambient temperature was increased.

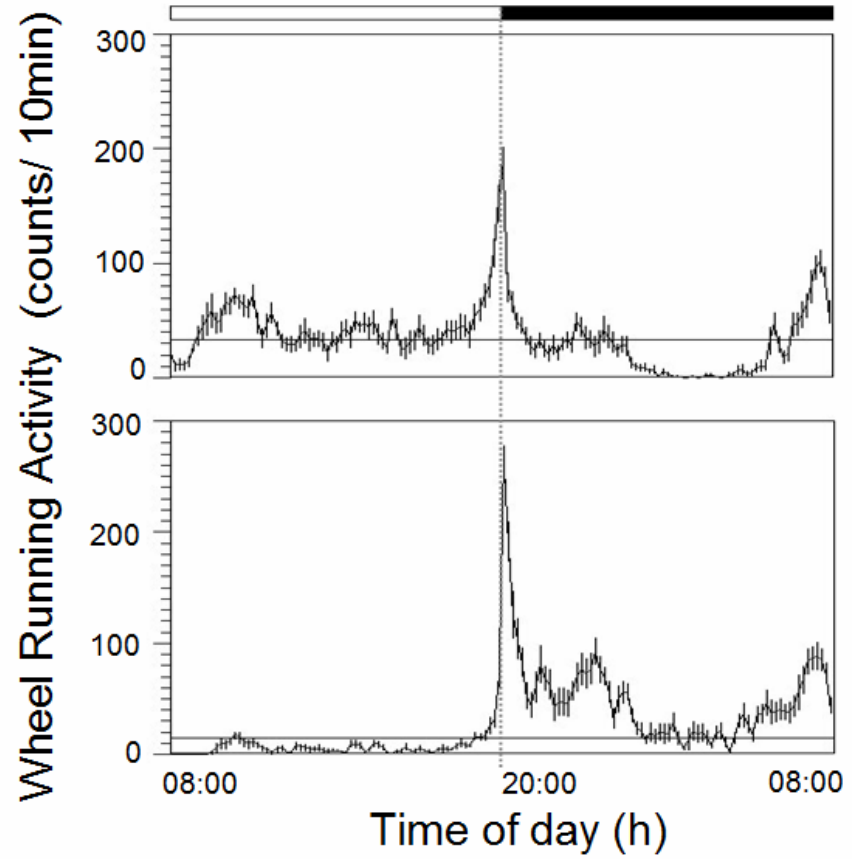

Figure 3. Mean waveforms for wheel running activity rhythms for diurnal (n=8, top panel) and nocturnal (n=7, bottom panel) degus subjected to 12:12 LD cycles. The horizontal line corresponds to the median for the 24h values. Vertical lines represent the standard error of the mean (SEM). The dotted vertical line indicates the light/dark transition for comparison purposes.

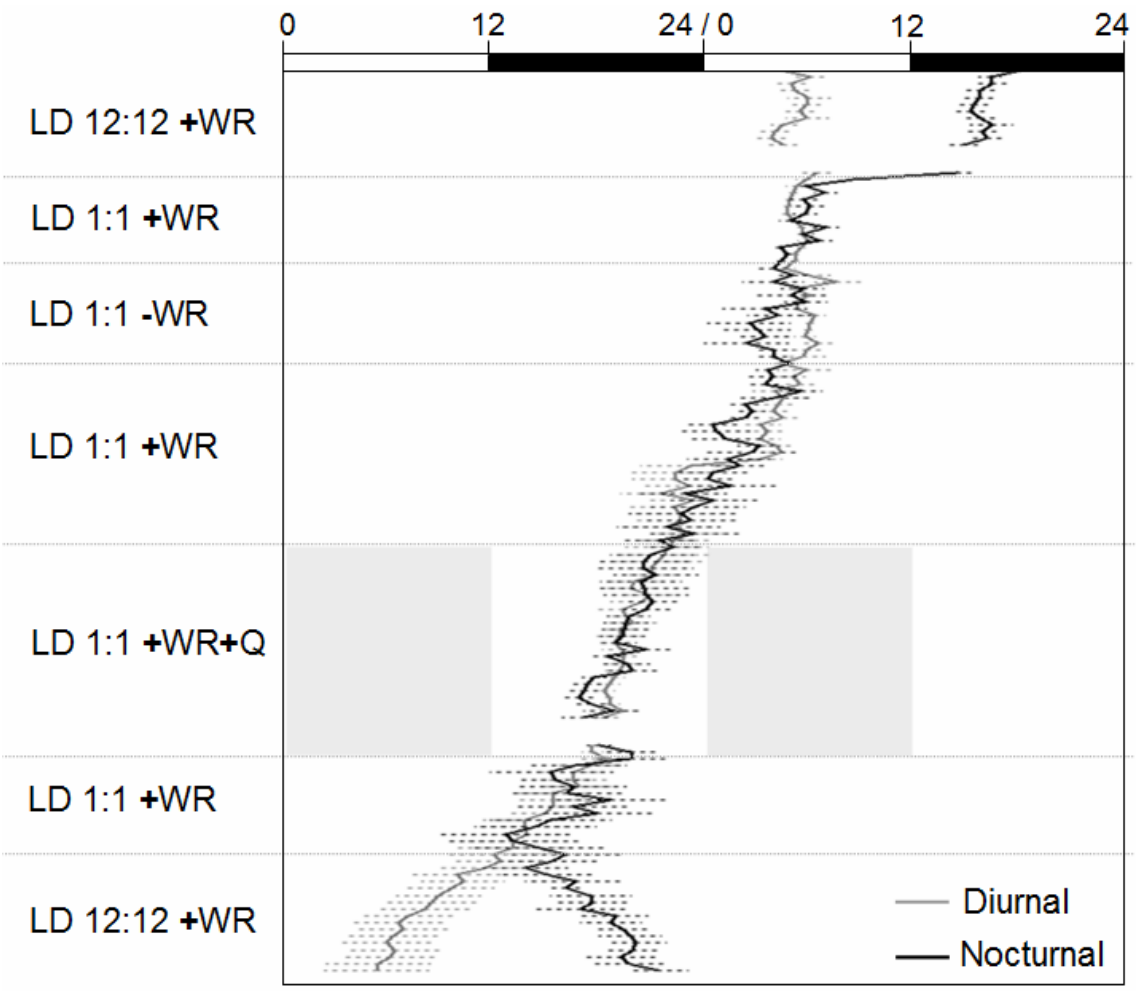

Figure 4. Daily mean acrophases for wheel running activity for diurnal (n=8) and nocturnal (n=7) degus during the different experimental phases, (see figure 1 legend for details). Mean acrophase for each variable, as calculated by cosinor analysis. The horizontal dotted line represents the SEM. The grey box on the actogram shows the period when the ambient temperature was increased.

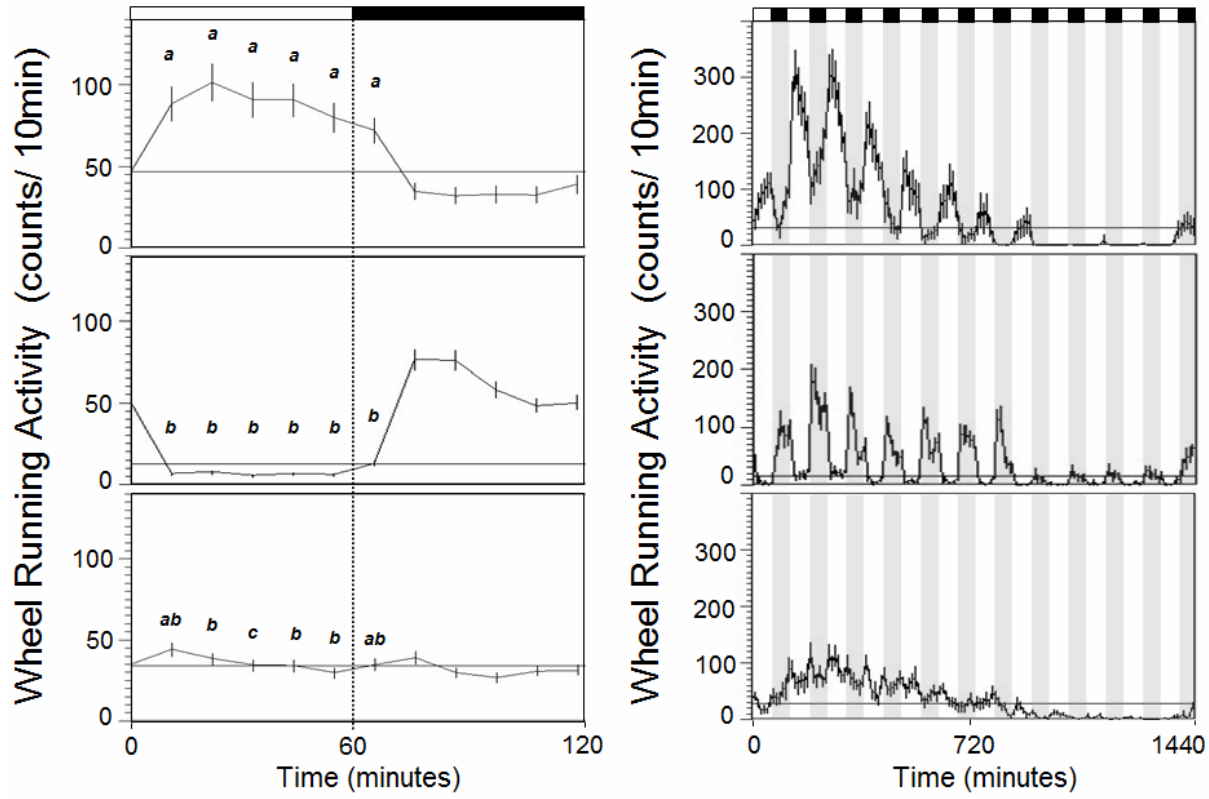

Figure 5. 120 min (left panel) and 1440 min (right panel) plotted mean waveforms of wheel running activity rhythms for three different masking by light responses under 1:1 LD cycles with wheel availability: Diurnal-like masking (top panels), nocturnal-like (center panels), and undefined (bottom panels). The horizontal line corresponds to the median for the 24h values. Vertical lines represent the standard error of the mean (SEM). The dotted vertical line indicates the light/dark transition for comparison purposes. Different letters indicate statistically significant differences between diurnal- and nocturnal-like types of masking responses (ANOVA, $p<0.01$).

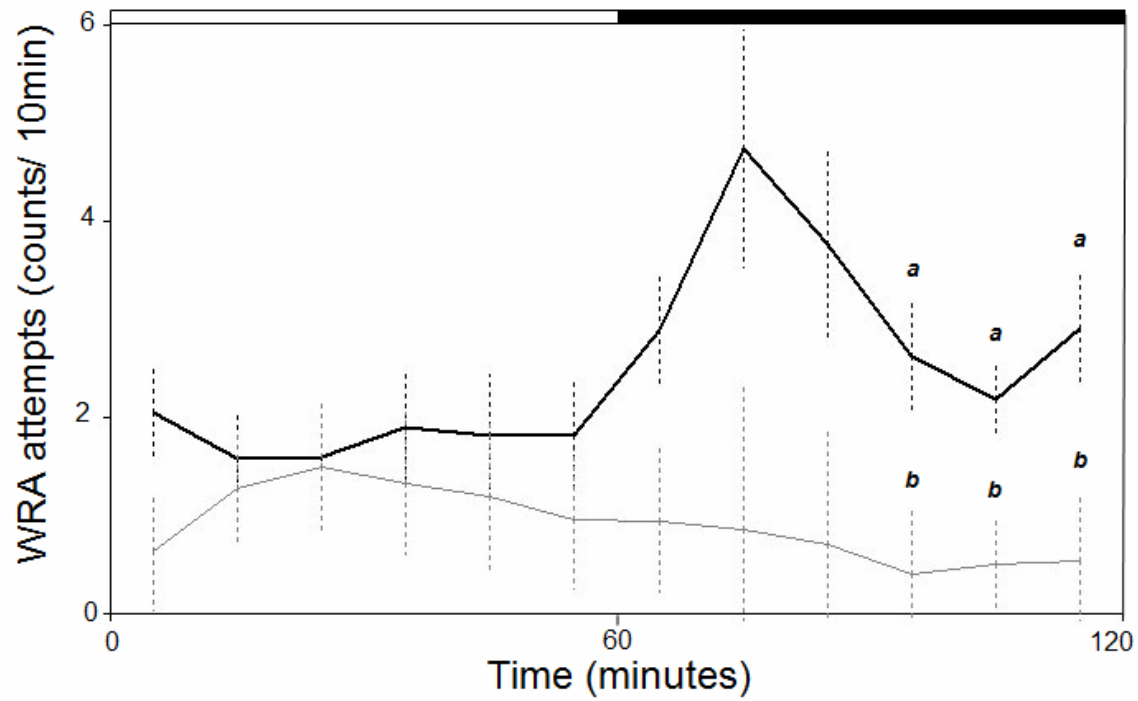

Figure 6. 120 min plotted mean waveforms of wheel running attempts showing diurnal-like (grey line) and nocturnal-like (black line) masking by light responses when degus were subjected to 1:1 LD cycles with a locked wheel. The horizontal line corresponds to the median for the 24h values. Vertical lines represent the standard error of the mean (SEM). The dotted vertical line indicates the light/dark transition for comparison purposes. Asterisks indicate statistically significant differences between diurnal- and nocturnal-like types of masking responses (ANOVA, $p<0.05$).

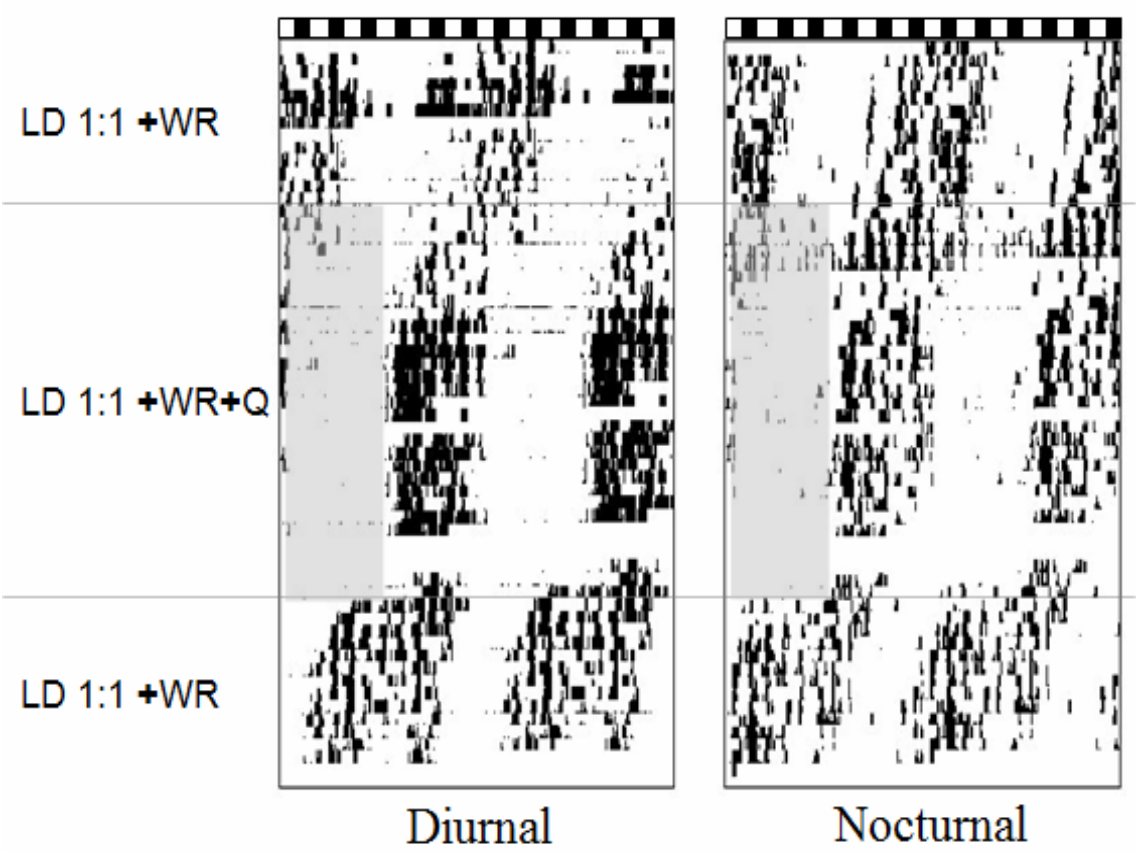

Figure 7. Double-plotted representative wheel running activity (WRA) actograms for a diurnal and a nocturnal degu during the experimental phases: 1:1 LD with WR available; 1:1 LD, WR available and high diurnal ambient temperature cycle; 1:1 LD, WR available and baseline ambient temperature. The grey box on the actogram shows the period when the ambient temperature was increased.

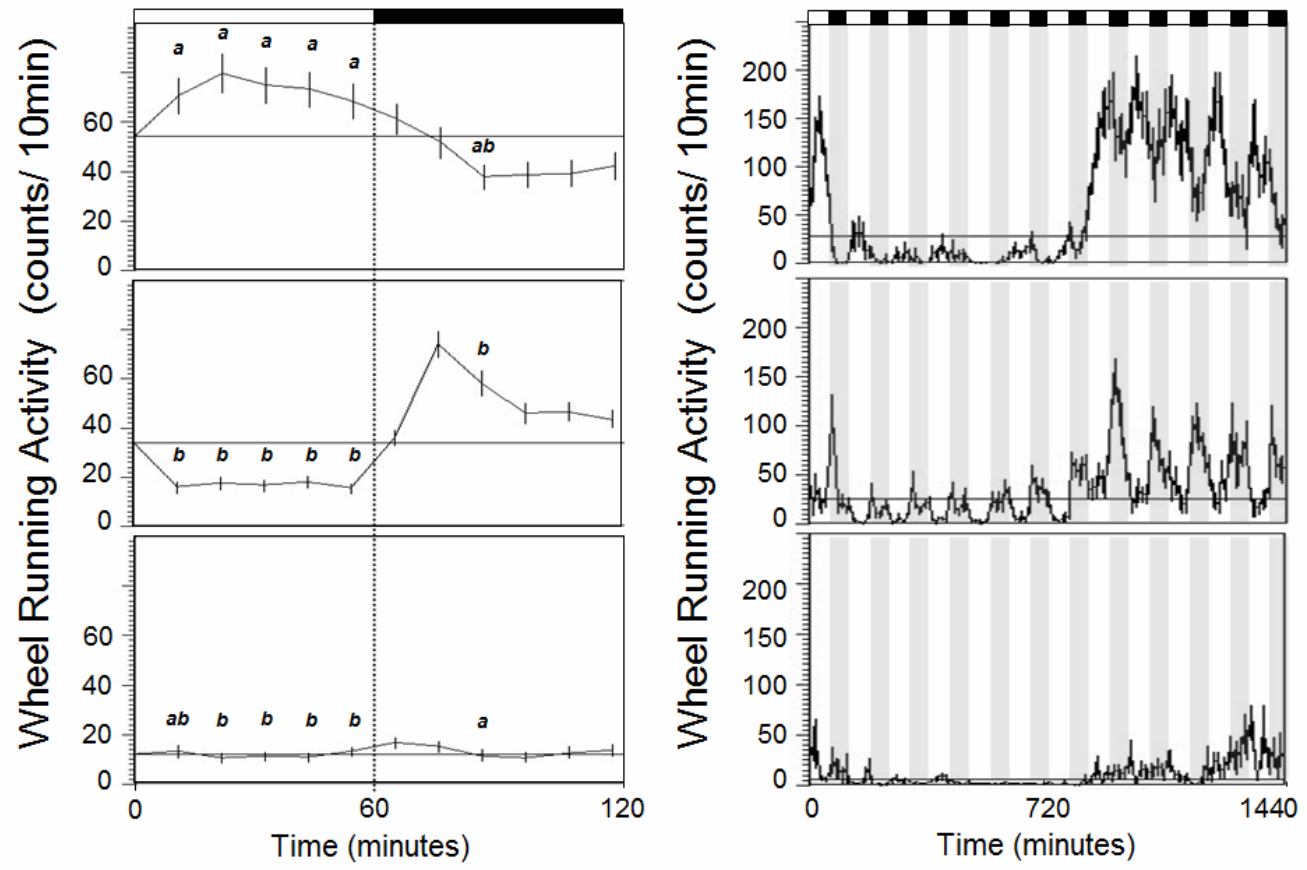

Figure 8. 120 min (left panel) and 1440 min (right panel) plotted mean waveforms for wheel running activity rhythms for three different masking by light responses under 1:1 LD cycles with wheel availability and high diurnal ambient temperature cycle: Diurnal-like masking (top panels), nocturnal-like (center panels), and undefined (bottom panel). The horizontal line corresponds to the median for the 24h values. Vertical lines represent the standard error of the mean (SEM). Different letters indicate statistically significant differences between diurnal- and nocturnal-like types of masking responses (ANOVA, p<0.05).

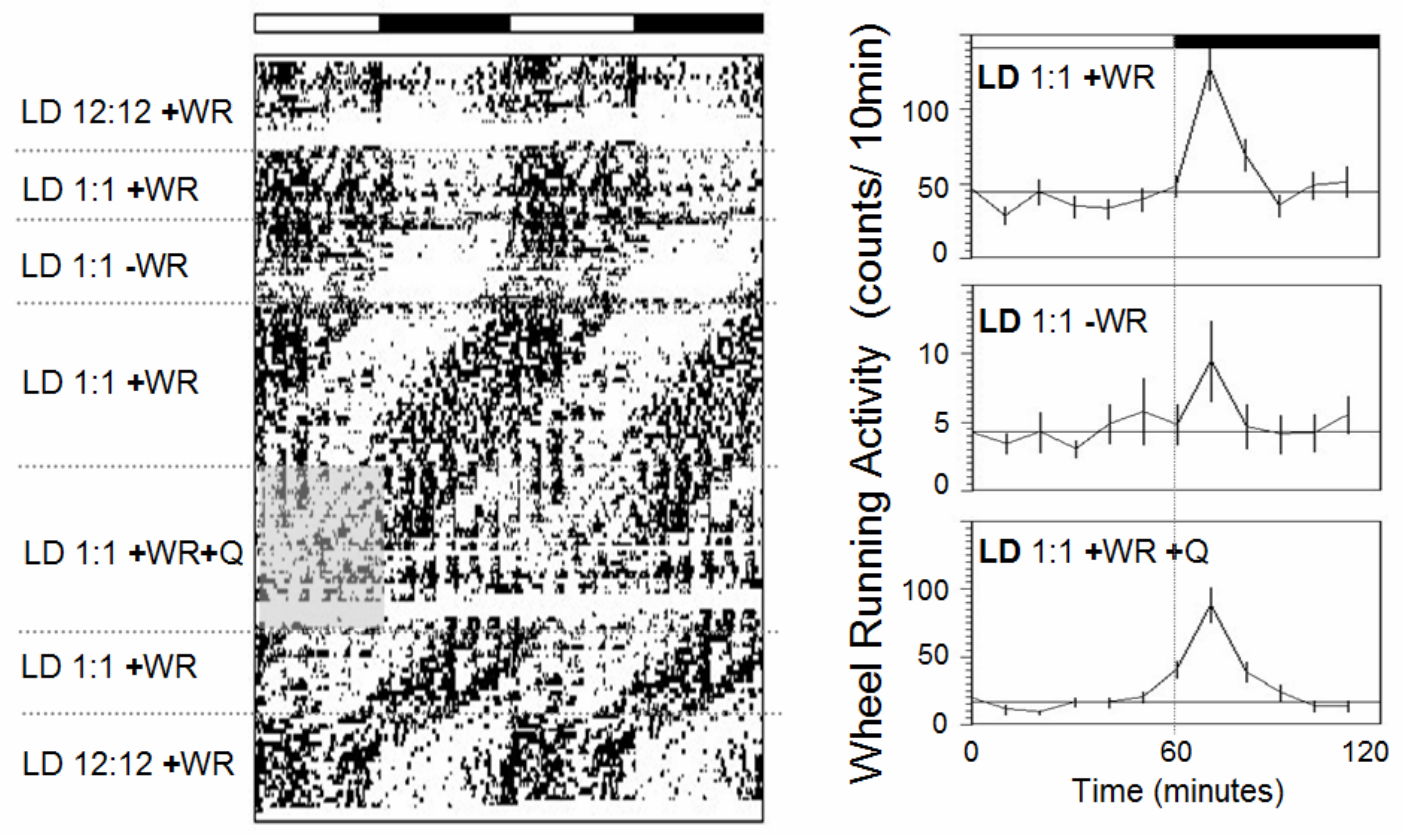

Figure 9. Representative actogram (left panel) and mean waveform (right panels) for one diurnal degus that presented a paradoxical masking by light throughout the entire experiment: 12:12 LD with wheel running (WR) available; 1:1 LD with WR available; 1:1 LD with WR locked; 1:1 LD with WR available once again; 1:1 LD, WR available and high diurnal temperature; 1:1 LD, WR available and constant ambient temperature; 12:12 LD with WR available. The grey box on the actogram shows the period when the ambient temperature was increased. On the waveforms, the horizontal line corresponds to the median for the 24h values; vertical lines represent the standard error of the mean (SEM); and the dotted vertical line indicates the light/dark transition for comparison purposes.

Experimental Chapter 5

Title: "Dissociation of the circadian system in *Octodon degus* induced by T28 and T21 LD cycles"

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ABSTRACT

Octodon degus is a primarily diurnal rodent which presents a great variability of circadian chronotypes due to the interaction between two phase angle of entrainment, diurnal and nocturnal, and graded masking effects by light and temperature. The aim of this study was to test whether the circadian system of this diurnal rodent can be internally dissociated by cycles shorter and longer than 24 hours and to determine the influence of degu's chronotypes and wheel running availability on such dissociation.

