UNIVERSIDAD DE MURCIA

FACULTAD DE MEDICINA

Circadian system functionality in physiological and pathological rodent models of chronodisruption. Chronoenhancement by melatonin.

Funcionamiento del sistema circadiano en modelos fisiológicos y patológicos de cronodisrupción en roedores. Cronopotenciación por melatonina.

Dª Beatriz Baño Otálora

2012

UNIVERSITY OF MURCIA

Department of Physiology

Animal Physiology Unit

CIRCADIAN SYSTEM FUNCTIONALITY IN PHYSIOLOGICAL AND PATHOLOGICAL RODENT MODELS OF CHRONODISRUPTION. CHRONOENHANCEMENT BY MELATONIN

Dissertation submitted by Beatriz Baño Otalora to obtain the PhD. degree by the University of Murcia

Murcia 2012

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Departamento de Fisiología

Unidad de Fisiología Animal

FUNCIONAMIENTO DEL SISTEMA CIRCADIANO EN MODELOS FISIOLÓGICOS Y PATOLÓGICOS DE CRONODISRUPCIÓN EN ROEDORES. CRONOPOTENCIACIÓN POR MELATONINA

Memoria de la Tesis Doctoral presentada por Dña. Beatriz Baño Otálora para optar al grado de Doctor en Biología por la Universidad de Murcia

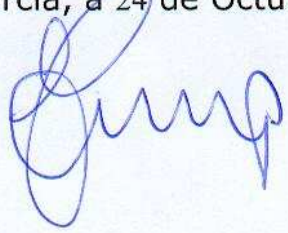
Murcia 2012

UNIVERSIDAD DE
MURCIA

D. JUAN ANTONIO MADRID PÉREZ, Catedrático de Universidad del Área de Fisiología en el Departamento de Fisiología, AUTORIZA:

La presentación de la Tesis Doctoral titulada "FUNCIONAMIENTO DEL SISTEMA CIRCADIANO EN MODELOS FISIOLÓGICOS Y PATOLÓGICOS DE CRONODISRUPCIÓN EN ROEDORES. CRONOPOTENCIACIÓN POR MELATONINA."Circadian system functionality in physiological and pathological rodent models of chronodisruption. Chronoenhancement by melatonin"", realizada por Dª. BEATRIZ BAÑO OTÁLORA, bajo mi inmediata dirección y supervisión, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

En Murcia, a 24 de Octubre de 2012



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Dª. MARIA ÁNGELES ROL DE LAMA, Profesora Titular de Universidad del Área de Fisiología en el Departamento de Fisiología, AUTORIZA:

La presentación de la Tesis Doctoral titulada "FUNCIONAMIENTO DEL SISTEMA CIRCADIANO EN MODELOS FISIOLÓGICOS Y PATOLÓGICOS DE CRONODISRUPCIÓN EN ROEDORES. CRONOPOTENCIACIÓN POR MELATONINA. "Circadian system functionality in physiological and pathological rodent models of chronodisruption. Chronoenhancement by melatonin"", realizada por Dª. BEATRIZ BAÑO OTÁLORA, bajo mi inmediata dirección y supervisión, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

En Murcia, a 24 de Octubre de 2012

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Date: October 23, 2012

To whom it can be concern,

I am very much delighted to write this letter of support of the PhD candidate Beatriz Baño Otalora in view of submitting and defending her PhD thesis, entitled, "Circadian system functionality in physiological and pathological rodent models of chronodisruption. Chrono-enhancement by melatonin." as a European thesis.

The PhD thesis consists of 8 major experimental chapters in which circadian disruption in diurnal and nocturnal rodent species is being systematically investigated. Furthermore, the efficacy of the hormone melanin has been studied in transgenic rodent models. Four of those 8 experimental 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 and on how melatonin may be an important factor in the maintenance of synchronization to the 24 hour light/dark cycle.

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

Yours Sincerely,

Martien Kas, PhD

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The dissertation submitted by Beatriz Baño Otalora to obtain the Ph.D. degree from the University of Murcia titled « **CIRCADIAN SYSTEM FUNCTIONALITY IN PHYSIOLOGICAL AND PATHOLOGICAL RODENT MODELS OF CHRONODISRUPTION. CHRONOENHANCEMENT BY MELATONIN** » and supervised by Marian Rol and Juan Antonio Madrid, is an original work.

This thesis has two main objectives 1) investigate if a disruption in the internal temporal organization of the degu's circadian system emerges when the animals voluntary reverse their activity-phase preference fromday to night, 2) study the circadian system functionality in pathologies associated with chronodisruption, and its possible chronoenhancement by exogenous melatonin. To reach these objectives, the candidate addressed some questions using behavioural, physiological and molecular approaches. The manuscript is composed of 8 experimental chapters, 4 already published (2 in Journal of Pineal Research and 2 in Chronobiology International), and 4 being submitted. This highlights the scientific quality of this report.

After the general introduction, Beatriz Baño Otalora presents her results with scientific publications leading to a clear and didactic report. The thesis ends with a general discussion summarizing the arguments exposed in the different chapters. Note that the whole thesis is written in English, including the general introduction and discussion, emphasizing the European dimension of this work.

I wish to congratulate Miss Beatriz Baño Otalora for her work and give, without any doubt, a favourable recommendation as an European thesis.

In conclusion, the doctoral thesis submitted by Miss Beatriz Baño Otalora fulfils the European standards to obtain the Ph. D. degree.

M. Beauchaud

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For my family and friends

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“Esta tesis doctoral está sometida a procesos de protección o transferencia de tecnología o de conocimiento, por lo que los siguientes contenidos están inhibidos en la publicación en los repositorios institucionales.

Autorizado por la Comisión General de Doctorado de la Universidad de Murcia con fecha 15 de febrero de 2013”

1. INTRODUCTION

From the origin of life on our planet, organisms have been exposed to recurring environmental fluctuations. The rotation of the Earth on its own axis generates the day and night or the light-dark (LD) cycle with a periodicity of 24 hours, while the Earth's revolution around the sun gives way to the seasons along a yearly cycle. The presence of endogenous timing mechanisms within the organisms, that allow anticipation of daily cyclical changes in the environment, certainly provides an adaptive advantage to the individual. Thus, it is not surprising that natural selection has favoured the presence of biological clocks in all living organisms across all lines of evolution, from prokaryotes to humans. These biological clocks generate oscillations with periods of approximately 24 hours (circadian), orchestrating endogenous rhythmicity in physiology, behavior, and metabolism, allowing living organisms to anticipate and prepare in advance for daily changes in their environment.

The scientific field that studies these timing processes (biological rhythms) is termed Chronobiology, a word derived from three Greek stems: "*kronos-bio-logos*", meaning "time-life-study" (DeCoursey, 2004).

1.1. BIOLOGICAL RHYTHMS, CLASSIFICATION AND PROPERTIES

A biological rhythm is the recurrence of a biological phenomenon in a periodic and predictable manner. One parameter that characterizes the biological rhythms is the period (the time after which a defined phase of an oscillation recurs) or its inverse, called frequency (number of cycles per time unit). The biological rhythms can be classified into three categories using day (24h) as the unit of frequency,

-**Circadian**: rhythms whose frequency of oscillation is close to a day (period between 20 and 28h), such as rhythms in motor activity (sleep-wake), body temperature, and melatonin secretion.

-**Ultradian**: rhythms whose frequency of oscillation is higher than one cycle per day (period <20h), such as the rhythms in the secretion of some hormones (e.g. Follicle Stimulating Hormone (FSH), and Luteinizing Hormone (LH)), brain wave electrical activity and heart beats.

-**Infradian:** rhythms whose frequency of oscillation is lower than one cycle per day (period >28h). This category includes circa-lunar rhythms (approx 28 days), such as the menstrual cycle, and circa-annual (period approx 365 days), such as the reproduction cycles in some species.

1.1.1. Properties of the circadian rhythms

One of the main features of the biological rhythms is their persistence under constant conditions. That is, in the absence of any external time cues. This demonstrates that biological rhythms are **endogenously generated** by an internal clock (Pittendrigh, 1960). Their endogenous period, known as the free-running period (tau, **τ**) is slightly different from the period of the environmental cycles (**T**). Tau is species-specific and its value is around 24h in rodents and humans.

Another important feature of endogenous rhythmicity generated by an internal circadian clock is that it shows **temperature compensation**. That is, circadian clock oscillates with a period that is not significantly affected by changes in temperature (Pittendrigh, 1960).

Endogenous rhythms can be **entrained** by 24h environmental cues. Under these circumstances, the period of the biological rhythm (τ) becomes equal on average to that of the entraining stimuli (T), with a stable phase relationship (psi, **ψ**) between the entraining and entrained oscillations (Figure 1) (Pittendrigh, 1981).

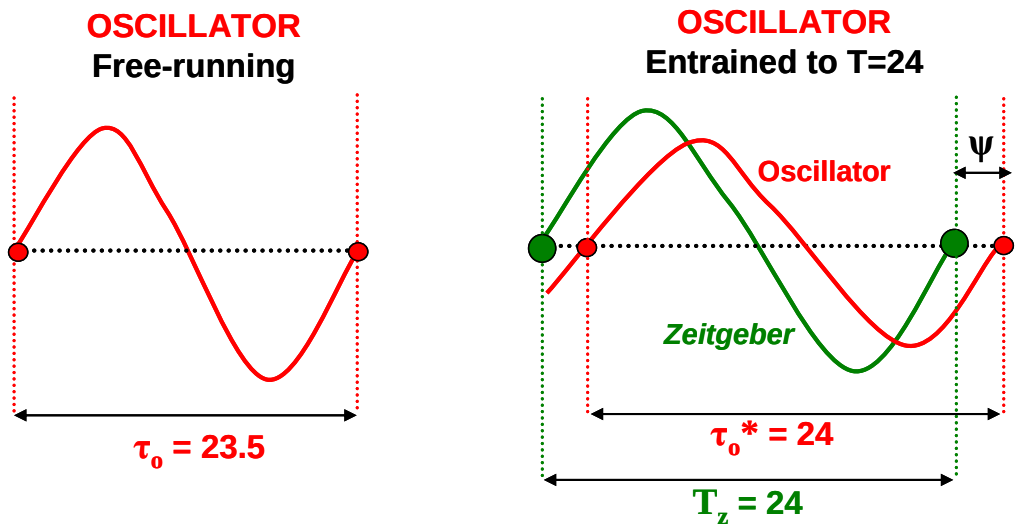

Figure 1. Entrainment. In the absence of external time cues, the oscillator exhibits a free-running period close to 24h (for example, τ_o=23.5h). This oscillator can be entrained by a *zeitgeber* with a period of 24 (T_z=24), such as the light/dark cycle. Then, the period of the oscillator changes from τ_o to τ_o* and becomes equal to the period of the *zeitgeber* (T_z=τ_o*). In the entrained steady-state, a unique phase relationship (ψ) between the *zeitgeber* and the oscillator is established. Modified from Pittendrigh, 1981.

An environmental agent that can synchronize the circadian clocks is called a **synchronizer** or ***zeitgeber,*** which is from the German term "*time giver*". The main *zeitgeber* for all living organisms is the LD cycle, but temperature cycles, social cues, food availability, novel wheel running can also operate as *zeitgebers*. To determine if an environmental factor can act as a *zeitgeber* or synchronizer, several criteria are proposed (Moore-Ede et al., 1982).

- In absence of other environmental agents, the rhythm must free-run with a determinate period (τ) before applying the *zeitgeber*. Following the removal of the *zeitgeber*, the rhythm has to revert to its free-running periodicity.
- Period control: when the *zeitgeber* is present, the period of the rhythm must be equal to that of the entraining cycle (τ=T).
- Stable phase relationship between the rhythm and the *zeitgeber* must exist.
- Phase control: when the *zeitgeber* is removed, the rhythm must start to free run from a phase previously determined by the *zeitgeber*.

Frequently, organisms respond to stimuli reactively. That is, the organism cannot anticipate when the stimuli will be present and/or absent (Cambras, 2006). If the stimuli occur cyclically, for example every 24 hours, the internal reaction generated in the animal can affect the manifestation of the rhythm, overriding the endogenous rhythmic signals from the pacemaker. This phenomenon is known as **masking**. In these circumstances, the overt rhythms are as a result of the interaction between two processes, masking and entrainment. Masking can be considered as part of the circadian system which, together with rhythms that emerge from the entrained circadian pacemaker, plays an important role in the adaptability of animals to temporal niches (Mrosovsky, 1999).

Masking can be classified as positive or negative, depending on if the masked process exhibits an increase or decrease by the stimulus, respectively. For example, a diurnal animal usually shows positive masking (increase in activity) in the presence of light or after increased illumination; on the contrary, a negative masking occurs (decrease in activity) with darkness or after decreased in illumination. For nocturnal animals, positive masking is displayed in response to darkness whereas negative masking occurs in response to light. In addition, there are cases known as paradoxical masking in which light decreases activity in diurnal animals and increases it in nocturnal ones (Mrosovsky, 1999).

The best way to differentiate if an overt rhythm is entrained or masked is to show the existence of phase control after removing the cyclical stimuli. Thus, a rhythm is entrained if after removing the periodic stimuli it begins to free-run with the phase determined by the

zeitgeber. By contrast, if the rhythm stars to free-run from a different phase, the rhythm was masked (Figure 2) (Johnson et al., 2004).

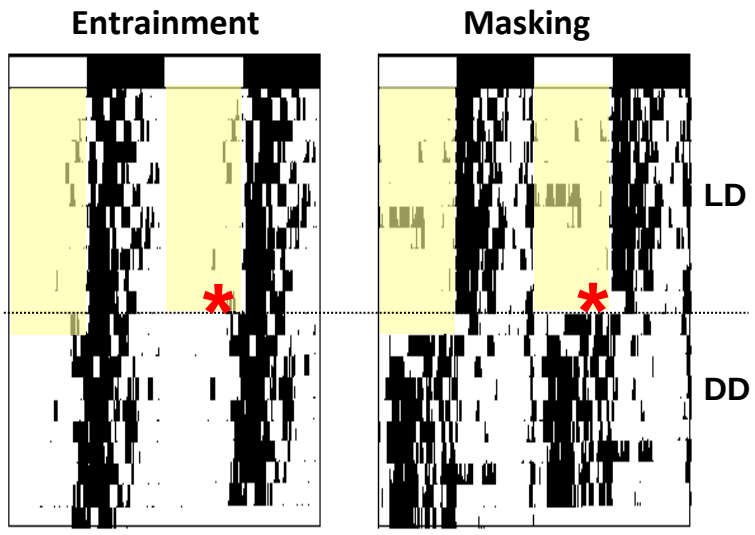

Figure 2. Differences between entrainment and masking. These actograms represent the wheel running activity rhythms of two animals. Both of them exhibit a nocturnal pattern under a 12:12 light-dark cycle (LD). When they are released into constant darkness (DD), the animal on the left starts to free-run from its former LD phase, showing that it was entrained. By contrast, the animal on the right free-runs from a different phase (phase advance) demonstrating that its activity rhythm was masked (Data from our lab).

1.2. THE MAMMALIAN CIRCADIAN TIMEKEEPING SYSTEM

The circadian system consists of a set of brain and peripheral structures that generate and maintain a temporal organization in the organisms, and their synchronization to the 24h environmental cycles. The circadian system can be conceptualized with three main components: the **inputs**, the **pacemaker** and the **outputs** (Figure 3) (Golombek & Yannielli, 2006). The central pacemaker receives inputs from the environmental cues or *zeitgebers,* which reset and entrain its activity. Here, the synchronized activity of the pacemaker generates a coherent circadian signal that is communicated, through different output pathways, to the effector system in order to regulate overt circadian rhythms. These rhythms can have a feedback effect onto the circadian system that generates them, modifying its activity. Finally, external cues can directly or indirectly affect the overt rhythms generated by the circadian system in a phenomenon called “masking” (see above).

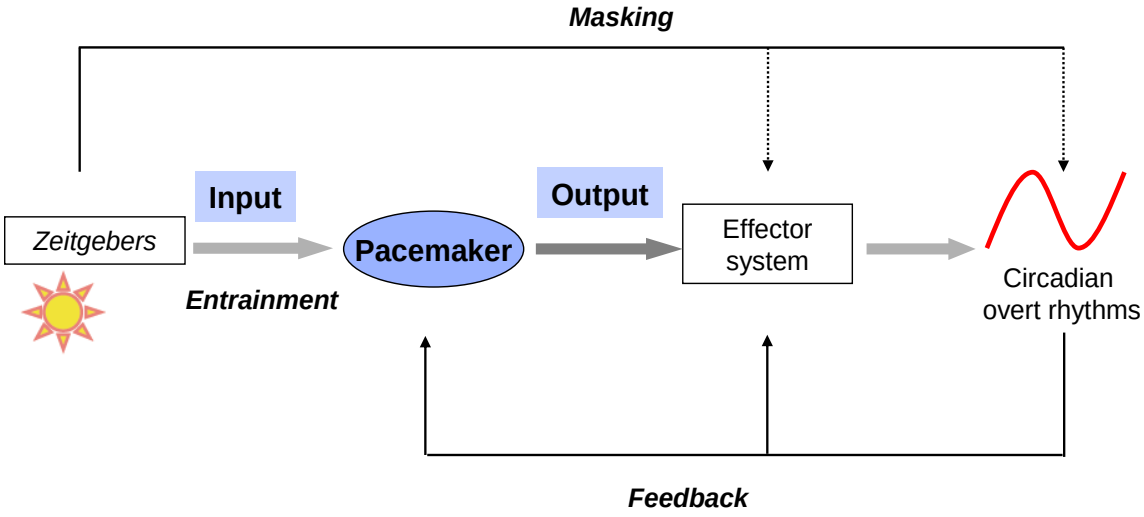

Figure 3. Diagram of the basic circadian system organization showing its main components: inputs, pacemaker, and outputs. See the text for more details.

1.2.1. Central Pacemaker

In 1972, two different research groups independently localized the mammalian central pacemaker in the hypothalamic suprachiasmatic nuclei (SCN) (Moore & Eichler, 1972; Stephan & Zucker, 1972). This brain structure is located immediately dorsal to the optic chiasm. SCN lesion eliminates a variety of circadian rhythms, including motor activity, drinking and feeding, body temperature, blood pressure, heart rate and the rhythmic secretion of a variety of hormones. Further, transplantation experiments have provided unequivocal evidences that the central pacemaker is situated in the SCN. Indeed, SCN-lesioned mutant animals, with original period of 20h, started to show 24h rhythms after receiving fetal tissue from a donor animal with a 24h period (Ralph et al., 1990).

The SCN consists of a pair of nuclei located bilaterally to the third ventricle. It is made up of approximately 20,000 densely packed neurons that are heterogeneous and small (10-15µm in diameter), as well as many populations of glial cells (e,g, astrocytes). The densely packed arrangement of its neurons and glia makes the SCN histological easy to locate and identify. Anatomically, the SCN can be divided into two distinct parts, the **dorsomedial area** or "*shell*", and the **ventrolateral area** or "*core*" (Figure 4).

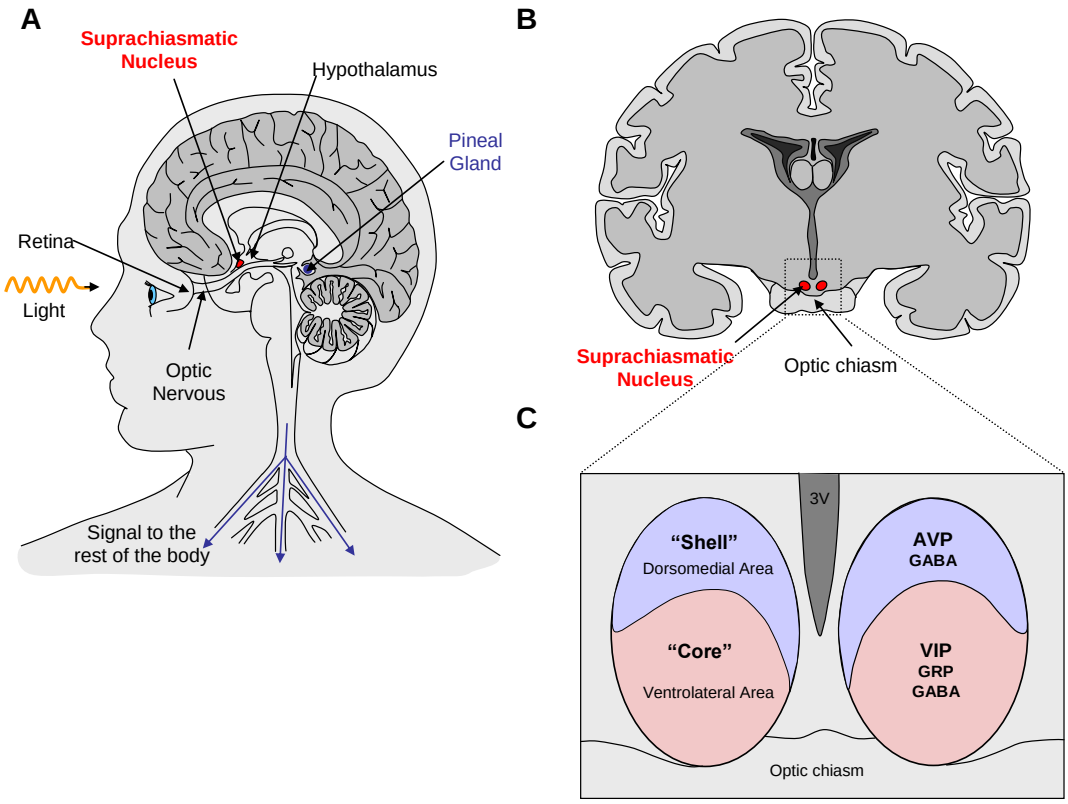

Figure 4. The **master circadian clock: suprachiasmatic nucleus (SCN).** (A) Location of the SCN in the human brain. The SCN receives light information from the retina via the retinohypothalamic tract and conveys timekeeping signal to the rest of the body. (B) Coronal section of the human brain containing the SCN immediately dorsal to the optic chiasm. (C) Organization of the SCN. The dorsomedial area or "shell" and the ventrolateral area or "core" and the major neuropeptides synthesized in each part. 3V, third ventricle; AVP, neuropeptide arginine vasopressin; GABA, γ-aminobutyric acid; VIP, vasoactive intestinal peptide; GRP, gastrin releasing peptide.

Although most SCN neurons synthesize GABA, the ventrolateral region is characterized by a high density of neurons which express vasoactive intestinal polypeptide (VIP) and gastrin releasing peptide (GRP). In the dorsomedial area, the cell density is lower and is characterized by the presence of neurons expressing mainly arginine vasopressin (AVP) (Moore, 1996; Abrahamson & Moore, 2001).

The core of the SCN acts as an integrator and receives multiple inputs originating from the retina, through the retinohypothalamic tract (RHT), the thalamic intergeniculate leaflet (IGL) by the geniculohypothalamic pathways, as well as from the raphe nuclei. By contrast, the shell region receives inputs mainly from the SCN core, but communicates with multiple areas of the brain and body using several neuropeptide signaling (see below). Although the specific functions of the core and shell regions of the SCN are not fully known, the ventrolateral area seems to play an important role in synchronizing SCN timekeeping processes with the LD cycle, whereas the shell may be more involved in the modulation of the clock outputs.

Individual neurons of the SCN contain the molecular clock which drives self-sustained oscillations in their firing rates. Electrical activity in the SCN follows a circadian rhythm; neurons display higher firing rate during the day than at night. Thus, the SCN represents a multi-oscillator network of neurons whose electrical activity are synchronized by a high degree of cell-to-cell couplings in order to generate a coherent rhythm output that conveys timekeeping signal to the rest of the body (Brown & Piggins, 2007). This intercellular synchronization in the SCN is achieved mostly by chemical synapses using GABA and VIP as their main neurotransmitters, but some gap junction connections have also been reported (Aton & Herzog, 2005).

1.2.2. Input pathways to the SCN

Inputs pathways to the SCN transmit both photic and non-photic information from *zeitgebers* to the central pacemaker in order to synchronize its activity and maintain a stable phase-relationship with the environmental cycles. Light is the most important *zeitgeber* for the circadian system in living organisms (Morin & Allen, 2006).

Light perception occurs in the retina. Here, the classical photoreceptors (the rods and cones) are the main sensors of light. The light signal is then sent to the brain by the retinal ganglion cells (RGCs) (Wassle, 2004). Most of the RGCs are involved in visual processing and project their axons to the visual cortices. However, a subpopulation of RGCs containing a functional photopigment known as melanopsin forms the non-image forming **retinohypothalamic tract** (RHT) and sends its projections to some key areas of the hypothalamus including the SCN (Berson et al., 2002; Hattar et al., 2002). Melanopsing-containing-RGCs not only receive light information from the cones and rods, but they are themselves intrinsically photosensitive, particularly to light ranging from 460-480 nm (Turner & Mainster, 2008); hence they are termed (**ipRGCs**) (Figure 5). Glutamate and pituitary adenylate cyclase-activating polypeptide (PACAP) are the primary neurotransmitters released by the RHT terminals in the SCN. The discovery that these ipRGCs are able to fire action potentials in response to light, independently of the rods and cones, explain why blind mice lacking rods and cones can entrain to photic cues (Freedman et al., 1999).

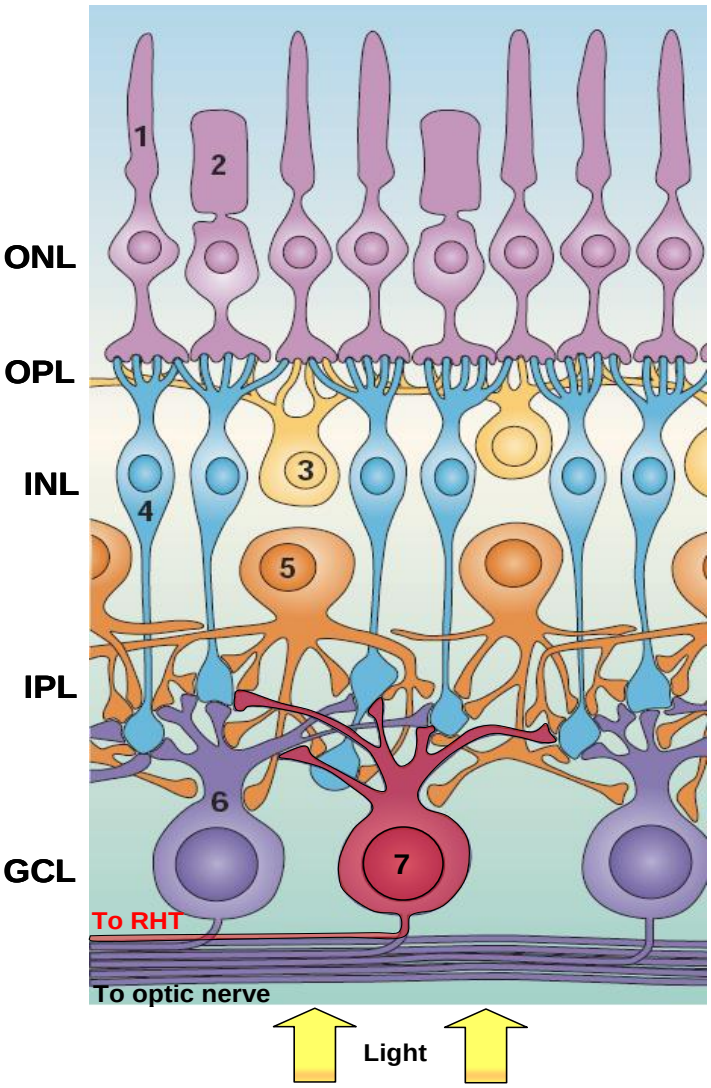

Figure 5. Retinal photoreception. The mammalian retina includes six basic cell types. The classical photoreceptors, rods (**1**) and cones (**2**) which transmit light information to the retinal ganglional cells (RGCs, **6**) through bipolar cells (**4**). Lateral connections between rods and cones are mediated by horizontal cells (**3**), whereas, lateral connections between bipolar and RGCs are relayed onto amacrine cells (**5**). The axons from most of the RGCs form the optic nerve and project to brain areas involved in visual image forming. A subset of RGCs contains the photopigment melanopsin and is intrinsically photosensitive (**ipRGCs**, **7**). Their axons form the retinohypothalamic tract (RHT) and project mainly to the suprachiasmatic nucleus, mediating nonvisual photic input to the central pacemaker. The retina has a laminar distribution: ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglionar cell layer. Modified from Wässle, 2004.

The SCN also receives photic information indirectly from the retina through the **geniculohypothalamic tract** (GHT) originating in the IGL (Figure 6). The main neurotransmitters involved in these pathways are GABA and neuropeptide Y (Harrington, 1997). A third input pathway to the SCN comes from the median raphe nuclei (MRN) which project directly to the central clock, while inputs originating from the dorsal raphe nuclei (DRN) indirectly reach the SCN via the IGL. The neurotransmitter utilized by these midbrain pathways is serotonin (Morin, 1999). The IGL and raphe communicate non-photic signals to the SCN, such as exposure to a novel running wheel, interruption of sleep, and food restriction (Mrosovsky, 1996). The SCN also receives afferents from the limbic system, many regions of the hypothalamus and the paraventricular thalamus (see below).

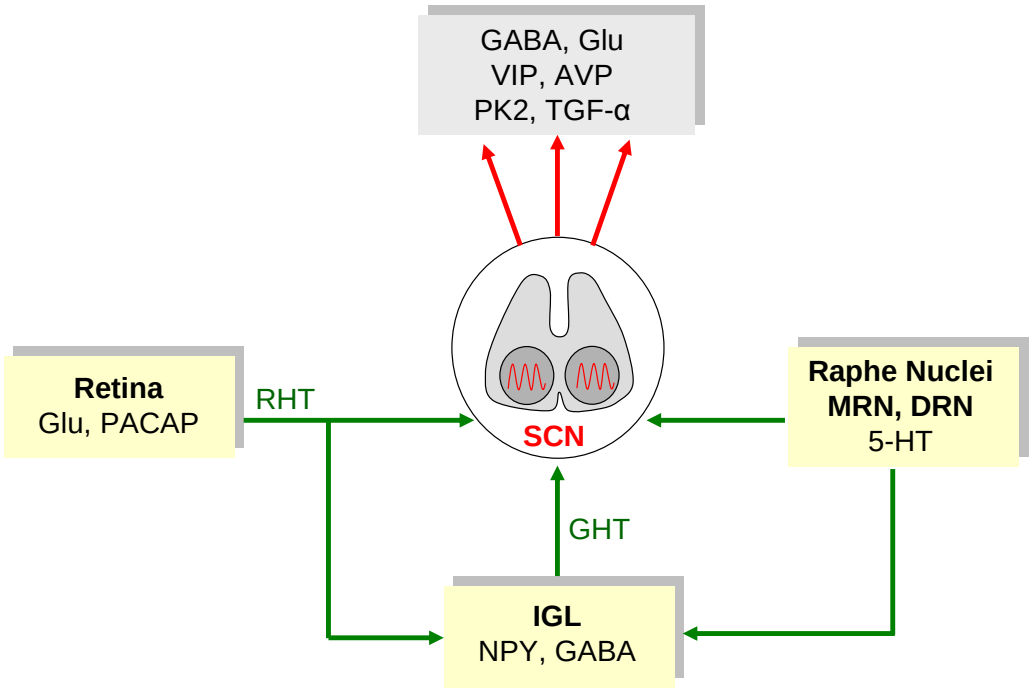

Figure 6. Input pathways to the suprachiasmatic nucleus (SCN). The SCN receives glutamatergic and peptidergic projections from the retina, GABAergic and peptidergic projections from the thalamic intergeniculate leaflet (IGL) and serotonergic projections from the raphe nuclei. RHT, retinohypothalamic tract; GHT, geniculohypothalamic tract; Glu, glutamate; PACAP, pituitary adenylate cyclase-activating polypeptide; NPY, neuropeptide Y; GABA, γ-aminobutyric acid; AVP, neuropeptide arginine vasopressin; VIP, vasoactive intestinal peptide; PK2, prokineticin-2; TGF-α, transforming growth factor α; 5-HT, 5-hydroxytryptophan; MRN: Median raphe nuclei; DRN: Dorsal raphe nuclei.

1.2.3. Molecular clock

The core molecular clock machinery is an autoregulatory genetic oscillator integrating negative and positive transcriptional/translational feedback loops (TTFL) that drive recurrent rhythms in mRNA and protein levels of key clock components with a rhythmicity close to 24 hours (Ko & Takahashi, 2006). The molecular clock components are defined by the genes whose protein products are needed for the generation and regulation of circadian rhythms within individual cells in the SCN and peripheral oscillators. The main clock genes identified in mammals are transcriptional activators (positive elements) ***Clock*** and ***Bmal1***, and the regulator products of the ***Period*** (*Per1-2-3*) and ***Cryptochrome*** (*Cry1-2*) genes which drive the negative arm of the feedback loop (Reppert & Weaver, 2002)(Figure 7).

CLOCK and BMAL1 are transcription factors containing bHLH domains ("basic Helix-Loop-Helix") which confer the ability to bind to DNA. These proteins heterodimerize in the cytoplasm and translocate to the nucleus where they activate transcription by binding to an E-box (nucleotide sequence CACGTG). Specifically, CLOCK:BMAL1 heterodimers activate the transcription of *Per*, *Cry, Rev-erbα* as well as other clock-controlled genes (CCG), including those that are key regulators of the cell cycle and metabolism.

The negative feedback loop is composed of the heterodimers PER:CRY that translocate to the nucleus to inhibit the activity of CLOCK:BMAL1 which in turn represses their own transcription. On the other hand, REV-ERBα represses *Bmal1* transcription by binding to Rev-erbα/ROR response elements in its promoter region. Consequently, *Bmal1* mRNA levels decline while *Per* and *Cry* levels increase (Figure 8). When heterodimers CRY:PER repress their own transcription in the nucleus (through actions on CLOCK:BMAL1), *Rev-erbα* transcription is also inhibited resulting in de-repression (activation) of *Bmal1* transcription.

The rhythmicity of the molecular clock is kept close to 24h mainly by post-translational modifications such as phosphorylation and ubiquitination (Lee et al., 2001); where proteins such us Casein kinase 1 epsilon (CK1ε) and Casein kinase 1 delta (CK1δ) play a critical role. Theses processes affect the stability and translocation of proteins to the nucleus and therefore modulate the functioning of the clock (Gallego & Virshup, 2007).

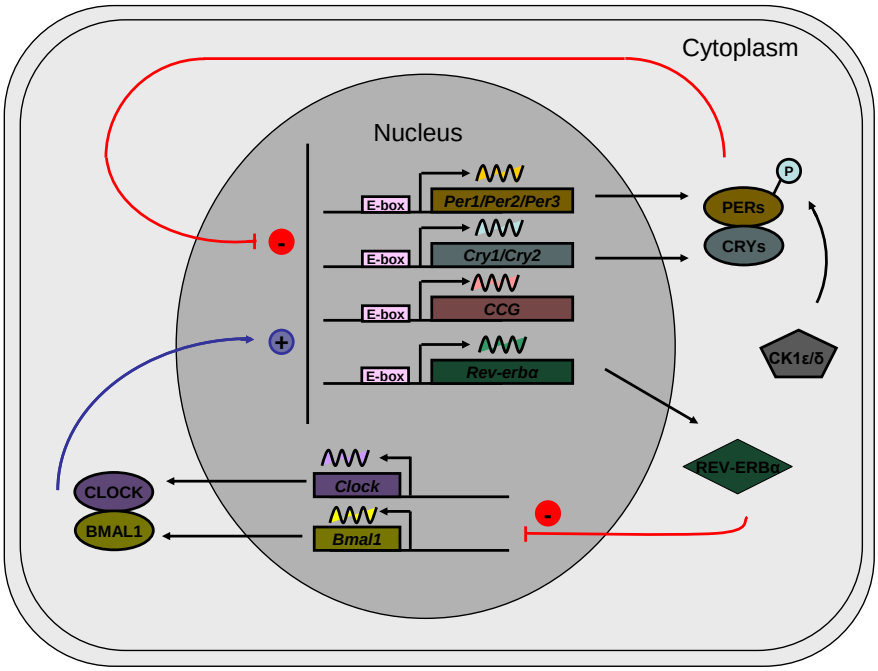

Figure 7. Simplified model of the mammalian molecular clock. The clock comprises of the interaction between two feedback loops: one positive (blue) including CLOCK and BMAL1 proteins as main components, and one negative (red) which involves PER and CRY proteins. See the text for more details.

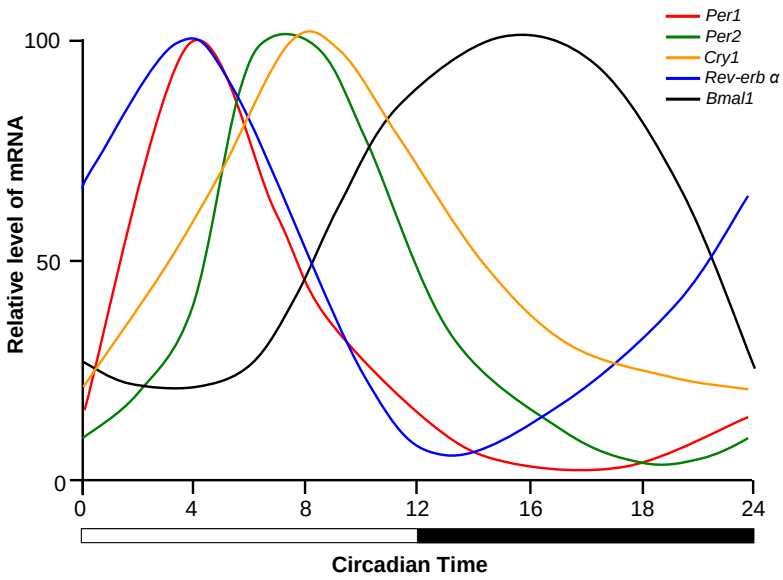

Figure 8. Relative levels of mRNA of the main key clock genes expression in the suprachiasmatic nucleus across one circadian cycle. *Per1* and *Rev-erbα* mRNA levels peak early in the subjective day, while *Bmal1* levels remains low; *Per2* and *Cry1* peak later in the subjective day; *Bmal1* peaks during the subjective night. Subjective day and night are indicated by the white and black bars, respectively, at the bottom of the graph. Modified from Guilding & Piggins, 2007.

1.2.4. Outputs of the central pacemaker

The timekeeping signals from the SCN are transmitted to the rest of the brain and body to orchestrate daily rhythms in physiology, behavior, and metabolism. The SCN conveys the temporal information by neural efferents to other brain regions and to peripheral organs and also by diffusible humoral signals. As mention above, SCN lesion results in a loss of rhythmic patterns of many physiological and behavioral functions while transplantation of fetal SCN tissue from a donor animal restores the rhythmicity. Furthermore, SCN transplantation was able to restore locomotor activity rhythmicity in SCN-lesioned animals, even when the graft was encapsulated in a semi-permeable membrane which prevented neural growth (Silver et al., 1996). Therefore, the SCN must coordinate the behavioral activity rhythms by humoral mediators. Some of the molecules described as SCN output signal includes the transforming growth factor (TGFα) (Kramer et al., 2001), cardiotropin (Kraves & Weitz, 2006), prokineticin-2 (PK2) (Cheng et al., 2002) and AVP.

The SCN connections with other brain areas have been elucidated by anterograde and retrograde neurotracers (Watts & Swanson, 1987). Within the hypothalamus, the SCN sends dense innervation to both the dorsal and ventral subparaventricular zone (dSPZ and vSPZ, respectively), and to the dorsomedial hypothalamus (DMH), as well as the paraventricular nucleus (PVN) (Saper et al., 2005). The dSPZ projects to the medial preoptic area (MPO) and controls circadian rhythms in body temperature. The DMH receives both direct and indirect inputs from the SCN via the vSPZ, and controls a range of circadian rhythms ouputs: sleep-wake cycle through projections to the ventrolateral preoptic area (VLPO); and activity and feeding cycles by connections with the orexinergic system and melanin-concentrating

hormone neurons in the lateral hypothalamus (LHA) (Figure 9). In the thalamus, the SCN neuronal efferents innervate the paraventricular nucleus of the thalamus and the IGL. Collectively, the main neurotransmitters released in these projections are GABA, VIP, and AVP (Kalsbeek & Buijs, 2002). The SCN also projects to many hypothalamic endocrine neurons, including gonadotropin-releasing hormone (GnRH) neurons, thereby modulating the reproductive cycle; corticotropin-releasing (CRH) neurons located in the paraventricular nucleus of the hypothalamus (PVN), which regulate corticosteroid release; and thyrotropin-releasing hormone (TRH) (Buijs & Kalsbeek, 2001).

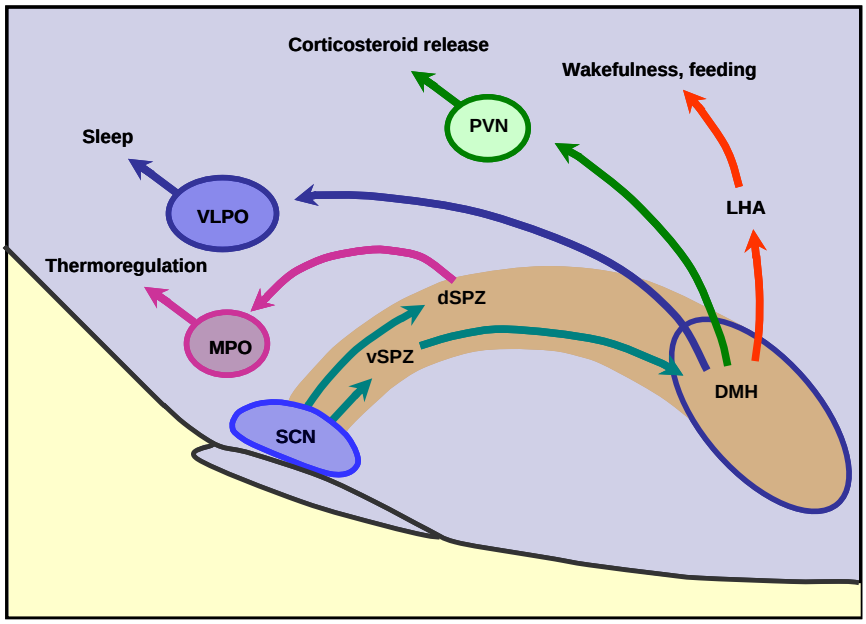

Figure 9. Suprachiasmatic nucleus (SCN) connections with other hypothalamic areas to orchestrate circadian rhythms in body temperature, sleep-wakefulness, corticosteroid level and feeding. See the text for more details. vSPZ, Ventral subparaventricular zone; dSPZ, dorsal subparaventricular zone; DMH, dorsomedial nucleus of the hypothalamus; MPO, medial preoptic area; VLPO, Ventrolateral preoptic area; PVN, Paraventricular nucleus; LHA, Lateral hypothalamic area. Modified from Saper et al., 2005.

Melatonin

Melatonin is a hormone primary synthesized by the pineal gland and transmits timekeeping signals of the SCN to the rest of the body (Pevet & Challet, 2011). The precursor for melatonin synthesis is aminoacid L-tryptophan which is converted to 5-hydroxytryptophan and then to serotonin. Next, serotonin is acetylated by aryl alkylamine N-acetyltransferase (**AA-NAT**) obtaining N-acetyl serotonin, which finally is methylated by Hydroxyindole-O-methyltransferase (HIOMT) to become melatonin. AA-NAT is the key enzyme in the synthesis of melatonin, and shows a circadian rhythm in its activity/levels that is indirectly controlled by

the SCN (Pandi-Perumal et al., 2006). The SCN controls the synthesis of melatonin via the PVN which sends projecting nerve fibers to the intermediolateral column of the spinal cord (IMC) and then to the pineal gland via the superior cervical ganglion (SCG) (Benarroch, 2008) (Figure 10A). Release of noradrenaline at night from postganglionic sympathetic fibers acts on β-adrenergic receptor in the pinealocytes membrane and triggers an intracellular cascade which promotes enzymatic activity of AA-NAT. The synthesized melatonin is then released into the bloodstream and CSF and conveys temporal signal of the SCN to the rest of the body (Figure 10B). During the light phase, increased SCN electrical activity inhibits neurons of the PVN which in turn decreased AA-NAT activity in the pinealocytes and melatonin production.

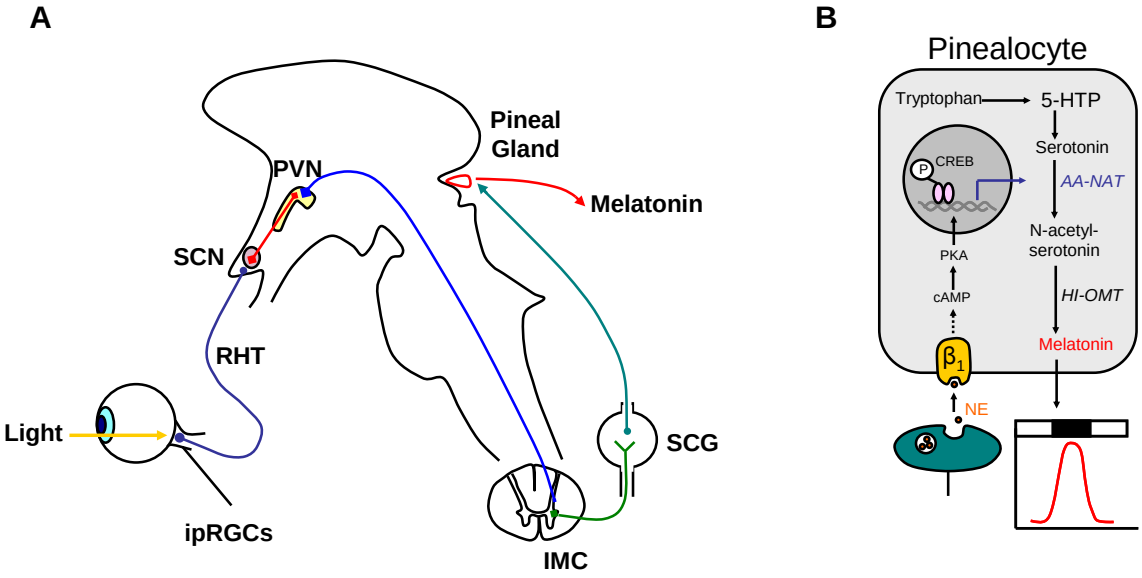

Figure 10. Circadian regulation of melatonin secretion. (A) Light information reaches the suprachiasmatic nucleus (SCN) through retinohypothalamic tract (RHT). The SCN sends projections to the paraventricular nucleus of the hypothalamus (PVN) which, after contacting the intermediolateral column of the spinal cord (IMC) and superior cervical ganglion (SCG), reach the pineal gland and control melatonin synthesis. (B) During the dark phase, the norepinephrine (NE) stimulates β1 receptors. This stimulation leads to the activation of adenylate cyclase and cAMP levels increase. This second messenger activates protein kinase A (PKA), which phosphorylates CREB (cAMP response element binding protein, CREB). CREB binds to cAMP response element (CRE) located in the gene promoter AANAT (arylalkylamine N-acetyltransferase), a key enzyme in the melatonin´s synthesis pathway. HI-OMT, hydroxyindole-O-methyltransferase; 5-HTP, 5-Hydroxytryptophan. Modified from Benarroch, 2008.

In all organisms thus far studied, melatonin secretion shows a robust circadian rhythm peaking at night and with low levels during the day; this has led melatonin to be known as the "*chemical expression of darkness*" (Reiter, 1991). Therefore, circadian changes in the daily production and secretion of melatonin are used as a daily clock which informs the organism that it is night-time; it also can be used as a calendar since the duration of night-time elevation

of melatonin is proportional to the duration of the dark phase (Reiter, 1993). Thus, the duration of melatonin secretion is longer in the short days of winter and shorter in the summer nights. Melatonin is a signal of darkness both in nocturnal and diurnal animals; in nocturnal animals, the peak of melatonin coincides with their active phase and is associated with arousal, physical activity and increased body temperature; whereas in diurnal animals, melatonin levels rise during their resting period and is associated with sleepiness, low body temperature and increased immune responses (van den Heuvel et al., 2005).

Melatonin exerts numerous physiological functions and displays pleiotropic effects throughout the body. It is involved in circadian rhythms regulation by modulating the SCN electrical activity, and by phase-shifting the circadian clock. These chronobiological effects of melatonin are mediated by its interactions with the MT1/MT2 membrane receptors (Pandi-Perumal et al., 2008). While MT1 activation modulates the SCN electrical activity, MT2 seems to be involved in the phase-shifting effects of melatonin that is dependent on circadian time, duration of exposure to melatonin, and melatonin receptors sensitivity. Thus, when exogenous melatonin is administered late in the subjective day (dusk) or in the early phase of the night, it phase-advances the circadian clock; by the contrast, delays of the circadian rhythms or no response are observed when melatonin is administered late in the subjective night or at early daytime (Lewy et al., 1998).

In addition to its role as a chronobiotic (Arendt & Skene, 2005), melatonin has antitumoral, neuroprotective, immunomodulatory, anti-inflammatory and antioxidant properties (Pandi-Perumal et al., 2006; Pandi-Perumal et al., 2008). Melatonin's protective effects against oxidative stress may partially involve its interactions with the enzyme quinone reductase 2 and the RORα receptors, as well as its capacity to act as a direct scavenger of free radicals (Galano et al., 2011).

Thus, melatonin can be used as a potential treatment for a variety of pathologies (See section 1.3.3).

Orexin/hypocretin system: integrator for arousal and circadian cues.

As mentioned above, the SCN sends projections to diverse hypothalamic areas in order to control key physiological and behavioral processes, such as the sleep-wake cycle and locomotor activity rhythm (Sakurai, 2007). Orexins are neuropeptides implicated in the regulation of sleep, wakefulness, arousal, and feeding. In mammals, orexins are mostly synthesized by neurons of the lateral hypothalamus, perifornical region and dorsomedial nucleus of the tuberal hypothalamus. Fibers containing orexins project to numerous brain

regions, which play important role in the regulation of the behavioral state, including the raphe nuclei, the locus coeruleus, and the tuberomammillary nucleus, and also innervate the SCN and the IGL (Peyron et al., 1998; Cutler et al., 1999; McGranaghan & Piggins, 2001; Nixon & Smale, 2007). The orexinergic system, therefore, contains reciprocal connections with the SCN which provides a well defined pathway for integrating arousal and circadian cues.

1.2.5. Extra-SCN oscillators

Results emerging in the past few years have challenged the concept of a uniclock model of the circadian system (Hastings et al., 2008). Clock gene expression and their protein products are not only confined to the SCN, but are found in many other brain regions (Yamamoto et al., 2001; Shieh, 2003). Daily rhythms in these genes/proteins are reported not only in extra-SCN brain sites (including nuclei in the hypothalamus, thalamus, olfactory bulb and cerebelum) but also in most peripheral tissues (liver, kidney, heart, spleen, lung, stomach, adipose tissue, pancreas and skeletal muscle) (Dibner et al., 2010) (Figure 11). It is believed that these extra-SCN oscillators form part of a network of "slave" oscillators that, despite of having their own endogenous rhythms, obtain essential circadian time-cues from the SCN (Guilding & Piggins, 2007). Multiple synchronization pathways for the setting up of local time in these peripheral clocks have been identified, and although, mainly include hormonal and neuronal outputs from the SCN, feeding time cues and local temperature are also important (Dibner et al., 2010). Collectively, these extra-SCN oscillators are believed to be important for daily tissue-specific timing in physiology and behavior (Mendoza & Challet, 2009). Thus, the circadian system is an organized hierarchy of multiple circadian oscillators, with the SCN as the main circadian pacemaker, synchronizing other oscillators residing in other regions of the nervous system and peripheral tissues.

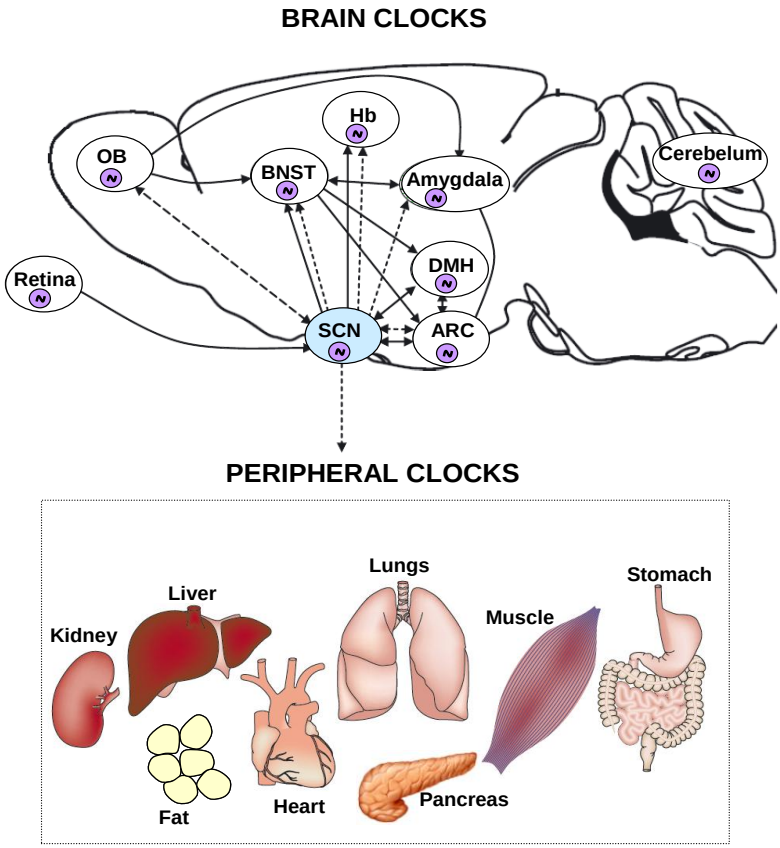

Figure 11. Extra-SCN brain oscillators and peripheral clocks. Clock genes are expressed not only in the master circadian pacemaker, the suprachiasmatic nucleus (SCN), but also in many extra-SCN brain regions and peripheral tissues as well. The entrained SCN coordinates the phase of these oscillators and couple them to maintain an oscillatory synchrony in the body's physiology, metabolism and behavior. In the diagram of the brain shown, solid arrows indicate direct connections, and dashed arrows show indirect connections; reciprocal connections are indicated by double arrows. OB, olfactory bulb; ARC, arcuate nucleus of the hypothalamus; BNST, bed nucleus of the stria terminalis; DMH, dorsomedial hypothalamus; Hb, habenula.

1.3. CIRCADIAN SYSTEM AND HEALTH

The circadian system plays a critical role not only in the generation of daily rhythms but also in the orchestrating of these rhythms to maintain a stable internal temporal order in the physiology and behavior of the organism. It is agreed that the maintenance of this temporal order is a necessary condition for good health (Waterhouse & DeCoursey, 2004). By analogy, we could compare this internal temporal order with an orchestra whereby each rhythm represents a musician playing an orchestral instrument. Thus, all of them have to play in harmony at the right moment in order to produce Beethoven's beautiful 5th symphony, for example. One could imagine the horrible sound of an out-of-tune orchestra in which a beautiful piece of music can turn into unrecognizable noise. Within the circadian system, this disruption in the harmony of its rhythms is called **chronodisruption** (CD). Therefore, CD is defined as a significant disturbance in the internal temporal order of biochemical, physiological

and behavioral circadian rhythms. It is also considered as the misalignment between our internal temporal organization and environmental time cues (Garaulet & Madrid, 2010; Ortiz-Tudela et al., 2012).

Disruption of the circadian system manifests in several ways, including dampening in the amplitude of the rhythms, total loss of rhythmicity, lower inter-daily stability, fragmentation of the overt circadian rhythms, phase advances or delays between different peripheral clocks and the SCN, as well as a partial inversion of circadian rhythms, as seen in night shift workers.

Epidemiological studies show that CD induced by shift work, chronic jet lag, social jet lag and exposure to bright light at night is associated with an increased incidence of pathological states, including metabolic syndrome, cardiovascular disease, cognitive and affective impairments, sleep disorders, some forms of cancers and premature aging and reproductive abnormalities.

1.3.1. Factors involved in chronodisruption

CD can result from disturbances in **the inputs to the clock.** As discussed above, the LD cycle is the most important *zeitgeber* for the circadian system. Since the origin of life on earth, living organisms have been exposure to natural LD cycles in parallel with high environmental temperature during the day and lower level at night. However, the invention of artificial light allows human beings to extend their active phase well into the night creating 24h-societies (Rajaratnam & Arendt, 2001). This 24h society has lead to many alterations in the inputs to the biological clock including:

Exposure to bright light at night (**LAN**). One of the main consequences of LAN is the inhibition of the melatonin secretion (Rahman et al., 2008; Rahman et al., 2011), a hormone which transmits timekeeping signals from the SCN to the rest of the body, as described above. This exposure to LAN not only disrupts the rhythmicity of different behavioral and physiological rhythms, such as the sleep-wake cycle, but also deprives the body of the night-time melatonin surge and its antitumoral, antioxidant and immunostimulating properties (Erren et al., 2003; Reiter et al., 2007).

Further, it is not only that we are being exposure to LAN, during the day we spend most of our time indoors (Hebert et al., 1998) under artificial lighting conditions which provide only around 1% of the intensity of sunlight. Exposure to LAN, together with exposure to reduced light intensity and inappropriate spectrum during the day, lead to a decrease in the contrast of the LD cycle. This, in turn renders a LD cycle with a reduced amplitude/strength to synchronize daily rhythms in our bodies (van Someren & Riemersma-Van Der Lek RF, 2007).

Indeed, it is known that a high contrast in the synchronizers is associated with high amplitude oscillations in circadian rhythms (Martinez-Nicolas et al., 2011).

Jet lag and shift work. Intercontinental travel and rotating shift work cause abrupt shifts in the LD cycle. In the last few years, social jet lag has also been added to this list (Wittmann et al., 2006). During re-entrainment to the new phase-shifted LD cycle there is a transient period of internal desynchronization within the SCN (Reddy et al., 2002; Nagano et al., 2003) and between the SCN and peripheral oscillators (Davidson et al., 2009), due to different rates of resynchronization between these structures. This results in a disruption of the normal phase relationship between behavioral and physiological rhythms. Finally, the temporal order progressively re-emerges and the individual exhibits circadian rhythms that are entrained to the new LD cycle (Moore-Ede et al., 1977). However, when these abrupt shifts in the LD cycle become frequent, such as for flight attendants and pilots or rotating shift workers, there is a permanent misalignment between the circadian clock and the external cues; this repeated CD leads to health problems.

The photic input to the circadian system can be affected by ocular pathologies and blindness. The effectiveness of light as a synchronizer depends on the capacity of the lens to transmit light and on the neuronal integrity of the photic pathways (photoreceptors and optic nerve). The functional alteration of these structures can cause reduced light stimulation of the iRGCs leading to decreased photic input to the SCN and eventual alteration of the circadian rhythmicity. Indeed, studies have shown that reduced stimulation of the iRGCs reduces the likelihood of entrainment to external light cues. Further, in humans, blindness resulted from a total loss of functional photoreceptors (rods, cones and iRGCs) prevents entrainment of the circadian system to photic-inputs. Intriguingly, in these circumstances, these subjects rely on non-photic inputs, such us social contacts and scheduled meal times, and pharmacological treatments, to synchronize their rhythms (Mistlberger & Skene, 2004; Mistlberger & Skene, 2005). By contrast, visually impaired people with degenerated rod and cones but with functional iRGCs still have the capacity to photic entrainment and, therefore, should be exposed to bright light during the day and avoid light at night in order to optimally synchronize their circadian rhythms.

Meal times. Scheduled meals and food restriction are very important synchronizers for the peripheral clocks (Damiola et al., 2000), such as the liver and adipose tissue clocks, as well as for several extra-SCN brain oscillators (Feillet et al., 2008), including central amygdala, ventromedial hypothalamus and paraventricular nuclei. Food ingestion normally occurs during the active period of the organism; therefore food-elicited time signals and photic stimulation from the LD cycle have an appropriate phase relationship, providing inputs to the circadian

system which lead to coordination among the multiple oscillators (SCN and extra-SCN clocks) (Bass & Takahashi, 2010). However, shifts in the feeding schedule relative to the normal resting/sleep phase triggers shifts in the peripheral oscillators and in some extra-SCN brain oscillators resulting in an uncoupling between the SCN and extras-SCN oscillators (Garaulet et al., 2010; Escobar et al., 2011). This also results in an internal misalignment with the photic external cues.

CD can also result from **alterations of the central pacemaker.** As mentioned above, SCN lesion suppresses a range of circadian rhythms including motor activity, drinking and feeding, body temperature, blood pressure, heart rate and secretion of various hormones (Moore & Eichler, 1972; Stephan & Zucker, 1972). CD can occur due to impairments of the SCN clock itself or malfunctions in clocks in other brain regions. For example, aging in the SCN results in dampened amplitude of its electrical activity and rhythmic expression of the key clock genes, as well as a reduction in the number of neurons expressing AVP and MT1 melatonin receptors (Hofman & Swaab, 2006; Wu & Swaab, 2007). As a result, a reduction of the robustness of the SCN outputs is observed with aging. Some of these features are common in neurodegenerative diseases such as Alzheimer's disease (AD), Huntington's disease and Parkinson's disease (Kondratova & Kondratov, 2012). Development of animal models carrying mutations or knock-out for clock genes, has far-reaching implication for understanding the pathological consequences of clock dysfunction (Ripperger et al., 2011). For example, *Clock* mutant mice show alteration in circadian rhythmicity (Vitaterna et al., 1994), develop obesity and metabolic syndrome (Turek et al., 2005), and show mania-like behavior similar to those observed in patients with bipolar disorder (Wu & Swaab, 2005; Roybal et al., 2007).

Nocturnal secretion of melatonin by the pineal gland pineal acts as an output for the circadian system function. With aging and age-related pathologies like AD, there is a decreased in melatonin levels and dampened amplitude in the circadian rhythm of its synthesis and release (Reiter, 1995; Wu & Swaab, 2005). These changes can be responsible of the sleep-wake cycle disruptions observed in elderly people and, even more so, in AD patients.

1.3.2. Consequences of chronodisruption

An increasing amount of experimental evidences show that the disruption of the circadian system is related with a wide range of health problems. Chronic jet lag can accelerate malignant growth (Filipski et al., 2004) and increase mortality in aged mice (Davidson et al., 2006) and in animals with cardiomyopathic heart disease (Penev et al., 1998), as well as impair hippocampal memory formation in rats (Craig & McDonald, 2008), reduce pregnancy success (Summa et al., 2012), or alter innate immune system responses (Castanon-Cervantes et al.,

2010). It has been shown that the CD generated by food consumption during the normal resting phase leads to weight gain and it can be related with metabolic syndrome and obesity (Arble et al., 2009). These findings are also supported by epidemiological studies, which have shown that CD resulting from long term shift work in nurses or from frequent time-zone travellers (flight attendants and pilots) are associated with a higher incidence of health problems such as breast (Schernhammer et al., 2001; Davis et al., 2001), colorectal (Schernhammer et al., 2003) and prostate cancer (Kubo et al., 2006; Conlon et al., 2007), cognitive impairments (Cho et al., 2000), coronary heart disease (Knutsson & Boggild, 2000), deregulation of the inflammatory responses and higher overall mortality (Knutsson et al., 2004). Dysfunction of the circadian clock also contributes to aging and age-associated pathologies (Kondratov, 2007). Therefore, it is important to understand the impact of CD on the physiological systems.

1.3.3. Chronoenhancement

Health impairments associated with CD are becoming a public health issue. It is important to develop interventions and therapies to re-establish the internal temporal organization and strengthen the circadian system to combat the pathologies associated with CD. To date, most of the therapies are focused on reinforcing the *zeitgebers* and the clock outputs; some of these strategies include: methods to increase contrast between day and night, regular physical exercise, social interactions, feeding schedules and melatonin treatment (van Someren & Riemersma-Van Der Lek RF, 2007).

As already mentioned, the LD cycle is the primary *zeitgeber* for living organisms. Therefore, the exposure to bright light during the day and darkness at night can result in an increased amplitude and phase synchrony of the circadian oscillators and more robust overt rhythms (Martinez-Nicolas et al., 2011). Several studies have shown that exposure to bright light during the day improves the circadian rhythms disturbances (sleep-wake cycle fragmentation) seen in elderly people and Alzheimer patients (Satlin et al., 1992; Okumoto et al., 1998). In addition, bright light therapy improves depressive symptomatology in seasonal affective disorders (SAD) and depression (Wirz-Justice, 1986). Interestingly, exposure to bright light during the daytime enhances the evening rise in melatonin levels in elderly people (Lieverse et al., 2011). As far as possible, exposure to bright LAN should be limited due to its negative effects on melatonin synthesis and disruption of the internal temporal order (Erren et al., 2003). In the last years, controlling light spectrum has come as an important method of regulating the effects of light on physiological processes (Rahman et al., 2008; Rahman et al.,

2011). In those cases where night lighting is essential, it must be poor in the blue spectrum and enriched with warm colours.

Regular physical exercise in human acts as synchronizer of the circadian system (Atkinson et al., 2007) and enhances robustness of the overt rhythms. A significant reduction in the fragmentation of the rest-activity rhythm has been reported in healthy elderly following a fitness-training program (van Someren et al., 1997). In addition, exercise training improves physical health and depression symptoms in AD patients (Teri et al., 2003). In animal models, scheduled voluntary exercise acts a potent *zeitgeber*. Its therapeutic role for improving circadian rhythms in behavior has been demonstrated in mice with dysfunctional circadian pacemaker (Power et al., 2010).

As previously mentioned, scheduled meals is an important synchronizer for peripheral clocks and extra-SCN oscillators. Therefore, establishing regular meal times during the active period favour the internal synchronization between behavioral, physiological and metabolic rhythms (Escobar et al., 2011).

Social interaction may have a role as a synchronizer although its effects on the circadian system are likely to depend on age and species (Mistlberger & Skene, 2004). In human, as social interaction usually comes together with other temporal cues that synchronize the circadian system, such as meal time, exercise, time to sleep and to wake, it is difficult to determine its synchronizing role.

Melatonin treatment. As mentioned in section **1.2.4.**, melatonin has chronobiotic properties since it can phase shift the circadian clock and adjust the timing of internal biological rhythms (Pevet & Challet, 2011); therefore, melatonin has been widely studied as a pharmacological approach to treat circadian rhythms disturbances (Lewy et al., 2006). Daily melatonin administration at a fixed time entrains free-running rhythms in animals kept in the absence of environmental time cues (Redman et al., 1983; Chesworth et al., 1987). This ability to entrain free-running rhythms to a 24h cycle has also been demonstrated in most blind people together with an improvement of the nocturnal sleep and daytime alertness (Arendt & Skene, 2005; Skene & Arendt, 2007). Furthermore, numerous studies confirm the melatonin's usefulness in treating circadian disorders of delayed/advanced sleep phase syndromes (Lewy et al., 2006). In elderly people, melatonin treatment improves the robustness and consolidation of the sleep-wake cycle, both in healthy individuals and in those with AD, although not all studies show significant effects (Wu & Swaab, 2007). Melatonin treatment is also reported to strength the circadian rhythms in aged laboratory rodents (Vivanco et al., 2007). The therapeutic use of melatonin also extends to adjust the body clock and thereby reduce the symptoms of jet lag and shift work sleep disorders (Srinivasan et al., 2008; Brown et

al., 2009; Srinivasan et al., 2010). Indeed, melatonin has been shown to facilitate re-entrainment of the circadian rhythms to shifts of the LD cycles in both, animal models and humans. Over the last years, new pharmacological melatonin receptors agonists have been developed to treat sleep disorders. Ramelteon is a selective MT1/MT2 receptor agonist with a longer half-life compared to melatonin (Kato et al., 2005). It has been shown that ramelteon treatment reduces sleep latency and increases total sleep time in animal models (Miyamoto et al., 2004; Yukuhiro et al., 2004; Fisher et al., 2008) and in clinical trials for treating insomnia (Erman et al., 2006; Roth et al., 2006). However, unlike melatonin, ramelteon has no known direct free-radical-scavenging properties (Mathes et al., 2008).

1.4. ANIMAL MODELS USED IN THIS THESIS TO STUDY CHRONODISRUPTION

Experiments performed in the present PhD dissertation used four different animal models. The ***Octodon degus*** is increasingly used in Chronobiology, since, unlike most other laboratory species, it is a diurnal animal species with photopic vision (see below). Thus, studying the degu can reveal greater insight into human's circadian system in health and disease. Interestingly, the degus has also the ability to shift their activity from the day to the dark phase and become night-active (nocturnal). This provided us with a great opportunity to investigate the possible disruptions of the internal temporal organization of the animal's circadian system that are associated with the animals engaging activity during their normal resting phase. These changes in the internal temporal order that result from the shift from diurnal to nocturnal may be related with health problems associated with shift work in humans.

In addition, the present PhD dissertation also aims to study the circadian functionality in pathologies known to be associated with CD. To do this, it was necessary to focus on established animal models that are already bearing these pathologies. These include animals with: a) Retinitis pigmentosa (**P23H rhodopsin transgenic rat model**) which affects light inputs to the central pacemaker; b) Alzheimer disease (**APPSswe/PS1dE9 transgenic mouse model**) which results in neuronal loss and alterations in the normal functioning of the body circadian clock, as well as in its inputs and outputs; c) cancer (**melanoma-bearing C57BL/6 mouse**), which its progression disrupts the overt circadian rhythms.

Results that emerged from this PhD thesis demonstrate that the use of such variety of animal models provided a deeper understanding of how different pathological modalities disrupt the circadian system. This approach mimics closely many chronodisruptive conditions

in human and gather useful knowledge in the search for better therapeutic strategies to limit CD's negative effects.

1.4.1. *Octodon degus* (Waterhouse, 1848) Suborder: Hystricognatha; Infraorder: Caviomorpha.

The *Octodon degus* is an endemic rodent from Chile. It is found between Vallenar and Curico (28-35^{o} south latitude) on the west slope of the Andes up to 1200m (Woods & Boraker, 1975). The cheekteeth of these animals resemble a figures-of-eight, hence the Family (*Octodontidae*) and the Genus (*Octodon*) names (Figure 12A-B). Their life span in laboratory conditions is at least 5 years, but some can live up to 7 or 8 years (Lee, 2004). The degus exhibit some of human-like age-related diseases, such as atherosclerosis (Homan et al., 2010) diabetes (Varma et al., 1977), cataracts (Worgul & Rothstein, 1975) and Alzheimer (Inestrosa et al., 2005; van et al., 2011).

The degus have been characterized primarily as diurnal animals with two major activity bouts at dusk and dawn (Fulk, 1976). Observations in the wild reported an increase in the degus' diurnal activity during winter days, and a limitation of its activity to dawn and dusk during the hot and dry days of summer (Kenagy et al., 2002).

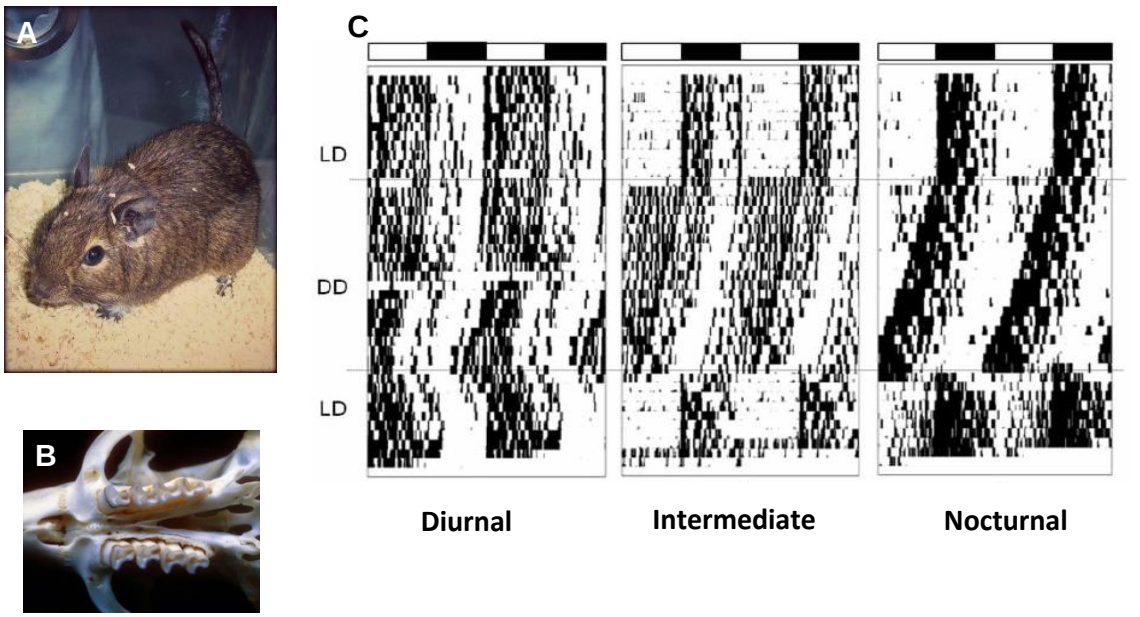

Figure 12. *Octodon degus*. (A) Photograph of an adult male degu from our lab. (B) The cheekteeth of the degus resembling a figures-of-eight, from which this species obtains its name. Photograph by Vladimír Motyčka. (C) Chronotype expression of degus. Wheel running activity actograms from a diurnal, intermediate with nocturnal behavior, and nocturnal degu chronotype. First, animals were kept under 12h light:12h dark (LD) cycle, then released into constant darkness (DD), and finally returned back into LD conditions. Modified from Vivanco et al., 2009.

The degus' flexibility to express a specific chronotype under the natural environment may be important, serving as an adaptive process to seasonal and environmental temperature changes, as well as predator-prey relationship cycle, or food availability (Lagos et al., 1995; Kenagy et al., 2002; Vivanco et al., 2010a; Vivanco et al., 2010d).