To this, wheel running activity and body temperature rhythms were studied in degus subjected to symmetrical LD cycles of T28h and T21h. The results show that both T-cycles can dissociate the circadian system of degus in two components, one light dependent component (LDC), influenced by the presence of the light; whereas the other, a non light dependent component (NLDC), free runs with a period different from the external lighting cycle. The LDC was more evident in the nocturnal than in the diurnal chronotype and when wheel running was available. Qualitative changes in masking effect by light were also observed in the LDC.

In conclusion, the present study indicates that in addition to rats and mice, degus have to be added to the species that can show an internal dissociation in their circadian systems. The existence of a multioscillatory circadian system with two groups of oscillators with low coupling strength could be a mechanism explaining the flexibility in degu's chronotypes.

KEYWORDS: T cycles, dissociation, *Octodon degus*, pacemaker, masking by light

INTRODUCTION

Many properties of the mammalian circadian system can be deduced by studying the characteristics of the overt rhythms. A circadian overt rhythm is the consequence of two main processes which interact mutually: the entrainment of the central pacemaker, the mammalian suprachiasmatic nucleus (SCN), and masking effects by the direct action of external or internal variables (Mrosovsky, 1999; Minors & Waterhouse, 1989).

The circadian system is based on a multi-oscillatory network of individual neurons (or clusters) that through intercellular coupling mechanisms generate and spread an integrated and synchronous high-amplitude output signal driving overt rhythms (Pittendrigh & Daan, 1976). However, a dissociation of rhythmicities could be obtained when using exotic lighting conditions, called T-cycles, as demonstrated in rats and mice under shorter (Campuzano et al., 1999, de la Iglesia et al., 2004 and 2008) and longer (Scannapieco et al., 2009) than 24h LD cycles. In all the cases, the whole output signal was dissociated into two rhythmical components: a **L**ight **D**ependent **C**omponent (LDC) which was influenced by light and a **N**on-**L**ight **D**ependent **C**omponent (NLDC) which was presented under free running with a different periodicity from that of the T-cycle (Campuzano et al., 1998; Cambras et al., 2004). While the NLDC has been related to the pacemaker control, the LDC has been associated to both, pacemaker entrainment and masking by light.

From initial reports to date, masking has been defined as a direct effect upon the overt rhythmicity (Aschoff, 1960; Mrosovsky, 2003). Frequently, the same natural agents that entrain the pacemaker, namely *zeitgebers*, mask at the same time the overt rhythms, overriding the information of the pacemaker. Thus distinguish between masking and entrainment becomes a very difficult task. Traditionally, masking has been classified according to the response of the overt rhythm: if an increase in a biological variable, for example wheel running activity (WRA) occurs, masking is defined as positive, while a decrease is defined as negative (Mrosovsky, 1999). In most cases masking and entrainment act in the same direction, i.e. light induces a negative masking (decreasing WRA) in nocturnally entrained rodents. However, paradoxical masking, with masking and entrainment counteracting in opposite directions, has also been observed in the diurnal Nile grass rat (Mrosovsky, 1999) and in degus (unpublished data from our group).

Octodon degus is a dual phasing rodent from Central Chile which presents a great variability of circadian chronotypes due to a continuous gradient of interaction between the pacemaker and masking effects (Vivanco et al., 2007 and 2009). Moreover, sometimes a phase inversion from diurnal to nocturnal chronotype occurs when a wheel running is available on their cage. Several hypotheses have been raised for this inversion, from ecological to physiological ones (Lagos et al., 1995; Kenagy et al., 2002); however, experimental evidences point to thermoregulatory constraints involving the circadian system (Vivanco et al., 2010b).

The aim of this study was, first, to evaluate the flexibility of the degus' circadian system using a forced-desynchronization protocol by exposure to T-cycles of 28 and 21 h, and additionally to determine if masking by light was dependent on the degus´ chronotype and wheel running availability.

MATERIAL AND METHODS

Twenty-nine *Octodon degus* (18 months of age) were obtained from the Animal Facilities at the University of Alicante (Spain). The animals were individually housed in polycarbonate cages (Panlab, S.L.) equipped with wheels (52 x 15 x 27 cm, L x H x W) in an isolated room ("Chronolab") with controlled humidity (60%), temperature (26.3 ± 0.8 ºC) and photoperiod (LD 12:12) unless otherwise specified. Light was provided by two lateral fluorescent lamps controlled by an electronic timer (Data Micro, Orbis), with an intensity of 350-400 lux at cage level. The degus were fed *ad libitum* with commercial rat chow (A04 rat-mouse maintenance Panlab). All experimental procedures were performed in accordance with the "Principles of Animal Care" (Portaluppi et al., 2008; NIH publication No. 86-23, revised 1985) and Spanish laws.

Data Recording

Wheel running activity was recorded as wheel turns per 10 min interval using a data acquisition system (Electronic Service at the University of Murcia, Spain). Body temperature (T_b) was measured at 60 min intervals using a miniature data logger (ThermoChron®, Data loggers iButton, Maxim Integrated Products, Sunnyvale, California, USA) having an accuracy of 0.1ºC. For this purpose, sterilized data loggers were implanted intraperitoneally, under aseptic conditions, using fluothane as anaesthesia (Forane®, Abbot Laboratories S.A., Madrid, Spain). Reabsorbable suture

material (2/0, Safil®Quick B/Braun, Barcelona) was used to close the abdominal layers, and non-reabsorbable silk was used to suture the skin. No mortality or morbidity was observed after the surgery. The experiment began following a two-week recovery period. At the end of the experiment, the data logger was removed under the same conditions in which it was implanted. IButton readout hardware was used to transfer temperature data to a computer.

Experimental Design

Experiment I. 28h T-cycle and WR available

Sixteen male degus were individually housed with wheel running availability under LD 12:12. After 7 days, degus were subjected to a T28 cycle (14:14 LD) for 24 days. The animals were then transferred to a DD cycle during 11 days, again returning to a 12:12 LD cycle for 12 days.

Experiment II. 28h T-cycle and WR blocked

In order to study the effect of WRA on the masking by light component, the running wheel was blocked and, therefore, unavailable in the same sixteen degus. Animals were again maintained under 12:12 LD cycle during two months for stabilising the circadian rhythmicity. After that, animals were subjected to a DD cycle for 6 days, and again a 12:12 LD cycle for 6 days. Afterwards, degus were subjected to a T28 cycle (14:14 LD) for 21 days. Finally, animals returned to the initial LD 12:12 during 12 days.

Experiment III. 21h T-cycle and WR available

Thirteen male degus were individually housed with wheel running availability under LD 12:12 for 8 days. After that, degus were subjected to a T21 cycle (10.5:10.5 LD) for 87 days. Finally, animals returned to the initial LD 12:12 during twelve days.

Chronotype Characterization and Data Analysis

To characterize each degus´ chronotype, a numerical criterion based on the percentage of diurnal versus total activity was used. Thus, when an animal under LD conditions showed a diurnal/total activity ratio above 60%, it was considered diurnal. A

nocturnal animal was defined as one whose diurnal/total activity ratio was less than 20%.

Individual actograms, mean waveforms and chi-square periodograms of WRA and T_b rhythms were obtained using software specifically designed for chronobiological analysis (El Temps, version 1.228; © Díez-Noguera, University of Barcelona). The analysis of variance explained by the rhythm (%Var.) was obtained by Sokolove-Bushell periodograms. A repeated measures ANOVA was performed to compare the percentages of variance between diurnal and nocturnal individuals under T28 with the wheel available. A linear regression was performed to establish the relationship between the percentage of diurnalism of each animal with respect to the %Var. per circadian component (NLDC, LDC or both). The statistical analysis was performed with SPSS 15.0 software.

RESULTS

Experiment I. 28h T-cycle and WR available

From the initial sixteen degus under 12:12 LD with wheel running availability, two individuals were removed due to their irregular and unclassifiable chronotype. Six out of fourteen presented clear diurnal WRA and T_b rhythms and eight individuals presented nocturnal ones. A gradient of circadian expression, from diurnal to nocturnal one, was apparent under this light schedule (Figs. 1 & 2).

When animals were subjected to T28 cycles, different responses were found according to the degus' chronotype. In diurnal individuals, WRA and T_b rhythmicities started to free-run from in accordance to the previous circadian phase in 12:12 LD (Figs. 1A and B & 2A). However, in nocturnal degus, 5 out of 8 individuals presented a large phase shift from the nocturnal to the diurnal phase (Figs. 1C & 2B and 2C), indicating that their nocturnalism was only induced by masking effects by light. The remaining three nocturnal degus (see Fig. 1D as an example) were nocturnal-entrained animals and therefore, they did no present such phase shift.

T28 cycles permitted to dissociate the central rhythmicity into two circadian components: a light dependent component (LDC) with a period similar to that of the external cycle and a non-light dependent component (NLDC), which started to free run

the first day under the T-cycle. The periodogram analysis showed a significant peak of rhythmicity at 1461 ± 1 and 1455 ± 1 min for WRA and T_b, respectively in diurnal degus. In the case of nocturnal degus, two significant differentiated components appeared: one at 1456 ± 3 min for WRA and 1462 ± 4 min for T_b, associated to the NLDC, and 1665 ± 5 min for WRA and 1653 ± 7 min for T_b associated to the LDC. A closer inspection of the rhythmicity under T28 permits to observe a relative coordination of the NLDC when both LDC and NLDC are in phase. It is interesting to note that sometimes, when the LDC coincided with the subjective night of the NLDC; the light dependent component disappeared (Figs. 1C and D).

Figure 3 shows the relationship between the percentage of variance (%Var.) of NLDC and LDC components, and the percentage of diurnalism per animal. While diurnal individuals present significant differences in the %Var. between NLDC and LDC in both WRA (p=0.001) and T_b (p=0.037) rhythms; nocturnal ones do not (Fig. 3, upper panels).

It is also interesting to note that LDC, in the T_b rhythm, is significantly higher in nocturnal than in diurnal degus (p=0.044). The %Var. of each circadian component for WRA and T_b according to the percentage of diurnal WRA (Fig. 3, lower panels) shows that increasing diurnal activity implies an increase in the NLCD (R^2= 0.335, p= 0.030 for WRA, and R^2= 0.115, p= 0.511 for T_b rhythm) while decreasing the LDC (R^2=0.154, p= 0.185 for WRA, and R^2= 0.343, p= 0.222 for T_b rhythm). However, WRA and T_b remind constant when both circadian components are considered together (R^2= 0.064, p= 0.405 for WRA, and R^2= 0.002, p= 0.928 for T_b rhythm, suggesting a circadian compensation between them (Fig. 3, lower panels).

In both diurnal and nocturnal individuals was present an increase in WRA and T_b in response to light dark transition, as indicate their waveforms (Fig. 4, second panel). It is also interesting to note that the LDC did not appear in T28 as a simple response to light. Indeed, the LDC slowly achieved its final phase relationship in 3-4 days (Fig. 5 left). Furthermore, when T28 started, LDC rhythmicity stars on phase with the dawn component of the previously entrained rhythm under 12:12 LD (Figs. 1B & 5 left panel). In the case of the dusk component, under T28 it free ran in the NLDC (Fig. 1B & 5 left panel).

When animals were transferred to DD, LDC was in most of the individuals not detected, then it disappeared or was part of the whole residual rhythm which free ran in

diurnal and nocturnal animals with a periodicity of 1438 ± 4 min for WRA and 1435 ± 1 min for T_b in diurnal animals, and 1424 ± 5 min for WRA and 1430 ± 1 min for T_b in nocturnal ones. However, in some individuals (Fig. 1B) LDC did not disappear under DD but it started to free ran with its own periodicity. Finally, when a 12:12 LD cycle was imposed, all degus slowly re-synchronized until they achieved their final phase.

Experiment II. 28h T-cycle and WR blocked

After two months with the wheel running blocked, all degus (sixteen) presented a clear diurnal T_b rhythm under 12:12 LD cycle (Fig. 6). When animals were subjected to T28 cycles, in all of them T_b rhythm started to free run from the same phase relationship that in the previous light schedule. The analysis of the %Var. determined a significant peak of rhythmicity around 1459 ± 3 min for T_b rhythm (NLDC) in these diurnal degus (see also Fig. 6). Some individuals presented, apart from the significant NLDC, another component although not statistically significant, as the individual showed in Fig. 6B with a periodicity of 1720 min and the individual of Fig. 6C with around 1565 min (this last measured directly on the actogram).

The mean waveform analysis of T_b under T28 with wheel blocked exhibited a non-defined circadian rhythmicity which also was no influenced by the light or dark transitions, contrary to that observed when wheel running was available (Fig. 4, third panel). Finally, when 12:12 LD cycle was restored, T_b rhythm presented a brief disorganization with part of the rhythmical components resynchronizing by delay and other part by advance, as shows in Figs. 6 B and C, respectively.

Experiment III. 21h T-cycle and WR available

When degus, diurnal all of them under 12:12 LD with wheel running availability, as those representatives showed in Fig. 7, were subjected to T21 cycles, WRA rhythm started to free run in accordance with the previous phase under 12:12 LD. Under this T-cycle the resistance to the apparition of the LDC was higher than previously under T28. The LDC was patent in 6 out 12 degus after spending the half of the experimental phase. It is interesting to observe the analysis of variance explained by the rhythm measured on two different moments of the experiment (Fig. 7). The first analysis (upper panel of the periodograms) considered 20 to 30 days after starting the T21 cycle and shows a mean peak of 1446 ± 4 min associated to the NLDC, and a very small peak in 1255 min, this last being the LDC. The second analysis (lower panel of the

periodograms) considered 60 to 70 days after starting the T21 cycle and it shows a shorter NLDC located about 1435 ± 5 min and a LDC increased in the %Var. on 1255 min.

Only one individual evidenced the LDC from the first day (Figure not included), however, it disappeared in just one week. However, in this individual, LDC started from the dusk component of the previous 12:12 LD rhythmicity. Under the T21 cycle the phase relationship between the LDC and NLDC was advanced when compared with the T28 (Fig. 5, right panel). Moreover, the waveform of the WRA shows again an increase of the WRA after the light-dark transition (Fig. 4, last panel).

DISCUSSION

The results show the presence of a non-light dependent component (NLDC) dissociated from a light dependent component (LDC) under both T28 and T21 cycles. While the first one is associated to the intrinsic pacemaker rhythmicity, the later one is due to the presence of the external lighting, and related to the nocturnalism status of the animal. The wheel running appears as a stimulus that directly triggers the appearance of the LDC in degus. Moreover, LDC does not act as a simple light-reactive stimulus but it is phase related with the central pacemaker rhythmicity.

On first notes on masking, appears the citation from Aschoff in 1960 as a “suppression or accentuation of the observed function (e.g., locomotor activity) by the environmental stimulus”. Subsequently, masking definition was elaborated on positive or negative one, according to the result of the masking action: positive when the stimulus enhances/activates the rhythmicity or negative when the stimulus decreases/inhibits it (Aschoff & Goetz, 1988 and 1989).

In 1989, Minors & Waterhouse, more oriented to human studies, also expanded the term by classifying masking responses according to the causal source: Masking type 1 was defined as “a direct effect of the normal environment”; masking type 2A as “behavioural changes in the experimental plant or animal in response to normal environmental changes”; and, finally, masking type 2B as “behavioural changes in the experimental plant or animal in response to abnormal environmental changes”.

Nicholas Mrosovsky, the main researcher on masking, introduced in his review (1999) the term “paradoxical masking” as a not predictable response when considering the chronotype of the individual. In this sense, a paradoxical positive masking occurs when, for example, a nocturnal animal increases its activity in response to light.

In our opinion, in the literature, masking has been referred in a wide diversity of situations. During light transitions, as dawn and dusk, it is said that masking causes the expression of the animals’ chronotype. For example, in a nocturnal animal, dark perception triggers activity while in a diurnal one, it slows or abruptly cesates activity (Mrosovsky, 1999). This “tonic” concept of masking could differ from a “phasic” one which is found when an animal is subjected to brief pulses of one stimulus, i.e. the light, through the circadian cycle, as in the case of ultradian cycles of lighting (Redlin & Mrosovsky, 2004). In this sense, an interesting recent study shows how a series of flash light pulses induces a complex behavioural response in mice, starting with a locomotor quiescence and finishing with a sleep status. This process has been referred as photosomnolence (Morin & Studholme, 2009).

Masking is also involved in temporal niche switching, due to seasonal rhythms, thermoregulatory constraints or any other causality (Gwinner, 1996; Mrosovsky, 2003; Vivanco et al., 2010a and b). This “qualitative” change in rhythmicity occurs as a physiological response, involving the circadian system, against a potentially dangerous causal agent (Kronfeld-Schor & Dayan, 2003). The common point in all of these references to masking is the existence of an overt rhythmicity change. To date, masking has been only considered as a “quantitative” change that disturbs the endogenous signal from the circadian pacemaker; however, experimental evidences in *Octodon degus* points out that masking is a more complex player for the rhythm generation than previously considered.

Under 28h and 21h T-cycles, the degus circadian system dissociated into two components: a NLDC which can be assumed as the intrinsic rhythmicity generated by the pacemaker, and a LDC.

The LDC can be considered in different ways: one possibility is to assume that LDC is only the result of masking effects by light. Other possibility consist in that LDC is a part of a multioscillatory pacemaker and whose activity is influenced by light, it can receive photic innervation, but it is not, in any way, related to masking. A third possibility could be a mixture of both, in which masking and pacemaker are completely

interrelated to each other. In this sense, LDC would be the consequence of masking by light acting only on a subpopulation of oscillators (from the pacemaker) entrained to LD cycle.

Assuming the first possibility means entrainment properties that would have been already recognized. Campuzano et al. (1998) found that, under T-cycles shorter than 24 h, rats dissociated their locomotor activity rhythm into a non-light entrainable and a light-entrainable component. This later entrainable component could no be differentiated from normal masking effects by light.

The second possibility implies that masking is not involved in any way. Under our opinion, this option is no realistic as the light is the main masking agent; therefore its presence upon the dissociated rhythmicity is compulsory in any way. Therefore, we support the third hypothesis based on the next experimental evidences:

a) LDC did not appear directly by light stimulation but it comes from the previous rhythmicity and it slowly resynchronizes until achieving a stable phase, a fact that points out entrainment involvement;

b) In DD, after removing the T28 light cycle, the LDC completely disappeared in most of the individuals; however, did not in others. If LDC was solely a pacemaker component, it should have phase shifted or resynchronized in all of the animals;

c) LDC expression sometimes disappeared when it was in synchrony with the subjective night of the NLDC, as described by Aschoff & Goetz (1988 and 1989), showing the masking response dependence on the pacemaker phase;

d) The shorter periodicity of the NLDC was associated to the appearance of the LDC under T21. This fact again links the pacemaker with the LDC;

e) LDC was related to the nocturnalism status of the animal, which is demonstrated to be related to masking processes in degus (Vivanco et al., 2009, 2010a and b);

f) Wheel running availability, the main stimulus that triggers nocturnalism in degus, was directly associated to the significant appearance of the LDC; and

g) The increase of WRA or T_b immediately after dusk transition in T28 and T21 indicates that masking processes were involved.

Whatever the relationship between pacemaker and masking processes in the LDC, the evidence demonstrates a high flexible degu's circadian system. Neuroanatomically, the dissociation process could be explained by a forced

desynchronization of zones inside the pacemaker, as the dorsomedial and ventrolateral zones. In fact, it was possible dissociate both pacemaker areas by means of 22h T-cycles in rats (de la Iglesia et al., 2004). In this sense, it would make sense that while the LDC would be located in, or related to, the ventrolateral zone of the suprachiasmatic nucleus (zone directly innervated by retinal afferences through the retinohypothalamic tract); the NLDC would be located in, or related to, the dorsomedial zone, the real self-sustaining timekeeping of the pacemaker. Moreover, it cannot be discarded the possibility of a differential involvement of the right and left side of the SCN, as demonstrated in hamsters subjected under LL conditions (de la Iglesia et al., 2000).

In conclusion, our results show that, in addition to rats and mice, degus has to be added to the species that show an internal dissociation in their circadian systems under forced desynchronization protocols by exposure to T-cycles, as T28h and T21h. When dissociated, a non-light dependent component free runs and a light-dependent, characterized by a combination of masking by light and entrainment properties, emerges. The ability to dissociate depends on the degu's chronotype and wheel running availability. The existence of a multioscillatory circadian system with two groups of oscillators with low coupling strength and different reaction to light could be a mechanism explaining the high flexibility in degu's chronotypical differentiation.

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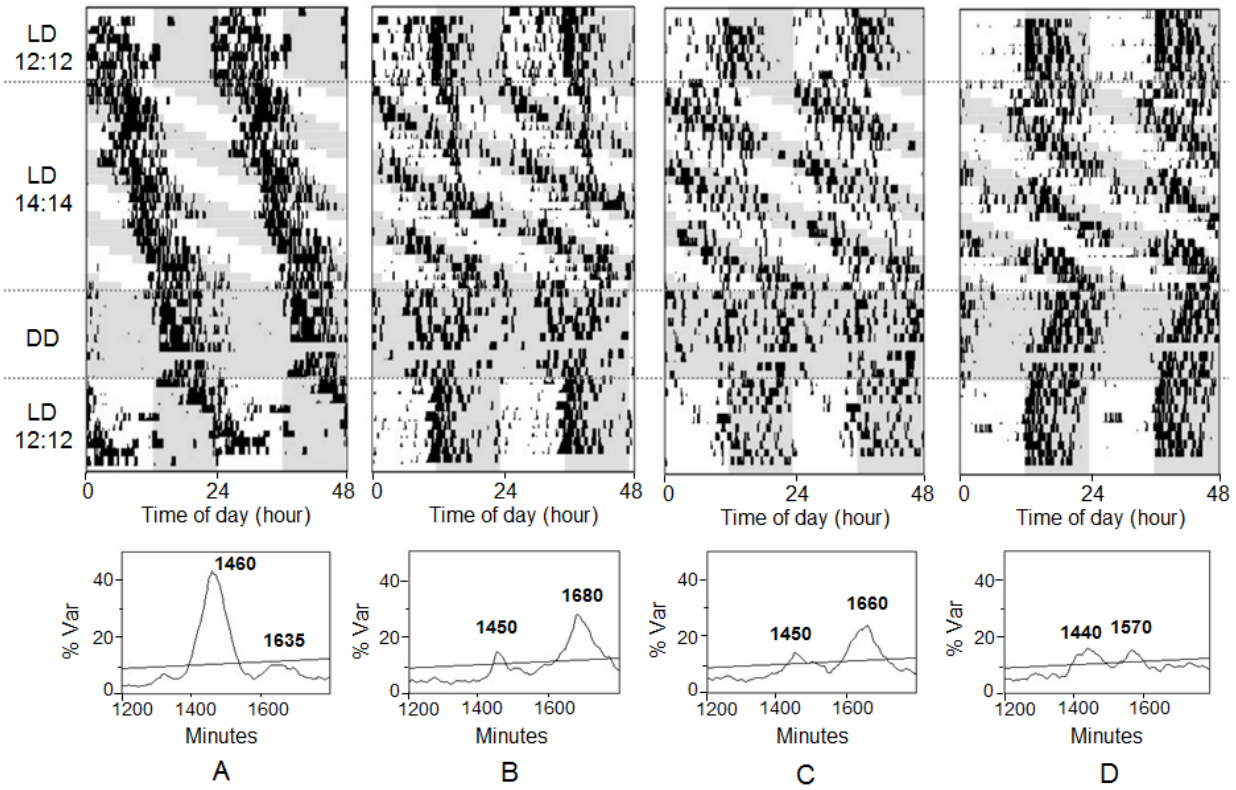

Figure 1. Double-plotted representative wheel running activity actograms for the different experimental phases: 12:12 LD cycle, 14:14 LD cycle (T28), DD, and 12:12 LD cycle, and chronotypes (A) a diurnal individual with low expression of the light dependent component (LDC) under T28 cycles; (B) a diurnal degus with high expression of LDC; (C) a nocturnal degus with high expression of the LDC and phase shifted to the diurnal phase when it was subjected to the T28 cycle; and (D) a nocturnal degus with few expression of LDC and no phase shifted under the transition to T28. Actograms are represented on a 24h periodicity. Grey areas represent light off phase. Underneath each actogram is included its correspondent analysis of variance (%Var) under the T28 cycle, with its period in abscises. Values on the periodogram indicate the significant periods detected.