In the laboratory, some degus have the ability to switch from day to night–time activity when free access to running wheels is provided (Kas & Edgar, 1999). Recently, it was showed that both masking and entrainment processes were involved in the nocturnalism of the degus (Vivanco et al., 2009) and that room temperature plays an important role in the variations of the degus' activity pattern (Lee, 2004; Vivanco et al., 2010d) (Figure 12C). Therefore, degus offers us the opportunity to study within the same species the basic mechanisms underpinning activity phase preferences in mammals.

Along four of the experimental chapters presented in this dissertation, we investigated whether oppositely phased activity rhythms in diurnal and nocturnal degus also implies a complete shift in their internal temporal organization. We looked at the nocturnalism of the degus from a new point of view, as a potential model to study disruption in the temporal organization.

1.4.2. P23H-3 rhodopsin transgenic rat model of retinitis pigmentosa (RP)

LD cycle is the primary *zeitgeber* for the circadian system, but its effectiveness to synchronize the activity of the central pacemaker depends on the degree to which the light perception is conserved. Therefore, it is common that people with ocular pathologies and blindness shows alterations in their circadian rhythms (Skene & Arendt, 2007; Drouyer et al., 2008). RP is a heterogeneous group of retinal degenerative disorders which causes a progressive loss of retinal function and represents a major cause of blindness. Mutations in the rhodopsin gene have been linked with autosomal dominant RP. One of the most common mutations is the P23H, a transversion in codon 23 corresponding to a proline-histidine substitution (Dryja et al., 1990). P23H transgenic rats bearing this mutation are useful model to assess the circadian rhythms alterations associated with ophthalmic degeneration (Machida et al., 2000; Cuenca et al., 2004; Kolomiets et al., 2010) (Figure 13) as well as monitoring the effectiveness of treatments to prevent those alterations (Fernandez-Sanchez et al., 2012).

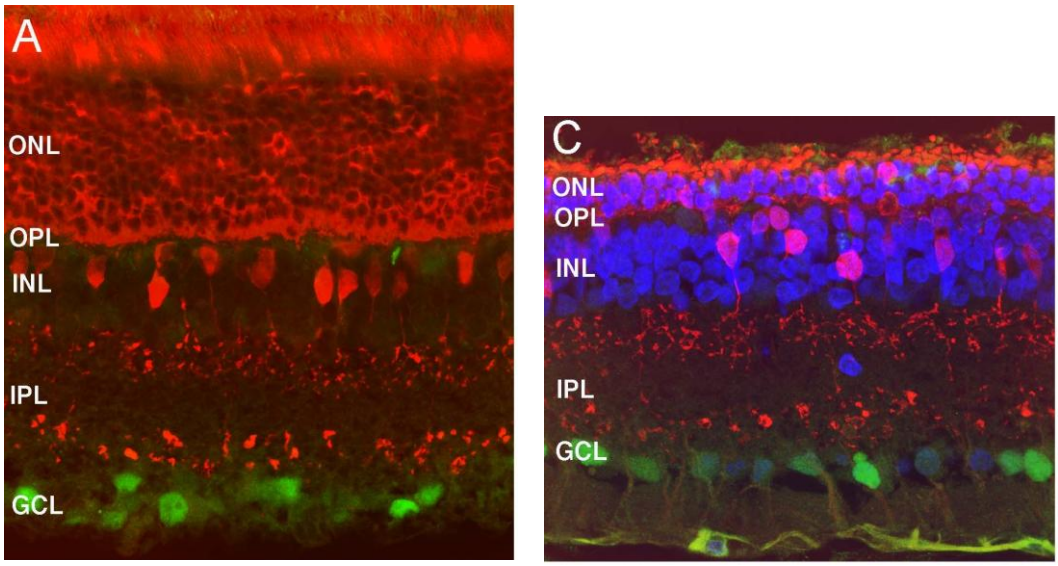

Figure 13. Retina degeneration of P23H rhodopsin transgenic rats. Sections of the retina from Sprague–Dawley (A) and P23H-3 homozygous (C) rats at 6 months of age stainined with recoverin and Brn3a (A) or with recoverin, Brn3a and TO-PRO (C). Note the loss of photoreceptors in P23H. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar= 20 µm. From (Lax et al., 2011).

1.4.3. APPSswe/PS1dE9 transgenic mouse model of Alzheimer disease

Alzheimer disease (AD) is a neurodegenerative disorder characterized by β-amyloid deposits and neurofibrillary tangles in the brain (Duyckaerts et al., 2009), resulting in cognitive and behavioral deficits. In recent years, a wide variety of transgenic mouse models of AD have been developed (Duyckaerts et al., 2008) with the aim to replicate the neuropathology and cognitive impairments commonly observed in AD patients. Mutant mouse models have mainly focused on two genes, amyloid precursor protein (APP) and/or presenilin 1 (PSEN1), which are linked with the early onset of AD (Hardy, 1997).

It has been shown that circadian dysfunction, including alteration in the SCN, reduced systemic melatonin levels and sleep disturbances, also occurs in AD patients (van Someren et al., 1996; Wu & Swaab, 2005; Wu & Swaab, 2007). Therefore, it is important to characterize the possible circadian disruptions in the AD mouse models that are widely used today and trial strategies to fix them.

In the present PhD dissertation we used mice carrying a chimeric human/murine APP construct bearing the Swedish mutation and the exon-9-deleted PSEN1 mutation (the APP695swe and PS1dE9 mutations). These double transgenic mice begin to develop amyloid deposition in the hippocampus and neocortex at the age of 6-7 months, and have abundant plaque deposits by 9 months (Jankowsky et al., 2004) (Figure 14).

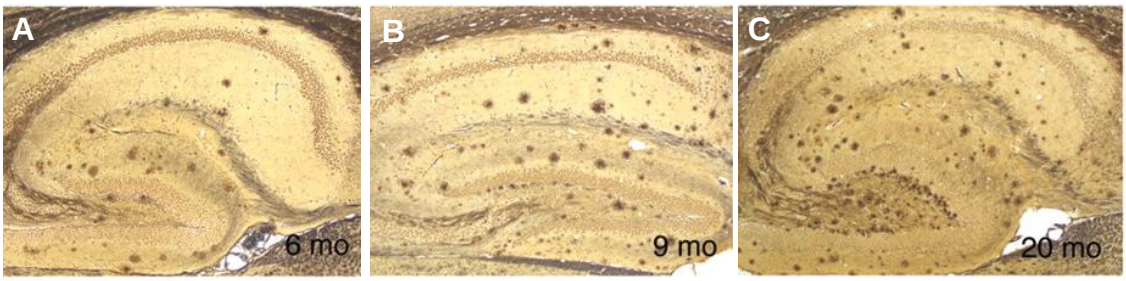

Figure 14. Amyloid pathology in the APPswe/PS1dE9 transgenic mice. Substantial hippocampal amyloid deposition is visible by 6 mo (months) of age (A) and amyloid burden increases progressively with age (B-C), as shown by Hirano silver stain. From Jankowsky et al., 2004.

1.4.4. Melanoma-bearing C57BL/6 mouse

Circadian rhythms disruption resulting, from long-term shift work or jet lag, for example, has been associated with a higher incidence of some types of cancer (Schernhammer et al., 2001; Rafnsson et al., 2001; Schernhammer et al., 2003). At the same time, rhythm robustness represents a prognostic factor for survival in cancer patients, in such a way that the better the circadian rhythm status the better the survival prognostics (Mormont et al., 2000; Mormont et al., 2002). In the present PhD dissertation we selected the melanoma cancer to investigate this bidirectional relationship between cancer development and circadian system functionality. Melanoma is a tumor derived from the malignant transformation of melanocytes in the basal layer of the epidermis that occurs mainly in white populations. Despite being one of the less common skin cancers, it is one of the most aggressive (Garbe & Leiter, 2009).

2. OBJECTIVES

The overall objective of this Doctoral Thesis was twofold:

1. To investigate if a disruption in the internal temporal organization of the degu's circadian system emerges when the animals "spontaneously" reverse their activity-phase preference from day to night.

1.1. To evaluate whether shifting diurnal activity-phase preference to the night in degus also implies a complete shift in their physiological, biochemical and hematological rhythms.

1.2. To study *Period* gene (*Per1* and *Per2*) day-night expression within the suprachiasmatic nucleus (SCN) and in extra-SCN brain areas in diurnal and in masked or entrained nocturnal degus.

1.3. To establish the temporal and spatial pattern of orexin neuronal activation in diurnal and nocturnal degus over the light-dark cycle.

1.4. To investigate the phase-switch of behavioral and physiological rhythms in response to high ambient temperatures and wheel running access.

1.5. To assess the desynchronizing effects of shifting light-dark schedules that mimic shift work conditions in humans on the degu's circadian system, and the chronobiotic effect of melatonin treatment.

2. To study the circadian system functionality in pathologies associated with chronodisruption, and its possible chronoenhancement by exogenous melatonin.

2.1. To determine the circadian system dysfunction in a model of retinitis pigmentosa (P23H rhodopsin transgenic rats) and melatonin's possible restoring effects on circadian impairment.

2.2. To evaluate the circadian system functionality and hippocampal oxidative stress levels in the APPswe/PS1dE9 transgenic mouse model of Alzheimer Disease, and assess the effects of melatonin or ramelteon long-term treatment.

2.3. To investigate how melanoma progression impairs the circadian system rhythmicity in the mouse, and test whether exogenous melatonin enhances circadian system functionality, restricts tumor growth and increases survival.

3. EXPERIMENTAL CHAPTERS

3.1. Experimental Chapter 1

INTERNAL TEMPORAL ORDER IN THE CIRCADIAN SYSTEM OF A DUAL-PHASING RODENT, THE *OCTODON DEGUS*

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3.1. INTERNAL TEMPORAL ORDER IN THE CIRCADIAN SYSTEM OF A DUAL PHASING RODENT, THE *OCTODON DEGUS*

ABSTRACT

Daily rhythms in different biochemical and hematological variables have been widely described in either diurnal or nocturnal species, but so far no studies in the rhythms of these variables have been conducted in a dual phasing species such as the *degus*. The *Octodon degus* is a rodent which has the ability to switch from diurnal to nocturnal activity under laboratory conditions in response to wheel running availability. This species may help us discover whether a complete temporal order inversion occurs parallel to the inversion that has been observed in this rodent's activity pattern. The aim of the present study is to determine the phase relationships among 26 variables, including behavioral, physiological, biochemical, and hematological variables, during the day and at night, in diurnal and nocturnal degus chronotypes induced under controlled laboratory conditions through the availability of wheel running. A total of 39 male degus were individually housed under a 12:12 LD cycle, with free wheel running access. Wheel running activity (WRA) and body temperature (Tb) rhythms were recorded throughout the experiment. Melatonin, hematological, and biochemical variables were determined by means of blood samples obtained every 6 h (ZT 1, ZT 7, ZT 13, and ZT 19). In spite of great differences in WRA and Tb rhythms between nocturnal and diurnal degus, no such differences were observed in the temporal patterns of most of the biological variables analyzed for the two chronotypes. Variation was only found in plasma urea level and lymphocyte number. A slight delay in the phase of the melatonin rhythm was also observed. This study shows that the internal temporal order of a dual phasing mammal does not show a complete inversion in accordance with its activity and body temperature pattern; it would appear that the switching mechanism involved in the degu's nocturnalism is located downstream from the pacemaker.

Keywords: *Octodon degus*; body temperature rhythm; wheel running activity; diurnalism; nocturnalism; hematological parameters; biochemical parameters; melatonin

INTRODUCTION

It is generally accepted that one of the most important functions of the circadian system is to maintain a stable internal temporal relationship in an animal's daily behavioral and physiological rhythms. This order is closely synchronized to the environment and is maintained despite changes in key *zeitgebers*, such as the light-dark (LD) cycle. After an abrupt shift in the LD cycle, e.g., jet lag or shiftwork, there is a transient period of internal desynchronization in which the normal relationships among behavioral and physiological variables are disrupted. However, the temporal order progressively re-emerges, and the individual exhibits circadian rhythms in this regard that are adapted to the new LD cycle (Moore-Ede et al., 1977). This is significant, as most experts would agree that the maintenance of the internal temporal order is a necessary condition for good health (Waterhouse & DeCoursey, 2004). Furthermore, and not surprisingly, chronodisruption has been associated with many pathological states (Penev et al., 1998; Cho.,2001; Filipski et al., 2004; Davidson et al., 2006) in response to conflicting *zeitgebers*, for example, when food is restricted to an unusual feeding time during the LD cycle (Howell et al., 2009).

So far, all animal species have been categorized as diurnal, nocturnal, or crepuscular, depending primarily on their behavioral and physiological rhythms. However, some species, including fish (Sánchez-Vázquez et al., 1995, 1996) and mammals, such as the mole rat, *Spalax ehrenbergi* (Oster et al., 2002); Nile grass rat, *Arvicanthis niloticus* (Blanchong et al., 1999); gerbil, *Meriones unguiculatus* (Weinert et al., 2007); golden spiny mice, *Acomys russatus* (Cohen & Kronfeld-Schor, 2006; Cohen et al., 2009), and *Octodon degus* (Kas & Edgar, 1999) exhibit the ability to switch their diurnal phase preference for feeding and locomotor activity to a nocturnal phase in response to certain environmental changes, such as seasons, wheel running availability, food restriction, and temperature cycles. These dual-phasing species make excellent animal models to study whether oppositely-phased activity rhythms in diurnal and nocturnal animals also imply a complete inversion in their internal circadian order (Levy et al., 2007).

Most studies in dual-phasing rodents have focused on the relationship between rhythms in locomotor activity and central processes, such as neural activity, neurochemistry, and levels of awake-promoting neuropeptides (Castillo-Ruiz et al., 2010; Mahoney et al., 2000; Nixon & Smale, 2004; Smale et al., 2001a, 2001b). To date, most of the studies investigating the link between behavioral rhythms and the daily pattern of hematological and biochemical variables have focused on either diurnal or nocturnal species (Cuesta et al., 2009; Dunlap et al., 2004; García-Rodriguez et al., 1987; Halberg et al., 1979; Haus et al., 1983; Piccione et al., 2005, 2007; Refinetti, 1999). To the best of our knowledge, no studies have examined the

relationship between multiple biochemical and hematological variables and chronotypes in O*ctodon degus.*

The *Octodon degus* is a dual phasing rodent, and although it is defined primarily as a diurnal animal (Fulk, 1976), a subset of animals switches to nocturnal activity under laboratory conditions in response to wheel running exercise (Kas & Edgar, 1999), which is maintained after the wheel has been removed (Refinetti, 2006). Recently, we have demonstrated that nocturnal inversion in wheel running activity in this species is a consequence of the appearance of a negative masking effect by light, together with a shift in the phase angle of entrainment in a subset of animals (Vivanco et al., 2009). The aim of the present study was to determine the phase relationships among 26 variables, including behavioral (wheel running activity), physiological (body temperature), biochemical, and hematological variables, during the day and at night, in diurnal and nocturnal degus chronotypes that have been induced under controlled laboratory conditions by wheel running availability. In addition, the daily pattern for these variables was also investigated, independently of the chronotype.

MATERIAL AND METHODS

Animals and housing conditions

All experimental procedures were performed in accordance to the Principles of Animal Care (Portaluppi et al., 2008) and Spanish laws. A total of thirty-nine male *Octodon degus* between 24 and 36 months of age were obtained from a colony maintained by the Animal Service at the University of Alicante (Spain). The animals were individually housed in plexiglas cages equipped with running wheels (52 x 15 x 27 cm, L x H x W) in an isolated room ("Chronolab") with controlled temperature (27.4±0.5^{o}C), relatively humidity (60%±10), and photoperiod (12:12 LD cycle, with lights on from 08:00 to 20:00 h). Light was provided by two lateral fluorescent lamps controlled by an electronic timer (Data Micro, Orbis, Madrid, Spain), with an intensity of 350-400 lux at cage level. Animals were fed commercial rat chow (A04 rat-mouse maintenance Panlab, Barcelona, Spain) *ad libitum.* After the wheel running activity rhythms had been stabilized for at least 4 wks, the animals were classified as diurnal, nocturnal, or intermediate, based on the percentage of activity they demonstrated during the scotophase or photophase throughout the experiment. Animals were considered as diurnal when their diurnal activity was > 60%. When that percentage was < 40%, animals were included in the nocturnal chronotype category. Animals with a diurnal activity between 40 and 60% were classified as intermediate.

When these criteria were applied, 43% of degus (17 out of 39) exhibited a diurnal chronotype, whereas a nocturnal pattern was displayed by 28% of degus (11/39). The 11 remaining animals were classified as intermediate.

Data recording

Wheel running activity (WRA) was recorded as wheel revolutions/10-min intervals using a data acquisition system (Electronic Service at the University of Murcia, Spain).

Body temperature (Tb) was recorded every 60 min using a datalogger (ThermoChron®, Data loggers iButton, Maxim Integrated Products, Sunnyvale, California), with an accuracy of ±0.125 ^{o}C. Sterile data loggers were implanted intraperitoneally under aseptic surgical conditions. Fluothane was used as anesthetic (Forane®, Abbot Laboratories S.A., Madrid, Spain) and iodine solution (Betadine®, Viatris, Madrid, Spain) as a surgical scrub. Absorbable sutures (2/0, Safil®Quick B/Braun, Barcelona,Spain) were used to suture the abdominal muscle layer, and nonabsorbable silk (2/0, Silkam®, B/Braun, Barcelona, Spain) was used to suture the skin. No mortality or morbidity was observed after the surgery. At the end of the experiment, the animals were sacrificed and the data loggers were removed and the data transferred to a computer.

Blood sampling procedure

Animals were slightly anesthetized (2.5% fluothane mixed with oxygen for induction, and 1.5% fluothane for maintenance). Blood samples for biochemical variables were extracted by jugular venopuncture at different *zeitgeber* times (ZTs 1, 7, 13 and 19, lights-on = ZT0) on different days for each animal, using heparin as an anticoagulant. Animals were allowed to recover for five days between each blood sampling. Blood samples for hematological variables were extracted by cardiac puncture, using EDTA as an anticoagulant, prior to the time the animals were sacrificed at ZT1, ZT7, Z13 and ZT19. Hematological parameters were determined immediately after sampling. Biochemical variables were determined from blood samples immediately after they were obtained. The remaining blood samples were then centrifuged for 15 min at 4^{o}C to 2026 g. The plasma obtained was stored at -80^{o}C until the time the melatonin analysis was carried out.

Blood cell analysis

Hematological parameters were measured using an automatic hematology analyzer (Abacus Junior Vet, CVM S.L., Navarre, Spain).

The analyzed hematological variables were white blood cells (WBC), lymphocytes (LYM), monocytes (MID), granulocytes (GRA), red blood cells (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), and platelets (PLT).

Clinical biochemistry

Albumin, globulins, total protein, total bilirubin, creatinine, amylase, alanine aminotransferase (ALT), alkaline phosphatase (ALP), sodium, potassium, calcium, phosphate, and urea were determined in blood using an automatic analyzer (VetScan® Classic, CVM S.L., Navarre, Spain). Blood glucose concentration was measured by a glucometer (Glucocard G meter, Menarini, Italy).

Melatonin analysis

Plasma melatonin (MEL) was determined by RIA, using a commercial kit (Melatonin direct RIA kit, IBL Hamburg®; Hamburg, Germany).

Data analysis

WRA and Tb actograms and mean waveforms were calculated using a software specifically designed for time series analysis (El Temps version 1.228; © Díez Noguera, University of Barcelona). Data were expressed as mean ± SEM. Statistical differences between chronotypes and timepoints were evaluated by means of a factorial analysis of variance (ANOVA) followed by *post-hoc* pairwise comparisons by Fisher's LSD test. A paired Student's *t*-test was performed when only two groups were compared. Values of $p < 0.05$ were considered to be statistically significant. All statistics were calculated using Statgraphics® Plus 5.1., Statistical Graphics Corp.

RESULTS

Wheel running activity (WRA) and body temperature (Tb) mean actograms for all degus are shown in Figure 1. Overall, under a 12:12 LD cycle, the animals exhibited a predominantly diurnal pattern, with low amplitude and two crepuscular peaks of activity around dusk and before lights-on.

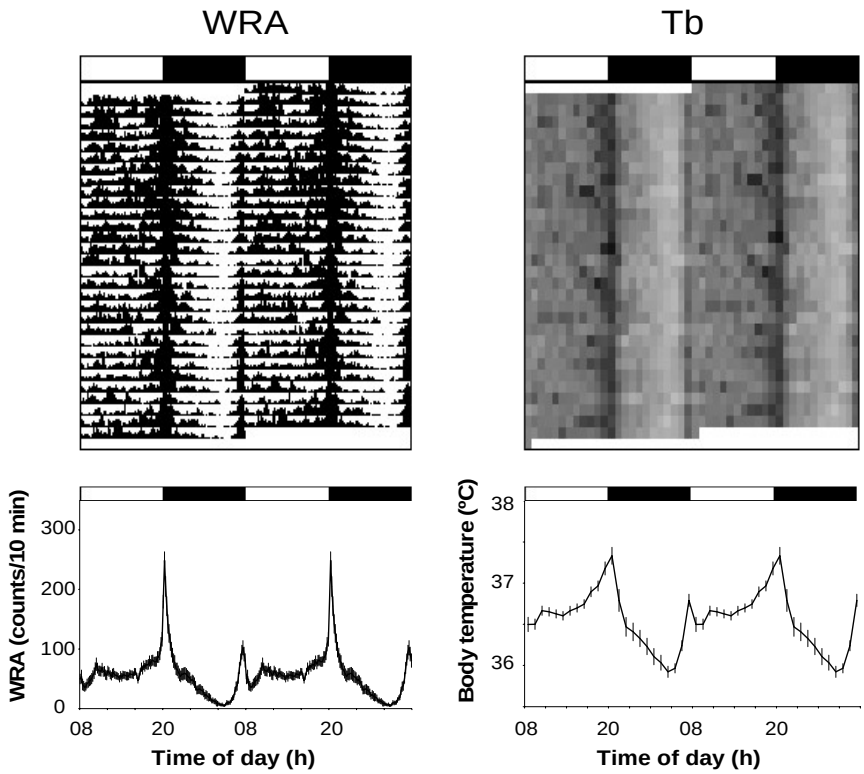

Figure 1. Wheel running activity (WRA) and body temperature (Tb) mean actograms (upper panels) and WRA and Tb mean waveforms (lower panels) for 39 degus exposed to a 12:12 LD cycle. Light and dark schedule is represented at the top by white and black bars.

The mean values per measured variable at the different *zeitgeber* times are shown in Table 1. Plasma melatonin concentration showed a circadian pattern that peaked during the night (228.54 ± 15.91 pg/ml at ZT19), whereas the lowest level was observed at mid-light (141.92 ± 9.10 pg/ml at ZT7).

Most of the hematological variables, with the exception of MCV and PLT, exhibited a maximum level at mid-dark, but the differences were only statistically significant (ANOVA, Fisher's test, p <0.05) for MID, RBC, HGB, and HCT. On the contrary, most of the biochemical variables showed maximum levels at mid-light, and significant differences were only found in creatinine and sodium plasma levels, with maximum values at ZT7 and ZT19, respectively (paired t-test, p = 0.001 and p = 0.009, respectively).

Figures 2A and 2C show WRA and Tb rhythms from a representative diurnal and nocturnal degus, respectively, which were exposed to a 12:12 LD cycle. Both chronotypes exhibited two crepuscular peaks of activity, but the diurnal type confined its WRA to the photophase, whereas the nocturnal type was active during the scotophase. However, between these two "extreme" chronotypes, there was a gradient of intermediate chronotypes (Figure 2B), as previously described by our group (Vivanco et al., 2009). Tb displayed the same pattern as WRA rhythms, with high values during the day in the diurnal chronotype, and at night in the nocturnal chronotype.

Table 1. Behavioral, physiological, hematological, and biochemical variables at different *Zeitgeber* Times in degus.

	ZT1	ZT7	ZT13	ZT19
WRA	47.55 ± 5.21^{a}	57.74 ± 5.50^{a}	95.17 ± 6.74^{b}	28.00 ± 5.55^{c}
Tb (°C)	36.44 ± 0.05^{a}	36.66 ± 0.05^{b}	36.94 ± 0.07^{c}	36.17 ± 0.09^{d}
MEL (pg/mL)	160.13 ± 15.41^{ab}	141.92 ± 9.10^{a}	183.36 ± 11.95^{b}	228.54 ± 15.91^{c}
Hematological (n = 10)				
WBC (10^9cells/L)	2.99 ± 0.76	3.41 ± 0.52	3.42 ± 0.54	4.53 ± 0.62
LYM (10^9 cells/L)	1.25 ± 0.44	1.62 ± 0.27	1.78 ± 0.33	1.87 ± 0.30
MID (10^9 cells/L)	0.20 ± 0.06^{ab}	0.26 ± 0.08^{ab}	0.17 ± 0.03^{a}	0.37 ± 0.11^{b}
GRA (10^9 cells/L)	1.54 ± 0.44	1.53 ± 0.23	1.46 ± 0.25	2.29 ± 0.76
RBC (10^{12} cells/L)	6.48 ± 0.16^{ab}	6.38 ± 0.14^{a}	6.94 ± 0.24^{bc}	7.29 ± 0.28^{c}
HGB (g/dL)	9.83 ± 0.28^{a}	9.68 ± 0.24^{a}	10.37 ± 0.42^{ab}	11.07 ± 0.56^{b}
HCT (%)	34.23 ± 0.85^{a}	33.66 ± 0.73^{a}	35.64 ± 1.14^{ab}	37.39 ± 1.28^{b}
MCV (μm^3)	52.90 ± 0.43	52.80 ± 0.93	51.44 ± 0.77	51.60 ± 0.85
PLT (10^9 cells/L)	255.20 ± 35.37	219.00 ± 12.11	223.73 ± 23.79	190.80 ± 39.51
Biochemical (n = 13)				
Glucose (mg/dL)	100.46 ± 4.70	107.79 ± 5.03	95.33 ± 4.86	97.38 ± 5.22
Albumin (g/dL)	—	4.58 ± 0.15	—	4.12 ± 0.23
ALP (units/L)	—	69.03 ± 7.55	—	59.92 ± 6.58
ALT (units/L)	—	17.69 ± 1.59	—	15.77 ± 0.85
Amylase (units/L)	—	480.38 ± 24.97	—	452.77 ± 31.03
Bilirubin (mg/dL)	—	0.42 ± 0.01	—	0.43 ± 0.01
Urea (mg/dL)	—	28.85 ± 1.13	—	28.08 ± 1.52
Calcium (mg/dL)	—	10.82 ± 0.22	—	11.02 ± 0.19
Phosphate (mg/dL)	—	4.11 ± 0.63	—	3.55 ± 0.25
Creatinine (mg/dL)	—	0.56 ± 0.03 *	—	0.39 ± 0.03
Sodium (mg/dL)	—	160.31 ± 1.73*	—	167.69 ± 1.90
Potassium (mg/dL)	—	4.62 ± 0.17	—	4.05 ± 0.22
Total protein (g/dL)	—	5.74 ± 0.15	—	5.28 ± 0.29
Globulins (g/dL)	—	1.12 ± 0.15	—	1.13 ± 0.15

Values are expressed as mean ± SEM. (n = 39 for WRA, Tb, MEL, and glucose). Different letters indicate significant differences (ANOVA, Fisher's test, $p < 0.05$). *$p < 0.01$, paired t-test (ZT7 vs. ZT19). See Material and Methods for more details.

Figure 2 also shows the plasma melatonin profiles for three representative animals. As we have previously described, melatonin peaked at ZT19 in diurnal degus (Figure 2A). However, in the case of the nocturnal degus (Figure 2C), the acrophase was delayed (peaked at ZT1). In addition, mean melatonin concentration in nocturnal degus was higher than in diurnal degus (246.47 ± 42.46 vs. 115.31 ± 46.93 pg/ml, respectively, paired t-test, $p = 0.042$). In intermediate chronotype degus, the mean melatonin level was quite similar to that of diurnal chronotype (145.59 ± 25.84 pg/ml).

Double-plotted WRA and Tb mean actograms and WRA and Tb mean waveforms for diurnal and nocturnal chronotypes are represented in Figure 3. As previously shown in the Figure 2A and 2B, most of the WRA was concentrated during the photophase for diurnal degus and during the scotophase for nocturnal animals. Thus, WRA at mid-light was higher for the

diurnal chronotype, whereas the nocturnal chronotype exhibited much higher values at mid-dark (see Table 2).

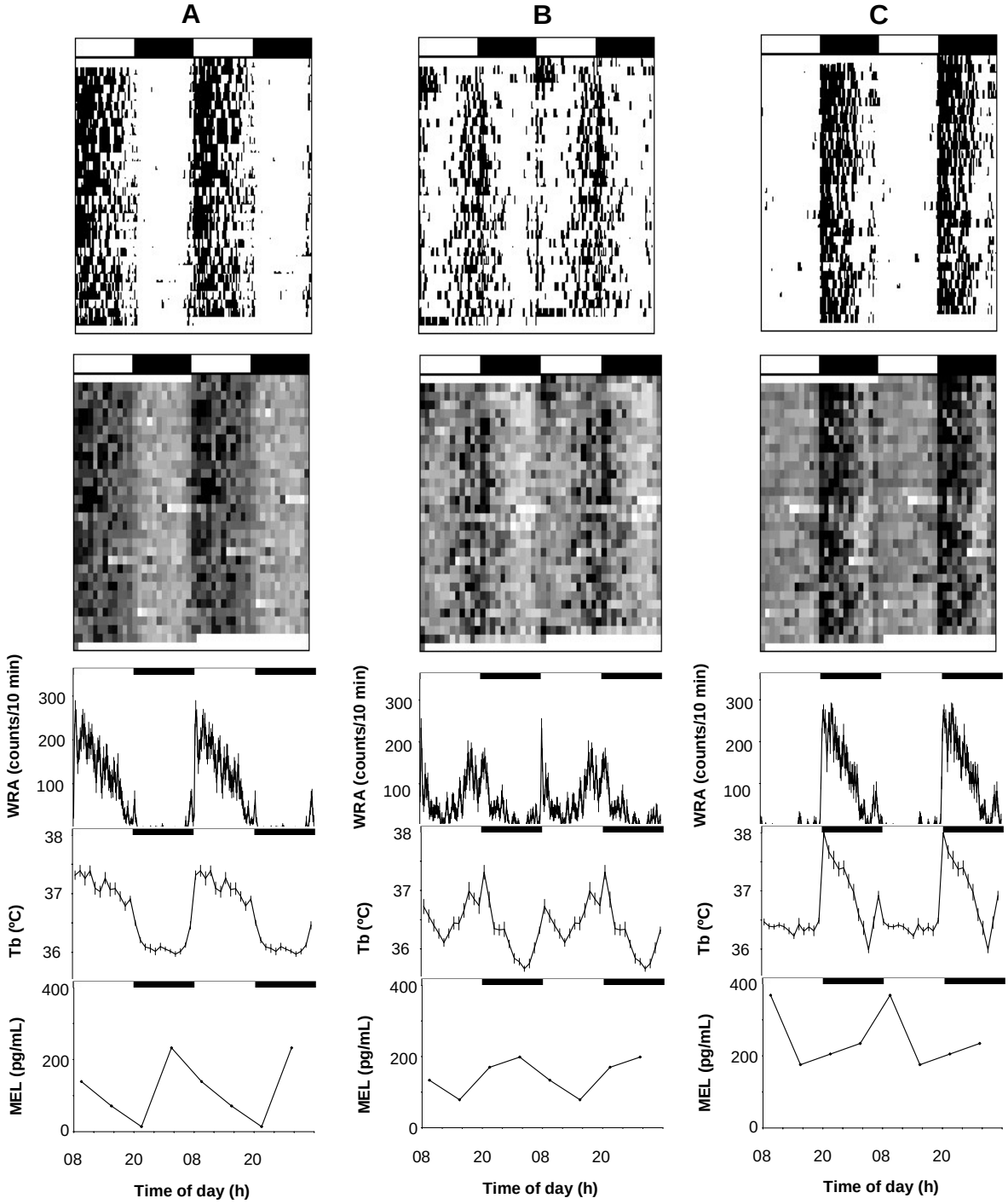

Figure 2. Wheel running activity (WRA), and body temperature (Tb) actograms (panels in the first and second rows), WRA and Tb mean waveforms (panels in the third and fourth rows), and plasma melatonin concentration (panels in the fifth row) for a representative diurnal (A), intermediate (B), and nocturnal (C) *degus* exposed to a 12:12 LD cycle. Light and dark schedule is represented at the top of the graphs by white and black bars.

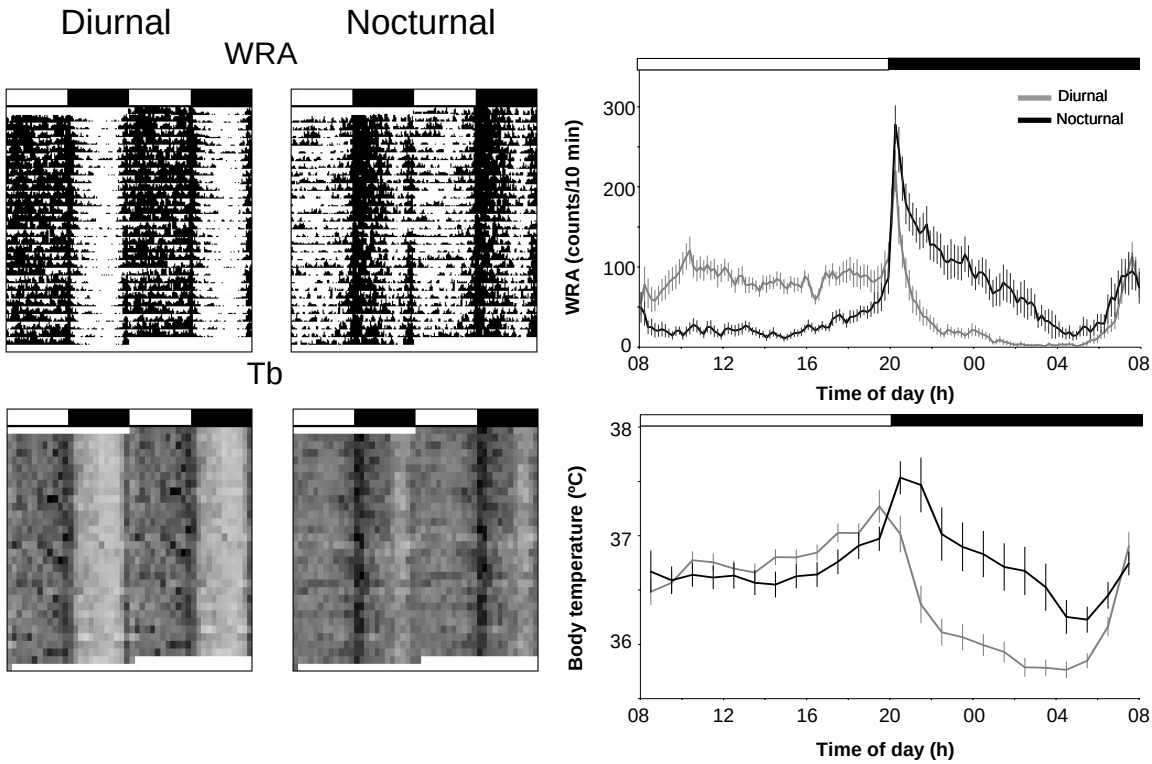

Figure 3. Wheel running activity (WRA) and body temperature (Tb) mean actograms (left) and WRA and Tb mean waveforms (right) for diurnal (n = 17) and nocturnal (n = 11) *degus* under a 12:12 LD cycle. Light and dark schedule is represented at the top of the graphs by white and black bars.

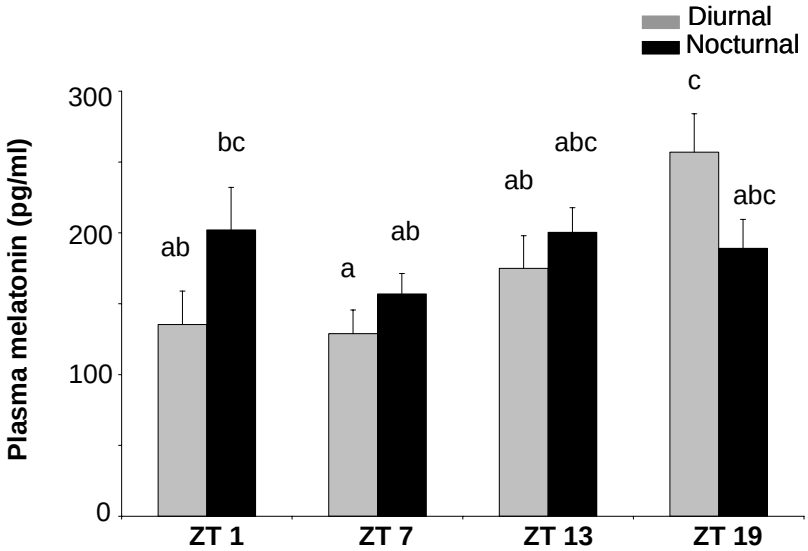

Figure 4. Plasma melatonin levels in both diurnal (n = 17) and nocturnal (n = 11) degus chronotypes subjected to a 12:12 LD cycle. Values are expressed as mean ± SEM. Different letters indicate significant differences (ANOVA, Fisher's test, $p < 0.05$).

Tb also exhibited two crepuscular peaks (Figure 3). The temperature patterns were quite similar in both chronotypes during the photophase, with the mean temperature during this phase measuring 36.85 ± 0.06 and 36.68 ± 0.05 ºC for the diurnal and nocturnal types,

respectively. However, the temperature during the dark period was higher for the nocturnal chronotype than for diurnal degus (36.60 ± 0.08 vs. 36.04 ± 0.11 °C, paired t-test, $p < 0.0001$) (Figure 3 and Table 2).

Table 2. Behavioral, physiological, hematological and biochemical variables at ZT7 and ZT19 in nocturnal and diurnal degus.

	Diurnal		Nocturnal	
	ZT7	ZT19	ZT7	ZT19
WRA	82.68 ± 6.26^{a}	7.85 ± 2.34^{b}	19.92 ± 4.06^{b}	61.52 ± 14.16^{c}
Tb (°C)	36.76 ± 0.06^{a}	35.89 ± 0.08^{b}	36.61 ± 0.11^{a}	36.65 ± 0.20^{a}
Hematological (n = 4)				
WBC (10^9 cells/L)	1.99 ± 0.23^{a}	4.94 ± 1.48^{b}	3.90 ± 0.23^{ab}	4.69 ± 0.42^{b}
LYM (10^9 cells/L)	0.76 ± 0.13^{a}	1.70 ± 0.43^{b}	2.06 ± 0.30^{b}	1.88 ± 0.10^{b}
MID (10^9 cells/L)	0.16 ± 0.04^{a}	0.40 ± 0.11^{bc}	0.23 ± 0.08^{ab}	0.52 ± 0.04^{c}
GRA (10^9 cells/L)	1.07 ± 0.09	2.84 ± 1.29	1.61 ± 0.19	2.30 ± 0.44
RBC (10^{12} cells/L)	6.10 ± 0.23^{a}	7.50 ± 0.44^{b}	6.38 ± 0.07^{a}	7.28 ± 0.16^{b}
HGB (g/dL)	9.13 ± 0.32^{a}	11.33 ± 0.97^{b}	9.83 ± 0.29^{ab}	11.08 ± 0.33^{b}
HCT (%)	31.69 ± 0.57^{a}	37.43 ± 2.11^{b}	34.36 ± 1.09^{ab}	37.82 ± 0.70^{b}
MCV (μm^3)	52.00 ± 1.73	50.25 ± 0.75	54.00 ± 1.58	52.25 ± 1.03
PLT (10^9 cells/L)	198.25 ± 18.38	178.75 ± 71.03	245.75 ± 17.02	219.50 ± 6.51
Biochemical (n = 5)				
Glucose (mg/dL)	106.18 ± 6.37	106.06 ± 9.29	97.82 ± 7.60	94.90 ± 9.64
Albumin (g/dL)	4.64 ± 0.14	3.98 ± 0.48	4.88 ± 0.14	4.10 ± 0.32
ALP (units/L)	71.40 ± 14.96	65.80 ± 15.70	78.00 ± 11.65	57.40 ± 8.26
ALT (units/L)	17.60 ± 4.18	15.40 ± 1.63	17.80 ± 1.39	15.60 ± 1.47
Amylase (units/L)	503.60 ± 20.42	471.40 ± 71.12	478.60 ± 61.99	417.40 ± 40.48
Bilirubin (mg/dL)	0.40 ± 0.00	0.42 ± 0.02	0.42 ± 0.02	0.42 ± 0.02
Urea (mg/dL)	31.80 ± 1.16^{b}	27.20 ± 3.61^{ab}	25.20 ± 1.66^{a}	28.40 ± 1.03^{ab}
Calcium (mg/dL)	10.94 ± 0.28	11.04 ± 0.51	11.20 ± 0.25	10.88 ± 0.15
Phosphate (mg/dL)	3.38 ± 0.51	3.26 ± 0.47	5.30 ± 1.78	3.72 ± 0.40
Creatinine (mg/dL)	0.56 ± 0.02^{b}	0.38 ± 0.06^{a}	0.52 ± 0.07^{ab}	0.36 ± 0.07^{a}
Sodium (mg/dL)	161.60 ± 2.50^{a}	166.20 ± 3.65^{ab}	159.00 ± 2.83^{a}	171.00 ± 2.37^{b}
Potassium (mg/dL)	4.70 ± 0.22^{ab}	4.02 ± 0.39^{a}	5.04 ± 0.20^{b}	4.00 ± 0.37^{a}
Total protein (g/dL)	6.02 ± 0.19	5.22 ± 0.68	5.82 ± 0.18	5.24 ± 0.40
Globulins (g/dL)	1.34 ± 0.19	1.22 ± 0.25	0.90 ± 0.29	1.12 ± 0.21

Values are expressed as mean ± SEM. (n = 17 diurnal and n =11 nocturnal degus for WRA, Tb, and glucose). Different letters indicate significant differences (ANOVA, Fisher's test, $p < 0.05$). See Material and Methods for more details.

In diurnal degus, plasma melatonin concentration was higher at night than during the day. When the plasma melatonin profile (Figure 4) was compared between the chronotypes, differences were only found at ZT1 (nocturnal vs. diurnal; 202.04± 30.08 vs. 135.34 ± 23.56 pg/ml), probably due to a slight delay in the melatonin rhythm phase in nocturnal animals.

The mean values of all the variables measured for both diurnal and nocturnal chronotypes are shown in Table 2. Most of the hematological and biochemical values for both

chronotypes were higher at ZT 19 and ZT 7, respectively, coinciding with the observations recorded in Table 1. Significant differences were only found in lymphocyte number (LYM) and blood urea level (BUN), with the LYM for diurnal degus being lower than for nocturnal degus at ZT7 (0.76 ± 0.13 vs. 2.06 ± 0.30 10^9 cells/l, ANOVA, Fisher's test, $p < 0.05$), whereas the BUN was higher for the diurnal chronotype at that same point in time (31.80 ± 1.16 vs. 25.20 ± 1.66 mg/dl, ANOVA, Fisher's test, $p < 0.05$).

DISCUSSION

In spite of great differences in WRA and Tb rhythms between nocturnal and diurnal degus chronotypes, the temporal pattern of most of the biological variables analyzed (21/24) remained unchanged when the two chronotypes were compared. Differences were only found in plasma urea level and lymphocyte number. A slight delay in the phase of the melatonin rhythm was also observed.

Some studies have documented the existence of daily rhythms in a number of hematological and biochemical variables that have been simultaneously monitored in nocturnal animals (Ohkura et al., 2007); however, much less information is available about diurnal rodents (Cuesta et al., 2009), and no data are available for *Octodon degus*, apart from a single timepoint measurement involving this species (Murphy et al., 1978).

Our results indicate that WRA and Tb show a predominant diurnal pattern for all degus, with low amplitude and two crepuscular peaks associated with light transitions. Plasma melatonin concentration showed a circadian rhythm, with high levels at night and low levels during the day, as previously reported (Reiter, 1991; Vivanco et al., 2007). To date, this is the first time that the degu's daily hematological and biochemical patterns have been reported. However, the values obtained here fall within the normal range of the single timepoint sampled in degus as earlier reported by Murphy et al. (1978). Most of the hematological variables measured exhibited maximum values around mid-dark, the phase opposite to hematological rhythms in nocturnal rodents (Ohkura et al., 2007; Oishi et al., 2006), coinciding with what has been described for humans (Haus, 1996; Haus et al., 1983). Blood glucose concentration in our degus was found to be similar to that previously described for this species (Opazo et al., 2004), with no difference according to chronotype, consistent with both *Octodon bridgesi* (nocturnal) and *O. degus* (diurnal) (Opazo et al., 2004).

The low amplitude observed in WRA and Tb rhythms in degus is probably the result of averaging a chronobiologically heterogeneous population. The differentiation of degus into nocturnal and diurnal chronotypes in response to wheel running availability (Kas & Edgar,

1999; Vivanco et al., 2009) was also observed in our experiment. Whereas a nocturnal chronotype was found in 28% of our degus, 43% displayed a diurnal pattern.

In spite of the fact that WRA and Tb rhythms were phase-opposed in nocturnal and diurnal degus, most variables exhibited a very similar profile in both chronotypes, except for the melatonin rhythm, plasma urea level, and lymphocyte number, as mentioned earlier. Plasma melatonin in degus is typically high at night and low during the day, regardless of their activity pattern (Vivanco et al., 2007). In nocturnal rodents, melatonin secretion is delayed until after the onset of darkness, whereas in humans and diurnal degus, this occurs earlier (Lee et al., 2009; Wehr, 1991). Our results also showed this type of phase delay of the melatonin rhythm in the nocturnal chronotype, so much so that it peaked early in the morning. Despite this slight difference in the phase of endogenous melatonin secretion between nocturnal and diurnal degus, it is unlikely that melatonin is the key to nocturnal and diurnal chronotypes, since the exogenous administration of melatonin alone to diurnal degus fails to generate nocturnal chronotypes in this species (Vivanco et al., 2007). As has been described for other diurnal mammals, such as horses and sheep (Piccione et al., 2005), diurnal degus exhibited high urea values during the day. However, urea levels in nocturnal degus were lower during the photophase, coinciding with what has been shown for a nocturnal animal, such as the rabbit (Piccione et al., 2007). The LYM rhythm, which is associated with the activity pattern, peaks during the early morning in nocturnal rodents (Ohkura et al., 2007; Oishi et al., 2006) and at night in humans (Haus et al., 1983); similarly, our data showed that in degus, LYM peaks at ZT7 or ZT19 for nocturnal and diurnal chronotypes, respectively. Thus, the WRA and Tb shift from diurnal to nocturnal chronotype in degus fails to trigger a complete inversion in the internal temporal order. Therefore, our results indicate that the switching mechanism should be located downstream from the suprachiasmatic nucleus (SCN), rather than upstream from the clock, as has been proposed earlier (Smale et al., 2003).

Studies on the SCN show that the phasing of core clock gene expression, temporal patterns of expression of major neuropeptides, and electrical activity are not significantly different between diurnal and nocturnal rodents (Challet, 2007; Schwartz et al., 1983; Smale et al., 2008; Vosko et al., 2009). However, neural activity in the lower subparaventricular zone (LSPV), immediately dorsal to the SCN exhibits different rhythms in diurnal grass rats and nocturnal laboratory rats (Nunez et al., 1999). This finding raises the possibility that the LSPV could play an important role in the circadian regulation of diurnality (Smale et al., 2003). To understand the mechanism underpinning diurnalism and nocturnalism in a broader sense, the outputs signals and neural projections from the SCN should be taken into account (Smale et al., 2003).

One possible explanation for this partial inversion in degus is based on the existence of multiple synaptic relays in the SCN output that would differ between nocturnal and diurnal degus. The melatonin rhythm depends on a direct projection from the SCN to the paraventricular nucleus of the hypothalamus, whereas the Tb rhythm is managed predominantly by a direct projection from the SCN to the dorsal subparaventricular zone (dSPZ) (Saper et al., 2005). However, the locomotor, and probably the feeding and corticosterone-cortisol rhythms depend on two relays, one from SCN to ventral subparaventricular zone (vSPZ) and a second from the vSPZ to the dorsomedial nucleus of hypothalamus (DMH). This being the case, wheel running exercise may be affecting these relays (the DMH and the dSPZ), shifting the activity and temperature patterns, respectively, without affecting other variables (Saper et al., 2005). The ability to invert the wake-sleep, locomotor activity, feeding, and corticosteroid rhythms may correlate with a shift in DMH activation to a new temporal niche. It would, therefore, seem that the DMH would be a good candidate for the location of the partial switch from diurnal to nocturnal behavior, as it receives inputs from the SCN and is involved in the maintenance of the sleep-wake cycle and feeding rhythms; furthermore, it is also indirectly involved in the Tb rhythm. This model of a hypothalamic circadian integrator, as proposed by Saper et al. (2005), would explain the high degree of flexibility in the circadian system in dual phasing species, and it would justify the existence of the gradual phenotypic expressions, from diurnalism to complete nocturnalism, in degus chronotypes. A second possible explanation for diurnalism-nocturnalism is based on the presence of dual oscillators (or groups of oscillators), one for morning activity and another for evening activity. Each of these oscillators would most likely have a particular phase angle of entrainment to light-dark transitions and a particular period (García-Allegue et al., 1999).

Light and temperature masking induced by wheel running availability has been observed in degus (Vivanco et al., 2009, 2010). Direct clock-independent responses to light, i.e., negative masking by light, was proposed by Kas and Edgar (1999) as being responsible for nocturnalism in degus when wheel running access is provided. In fact, this masking effect was involved in most cases of nocturnal chronotypes described in our previous papers (Vivanco et al., 2009, 2010). In addition, high-low environmental temperature cycles seem to exert a negative masking effect on the activity pattern, confining activity in degus to the cooler phase, without affecting masking by light (Vivanco et al., 2010).

However, the two hypotheses (different oscillators and synaptic relays on the SCN outputs) are not mutually exclusive. Two groups of oscillators, one for dawn and one for dusk, with a different grade of coupling between them and different relays for melatonin, temperature, and activity outside the SCN, could explain why a continuous gradient of

phenotypic expression for degus activity patterns (for details, see Vivanco et al., 2009) can coexist with other biochemical and hematological variables that remain predominantly unshifted. Furthermore, the particular propensity of WRA to masking by light without the masking of other variables also points in the same direction.

The present multivariable analysis involving behavioral, physiological, hematological, and biochemical variables shows that a complete inversion in the temporal order does not occur parallel to a WRA and Tb pattern shift. The observation that this species can partially switch its temporal physiology would indicate a switching mechanism located downstream from the SCN, which implies the involvement of different relays.

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3.2. Experimental Chapter 2

PERIOD GENE EXPRESSION IN THE BRAIN OF A DUAL PHASING RODENT, THE *OCTODON DEGUS*

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3.3. Experimental Chapter 3

TEMPORAL AND SPATIAL PATTERNS OF ACTIVATION OF OREXIN NEURONS IN DIURNAL AND NOCTURNAL *OCTODON DEGUS* CHRONOTYPES

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3.4. Experimental Chapter 4

AMBIENT TEMPERATURE, THERMOREGULATORY CONSTRAINTS AND WHEEL RUNNING AVAILABILITY IN THE NOCTURNALISM OF THE *OCTODON DEGUS*

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3.5. Experimental Chapter 5

DISRUPTION OF THE CIRCADIAN SYSTEM IN THE *OCTODON DEGUS* UNDER LIGHT:DARK CYCLES THAT SIMULATE SHIFT WORK LIGHTING CONDITIONS IN HUMANS. EFFECTS OF EXOGENOUS MELATONIN

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3.6. Experimental Chapter 6

CIRCADIAN DYSFUNCTION IN P23H RHODOPSIN TRANSGENIC RATS: EFFECTS OF EXOGENOUS MELATONIN

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BB Otalora performed body temperature recordings and chronobiological data analysis. All authors contributed to the writing and editing of the manuscript

3.6. CIRCADIAN DYSFUNCTION IN P23H RHODOPSIN TRANSGENIC RATS: EFFECTS OF EXOGENOUS MELATONIN

ABSTRACT

This study focuses on the effects of retinal degeneration on the circadian patterns of P23H rats, as well as on the effect of exogenous melatonin administration. To this end, the body temperature of P23H and Sprague–Dawley rats was continuously monitored and their retinas examined at different stages of degeneration, by means of histological labeling and electroretinogram recordings. Melatonin (2 mg/ kg BW/ day) was supplied ad libitum throughout the experiment to a subset of animals. The body temperature recordings from wild-type and mutant animals showed no differences in the periodogram and the pattern of the mean waveform. However, a progressive decrease in the relative amplitude of the rhythm (RA), a decline in the coupling strength of the rhythm to environmental *zeitgebers* (interdaily stability, IS) and increased rhythm fragmentation (intradaily variability, IV) were observed in P23H rats, when compared to wild-type animals. The P23H animals showed a progressive decrease in light-induced retinal responses until reaching 18 months of age. By this age, all photoreceptors had already disappeared, and no responses were found in the EGRs. Exogenous administration of melatonin improved the visual response of P23H rats. In fact, the maximum b-wave recorded at 14 months of age was significantly higher in melatonin-treated P23H rats than in the control animals. Furthermore, the maximum b-wave recorded for P23H rats at the age of 14 months significantly correlated with RA, IS, and IV. This leads us to conclude that vision loss in P23H rats is correlated with a progressive fragmentation of their circadian patterns. Both effects are partially reversed by melatonin administration.

Keywords: chronodisruption; core temperature rhythm; electroretinogram; melatonin; P23H; retinitis pigmentosa

INTRODUCTION

The circadian system adjusts the timing for different oscillating variables over the 24-hr day and regulates the body's internal temporal organization. Circadian rhythms are timed by the suprachiasmatic nuclei (SCN, the master circadian clock), which coordinates tissue-specific rhythms according to the light input it receives from the outside world (Reppert & Weaver, 2002). In mammals, the photosensory brightness-sensing system provides direct photic information to the SCN (Czeisler et al., 1986; Czeisler et al., 1989) through a subset of melanopsin-expressing retinal ganglion cells (RGCs) (Gooley et al., 2001; Hattar et al., 2002), which also receive synaptic inputs from rod and cone photoreceptors (Hattar et al., 2003). In general terms, the period of the clock is genetically determined, whereas its phase is heavily influenced by environmental stimuli, such as light.

The functional integrity of the circadian system is essential to maintaining good health (Rajaratnam & Arendt, 2001). The effects of 24-hr shift work on general health have been the topic of a great deal of research over the last three decades (Smith et al., 1994; Costa, 1996). Moreover, circadian rhythm alterations (amplitude damping, phase shifts, and/or period changes) are common in aging and pathologic processes (Turek et al., 1995; Zhang et al., 1996). In fact, animal and human endocrine, metabolic, and immunologic circadian rhythms can serve as reliable markers for tumors (Mormont & Levi, 1997).

Ocular pathologies and blindness in humans are also associated with circadian disorders that depend on the degree to which light perception is conserved (Drouyer et al., 2008; Jean-Louis et al., 2008). In general, the lower the degree of light perception a subject has, the less likely his/her circadian system is to be entrained to normally phased circadian rhythms (Skene & Arendt, 2007). Moreover, the effects of ophthalmic degeneration on the circadian timing system may be worsened by the indirect impact of social isolation as the result of blindness. On the other hand, research conducted on visually impaired people has shown that, when appropriately administered, exogenous melatonin is able to entrain circadian desynchronization (Lockley et al., 2000; Hack et al., 2003) and decrease the magnitude of circadian rhythm disturbances (Skene et al., 1999).

Retinitis pigmentosa (RP) is a heterogeneous group of retinal degenerative disorders with a polymorphic hereditary basis which cause a progressive loss of retinal function and represent a major cause of blindness. Approximately 20–25% of patients with autosomal dominant RP have a mutation in the rhodopsin gene, one of the most common rhodopsin mutations being the P23H (Dryja et al., 1990), which accounts for approximately one-third of such cases in the USA (Dryja et al., 2000). In an effort to better understand this condition, animal RP models have been developed. P23H transgenic albino rats, both line 1 (faster

degeneration) and line 3 (slower degeneration), suffer from a progressive rod degeneration initially associated with normal cone function, which is consistent with the clinical findings in P23H patients (Berson et al., 1991; Machida et al., 2000; Cuenca et al., 2004; Pinilla et al., 2005). The loss of photoreceptors is accompanied by degenerative changes in the inner retina (Marc et al., 2003; Cuenca et al., 2004; Puthussery & Taylor, 2010) and a substantial degeneration of retinal ganglion cells (Jones et al., 2003; Kolomiets et al., 2010).

Considering the relative prevalence of ophthalmic diseases in aging humans (Congdon et al., 2004; Yamada et al., 2010), it is important to study the link between ophthalmic dysfunctions and circadian rhythm dysfunctions. The aim of this research is therefore to determine the effects of retinal degeneration on the circadian patterns of P23H rats. The effect of exogenous melatonin administration is also analyzed.

MATERIALS AND METHODS

Animals

The study included two groups (six animals per group) of male Sprague–Dawley (SD) rats, used as wild-type animals, and two groups (nine animals per group) of male laboratory rats homozygous for the P23H-3 rhodopsin mutation. All animals were bred in a colony at the University of Alicante and maintained under controlled humidity (60%), temperature (23 ± 1^{o}C), and photoperiod (LD 12:12) conditions. Light was provided by two fluorescent lamps with an intensity of 350–400 lux at cage level. At 2 months of age, the animals were individually housed in polycarbonate cages (Panlab SL, Barcelona, Spain), and their body temperature was recorded over the following 16 months. Body weight and food and water intake were measured every 2 wk. Electroretinogram (ERG) responses were recorded at the ages of 6, 10, 14, and 18 months. Melatonin (2 mg/kg BW/day) was administered to a group of SD and P23H animals during the 16 months of data collection. Experimental procedures were performed in accordance with the European Union guidelines for the use of laboratory animals, minimizing animal suffering and the number of animal specimens used for experimentation.

Body temperature recording

Body temperature (Tb) was continuously monitored at 60-min intervals throughout the experiment (16 months), using a miniature data logger (ThermoChron, Data loggers iButton; Maxim Integrated Products, Sunnyvale, CA, USA) with an accuracy of ±0.125^{o}C, as has been previously described (Otalora et al., 2008). Sterilized data loggers were implanted i.p. under aseptic conditions, using a solution of ketamine (100 mg/kg) and xylazine (4 mg/kg) as

anesthesia. Data loggers were replaced at the ages of 6, 10, and 14 months. No mortality or morbidity was observed after the surgery. iButton readout hardware was used to transfer temperature data to a computer.

Melatonin administration

Melatonin (M-5250; Sigma, Milwaukee, WI, USA) was dissolved weekly in ethanol (98%), and this stock solution was stored in darkness at) -20ºC until use. Every 2 days, the stock of melatonin was used to prepare 200 mL of drinking water (0.02% ethanol) per animal, which was supplied in light-sealed bottles (Vivanco et al., 2007; Otalora et al., 2008). Melatonin was ingested between 2 and 18 months of age. To adjust the amount of melatonin administered to each individual to 2 mg/ kg BW/ day, daily water intake was measured every 14 days throughout the experiment. Untreated animals were supplied drinking water containing 0.02% ethanol throughout the duration of the experiment.

ERG recordings

Electroretinogram (ERG) responses were recorded at ages 6, 10, 14, and 18 months. Following overnight adaptation, the animals were prepared for recordings under dim red light. Under anesthesia with a mixture of ketamine (100 mg/kg) and xylazine (4 mg/kg), the animals were placed in a Faraday cage, and their Tb was monitored using a rectal thermometer and maintained by a homeothermic blanket set at 38 ºC. The animal's pupils were dilated by a topical application of 1% tropicamide (Colircusí Tropicamida, Alcon Cusí, SA, El Masnou, Barcelona, Spain). One drop of 0.2% carbomer (polyacrylic acid; Viscotears, Novartis, Barcelona, Spain) was placed on the cornea to prevent dehydration and provide electrical contact with the recording electrode (DTL fiber electrodes, X-Static silver-coated nylon conductive yarn; Sauquoit Industries, Scranton, PA, USA). A 25-gauge platinum needle was inserted under the scalp, between the eyes; this served as the reference electrode. A ground electrode (gold) was placed in the mouth. Scotopic flash ERGs were recorded from each eye in response to light stimuli produced by a Ganzfeld stimulator. Light stimuli were delivered for 10 ms at six different increasing intensities (ranging from 3 x 10^{-3} to 10^{2} cd/m^{2}). Three to ten consecutive recordings were averaged for each light application. The interval between light flashes ranged from 10 s for dim flashes up to 20 s for those with the highest intensity. The ERG signals were amplified and band-pass filtered (1– 1000 Hz, without notch filtering) using a data acquisition board (DAM50; WPI Inc, Aston, UK). Stimulus application and data acquisition (4 kHz) were provided by a PowerLab system from ADInstruments (Oxfordshire, UK).

Retinal histology

Histological studies of the retinas were performed once the animals reached 6 months of age (10 animals were sacrificed) and at 18 months, at the end of the experiment. Cryostat vertical sections were obtained and processed for immunohistochemistry. For objective comparison, retinas from wild-type and P23H rats (both treated and untreated with melatonin) were fully processed in parallel. Their eyeballs were fixed in 4% paraformaldehyde and sequentially cryoprotected in 15%, 20%, and 30% sucrose. After being washed in 0.1 m PB (pH 7.4), the cornea, lens, and vitreous body were removed, and the retinas were processed for vertical sections. For Cryostat sections, the retinas were embedded in OCT and frozen in liquid nitrogen. Sections of 14 µm thick were obtained at -25^{o}C, mounted on superfrost slides, and then air-dried. The sections were incubated in 10% normal donkey serum for 1 hr (Jackson, West Grove, PA, USA) to avoid nonspecific staining and then double-immunostained overnight at room temperature, with combinations of antibodies against recoverin antirabbit 1:500 (Chemicon International Inc., Billerica, MA, USA) and Brn3a anti-mouse 1:100 (Chemicon International Inc.). To identify retinal cells, we used TO-PRO three iodide 1:1000 (Molecular Probes, Eugene, OR, USA). Subsequently, the sections were washed in PBS and exposed to secondary antibodies, Alexa 546-conjugated donkey anti-rabbit IgG (red, Molecular Probes), and Alexa Fluor 488-conjugated donkey anti-mouse IgG (green, Molecular Probes) at a 1:100 dilution for 1 hr. The sections were finally washed in PBS, mounted in citifluor (Citifluor Ltd., London, UK), and coverslipped for viewing by laserconfocal microscopy (Leica TCS SP2; Leica Microsystems, Wetzlar, Germany). Unless otherwise indicated, images were obtained from central retinal sections, and all images were obtained from the projections of four to six single frames.

Data analysis

To better understand the temporal evolution of Tb and to allow comparisons with ERG recordings, Tb records have been processed for four different experimental periods (4 months each in duration), with the end of each period corresponding to the ERG recordings. Tb records were analyzed for each experimental period using software specifically designed for chronobiological analysis (El temps, Diez-Noguera, University of Barcelona). Actograms, mean waveforms, and Sokolove–Bushell periodograms were calculated. Furthermore, a nonparametric analysis of the recordings was performed considering the last 15 days of each experimental period. The relative amplitude (RA) of the rhythm represents the ratio of the most active 10-hr period to the least active 5-hr period. Interdaily phase stability (IS) values (between 0 and 1) are considered proportional to the degree of phase homogeneity during the

corresponding experimental period and can be considered a measure of the phase stability of the rhythm over successive days. The intradaily variability (IV), which varies between 0 and 2, indicates the fragmentation of the rhythm, assessing the frequency and extent of transitions between rest and activity. The circadian function index (CFI) is an integrated parameter that is set taking into account the RA, IS, and IV values already described by our group (Ortiz-Tudela et al., 2010). This parameter oscillates between 0 (the absence of circadian rhythmicity) and 1 (a robust circadian system).

A repeated measures factorial analysis of variance (ANOVA) was performed to evaluate the effects of genotype (SD versus P23H), treatment (CON versus MEL), and experimental stage (6, 10, 14, and 18 months), as well as the interactions between them. When a 0.05 level of significance was found, post hoc pairwise comparisons using Bonferroni's test were made. Normal distributions and homogeneity of variance were found for the categories of the previously defined variables. A paired Student's t-test was used when only two groups were compared. A regression analysis was performed to establish the relationship between ERG b-wave amplitude and nonparametric circadian values. Data are reported as mean ± S.E.M. Values of $P < 0.05$ were considered to be statistically significant. All statistical analyses were performed using SPSS 15.0 software (IBM Corporation, Somers, NY, USA).

RESULTS

Figure 1 shows representative actograms for core Tb from each experimental group: P23H rats treated with melatonin (P23H-MEL), P23H rats untreated with melatonin (P23HCON), SD rats treated with melatonin (SD-MEL), and SD rats untreated with melatonin (SD-CON). Throughout the experiment, BT exhibited a robust circadian rhythm in all animals, regardless of the type or treatment, with no differences in the periodogram or in the pattern of the mean waveform (not shown). During the final experimental period, however, we observed a decrease in the amplitude of the BT rhythm in P23H rats, when compared to SD (0.48 ± 0.03 and 0.65 ± 0.04, respectively, ANOVA, Bonferroni's test, $P<0.01$). No significant differences in BT amplitude were found between SD-CON and SD-MEL animals (0.65 ± 0.04 and 0.58 ± 0.04, respectively) or between P23H-CON versus P23H-MEL (0.48 ± 0.03 and 0.49 ± 0.01, respectively).

To better understand the effects of visual degeneration on the circadian patterns of P23H rats, we evaluated the degree of phase homogeneity during each experimental period by means of IV and IS.

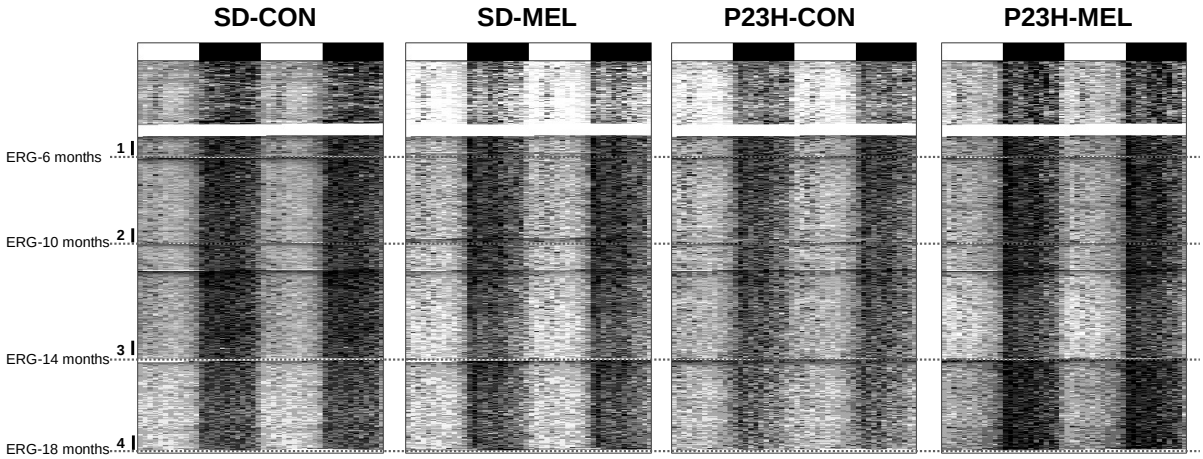

Figure 1. Double-plotted body temperature actograms for a representative animal from each experimental group exposed to a 12:12 LD cycle. SD rats untreated with melatonin (SD-CON), SD rats treated with melatonin (SD-MEL), P23H rats untreated with melatonin (P23H-CON) and P23H rats treated with melatonin (P23H-MEL). Light and dark schedules are represented by white and dark bars at the top of the graphs. Four 15-day periods (indicated on the left side of the actograms) were used to performed non-parametric tests. The end of each period corresponds to the ERG recordings performed on the animals at the age of 6, 10, 14 and 18 months.

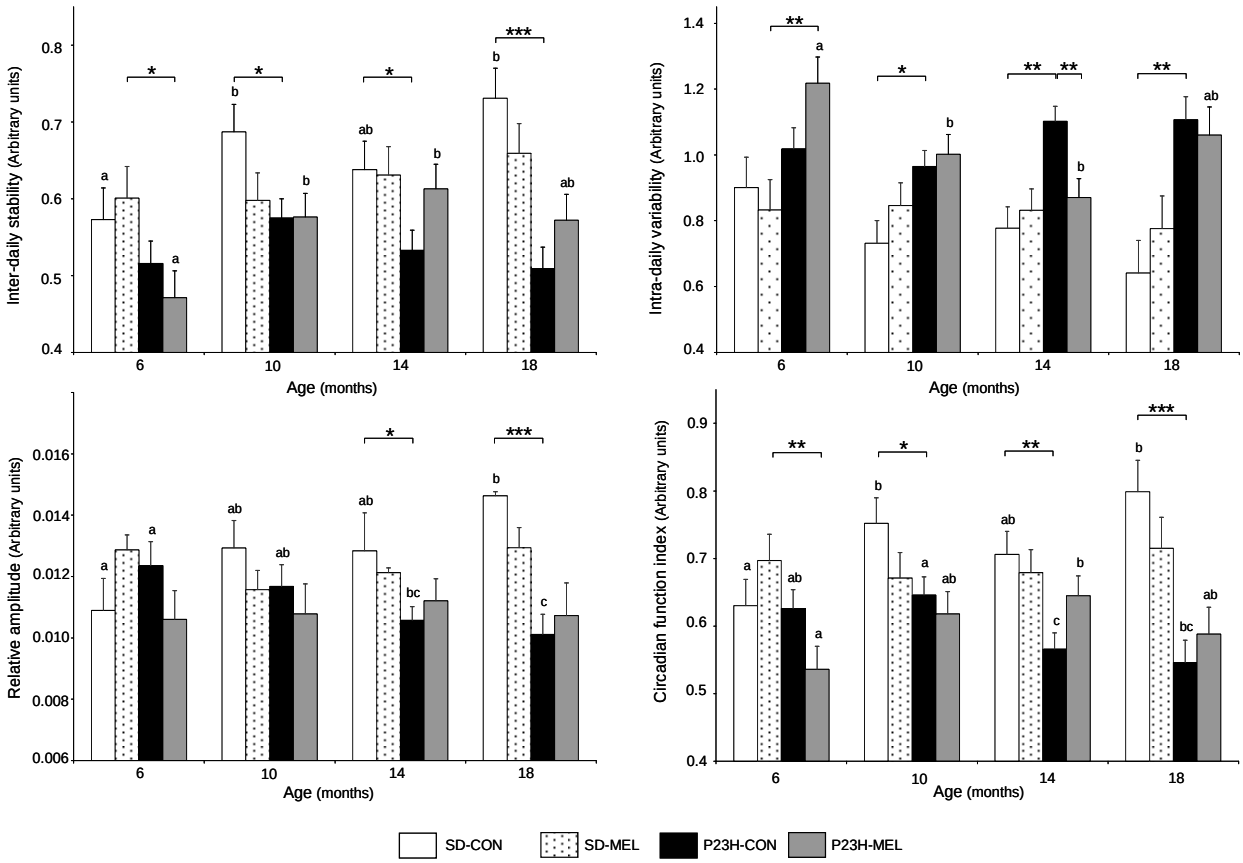

Figure 2. Non-parametric variables: body temperature inter-daily stability (A), intra-daily variability (B), relative amplitude (C) and circadian function index (D) for each experimental group at the age of 6, 10, 14 and 18 months. Values are expressed as mean ± SEM. Asterisks indicates statistically significant differences between the different treatments or genotypes at a given time point (*P < 0.05, **P < 0.01, ***P < 0.001, repeated measures factorial ANOVA, Bonferroni's test). Different letters indicate significant differences for the same group throughout time (6, 10, 14 and 18 months).

Repeated measures ANOVA for IS, IV, RA, and CFI showed significant interactions between genotype, treatment, and experimental stages (IS: $F = 3.915$, $df = 3$, $P = 0.016$; IV: $F = 4.344$, $df = 3$, $P = 0.010$; RA: $F = 8.224$, $df = 3$, $P < 0.001$; CFI: $F = 8.920$, $df = 3$, $P < 0.001$). Paired comparisons found that the coupling strength of the rhythm to environmental *zeitgebers* (IS) was weaker for P23H than for SD rats, during all experimental periods (Figure 2).

The analysis of the rhythm fragmentation (IV) showed a significantly higher intradaily variability in P23H rats than in SD animals (Figure 2). In addition, a decrease in the relative amplitude of the BT rhythm (RA) was observed in P23H rats during the second half of the experiment, when compared to SD specimens (Figure 2). The CFI, a parameter that takes into account RA, IS, and IV, also revealed a weaker strength of the circadian patterns recorded in P23H rats, when compared to those obtained for SD animals (Figure 2).