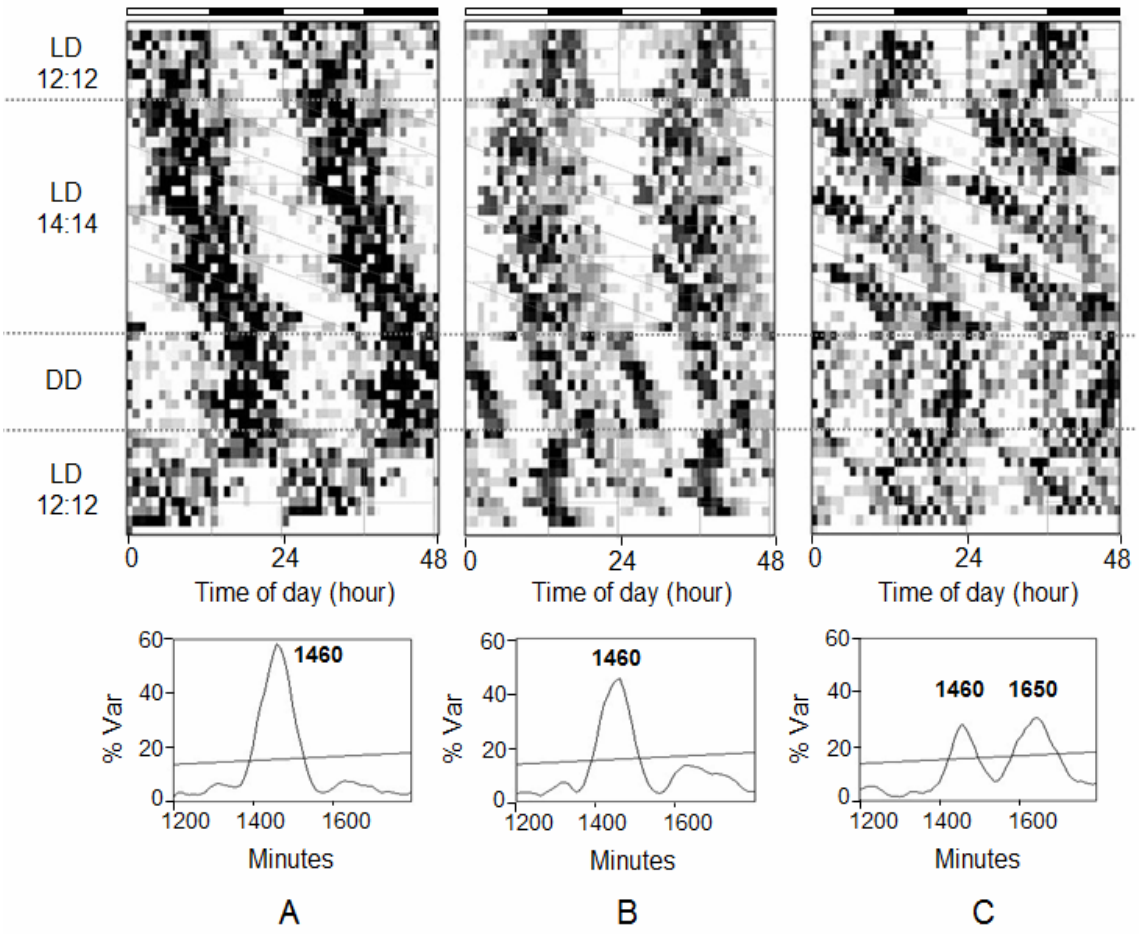

Figure 2. Double-plotted representative body temperature actograms for the different experimental phases: 12:12 LD cycle, 14:14 LD cycle (T28), DD, and 12:12 LD cycle, and chronotypes: (A) a diurnal individual with no expression of the LDC under T28 cycles; (B) a nocturnal degus with no expression of the LDC and phase shifted to the diurnal phase when it was subjected to the T28 cycle; and (C) a nocturnal degus with high expression of the LDC and no phase shifted under the transition to T28. Actograms are represented on a 24h periodicity. Lined-boxes drawing on the actograms represent the light off phase. Underneath each actogram is included its correspondent analysis of variance (%Var) under the T28 cycle, with its period abscises. Values on the periodogram indicate the significant periods detected.

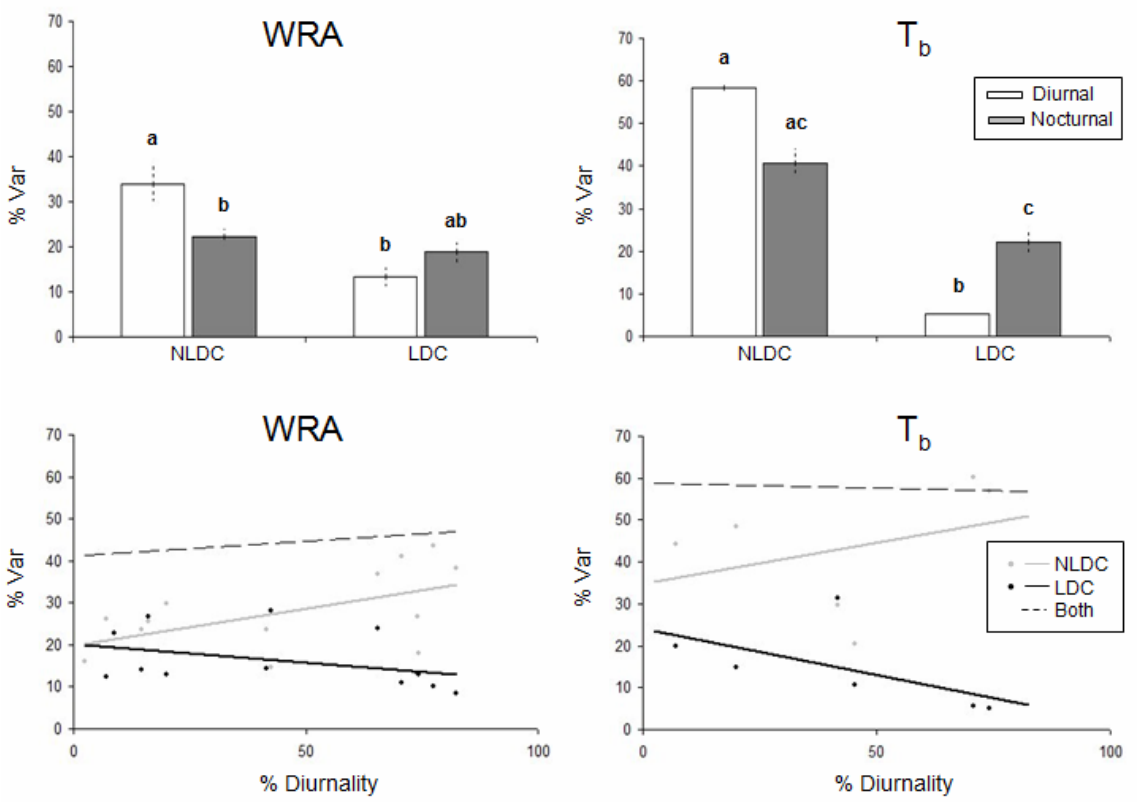

Figure 3. Relationship between each differentiated circadian component, non-light dependent component (NLDC) and light dependent component (LDC), with respect the diurnal or nocturnal degus´ chronotype (upper panels) and with the percentage of diurnalism (lower panels) of each animal, studied for both wheel running activity (WRA, left panels) and body temperature rhythms (T_b, right panels) under T28 cycles. Different letters indicate statistical significant differences between chronotypes and circadian components (p<0.05). On the lower panels, apart from the NLDC and the LDC, the sum of both rhythmicities, ("both" in the legend) was also included.

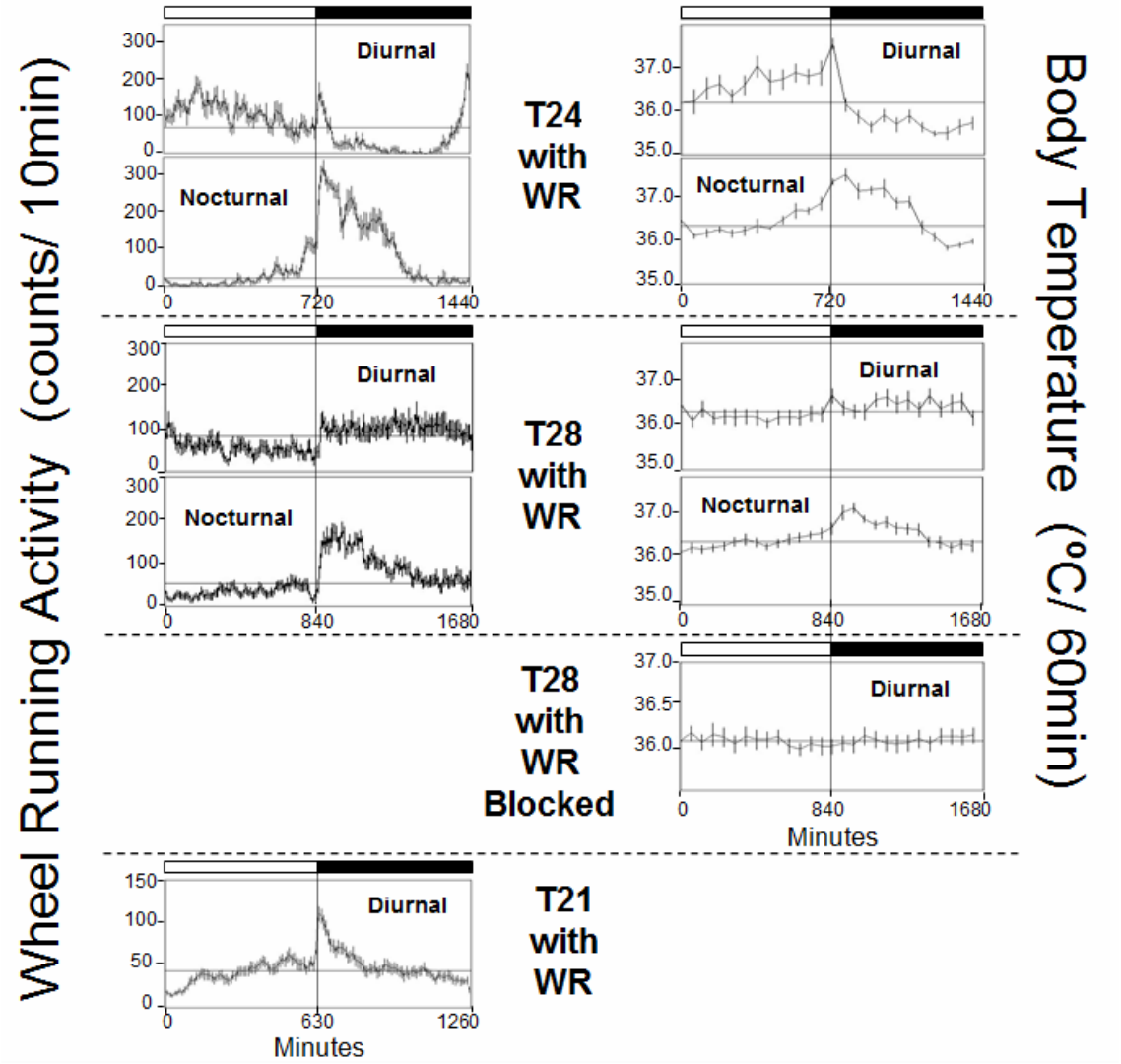

Figure 4. Mean waveforms for wheel running activity (WRA) and body temperature (T_b) rhythms for diurnal and nocturnal degus subjected to different experimental phases: (first panel) 12:12 LD (T24) with the wheel running (WR) available, represented on a 1440min periodicity; (second panel) 14:14 LD (T28) with the WR available, represented on a 1680min periodicity; (third panel) 14:14 LD (T28) with the WR blocked, represented on a 1680min periodicity; (last panel) 10.5:10.5 LD (T21) with the WR available, represented on a 1260min periodicity. The horizontal line corresponds to the median for the circadian day values. Vertical lines represent the standard error of the mean (SEM). A vertical line marks light/dark transition for comparison purposes.

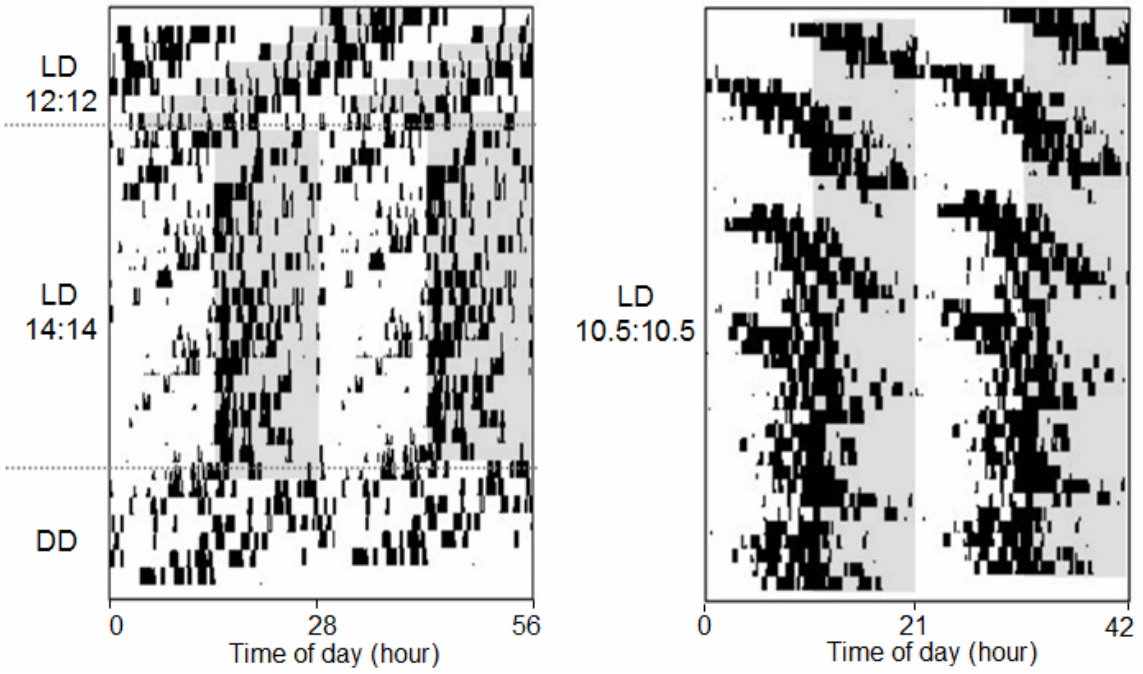

Figure 5. Double-plotted representative wheel running activity actograms of: a diurnal degus (left panel) subjected to the different experimental phases: 12:12 LD cycle, 14:14 LD cycle (T28), and DD, on a 28h periodicity; and a diurnal degus (right panel), subjected to a 10.5:10.5 LD cycle (T21), represented on a 21h periodicity. Grey area indicates the light off phase.

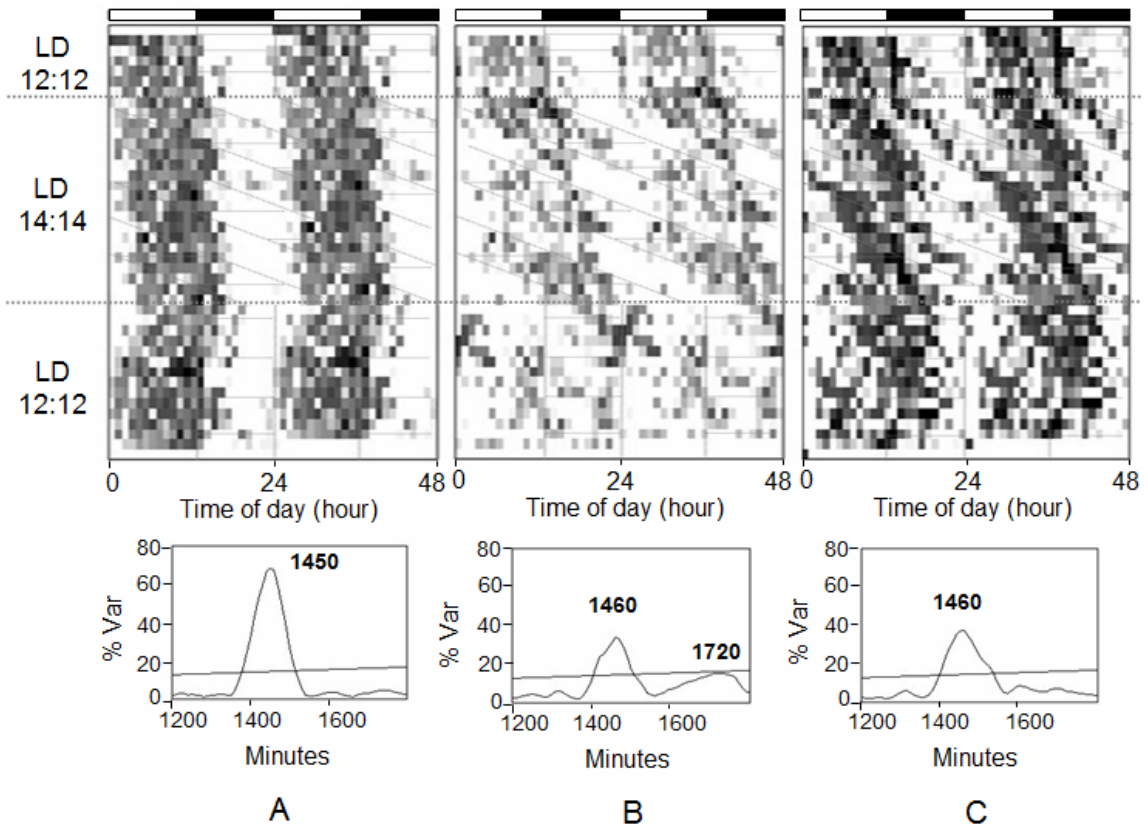

Figure 6. Double-plotted representative body temperature actogram of the different experimental phases: 12:12 LD cycle, 14:14 LD cycle (T28), and 12:12 LD cycle, and chronotypes with the wheel running blocked: (A) a diurnal individual with no expression of LDC under T28 cycles; (B) a diurnal degus with low expression of the LDC; and (C) another diurnal degus with no expression of the LDC. Actograms are represented on a 24h periodicity. Lined-boxes drawing on the actograms represent the light off phase. Underneath each actogram is included its correspondent analysis of variance (%Var) under the T28 cycle, with its period abscises. Values on the periodogram indicate the significant periods detected.

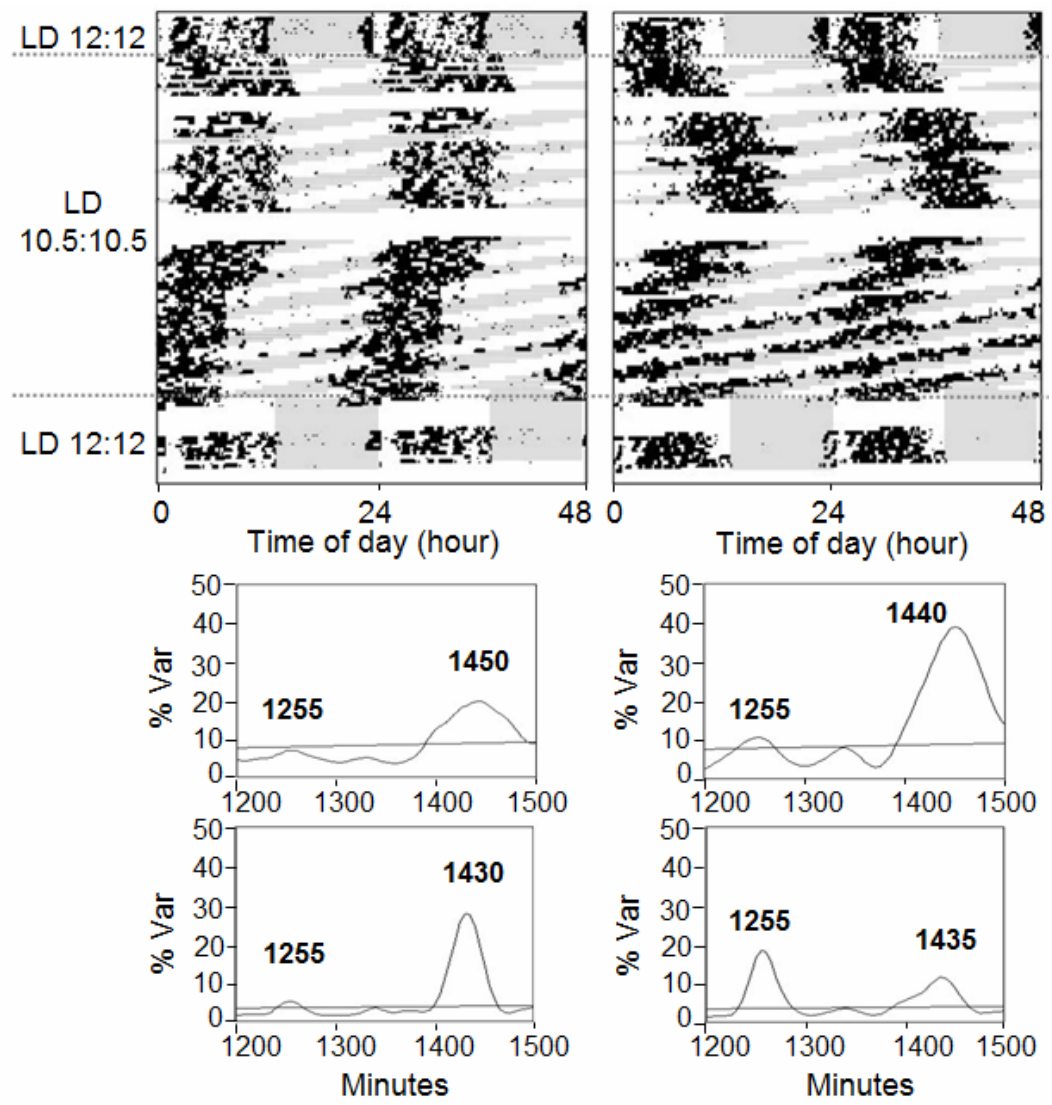

Figure 7. Double-plotted representative wheel running activity actograms of the different experimental phases: 12:12 LD cycle, 10.5:10.5 LD cycle (T21), and 12:12 LD cycle, for: a diurnal individual (left) with low expression of the LDC that was only present at the end of the T21 cycles; a diurnal degus (right) with high expression of the LDC, especially at the end of the T21 cycles. Actograms are represented on a 24h periodicity. Grey area represents the light off phase. Each actogram is included its correspondent analysis of variance (%Var) under the T28 cycle, with its period abscises.

Underneath each actogram two graphs for their correspondent analysis of variance (%Var) explained by the rhythm under the T21 cycle (on the upper part for the 20 to 30 days and in the lower part for the 60-70 days of this experimental stage), whose period is represented in abscises.. Values on the graph indicate the significant periods detected.

Experimental Chapter 6

Title: "Looking for the keys to diurnality downstream from the circadian clock: role of melatonin in a dual-phasing rodent, *Octodon degus"*

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Looking for the keys to diurnality downstream from the circadian clock: role of melatonin in a dual-phasing rodent, *Octodon degus*

Abstract: Melatonin is an essential component for circadian system function, whose daily plasma secretory rhythm is driven by the suprachiasmatic nucleus (SCN), contributing to the communication of temporal messages from the central circadian clock to all cells. Melatonin secretion peaks in the dark, regardless of whether animals are diurnal or nocturnal. To date, the precise mechanisms that explain how the circadian system is configured as nocturnal or diurnal remain unknown. The present study examines mid-day and midnight melatonin plasma levels and the influence of exogenous melatonin on the circadian system phasing of *Octodon degus*, a diurnal rodent, which exhibits nocturnal and diurnal chronotypes when free access to a wheel is provided. Plasma levels of melatonin were determined by RIA in blood samples taken from the jugular vein at mid-light (ML) and mid-dark (MD). Melatonin (0.5 mg/kg b.wt.) was orally administered in their drinking water for 30 days, 2 hr before the onset of darkness. The results showed that plasma melatonin levels and their qualitative effects, hypothermia and improved synchronization with no modification in the 24-hr wheel running activity (WR), were similar in both nocturnal and diurnal degus. Furthermore, melatonin can be used to improve the impaired circadian rhythmicity observed in aged animals, with no rebound effect after ceasing the treatment. It is concluded that plasma melatonin levels and the differential responses to melatonin do not seem to be responsible for nocturnal and diurnal chronotypes, and thus other mechanisms upstream, within, or downstream from the SCN should be investigated.

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Introduction

Throughout their lifetimes, the daily light–dark transition has served for almost all species as a Zeitgeber to entrain their internal clocks, and thus accommodate their behavior to a 24-hr period to optimize the survival. Although most animals display stable nocturnal or diurnal behaviors conditioned by physiological adaptation to light or darkness, some rare species, including fish (European sea bass, *Dicentrarchus labrax*; goldfish, *Carassius auratus*) and mammals (mole rat, *Spalax ehrenbergi*; Nile grass rat, *Arvicantis niloticus*) [1–4] can be considered dual species because of their ability to switch from a nocturnal to a diurnal behavior and vice versa in just a few days. *Octodon degus* is also able to phase shift its daily activity cycle from diurnal to nocturnal [5], with predator avoidance, food availability and thermoregulatory constraints being some of the causes proposed to explain this locomotor rhythm flexibility in natural environments [6, 7].

Laboratory experiments suggest the presence in these rodents of a switch to control their phase preferences for diurnal or nocturnal activity; however, the molecular and neural mechanisms underlying diurnality remain unclear. Several lines of evidence indicate that the pacemaker in the suprachiasmatic nucleus (SCN) operates quite similarly in both nocturnal and diurnal species [8]. Therefore, it has been suggested that the mechanism determining the activity phase must be located downstream from the clock, that is, in the humoral output molecules of the SCN, i.e. prokineticin 2, transforming growth factor alpha [8–11] and, probably, melatonin [12].

In all vertebrate species, plasma melatonin peaks during the night, regardless of whether the animal shows nocturnal or diurnal behavior. This hormone is therefore considered to be the 'chemical expression of darkness' [13]. In diurnal species, the nocturnal rise in melatonin coincides with the physiological rest period (increased sleepiness, decreased body temperature (BT) and locomotor activity, and increase in the immune responses) [14]. By contrast, in nocturnal species, the stable endogenous signal of nocturnal melatonin is associated with wakefulness (increased locomotor activity and BT and decreased sleepiness). According to Mendelson et al. [15], endogenous melatonin should promote those behaviors associated with night, in other words, resting in diurnal and activity in nocturnal species. The existence of a dual phasing behavior within the same animal species constitutes a unique animal model for determining this antagonistic response to melatonin in nocturnal and diurnal animals.

The circadian rhythmicity of plasma melatonin is significantly impaired by aging in several species, including humans. In most species that have been studied, it is specifically the nocturnal melatonin that decreases as the animal ages [16, 17]. These findings, together with its documented antioxidant, immunostimulant, antitumoral and hypnotic effects, lead to propose the use of melatonin as an anti-aging therapy for human beings; however, most animal studies using exogenously administered melatonin have been performed with nocturnal rodents as models. In these species, free radicals should be produced in phase with their melatonin rhythm, a significant difference with respect to diurnal animals, in which melatonin and oxidative stress are in phase opposition. In recent years, considerable effort has been made to study diurnal animal models, such as Nile rat, mole rat and degus as alternatives to nocturnal rodents. Among them, the degus, a primarily diurnal active rodent from semi-arid environments in Central Chile, constitutes an interesting model for aging studies, as it develops degenerative diseases such as diabetes, cataracts and Alzheimer-like diseases common in the human [18]. However, to date no information is currently available about its plasma melatonin levels, whether this hormone decreases with aging, or the potential efficacy of exogenously administered melatonin to reverse this age-associated decline in circadian rhythmicity.