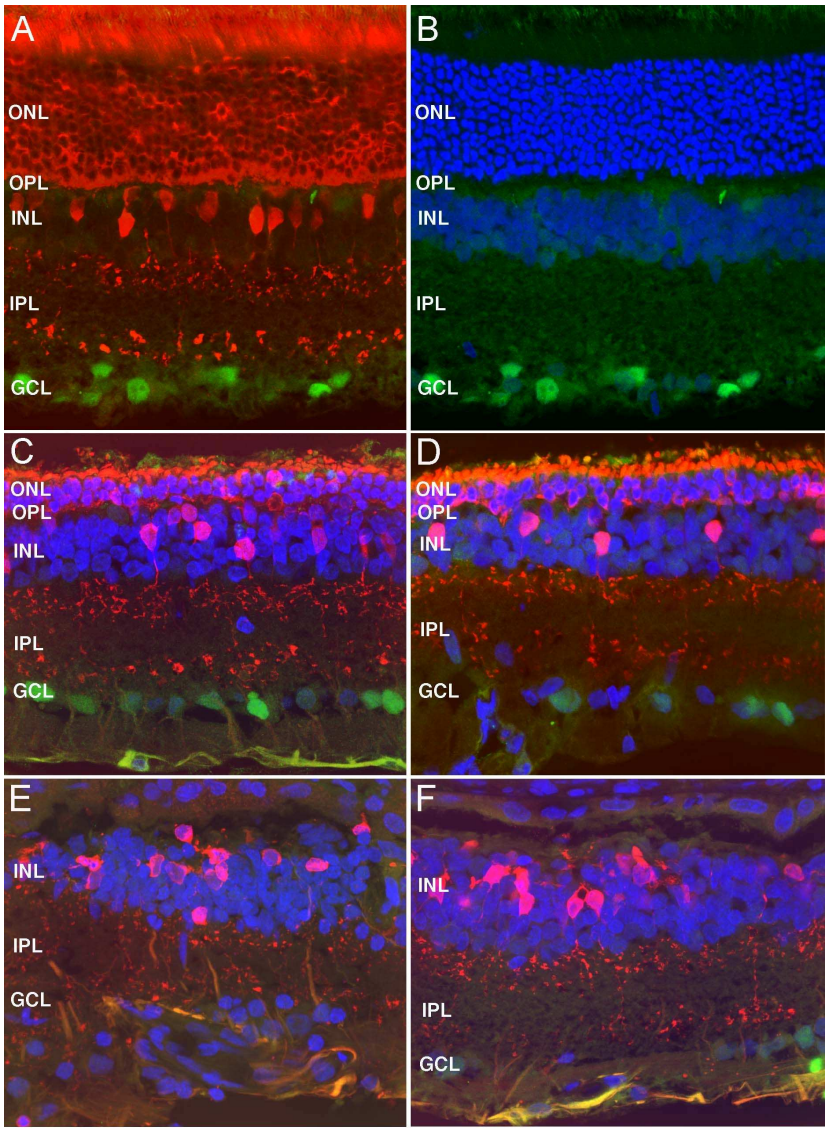

Figure 3. Histological labeling. (A, B) Vertical sections of retina from an SD control rat at 6 months of age (SD 6 m) labeled with recoverin and Brn3a (A) or Brn3a and TO-PRO (B). (C–F) Immunolabeling with recoverin, Brn3a and TO-PRO in retinal vertical sections from control (C, E) and melatonin-treated (D, F) P23H rats at 6 months (C, D) and 18 month of age (E, F). Note the absence of photoreceptors in the outer nuclear layer and the loss of ganglionar cells in P23H rats at 18 months. [abbreviations: outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL)]. Scale bar = 20 µm.

Retinas from P23H animals showed a progressive degeneration during the experiment (Figure 3). Vertical sections of the retinas immunostained with recoverin, which labels rod and cone photoreceptors, showed a pronounced reduction in photosensitive cells in the outer nuclear layer (ONL) of P23H rats at 6 and 18 months of age (Figure 3C,E, respectively), when compared to SD animals (Figure 3A). To quantify the degree of this degeneration, we stained the nuclei of retinal cells with TO-PRO. As shown in Figure 3, only two to three rows of photoreceptor cell nuclei remained in P23H rats at 6 months of age (Figure 3C), and no photoreceptors were present in the ONL of these animals at 18 months (Figure 3E). The immunostaining of cryostat section with Brn3a, which labels ganglionar cells (RGC), showed a noticeable loss of RGC at 18 months of age in P23H animals (Figure 3E).

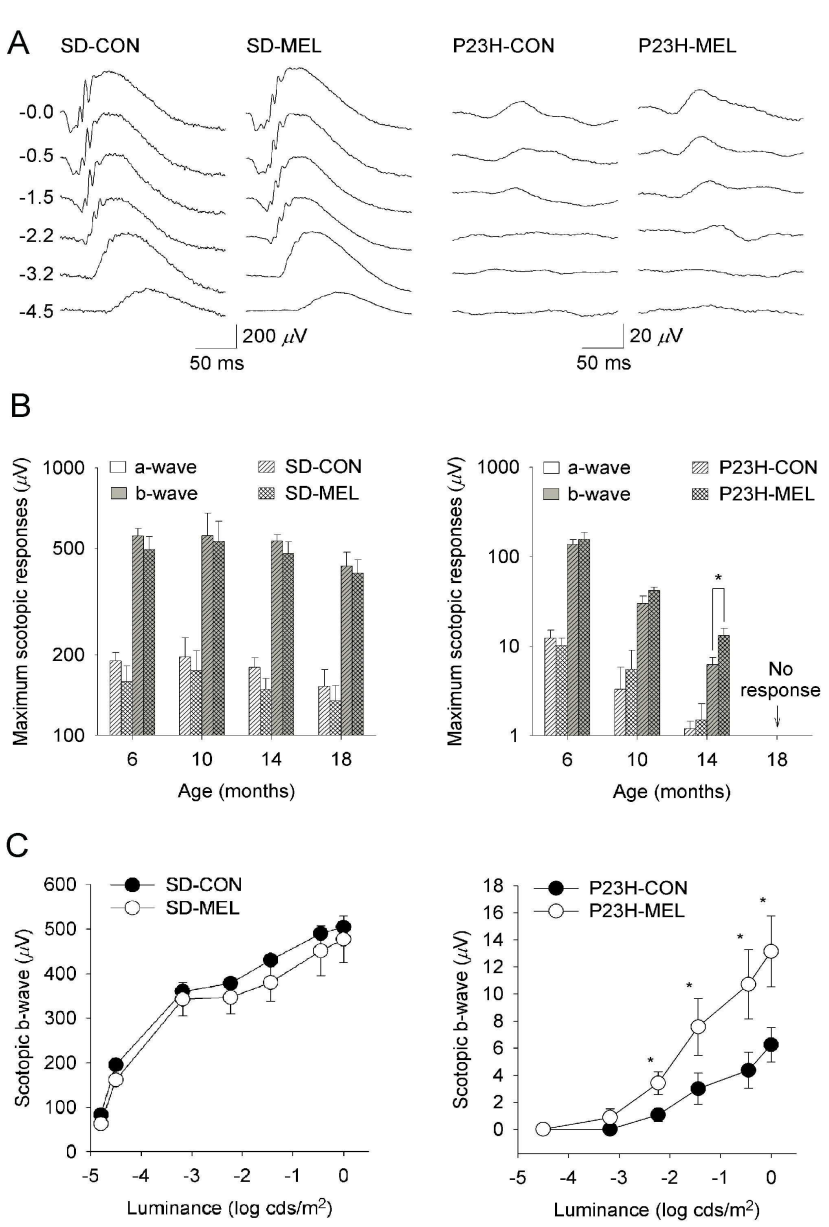

Figure 4. (A) Example of scotopic ERG traces from a 14-month-old SD (left) and P23H rat (right) treated or untreated with melatonin (MEL vs. CON). Units on the left of the panel represent the luminance of the flash in log cds/m2. (B) Maximum scotopic a-wave and b-wave recorded at 6, 10, 14 and 18 months in SD (left) and P23H animals (right), both treated and untreated with melatonin. Note that the scotopic b-wave recorded in P23H rats at the age of 14 months reached higher values in melatonin-treated animals than in the control specimens. (C) Stimulus intensity curve for scotopic b-waves from 14-month-old SD (left) and P23H rats (right), treated and untreated with melatonin. *P < 0.05, Student's t-test.

Retinal degeneration in P23H rats was reflected by their light-induced retinal responses (Figure 4). The dark-adapted ERG was characterized by measuring the mixed (cone- and rod-driven) a-wave (Va) and b-wave (Vb). Va and Vb were controls at all time points measured (6, 10, 14, and 18 months; $P < 0.01$, Student's t-test). Va and Vb values for P23H animals progressively decreased with age, reaching undetectable values at the age of 18 months.

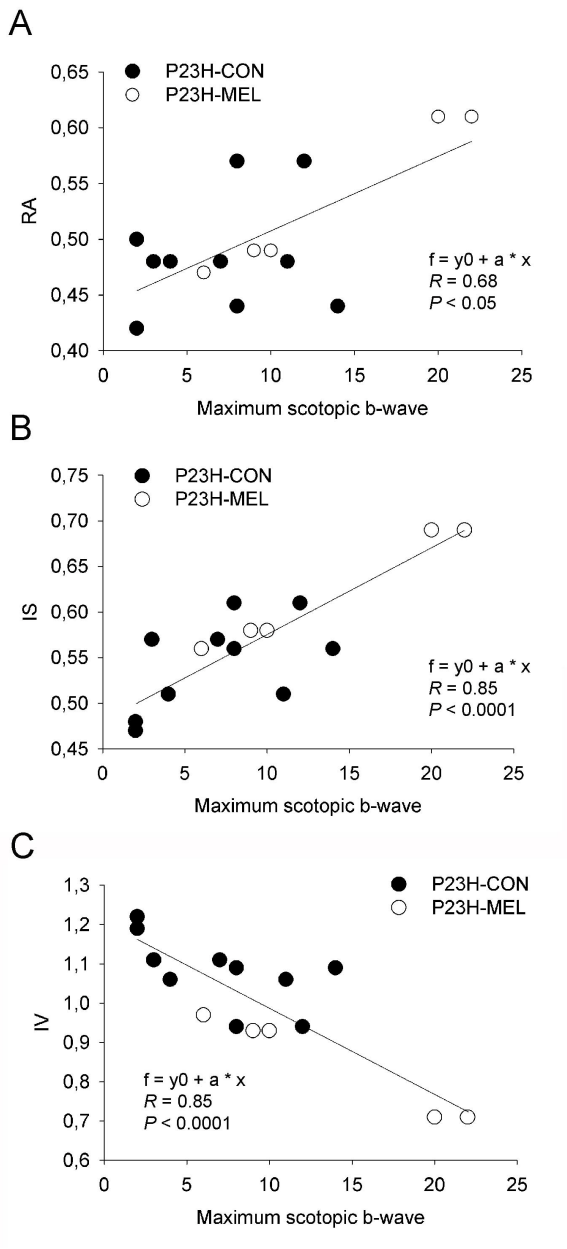

Figure 5. Correlation between the maximum scotopic b-wave recorded on P23H rats (treated or not with melatonin) at the age of 14 months and values of the chronobiological analysis obtained at the same age: (A) relative amplitude (RA), (B) interdaily phase stability (IS) and (C) intradaily variability (IV) of the BT rhythm.

Figure 4B shows how the maximum scotopic a-wave (Vamax) and b-wave (Vbmax) amplitude, obtained at the highest stimulus intensity, decreased with age in P23H rats, regardless of whether they were treated with melatonin.

Exogenous melatonin produced no changes in the ERG responses of wild-type animals (Figure 4). However, melatonin administration (2 mg/kg BW/day) caused an improvement in the visual response of P23H rats (Figure 4B). In fact, the Vbmax recorded at 14 months of age demonstrated higher values in melatonin-treated animals than in control rats (13.1 ± 2.6 versus 6.3 ± 1.3 lV; $P < 0.05$, Student's t-test). Figure 4A,C show how the P23H scotopic Vb was higher at 14 months of age in P23H rats treated with melatonin, when compared to the control animals.

In parallel, melatonin administration produced a noticeable improvement in the BT circadian patterns recorded in P23H rats. Figure 2 shows how the intradaily variability decreased more markedly in P23H-treated animals, a difference that became statistically significant at the age of 14 months.

Histological studies of the retinas showed no apparent differences between SD-CON and SD-MEL animals at any ages. No differences in the number of rows of retinal photoreceptors and in the retinal structure were also found between P23H-CON and P23-MEL at the age of 6 months (Figure 3C,D). However, at 18 months, the retinal layers in P23H-MEL rats were more organized, well defined, and structured than in P23H-CON animals (Figure 3E,F).

When ERG values were compared to the results obtained during the chronobiological evaluation, a correlation was found between the scotopic b-wave recorded on P23H and nonparametric values (Figure 5). In fact, the maximum b-wave recorded for P23H rats (both treated and untreated with melatonin) at the age of 14 months significantly correlated with RA and IS (R = 0.68 and 0.85, respectively), while the opposite was found for IV (R = 0.85).

DISCUSSION

The present work provides evidence that the retinal degeneration described by the retinitis pigmentosa model P23H positively correlates with the occurrence of circadian dysfunctions in these animals. Decreased amplitudes, a weaker coupling strength of the rhythm to environmental *zeitgebers* and higher rhythm fragmentation were found in P23H rats after severe retinal degeneration. We have also shown that administration of melatonin in the drinking water reduces both visual degeneration and circadian rhythmicity impairment.

The present study concerns P23H transgenic rats, which have been engineered to mimic a naturally occurring human mutation manifesting as a gradual loss of photoreceptors (Berson et al., 1991; Machida et al., 2000; Cuenca et al., 2004; Pinilla et al., 2005). P23H rats show a tight correlation between photoreceptor responses and histopathologic retinal changes (Machida et al., 2000; Cuenca et al., 2004), making them a valuable model of the human condition. In our results, retinas from P23H animals showed a progressive degeneration, in accordance with previously described data (Machida et al., 2000; Cuenca et al., 2004; Pinilla et al., 2005). Along the same lines, the absence of both photoreceptors and ERG responses was observed in 18-month-old P23H rats, which also showed an evident loss of RGCs.

By 50 yr of age, 95% of people with RP experience intermittent insomnia, daytime sleepiness, and reduced alertness (Gordo et al., 2001; Ionescu et al., 2001), suggesting that photoreceptor degeneration has an influence on the circadian cycle. Moreover, a previous study carried out on rds/rds mice (Mrosovsky & Thompson, 2008), an animal RP model, demonstrated a severe reduction in the positive masking response to dim light in this animal at

1 yr of age. In our results, P23H rats showed impaired circadian rhythmicity, consisting of decreased amplitude, weaker coupling strength, and higher rhythm fragmentation, than that observed in wild-type animals. This would suggest that the disturbance of circadian rhythms in P23H rats may be associated with the aforementioned visual loss.

Our results also show a correlation between the dark adapted ERGs recorded on 14-month-old P23H rats (both treated and untreated with melatonin) and both the relative amplitude of the BT rhythm and the degree of phase homogeneity during the corresponding experimental period. These results represent further evidence of the existence of a positive correlation between the animals' visual capacity and the strength of their circadian rhythmicity.

Despite the circadian dysfunctions found in P23H rats, all rhodopsin-mutant animals displayed 24-hr-entrained rhythms, even after the total loss of both photoreceptors and measurable ERG responses. This agrees with a previous study that found normal melatonin secretion rhythms in patients with retinitis pigmentosa (Banas et al., 1995). This continued rhythm entrainment could be attributed to the existence of nonphotic cues, such as temperature (oscillating in ± 1^{o}C) and social behavior (despite animals were individually housed). However, photic input to the retinothalamic tract is known to persist in visually impaired individuals if retinal ganglion cell photoreceptors and their suprachiasmatic connections are intact (Turner & Mainster, 2008). Retinitis pigmentosa may affect ganglion cells as well as rod and cone photoreceptors. owever, previous studies on P23H rats (line 1) have provided evidence that, despite the retinal degeneration found at 14 months of age, RGCs maintain their basic physiologic and networking properties (Kolomiets et al., 2010). In our results, retinal sections from 18-month-old P23H rats (line 3) showed the presence of RGCs, despite the total absence of photoreceptors.

Research conducted on visually impaired subjects has shown that, when appropriately administered, exogenous melatonin is able to entrain circadian desynchronization (Lockley et al., 2000; Hack et al., 2003) and improve circadian rhythm disturbances (Skene et al., 1999). On the other hand, experiments using glaucoma models have shown that melatonin diminishes the vulnerability of retinal ganglion cells to the deleterious effects of ocular hypertension (Belforte et al., 2010), which may improve the functionality of the photosensory brightness-sensing system. In our results, the administration of melatonin improved the BT circadian patterns recorded in P23H rats. Although significant differences were observed only with respect to the intradaily variability, melatonin administration improved the relative amplitude and the interdaily phase stability of the BT rhythm recorded in P23H rats.

Melatonin has been shown to act directly as a free radical scavenger and to stimulate a

number of antioxidative enzymes (Tan et al., 1993; Liang et al., 2004; Paradies et al., 2010; Jou et al., 2010). The potential antioxidant role of melatonin in ocular tissues has been extensively demonstrated (Siu et al., 1998; Lundmark et al., 2006; Siu et al., 2006). Properly administered, external melatonin has been shown to prevent retinal degeneration in ocular diseases such as glaucoma (Lundmark et al., 2007; Belforte et al., 2010; Rosenstein et al., 2010), uveitis (Sande et al., 2008; Rosenstein et al., 2010), age-related macular degeneration (Yi et al., 2005), and cataracts (Abe et al., 1994; Li et al., 1997; Yagci et al., 2006). In a previous study conducted on rds/rds mice, melatonin administration significantly delayed photoreceptor loss and reduced the number of apoptotic photoreceptors (Liang et al., 2001). Moreover, mitochondrial membrane depolarization and reactive oxygen species have been detected at the peak of cell death in postnatal cultured rd retinas (Doonan et al., 2005). In the present study, exogenous administration of melatonin prevented the visual function decline previously described for P23H rats, improving the scotopic Vb obtained in P23H rats and their retinal structure.

Previous studies have shown that chronic exposure of the retina to melatonin at inappropriate times of the day may be injurious to the photoreceptors (Wiechmann et al., 2008). In our experiments, melatonin was administered dissolved in the drinking water. About 90% of the drinking activity in rats is known to occur at night (Yuan, 1995). Accordingly, although available around the clock, most melatonin intake occurred during the night, thereby eliminating any possible adverse effects from melatonin.

Based on the evidence presented here, we conclude that exogenous melatonin administered in the drinking water prevents vision loss and circadian rhythmicity impairment in P23H rats. This would indicate its potential use in the clinical treatment of retinitis pigmentosa. However, further studies are required to elucidate the mechanism behind these effects.

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3.7. Experimental Chapter 7

CIRCADIAN SYSTEM FUNCTIONALITY, HIPPOCAMPAL OXIDATIVE STRESS, AND SPATIAL MEMORY IN THE APPSWE/PS1DE9 TRANSGENIC MODEL OF ALZHEIMER DISEASE: EFFECTS OF MELATONIN OR RAMELTEON

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3.7. CIRCADIAN SYSTEM FUNCTIONALITY, HIPPOCAMPAL OXIDATIVE STRESS, AND SPATIAL MEMORY IN THE APPswe/PS1dE9 TRANSGENIC MODEL OF ALZHEIMER DISEASE: EFFECTS OF MELATONIN OR RAMELTEON

ABSTRACT

Alzheimer disease (AD) is a neurodegenerative disorder that primarily causes β-amyloid accumulation in the brain, resulting in cognitive and behavioral deficits. AD patients, however, also suffer from severe circadian rhythm disruptions, and the underlying causes are still not fully known. Patients with AD show reduced systemic melatonin levels. This may contribute to their symptoms, since melatonin is an effective chronobiotic and antioxidant with neuroprotective properties. Here, we critically assessed the effects of long-term melatonin treatment on the circadian system function, hippocampal oxidative stress, and spatial memory performance in the APPswe/PS1 double transgenic (Tg) mouse model of AD. To test if MT1/MT2 receptor activation, alone, was involved, we chronically treated some mice with the selective MT1/MT2 receptor agonist, ramelteon. Our results indicate that many of the circadian and behavioral parameters measured, including oxidative stress markers, were not significantly affected in these AD mice. During the day, though, Tg controls (Tg-CON) showed significantly higher mean activity and body temperature (Tb) than wild-type (WT) mice. Overall, Tb rhythm amplitude was significantly lower in Tg than in WT mice. Although melatonin treatment had no effect, ramelteon significantly reduced the amplitude of the Tb rhythm in Tg mice. Towards the end of the experiment, Tg mice treated with ramelteon (Tg-RAM) showed significantly higher circadian rhythm fragmentation than Tg-CON and reduced circadian Tb rhythm strength. The free-running period (τ) for the Tb and locomotor activity (LA) rhythms of Tg-CON was <24 h. While melatonin maintained τ at 24 h for Tb and LA in both genotypes, ramelteon treatment had no effect. In the behavioral tests, the number of approaches and the time spent exploring novel objects were significantly higher in Tg-CON than WT controls. Brain tissue analysis revealed significant reduction in hippocampal protein oxidation in Tg-MEL and Tg-RAM compared to Tg-CON animals. Our results suggest that not all aspects of the circadian system are affected in the APPswe/PS1 mice. Therefore, care should be taken when extending the results obtained in Tg mice to develop new therapies in humans. Our study also revealed the complexity in the therapeutic actions of melatonin and ramelteon in this mouse model of AD.

Key words: Alzheimer disease; melatonin; ramelteon; body temperature rhythm; circadian system; hippocampus; oxidative stress; spatial memory

INTRODUCTION

Alzheimer disease (AD) is a neurodegenerative disorder characterized by β-amyloid deposits and neurofibrillary tangles in the brain (Duyckaerts et al., 2009). Mitochondrial dysfunction and the resultant oxidative stress accumulation are believed to play a major role in the pathogenesis of AD (Marques et al., 2010). The most common symptoms of the disease are cognitive impairments and behavioral abnormalities that strongly affect autonomy and self-care of AD patients, and are the most frequent reason for difficulties in providing them with care.

Patients with AD also show disruption in their circadian rhythms (Wu & Swaab, 2007), including severe sleep disturbances that are characterized by increased nocturnal activity and daytime sleep (Lee et al., 2007; Merlino et al., 2010; van Someren et al., 1996). AD patients also display increased agitation and arousal in the late afternoon or evening, a condition known as "*sundowning*" (Bliwise, 1994; Vitiello et al., 1992). These alterations in their sleep-wake cycle are associated with phase shift in the core body temperature rhythm (Satlin et al., 1995; Volicer et al., 2001), presumably caused by alterations in the activity of the central pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus. Indeed, significant reduction in the number of vasointestinal polypeptide- and vasopressin-expressing neurons is reported in the SCN of AD patients, when compared to healthy aged-matched individuals (Wu et al., 2007).

Interestingly, changes in plasma levels of pineal-derived melatonin, one of the main output signals of the SCN, have also been found in AD patients. The nocturnal synthesis of pineal melatonin is regulated by the cyclic release of noradrenalin onto the pinealocytes in response to neural signals from the SCN. In AD patients, profound reduction in melatonin secretion, and changes in SCN and hippocampal MT1/MT2 receptor expression occur (Savaskan et al., 2002, 2005; Wu & Swaab, 2005; Wu et al., 2007). These changes in melatonin signaling may contribute to the circadian rhythm alterations and cognitive impairments associated with AD.

In addition to its important role as a chronobiotic, melatonin has antitumoral, neuroprotective, immunomodulatory, anti-inflammatory, and antioxidant properties (Galano et al., 2011; Reiter et al., 2010). Thus, melatonin can be used as a potential treatment for the circadian rhythm disturbances, high oxidative stress levels, and increased inflammatory

responses that typically occur in the pathogenesis of AD (Cheng et al., 2006; Dragicevic et al., 2011; Mahlberg et al., 2004; Pappolla et al., 2000). The chronobiological effects of melatonin are mediated by its interactions with the MT1/MT2 membrane receptors. While MT1 activation modulates SCN electrical activity, MT2 seems to be involved in phase shifting effects of melatonin that are dependent on a specific phase-response curve of the circadian system (Lewy et al., 1998).

Melatonin's protective effects against oxidative stress may partially involve its interactions with the enzyme quinone reductase 2 (Foster et al., 2000) and RORα receptors that results in stimulation of antioxidative enzymes (Tomás-Zapico & Coto-Montes, 2005). Further, melatonin directly acts as an antioxidant by scavenging a variety of free-radicals, actions that do not require receptor mediation (Galano et al., 2011; Reiter et al., 2009; Tan et al., 1993). That is, this neurohormone can act in several ways to reduce oxidative stress. Specific agonists, such as ramelteon, that selectively activates the MT1/MT2 receptors (Kato et al., 2005), but has no direct free-radical scavenging properties (Mathes et al., 2008), represent useful tools for discerning the pathways underlying melatonin's actions (Boutin et al., 2005).

In recent years, a wide variety of transgenic mouse models of AD have been developed (Duyckaerts et al., 2008). These models aim to replicate the AD-like neuropathologies (β-amyloid deposits and neurofibrillary tangles), behavioral abnormalities, and cognitive impairments commonly observed in patients with AD. Mutant mouse models have mainly focused on two genes, amyloid precursor protein (APP) and/or presenilin 1 (PSEN1) that are linked with the early onset of AD (Hardy, 1997). The double transgenic mice, co-expressing APP and PSEN1 mutant transgenes, begin to develop amyloid deposition in the hippocampus and neocortex at the age of 6-7 mo, and have abundant plaque deposits by 9 mo (Jankowsky et al., 2004). To date, although these transgenic animal models are increasingly used to study the neuropathology of AD (Arendash et al., 2001; Gordon et al., 2002; Reiserer et al., 2007; Savonenko et al., 2005), no studies have described in detail the circadian rhythm disruptions that can occur in this double transgenic model.

In the present study, we evaluated the circadian system function, hippocampal oxidative stress levels, and spatial memory performance in the double transgenic mouse model of AD. These mice carry a chimeric human/murine APP construct bearing the Swedish mutation and the exon-9-deleted PSEN1 mutation (the APP695swe and PS1dE9 mutations). We also evaluated the effects of long-term melatonin or ramelteon treatment in these AD mice in order to determine whether activation of MT1/MT2 receptors is sufficient to minimize impairments associated with AD progression. This study measured rhythmicity in two circadian outputs, body temperature (Tb) and locomotor activity (LA), and assessed spatial memory

performance during the active phase of the mice.

MATERIALS AND METHODS

Animals

A total of 33 male B6C3-Tg double transgenic mice (APPswe, PSEN1dE9 or APPswe/PS1) and 19 male wild-type littermates between 3.5 and 5.5 mo of age were used. Breeding pairs for the B6C3-Tg mice were purchased from the Jackson Laboratory and bred at the University of Valencia (Spain). Animals were individually housed in cages kept in an isolated room with controlled temperature, relative humidity and photoperiod (12 h light: 12 h dark (LD) cycle, lights-on at 17:00 h). Mice were fed commercial rat chow (A04-rat-mouse maintenance; Panlab, Barcelona, Spain) and were provided with drinking water *ad libitum*. All experimental procedures were performed in accordance to the Principles of Animal Care (Portaluppi et al., 2010), the European Communities Council Directive of November 24, 1986 (86/609/EEC), and guidelines issued by the Spanish Ministry of Agriculture, Fishing and Feeding (Royal Decree 1201/2005 of October 21, 2005), and were approved by the Bioethical Committee at the University of Murcia. Efforts were made to minimize the number of animals used, as well as their suffering.

Experimental procedures

After 1 mo of acclimation to laboratory housing conditions, mice were divided into five experimental groups: wild-type untreated (WT-CON, n = 9) and melatonin treated (WT-MEL, n = 10) groups; transgenic untreated (Tg-CON, n = 11) and melatonin treated (Tg-MEL, n = 11) groups, and transgenic mice treated with ramelteon (Tg-RAM, n = 11). Melatonin and ramelteon treatments were provided at an early age because it is suggested that starting the treatment before plaque deposition produces greater beneficial effects (Feng et al., 2006; Quinn et al., 2005).

Melatonin (5 mg/kg BW/day) and ramelteon (2 mg/kg BW/day) were administered in the drinking water or in the re-pelleted food, respectively, for 5.5 mo. Previously, we showed that 2 mg/kg BW/day of melatonin was sufficient to restore circadian system functionality in mice with a similar genetic background (C57BL6) as those used in the present study. The chosen dose of melatonin used here takes into account the reported doses (2.6 to 20 mg/kg BW/day) of melatonin commonly used in AD mice models (Feng et al., 2004; Furio et al., 2002; Olcese et al., 2009; Quinn et al., 2005), and that used in our previous work (Otalora et al., 2008).

To test their circadian system function after 2 mo of treatment, all mice were subjected to a 6-h phase advance in their LD cycle, and 2 wks later they were transferred to constant darkness (DD) for 3 wks. Finally, they were returned to their previous 12:12 LD cycle until the end of the experimental period. The batteries of the behavioral tests were performed at two stages, stage 1 and 2, corresponding to 5 wk and 5.5 mo into treatment, respectively.

Melatonin and ramelteon treatment

Every 2 days a stock solution for melatonin (Fagron Iberica, 33457-24; Barcelona, Spain) was prepared in 100% ethanol. Final concentration of melatonin (5 mg/kg BW/day) was achieved by adding stock solution to drinking water. Final ethanol concentration in the drinking water was .04%. Water with melatonin was supplied *ad libitum* for 5.5 mo in light-protected bottles. Water intake was measured throughout the experiment, and the concentration of melatonin provided was adjusted accordingly. WT-CON, Tg-CON, and Tg-RAM were supplied with drinking water containing .04% ethanol throughout the experiment.

Weekly, the pelleted maintenance rat chow was supplemented with ramelteon (TAK-375; Rozerem®, Takeda Pharmaceuticals America, Inc. Deerfield, IL 60015), and the calculated ramelteon intake was 2 mg/kg BW/day. Control animals for this group were fed unsupplemented diet. Drug-treated and untreated animals consumed similar amount of food (3.3-3.7 g/d).

Data recordings

Tb was recorded every 2 h throughout the experiment using a data logger (ThermoChron®, Data loggers iButton; Maxim Integrated Products, Sunnyvale, California). Data loggers were implanted intraperitoneally under aseptic surgical procedures, as previously described (Otalora et al., 2008). Fluothane was used as anesthetic (Forane®; Abbot Laboratories S.A., Madrid, Spain) and an iodine solution (Betadine®; Viatris, Madrid, Spain) as a surgical scrub. Absorbable sutures (2-0; Safil®Quick; B/Braun, Barcelona, Spain) were used to suture the abdominal muscle layer, and non-absorbable silk (2-0; Silkam®; B/Braun) was employed to suture the skin.

LA was recorded in 10 min bins during the last 4 mo using infrared motion sensors (Omron, photoelectric switches, E3S-AD62; Kyoto, Japan) installed on the long side of each plastic cage. Activity sensors were connected to a data acquisition system (Electronic Service at the University of Murcia, Spain).

Behavioral tests

All behavioral tests were conducted in the animal's active phase under dim red light

(<10 lux). Tests were recorded by a ceiling-mounted video-camera, and these recordings were used for analysis. The test apparatus was cleaned between experimental sessions with 70% ethanol to eliminate any possible odor cues.

Y maze test

Before any treatment began, mice underwent a Y-shaped maze test to evaluate their general exploratory activity. The mice were placed inside the "vertical" arm of the maze. Latency to cross the intersection of the three arms was used to assign the mice to the five balanced experimental groups: WT-CON, WT-MEL, Tg-CON, Tg-MEL, and Tg-RAM.

Object novelty test

The object novelty test was the first assessment performed in the three 5-min trial sessions series (object novelty test, object recognition, and object location tests), where each test was separated by a 60-min interval. The protocols for these behavioral tests were modified from those described by Ennaceur et al. (2005). The tests were performed in a circular open arena (diameter 1 m, height 35 cm) made of white polyvinyl chloride. The object novelty test allowed us to both determine the animal's exploratory behavior and its level of anxiety. For this purpose, six identical objects, made of neutral material, were equally distributed on the border of the test arena of the apparatus. The mice were placed in the center and released for 5 min to explore the objects. Exploration of an object was satisfied when a mouse directed its nose to the object at a distance ≤2 cm and/or touched it with its nose. Conversely, sitting on or turning the object around was not considered as exploratory behavior. The following parameters were measured: frequency of object exploratory approaches, which refers to the total number of times a mouse had approached any of the six objects; total duration of object exploration, defined as the total time spent in exploring all objects; and latency of first approach, defined as the time spent to approach any of the six objects.

Object recognition test

This experiment was the second trial in the series of the three trials of memory. One of the objects was replaced by a novel object. The mouse was re-introduced to the open arena and the "choice" phase continued for a further 5 min. In the object recognition test, we evaluated the ability of the mouse to distinguish the new object among familiar objects by analyzing: latency and incidence of first approach to the new object, time spent for the new object exploration, and frequency of approaches to the new object. Discrimination between novelty and familiarity in object recognition was assessed by the following indexes, modified from Ennaceur et al. (2005): index of discrimination based on the duration of approaches (the percentage of time spent exploring the new object compared to the time spent exploring all

objects) and index of discrimination based on the frequency of approaches (frequency of approaches to the new object compared to exploration frequency for all objects, expressed as percentage).

Object location test

This was the last memory test of the trails. The novel object was displaced to a new location in the tested arena, substituting one of the familiar objects. To evaluate the animal´s ability to recognize the new position (spatial memory), incidence, frequency, and duration of approaches to the object, both in the new and old location were measured. Discrimination between novelty and familiarity in object location was assessed by the following indices, modified from Ennaceur and co-authors (2005): index of discrimination based on the duration of approaches (the percentage of time spent exploring the object in the new or original location compared to the time spent exploring all objects) and index of discrimination based on the frequency of approaches (frequency of approaches to the new or original location compared to total exploration frequency for all objects, expressed as percentage).

A different set of objects was used when mice were retested after 5.5 mo of treatment. Positions of the new object in the object recognition and object location tasks were the same in the trials repeated after 5.5 mo of treatment.

Tissue collection

At the end of the experiment (after 5.5 mo of treatment), mice were sacrificed by decapitation. Brains were removed and immediately placed on ice. The hippocampus was dissected and quick-frozen in liquid nitrogen. All samples were stored at −80°C until required for analysis.

Lipid and protein damage

Protein carbonyls

Oxidative modification of proteins in the hippocampus was measured by Oxyblot^{TM} Protein Oxidation detection kit S7150 (Millipore, Temecula, CA 92590, USA). This assay detects oxidation-dependent carbonyl groups within proteins. After derivatization of these sites with dinitrophenyl hydrazones, oxidatively-modified proteins were detected by an antibody-mediated process. Protein preparation, processing, and detection by western blot were performed according to the manufacturer's instructions.

Determination of malondialdehyde (MDA)

Lipid peroxidation was determined in hippocampal samples as levels of MDA, assessed by a

high-performance liquid chromatography method (Wong et al., 1987), using a UltiMate® 3000 HPLC system (Dionex, Barcelona, Spain) with a 15 x .46 cm C-18 Spherisorb column (Teknokroma, Barcelona, Spain).

Data analysis and statistics

To better understand the evolution of Tb and to facilitate its comparison with behavioral test results, Tb data were analyzed at two stages (stage 1 and stage 2) corresponding to 15 d prior to each behavioral test period. That is, after 5 wk (stage 1) and after 5 mo (stage 2) into the treatment regime. LA was also analyzed at stage 2. Results obtained for LA were normalized between 0 and 1, considering the data above the 95% percentile as 1.

Tb and LA data were analyzed using El Temps, a software package specifically designed for time series analysis (El Temps version 1.228; © Díez Noguera, University of Barcelona). Actograms, Fourier analysis, and mean waveforms were performed. The free-running period was calculated using the Sokolove-Bushell periodogram, based on the last 10 d when animals were under DD conditions. In addition, a non-parametric analysis of Tb and LA rhythms was performed. Inter-daily stability (IS, values between 0 and 1), a measure of the phase stability of the rhythms over days; intra-daily variability (IV, values between 0 and 2) which indicates the rhythm fragmentation within a day; relative amplitude (RA) calculated from the most active 10-h period and the least active 5-h period; and circadian function index (CFI, values between 0 and 1) as an integrated measure of circadian robustness (Ortiz-Tudela et al., 2010; Lax et al., 2011) were evaluated.

Based on the mean LA waveform, activity-onset was determined by the first 10-min bin when activity was higher than the mean value and followed by at least three out of the next six bins being above the mean value. Activity-offset was defined by the last bin when activity was greater than the mean value preceded by three out of six bins being above the mean value (Valentinuzzi et al., 1997). The length of the activity phase (α) was calculated as the time between activity-onset and activity-offset. Percentage of activity during the light phase, and the L/D activity ratio were also calculated.

The effect of genotype (WT vs. Tg), treatment (CON, MEL, RAM), and stage (stage 1 vs. stage 2), as well as their interactions on Tb were evaluated by factorial analysis of variance (ANOVA) with repeated measures, followed by Bonferroni post hoc pairwise comparisons. LA data and oxidative stress markers were analyzed by two-way ANOVA. Behavioral data were analyzed by a non-parametric analysis, the Kruskal-Wallis test, and inter-groups differences were revealed by Mann-Whitney U-test. Values of $p < .05$ were considered to be statistically

significant. Data are expressed as mean ± SEM. All statistical tests were performed using SPSS 15.0 (Statistical Package for Social Sciences, Chicago, IL, USA). Data from those animals that died during the experiment were excluded from the statistical analysis.

RESULTS

Circadian rhythmicity in Tb and LA under a 12:12 LD cycle and in constant darkness (DD) were similar in WT and Tg mice. The way in which WT and Tg mice responded to a 6-h phase advance, and the time needed for both groups to re-synchronize to the new LD cycle also did not differ (Figure 1). However, slight effects on circadian system function were seen during aging, and following chronic melatonin or ramelteon treatments.

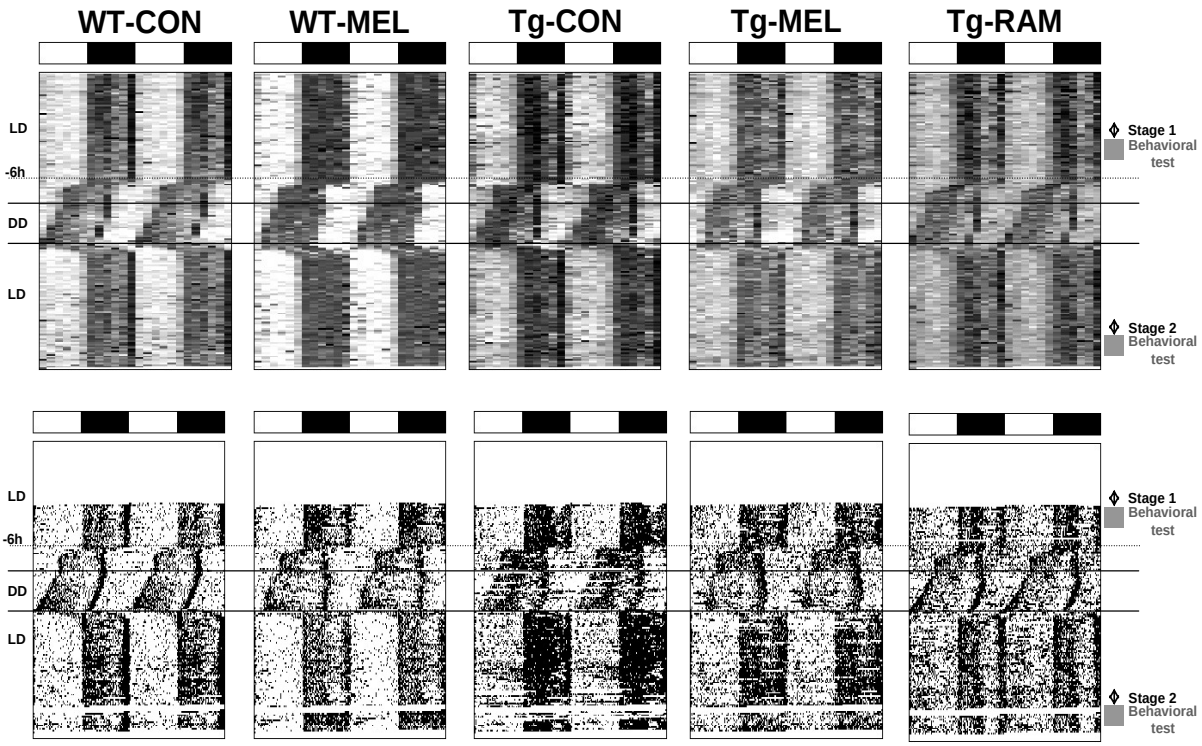

Figure 1. Double-plotted body temperature (top) and locomotor activity (below) actograms for a representative animal from each experimental group. After 3 mo under 12 L:12 D cycle, mice were subjected to a 6-h phase advance and then were transferred to constant dark (DD) conditions, as indicated on the left side of the actograms. Stage 1 and 2 correspond to 15 d prior to behavioral performance testing, that is, after 5 wk and after 5 mo into the treatments, respectively.

When the Tb rhythm was analyzed, no significant differences were found between genotypes in inter-daily stability (IS), intra-daily variability (IV), and circadian function index (CFI) (Figure 2A, B, and D). Further, melatonin treatment had no effect on rhythm

fragmentation (measured by IV), stability (measured by IS), and in overall circadian robustness (assessed by CFI). Transgenic mice treated with ramelteon (Tg-RAM), however, showed a trend to higher fragmentation values when compared with transgenic controls (Tg-CON) at stage 1, which reached statistical significance at stage 2 (Tg-CON = .84 ± .05; Tg-RAM= 1.10 ± .04, p = .01). The Tg-RAM group also showed significant reduction in circadian Tb rhythm strength when compared with Tg-CON (CFI: Tg-CON = .68 ± .02; Tg-RAM = .60 ± .01, p = .03).

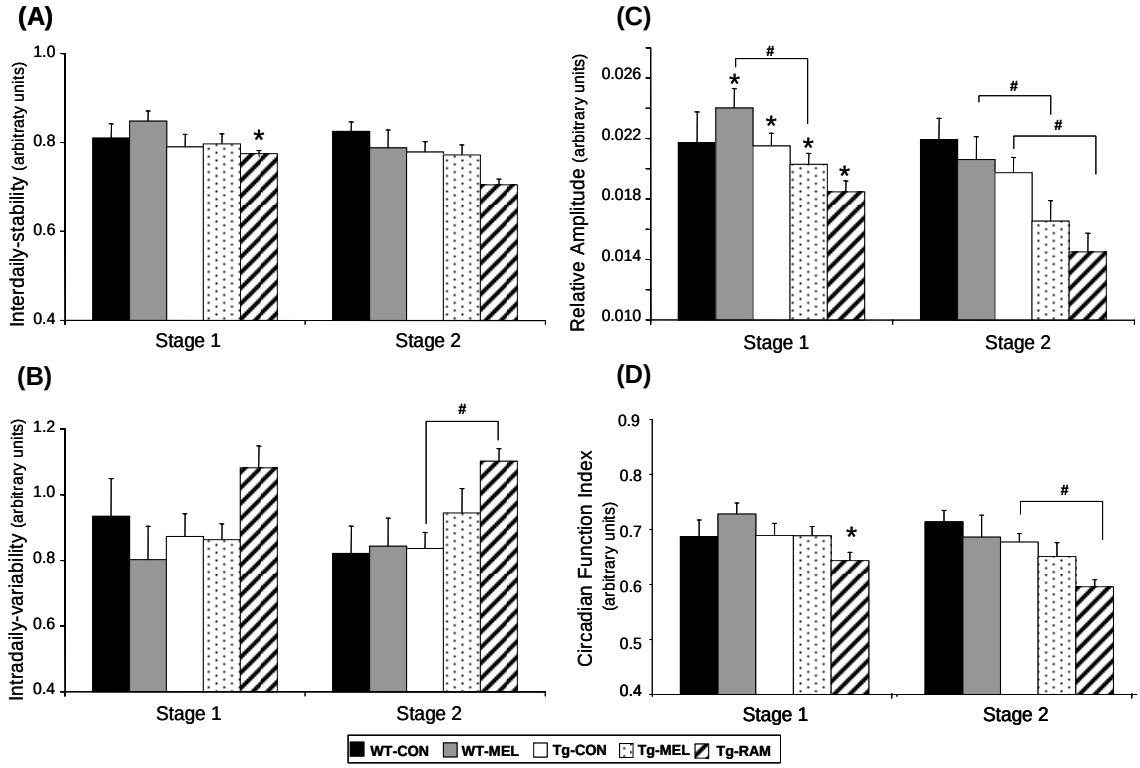

Figure 2. Non-parametric variables for body temperature rhythm: inter-daily stability (A), intra-daily variability (B), relative amplitude (C), and circadian function index (D) for each experimental group at stages 1 and 2. Values are expressed as mean ± SEM. Asterisks indicate statistically significant differences between stages 1 and 2 for each experimental group. Hashes indicate statistically significant differences between different treatments or genotypes. (*, #, p < .05, repeated two-way ANOVA, Bonferroni's test) (WT-CON = 4, WT-MEL = 5, Tg-CON = 8, Tg-MEL = 6, Tg-RAM = 5).

In general, the relative amplitude of the Tb rhythm in all mice was significantly lower at stage 2 than at stage 1 (F = 28.183, df = 1, p< .001; Figure 2C). This significant effect on relative amplitude across the stages was due to the lower peak values in Tb at stage 2 (stage 1 = 37.49 ± .05^{o}C and stage 2 = 37.22 ± .05^{o}C, F = 49.175, df = 1, p< .001).

Overall, the minimum Tb values during the light phase (henceforth referred to as minimum Tb) was significantly higher in Tg than in WT mice (WT = 35.19 ± .03^{o}C and Tg= 35.46 ± .03^{o}C, F = 17.051, df = 1 p< .001; Figure 3A). Since the maximum or peak Tb during the dark phase for both groups reached similar values, the resulting relative amplitude of the circadian

rhythm in Tg mice was significantly lower than in WT animals (F = 6.811, df = 1, p = .016; Figures 2C and 3A).

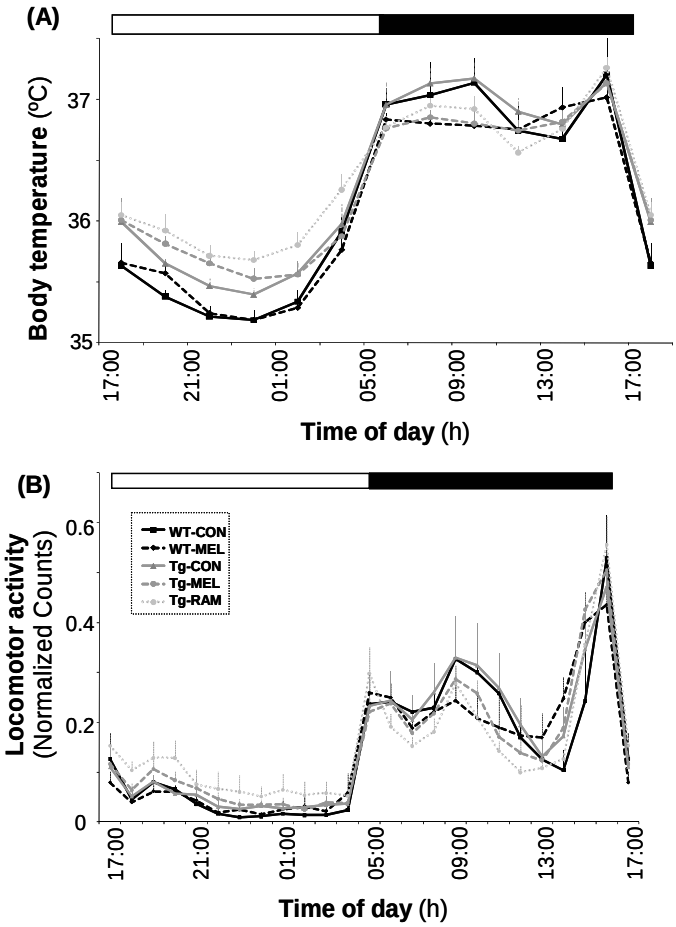

Figure 3. Body temperature (top) and locomotor activity (below) mean waveforms for each experimental group at stage 2. Values are expressed as mean ± SEM. (WT-CON = 4, WT-MEL = 5, Tg-CON = 5, Tg-MEL = 6, Tg-RAM = 5).

Although, melatonin administration to Tg or WT mice had no significant effects, chronic ramelteon treatment produced significant reduction in the relative amplitude of the Tb rhythm in Tg mice when compared to Tg-CON (p = .009). This was due to significantly higher values for the minimum Tb in Tg-RAM animals (Tg-CON = 35.36 ± .04ºC, Tg-RAM = 35.65 ± .05ºC, p = .008; Table 1 and Figure 3A).

Table 1. Maximum, minimum, and mean body temperature (Tb) values for each experimental group at stage 1 (upper row) and stage 2 (lower row).

Parameters	WT-CON n=4	WT-MEL n=5	Tg-CON n=8	Tg-MEL n=6	Tg-RAM n=5
Max Tb (ºC)	37.5 ± 0.22	37.5 ± 0.15*	37.6 ± 0.10*	37.4 ± 0.12*	37.4 ± 0.10
	37.3 ± 0.27	37.1 ± 0.06	37.3 ± 0.13	37.1 ± 0.07	37.3 ± 0.09
Min Tb (ºC)	35.3 ± 0.05	35.2 ± 0.07†	35.4 ± 0.04	35.4 ± 0.09	35.5 ± 0.07*
	35.2 ± 0.07	35.1 ± 0.07†	35.4 ± 0.05#	35.5 ± 0.10	35.6 ± 0.05
Mean Tb (ºC)	36.3 ± 0.13	36.3 ± 0.06*	36.4 ± 0.07	36.4 ± 0.09	36.4 ± 0.08
	36.2 ± 0.10	36.1 ± 0.04	36.3 ± 0.09	36.3 ± 0.03	36.4 ± 0.06

Values are expressed as mean ± SEM. Statistically significant differences are shown by asterisks (stages 1 vs. 2); crosses (WT-MEL vs. Tg-MEL), and hashes (Tg-CON vs. Tg-RAM) (*, † ,#, p < .05. repeated two-way ANOVA, Bonferroni's test).

No significant effect of genotype or treatment were found for activity-onset and activity-offset, activity duration (α) (Table 2 and Figure 3B), daytime activity, and L/D ratio (Figure 4A and B). When the entire daytime activity was averaged, no significant differences were found between genotypes. However, when activity was separately averaged during the early light phase (average from the first 5 h into the light phase) and late light phase (average from the next 5 h into the day), Tg-CON showed significantly higher mean activity values than WT mice in the late light phase (Tg-CON = .029 ± .006 and WT = .014 ± .001, *p*= .03, *t*-test; Figure 3B).

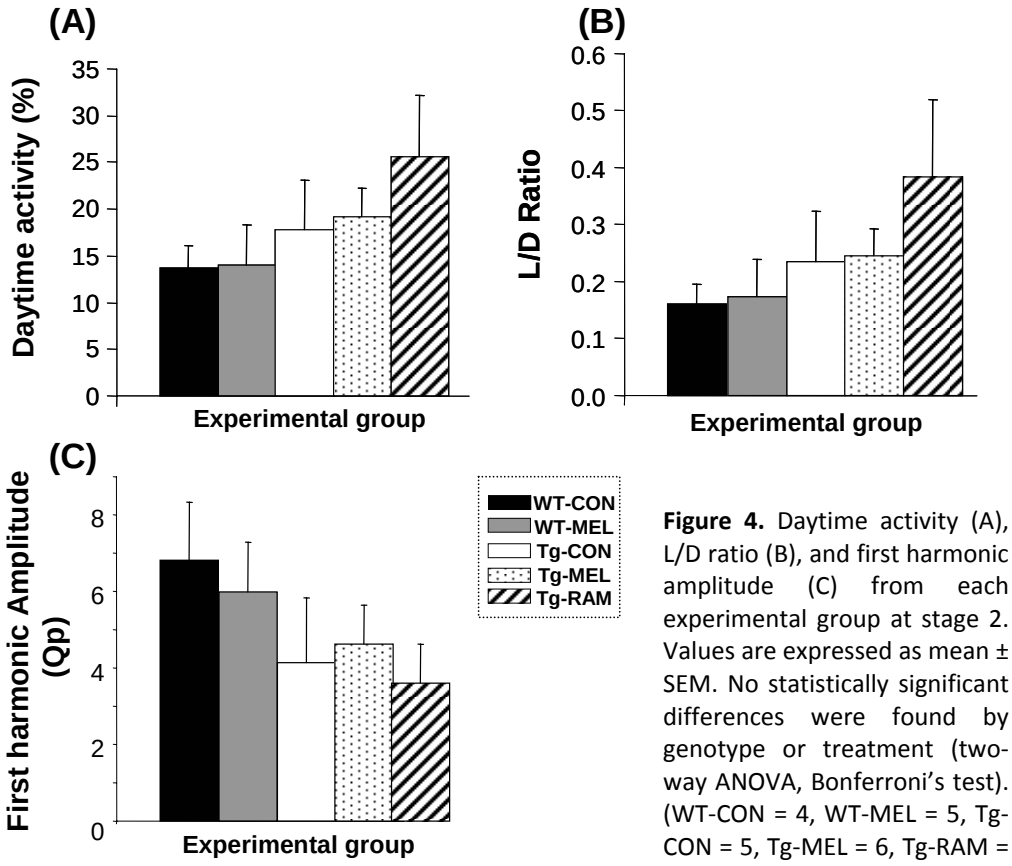

Figure 4. Daytime activity (A), L/D ratio (B), and first harmonic amplitude (C) from each experimental group at stage 2. Values are expressed as mean ± SEM. No statistically significant differences were found by genotype or treatment (two-way ANOVA, Bonferroni's test). (WT-CON = 4, WT-MEL = 5, Tg-CON = 5, Tg-MEL = 6, Tg-RAM = 5).

In general, Tg mice exhibited a slight reduction in circadian rhythm robustness, as measured by the power of first harmonic (F = 4.367, df = 1, *p*= .05; Figure 4C). Transgenic mice treated with ramelteon (Tg-RAM) tended to have a higher percentage of activity during the light phase and a higher L/D ratio when compared with the other groups (Figure 4A and B). Non-parametric analysis revealed that the differences between genotypes and treatment for IS, IV, RA, and CFI were not significant (Table 2).

Table 2. Summary of circadian locomotor activity (LA) parameters (mean ± SEM) for each experimental group at stage 2.

Parameters	WT-CON n=4	WT-MEL n=5	Tg-CON n=5	Tg-MEL n=6	Tg-RAM n=5
Interdaily-stability	0.27 ± 0.06	0.25 ± 0.02	0.28 ± 0.05	0.24 ± 0.04	0.22 ± 0.04
Intradaily-variability	1.02 ± 0.16	1.23 ± 0.16	1.11 ± 0.21	1.20 ± 0.09	1.16 ± 0.12
Relative amplitude	0.91 ± 0.01	0.85 ± 0.07	0.82 ± 0.04	0.73 ± 0.08	0.66 ± 0.13
Circadian function index	0.55 ± 0.05	0.49 ± 0.05	0.50 ± 0.06	0.45 ± 0.04	0.42 ± 0.07
Onset (min)	723.4 ± 8.15	715.1 ± 13.37	730.2 ± 7.91	726.1 ± 7.40	696.2 ± 22.67
Offset (min)	1451.3 ± 7.84	1442.1 ± 14.80	1453.4 ± 5.39	1452.5 ± 2.68	1458.2 ± 3.51
Alpha (min)	727.9 ± 14.95	727.0 ± 24.36	723.2 ± 10.83	726.4 ± 8.53	762.0 ± 24.30

No statistically significant differences were found between the different treatments or genotypes (two-way ANOVA, Bonferroni's test).

When animals were subjected to a 6-h phase advance in their LD cycle, no differences were found between Tg and WT mice in the time they needed to re-entrain to the new LD cycle. In all animals, the Tb and LA rhythms re-entrained to the new LD schedule within 4 d (Figure 5).

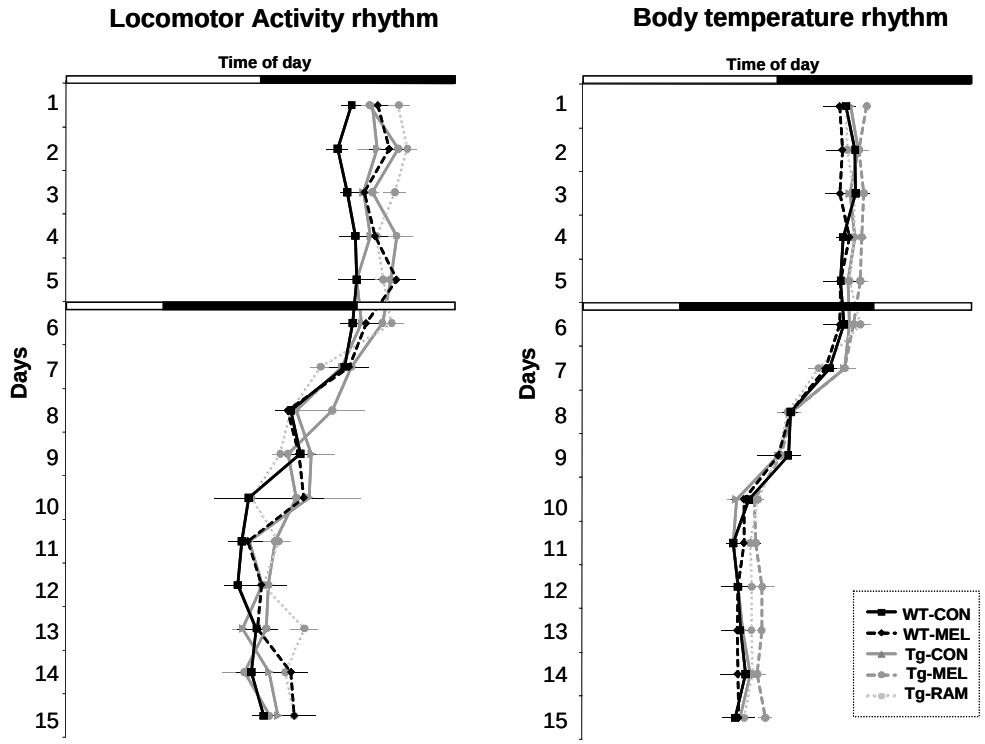

Figure 5. Daily evolution of locomotor activity and body temperature rhythm acrophases (peak times) per experimental group. Animals were subjected to 6-h phase advance of the LD cycle on d 6 (WT-CON = 4, WT-MEL = 5, Tg-CON = 5, Tg-MEL = 6, Tg-RAM = 5).

Figure 6 shows the free-running period for the animals under constant darkness (DD). Two-way ANOVA revealed significant effect of treatment on the free-running period (τ) ($F = 11.151$, df = 2, $p = .001$ for the Tb rhythm; $F = 10.279$, df = 2, $p = .001$ for the activity rhythm), but no differences were found by genotype, and there was no interaction. As in WT-CON, the τ in Tg-CON animals under DD was <24 h, both for Tb (1423.7 ± 2.81 min) and LA (1426.0 ± 2.74 min) rhythms. Surprisingly, while melatonin administration maintained τ at 24 hours for Tb and LA in both genotypes, ramelteon treatment had no effect (LA = 1427.5 ± 5.53 min, Tb= 1424.0 ± 2.74 min).

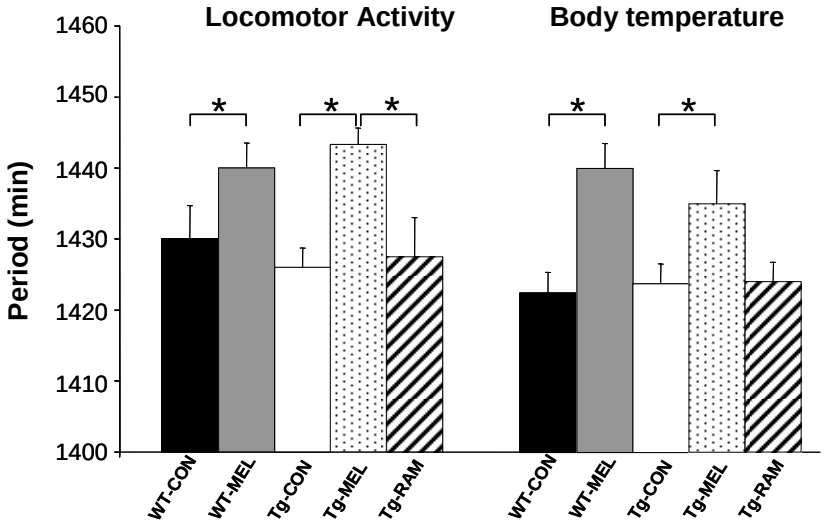

Figure 6. Free-running period for activity and body temperature rhythms under constant darkness (DD) conditions. Values are expressed as mean ± SEM. Asterisks indicate statistically significant differences (* $p < .05$, two-way ANOVA, Bonferroni's test). (WT-CON = 4, WT-MEL = 5, Tg-CON = 5, Tg-MEL = 6, Tg-RAM = 5).

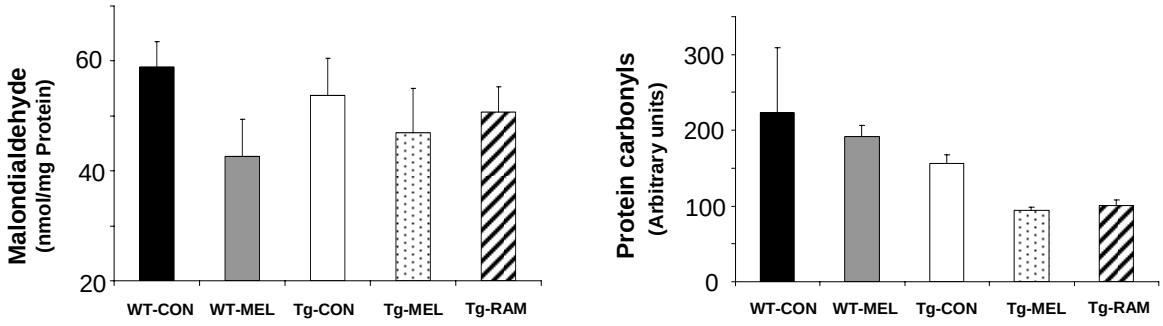

Figure 7. Oxidative stress markers -- malondialdehyde and protein carbonyls levels in the hippocampus -- of each experimental group. Values are expressed as mean ± SEM (WT-CON = 7, WT-MEL = 5, Tg-CON = 7, Tg-MEL = 7, Tg-RAM = 6).

AD progression is associated with increase in oxidative stress. Figure 7 shows the levels of two oxidative stress biomarkers, malondialdehyde (MDA) and protein carbonyls, in the hippocampus at the end of the experiment, after 5.5 mo of chronic treatment with melatonin or ramelteon. Interestingly, two-way ANOVA revealed no significant differences in the levels of MDA and protein carbonyls by genotype or by treatment, when results from all groups were considered. However, when only transgenic mice were compared, one-way ANOVA indicated a significant effect of treatment (F = 17.26, df = 2, p< .0001), showing a reduction in protein oxidation in the hippocampus of Tg-MEL and Tg-RAM animals with respect to Tg-CON.

Figures 8, 9, and 10 show cognitive performance results by genotype, age, and treatment. Before treatment, and to avoid potential differences ascribed to anxiety on cognitive functions, mice were distributed in groups according to similar anxiety level evaluated by the latency to cross the intersection of the three arms of the Y maze (see Materials and Methods; data not shown).

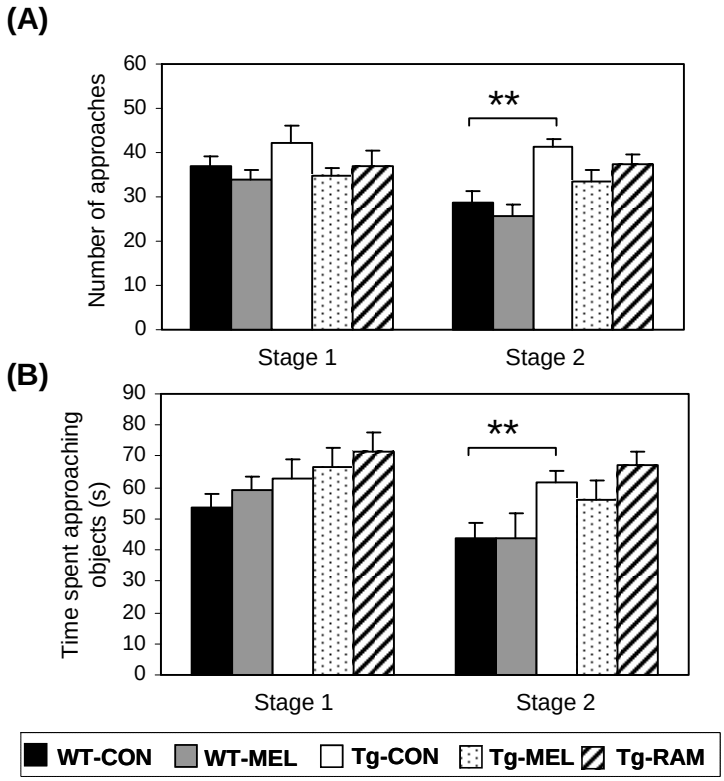

Figure 8. Object novelty test performed after 1.5-mo (stage 1) and 5.5-mo (stage 2) of treatment. The object exploratory behavior is presented by total frequency (A) and duration (B) of object approaches. Data are expressed as mean ± SEM. The intergroup differences are presented as *p < .05 and ** p< .01 (WT-CON = 8, WT-MEL = 5, Tg-CON = 11, Tg-MEL = 11, Tg-RAM = 9).

In the object novelty test performed at the stage 1, there were no significant differences between groups in any parameters tested (Figure 8). However, at stage 2, there were significant differences between groups in the total frequency and duration of object approaches (H = 15.366, df = 4, p< .01; H =11.915, df = 4, p < .05, respectively). Tg-CON approached more frequently to the objects and spent more time exploring them (Tg-CON vs.

WT-CON, $p < .01$). No effects of aging on the animal's performances (stage 1 vs. stage 2) were detected.

In the object recognition test, the latency, frequency, and duration of approaches to the new object were similar between groups at stage 1 and stage 2 (data not shown). Only an increase in the indexes of discrimination, based on the duration and on the frequency of approaches to the new object, was seen with aging (stage 1 vs. stage 2) (Figure 9).

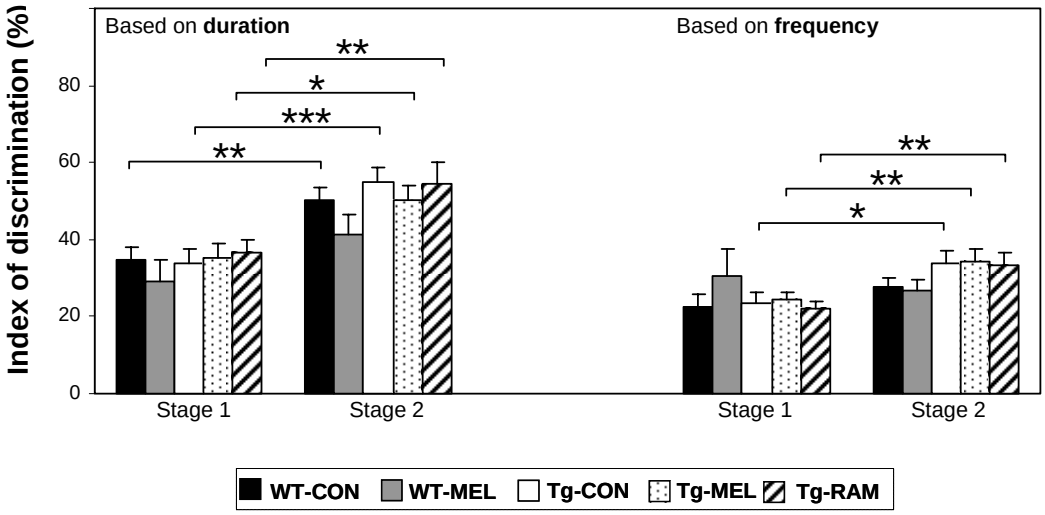

Figure 9. Object recognition test performed after 1.5-mo (stage 1) and 5.5-mo (stage 2) of treatment. Discrimination between novelty and familiarity in object recognition was assessed by the index of discrimination, based on the duration of approaches, and by the index of discrimination, based on the frequency of approaches expressed in percentage. Intra-group differences between stage 1 and 2 are presented as *p< .05, **p< .01, and ***p< .001 (WT-CON = 8, WT-MEL = 5, Tg-CON = 11, Tg-MEL = 11, Tg-RAM = 9).

In the object location test, the index of discrimination, based on the duration of approaches to the novel location, was higher at stage 2 compared to stage 1 in WT animals (WT-CON: $p < .05$ and WT-MEL: $p < .05$) (Figure 10A). However, no statistically significant differences were found between treatments or genotype. Similar results were obtained when the index of discrimination, based on the frequency of approaches to the novel position, was considered. That is, WT-CON ($p < .01$) and WT-MEL ($p < .05$) groups showed a higher number of approaches to a novel position at stage 2 (Figure 10B). No significant differences were detected in the duration or in the number of approaches to the familiar position (Figure 10A and B).

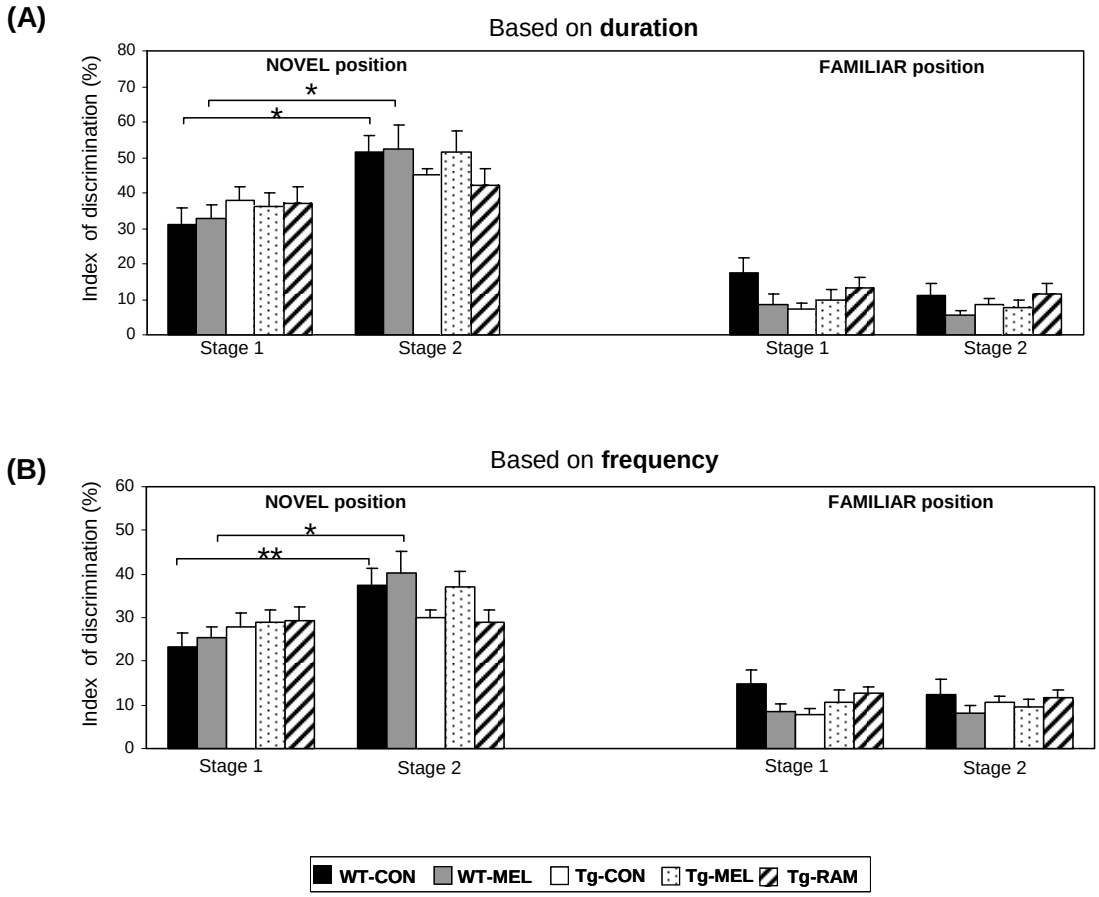

Figure 10. Object location test performed after 1.5-mo (stage 1) and 5.5-mo (stage 2) of treatment. Index of discrimination based on the duration of approaches (A) and based on frequency of approaches (B) expressed in percentage. The intra-group differences between stage 1 and 2 are presented as *p< .05, **p< .01 (WT-CON = 8, WT-MEL = 5, Tg-CON = 11, Tg-MEL = 11, Tg-RAM = 9).

DISCUSSION

The present study evaluates the circadian system functionality, hippocampal oxidative stress, and cognitive performance in the APPswe/PS1 double transgenic model of AD. To the best of our knowledge, this is the first report investigating the possible therapeutic effects of long-term ramelteon treatment, alongside those of melatonin, in an animal model of AD. We also report spatial memory performance in these Tg mice during their active phase.

Previous studies comparing the circadian system functionality in Tg models of AD with WT animals assessed activity records only (Gorman & Yellon, 2010; Sterniczuck et al., 2010; Vloeberghs et al., 2004; Wisor et al., 2005). To obtain a more in-depth overview of the circadian system functionality in AD mice, and to compare this with WT animals, in addition to activity records, we extended our investigation to include Tb measurements, one of the best markers for assessing the circadian system function. Overall, our findings show that in many

aspects, the circadian timekeeping system in Tg and WT mice functions in a similar way. For example, there were no differences in the onset, offset, and duration of activity between genotypes. These observations are consistent with a previous report using the Tg 2576 transgenic model of AD (Gorman & Yellon, 2010). When assessing τ in the different models of AD under DD, some studies reported longer (Gorman & Yellon, 2010; Wisor et al., 2005) while other claimed shorter (Sterniczuk et al., 2010) values. In the current study, the free-running period did not differ between WT and Tg mice. Thus, it was not surprising to find that both genotypes took a similar number of days to resynchronize to an advance in their LD cycle. Collectively, the findings suggest that in the age-range studied, our AD mouse model shares common, albeit unexpected, normality in their circadian system function with WT animals.

However, we noticed some key differences between WT and Tg mice used in the current study. Analysis of Tb shows that in the middle of the light phase, when Tb for both groups was at minimum, Tg mice had higher Tb than WT. At night, though, the Tb of both groups reached equivalent peak levels. As a result, we observed a dampened circadian rhythm in the core Tb of Tg mice, when compared to WT mice. Altered metabolic states coupled with hepatic impairment may account for the abnormally high Tb seen in the Tg mice during the day. But, the higher Tb in these animals during the rest phase could also be related with the elevated activity exhibited by these animals in the later part of the light phase. This higher daytime activity has also been reported in the 3xTg-AD model (Sterniczuk et al., 2010).