Thus, the aims of the present study were: (a) to determine the melatonin plasma levels related to the *degus*' chronotypes and age; (b) to determine whether exogenous melatonin can induce different effects on nocturnal and diurnal chronotypes; and (c) to determine the effect of melatonin on improving circadian rhythmicity in aged animals.

Materials and methods

Animals and housing conditions

A total of 18 *O. degus* of both sexes (10 males and eight females), with ages between 24 and 60 months, were obtained from a colony maintained at the Animal Service of the University of Murcia. Animals were individually housed in Plexiglas cages equipped with wheels ($52 \times 15 \times 27$ cm, L × H × W) in an isolated room (Cronolab), with controlled humidity (60%), temperature (23 ± 1°C) and photoperiod (LD 12:12). Light was provided by four fluorescent lamps controlled by an electronic timer (Data Micro, Orbis), with an intensity of 350–400 lux at the level of the cages. The *degus* were fed *ad libitum* throughout the experiment, using commercial rat chow (A04 rat-mouse maintenance Panlab). Water and food intake were recorded weekly. All experimental procedures were performed in accordance with the 'Principles of Animal Care' (NIH publication N° 86–23, revised 1985) and Spanish laws.

Data recording

Running wheel activity (WR) was recorded as turns on a wheel with a circumference of 25 cm and a width of 10 cm, at 10 min intervals, using a data acquisition system (Electronic Service at the University of Murcia).

Body temperature was measured by using a miniature data logger (ThermoChron®, Data loggers iButton, IDC S.A., Spain) with an accuracy of 0.1°C. It was programmed with a sampling period of 60 min [19]. Sterilized data loggers were implanted i.p., under aseptic conditions, using fluothane as anesthesia (Forane®, Abbot Laboratories S.A., Madrid, Spain). Reabsorbable silk (2/0, Safil® Quick, B/Braun Barcelona, Spain) was used to suture the abdominal layers, and nonreabsorbable silk was used to suture the skin. No mortality nor morbidity was observed after the surgery. The experiment began following a 2-week recovery period. At the end of the experiment, the data logger was removed under the same conditions in which it was implanted. iButton readout hardware was used to transfer temperature data to a computer.

Melatonin administration and analysis

Melatonin (Sigma, M-5250; Milwaukee, WI, USA) stock solution was dissolved weekly in ethanol, and then mixed in drinking water to obtain the appropriate concentration (0.6 μg/mL in 0.01% ethanol). It was then stored in darkness at −20°C until use. Every 2 days, stocked melatonin was used to prepare 225 mL of drinking water per animal, which was supplied in light-sealed bottles for 2 hr before lights off. To adjust the amount of melatonin administered to each individual to 0.5 mg/kg. b.wt. per day, daily water intake was recorded for 1 wk before starting the melatonin treatment. To this end, animals were supplied with drinking water containing 0.01% ethanol for 2 hr immediately before lights off. Water intake was measured throughout the experiment, in order to individually readjust the melatonin doses and keep them constant.

Plasma melatonin was determined by RIA (Melatonin direct RIA kit, IBL Hamburg®; Hamburg, Germany) in venous blood samples obtained from slightly anesthetized animals (fluothane 2.5% mixed with oxygen for induction and 1.5% for maintenance) through jugular venopuncture during the ML periods and, on a different day, in the MD periods. Blood samples (with the anticoagulant Heparin Leo©) were immediately centrifuged to 2026 g for 15 min at 4°C. The plasma obtained was stored frozen at −80°C until its quantification.

Experimental procedure

Experiment 1. Influence of chronotype and age on plasma melatonin levels

After acclimation to wheel running, 18 *degus* that had been previously classified according to their chronotype (determined through analysis of wheel running activity, see chronotype characterization) and age were anesthetized and sampled by jugular venopuncture at 02:00 hr (MD) and 14:00 hr (ML) in November and March. Animals were allowed 1 wk of recovery between the two samplings.

Experiment 2. Role of exogenous melatonin on wheel running and BT rhythms

After 1 month of acclimation to laboratory conditions and wheel availability, 13 adult *degus* (24–42 months old) that had been classified as nocturnal (n = 5) or diurnal (n = 8) were surgically implanted with a miniature data logger for temperature recording. Two weeks later, they were subjected sequentially to four experimental stages: (a) Control stage (CON). WR and BT were recorded for 2 wk in animals provided with water *ad libitum*. (b) Vehicle stage (VEH). Water containing 0.01% ethanol was restricted to 2 hr before darkness, for 2 wk. (c) Melatonin stage (MEL). Melatonin was supplied in drinking water for 2 hr before darkness at a dose of 0.5 mg/kg b.wt. per day, for 30 days. (d) Recovery stage (REC). Drinking water was provided *ad libitum* for 2 wk.

Chronotype characterization and data analysis

After stable wheel running rhythms had settled for at least 2 wk, degus were classified as day- or night-active. To avoid interference with masking effects induced by light–dark transitions, data from 20 min before and 20 min after light–dark transitions were removed from the calculations. A diurnal chronotype was considered to exist when the ratio between diurnal/nocturnal activity was above 60%. All animals who demonstrated a diurnal/nocturnal ratio below 40% were included in the nocturnal chronotype category. One degus from the initial population was excluded, because of its unstable wheel running pattern.

Wheel running and BT were analyzed by using software specifically designed for chronobiological analysis (El Temps, version 1.192; © Díez-Noguera, University of Barcelona). Averaged actograms, mean waveforms, Fourier analysis and Rayleigh *z*-tests were performed.

A mean daily waveform for each animal and experimental stage was constructed based on the 24-hr LD cycle. The average waveform per experimental group and stage was then calculated. To compare exogenous melatonin responses among chronotypes, mean light and dark activity was then computed.

The rhythm amplitude was determined from the mean waveform of each experimental phase as the difference between the actual maximum and minimum values for the variable.

Interdaily phase stability was calculated as follows: firstly, daily acrophases of WR and BT rhythms per experimental stage were obtained, using least-squares data fitting to a cosine function within a period of 24 hr. Acrophase distribution within a 24-hr period were assessed, using a Rayleigh *z*-test [20]. This test obtains an ***r*** vector with its origin at the center of a circumference of radius one. The *r* length (between 0 and 1) is proportional to the degree of phase homogeneity during the corresponding experimental stage, and can be considered a measure of the rhythm's phase stability during successive days.

A statistical analysis of variance (ANOVA) with repeated measures was performed, to make comparisons between experimental stages. Normal distributions and homogeneity of variances for categories of the previously defined variables were found. A regression analysis was performed to establish the relationship between plasma melatonin levels and age. Data of melatonin and their respective VEH were analyzed by using a paired *t*-test.

Results

In LD conditions, plasma melatonin levels displayed a daily rhythm with a wide amplitude in both diurnal and nocturnal degus (Fig. 1). MD values ranged from 100 to 120 pg/mL, whereas ML levels were lower than 20 pg/mL. No statistically significant differences were detected between chronotypes (Fig. 1) or sexes (10 males versus eight females). Therefore, male and female *degus* were pooled for further analysis. Similar to what has been reported for other species, MD melatonin values exhibited a significant negative correlation with age (Fig. 2A), and in contrast, diurnal levels showed a slight increase with age contributing to the observed reduction in amplitude (MD–ML difference) as age increased (Fig. 2B).

When degus were kept in cages and provided with free access to running wheels, eight animals out of 14 displayed more than 60% of their WR activity during the photophase (diurnal chronotype), whereas five animals exhibited more than 90% of their activity during the dark (nocturnal chronotype), and one animal showed no clear chronotype.

A superimposed double plot actogram of wheel running activity (WR) for all adult animals classified according to their chronotype is depicted in Fig. 3. Both chronotypes exhibited two crepuscular peaks of activity, found exactly after dark-on and just before light-on. However, the diurnal chronotype showed a sustained high level of activity between these two peaks during the photophase, while the nocturnal chronotype concentrated most of its WR between both peaks, during the scotophase. This resulted in a daily activity pattern that was much more defined in the nocturnal than in the diurnal chronotype. On average, 96% of the total daily

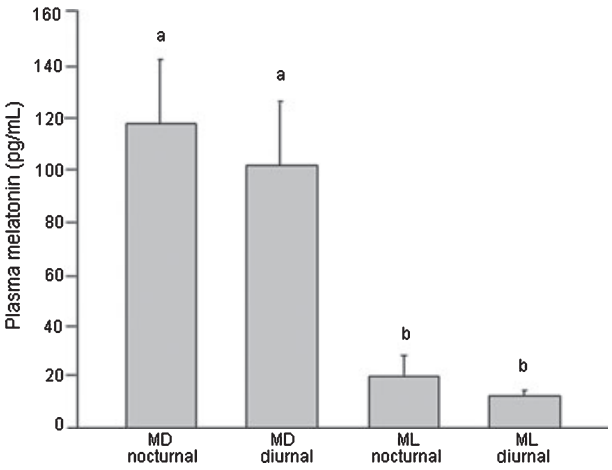

Fig. 1. Plasma melatonin levels at mid-dark (MD) and mid-light (ML), in both chronotypes of *Octodon degus*. Values are expressed as mean ± S.E.M of 10 diurnal and eight nocturnal animals. No statistically significant differences were observed between the chronotypes. Different letters indicate statistically significant differences (ANOVA, $P < 0.05$).

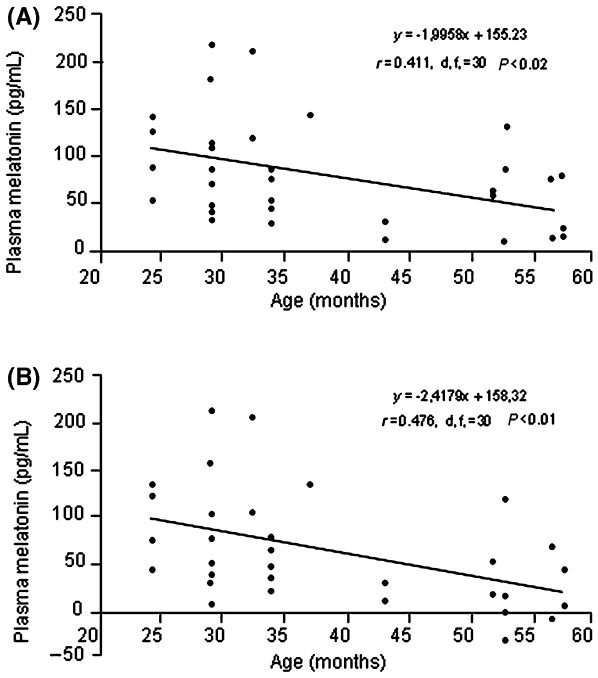

Fig. 2. Relationship between plasma melatonin and age in *Octodon degus*. (A) Correlation between MD values and age calculated from 16 degus sampled two times, in November and March. (B) Correlation between plasma melatonin rhythm amplitude (MD-ML) and age for the same degus represented in (A).

locomotor activity was restricted to the scotophase in nocturnal animals, while only 63% of the activity appeared during the photophase for the diurnal chronotype.

Regarding temperature rhythms (Fig. 4), the superimposed actogram revealed that, again, both chronotypes exhibited two peaks coinciding with the crepuscular peaks of the WR. However, whereas the temperature profile was very similar among chronotypes during the scotophase, the pattern during the photophase was quite different, with high sustained BTs in diurnal *degus* and low values in nocturnal animals. It is surprising that nocturnal animals, despite high levels of locomotor activity during the night, did not show large differences between night and day temperatures.

No significant differences in wheel running and BT rhythms were detected according to gender. Males and females were therefore pooled for further analysis.

Population daily waveform means for wheel running and BT for both nocturnal and diurnal animals throughout the four experimental phases are shown in Figs 5 and 6, respectively. During the control period, locomotor activity was higher in nocturnal chronotypes than it was in diurnal ones, while the opposite was true for BT, however, these differences were not statistically significant.

To reproduce the nocturnal melatonin increase, drinking water remained available for only 2 hr before the onset of darkness, and melatonin intake occurred at the same time for all animals, regardless of their chronotype. Although the coexistence of animals with nocturnal and diurnal behavior in the same population could imply differences in spontaneous water intake patterns, mean water consumption was not affected by either the 2-hr restriction or the chronotype (mean water intake: 19.38 ± 2.78 mL and 17.05 ± 3.41 mL in control, 19.25 ± 3.53 mL and 19.70 ± 1.79 mL in vehicle, and 20.53 ± 1.95 mL and 20.65 ± 2.51 mL in MELs for diurnal and nocturnal chronotypes, respectively).

Restricting the availability of water containing only the vehicle (dissolved in 0.01% ethanol) increased the amplitude of locomotor activity rhythms, with a clear period of anticipatory activity in diurnal, but not in nocturnal, animals. Vehicle administration induced small effects on BT, but it should be noted that a 1 hr advance in BT increase associated with the dusk peak was observed in both chronotypes (Fig. 6).

The oral administration of melatonin 2 hr before darkness induced a slight increase of anticipatory activity in wheel running in diurnal animals (Fig. 5, left), and the appearance of such anticipatory activity in nocturnal animals, when compared with vehicle (Fig. 5, right). Melatonin did not alter the short burst of high activity immediately after lights-off or the burst prior to lights-on for either chronotype.

The temperature waveform induced by melatonin was, in general, quite similar to that of vehicle; however, the difference between maximal–minimal values increased, and a generalized hypothermic effect was observed (Fig. 6). The

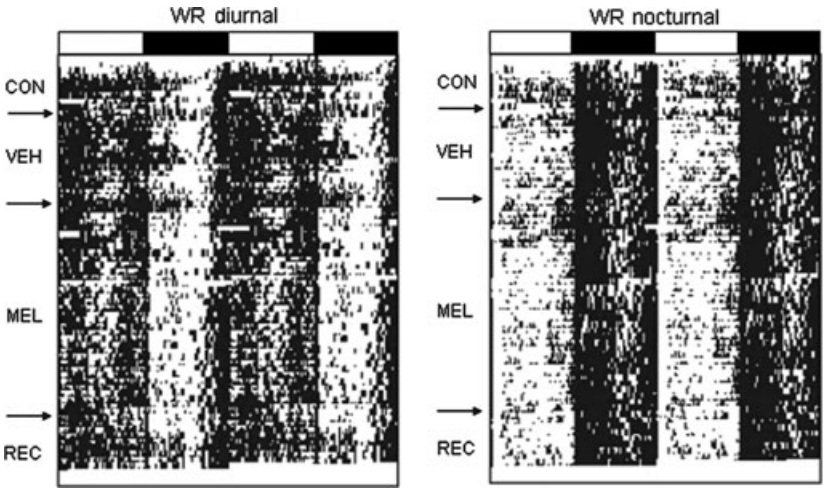

Fig. 3. Superimposed wheel running activity (WR) actogram from eight diurnal and five nocturnal degus exposed to LD 12:12 and subjected to four experimental stages. CON (control stage) animals received drinking water *ad libitum*; VEH (vehicle stage) animals received drinking water with 0.01% ethanol, which was made available for 2 hr before darkness; MEL (melatonin stage) animals received drinking water with melatonin added to reach a daily intake of 0.5mg/kg b.wt.; REC (recovery stage) animals received drinking water *ad libitum*.

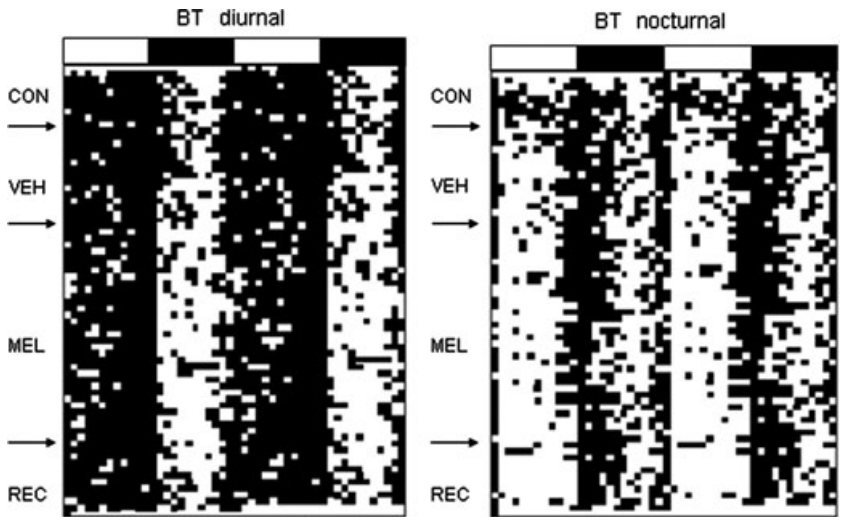

Fig. 4. Superimposed body temperature (BT) actogram from eight diurnal and five nocturnal degus exposed to LD 12:12 and subjected to four experimental stages (see Figure 3 for details).

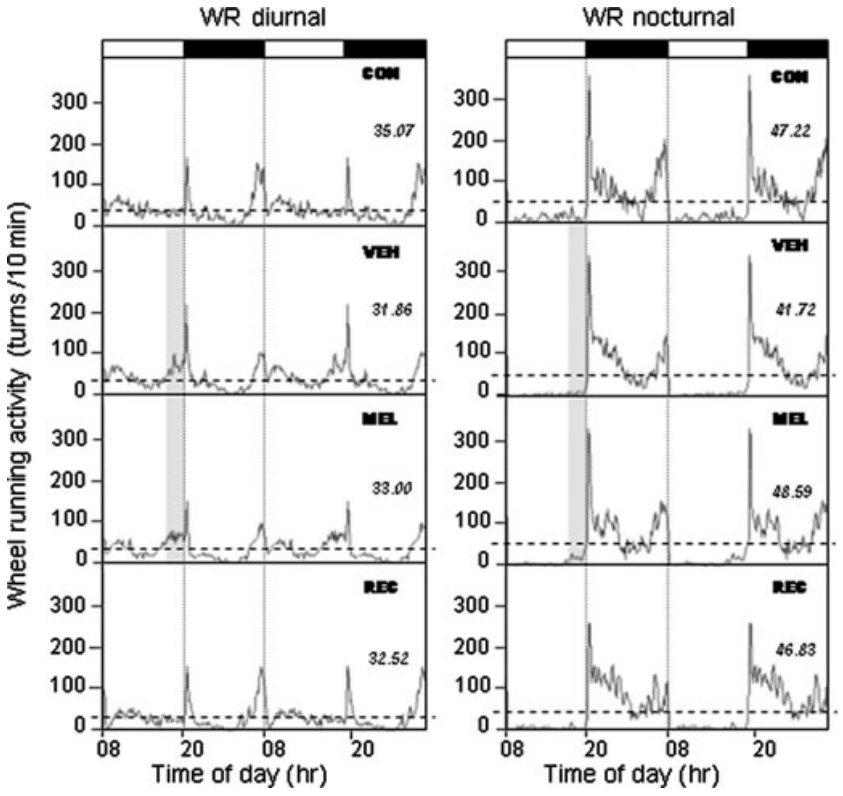

Fig. 5. Mean wheel running (WR) waveforms from diurnal (n = 8) and nocturnal (n = 5) degus in four experimental stages. Mean waveforms were calculated from individual waveforms averaged over the entire experimental period (except for the VEH stage, in which the first week was discarded). The dotted line and the numbers above it indicate the 24-hr mean. The gray vertical bars on the VEH and MEL graphs indicate the 2-hr period of drinking water availability (containing vehicle and melatonin, respectively).

specific effect induced by melatonin with respect to vehicle administration is shown in detail in Figs 7 and 8. As can be observed, BT was more affected than wheel running activity by melatonin administration. Exogenous melatonin produced a general reduction of BT in both chronotypes, with respect to the vehicle (Table 1), as demonstrated by the maximum, minimum and 24-hr mean values for BT. Furthermore, it would seem that the reduction was particularly marked in the case of nocturnal *degus*. Therefore, to establish whether BT reduction was particularly effective depending on the time of the day, nocturnal and diurnal means (Fig. 7) and 2-hr means (Fig. 8) were calculated. Our data show that nocturnal animals presented a more marked reduction in both photophase and scotophase temperatures (Fig. 7), but no particular sensitivity related to the time of the day or chronotype was detected (Fig. 8). Melatonin did not induce any significant effects on the overall WR values during the day or night in either chronotype.

As one of the main effects of melatonin is its ability to improve circadian system synchronization, a statistical analysis of the *r*-Rayleigh index, considered a measure of rhythm phase stability, was used to determine the degree of phase homogeneity during each experimental phase. The ***r*** vector can oscillate between 0 (maximum variability) and 1 (complete stability). We found that the ***r*** vector was higher for WR activity in nocturnal animals than it was in diurnal

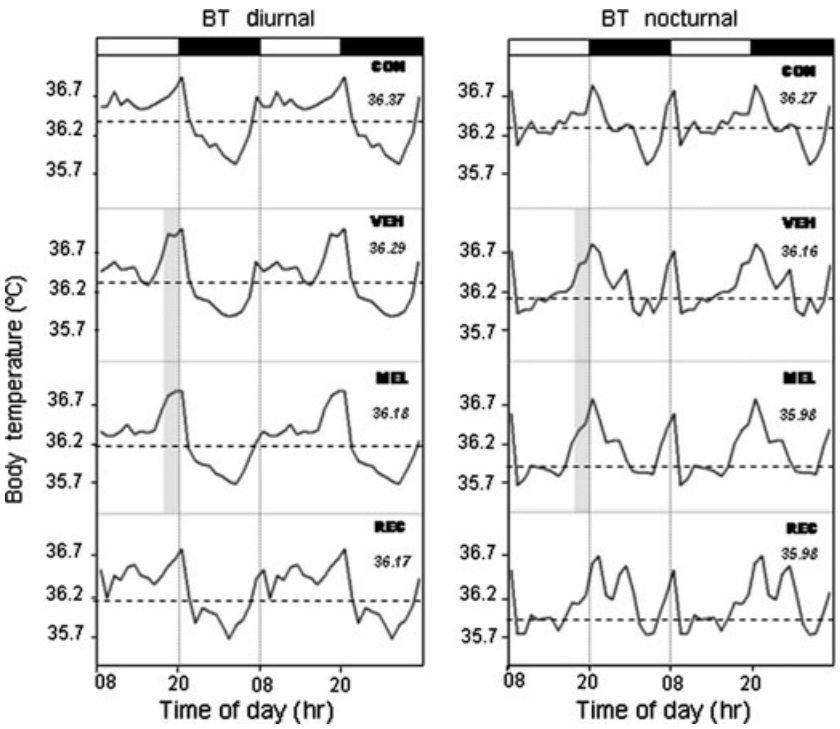

Fig. 6. Mean body temperature (BT) waveforms from diurnal (n = 8) and nocturnal (n = 5) degus in four experimental stages. Mean waveforms were calculated from individual waveforms averaged over the entire experimental period (except for the VEH stage, in which the first week was discarded). The dotted line and the numbers above it indicate the 24-hr mean. The gray vertical bars on the VEH and MEL graphs indicate the 2-hr period of drinking water availability (containing vehicle and melatonin, respectively).

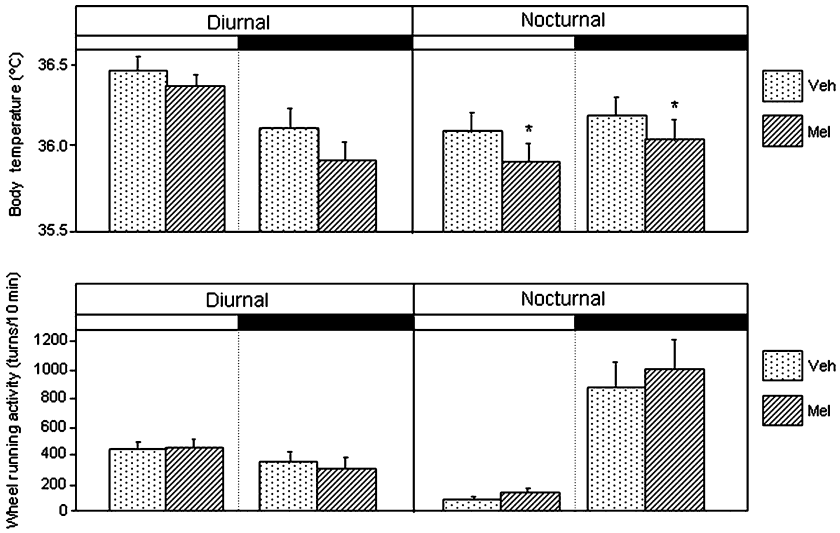

Fig. 7. Effect of exogenous melatonin on the diurnal/nocturnal relationship of wheel running and body temperature in diurnal (n = 8) and nocturnal (n = 5) degus. Values are expressed as the 12 h -mean (±S.E.M), calculated from individual waveforms averaged over the entire experimental stage (except for the VEH stage, in which the first week was discarded). Statistically significant differences between melatonin and vehicle (paired *t*-test) are marked with an asterisk ($P < 0.05$). The horizontal white and black bar in the upper part of graph represents means obtained during the 12 light and 12 dark hours, respectively.

animals ($r = 0.95$ versus 0.62, respectively). Vehicle and melatonin tended to improve ($P = 0.07$) interdaily stability in diurnal animals, but not in nocturnal ones (Table 1). With regard to temperature rhythms, diurnal animals displayed better stability than nocturnal animals in the control phase ($r = 0.88$ versus 0.63, respectively, $P < 0.05$), and only nocturnal animals improved their stability index after melatonin treatment when compared with CON ($P < 0.05$).

Interestingly, melatonin effects on WR and BT phase stability persisted for some days after the end of the treatment.

Because the aged *degus* used in this experiment did not exhibit clear chronotypes, the results for this experimental group were processed together. As can be seen in the mean waveform of Fig. 9, WR activity is reduced when compared with adult animals (Fig. 5, note the difference in the scale), and these animals did not exhibit a clear circadian pattern. Circadian rhythmicity was improved in the VEH, and it was potentiated by melatonin administration; however, no significant differences in mean or maximum WR were observed after melatonin administration.