In general, Tg mice showed a slight reduction in activity rhythm robustness, as measured by the power of the first harmonic. This observation is not consistent with the results obtained in Tg2576 mice which showed a higher power than WT animals (Gorman & Yellon, 2010), suggesting that the activity rhythm in this AD model was more robust than in WT mice. This discrepancy could result from the great variability between the different transgenic AD mice models, where strain/model-specific phenotypes can emerge.

Tb rhythm analysis at the beginning and at the end of the experiment (stages 1 and 2) allows for the assessment of circadian function with age. Our results show that in both genotypes, only the reduction in the amplitude of the Tb rhythm was associated with aging. The lack of a significant interaction between stage and genotype for the Tb rhythm amplitude indicates there was no difference in the aging process between Tg and WT animals. This is consistent with the results reported by Gorman & Yellon (2010), suggesting the aging process was also not accelerated in the Tg 2576 mice.

In elderly people, melatonin treatment may improve the robustness and consolidation of the sleep/wake cycle, both in healthy individuals and in those with AD, although not all studies show significant effects (Wu & Swaab, 2007). Melatonin treatment (.5 mg/kg BW/d

and 2 mg/kg BW/d) is also reported to strengthen the circadian rhythms of some laboratory rodents (Otalora et al., 2008; Vivanco et al., 2007). These findings suggest melatonin may have beneficial effects on the circadian system, and may have therapeutic value in AD mice. However, treating WT and Tg mice with melatonin did not produce expected results. As shown by the circadian function index, inter-daily stability, and intra-daily variability, among other parameters measured, melatonin did not improve the function of the circadian system in these animals. Although these results are surprising, we have to take into account that in both genotypes the circadian system was functioning well. Thus, this lack of melatonin's effects on the circadian system may simply be because no further benefits could be gained by its administration to these animals. Melatonin, however, did have beneficial effects on controlling several pathological markers of AD (See below).

The effects of selective MT1/MT2 receptor activation by ramelteon (.003 to 30 mg/kg) on sleep and sensory and motor systems have been reported (Fisher et al., 2008; Miyamoto, 2006; Yukuhiro et al., 2004); herein, however we extend these observations by assessing ramelteon's possible therapeutic value in AD. Ramelteon treatment worsens activity and Tb rhythms in Tg mice. By the end of the experiment ramelteon treatment caused elevation in daytime activity and Tb which dampened the overall daily amplitude in these rhythms. This impairment of the circadian system function by ramelteon was surprising, and may be specific to this AD mouse model. The body weight and lipid deposit in Tg mice treated with ramelteon were significantly higher than in non-treated Tg animals, and Tg mice also suffered from hepatic hypertrophy (data not shown). It is, therefore, tempting to speculate that ramelteon affected the circadian system function in Tg mice by acting on other physiological factors that are linked with circadian rhythm generation.

Although melatonin treatment did not improve the LA and Tb rhythms under the 12 L:12 D cycle, its effect was apparent when animals were released under DD. In both genotypes, melatonin treatment maintained τ within 24 h, while mice that were not treated with melatonin or those treated with ramelteon exhibited shorter periods. This effect of melatonin was unexpected and warrants further investigation to clarify the involved mechanisms.

Melatonin or ramelteon administration did not accelerate re-entrainment to an LD phase advance. However, previous studies reported that melatonin and/or ramelteon facilitated re-entrainment (Hirai et al., 2005; Redman & Armstrong, 1988). In these studies melatonin and/or ramelteon were administered at set times, whereas here they were provided ad libitum, which may account for the differences in the results found.

It is believed that increased brain oxidative stress plays a major role in the pathogenesis of AD (Marques et al., 2010). It is well documented that melatonin reduces

oxidative stress by its direct free-radical scavenging properties (Poeggeler et al., 2002), and indirectly by possibly interacting with RORα receptors to activate antioxidant enzymes (Tomás-Zapico & Coto-Montes, 2005). Other studies (e.g., Feng et al., 2006; Quinn et al., 2005) have reported this therapeutic effect of melatonin is effective only when given at the early stages of AD. Our results showed a non- significant effect of melatonin treatment on damaged protein levels in the hippocampus of WT mice, whereas in Tg animals, melatonin caused marked reduction in protein oxidation in this brain region. Treatment with ramelteon, a selective MT1/MT2 receptor agonist, also reduced protein oxidation in the hippocampus of Tg mice. Unlike melatonin, ramelteon has no known direct free-radical scavenging properties (Mathes et al., 2008). Therefore, our results support previous views (e.g., Mathes et al., 2008) that activation of the melatonin receptors (MT1/MT2) may be sufficient to provide protection against oxidative stress in some situations. However, further work is needed to understand how ramelteon reduces oxidative stress levels in the brain of AD mice.

Impairments in face and object recognition, as well as diminished spatial memory, are early indicators of AD in humans (desRosiers et al., 1995). By extension, from the age of 6-9 mo, deficits in object discrimination (Howlett et al., 2004; Scullion et al., 2011) and spatial memory (Minkeviciene et al., 2004; Scullion et al., 2011) are reported in several lines of APP/PS1 mice. Our study indicates, however, that at 7 mo of age, exploratory behavior as well as cognitive performance were similar between Tg and WT age-matched littermates.

Although, at 12 mo of age, Tg mice showed more object exploratory behavior than WT animals, no significant differences in cognitive performance were observed between the two groups. These observations are in accordance with results obtained from 12-mo old APPswe+PSEN1De9 mice (Frye & Walf, 2008), and with previous finding from double transgenic APP/PS1 animals (Gruart et al., 2008). This suggests that all measured spatial/object recognition memory processes are preserved in these Tg animals. Our results, therefore, support the conclusion by these authors that the presence of amyloid plaques or genetic manipulations in AD mice is not related to learning and memory deficits seen in aged WT and Tg mice.

The effect of melatonin on spatial memory in AD transgenic mouse models has been reported in a few studies. Chronic melatonin treatment improved spatial performance of APP/PS1 mice in the 6-radial arm maze and 16-holes circular platform test, but not in the Morris water maze (García et al., 2009; Olcese et al., 2009) and in the platform recognition task (Olcese et al., 2009). Our results agree with the latter two studies, since no effect of melatonin treatment on cognitive performance was found. To date, no study has investigated the effect of ramelteon on the behavior and cognitive performance of transgenic AD mice.

Nevertheless, our results show chronic ramelteon treatment had no effect on the spatial/object recognition memory of Tg mice, both at 7 and 12 mo of age.

Altogether, our study shows that only specific aspects of the circadian system are affected in this double transgenic model of AD. Since the APPswe/PS1 transgenic mice failed to mimic most of the common symptoms and deficits that are normally present in humans with AD, care should be taken when extending the results obtained in these Tg mice to humans. Our results also demonstrate the effects of melatonin and ramelteon treatment in these AD mice are complex, and that further research is needed to fully understand their therapeutic values in AD.

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3.8. Experimental Chapter 8

EFFECTS OF EXOGENOUS MELATONIN AND CIRCADIAN SYNCHRONIZATION ON TUMOR PROGRESSION IN MELANOMA-BEARING C57BL/6 MICE

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3.8. EFFECTS OF EXOGENOUS MELATONIN AND CIRCADIAN SYNCHRONIZATION ON TUMOR PROGRESSION IN MELANOMA-BEARING C57BL6 MICE

ABSTRACT

Circadian rhythmicity impairment reportedly becomes significant as a tumor progresses, while the incidence of cancer can be affected by disruption of the circadian system. Melatonin has oncostatic effects on several types of cancer (breast, prostate and colorectal cancers), while it can be self-defeating in others, such us lymphoma. Melanoma is one of the most aggressive cancers in humans, however, it seems to respond positively to melatonin *in vitro.* The present work tested whether body temperature rhythms are impaired by tumor progression, and whether exogenous melatonin restricts tumor growth and restores circadian rhythmicity, therefore enhancing survival. To this end, C57BL6 mice were i.p. implanted with a temperature data logger and subcutaneously inoculated with melanoma cells. Animals were then submitted to LD 12:12 cycles or continuous light (LL), with or without melatonin administration. Under LD light conditions, the Tb rhythm exhibited a marked reduction in the 1st circadian harmonic amplitude, and increased phase instability (Rayleigh vector) as the tumor progressed. Melatonin administration (2 mg/kg BW/ day), on the other hand, increased the Tb rhythm amplitude and phase stability, reduced tumor weight and prevented intraperitoneal dissemination. Exposure to LL induced a free-running rhythm (1500 min), significantly increasing tumor malignity, and therefore reducing survival. Surprisingly, the highest tumor weights and morbidity by metastasis were seen in the LL group treated with melatonin probably because this indoleamine was being administered at different subjective hours to free-running animals. Circadian rhythmicity can thus be used as a marker rhythm for tumor progression, since rhythm impairment increases along with tumor malignancy. While melatonin administration improves rhythmicity and enhances survival under LD conditions, the results are self-defeating when they coexist with circadian disruption as it occurs under LL. This emphasizes the importance of taking into account endogenous rhythmicity and limiting melatonin administration to the subjective night in order to restrict melanoma progression.

Keywords: chronodisruption; continuous light (LL); core temperature rhythm; desynchronization; melanoma; melatonin; mice

INTRODUCTION

The main circadian pacemaker in mammals is located in the hypothalamic suprachiasmatic nucleus (SCN), which generates and coordinates locomotor activity, body temperature, and cortisol and melatonin circadian rhythms, among others (Moore & Eichler, 1972; Stephan & Zucker, 1972). The cyclic activity of the SCN is reset every day by periodic environmental inputs or *zeitgebers* (Mrosovsky, 1996); among which the light-dark cycle, with a period of 24 h, is the most relevant. However, other periodic cues, such as feeding restriction, are also *zeitgebers* (Lax et al., 1999; Sanchez-Vazquez et al., 2001).

Circadian system functional protection is a requisite for individual survival and normal aging. Lifespan and survival, respectively, are reduced in clock-gene mutant hamsters (Ralph & Menaker, 1988) and in SCN-ablated animals (DeCoursey et al., 1997), in this latter case due to increased predation. Moreover, circadian rhythm alterations (amplitude damping, phase shifts and/or period changes) are common in aging and pathological processes, such as those that occur during the later stages of cancer development. In fact, it has been documented that circadian cortisol and activity rhythm robustness are a prognostic factor for survival in colorectal cancer patients, in such a way that the better the circadian system status, the better the survival prognostics (Mormont et al., 2000; Mormont et al., 2002). Therefore, animal and human endocrine, metabolic and immunologic circadian rhythms can be reliable markers for tumor status (Mormont & Levi, 1997).

An increased amount of experimental evidence suggests that the disrupted circadian function generated by SCN lesions (Filipski et al., 2003) or chronic jet lag (Filipski et al., 2004) can accelerate tumor progression in tumor-bearing animals. These findings are also supported by several epidemiological studies, which have shown that chronodisruption resulting from prolonged exposure to shift work in nurses (Schernhammer et al., 2001), or from chronic jet lag in flight attendants (Rafnsson et al., 2001), is associated with a higher incidence of breast cancer. According to Schernhammer *et al.* 2003 this could be the result of the suppression of nocturnal melatonin secretion due to exposure to night light. The same hypothesis has also been proposed to explain the increased risk of colorectal cancer in patients subjected to rotating shifts (Schernhammer et al., 2003).

It has been widely documented that melatonin has oncostatic effects, both *in vivo* and *in vitro*, (Reiter et al., 2006). Melatonin has demonstrated significant antitumoral effects on the ovarian carcinoma cell line (Petranka et al., 1999), cultured human uveal melanoma cells (Hu & Roberts, 1997), metastatic cell sublines of mouse melanoma (B16BL6 y PG 19) (Cos et al., 2001), hepatomas (Blask et al., 2005) and positive estrogen-receptor human mammary tumors (Kiefer et al., 2002).

Oxidative stress contributes to the etiology of carcinogenesis (Klaunig et al., 1998). Over the last few years, hundreds of papers have been published reporting that melatonin (Tan et al., 1993) as well as its metabolites (Rosen et al., 2006; Manda et al., 2007; Tan et al., 2007) are highly effective free radical scavengers and potent antioxidants. In fact, melatonin's antitumoral effects are in part mediated by protecting DNA from oxidative damage (Karbownik et al., 2001). However, its oncostatic actions have also been attributed to limiting linoleic acid transport to tumor cells (Blask et al., 2002) and to its immunostimulating properties (Miller et al., 2006) and other actions (Shiu, 2007). There is also some evidence suggesting that the oncostatic effects of melatonin can be dependent on the dosage schedule, with melatonin being more effective when it is administered at the end of the light or at the beginning of the dark phase (Sauer et al., 2001; Reiter et al., 2006).

In spite of these favorable results, not all tumors respond positively to melatonin. Some of the negative results could be explained by an inappropriate administration time or by its immunoenhancing effects (Pawlikowski et al., 2002; Anisimov, 2003). As a result, more studies testing the effects of melatonin on different cancer models are needed.

Since rhythm robustness and internal temporal maintenance seem beneficial to cancer prognosis, we must take into account that melatonin, apart from being a potent antioxidant, is also a chronobiotic that can synchronize other circadian rhythms, as also occurs with feeding time. To date, very few studies have explored this possibility, especially with regards to melanoma, one of the most aggressive skin cancers (Gómez-Zapata et al., 1987; Pawlikowski et al., 2002). Moreover, conflicting results have been obtained regarding melanoma growth (Pawlikowski et al., 2002), perhaps due to differences in administration timing and photoperiodic regimen.

The aim of the present research is, therefore, to determine the effects of exogenous melatonin and circadian synchronization on survival and malignancy in melanoma-bearing mice. To this end, melatonin was orally supplied to C57BL6 mice that had been injected with the melanoma cell line and surgically implanted with a wireless data logger (Thermochron, iButton) to record body temperature.

MATERIALS AND METHODS

Animals

A total of 92 male C57BL6 mice with an initial body weight of 22 ± 2g were obtained from the animal facilities of the University of Murcia. The animals were individually housed in polycarbonate cages in an isolated room (with a temperature of 23 ± 2^{o}C, and a humidity of 60

± 10%). Light conditions, with an intensity of 200-300 lux, were scheduled by an electronic timer (Data Micro, Orbis, Madrid, Spain), according to the experimental design. Mice were fed commercial rat chow (A04 rat-mouse maintenance Panlab). All experimental procedures were performed in accordance with the "Principles of Animal Care" (NIH publication Nº 86-23, revised 1985) and Spanish laws and has approved by the Bioethical Committee from the University of Murcia.

Melatonin administration

Melatonin (Sigma, M-5250 USA) was dissolved in 0.02% ethanol to prepare a final stock solution of 80 mg/ml. It was then stored in darkness at -80^{o}C until use. The stock solution was diluted every two days by adding drinking water until reaching the appropriate concentration (2 mg/ kg BW/ day). Animals were allowed to drink from lightproof bottles from 19:30 to 08:30h. Water intake was recorded throughout the experiment, in order to calculate and maintain constant individual melatonin doses.

Data recording

Body temperature (Tb) was recorded every hour using a data logger (Thermochron®, Data loggers iButton DS1921H-F50, IDC S.A., Dallas) with an accuracy of 0.125^{o}C. Sterile data loggers were implanted intraperitoneally in 63 mice using aseptic surgical procedures. The rest of the animals served as controls, and did not have data loggers implanted. Fluothane was used as anesthetic (Forane®, Abbot Laboratories S.A., Madrid, Spain), and iodine solution (Betadine®) as a surgical scrub. Absorbable sutures (2/0, Safil®Quick B/Braun, Barcelona, Spain) were used to suture the abdominal muscle layer, and non-absorbable silk (2/0, Silkam®, B/Braun, Barcelona, Spain) was used to suture the skin. No mortality or morbidity was observed after the surgery. At the end of the experiment, as the mice died or were sacrificed by cervical dislocation, data loggers were removed and their data transferred to a computer.

Cancer cell lines and transplantation

The murine B16 melanoma line (European Collection of Cell Cultures, UK) was cultivated using Eagle's minimum essential medium (EMEM; Gibco, Langley, VA USA), buffered at pH 7.2 - 7.4 and supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich Chemicals, Madrid, Spain), penicillin (100 U/mL) and streptomycin (100 µg/mL) (Gibco, Langley, VA, USA). The absence of *Mycoplasma spp*. was verified by direct fluorescence with DNA specific colorant H33233 (Hoecht, Germany). Twenty-one days after the data loggers were implanted,

all animals were subcutaneously inoculated with $5x10^5$ viable B16 cells in the left flank. Survival was checked daily.

Experimental procedure

After a 20-day acclimation period to laboratory housing conditions, all animals were subjected to three experimental stages: I). After implanting the data loggers, all mice were maintained for ten days under an LD 12:12 (light off from 20:00 to 08:00h) photoperiod. Food and water were available *ad libitum*. II). Ten days before tumor cell inoculation, the housing conditions (light schedule and food and water availability) were changed according to the corresponding experimental design. III). Twenty-one days postinoculation, the animals were sacrificed by cervical dislocation. At this moment, body and tumor weight were determined and data logger recordings recovered. Housing conditions depended on the experimental group, as in stage II. This stage was subdivided into three consecutive periods of seven days each (labeled as 3-1, 3-2 and 3-3), in order to study tumoral evolution. The last period was excluded from the statistical analysis due to the early death of some animals as the result of intraperitoneal tumor dissemination. The mice were randomly assigned to one of these eight experimental groups:

LD-CON (n= 12), with data loggers surgically implanted, and LD-CONno (n= 10), with no data loggers, served as the control groups. Both were subjected to an LD 12:12 cycle (20:00-08:00h) and food and water *ad libitum* during all experimental stages.

LD-MEL (n= 10), with data loggers surgically implanted, and LD-MELno (n= 10), with no data loggers, were treated with melatonin in their drinking water (2 mg/kg BW/ day) throughout stage III. They were submitted to an LD 12:12 cycle (20:00-08:00h) during the entire experiment. Food and water were available from 19:30 to 8:30 throughout stages II and III.

LL-CON (n=21), with data loggers surgically implanted, and LL-SHAM (n=9), submitted to laparotomy, but with no data loggers implanted, were both subjected to an LD 12:12 cycle (20:00-08:00h) during postoperative recovery (stage I), and to LL from stage II until the end of the experiment. Food and water were available *ad libitum*.

LL-MEL (n=10) and LL-RES (n=10), with data loggers surgically implanted, were submitted to an LD 12:12 cycle (20:00-08:00h) and food and water *ad libitum* during stage I. During stage II they were maintained under an LL cycle. Food and water were only available between 20:30 and 08:30 h. Mice from LL-MEL were treated with melatonin in their drinking water (2mg/ kg BW/ day) throughout stage III.

Malignancy score

Five categories were defined (I-V), according to tumor aggressiveness. Tumor intraperitoneal dissemination that caused an animal's death before the date established for its sacrifice was considered as V. The rest of categories were established in terms of a tumor weight frequency histogram, in such a way that categories I and IV each included a sample size of 17% and included the smallest and largest tumor sizes, 2.0 - 6.9 g and 11.5 - 15.5 g, respectively. Categories II and III each included a sample size of 33%, and medium-sized tumors from 7.0 - 8.9 g to 9.0 -11.4 g, respectively.

Data analysis

Body temperature data were analyzed using software specifically designed for time series analysis (El Temps version 1.192; © Díez Noguera, University of Barcelona). Actograms, mean waveforms, periodograms, Fourier analysis and Rayleigh tests were performed.

The free-running period was calculated using the Sokolove-Bushell model. Rhythm amplitude was determined as the difference between the maximum and minimum temperature values from the mean waveform for each experimental stage.

Statistical differences between groups and experimental stages were evaluated by factorial analyses of variance (ANOVA). Values of $p \leq 0.05$ were considered statistically significant. Lineal regression analyses were performed to establish the relationship between tumor progression and rhythm stability. Mortality data were analyzed using the χ^2 test. All statistics were performed using SPSS 13.0 (Statistical Package for Social Sciences, Chicago, IL).

RESULTS

Superimposed actograms of Tb from all C57BL6 mice are shown in figure 1. Under LD 12:12 (LD-CON), Tb exhibited a robust circadian rhythm (Figure 1A) with a bimodal pattern, demonstrating peaks immediately after light off and before light on (Figure 2A). This general pattern was maintained in LD-MEL (Figure 1B), with an increase in the amplitude of the Tb rhythm being the most evident effect observed in melatonin-treated animals (Figure 2B).

When animals were subjected to LL conditions (LL-CON), they exhibited a free-running rhythm with a mean period of 1500 min appeared (Figure 1C), and no significant rhythmicity with 24 h period (Figure 2C) was observed. Food and water restriction for 12 h out of every 24 h (LL-RES) was not able to entrain this free-running rhythm (which maintained a tau of 1500 min, Figure 1D), although a masking effect with a 1440 min period, induced by providing and removing food and water every 12 h, was observed (Figure 2D). When melatonin was added to

the drinking water, still only available for 12 h, besides the masking effect just mentioned, most animals displayed a relative degree of coordination between the free-running rhythm and the rhythm induced by melatonin administration (Figure 1E). A complete entrainment to the 24 h period of melatonin administration was not achieved, however (Figure 2E).

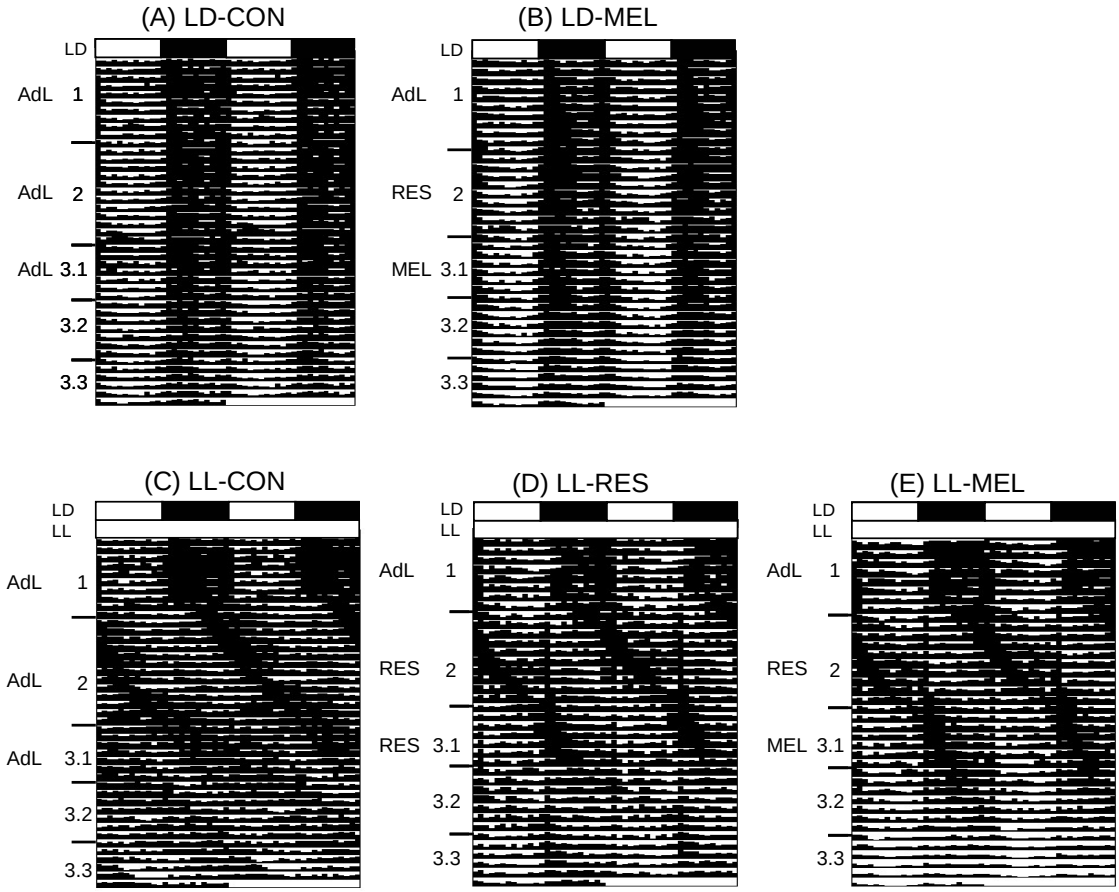

Figure 1. Superimposed body temperature (Tb) actograms from LD-CON (A, n=12), LD-MEL (B, n=10), LL-CON (C, n=11), LL-RES (D, n=10) and LL-MEL (E, n=10) groups subjected to three experimental phases. Stage 1: 10 days after data logger implant; Stage 2: 10 days before tumor cell inoculation; Stage 3: 21 days postinoculation, subdivided into 3.1: days 1-7; 3.2: days 7-14 and 3.3: days 14-21 following melanoma cell implant. Light and dark cycles are represented at the top of the graph by white and black bars (LD 12:12) and a white bar (LL). LL-CON, LL-RES and LL-MEL groups were exposed to continuous light from stage 2 until the end of the experiment. The feeding regimen is indicated on the left side as AdL (food and water *ad libitum*), RES (food and water available from 20:30 to 08:30h), and MEL (food and water with 2mg/ kg BW of melatonin added available from 20:30 to 08:30h).

The evolution of rhythmic parameters after tumor inoculation is shown in figure 3 and table 1. Tumor growth induced a progressive dampening of circadian rhythmicity, seen as a trend towards reduced mesor, amplitude, phase stability (as measured by the r vector) and the power of the first harmonic, with no changes in the free-running period.

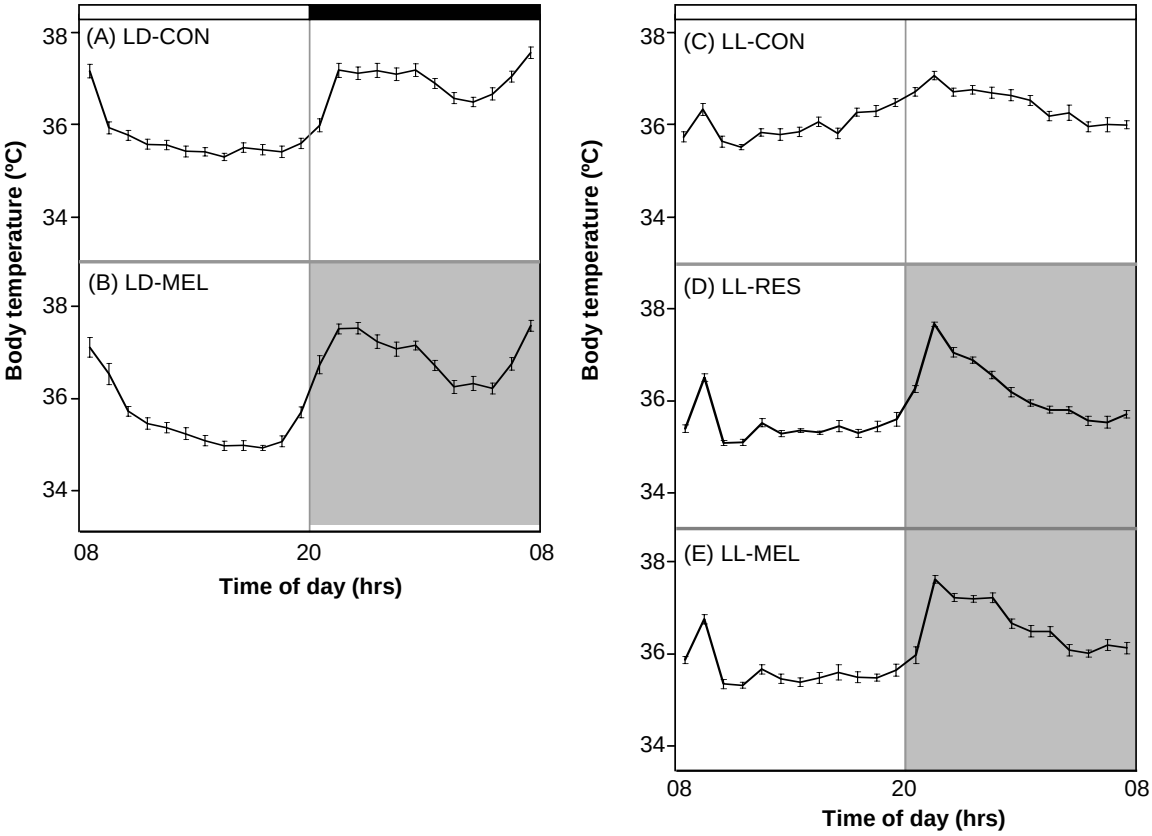

Figure 2. Mean body temperature (Tb) waveforms from LD-CON (A, n=12), LD-MEL (B, n=10), LL-CON (C, n=11), LL-RES (D, n=10) and LL-MEL (E, n=10) groups. Mean waveforms for stage 3.1 were averaged from individual waveforms (days 1-7 after implanting melanoma cells). The shaded area indicates food and water availability for the LL-RES group, and melatonin administration in the drinking water for LL-MEL and LD MEL groups. Values are expressed as mean ± SEM. The light and dark regimen (LD 12:12) is shown at the top of the graphs on the left by a black and white bar. Continuous light (LL) is indicated by a white bar at the top of the graphs on the right.

Table 1. Core temperature circadian parameters.

	Stage	LD-CON	LD-MEL	LL-CON	LL-MEL	LL-RES
Max. BT	2	37.74 ± 0.08^{ab}	37.90 ± 0.09^{a}	37.88 ± 0.08^{a}	$37.68 \pm 0.08^{a-c}$	37.39 ± 0.07^{cd}
	3.1	$37.60 \pm 0.12^{a-c}$	37.82 ± 0.08^{a}	$37.43 \pm 0.07^{b-d}$	$37.45 \pm 0.07^{b-d}$	37.25 ± 0.10^{d}
	3.2	36.86 ± 0.25^{e}	$37.60 \pm 0.09^{a-d}$	36.57 ± 0.20^{ef}	36.45 ± 0.14^{fg}	36.18 ± 0.16^{g}
Min. BT	2	35.16 ± 0.08^{ab}	$35.05 \pm 0.10^{a-c}$	35.50 ± 0.07^{d}	35.48 ± 0.09^{d}	$35.06 \pm 0.07^{a-c}$
	3.1	35.19 ± 0.09^{ab}	$34.85 \pm 0.08^{c-f}$	35.25 ± 0.07^{ad}	$35.1\,7 \pm 0.08^{ab}$	$34.93 \pm 0.04^{b-e}$
	3.2	34.68 ± 0.21^{ed}	$34.83 \pm 0.09^{c-f}$	34.97 ± 0.12^{bce}	$34.76 \pm 0.10^{d-f}$	34.64 ± 0.11^{f}
Amplitude BT	2	2.58 ± 0.06^{ab}	2.85 ± 0.08^{c}	2.38 ± 0.07^{ad}	2.20 ± 0.08^{d}	2.32 ± 0.10^{d}
	3.1	2.41 ± 0.07^{ad}	2.97 ± 0.07^{c}	2.18 ± 0.06^{d}	2.28 ± 0.06^{d}	2.32 ± 0.10^{d}
	3.2	2.18 ± 0.13^{d}	2.76 ± 0.08^{bc}	1.61 ± 0.12^{e}	1.70 ± 0.11^{e}	1.54 ± 0.12^{e}
Mesor	2	$36.31 \pm 0.07^{a-c}$	36.39 ± 0.08^{ab}	36.43 ± 0.06^{a}	$36.24 \pm 0.07^{a-d}$	$35.95 \pm 0.02^{d-f}$
	3.1	$36.28 \pm 0.09^{a-c}$	$36.25 \pm 0.08^{a-c}$	$36.19 \pm 0.06^{a-d}$	$36.08 \pm 0.07^{c-e}$	$35.81 \pm 0.03^{e-g}$
	3.2	35.68 ± 0.23^{fg}	$36.11 \pm 0.08^{b-d}$	$35.69 \pm 0.1\,4^{fg}$	35.59 ± 0.11^{gh}	35.32 ± 0.12^{h}

Intraperitoneal temperature circadian parameters (mean ± SEM) throughout different experimental stages (2, 3.1 and 3.2) for LD-CON, LD-MEL, LL-CON, LL-MEL and LL-RES groups. Different letters indicate statistically significant differences (ANOVA, $p \leq 0.05$). Max Tb: Maximum body temperature, Min Tb: Minimum body temperature. (See Figure 1 legend for group details).

In LD-CON mice, no significant changes were observed in either the r vector (Figure 3A), or the amplitude or mesor (Figure 3B, Table 1) during the first few days following tumor inoculation (stage 3.1, as compared to stage 2). However, statistically significant reductions were detected in the amplitude, mesor (Table 1), phase stability and the power of the first harmonic (Figure 3) later on in tumor development (stage 3.2).

Melatonin administration under LD conditions prevented Tb rhythm dampening due to tumor growth, and therefore no significant changes were observed in mesor, amplitude (Table 1), phase stability or the power of the first harmonic (Figure 3).

In animals subjected to LL conditions, no differences were observed between stage 2 and 3.1 (Table 2, Figure 3), except for the power of the first harmonic, which was significantly increased in LL-MEL and LL-RES. Again, it was in stage 3.2 when circadian rhythmicity was impaired. Mesor, amplitude (Table 1) and phase stability during this latter stage were significantly reduced with respect to stage 2 and 3.1, the only exception being LL-MEL, which maintained a high r value (Figure 3A). The power of the first harmonic decreased in LL-MEL and LL-RES, with respect to stage 3.1 (Figure 3B).

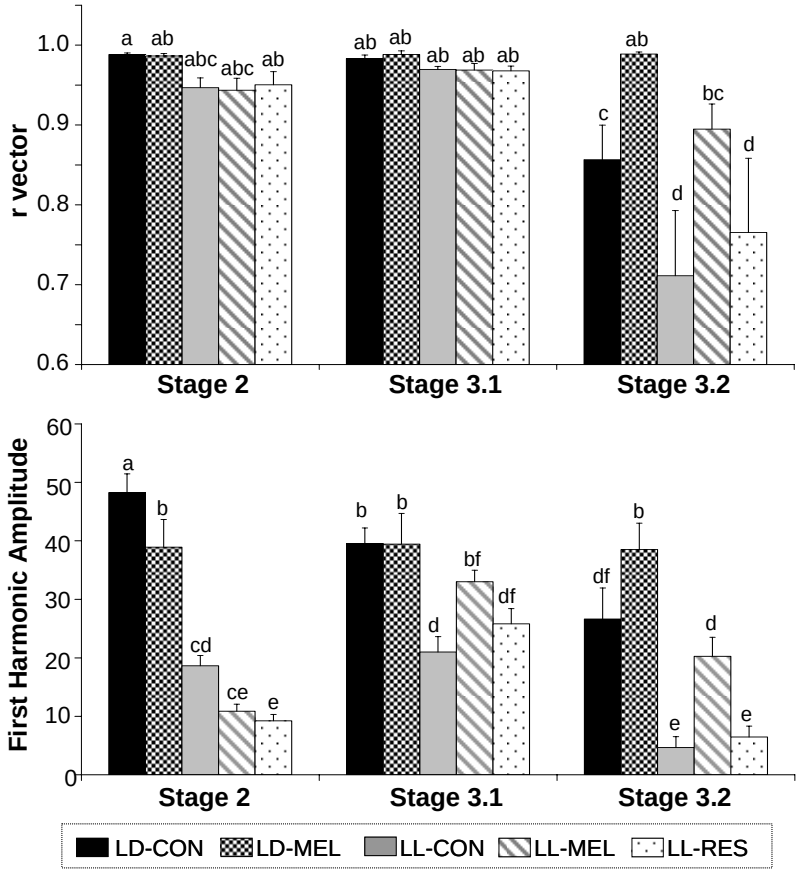

Figure 3. Phase stability as measured by Rayleigh test (A) and first harmonic amplitude (1440 min) by Fourier analysis (B) from LD-CON, LD-MEL, LL-CON, LL-MEL and LL-RES groups throughout the different experimental phases (see Figure 1 for details). Values are expressed as mean ± SEM. Different letters indicate statistically significant differences (ANOVA, $p \leq 0.05$).

The 24-h rhythm in Tb of LD-CON animals without intraperitoneal dissemination, sacrificed 21 days after tumor inoculation, showed a slight amplitude dampening and a

progressive decrease in mesor during the five days prior to sacrifice (Figure 4B). These effects were partially prevented by melatonin administration (Figure 4C).

When the animals died prematurely from intraperitoneal dissemination, as with LD-CON mice (Figure 4A), a progressive hypothermia and circadian rhythmicity impairment developed during the days prior to death. These were particularly marked in animals from to the LL-CON and LL-RES groups (subjected to LL conditions) during the last five days of their lives (Figure 4D and E). Again, melatonin administration under these conditions (Figure 4F) partially prevented the loss of rhythmicity as compared to LL-RES.