A significant improvement in circadian rhythmicity was observed for BT during the vehicle and MELs, with these effects persisting even after the end of melatonin treatment (Fig. 9). Melatonin administration caused a statistically significant decrease in the mean BT, although the observed reduction in the maximum and minimum BT was not statistically significant (Table 2).

The synchronizing effects of melatonin on the BT rhythm were more evident after calculating the ***r*** vector. Melatonin

Table 1. Locomotor and temperature circadian parameters in the four experimental stages for diurnal and nocturnal chronotypes

	Control	Vehicle	Melatonin	Recovery
Mean locomotor activity	35.07 ± 6.00	31.86 ± 4.94	33.00 ± 5.76	32.52 ± 6.19
	47.22 ± 7.59	41.72 ± 6.25	48.59 ± 7.28	46.83 ± 7.82
Max. locomotor activity	480.50 ± 33.48	458.37 ± 38.83	461.37 ± 30.85	436.75 ± 30.87
	531.60 ± 42.35	526.80 ± 49.12	500.60 ± 39.02	473.80 ± 39.06
Mean body temperature (BT)	36.37 ± 0.08[a]	36.29 ± 0.09[ab]	36.18 ± 0.09[ab]	36.17 ± 0.09[b]
	36.27 ± 0.10[a]	36.16 ± 0.11[ab]	35.98 ± 0.11[b]	35.99 ± 0.11[ab]
Min. BT	35.04 ± 0.15	35.06 ± 0.13	34.82 ± 0.18	34.77 ± 0.18
	34.88 ± 0.19[ab]	34.98 ± 0.17[a]	34.38 ± 0.22[ab]	34.30 ± 0.23[b]
Max. BT	37.92 ± 0.11	37.85 ± 0.14	37.68 ± 0.10	37.85 ± 0.13
	38.02 ± 0.14[a]	37.86 ± 0.18[ab]	37.60 ± 0.13[b]	37.76 ± 0.16[ab]
Amplitude BT	2.88 ± 0.20	2.79 ± 0.16	2.85 ± 0.14	3.07 ± 0.16
	3.14 ± 0.25	2.88 ± 0.20	3.22 ± 0.18	3.46 ± 0.21
Rayleigh locomotor activity	0.62 ± 0.07	0.85 ± 0.03	0.85 ± 0.07	0.81 ± 0.07
	0.95 ± 0.09*	0.96 ± 0.04	0.88 ± 0.08	0.93 ± 0.08
Rayleigh BT	0.88 ± 0.06	0.79 ± 0.09	0.91 ± 0.03	0.90 ± 0.03
	0.63 ± 0.07*[a]	0.76 ± 0.11[ab]	0.81 ± 0.03[b]	0.83 ± 0.04[ab]

[a]Mean ± S.E.M of locomotor and temperature circadian parameters in the four experimental stages for diurnal (upper values, white background) and nocturnal chronotypes (lower values, gray background).
[b]Different letters in the same row indicate statistically significant differences among experimental phases (ANOVA, $P < 0.05$). Asterisks indicate statistically significant differences between nocturnal and diurnal chronotypes in the same experimental phase ($P < 0.05$).

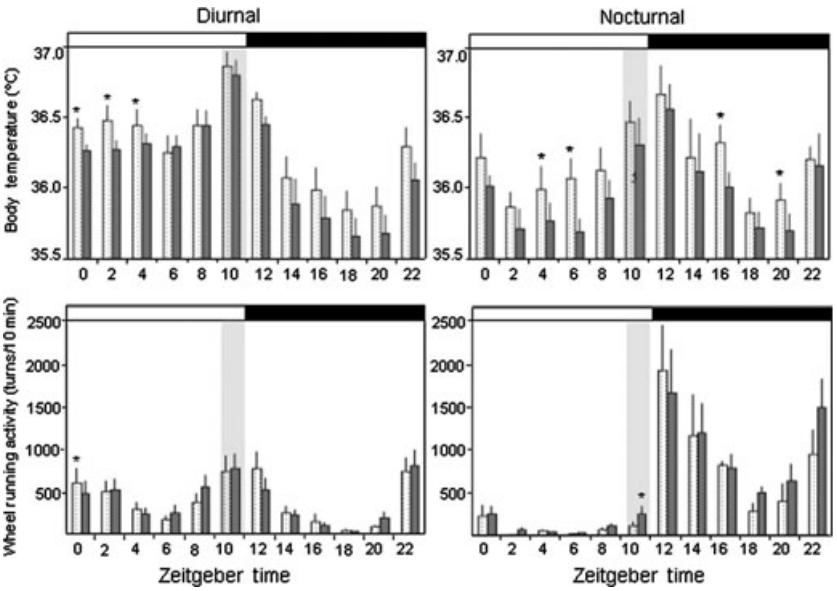

Fig. 8. Effect of exogenous melatonin (gray bars) versus vehicle (dotted bar) on the diel pattern of wheel running and body temperature in diurnal (n = 8) and nocturnal (n = 5) degus. Values correspond to 2 hr mean (±S.E.M) calculated from individual waveforms averaged over the entire experimental stage (except for the VEH stage, in which the first week was discarded). Statistically significant differences (paired *t*-test) between melatonin and vehicle are marked with an astersik ($P < 0.05$). The vertical pale gray bar indicates 2-hr period of drinking water availability, containing the vehicle or melatonin. The horizontal white and black bar in the upper part of the graph represents the LD 12:12 photoperiod.

improved interdaily stability (0.77 vehicle and 0.93 melatonin in aged animals, versus 0.78 vehicle and 0.87 melatonin in adults, $P < 0.05$), synchronization, which persisted for a number of days after the melatonin treatment.

Discussion

The present study was performed to examine whether differences in responses to endogenous and exogenous melatonin might be a key factor for defining diurnality in a dual rodent species (*O. degus)*. In addition, the synchronizing effects of melatonin in aged animals were also analyzed.

Our data on adult animals indicate that the degus circadian rhythm phase is not defined by the plasma level of nocturnal melatonin, nor is exogenous melatonin able to induce differential responses in terms of diurnal–nocturnal chronotype changes. This indoleamine had similar effects on both chronotypes, including a generalized hypothermic effect that was slightly more marked in nocturnal animals, and increased interdaily stability of the circadian phase of BT for nocturnal animals and WR for diurnal ones. Oral administration of melatonin to aged animals improved their circadian rhythmicity, both in locomotor activity and BT, with these effects persisting for a number of days after stopping the treatment.

Although there are many papers dedicated to clarifying the molecular mechanisms underlying endogenous oscillators, little is known about what defines diurnality or nocturnality. From a theoretical point of view, diurnalism–

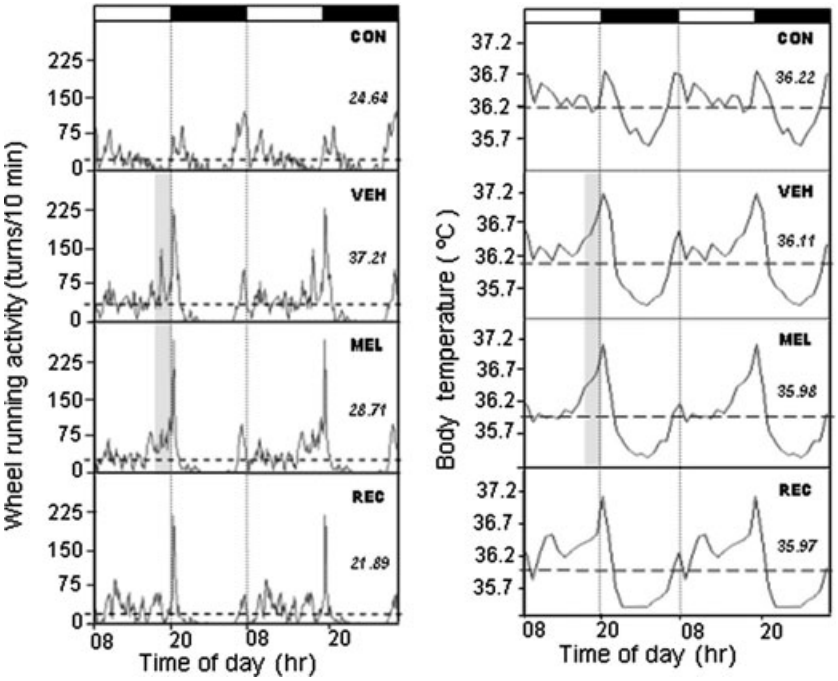

Fig. 9. Mean wheel running (WR, left panel) and body temperature (BT, right panel) waveforms from aged degus (n = 4, 57–60 months old) in the four experimental stages. mean waveforms were calculated from individual waveforms averaged over the enitre experimental period (except for the VEH stage, in which the first week was discarded). The dotted line and the numbers above it indicate the 24-hr mean. The gray vertical bars on the VEH and MEL graphs indicate the 2-hr period of drinking water availability (containing vehicle and melatonin, respectively).

Table 2. Loco motor and temperature circadian parameters in the four experimental stages for aged and adult animals

	Control	Vehicle	Melatonii	Recovery
Mean locomotor activity	24.64 ± 11.37	37.21 ± 9.94	28.71 ± 10.33	21.89 ± 10.58
	39.74 ± 5.46	35.65 ± 4.77	38.99 ± 4.96	38.03 ± 5.08
Max. locomotor activity	414.33 ± 59.37	391.33 ± 65.50	367.83 ± 55.51	340.00 ± 57.19
	500.15 ± 28.52	484.69 ± 31.46	476.46 ± 26.67	451.00 ± 27.47
Means body temperature (BT)	36.22 ± 0.14	36.11 ± 0.16	35.98 ± 0.17	35.97 ± 0.15
	36.34 ± 0.07[a]	36.24 ± 0.08[a]	36.11 ± 0.08[b]	36.10 ± 0.07[b]
Min. BT	34.77 ± 0.23	34.67 ± 0.21	34.68 ± 0.30	34.47 ± 0.30
	34.98 ± 0.11[ab]	35.03 ± 0.10[a]	34.65 ± 0.14[b]	34.59 ± 0.14[b]
Max. BT	37.60 ± 0.23	37.90 ± 0.23	37.72 ± 0.20	37.80 ± 0.20
	37.96 ± 0.11[a]	37.85 ± 0.11[a]	37.65 ± 0.10[b]	37.81 ± 0.09[ab]
Amplitude BT	2.83 ± 0.34	3.23 ± 0.23	3.03 ± 0.23	3.33 ± 0.26
	2.98 ± 0.16	2.82 ± 0.11	3.00 ± 0.11	3.22 ± 0.12
Rayleigh locomotors activity	0.71 ± 0.14	0.78 ± 0.09	0.91 ± 0.10	0.76 ± 0.11
	0.75 ± 0.07	0.89 ± 0.04	0.86 ± 0.05	0.86 ± 0.05
Rayleigh BT	0.63 ± 0.14	0.77 ± 0.15	0.93 ± 0.05	0.95 ± 0.05
	0.79 ± 0.07	0.78 ± 0.07	0.87 ± 0.02	0.88 ± 0.02

[a]Mean ± S.E.M of locomotor and temperature circadian parameters in the four experimental stages for aged (upper values, white background) and adult animals (lower values, gray background).
[b]Different letters in the same row indicate statistically significant differences among experimental phases (ANOVA, $P < 0.05$). Asterisks indicate statistically significant differences between aged and adult animals in the same experimental phase (ANOVA, $P < 0.05$).

nocturnalism duality might be the result of processes at three possible levels. (a) upstream from the clock, in the light input coupling with the SCN neurons; (b) in the clock itself, that is, in the SCN's functional mechanism, or (c) downstream from the clock, in the neural or humoral output connecting the SCN to the physiological or behavioral rhythmic processes. With certain exceptions [4], based on the similarities in the SCN functionality between diurnal and nocturnal animals, it is generally accepted that mechanisms downstream from the SCN specify nocturnal or diurnal behavior. In this context, studies on animals that switch from diurnal to nocturnal behavior are of special interest. *Degus*, for example, is a Chilean rodent that, although generally considered as a diurnal or crepuscular animal in the wild [21] and in the laboratory [22], some animals invert their phase preference from day to night when provided with nonrestricted wheel running [5]. Both diurnal and nocturnal chronotypes share a common crepuscular pattern, with a peak of activity after the onset of darkness and a second peak just before the onset of light. As a result, while diurnal animals run between these two peaks in the photophase, nocturnal degus run only during the scotophase. This dual pattern is particularly clear for wheel running activity, but not for BT, as a less defined

diurnal–nocturnal difference was observed. Inexplicably, nocturnal animals which ran more than 96% of their total wheel running activity during the night, only exhibited a difference of 0.1°C between their nocturnal and diurnal mean temperatures, while this difference reaches 0.3°C in diurnal animals. Thermoregulatory constraints have been suggested as one of the adaptive reasons for the existence of chronotypes [7].

The chronotypes observed were not the result of different plasma melatonin profiles, as no differences were detected in MD or ML melatonin levels between nocturnal and diurnal chronotypes, as occurs with strictly diurnal and nocturnal species. In both cases, melatonin peaked during the night, regardless of their activity pattern [13]. Plasma melatonin values and nocturnal–diurnal differences in *degus* are similar to those described for other mammals. However, to our knowledge, this is the first time that this hormone has been examined in this species.

If, as our data show, nocturnal and diurnal animals display similar plasma melatonin levels, we might ask ourselves whether this animal's physiological response to exogenous melatonin could still be equal in terms of chronotypes. Opposite effects of exogenous melatonin on sleep propensity, locomotor activity and BT have been reported in both diurnal and nocturnal species [23]; however, some discrepancies to this general trend have also been published [14]. These discrepancies could be due to the use of different doses, timing, administration methods or species-related responses to melatonin. Therefore, the use of this animal model allows us to examine, for the first time, the influence of melatonin on two different chronotypes of the same species, eliminating the above-mentioned interfering factors.

Oral administration of melatonin in drinking water was selected for our experiments, to reduce the stress associated with other procedures, such as i.p. injection. Similar timing for melatonin administration, regardless of the chronotype, was achieved by scheduling water availability. Two hours of water availability before the onset of darkness was enough to allow this species to ingest all the water it needed for the 24-hr period, without inducing any water or indirect food restriction compared with *ad libitum* water availability. The special water conservation adaptation of degus, who live in semiarid environments, probably explains this tolerance to narrow water availability. In any case, our data indicate that water restriction induces some improvement in synchronization, as measured by the r vector of the Rayleigh z-test, over the control period, at least in those animals with a lower Rayleigh value. Water as well as food restriction has been described as a synchronizing agent in other mammals [24].

Melatonin administration did not significantly modify the averaged wheel running activity, nor the maximum WR in either of the chronotypes, when compared with vehicle. This lack of response indicates that a differential sensitivity to melatonin of those neural centers that regulate locomotor activity is not the reason for diurnalism in this species. It has been reported that the SCN is a target site for melatonin effects on locomotor activity [25], with these effects being mediated by melatonin high-affinity receptors [26, 27].

A hypothermic effect was observed in both *degus* chronotypes during melatonin treatment. The reduction of BT was observed in the 24-hr mean, maximum, minimum and day and night BT mean. The hypothermia was more pronounced in the nocturnal (0.6°C) than in the diurnal chronotype (0.24°C) (Table 1). A similar BT reduction was observed in humans after melatonin intake [28], while the opposite occurs in a nocturnal species like the rat, i.e. melatonin administration during the night increases BT [29]. Thus, nocturnal chronotype degus respond to exogenous melatonin as a diurnal animal, whereas the diurnal chronotype is less sensitive to the hypothermic effects of melatonin. As this hypothermia is not accompanied by a parallel reduction in locomotor activity, it would seem to be a direct effect of melatonin on thermoregulation.

The role of melatonin as an internal Zeitgeber for the circadian system justifies its use as a chronobiotic. Our results show that melatonin administration increases interdaily phase stability of circadian rhythms. These effects are particularly evident in those chronotypes and stages which exhibit reduced interdaily stability before melatonin treatment. In addition, a slight phase advance in WR and BT rhythms was observed during melatonin administration compared with vehicle. In both nocturnal and diurnal species, the sensitivity window for exogenous melatonin is located near the activity/rest transitions, and the entrainment capacity of exogenous melatonin is similar to those of other nonphotic stimuli [30]. In humans, the PRC to melatonin exhibits phase advances when melatonin is administered late in the subjective day, and phase delay when the indole is administered late in the subjective night [31].

Locomotor activity and BT rhythms can be considered as marker rhythms of the circadian system [32]. Throughout the aging process, changes in the circadian rhythmicity of a variety of functions have been found in animals and humans [33]. Among these changes, a decreased activity-to-rest ratio [34], fragmentation of the activity rhythm [35, 36], a longer free-running period [37, 38], internal desynchronization and interdaily instability [33] are common alterations in aged animals.

Oral melatonin improves circadian rhythmicity in old degus, particularly through an increase in interdaily phase stability of WR and BT rhythms. In addition, hypothermia was also observed after melatonin treatment in old *degus*, but what is more important, melatonin effects persisted for at least 10 days after the end of the treatment. These results suggest that melatonin can be useful to restore some characteristics of an impaired circadian system in aged animals. In addition, and as no rebound effect was observed after melatonin administration period, it seems that no habituation and/or reduced endogenous melatonin production is induced by long-term exogenous melatonin treatment. This result is consistent with the seeming lack of a negative feedback between plasma melatonin levels and pineal synthesis and/or release of melatonin.

Based on our results, melatonin does not seem to be involved in the mechanisms implicated in diurnal/nocturnal shifts downstream from the central pacemaker, as no qualitative differences were observed in nocturnal and diurnal chronotypes, either in their plasma melatonin levels or in their response to exogenous melatonin. Both chrono-

types react similarly to melatonin treatment, with no differential changes in locomotor activity, interdaily phase stability, or hypothermia, although this last condition was more pronounced in the nocturnal chronotype. Our data further suggest that melatonin acts as an effective chronobiotic in old animals, improving some characteristics of the rhythmicity impaired by aging in the circadian system.

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Experimental Chapter 7

Title: "Temporal expression of arginine vasopressin, vasoactive intestinal peptide and Fos protein in the hypothalamus of diurnal and nocturnal *Octodon degus*"

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ABSTRACT

Diurnal and nocturnal animals are easily differentiated by choosing opposite temporal niches; however, the neural mechanisms involved in that phase preference are still unknown. To date, experimental findings point out that the pacemaker function is the same in both chronotypes. Dual phasing species, as the Chilean *Octodon degus*, are useful tools for understanding basic mechanism for the predilection of the individual activity phase. Although the degus' nocturnalism is very influenced by masking effects, both diurnal and nocturnal steady-entrainment phases have been demonstrated.

The aim of this work was to characterize and investigate differences in the temporal expression of, arginine-vasopressin (AVP), vasoactive-intestinal polypeptide (VIP) and c-Fos in the suprachiasmatic nuclei of diurnal and nocturnal degus. Results show that VIP and AVP immunorreactivity displayed similar values during the day and night; however, the number of Fos-IR cells was higher during the day than during the night in the SCN. Neither, of these variables differed among nocturnal and diurnal chronotype, indicating that degu's chronotype differentiation could be generated outside the SCN.

KEYWORDS: Arginine vasopressin (AVP), Vasoactive intestinal polypeptide (VIP), Fos protein, Suprachiasmatic nucleus (SCN), Paraventricular nucleus (PVN), nocturnalism, *Octodon degus*

INTRODUCTION

Animals display a wide expression of temporal niche preferences which researchers have classified into diurnal, nocturnal and crepuscular. The neural mechanisms involved in that activity phase preference are still unknown.

Initially, it was thought that the phase difference could be generated by a differential function of the central pacemaker located in the mammalian suprachiasmatic nuclei (SCN) of the hypothalamus (Moore & Eichler, 1972; Stephan & Zucker, 1972). Neuronal firing rate (Kubota et al., 1981; Sato & Kawamura, 1984) and SCN metabolic activity (Schwartz et al., 1983) peak at the same time in both diurnal and nocturnal animals. However, some differences in the immediate-early gene c-Fos expression have been found (Krajnak et al., 1997). Furthermore, neurons within and outside the SCN present a response differently to a direct light stimulus (Jiao & Rusak, 2003).

The pacemaker has been traditionally divided into two main anatomical-functional regions, the dorsomedial region ("shell") and the ventrolateral region ("core"). The shell is characterized by a high expression of arginine vasopressin (AVP) neurons; and the core, mainly expresses vasoactive intestinal polypeptide (VIP) and the gastrine-releasing peptide (GRP) neurons (Sofroniew & Weindl, 1980; Stopa et al., 1988). The dorsomedial region receives projections from the ventrolateral SCN and communicates that information to the rest of the brain, such as the dorsomedial hypothalamus (DMH) and the paraventricular nucleus (PVN) of the hypothalamus (Reghunandanan & Reghunandanan, 2006).

While AVP expression in the shell is directly under molecular clock control in the central pacemaker (Jin et al., 1999), neuropeptide expression in the core is more dependent on external conditions, such us light conditions (Shinohara & Inouye, 1995). The core is considered as an integration centre of inputs which receives photic information directly from the retina by the retinohypothalamic tract (Johnson et al., 1988) and indirectly from the intergeniculate leaflet (IGL) [Harrington, 1997]. The IGL pathway is also involved in relaying non-photic information to the SCN (Mrosovsky, 1996). Moreover, a third important input to the SCN comes from the serotoninergic neurons from the dorsal raphe nuclei of the brainstem, and it has been related to photic and non-photic entrainment (Morin, 1999).

To date, no studies have reported major differences in the temporal expression of the main neuropeptides in the pacemaker between diurnal and nocturnal species (Dardente et al., 2004; Mahoney et al., 2009b). Therefore, it has been hypothesized that activity phase control might be located upstream or downstream from the SCN. Although many studies suggest that the phase preference is controlled downstream from the pacemaker (Mrosovsky, 2003), in few rodents, an upstream control has been described (Oster et al., 2002; Mrosovsky & Hattar, 2005).

To better understand the mechanisms involved in the temporal niche preference dual species are useful tools. *Octodon degus* is a Chilean caviomorph rodent characterized mainly as diurnal (Lee, 2004). However, under specific conditions, such as wheel running availability (Kas & Edgar, 1999), nocturnal feeding schedule (Vivanco et al., 2010a) or high diurnal ambient temperature (Vivanco et al., 2010b), some individuals shift from a diurnal to a nocturnal phase. Although degus' nocturnalism is very influenced by masking effects (Kas & Edgar, 1999), both diurnal and nocturnal steady-entrainment phases have been demonstrated (Vivanco et al., 2009).

The aim of this work was to characterize the possible differences in the temporal expression of AVP, VIP, and Fos in the SCN of diurnal and nocturnal degus.

MATERIAL AND METHODS

Thirty-nine male *Octodon degus* 24-36 months of age were obtained from the Animal Facilities at the University of Alicante (Spain). The animals were individually housed in polycarbonate cages (Panlab, S.L.) equipped with wheels (52 x 15 x 27 cm, L x H x W) in an isolated room ("Chronolab") with controlled humidity (60%), temperature (27.4 ± 0.5 ºC) and photoperiod (LD 12:12). Light was provided by two lateral fluorescent lamps controlled by an electronic timer (Data Micro, Orbis), with an intensity of 350-400 lux at cage level. The degus were fed *ad libitum* with commercial rat chow (A04 rat-mouse maintenance Panlab). All experimental procedures were performed in accordance with the "Principles of Animal Care" (Portaluppi et al., 2008; NIH publication No. 86-23, revised 1985) and Spanish laws.

Data Recording

Wheel running activity (WRA) was recorded as wheel turns per 10 min interval using a data acquisition system (Electronic Service at the University of Murcia, Spain). Body temperature (T_b) was measured at 60 min intervals using a miniature data logger (ThermoChron®, Data loggers iButton, Maxim Integrated Products, Sunnyvale, California, USA) with an accuracy of 0.1ºC. For this purpose, sterilized data loggers were implanted intraperitoneally, under aseptic conditions, using fluothane as anaesthesia (Forane®, Abbot Laboratories S.A., Madrid, Spain). Reabsorbable silk material (2/0, Safil®Quick B/Braun, Barcelona) was used to suture the abdominal layers, and non-reabsorbable silk was used to suture the skin. No mortality or morbidity was observed after the surgery. The experiment started after a two-week recovery period. Data logger was removed when animals were sacrificed. IButton readout hardware was used to transfer temperature data to a computer.

Experimental Design

Degus were individually housed with wheel running availability under LD 12:12. In order to characterize each individual chronotype, degus were subjected to a DD cycle for 5 days and returning to the previous LD cycle for 7 days. Then, degus were sacrificed at one hour after lights on (*Zeitgeber Time* - ZT 1), mid-light (ML, ZT6), one hour after lights off (ZT13), and finally at mid-dark (MD, ZT18). Nocturnal animals were only sacrificed at ML and ML time points.

Brain tissue collection and immunohistochemistry procedure

Animals were deeply anesthetized by fluothane and intracardially perfused with 250mL of 0.9% saline, and then with 500mL of 4% paraformaldehyde solution in 0.1M phosphate buffer pH 7.4. Each brain was carefully removed and post fixed in 4% paraformaldehyde solution for another 24 hours. Then, they were stored in sucrose 30% in 0.1M phosphate buffer pH 7.4 at 4ºC for 24h. Finally, brains were stored at -80 ºC till its analysis.

Brain sections containing SCN or PVN were cut at 40µm in the coronal plane by using a sledge microtome (Bright Instruments Ltd., Huntingdon, UK). SCN or PVN consecutive sections were treated with the following primary antibodies: either Rabbit anti-AVP (dilution 1:15000, Fitzgerald Industries International Inc., Concord, MA, USA),

rabbit anti-VIP (dilution 1:2000, Biomol International, LP, Exeter, UK) or rabbit anti-Fos protein (dilution 1:5000, Santa Cruz Biotechnology Inc., CA, USA). Briefly, brain sections were washed in 0.1M phosphate buffer with the last one containing 0.1% Triton X. Then, they were incubated in 1.5% hydrogen peroxide for 30min followed by serial washes. Sections were transferred in 5% normal goat serum in 0.1 M phosphate buffer with 0.1% Triton X for 1h and finally, they were incubated with respective primary antibodies prepared in blocking solution overnight at 4ºC. Then, sections were rinsed and placed in goat anti-rabbit IgG biotinylated secondary antibody (1:400) for 90 min (Vector Laboratories, Inc., CA, USA). After several washes sections were incubated with avidin-biotin complex solution (Vector Laboratories). and visualized by a nickel solution as chromogen (DAB substrate kit; Vector Laboratories). Finally, sections were mounted onto gelatine-coated glass slides, dried, dehydrated and coverslipped.

In order to characterize the spatial distribution of the SCN in the hypothalamus, two degus brains, not from the experimental group, were collected following the same experimental conditions above explained, cut in coronal sections with a sledge microtome at 40µm and stained with Cresyl violet.

Inmunohistochemistry analysis

All sections were visualized with a Leica DM2000 digital photomicroscope (Leica Microsystems, Wetzlar, Germany), and high-resolution images were digitally captured and stored in a computer. In order to objectively quantify (normalized immunoreactive staining) the AVP and VIP expression by optical density, background staining was subtracted using a specialized image software (IMAGEJ, version 1.38 NIH, Bethesda, MD, USA). In the Figure 1A the SCN is outlined and background area is depicted.

Neither AVP nor VIP immunoreactivities (AVP-IR and VIP-IR, respectively) presented a differential expression in the anterior, medial or posterior part of the SCN, so a pool was performed in order to facilitate the measures. In the case of Fos protein and AVP expression in the PVN of the hypothalamus, the number of immunoreactive positive-cells for each antibody were blind counted by one independent researcher using high-resolution pictures and filtering the background, non-useful, information by light contrast, and an average value was obtained for each section. Examples of AVP-ir cells in the PVN and Fos immunoreactive (Fos-IR) neurons in the mid SCN are shown in Figs. 1B and 1C, respectively.

Chronotype Characterization and statistical Analysis

To characterize the chronotype of each degus, a numerical criterion based on the percentage of diurnal versus total activity was used. Thus, when an animal under LD conditions showed a diurnal/total activity ratio above 60%, it was considered diurnal. A nocturnal animal was defined as one whose diurnal/total activity ratio was less than 20%. Due to crepuscular rhythms and intermediate locomotor activity values, in six degus it was necessary a closer inspection of their WRA actogram for considering their final classification. Moreover, because the existence of degus with nocturnal steady-entrainment phase and individuals with nocturnalism generated by masking, we included the analysis of the entrainment phase angle observed in the first days under DD after a previous LD schedule (Vivanco et al., 2009).

WRA and T_b actograms were performed using software specifically designed for chronobiological analysis (El Temps, version 1.228; © Díez-Noguera, University of Barcelona).

A repeated measures ANOVA (p<0.05) was performed to compare the expression of AVP, VIP and Fos protein between diurnal and nocturnal animals at ML and MD. All statistical analyses were performed with SPSS 13.0 software.

RESULTS

Cresyl-violet stain indicated the shape and size of the suprachiasmatic nuclei in the anterior hypothalamus of a degus brain (Fig. 2). SCN is presented as a bilateral structure situated on the optic chiasm and physically separated by the third ventricle. In rostral sections its shape is horizontally elongated and its size is small. Mid SCN is vertically elongated and bigger than in rostral sections. Finally, SCN neurons project their axons to the paraventricular nucleus of the hypothalamus, so in caudal sections the SCN starts to disappear at the same time that it presents longer axonal projections to upper structures.

WRA and T_b actograms from a representative diurnal and a nocturnal degus are shown in Fig. 3. Diurnal animal limited its activity to the light phase whereas the nocturnal one was particularly active during dark phase. The temperature patterns were quite similar to the WRA rhythm in both chronotypes.

Based on the percentage of diurnal versus total activity in LD, 26 out of 39 exhibited predominantly diurnal patterns. Among nocturnal degus, the phase angle of entrainment from the first day under DD, allows differentiating 2 individuals with nocturnal steady-entrainment phase and 11 individuals with nocturnalism generated by masking. Individuals with nocturnal steady-entrainment phase and those generated by masking did not present significant differences in the posterior analysis of immunohistochemistry, so a pool of nocturnal degus was performed to facilitate the comparisons.

The AVP and VIP immunoreactive (IR) staining in the SCN did not show a circadian profile in diurnal degus (Fig. 4). No significant differences in AVP expression in the PVN were found across *Zeitgeber times* in those animals (Fig. 4). When AVP and VIP expression at ML and MD were compared between nocturnal and diurnal chronotypes, no differences were observed either in the SCN or in the PVN (Figs. 5 and 6).

However, the analysis of the number of Fos-IR neurons (Fig. 4) in the SCN, showed a circadian rhythmicity with higher levels during the light phase (ZT1 and ZT6) than during the dark one (ZT13 and ZT18). The number of Fos-IR cells in the SCN was significantly elevated at ML when compared to MD ($p<0.001$) in both, nocturnal and diurnal chronotypes without statistical differences between them (Fig. 6).

DISCUSSION

In the present study we found that the number of Fos-IR cells in the SCN was higher at midlight than at middark regardless of the nocturnal or diurnal chronotype. However, the number of AVP-IR and VIP-IR neurons in the SCN and PVN remained unchanged through the day in both chronotypes without differences between them.

Animals can be active during a wide variety of temporal niches; however, the neural mechanisms involved in the temporal preference have not been yet identified. The majority of studies have been performed by comparing diurnal and nocturnal species. In this sense, dual phasing species, such as the Nile grass rat, *Arvicanthis niloticus* (Rose et al., 1999), or the degu, *Octodon degus* (Vivanco et al., 2007), become as useful tools for understanding the neural basis of the phase control.

Among the different possibilities to locate the diurnalism or nocturnalism in an animal, the pacemaker has been the main focus of attention. However, it has been described that SCN is more active during the day in both nocturnal and diurnal species (Schwartz et al., 1983), and the clock machinery's expression profiles are also very similar in both species (Smale et al., 2008). One neuropeptide directly controlled by the oscillation of the clock is the AVP. It exhibits, in the SCN of most of the studied animals, a daily rhythmicity with high levels during the day, regardless diurnal or nocturnal chronotype (Dardente et al., 2004).

Our results in degus did not show significant differences in the number of AVP-IR cells neither throughout the day nor between chronotypes. In this sense, it has been documented in several nocturnal rodents, such as the common vole (*Microtus arvalis*), the relationship between the circadian organization and the amount of AVP-IR neurons in the SCN (Gerkema et al., 1994). Furthermore, in some strain of rats whose motor activity was higher and more concentrated during the night, it was found a higher amount of AVP-IR neurons than in those whose activity was more reduced and diffused (Wollnik & Bihler, 1996).

Although it have been demonstrated two steady phases of entrainment, diurnal and nocturnal, in the degus, there is a great influence of masking effects on the behavioural rhythms which generate a graded circadian chronotypes (Vivanco et al., 2009). However, the tentative circadian rhythm in AVP expression could be faded by averaging a heterogeneous population with high intra- and inter-individual variability in locomotor activity expression. Whatever the explanation, this fact discards AVP temporal signal in the SCN as a candidate to establish the phase preference of degus.

On the other hand, the light-induced c-Fos expression in SCN neurons provides useful information about the functional state of the central pacemaker by indicating the cellular transcription activity (Rea, 1989). Under LD cycles, Fos protein presents maximum values during the light phase and minimum at night in both diurnal and nocturnal rodents (Katona et al., 1998; Schwartz et al., 1994). Our results in degus are in agreement with those studies. However, we have found that the number of Fos-IR neurons peaked at mid-light instead of by the time of lights off as it has been previously described for this species (Mahoney et al., 2009a).

VIP and GRP are both the main neuropeptides located in the ventrolateral zone of the SCN. This region receives direct photic innervation from the retina. This light

information influences Fos and VIP expressions (Piggins & Cuttler, 2003). For this reason, it has been suggested that VIP release in the SCN could not be under a strong circadian control (Dardente et al., 2004). VIP expression profile has been described mainly in nocturnal species, such as mice and rats (Dardente et al., 2004; Okamoto et al., 1991); showing highest levels at night. However, there are other studies in which VIP oscillations were not found, as in the Syrian hamster (Duncan et al., 2001) or even in humans (Hoffman et al., 1996). In our animals, and as occurred for AVP, we could not find a rhythmic VIP-IR either in diurnal or in nocturnal degus, probably due to the strong light influence in this species.

Similar results have been reported in nocturnal and diurnal grass rats. In this species no differences in the number of AVP-IR and VIP-IR neurons in the SCN were found in those animals sacrificed during the day or during the night. However, Fos expression within those VIP and AVP neurons showed a circadian rhythmicity with higher values during the day than during the night (Rose et al., 1999; Smale et al., 2001). Likewise, we have found in the degus that the number of Fos-IR cells in the SCN is higher at midday than at midnight regardless of the nocturnal or diurnal chronotype.

VIP neurons have projections to AVP neurons located in the shell, and from here is spread the information to other brain areas outside the SCN, such as the lower subparaventricular zone (LSPV) of the hypothalamus, the dorsomedial hypothalamus (DMH) and the paraventricular nucleus (PVN) of the hypothalamus (Reghunandanan & Reghunandanan, 2006). In addition, VIP-IR neurons project directly to the same brain areas (Piggins & Cuttler, 2003). Therefore, if the pacemaker works similarly in diurnal and nocturnal animals, the phase control might be located upstream or downstream the clock. Although the downstream neural substrates are the candidates to harbour this phase switching, upstream mechanisms should not be discarded (Oster et al., 2002; Mrosovsky & Hattar, 2005).

PVN is a primary target of innervation from AVP-expressing dorsomedial SCN, and its activity is involved in the coordination of neuroendocrine and autonomic homeostatic responses (Swanson & Sawchenko, 1983). Since PVN is a relay centre for temporal signal directly spread by AVP-expressing neurons of the dorsomedial SCN to several organs, it could be a candidate to control the behavioural preference in animals. However, very few studies have been conducted in this sense (Smith & Canal, 2009). In degus, the amount of AVP-IR cells in PVN did no present differences ML-MD

nor between chronotypes. Therefore, PVN seems not to be the key of the phase control, at least in these animals.

Finally, SCN also transmits the temporal information to other structures by humoral mediators, such as melatonin, Prokineticin-2 or Tumoral Necrosis Factor-α. An interesting study demonstrated that tissues of SCN inside semipermeable capsules and transplanted into ablated-SCN hamsters could restore their lost circadian activity rhythms (Silver et al., 1996) suggesting that an humoral, non neural, signal was implicated. Still, and although the role of these humoral factors on activity phase control remains unclear, no differences in plasma melatonin levels have been found between nocturnal and diurnal degus (Vivanco et al., 2007),

In conclusion, although diurnal and nocturnal degus show clear temporal differences in their physiological and behavioural rhythms, no major differences could be found in the temporal pattern of AVP, VIP and Fos inmunoreactivity in the SCN or in AVP-IR in PVN. Thus, the temporal niche control should be located outside the SCN in one, or more, nearby neural structures and/or, may be dependent on some humoral factor.

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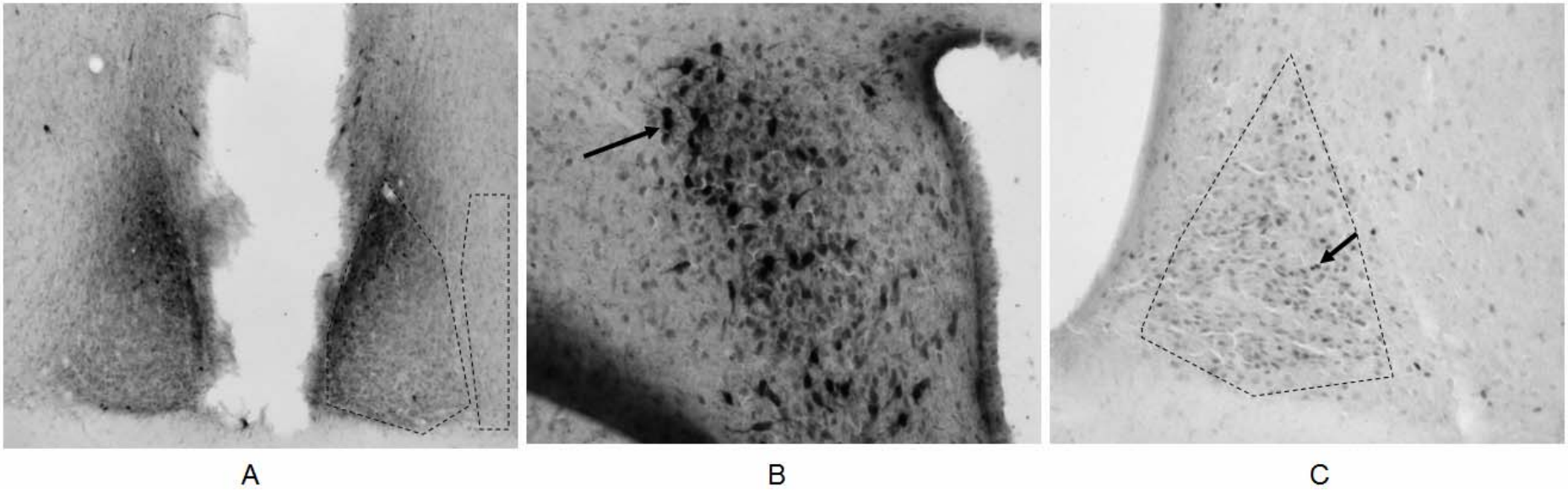

Figure 1. Immunohistochemistry quantification methodology. Photomicrographs of a section stained for AVP (A) showing the SCN and background area outlined for counting; (B) a section containing AVP-IR cells in the PVN and, (C), a section showing Fos-IR cells in the outlined SCN. Arrows indicate cells expressing AVP or Fos in (B) and (C) respectively.

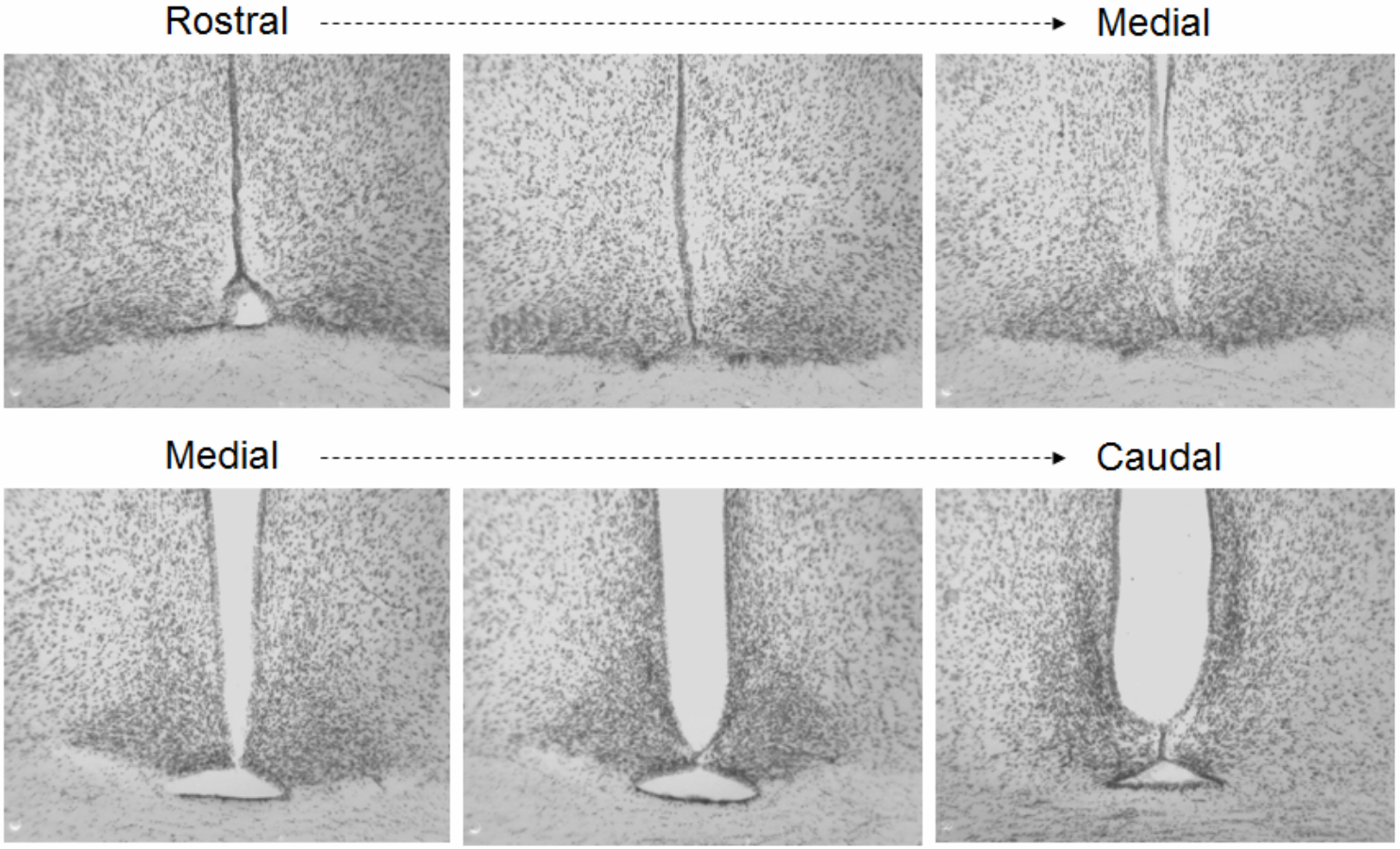

Figure 2. Photomicrographs showing coronal cresyl-violet stained sections (40µm) through the SNC (rostral, medial and caudal). Note the shape and size of the SCN.

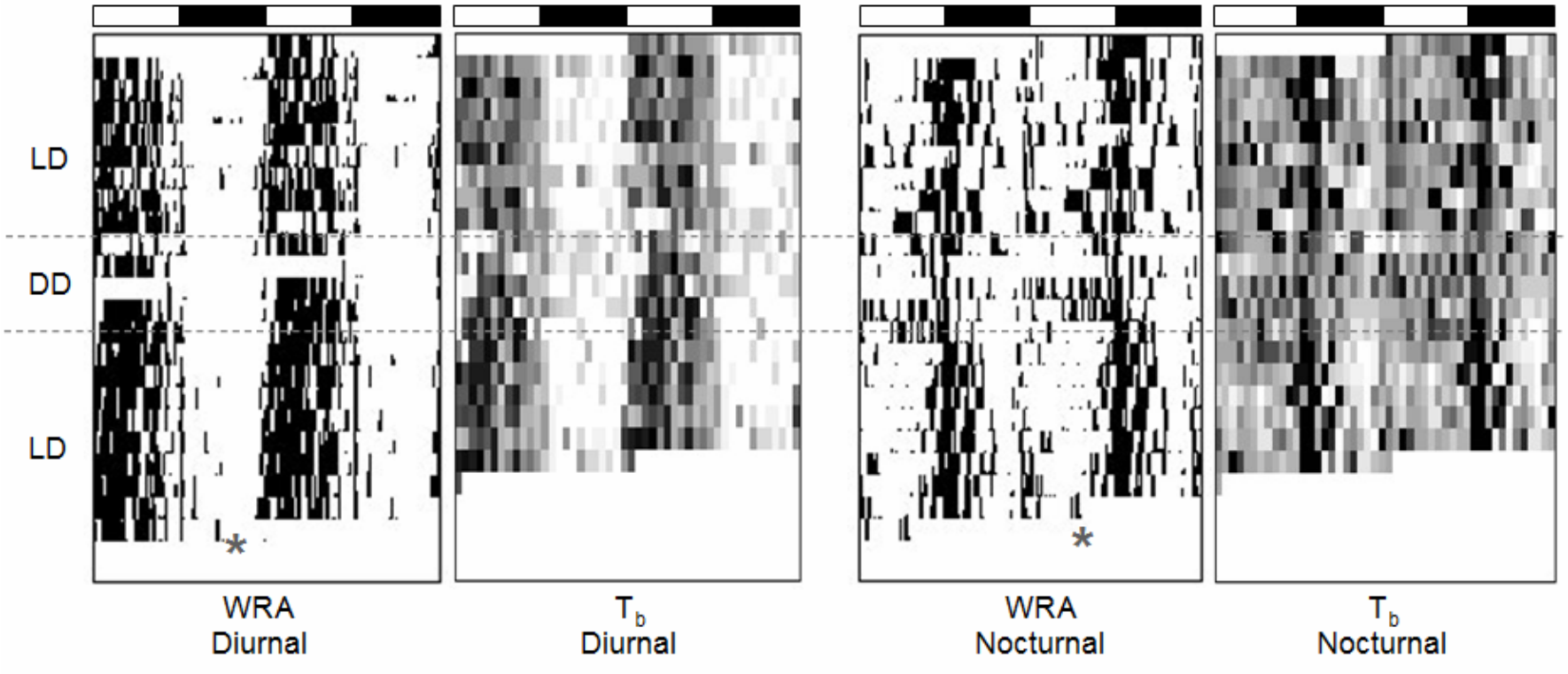

Figure 3. Double-plotted wheel running activity (WRA) and body temperature (T_b) actograms from a representative diurnal and nocturnal degus subjected to the different stages (LD 12:12, DD and LD 12:12 again). The asterisks on the WRA actograms indicate the moment of the animal's sacrifice.

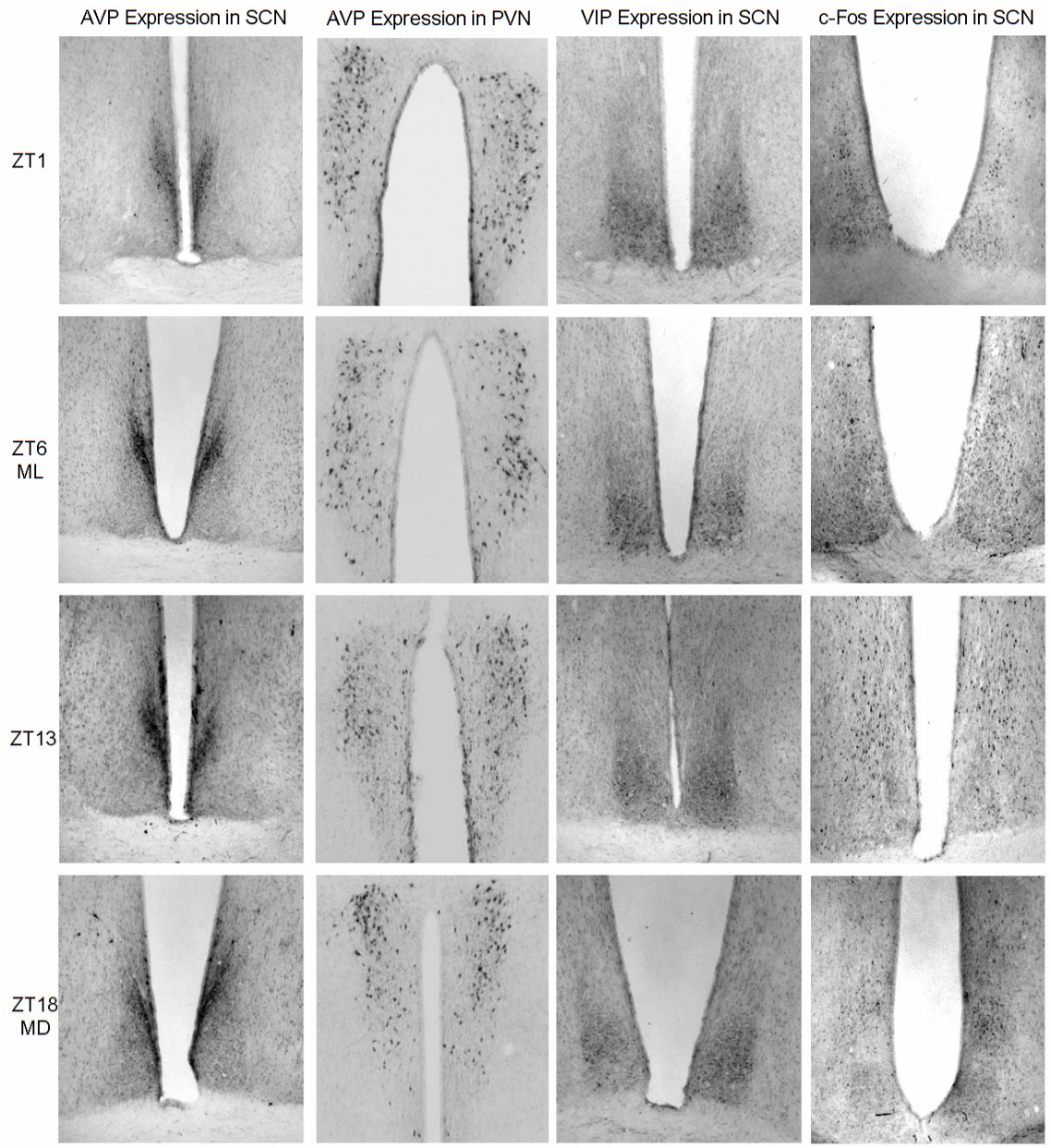

Figure 4. Representative photomicrographs illustrating AVP, VIP and Fos inmunoreactivity in the SCN and AVP in the PVN of the hypothalamus of diurnal degus at different *Zeitgeber Times* (ZT1, ZT6, ZT13 and ZT18).

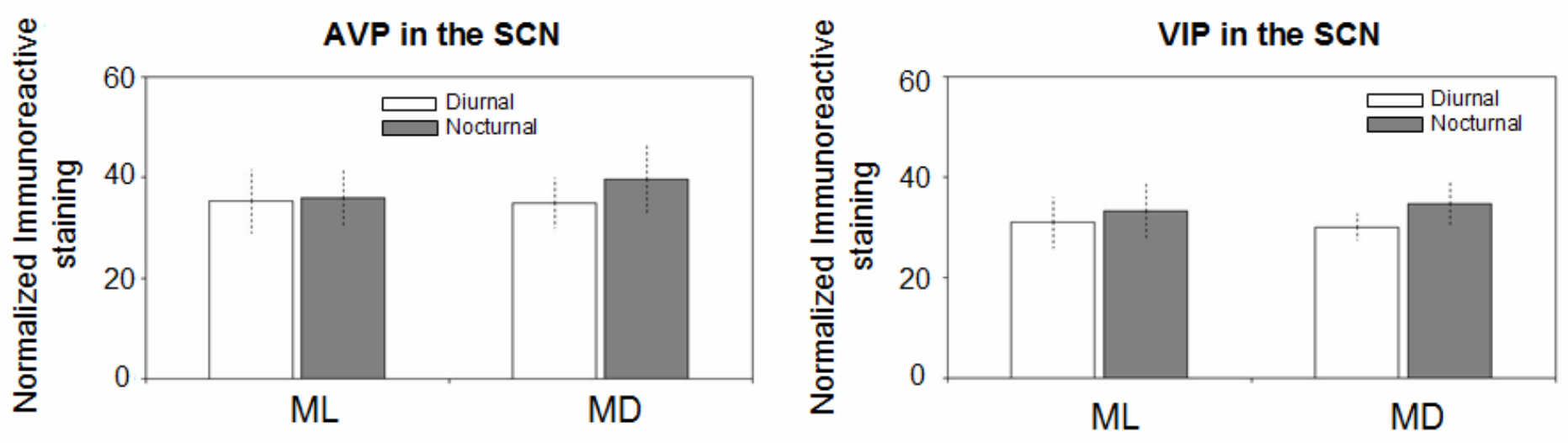

Figure 5. Normalized AVP (left panel) and VIP (right panel) immunoreactive staining at mid-light (ML) and mid-night (MD) in the SCN of diurnal and nocturnal degus. Values are expressed as mean ± S.E.M.

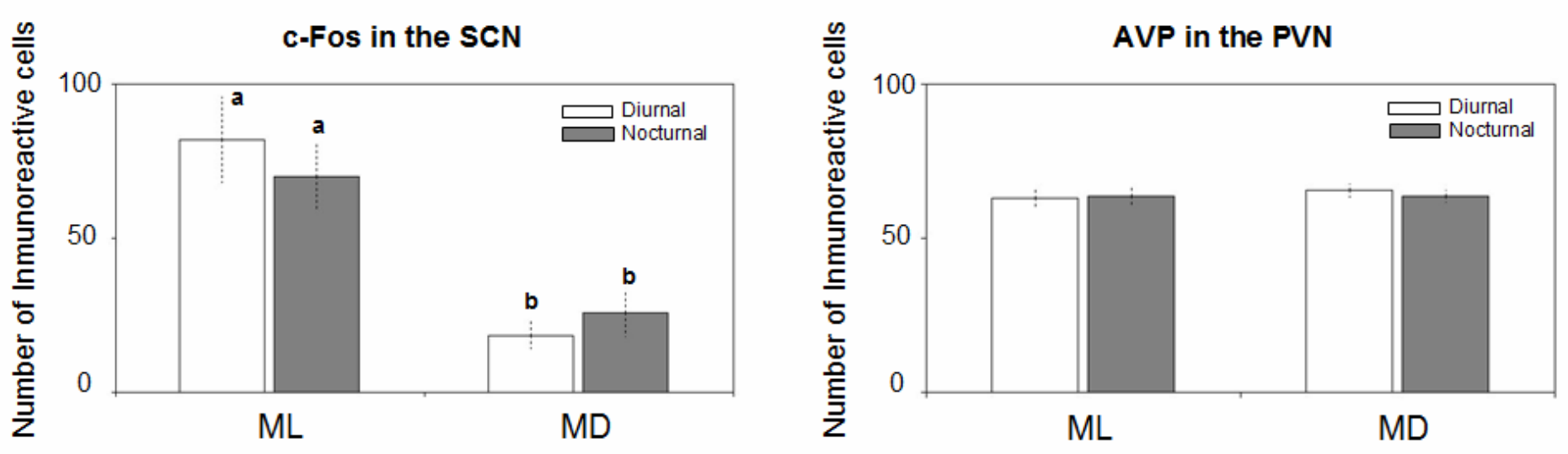

Figure 6. Number of Fos-IR cells in the SCN (left panel) and AVP-IR cells in the PVN (right panel) at mid-light (ML) and mid-dark (MD) in diurnal and nocturnal degus. Values are expressed as mean ± S.E.M. Different letters indicate statistically significant differences (ANOVA, $p<0.05$).

Discussion

The temporal niche selection reflects a circadian system adaptation, and specialization. Most animals display a well-defined phase relationship between their activities and the LD cycle, and therefore they can be classified as diurnal, nocturnal or crepuscular. In most species, nighttime or daytime behaviour is genetically determined, thus conditioning their circadian systems. This situation logically implies special sensory requirements, i.e. colour vision is necessary in diurnal animals in order to catch preys or avoid predators during the daytime (Osorio & Vorobyeb, 2008). However, a small number of animal species, including fish (Sánchez-Vázquez et al., 1995), migratory birds (Gwinner, 1996), and mammals (Mrosovsky, 2003), display a particular ability to shift their activity phase from diurnal to nocturnal, or *vice versa*, in few days. Between these dual phasing animals is the degus, *Octodon degus*, an endemic caviomorph rodent from Central Chile (Woods & Boraker, 1975).

From it first description in 1782 by Juan Ignacio Molina, the characterization of degus' circadian rhythmicity has been an arduous task due to a very high intra- and inter-individual behavioral variability. Early studies based on observations, both in nature and in captivity, considered degus to be a diurnal species, with morning and evening activity peaks (Fulk, 1976), or as a crepuscular-diurnal or crepuscular-nocturnal rodent (Iriarte et al., 1989). However, laboratory studies where the animals were able to exercise on a wheel running further increased the complexity of the issue. As a result, morning, evening, intermediate- (Labyak et al., 1997; García-Allegue et al., 1999), as well as predominantly nocturnal chronotype (Kas & Edgar, 1999) animals were additionally described. In fact, when a wheel running was available on their individual cage, some of the diurnal individuals shifted to a nocturnal phase in only few days. Due to the rapidity of the circadian changes, the main hypothesis for explaining the nocturnalism in degus consisted only in masking processes by light. This hypothesis has been accepted to date by the whole scientific community.

However, our group has demonstrated the existence of **two steady-entrainment phases**, diurnal and nocturnal, that combined with gradual masking by light effects explain the wide variability of chronotypes in this species (Vivanco et al., 2009; **Experimental Chapter 1**). In this way, considering the classical numerical criteria (such as the percentage of diurnal/total locomotor activity), together with the analysis of the entrainment phase angle observed in the first days under DD after a previous LD schedule, we demonstrated that the wheel running availability induced the immediate differentiation of degus in three different chronotypes:

- diurnal, with its pacemaker entrained to the photophase and no negative masking effect by light;
- nocturnal, characterized by a pacemaker entrained to the dark phase and a strong negative masking effect by light; and finally
- intermediate animals, a chronotype characterized by a gradual negative masking effect by light, overlapping a pacemaker entrained to the photophase as in the diurnal chronotype.

A possible explanation for the existence of entrainment in nocturnal degus consisted in feedback effects to the pacemaker by the vigorous nocturnal running activity, which was generated by masking by light. However, two arguments rose against this hypothesis. First, intermediate and nocturnal animals with the same apparent nocturnal locomotor activity phasing under LD condition exhibited different phase angles of entrainment after being transferred to DD conditions. Second, the feedback by WRA in degus was demonstrated not to be strong enough to entrain the pacemaker (Kas & Edgar, 2001). Moreover, since the lineage of octodontids (Order *Rodentia*, family *Octodontidae*) is composed of nocturnal species, such as *Octodon bridgesi* and *Spalacopus cyanus*; it has been suggested that the crepuscular-diurnal pattern of *Octodon degus* is a new, and perhaps incomplete, evolutionary acquisition (Ocampo-Garcés et al., 2006). The evolutionary inheritance of nocturnal steady-entrainment may still coexist with the recently acquired diurnalism.

Nocturnal degus held in cages without wheel availability have occasionally been described (Refinetti, 2006). In fact, Iriarte et al (1989), in a field study, captured 3.6% of the total of degus in the interval of time between 24:00-03:59h and also 9.7% between 04:00-07:59h. However, in other field study of Kenagy et al., (2002), they did not capture any degus during the dark period. Another reference comes from Ocampo-Garcés et al. (2006) in a study of the rest-activity pattern of degus, with no wheel running availability, and captured directly from the nature. In this work they found two individuals with more than 70% of activity counts concentrated almost entirely during the dark phase. Finally, a study on 19 free-ranging, radio-collared adults revealed that degus exhibit nocturnal activity inside, but not outside, their burrows (Personal Communication, Luis Ebensperger). In our opinion, if the nocturnalism is a reminiscent property of the degu's nature, according to Darwin's natural selection, it makes sense that it appear in a very low proportion, or even almost lost, in wild populations.

In nature, the particular ability of certain animals to shift the activity phase from diurnal to nocturnal, or *vice versa*, in few days has been suggested that can be triggered by:

- Seasonal variations in the photoperiod (Gwinner, 1996);
- Seasonal variations in the temperature (Aranda et al., 1999);
- Ecological intra- or interspecific competitions (Kronfeld-Schor, 2003);
- Food availability restrictions (Saper et al., 2005).

According to field studies in degus, a seasonal rhythmicity pattern in foraging time has been observed throughout the year (Kenagy et al., 2002). In summer, when the weather is hot and dry, some degus forage during the darkness, probably to avoid the higher daytime temperatures (Saper et al., 2005). In nature, this thermoregulatory constraint may be accompanied by a parallel shift in the predator-prey relationship, as pointed out by Lagos (1995) and as occurs with other mammals (Kronfeld-Schor, 2003). In fact, different degus predators have been found in both diurnal, as hawks and foxes, and nocturnal, as owls, temporal niches (Meserve et al., 1993).

Under laboratory conditions, the degus' ability to change their circadian phasing might be reminiscent of this adaptive ability, allowing degus to forage during either night or day with equal ease. However, results on nocturnal long and short (such as random or scheduled) **feeding availability** did not entirely support this hypothesis (Vivanco et al., 2010a *in press*; **Experimental Chapter 2**). Nocturnal food availability merely induced a nocturnal phase shift in diurnal degus, but was not able to induce a stable circadian inversion or generate the most characteristic feature of the nocturnal chronotype: the negative masking effect by the light. This incomplete nocturnalism could be explained by the involvement of the peripheral oscillator associated to feeding, the food entrainable oscillator (FEO).

In this sense, in an animal entrained to a LD condition and feeding *ad libitum*, the central pacemaker (the light entrainable oscillator, LEO) spreads a temporal signal through the whole organism. However, when an animal is subjected to restriction feeding times, FEO then uncouples from LEO information, and some rhythms, such as activity and temperature, can anticipate food intake (Damiola et al., 2000; Escobar et al., 2007). In the case of diurnal degus subjected to nocturnal feeding availability, the output of the light entrainable oscillator most likely did not shift by food availability schedules since the animals still showed a high level of activity during the light hours

when food was available during the night, and the phase of minimum body temperature was maintained throughout the experimental procedures. Therefore, since feeding restriction was not able to generate masking by light in diurnal individuals, it was hypothesized that this masking occurred only when LEO was involved. Indeed, if wheel running was able to generate a masking response by light by means of the direct effect of locomotor activity upon the circadian pacemaker (Chiesa et al., 2007); therefore, food restriction should generate another kind of masking (no light dependent), acting on a different relay stage of the circadian pathway.

On the other hand, the **temperature** has been always considered the most important key factor involved in the nocturnalism of degus. Temperature is a critical factor in the survival of organisms, so they have developed different mechanisms in order to control it within a physiological range. Behavioral strategies for thermoregulation involving the circadian system are common in homeothermic animals. These can vary from limited phase shifts in activity, with a modification of burrow entries and exits in order to avoid extremely hot periods, to complete temporal niche inversions. These behavioural shifts have been described in birds and mammals (Goldstein DL, 1984; Fielden et al., 1992).

In 1976, George Fulk demonstrated that degus present a circannual pattern in their general activity, correlated with the photoperiod and the environmental temperature. This work was directly corroborated by Kenagy et al. (2002), who showed that in summer, when the temperature rises to 40°C by around midday, the degus' out-burrowing activity presents a crepuscular pattern, while in winter it appears unimodally diurnal, as the animal seeks open areas with sunlight.

In 1977, Mario Rosenmann noted also that degus have a very small tolerance to high temperatures, above 32ºC, due to their limited ability for water evaporation, and concluded that a behavioral response would be necessary in order to avoid thermal stress. In fact, direct observations corroborated that high environmental temperatures inhibit degus activity and induce them to seek shady places when they are in the open, retreating into their burrow as temperatures rise above their thermoneutral zone, between 24 - 32 ºC, at midday (Kenagy et al., 2004). In addition, Theresa Lee (2004) suggested that the relative proportion of chronotypes may be dependent on ambient temperature, with nocturnalism in degus increasing as the environmental temperatures rise.

Based on this evidences, our group demonstrated that environmental temperature cycles, with high values during the day, produced 100% of nocturnalism in a previously diurnal population of degus through two mechanisms: steady entrainment to LD, and negative masking effects by high temperature (Vivanco et al., 2010b *in press*; **Experimental Chapter 3**).

Vigorous running activity, as occurred with the wheel availability, could overheat the animals during the day and generate the nocturnal phase inversion as an effective behavioral thermoregulatory response of avoidance. This thermoregulatory hypothesis provides a context to understand a number of experimental observations:

- First, it would explain the fact that high diurnal ambient temperature cycles generated 100% nocturnalism in the diurnal wheel runner population.
- Secondly, inter-individual thermal tolerance values and variable thermogenic effects of exercise would explain why circadian inversion occurred in some individuals and not in others when the exercise wheel was available under thermoneutral temperature conditions.
- Thirdly, the differential response of naïve and experienced wheel runner groups to the temperature cycle when the wheel was available pointed out the influence of acclimatization processes. Experienced animals were less sensitive to the masking effect of high temperatures and were resistant to synchronizing their WRA with the temperature cycles under DD. In this sense, acclimatization is a common physiological phenomenon in animals subjected to new environmental conditions, such as temperature, humidity or altitude (Shido et al., 1991; Frisancho, 1975).

Moreover, temperature cycles and exercise by wheel availability are non-photic stimuli for the circadian system. Based on a comparison between diurnal and nocturnal species, non-photic cues can be classified into two categories: arousal-independent and arousal dependent factors. Melatonin and brain GABA are both arousal-independent factors, whereas brain serotonin, feeding, locomotor and temperature rhythms belong to the arousal-dependent group. Since the nocturnalism induced by temperature cycles shared many of the characteristics of that induced by wheel running availability, it was hypothesised that these two non-photic stimuli could synchronize the

degu's circadian system by acting on a common element, such as the activation of centres serotonergic projected from the raphe nucleus of the midbrain on the suprachiasmatic nucleus or the intergeniculate leaflet (Challet, 2007).

Therefore, degus circadian expression is presented as the result of hierarchical interactions between the entrainment phase of the pacemaker, nocturnal or diurnal, and masking processes by light induced as consequence of physiological responses related to the protection against dangerous effects of the temperature.

Comparing with the entrainment properties of the pacemaker, **masking** processes have been always paid much less attention and little is known about its mechanisms. Masking has been traditionally classified according to the response generated in the overt rhythm. In the case of increasing or reducing a biological variable, as for example the wheel running activity, masking has been defined as positive or negative, respectively (Mrosovsky, 1999). In most cases masking and entrainment act in the same direction, i.e. the light induces a negative masking, decreasing the level of WRA, in nocturnally entrained rodents. However, paradoxical masking, with masking and entrainment counteracting in opposite directions, have been also observed, as in the diurnal Nile grass rat (Mrosovsky, 1999).

The concept of masking has been referred in a wide diversity of situations. The traditional "tonic" point of view of masking could differ from a "phasic" one which is found when an animal is subjected to brief pulses of one stimulus, i.e. the light, through the circadian cycle, as in the case of ultradian cycles of lighting (Redlin & Mrosovsky, 2004). In this sense, an interesting recent study shows that a series of flash light pulses induces a behavioural response in mice, starting with a locomotor quiescence and finishing with a sleep status. This process has been referred as photosomnolence (Morin & Studholme, 2009).

Masking is also involved in temporal niche switching, due to seasonal rhythms, thermoregulatory constraints or any other causality (Gwinner, 1996; Mrosovsky, 2003). This "qualitative" change in the rhythmicity occurs as a physiological response involving the circadian system against a potentially dangerous causal agent (Kronfeld-Schor & Dayan, 2003). The common point in all of these references to masking is the existence of a change of the overt rhythmicity. To date masking has been only considered as a "quantitative" change that disturbs the endogenous signal from the circadian

pacemaker; however, results found in *Octodon degus* present masking as a more complex player than assumed for the rhythm generation.

In this sense, what sets the circadian chronotype of an individual, the pacemaker phase control or masking by light?

Our results in degus demonstrated that masking effects by light did not involve the pacemaker phase control (**Experimental Chapter 4**). In fact, diurnal and nocturnal degus displayed stable masking by light, according to their respective chronotype when they were subjected to ultradian cycles (1:1 LD). Thus, while diurnal animals increased their activity in the presence of light, it induced a pronounced locomotor activity drop in nocturnal degus in spite of they shifted their activity phase of WRA to the "subjective day" once the 1:1 LD cycle started. These two types of masking appeared under ultradian LD cycles and persisted when the exercise wheel was locked and even when the animals were exposed to high diurnal ambient temperature cycles.

Chronotypic differentiation of degus could be potentiated by its higly flexible circadian system. Thus, a **dissociation of the circadian system** into two components, a non-light dependent component (NLDC) and a light dependent component (LDC) could be induced by subjecting degus under 28h and 21h T-cycles (**Experimental Chapter 5**). While the NLDC was associated to the intrinsic pacemaker rhythmicity, the LDC was liked to the presence of the external lighting, and also related to the nocturnalism status of the animal. The wheel running activity appeared as a stimulus that directly triggered the appearance of the LDC in degus. Moreover, LDC did not act as a simple light-reactive stimulus but it presents phase relationships with the central pacemaker rhythmicity. Therefore, if it is assumed, and there are several experimental evidences for doing so, that LDC and masking by light processes are directly related; the concept of masking should be then reconsidered.

Diurnal and nocturnal animals present clear temporal differences on their vital activities; however, little is known about the neural mechanisms that determine their chronotype. In this sense, what makes different a diurnal from a nocturnal animal? From a theoretical point of view, the diurnalism or nocturnalism might be the result of processes at three possible levels:

- Upstream from the master clock, in the light input coupling with the SCN neurons;

- Inside the clock, i.e. in the machinery of the pacemaker, or
- Downstream from the clock, in the neural or humoral output connecting the SCN to the physiological or behavioral rhythmic processes.

Between the candidates downstream from the clock are being investigated humoral molecules, as prokineticin-2, TGF-α or even melatonin, and also neural outputs such as the activation of sympathetic and parasympathetic pathways innervating tissues and organs. To this, SCN outputs relay in different neural structures such as the subparaventricular zone and the dorsomedial hypothalamus (Schwartz et al., 2004; Saper et al., 2005).

In all vertebrate species, plasma **melatonin** peaks during the night, regardless of the nocturnal or diurnal behavior of the animal. This hormone is therefore considered to be the "chemical expression of darkness" (Reiter, 1991). In diurnal species, the nocturnal rise in melatonin coincides with the physiological rest period, i.e. increased sleepiness, decreased body temperature and locomotor activity, and increase in the immune responses (Van den Heuvel et al., 2005). By contrast, in nocturnal species, the stable endogenous signal of nocturnal melatonin is associated with wakefulness, i.e. increased locomotor activity and body temperature and decreased sleepiness. According to Mendelson et al. (1980) endogenous melatonin should promote those behaviors associated with night, i.e. resting in diurnal and activity in nocturnal species. In this sense, the existence of a dual phasing behavior within the same animal species constitutes a unique animal model for determining this antagonistic response to melatonin in nocturnal and diurnal animals.

However, results of our study focused on searching differential responses to the endogenous and exogenous melatonin, as the factor involved in defining the chronotype on this dual species, pointed out that the temporal phase preference was not defined by the plasma level of nocturnal melatonin, nor by the exogenous melatonin administration (**Experimental Chapter 6**). In fact, the administration of this indoleamine had similar effects on both chronotypes, including a generalized hypothermic effect which was slightly more marked in nocturnal animals.

On the other hand, the central pacemaker, the mammalian suprachiasmatic nucleus, has been the main focus of study for locating differences between nocturnal and diurnal animals. Some results showed that circadian rhythms of neuronal activity and glucose utilization within the SCN were similar in both chronotypes (Schwartz et al.,

1983). However, were found differences in the immediate-early expression gene *c-Fos* in the SCN (Krajnak et al., 1997) and also in the neuronal electrical response inside and outside the SCN from light or optic nerve stimulation (Jiao & Rusak, 2003) between some diurnal and nocturnal mammalian species (van den Veen et al., 2008). Although differences were found, SCN function of diurnal rodents differed little from their nocturnal relatives (Smale et al., 2008).

Another set of experiments were focused on the anatomical regions of the **pacemaker**. In this sense, the pacemaker has been traditionally divided into two main anatomical-functional regions: the dorsomedial, or shell, characterized by the predominance of arginine-vasopressin (AVP) expressing neurons; and the ventrolateral, or core, mainly with vasoactive-intestinal peptide (VIP) expressing neurons (Sofroniew & Weindl, 1980; Stopa et al., 1988). While AVP expression of the shell is directly related with the clock-genes machinery of the circadian clock (Jin et al., 1999), the core is considered as an integration centre of input information which receives photic information directly from the retina (Johnson et al., 1988), thought the retinohypothalamic tract, but also non-photic one from the geniculohypothalamic tract or even from the median raphe cells of the brainstem (Harrington, 1997). Our inmunohistochemistry study of the temporal presence of the main neuropeptides, AVP and VIP, and the c-Fos protein in the SCN of diurnal and nocturnal degus showed no significant differences between chronotypes (**Experimental Chapter 7**). Moreover, we did not found differences in the AVP expression in one of the main first relay centre downstream from the pacemaker, the paraventricular nucleus of the hypothalamus. These results are in concordance with obtained from other studies performed between diurnal and nocturnal animals (Takeuchi et al., 1989; Smith & Carter, 1996; Dardente et al., 2004).

In conclusion, *Octodon degus* is a predominantly diurnal rodent that owns the circadian flexibility of inverting 180º its phase preference under certain experimental situations. Apart from a steady entrainment to diurnal phases, its central pacemaker is also able to entrain to nocturnal phases. This circumstance is accompanied by a slight *tau* shortening for these individuals. Moreover, the nocturnalism in degus can also be achieved without affecting the pacemaker but masking the overt rhythmicity. This possibility is the most extended both in degus under laboratory conditions and in the majority of dual animals studied to date. Degus show a wide range of masking by light effect on locomotor activity: from diurnal individuals which do not present any activity inhibition by light till other individuals that, although maintaining the pacemaker

entrained to a diurnal phase, present a maximum inhibition and, therefore, behave as nocturnal animals. Temperature seems to be the main physiological agent involved in the appearance of masking by light responses in degus. Intense exercise on a wheel or an increase in the environmental temperature, provoke an augment in the $T_{b,}$ above the individual threshold to tolerate high body temperature, and then a subsequent phase inversion to the night of locomotor rhythms occurs. Finally, a neurobiological correlate to explain this great diversity of chronotypes was not found, either in the pacemaker or in the SCN-pineal-melatonin axis, since SCN and melatonin seem to function in the same way regardless the chronotype of the animal.

Conclusions

1- Most degus show a diurnal phase preference, and inversion to a nocturnal preference occurs, in some individuals, when free access to a running wheel is provided. This inversion tends to occur more easily in some degus than in others. In addition, exposure to wheel novelty also facilitates the inversion.

2- Two steady-entrainment phases, nocturnal and diurnal, and gradual masking effect by light generate the wide variability of circadian chronotypes in degus.

3- Nocturnal feeding availability induces an incomplete nocturnalism which is not stable in time and do not generate the most characteristic feature of the nocturnal chronotype: the negative masking effect by light.

4- High diurnal environmental temperature produces a 100% of nocturnalism in a previously diurnal population of degus, including both steady entrainment and masking.

5- Thermoregulatory constraints, i.e. a response for overheating avoidance involving the circadian system together with physiological acclimatization constitute an important key for the nocturnal phase preference inversion in degus.

6.- Positive and negative masking effects of light on diurnal and nocturnal degus, respectively, seem to occur independently of relative phase control by the central pacemaker, wheel running availability or the negative masking induced by high environmental temperatures.

7- T28 and T21 cycles dissociate the circadian rhythmicity of degus into two components: a non-light dependent component, associated to the intrinsic pacemaker rhythmicity, and a light dependent component, that combines masking by light and entrained properties, and it is dependent on the nocturnalism of the animal.

8- The temporal phase preference in degus is not defined by nocturnal melatonin plasma level or by its exogenous administration.

9- The suprachiasmatic nucleus of diurnal and nocturnal degus does not present differences on the temporal expression of the two main neuropeptides, arginine-vasopressin polypeptide and vasoactive-intestinal peptide, or the Fos protein.

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Glossary

Actogram: Is a type of representation used in Chronobiology in which the values of the biological variable under study are drawn through the day, in the ordinate axis, and through the experimental days, in the abscise axis.

Acrophase: Is the phase (considered as a point in the time) when occurs the maximum value of a rhythm. In Chronobiology is considered as the phase of the maximum value of the rhythm resulted of adjusting the data from the biological variable into a sinusoidal curve.

Alfa (α): Is the time of activity when considered a cycle activity-rest.

Amplitude: Is the difference between the maximum and the minimum value, or between the maximum and the mean value, of an ondulatory system. In Chronobiology is considered as the difference between the maximum, or minimum, and the mean values of the rhythm resulted of adjusting the data from the biological variable into a sinusoidal curve.

Arrhythmic: Is considered a rhythm that oscillates with non apparent rhythmicity.

Batyphase: Is the phase (considered as a point in the time) when occurs the minimum value of a rhythm. In Chronobiology is considered as the phase of the minimum value of the rhythm resulted of adjusting the data from the biological variable into a sinusoidal curve.

Circadian: Is considered the rhythm that oscillates with a periodicity between 20-28h.

Circadian time (CT): Is a subjective method for expressing the endogenous time of an organism. It consists in adapting the endogenous periodicity of the organism into 24h. In this sense, if the period is 25h, each circadian hour is equivalent to 25/24h. As convention, in diurnal animals the CT0 is the onset of activity phase; however, in nocturnal ones CT is 12.

Circannual: Is considered the rhythm that oscillates with a periodicity of around one year.

Crepuscular: Is the animal that is mainly active during the dawn and dusk lighting conditions.

DD: It is the short form of designing a situation of complete darkness. It derives from Dark:Dark.

Entrainment: Is the final status or the process in which a natural pacemaker modulates its period of oscillation and its phase relationship with respect an environmental and rhythmical agent, as the light.

Free running: A rhythm is free running when is oscillating under its own periodicity. This situation occurs when the rhythm is not subjected to the influence of external agents, as the light.

Infradian: Is considered the rhythm that oscillates with a periodicity higher than 28h.

LD: It is the short form of designing a cycle of light and darkness. It derives from Light:Darkness.

LL: It is the short form of designing a situation of complete light. It derives from Light:Light.

Masking: Is the final status or the process in which an environmental agent directly modifies the overt expression of a biological rhythm. For example, if a person wants to measure his body temperature while having a shower or going running, the obtained body temperature is considered masked by the action of the temperature of the water or by the physical exercise, respectively.

Mesor: Is the mean value of the rhythm resulted of adjusting the data from the biological variable into a sinusoidal curve.

Nychthemeral: Is a synonymous of circadian.

Pacemaker: Is the structure that is able to generate oscillations by itself, and also to have control upon other rhythms.

Period: Is the time that lasts a rhythm in oscillating one complete cycle.

Phase: Is a specific point in the time during the oscillation of a rhythm.

Phase angle: Is the difference of phase (considered as a point in the time or grades) between a reference event and another one under study.

Phase response curve (PRC): Is the curve that shows the different phase effects that produce a brief pulse of a stimulus, as the light, through the circadian day of the animal.

Photophase: Is the light part of a light-dark cycle.

Relative coordination: Is a status of half-entrainment in which a synchronizer agent is able to produce phase shifts in the rhythm studied but is not too strong as entrain it.

Resynchronization: Is a state of transition in which a rhythm slowly adjusts its phase relationship with respect to another new rhythmicity.

Rho (ρ): Is the time of resting when considered a cycle activity-rest.

Scotophase: Is the part of night of a light-dark cycle.

Subjective day: Is the part of the cycle in which the organism considers as the day of the day-night cycle. For example, an animal that only is active during the day of a normal 12h light (day): 12h dark (night) cycle. When this animal is subjected to constant conditions, as DD, and the rhythm starts to free running. In this situation, the subjective day would be the part of the cycle in which the animal is active.

Subjective night: Is the part of the cycle in which the organism considers as the night of the day-night cycle. For example, an animal that only is active during the night of a normal 12h light (day): 12h dark (night) cycle. When this animal is subjected to constant conditions, as DD, and the rhythm starts to free running. In this situation, the subjective night would be the part of the cycle in which the animal is active.

Synchronization: Is the process in which a biological rhythm adopts a precise phase relationship with respect to another one.

Tau (τ): Is the endogenous periodicity of oscillation of a biological rhythm.

Ultradian: Is considered the rhythm that oscillates with a periodicity less than 20h.

Zeitgeber: German word which means “time-giver”, coined by Jürgen Aschoff to define the external agent able to generate a status of entrainment in an endogenous rhythmicity.

Zeitgeber time (ZT): Is the measurement of the time having as reference the *zeitgeber*. Normally, they are considered reference points when happens the transition between “on” or “off” in the exposition to the ***zeitgeber***. For example, if light “on” at 08:00h, that time is considered ZT0; therefore, 10:00h will be considered as ZT2.

Annex

Annex I: Scientific production resulting from the experiments on the present PhD thesis

Scientific Papers

- Looking for the keys to diurnality downstream from the circadian clock: Role of melatonin in a dual-phasing rodent, *Octodon degus*. **Vivanco P**, Ortiz V, Rol de Lama MA, Madrid JA. (2007). *J. Pineal. Res.* 42:280-290

- Two-steady entrainment phases and graded masking effects by light generate different circadian chronotypes in *Octodon degus*. **Vivanco P**, Rol MA, Madrid JA. (2009). *Chronobiol. Int.* 26:219-241

- Temperature cycles trigger nocturnalism in the diurnal homeotherm *Octodon degus*. **Vivanco P**, Rol MA, Madrid JA. (2010). *Chronobiol. Int* 27(3)- *In press*

- Age-related brain pathology in *Octodon degus*: blood vessel, white matter and Alzheimer-like pathology. van Groen T, Kadish I, Popovic N, Popovic M, Caballero-Bleda M, Baño-Otálora B, **Vivanco P**, Rol MA, Madrid JA. (2010). *Neurobiol. Aging- In press*

- Nocturnalism induced by scheduled feeding in diurnal Octodon degus. **Vivanco P**, Rol MA, Madrid JA. (2010). *Chronobiol. Int* 27(2)- *In press*

Sections in books

- Phase Inversions of the circadian rhythms. **Vivanco P**. Basic and clinic Chronobiology. (2006). pp 74

Annex II: Congress contribution resulting from the experiments on the present PhD thesis

- Nocturnalism induced by high environmental temperature during the photophase in the diurnal *Octodon degus*. **Vivanco P**, Rol MA, Madrid JA. *XI Congress of the European Biological Rhythms Society*. Strasbourg (France).

- Internal temporal order in a dual phasing rodent, *Octodon degus*. Otalora BB, **Vivanco P**, Madariaga AM, Rol MA, Madrid JA. *XI Congress of the European Biological Rhythms Society*. Strasbourg (France)

- Dissociation between the circadian pacemaker and the light-induced masking by 28h T-cycles in *Octodon degus*. **Vivanco P**, Rol MA, Madrid JA. *Congress by the Spanish Society of Physiological Sciences*. Valencia (Spain)

- Alteration of internal temporal order in the circadian system of a dual phasing rodent, *Octodon degus*. Otalora BB, **Vivanco P**, Madariaga AM, Rol MA, Madrid JA. *Congress by the Spanish Society of Physiological Sciences*. Valencia (Spain)

- Effects of exogenous melatonin on the circadian system of aged *Octodon degus*. **Vivanco P**, Rol MA, Madrid JA. *The first International Congress of Applied Chronobiology and Chronomedicine*. Antalya (Turkey)

- Plasma melatonin and DHEA in adult and aged *Octodon degus*. Ortiz V, **Vivanco P**, Cuesta A, Meseguer J, Esteban MA, Madrid JA, Rol de Lama MA. *The first International Congress of Applied Chronobiology and Chronomedicine*. Antalya (Turkey)

- *Octodon degus*, an animal model for studying aging on the circadian rhythms. Madariaga AM, **Vivanco P**, Ortiz V, Martínez-Nicolás A, Madrid JA, Rol de Lama MA. *ESLALV 2005 International Congress*. Elche (Spain)

- Effects of aging on the circadian rhythm of wheel-running activity in Octodon degus. Rol de Lama MA, **Vivanco P**, Madariaga AM, Sánchez-Vázquez FJ, Madrid JA. *First World Congress of Chronobiology, 10th Annual Meeting of Japanese Society for Chronobiology*. Sapporo (Japan)

Annex III: Placements in other laboratories during the realization of the PhD

- Chronobiology group of Antoni Díez-Noguera and Trinitat Cambras Riu. Faculty of Pharmacy. University of Barcelona (Spain)
Year: 2006 Duration: 2 months

- Chronobiology group of Maria Canal. Faculty of Life Sciences. Manchester (United Kingdom).
Year: 2008-2009 Duration: 7 months

Annex IV: Research Projects supporting the experiments on the present PhD

- Title of the Project: “Research network of aging and fragility - RETICEF”
Funded Institution: Health Institute Carlos III (RD06/0013/0019)
Duration: 2007-2011

- Title of the Project: “Premature aging due to an impaired circadian system: Role of melatonin”
Funded Institution: CICYT (BFU2007-60658)
Duration: 2007-2010

- Title of the Project: “Chronodisruption and aging: Animal models”
Funded Institution: Fundation SENECA (05700/PI/07)
Duration: 2007-2009

Resumen en Español

Teniendo en cuenta el conocimiento previo sobre la ritmicidad del *Octodon degus*, el objetivo de esta tesis es comprender los mecanismos implicados en el nocturnalismo de esta especie diurna. Para ello, fue necesario responder a una serie de preguntas que enfocaban el problema desde distintos puntos de vista:

Causal

¿Qué agentes externos desencadenan el nocturnalismo?

- ¿Es la actividad en rueda giratoria? (Capítulo 1)
- ¿Es la disponibilidad de alimento? (Capítulo 2)
- ¿Es la temperatura ambiental? (Capítulo 3)

Mecanicista

¿Quién controla la inversión hacia la fase nocturna?

- ¿Es el control de fase del marcapasos o el masking por luz? (Capítulo 4)
- ¿Puede la salida del marcapasos disociarse del masking por luz? (Capítulo 5)

Funcional

¿Qué es lo que hace diferente a un individuo nocturno de uno diurno?

- ¿Genera la secreción de melatonina una respuesta diferencial? (Capítulo 6)
- ¿Es el núcleo supraquiasmático diferente entre ambos cronotipos? (Capítulo 7)

Los objetivos específicos de esta tesis son:

1- Verificar si la actividad en rueda invierte la fase diurna del degus a una nocturna.

2- Verificar si el nocturnalismo del degus es debido exclusivamente a efectos masking por luz.

3- Probar si el nocturnalismo en degus puede ser inducido por una alimentación forzada en la noche.

4- Probar si la temperatura ambiental es el factor clave para la actividad nocturna del degus.

5- Caracterizar la respuesta masking por luz en los degus y su estabilidad con respecto a la disponibilidad de rueda giratoria y a la temperatura ambiental en individuos diurnos y nocturnos sujetos a ciclos ultradianos de una hora de luz y una de oscuridad.

6- Provocar la disociación circadiana entre el marcapasos y el componente enmascarado por luz usando ciclos T de 28 y 21 horas.

7- Probar si la melatonina endógena y/o la exógena es un factor fisiológico implicado en definir el cronotipo de las especies duales.

8- Evaluar las posibles diferencias en la expresión de los principales neuropéptidos (arginina vasopresina y el polipéptido intestinal vasoactivo) y la proteína Fos en el núcleo supraquiasmático de degus diurnos y nocturnos.

Capítulo Experimental 1. "Dos fases de encarrilamiento estables y efectos graduales de masking por la luz generan los diferentes cronotipos circadianos en el *Octodon degus*"

Mientras que los procesos implicados en el funcionamiento del marcapasos circadiano están bien caracterizados, se sabe poco sobre los mecanismos que controlan el comportamiento diurno, nocturno o crepuscular en las especies. En este sentido, los roedores duales, como el *Octodon degus*, aparecen como instrumentos muy útiles para descifrar estas claves. Basándose en el porcentaje de actividad del animal durante la fase de luz o de oscuridad, los degus de laboratorio se han descrito tradicionalmente bajo dos cronotipos principales, nocturno y diurno. Sin embargo, si además se tiene en cuenta el ángulo de fase de encarrilamiento los primeros días que siguen a un cambio de LD a DD, se puede distinguir un tercer cronotipo, intermedio, o mejor dicho, un gradiente continuo de expresiones circadianas entre los cronotipos diurnos y los nocturnos.

Nuestros experimentos apuntan a que el marcapasos de un degus diurno se encuentra encarrilado en las fases del día y, además, la ritmicidad final observable no está influenciada por ningún efecto masking inhibitorio ante la luz. Por otro lado, el marcapasos de un nocturno está encarrilado en las fases de noche, y en este caso la luz sí que ejerce un fuerte efecto masking negativo sobre su ritmicidad. Por último, el cronotipo intermedio se caracteriza por presentar un marcapasos encarrilado en las fases del día y cuya ritmicidad observable es superpuesta por un variado espectro de repuestas inhibitorias ante la presencia de la luz.

La inversión de cronotipo diurno a nocturno está relacionada con la disponibilidad de una rueda giratoria en la jaula del animal, y este efecto parece originarse en una posición por debajo del reloj circadiano. Sin embargo, los registros del ritmo de temperatura corporal, que se encuentran menos afectados por los efectos masking, apuntan a la implicación del marcapasos en la diferenciación del cronotipo ya que se detectaron ciclos transitorios de encarrilamiento, y no saltos bruscos de fase, tras disponer a los animales de la rueda. El diurnalismo en el degus parece ser el resultado de una variedad de mecanismos, que explicaría cómo distintos procesos pueden llegar a dar cronotipos similares.

Capítulo Experimental 2. "Nocturnalismo inducido por alimentación en *Octodon degus* diurnos"

El *Octodon degus* es un roedor principalmente diurno que se caracteriza porque en condiciones de laboratorio es capaz de cambiar su actividad locomotora hacia la noche. El objetivo de este estudio fue determinar si la restricción de la alimentación a la noche podría inducir nocturnalismo en los degus.

Para ello se registraron los ritmos de actividad en rueda, alimentación y temperatura corporal en degus diurnos que disponían de rueda giratoria y sujetos tanto a disponibilidad larga del alimento (12 horas) como a corta (2 horas). Para esta última se estudió cuando las 2h de disponibilidad del alimento fueron dispuestas de forma aleatoria en el tiempo y cuando fueron fijas.

Los resultados mostraron que la restricción del alimento a 2 horas (la disponibilidad aleatoria fue más efectiva que la fija), pero no a 12 horas, puede cambiar la fase diurna de actividad en rueda y temperatura corporal hacia una fase nocturna en los degus. Sin embargo, esta inversión fue inestable ya que los degus volvieron a la fase diurna en unos pocos días una vez que la restricción de alimento fue quitada. Además, el masking negativo inducido por la luz, que es característico del cronotipo nocturno en degus, no se observó cuando éstos fueron obligados a comer durante la noche.

Por lo tanto, ninguna disponibilidad de alimento durante la noche (larga, corta, aleatoria o fija), indujeron todas las características del cronotipo nocturno en *Octodon degus*.

Capítulo Experimental 3. "Ciclos de temperatura desencadena nocturnalismo en el homeotermo diurno *Octodon degus*"

La regulación de la temperatura corporal en rangos fisiológicos es un factor crítico para garantizar la supervivencia de los organismos vivos. La evitación de altas temperaturas ambientales es un mecanismo comportamental usado por los animales homeotermos que viven en condiciones ambientales extremas. En este sentido, se han descrito saltos de fase precisos e incluso inversiones completas del nicho temporal. Ambas situaciones justifican la implicación del sistema circadiano en estas respuestas termoreguladoras. *Octodon degus*, un roedor chileno principalmente diurno, presenta la capacidad de invertir su fase de actividad locomotora cuando dispone de una rueda giratoria en su jaula.

Los objetivos de este trabajo son dos: Determinar si ciclos de temperatura ambiental (con altos valores durante el día y bajos durante la noche) pueden inducir la aparición de cronotipos nocturnos en animales previamente caracterizados como diurnos. Y además comprender si estos ciclos de temperatura son capaces de actuar como *zeitgeber* en esta especie dual.

Para ello, los degus fueron sujetos a estos ciclos de temperatura bajo condiciones de 12:12 LD y DD. Se utilizaron dos grupos experimentales (uno de animales que tenían previamente la rueda giratoria, y otro al que se le introdujo en ese instante), con el fin de estudiar la influencia de los ciclos térmicos junto con la experiencia previa en correr en rueda sobre la dualidad del degus.

Los ciclos de temperatura (31.3 ± 1.5 ºC durante el día y 24.2 ± 1.6 ºC por la noche) indujeron que el 100% de los individuos diurnos invirtieran a nocturnos. De hecho, se encontraron tanto encarrilamiento con ángulos de fases nocturnos como ritmicidad nocturna inducida por masking. Además, estos ciclos de temperatura, a través de procesos masking, confinaron la ritmicidad de actividad en rueda en la fase fría cuando los individuos estuvieron sujetos a DD. En esta situación, el grupo al que se le introdujo recientemente la rueda presentó una mayor sensibilidad que el grupo de degus experimentados.

Capítulo Experimental 4. "Control de fase por el marcapasos vs. masking por la luz: marcando el cronotipo circadiano en *Octodon degus* duales"

En la expresión de la ritmicidad circadiana hay dos procesos implicados: encarrilamiento y masking. Mientras que el primero opera a través del marcapasos central para anticipar condiciones predecibles del medio ambiente, el masking (principalmente inducido por la luz), funciona directamente modulando la señal de salida circadiana, y es inducido por sucesos no predecibles. El roedor chileno *Octodon degus* presenta ambas preferencias, nocturna y diurna, en la actividad locomotora cuando se le dispone del acceso libre a hacer ejercicio en una rueda giratoria. Dos fases de encarrilamiento estable y efectos graduales de masking por luz parecen generar la amplia variabilidad de cronotipos en esta especie.

El objetivo de este trabajo fue caracterizar las distintas respuestas masking por luz acorde a los cronotipos individuales, su estabilidad en el tiempo y la influencia de la disponibilidad en rueda y la temperatura ambiental sobre la dualidad circadiana del degus. Para ello, degus diurnos y nocturnos fueron sujetos a ciclos ultradianos (1 hora de luz: 1 hora de oscuridad) con y sin disponibilidad de rueda giratoria, y bajo ciclos de temperatura ambiental normales y también altos.

Nuestros resultados mostraron que los degus diurnos y nocturnos presentan un masking por luz estable y acorde al correspondiente cronotipo. De esta manera, mientras que los animales diurnos aumentaban su actividad cuando se encendía la luz, a los nocturnos les inducía una bajada drástica de su actividad en rueda. Estos dos tipos de respuesta masking no solo fue patente cuando los animales estaban sincronizados al ciclo 12:12 LD sino que además bajo los ciclos ultradianos. Ambas respuestas masking se mantuvieron no solo cuando la rueda giratoria no estuvo disponible, sino que además cuando los animales movieron sus perfiles de actividad circadiana en respuesta a los ciclos ultradianos o a la exposición de altas temperaturas durante el día.

En conclusión, nuestros resultados muestran que los efectos positivos y negativos del masking por luz sobre los respectivos degus diurnos y nocturnos parecen ocurrir de manera independiente al control de fase impuesto por el marcapasos central, la disponibilidad de rueda giratoria o el masking negativo inducido por las altas temperaturas ambientales.

Capítulo Experimental 5. "Disociación del sistema circadiano del *Octodon degus* inducido por ciclos LD T28 y T21"

El *Octodon degus* es un roedor principalmente diurno que presenta una gran variabilidad de cronotipos circadianos debido a la interacción entre dos ángulos de fase de encarrilamiento, diurno y nocturno, y graduales efectos masking por luz y temperatura.

El objetivo de este estudio era probar si el sistema circadiano de este roedor diurno se puede disociar internamente mediante ciclos más cortos y más largos que 24 horas y además determinar la influencia del cronotipo y la disponibilidad de rueda giratoria en tal disociación. Para ello se estudiaron los ritmos de actividad en rueda y la temperatura corporal en degus sujetos a ciclos LD simétricos de duración 28 y 21 horas.

Los resultados muestran que ambos ciclos pueden disociar en dos componentes el sistema circadiano del degus: un componente dependiente de la luz, influenciado por la presencia de la luz, mientras que el otro es independiente de la luz y que entró en curso libre con un período diferente al del ciclo iluminación externo. El componente dependiente de luz fue más evidente en el cronotipo nocturno que en el diurno, y lo fue cuando la rueda giratoria estuvo disponible.

En definitiva, este estudio muestra como el sistema circadiano del degus, así como el de la rata y el ratón, puede presentar una disociación interna en su sistema circadiano. La existencia de un sistema multioscilatorio compuesto por dos grupos de osciladores con bajo grado de acoplamiento entre ellos podría ser el mecanismo que explique la flexibilidad de los cronotipos en el degus.

Capítulo Experimental 6. "Buscando las claves del diurnalismo aguas abajo del reloj circadiano: papel de la melatonina en un roedor dual, *Octodon degus*"

La melatonina es un componente esencial en la función del sistema circadiano, cuyo ritmo de secreción diaria está controlado por el núcleo supraquiasmático, y que contribuye a la dispersión del mensaje temporal desde el reloj central hasta todas las células. El pico de secreción de la melatonina ocurre durante la noche sin tener en cuenta si el animal es diurno o nocturno. Hasta el momento no se conocen los mecanismos que contribuyen al nocturnalismo o diurnalismo de los animales.

Este trabajo tiene como objetivo examinar los niveles de melatonina plasmática en mitad del día y en mitad de la noche, y la influencia de la administración exógena de melatonina sobre el sistema circadiano de esta especie dual, *Octodon degus*, que presenta ambos cronotipos, nocturno y diurno, cuando tienen disponible una rueda giratoria en su jaula. Los niveles plasmáticos de melatonina fueron determinados por radioinmunoensayo (RIA) en muestras sanguíneas tomadas desde la vena yugular en mitad de la noche y en mitad del día. La melatonina (0.5mg/kg b.wt.) fue administrada oralmente en el agua de bebida durante 30 días y cuya disponibilidad era tan solo de 2 horas al día antes del comienzo de la noche.

Los resultados mostraron que los niveles plasmáticos de melatonina y sus efectos cualitativos, como la hipertermia y la mejora de la sincronización sin la modificación de la actividad en rueda, fueron similares en degus diurnos y nocturnos. Además, la administración de melatonina pudo mejorar la ritmicidad circadiana desmejorada de los animales envejecidos sin el efecto rebote tras cesar con el tratamiento.

En definitiva, los niveles de melatonina plasmática y las respuestas diferenciales a la melatonina parecen no ser las responsables para la diferenciación del cronotipo nocturno o diurno. Por lo tanto, queda por investigar otros mecanismos dentro, por delante o por detrás del núcleo supraquiasmático.

Capítulo Experimental 7. "Expresión temporal de arginina vasopresina, polipéptido intestinal vasoactivo y la proteína Fos en el hipotálamo de *Octodon degus* diurnos y nocturnos"

A los animales diurnos y nocturnos se les diferencia fácilmente por elegir nichos temporales opuestos. Sin embargo, los mecanismos neurales implicados en la preferencia de fase no se conocen aún. Hasta el momento, los resultados experimentales apuntan a que la función del marcapasos es la misma en ambos cronotipos. En este sentido, especies duales, como el roedor chileno Octodon degus, son herramientas útiles para comprender los mecanismos básicos implicados en esta preferencia individual de fase. Aunque el nocturnalismo del degus está muy influenciado por efectos masking, se han demostrado en degus fases de encarrilamiento estables tanto diurna como nocturna.

El objetivo de este trabajo es caracterizar y localizar posibles diferencias en la expresión temporal de arginina vasopresina (AVP), polipéptido intestinal vasoactivo (VIP) y proteína Fos en el núcleo supraquiasmático de degus diurnos y nocturnos. Los resultados muestran que tanto la inmunoreactividad de VIP y de AVP presentan valores similares durante el día como durante la noche. Sin embargo, el número de células inmunoreactivas de Fos fue más alto durante el día que la noche. Ninguna de estas variables presentó diferencias entre los cronotipos diurno y nocturno, indicando que la diferenciación de cronotipo en el degus podría ser generado fuera del núcleo supraquiasmático.

Las conclusiones de esta tesis doctoral son las siguientes:

1- La mayoría de los degus muestran una preferencia por la fase diurna, y ocurre, en algunos individuos, una inversión hacia una fase nocturna cuando se les permite el acceso libre a una rueda giratoria. Esta inversión tiende a ocurrir más fácilmente en unos individuos que en otros. Además, la exposición novedosa a una rueda también facilita la inversión.

2- Dos fases estables de encarrilamiento, nocturno y diurno, y graduales efectos masking por luz generan la amplia variabilidad de cronotipos circadianos del degus.

3- La disponibilidad de alimentación nocturna induce un nocturnalismo incompleto que no es estable en el tiempo y no genera el rasgo más característico del cronotipo nocturno: el masking negativo por la luz.

4- Alta temperatura ambiental diurna produce un 100% de nocturnalismo en una población de degus previamente diurna, incluyendo encarrilamiento estable y masking.

5- La restricción por la temperatura, como la respuesta de evitación ante un sobrecalentamiento, implicando al sistema circadiano, junto con procesos de aclimatación fisiológica constituyen en degus la clave de su inversión de fase hacia la noche.

6- Efectos masking positivos y negativos por la luz en degus diurnos y nocturnos, respectivamente, parecen ocurrir independientemente del control de fase del marcapasos central, disponibilidad de rueda giratoria o el masking negativo inducido por altas temperaturas ambientales.

7- Ciclos T28 y T21 disocian la ritmicidad circadiana del degus en dos componentes: uno no dependiente de la luz, asociado intrínsecamente a la ritmicidad del marcapasos, y otro componente dependiente de la luz, que combina propiedades del masking por luz y de encarrilamiento, y es dependiente del nocturnalismo del animal.

8- La preferencia de fase temporal en el degus no está definida por el nivel de melatonina plasmática nocturna no por su administración exógena.

9- El núcleo supraquiasmático de degus diurnos y nocturnos no presentan diferencias en la expresión temporal de dos neuropéptidos principales, arginina vasopresina y polipéptido intestinal vasoactivo, o la proteína Fos.