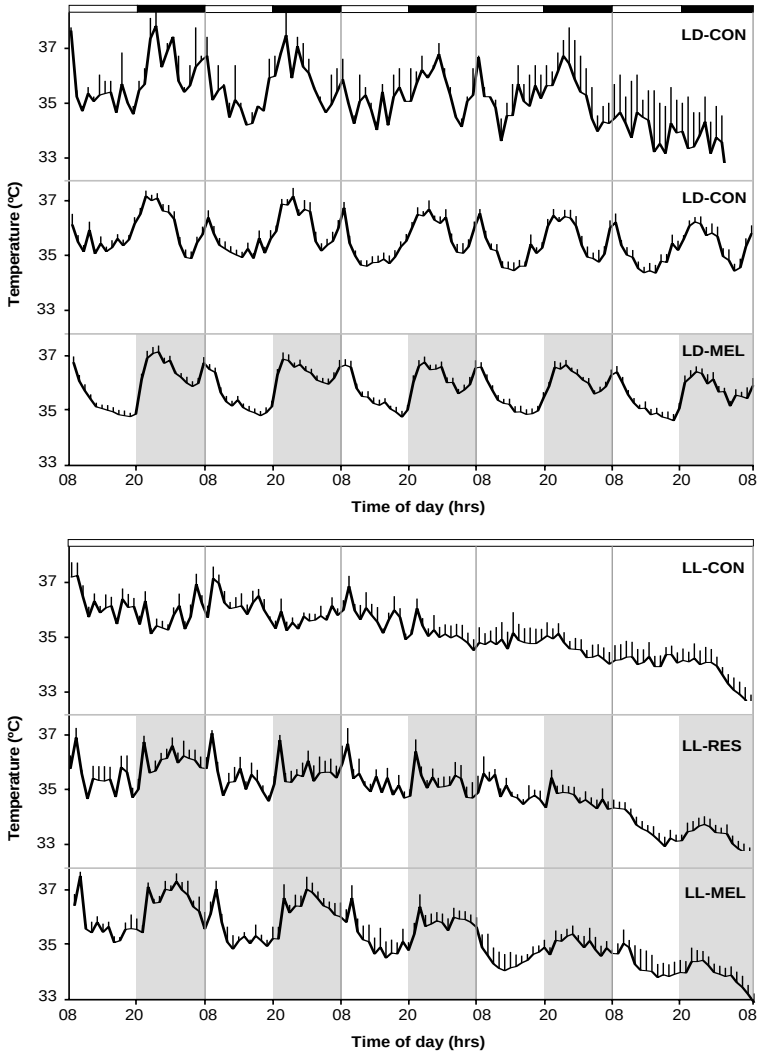

Figure 4. Body temperature during the last five days prior to death caused by intraperitoneal disseminated tumor in groups LD-CON (A, n=3), LL-CON (D, n=), LL-RES (E, n=5) and LL-MEL (F, n=7), or prior to animal sacrifice in groups LD-CON (B, n=9) and LD-MEL (C, n=10). The shaded area indicates food and water restriction and melatonin administration in the drinking water for LL-RES, LD-MEL and LL-MEL groups. Values are expressed as mean ± SEM. Light and dark (LD 12:12) and continuous light (LL) phases are represented at the top of the graphs by black and white bars or a white bar, respectively.

Subcutaneous inoculation of melanoma cells resulted in the appearance of a rapidly growing tumor that could evolve in one of two ways (Table 2, reference to figure 5 has been deleted). In most cases (64 out of 92; 69.6%), the tumor consisted of a solid, spheroid, subcutaneous mass (ST). However, 28 out of 92 animals (30.4%) showed a tumor mass that was widely disseminated into the peritoneum (IPT), and in some cases, even into the thoracic cavity. The malignancy of disseminated tumors was much higher than that of the

subcutaneous tumors, and none of the animals that developed one of them survived for more than 21 days after inoculation. As a result, all animals sacrificed at the end of the 21-day period that had been previously established had subcutaneous tumors.

Under our experimental conditions, only 3 out of 42 animals subjected to LD conditions died prematurely from IPT (Table 2). Conversely, 25 out of 50 mice (50%) held under LL conditions died before 21 days as the result of IPT. Neither the surgical procedure (LL-SHA) nor the presence of the iButton (LD-CONno, LD-MELno) could be associated with a higher mortality rate.

Table 2. Survival and malignancy score.

	CON	MEL	RES	CONno	MELno	SHA	∑
LD	3/12	0/10	X	0/10	0/10	X	3/42
LL	10/21	7/10*	5/10	X	X	3/9	25/50*
LD	3.25^{a-c}	2.30^{a}	X	2.70^{a-c}	2.60^{ab}	X	2.73
LL	3.62^{cd}	4.40^{d}	3.60^{b-d}	X	X	3.22^{a-d}	3.70*

Double input data table (treatment or light regimen, LD or LL). The first two lines indicate the number of animals that died within less than 21 days, compared to the total number of animals in that group. The next two lines indicate the mean malignancy score according to the categories described in the Materials and Methods section. Last column summarizes the data from each light regimen, LD or LL. *p<0.01: LL-MEL *vs*. LD-MEL; and LL *vs.* LD conditions (Chi Square test). Malignancy score values with different letters indicate significant differences (p<0.05, ANOVA).

Similar results were observed using tumor weight and IPT as a malignancy index (1 to 4 = four levels of increasing tumor size; 5=IPT). The mean score from all animals subjected to an LD cycle was 2.73, whereas it increased to 3.70 under LL conditions (Table 2). Melatonin administration to LD animals reduced malignancy score from 3.25 (LD-CON) to 2.30 (LD-MEL), and to 2.60 in the case of LD-MELno. No IPTs were observed in LD melatonin-treated mice. Again, neither the surgical procedure nor the presence of the data logger seemed to alter their malignancy score.

Neither the high percentage of mortality nor the malignancy score observed in LL-CON (50% and 3.62, respectively) and LL-RES (50% and 3.60, respectively) groups was significantly reduced by melatonin administration (70% and 4.40, respectively), nor were there any significant differences due to the presence or absence of the data logger (LL-CON vs. LL-SHAM) or to surgery under the same experimental conditions (LD-MEL vs. LD-MELno).

DISCUSSION

We have shown that nocturnal administration of melatonin in the drinking water

prevents the circadian rhythmicity impairment associated with cancer development and reduce melanoma malignancy in mice synchronized to an LD 12:12 cycle; however, no beneficial effects were observed when melatonin was administered for 12 h out of every 24 hours to free-running animals maintained under LL conditions with free or restrained food and water access.

Robust and stable circadian rhythms are now considered a reliable marker of good health. Conversely, severe functional disruption of the circadian system is associated with aging and illness (Erren et al., 2003), to the extent that biological rhythm impairment can be used to predict a poor survival rate in cancer patients (Mormont et al., 2000; Sephton et al., 2000; Mormont et al., 2002). However, to date, there is no agreement as to whether the impaired rhythmicity observed as an illness progresses is the result of disease evolution or whether it is a predisposing factor favoring the appearance of the disease and its progression.

Subcutaneous melanoma inoculation advanced in two different ways: either as a solid subcutaneous tumor (the most common type, 70%) or as a very aggressive intraperitoneal dissemination (30%). Disseminated tumors were more malignant, and as a result, all animals died before the 21 days that had been established for the end of the experiment. Therefore, only animals with subcutaneous tumors were sacrificed. Dissemination, a very rare phenomenon for this tumoral model (Gómez-Zapata et al., 1987), could be the result of three possible factors: a) surgical manipulation, required to implant the data logger intraperitoneally; b) temperature sensor presence in the peritoneum, or, c) animal exposure to a chronodisrupting environment of continuous light.

The surgical procedure would not seem to be the cause, since the surgical recovery period prior to tumor inoculation lasted for more than 20 days, and because no mortality or malignancy differences were detected between animals receiving the same treatment, regardless of whether or not they were subjected to surgery. Sensor presence could also be discarded, as once again, no differences were detected between experimental groups where the only difference was the presence of the sensor.

Therefore, continuous light exposure, a very well known chronodisrupting factor, would seem to be the most feasible cause to explain the high rate of melanoma dissemination observed in our experiments: 50 % of mice under LL compared to 7.1% under LD conditions.

The influence of lighting conditions on cancer development has been studied in several species, including rat (Dauchy et al., 1997) and mouse (Lang et al., 2003). Severe functional chronodisruption produced by *zeitgeber* alteration results in accelerated aging and malignant progression in animals injected with tumor cells (Erren et al., 2003). An increasing number of experimental and epidemiological studies have demonstrated the link between light and the

risk of cancer, however, it is not clear whether this increased risk can be attributed to diminished melatonin secretion or to conflictive input signals to the circadian pacemaker.

Plasma melatonin rhythm is regulated by the periodic output of the suprachiasmatic nucleus (SCN), whose activity is modulated by the entraining and acute inhibiting effect of bright light reaching the retina (Reiter et al., 2006). This indoleamine is considered to be an oncostatic agent in some cancers (Blask et al., 2002; Blask et al., 2005). In fact, it has been proposed that the reduction of melatonin levels can reasonably be considered as a human carcinogen (Portier, 2002). Should this be the case, nocturnal light exposure could predispose to cancer cell proliferation, tumor growth and the incidence of metastases due to the loss of a natural oncostatic agent (Blask et al., 2005).

The melatonin production maybe impaired and melatonin rhythm maybe absent in C57BL/6 mice. These inbred mice have a point mutation in the AANAT gene (Roseboom et al., 1998), which is responsible for decreased melatonin production (Kennaway et al., 2002). However, Vivien–Roels *et al.* (1998) observed a short-term (30 min) nocturnal melatonin peak in these mice when blood samples were taken every 15 min. Thus, it cannot be ruled out that the effects induced by continuous light on melanoma progression could be at least partially due to light-induced melatonin inhibition. On the other hand, continuous light or light at the subjective night could constitute a chronodisruptive signal per se. Besides inhibiting melatonin, continuous light also reduced the amplitude and lengthened the mice circadian rhythm. This is in contrast to rats, in which light induces total arrhythmicity (Lax et al., 1999).

To ensure melatonin administration in drinking water for 12 h per day, it was necessary to schedule cycles of water and food availability. Cycles of water and food restriction over short time periods (2-4 h of availability) during 24-h periods are considered to be important *zeitgebers* for some species (Mistlberger et al., 1990). However, in C57BL6 mice, food and water availability for 12 h out of every 24 failed to entrain Tb under LL conditions. The window for food and water availability was too wide, and probably could not reduce much food and water intake in restricted animals, which would diminish the entraining capacity of this *zeitgeber*, allowing the animals to continue with their free-running rhythms. This could explain why we did not find significant differences in Tb rhythm or melanoma progression when comparing LL-RES and LL-CON animals.

Exogenous melatonin administration during the night to our mice under LD conditions increased the amplitude and phase stability of the Tb rhythm, and also reduced intraperitoneal dissemination and tumor growth. Similar results on tumor growth have been obtained with other melanoma cells lines, such as B16 in athymic mice (Narita & Kudo, 1985) and HFH18 in C57BL/6 mice (Lang et al., 2003). In addition, direct antiproliferative effects of melatonin were

observed *in vitro,* using metastatic cell sublines of mouse melanoma B16BL6 and PG19 (Cos et al., 2001). To explain the oncostatic actions of melatonin, several hypotheses have been proposed: 1) modulation of the endocrine system, 2) immune system enhancement, and 3) a direct oncostatic action on tumor cells through its known antioxidant effects and the inhibition of linoleic uptake. However, the improvement of circadian rhythmicity through melatonin chronobiotic effects should not be discarded.

In our experimental model, the effects of exogenous melatonin on tumor growth seem to be dependent on the photoperiod and administration timing. Contrasting with the positive results of melatonin in mice under LD conditions, melatonin has no beneficial effect under LL conditions. To explain these results, it should be pointed out that the dose of melatonin we used (2 mg/kg BW/ day) failed to entrain the Tb rhythm under LL conditions, producing only a relatively strong coordination between the 24-h period generated by melatonin administration and the 25-h free-running period. Melatonin was therefore administered every day at a different subjective time. As a result, some days mice were administered melatonin during subjective day hours, while others they received it during their subjective night. Our results confirm that the efficacy of melatonin on tumor cell proliferation depends on the timing of the administration (Bartsch & Bartsch, 1981; Sauer et al., 2001; Anisimov, 2003; Reiter et al., 2006) as occurs with other antitumoral treatments, such us 5-fluorouracil (You et al., 2005; Wood et al., 2006). Moreover, melatonin administration with a period that differs from the free-running Tb period has a chronodisrupting effect, as the organism is exposed to two conflicting cues. In addition, as has been suggested by Reiter (2006), chronodisruption may have adverse health effects, including cancer initiation and promotion.

The Tb rhythm, considered to be a circadian system marker rhythm, was progressively impaired as the tumor progressed, as has been reported by other authors (Sephton et al., 2000; Filipski et al., 2002). The alterations observed in our study included a reduction in the r vector, amplitude, mesor and first circadian harmonic power in LD-CON animals.

Loss of rhythmicity associated with tumor growth in LD animals was prevented by melatonin administration. This chronoprotector effect of melatonin could be of interest in human medicine, since circadian rhythmicity impairment predicts poor survival in cancer patients (Mormont & Levi, 2003). Therefore, interventions that help patients improve their circadian rhythmicity, such as melatonin treatment or light therapy, could actually increase medical treatment effectiveness.

However, mice under LL conditions showed low amplitude free-running, as well as food and water and melatonin induced rhythms. No benefits from melatonin treatment similar to those seen under LD conditions could be established.

In addition, Tb circadian rhythmicity loss constitutes a good index for time of death. Indeed, LD-CON and LL-CON mice presenting intraperitoneal dissemination showed a progressive diminution of mesor and amplitude five days before their deaths, and total arrhythmicity the day before or five days prior to death under LD and LL conditions, respectively. In these animals, 12 h of melatonin administration, but not 12 h of food and water availability, significantly delayed circadian rhythmicity impairment, up until the last day of life. A similar situation was observed in animals presenting a solid subcutaneous melanoma five days before their sacrifice. In this instance once again, melatonin prevented circadian rhythmicity alterations. Therefore, it seems evident that melanoma progression, either subcutaneous or disseminated, is associated with an impairment of those parameters characterizing Tb rhythm. On the other hand, tumor size and malignancy scores increase under chronodisrupting conditions involving an internal desynchronization, such as LL or conflicting *zeitgebers,* as occurred when melatonin was administered with a period that differed from the endogenous free-running period.

Based on the evidence presented here, exogenous melatonin administered in the drinking water has oncostatic properties on melanoma when administered during the night in LD-synchronized mice. However, this effect seems to be photoperiod- and time-dependent, since no positive, but rather harmful, effects are observed when the indoleamine is administered under LL conditions. It thus becomes a conflicting input for these free-running animals circadian system. Moreover, melatonin prevents the loss of rhythmicity associated with tumor progression. The combination of oncostatic and chronobiotic effects of melatonin points out its potential use in melanoma clinical treatment. However, further studies will be necessary to elucidate the mechanism of this time-dependent effect of melatonin.

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4. DISCUSSION

The circadian system plays a critical role in the generation of daily rhythms in mammals, and it orchestrates these rhythms to maintain a stable internal temporal order in the physiology and behavior, ensuring an optimal phase-relationship with the external world. In the modern Western society, exposure to light pollution, rotational shift work, and intercontinental travel are steadily forcing a misalignment between the internal temporal organization of our physiological and behavioral circadian rhythms and environmental time cues. This disturbance is defined as chronodisruption (CD) (Erren et al., 2003; Garaulet & Madrid, 2010; Ortiz-Tudela et al., 2012). CD is believed to be associated with many known pathologies, such as obesity and metabolic syndrome, cognitive and affective impairments, reproductive abnormalities, premature aging, cardiovascular diseases and cancer. These health impairments are becoming a public health issue. To combat these CD-associated pathologies, it is important that we deepen our understanding of the basic mechanisms involved in the body's timekeeping systems. This will lead to more meaningful therapeutic interventions to re-establish the internal temporal organization of the circadian system, and its synchronization with the external world, of people experiencing CD.

In this PhD thesis, we adopted various animal models and experimental designs to study the consequences and some of the underling mechanisms of CD. Our approach used assessments at various physiological levels, ranging from the measurements of brain clock gene expression to systemic biochemical and behavioral analyses in order to define CD and assess its detrimental effects on health. We also used established transgenic rodent models bearing many of the pathological conditions found in humans to better our understanding of CD. Finally, we tested whether known synchronizing agents, such as melatonin, can be used as therapies for CD.

Unlike most animal species used in chronobiological studies, the *Octodon degus,* as humans, is primarily a diurnal species (Fulk, 1976; Lee, 2004). Therefore, studying the degu can reveal great insight into human's circadian system functionality. Interestingly, the degu also has the ability to shift their activity from the day to the dark phase and become night-active (nocturnal) when running wheels are provided under laboratory conditions (Kas & Edgar, 1999; Vivanco et al., 2009). It is proposed that this shifting of activity to the nocturnal phase is one way of attaining high levels of activity in running wheels while avoiding the danger of overheating (cooler at night). For example, Vivanco et al., (2010d) showed that temperature cycles, with high values during the day and low values at night, induce nocturnalism in degus previously characterized as diurnal. In the wild, degus increase diurnal activity during the cold winter days, and limit their activity at dawn and dusk, during the hot and dry days of summer (Kenagy et al., 2002). The expression of such specific chronotype by

this animal in the natural environment may served as an adaptive process to seasonal and ambient temperature changes (Lagos et al., 1995; Kenagy et al., 2002; Vivanco et al., 2010d). Together, both lab and field studies suggest the importance of thermoregulation as a key factor in the chronotype flexibility of the degus.

In **Chapter 4** we explored the thermoregulation constraints of the degus in order to gain greater understanding of the circadian system functionality in this animal, and begin to assess ways by which CD can emerge. We showed that by exposing the degus to constant high ambient temperature alone was not enough to induce switching in their activity phase preference. Thus, although under high ambient temperature naïve degus reduced their activity levels during the light phase, they still displayed a diurnal rhythm of activity. However, when running wheels were provided under high temperature conditions, all animals became active at night. It is interesting to note that, when wheels were introduced under constant high ambient temperature, the shift to a night-time activity pattern did not offer any "cooling" advantage to the degus since fluctuations in this daily ambient temperature were at minimal. That is, in our experimental paradigm, the night was just as warm as the day. The high ambient temperature, therefore, seems to provide additional facilitatory cues, forcing the degus to become active at night. This observation shows that the circadian system of the degus is highly adaptive and supports the view that an innate "protective" pre-program mechanism in this animal associates the dark phase with natural lower ambient temperature, thereby changes the timing of the behavioral activity in this animal species to reduce the potential risk of hyperthermia (Kas & Edgar, 1999).

The nocturnal pattern of activity in the degus results from two different processes, entrainment and masking; some animals have their pacemaker entrained to the scotophase (entrained nocturnal degus, EN), while in others, masked nocturnal degus (MN), their pacemaker is entrained to the photophase, but show activity at night because of the strong negative masking effect of light (Vivanco et al., 2009; Vivanco et al., 2010c).

In the following studies, we focussed on the nocturnalism of the degus as a potential model to study the disruption in the internal temporal organization in laboratory conditions. The main question here was: "**What happen to the internal temporal organization of the degu's circadian system when they "spontaneously" shift their activity-phase preference from day to night?**" Would there be a complete inversion in all their biological rhythms too, and are there differences between entrained and masked nocturnal animals?

To study this, we first performed a comprehensive study comparing overt rhythms in different physiological, biochemical, and hematological parameters to patterns of behavioral activity in both chronotypes. We showed that although nocturnal degus exhibited higher

wheel running activity (WRA) and body temperature (Tb) at night, most hematological and biochemical variables still remained similar to diurnal degus (**Chapter 1**). However, this lack of differences in most biological variables between nocturnal and diurnal animals could be as a result of nocturnal animals being masked, with their pacemaker still entrained to the photophase as in the diurnal degus. This led us to performed further experiments where we identify MN and EN degus and compared hematological and biochemical variables in these animals **(Chapter 4)**. We demonstrated, however, that most hematological and biochemical variables remained similar in diurnal and nocturnal chronotypes, even when nocturnal degus were entrained to the dark phase. Differences between these two chronotypes were only found in plasma urea levels and in the number of lymphocytes, which were associated with the activity patterns and, therefore, were phase reversed between diurnal and EN degus. Interestingly, MN degus showed an intermediate pattern between diurnal and EN. Thus, these results suggest that there is a continuous gradient of chronotype expression in the degus (from diurnal to strictly nocturnal), depending on masking by light and the degree of entrainment (Refinetti, 2006; Vivanco et al., 2009). This gradient is not only for activity and Tb rhythms but also for the temporal pattern in some biochemical and hematological variables (urea levels and lymphocytes count). The partial inversion in the overt rhythms supports the view that the switching mechanisms underlying activity phase-preference are located downstream from the central pacemaker, the SCN (Smale et al., 2003).

Indeed, many studies have shown that the phasing of core clock gene expression in the SCN and their protein products, the temporal pattern of expression of major neuropeptides, and its electrical activity are not significantly different across species with different activity patterns (Schwartz et al., 1983; Challet, 2007; Smale et al., 2008; Vosko et al., 2009; Ramanathan et al., 2010). Further, the discovery that clock gene expression was not confined to the SCN but is also found in many extra-SCN oscillators (Guilding & Piggins, 2007) posed the tantalizing question that the phase relationship between SCN and these extra-SCN oscillators may underlie differences between chronotypes (Lambert & Weaver, 2006; Vosko et al., 2009; Ramanathan et al., 2010). These extra-SCN oscillators are believed to be important for daily timing in physiology and behavior in a tissue specific manner (Mendoza & Challet, 2009).

In **Chapter 2** we investigated if there is a change in the phasing of some extra-SCN brain oscillators when diurnal degus engage their activity during their normal resting period, thus when they become nocturnal. Since these brain areas communicate circadian rhythms to many physiological processes of the body, the study of gene expression in these animals may reveal the underlying processes of temporal organization of their circadian rhythmicity. As expected, the core clock gene expression *Period (Per1* and *Per2)* in the degu's SCN peaked

during the day in all animals regardless of their chronotype. However, in extra-SCN brain areas (including hippocampus and cortices), *Per* expression peaked at the time the animals were active, that is during the day in diurnal and at night in nocturnal. Thus, when degus shift their activity to the dark phase, *Per* expression in extra-SCN brain areas phase-shifts accordingly, whereas its pattern of expression in the SCN remained unaffected. As seen for some biochemical and hematological variables (**Chapter 1 and 4**), masked nocturnal degus also represented an intermediate case between entrained diurnal and nocturnal degus at the molecular clock level in the brain, for example in the pattern of *Per* expression in the hippocampus.

The SCN forms reciprocal connections with diverse hypothalamic areas, including the orexinergic neurons in the tuberal hypothalamus, in order to control key physiological and behavioral processes, such as the sleep-wake cycle, arousal and locomotor activity rhythms (Sakurai, 2007). Recently, studies using pharmacogenetic and optogenetic manipulations have confirmed that activation or suppression of orexin neuronal activity can alter the behavioral state of animals (Sasaki et al., 2011; Tsunematsu et al., 2011). Therefore, the orexinergic system provides a well defined pathway integrating arousal and circadian cues. In **Chapter 3** we showed that the patterns of activation of orexin neurons in the degus were related to their behavioral state so they were phase-reverse between nocturnal and diurnal degus. Diurnal degus exhibited higher WRA levels and activation of their orexinergic system during the day. However, when degus shift their activity to the night, their orexin neuronal activation pattern also parallels this shift and occurs at night.

In summary, when the degus switch their activity-phase preference from day to the dark period, there is an alteration in their internal temporal organization as some but not all biological rhythms shift accordingly, whereas in their SCN, these features remained unaltered. The existence of some switching structures downstream from the SCN, which process the same SCN output signal as “positive” or as “negative” according to the chronotype, could explain these results. Thus, the degu is a good model to study the consequences associated with conditions causing phase reversal of activity pattern in humans, such as during night-time shift work.

The human population shows a continuum of chronotypes, ranging from larks to owls (Roenneberg et al., 2007; Adan et al., 2012). Recently, studies have suggested that some chronotypes are better at adjusting to shift work than others. However, although there are several reports on this issue, more studies are necessary to clearly establish the link between chronotype and the ease to adjust to shifting light/dark conditions (Adan et al., 2012). The heterogeneity of chronotypes (a continuum from strictly diurnal to nocturnal) present in the

degus (Labyak et al., 1997; Garcia-Allegue et al., 1999; Vivanco et al., 2009; Vivanco et al., 2010b) makes this species a well suited animal model to deepen our understanding of the effect of shift work in human. This may lead to improved strategies/therapies to cope with shift work.

In **Chapter 5,** we proposed a "5+2" paradigm which tried to simulate the lighting conditions experienced by humans under shift work schedules. This "5 working days + 2 weekends" paradigm placed the animals into a Morning, Afternoon and Night schedules each week, while in the weekends, the lighting conditions were always returned to Morning schedule. When degus were subjected to these conditions, their WRA and Tb patterns showed up to three different components. This disruption was more evident in nocturnal animals, probably because they exhibit a strong negative masking by light (Vivanco et al., 2009; Vivanco et al., 2010b; Vivanco et al., 2010c) which forces them to follow the shifted lighting paradigm. In addition, nocturnal chronotype exhibited a clear de-coupling between the WRA and the Tb rhythms under the Afternoon schedule.

The repeated disruption of the circadian rhythms has been associated with a wide range of health impairments. This situation calls for the development of strategies to re-align the internal temporal organization and external time cues. Most studies advising how to minimize the effects of jet lag, for example, have focused on accelerating the re-entrainment to the new LD cycle (Srinivasan et al., 2008; Brown et al., 2009). However, internal clocks in shift workers are disrupted on a more frequent basis, and it is not totally clear if accelerating the re-entrainment to the chronic shifting schedules it is the best strategy for strengthening the circadian rhythmicity and preventing health impairments.

Melatonin has chronobiotic properties since it can phase shift the circadian clock and adjust the timing of internal biological rhythms (Pevet & Challet, 2011). Based on these properties, in our study, we administered exogenous melatonin at a regular time-point every day regardless the lighting schedule **(Chapter 5)**. With this strategy, melatonin strengthened the 24h periodicity in WRA and Tb of the animals subjected to the 5+2 shifting schedule, and re-align the internal phase synchrony between WRA and Tb rhythms across the different schedules. Therefore, treatment with this hormone can be useful to cushion the disruptive effects of shift work.

Apart from jet lag and shift work conditions, there are other situations which can result in a disruption of the circadian rhythms. This includes: exposure to constant light (Erren et al., 2003; Reiter et al., 2007), ocular pathologies (Drouyer et al., 2008) and blindness (Skene & Arendt, 2007), aging and neurodegenerative diseases, such as Alzheimer Disease (AD)(Wu & Swaab, 2007) as well as tumorigenesis.

In the next part of this Thesis we focused on established animal models that are already bearing these pathologies to study their circadian system functionality. In these animals, we also tested the usefulness of melatonin treatment as a therapy to strength the circadian rhythmicity and limit the progression of the given pathology.

In **Chapter 6,** we showed that retinal degeneration in a model of Retinitis pigmentosa, P23H rhodopsin transgenic rat (Dryja et al., 1990), correlated with the occurrence of circadian dysfunctions in these animals. This highlights the key role of the light input to the circadian system as the main zeitgeber. P23H rats exhibited higher rhythm fragmentation and lower inter-daily stability and damped relative amplitude as the degeneration progressed. However, animals remained entrained to 24 hr period indicating that despite the total loss of photoreceptors in the retina, retinal ganglional cells were still present. Melatonin treatment, by its chronobiotic (Pevet & Challet, 2011) and antioxidant properties (Pandi-Perumal et al., 2006; Pandi-Perumal et al., 2008), improved circadian rhythms disturbances, and prevented the photoreceptor loss and retinal degeneration in these P23H rats, indicating its potential use in the clinical treatment of retinitis pigmentosa and probably in other ocular pathologies.

Neurodegenerative diseases, such as AD are associated with disruption of the overt circadian rhythms in human, and it is proposed that intrinsic changes in the functioning of the central pacemaker and its ability to drive outputs in other physiological processes, like melatonin production/secretion, may underlie these circadian rhythmicity impairments (Wu & Swaab, 2005; Wu & Swaab, 2007). In **Chapter 7,** we characterized the circadian system functionality of a double transgenic mice (APPSswe/PS1dE9) which expressed two mutant proteins associated with AD develop. Although these animals develop amyloid deposition (Jankowsky et al., 2004), we found no major dysfunction in the circadian system and that melatonin treatment, therefore, could offer no further benefits. These data show that this animal model does not recapitulate fully all of the pathological features of the AD in humans which limits its clinical relevance when extending results obtained in these mice to humans.

Epidemiological studies report a relationship between the CD resulting from shift work with a higher risk of cancer (Schernhammer et al., 2001; Rafnsson et al., 2001; Schernhammer et al., 2003). Nevertheless, this relationship is reciprocal, thus, as cancer progresses there is also a disruption of the circadian rhythms. In **Chapter 8**, we studied the bidirectional relationship between circadian system and cancer development in melanoma-bearing C57BL/6 mouse. An impairment of the body temperature rhythm occurred along with tumor development. Melatonin treatment improved rhythmicity and enhanced survival when provided to animals maintained in LD conditions. However, the results were self-defeating when melatonin was administered at inappropriate times to free running animals under LL

conditions. This data highlight the importance of considering the endogenous rhythmicity and the need to administer melatonin at a regular circadian time-point in order to restrict melanoma progression.

Together, our results show the existence of multiple ways to induce CD, including: a) spontaneous partial inversion of behavioral rhythms; b) internal circadian dissociation elicited by chronic shifts of the LD cycle that mimic the human rotating shift-work schedules; and c) chronodisruptive pathologies such as retinal degeneration, AD and cancer.

The appearance of internal phase dissociation between different rhythmic variables, simultaneous circadian periods, reduction in both, relative amplitude and inter-daily stability, together with an increase in rhythm fragmentation can be considered as reliable and objective indexes to assess the appearance of CD.

Melatonin treatment, through its chronobiotic properties, contributes to improve the circadian system functionality in the models of chronodisruption tested here, except when the timing of melatonin administration constitutes, by itself, a conflicting *zeitgeber*. These results highlight the importance of administering melatonin according to the body's internal time.

5. CONCLUSIONS

1. A complete inversion in the internal temporal order of physiological, hematological and biochemical rhythms does not occur in parallel to wheel running activity pattern shift in the degus.

2. Daily *Period* gene expression in extra-SCN brain regions in diurnal and nocturnal degus occurs in anti-phase, while their pattern of expression in the SCN in all chronotypes remains similar. There are fundamental differences between entrained and masked nocturnal degus.

3. The phase-reversed pattern observed in wheel running activity rhythms between nocturnal and diurnal degus also occurs in the pattern of orexin neuron activation in their brains. This profile of activation is similar in regions across the rostro-caudal and medial-lateral axes of the hypothalamus in both chronotypes.

4. Constant high ambient temperature is unable to induce nocturnalism in the degus. However, it facilitates activity-phase shift in the degus when wheel running is available.

5. Shifting LD schedule, an experimental condition that mimics lighting conditions experienced by shift-workers 5+2, causes disruption in WRA and Tb rhythms in the degus, mainly in those with nocturnal chronotype during the afternoon schedule. Melatonin treatment is an effective therapeutic strategy to strengthen the robustness of the 24h periodicity.

6. Retinal degeneration in a model of retinitis pigmentosa, P23H rats, positively correlates with circadian dysfunction. Melatonin treatment reduces both visual degeneration and circadian rhythmicity impairment in this model.

7. Unexpectedly, APPswe/PS1dE9 transgenic mice fail to mimic most of the circadian and behavioral impairments present in human patients with AD. As a consequence, no further benefits can be obtanined by melatonin or ramelteon treatment.

8. Circadian rhythm impairment increases alongside melanoma progression. Endogenous circadian rhythmicity must be taken into account for melatonin administration since it improves rhythmicity and enhances survival under LD conditions, but the results are

self-defeating when melatonin is not administered according to the body's internal time.

GENERAL CONCLUSIONS

The degu constitutes an excellent model to study CD. This species shows a "spontaneous" disruption in its internal temporal organization in response to wheel running, since although its activity phase is shifted to the night, not all biological rhythms shift accordingly. This probably is because different brain regions acting downstream from the SCN are involved in relaying circadian signals to the body. Beside the degus, other animal models are also useful to study specific chronodisruptive pathologies, such as retinal degeneration, AD and cancer.

Melatonin treatment, through its chronobiotic properties, contributes to improve the circadian system functionality in the models of chronodisruption tested here, except when the timing of melatonin administration acts, by itself, as a conflicting *zeitgeber*.

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7. ANNEX

7.1. ANNEX I. Scientific production resulting from the experiments performed in the present PhD thesis

7.1.1. Publications

1. **Otalora BB**, Madrid JA, Alvarez N, Vicente V, Rol MA. (2008). Effects of exogenous melatonin and circadian synchronization on tumor progression in melanoma-bearing C57BL6 mice. J Pineal Res. 44:307-315.

2. **Otalora BB**, Vivanco P, Madariaga AM, Madrid JA, Rol MA. (2010). Internal temporal order in the circadian system of a dual phasing rodent, the *Octodon* degus. Chronobiol Int. 27: 1564-1579.

3. Lax P, **Otalora BB**, Esquiva G, Rol MA, Madrid JA, Cuenca N. (2011). Circadian dysfunction in P23H rhodopsin transgenic rats: effects of exogenous melatonin. J Pineal Res. 50: 183-191.

4. **Otalora BB**, Popovic N, Gambini J, Popovic M, Viña J, Bonet-Costa V, Reiter RJ, Camello PJ, Rol MA, Madrid JA. (2012). Circadian system functionality, hippocampal oxidative stress and spatial memory in the APPswe/PS1dE9 transgenic model of Alzheimer disease: Effects of melatonin or ramelteon. Chronobiol Int. 29:822-834.

5. **Otalora BB**, Hagenauer MH, Rol MA, Madrid JA, Lee TM. *Period* gene expression in the brain of a dual phasing rodent, the *Octodon degus*. (*Close to submission*).

6. **Otalora BB**, Rol MA, Madrid JA. Ambient temperature, thermoregulatory constraints and wheel running availability in the nocturnalism of the *Octodon degus*. (*In preparation*).

7. **Otalora BB,** Vivanco P, Rol MA, Madrid JA, Canal MM, Piggins HD. Temporal and spatial patterns of activation of orexin neurons in diurnal and nocturnal *Octodon degus.* (*In preparation*).

8. **Otalora BB**, Rol MA, Madrid JA. Disruption of the circadian system in the *Octodon degus* under light:dark cycles that simulate shift work lighting conditions in humans. Effects of exogenous melatonin. (*In preparation*).

7.1.2. Communications to national and international congresses

1. **Otalora BB**, Álvarez-Sánchez N, Vicente V, Rol de Lama MA, Madrid JA. Body temperature rhythm in C57BL6 mice bearing a B16 melanoma. Influence of exogenous melatonin. II International Congress of Applied Chronobiology and Chronomedicine (ICACC). Gammarth (Tunisia) 2007. Oral communication.

2. **Otalora BB**, Álvarez-Sánchez N, Vicente V, Madrid JA, Rol de Lama MA. Alteration of body temperature rhythm in C57BL6 mice bearing a B16 melanoma. Influence of food and light. II International Congress of Applied Chronobiology and Chronomedicine (ICACC). Gammarth (Tunisia) 2007. Poster.

3. **Otalora BB**, Álvarez-Sánchez N, Vicente V, Rol de Lama MA, Madrid JA. Time-dependent effect of melatonin in C57BL6 mice bearing a B16 melanoma. II Iberoamerican Congress on Neuroimmunomodulation. Madrid (Spain) 2007. Poster.

4. Madrid JA, **Otalora BB**, Mondejar MT, Rol MA. Chronobiology of aging. XXXV Congress of the Spanish Society of Physiological Sciences. Valencia (Spain) 2009. Invited communication.

5. **Otalora BB**, Vivanco P, Madariaga AM, Rol MA, Madrid JA. Alteration of internal temporal order in the circadian system of a dual phasing rodent, the *Octodon degus*. XXXV Congress of the Spanish Society of Physiological Sciences. Valencia (Spain) 2009. Poster.

6. **Otalora BB**. Chronodisruption and aging. Young Researchers Symposium. 51st Congress of the Spanish Society of Geriatrics and Gerontology. Bilbao (Spain) 2009. Invited communication.

7. **Otalora BB**, Vivanco P, Madariaga AM, Madrid JA, Rol MA. Internal temporal order in a dual phasing rodent, the *Octodon degus.* XI Congress of the European Biological Rhythms Society: Strasbourg (France) 2009. Poster.

8. Rol MA, **Otalora BB**, Vivanco P, Madrid JA. *Octodon degus*: searching for the key of nocturnalism and diurnalism. 41st European Brain and Behaviour Society Meeting. Rhodes Island (Greece) 2009. Invited communication.

9. Lax P, **Otalora BB**, Esquiva G, Rol MA, Madrid JA, Cuenca N. Chronobiological dysfunction in rats homozygous for the P23H rhodopsin mutation. Effects of exogenous melatonin. 26th Conference of the ISC (International Society for Chronobiology). Vigo (Spain) 2010. Poster.

10. **Otalora BB**, Rol MA, Gambini J, Borrás C, Viña J, Madrid JA. Circadian system functionality in a model of Alzheimer Disease (B6C3-Tg transgenic mice). Effects of melatonin and ramelteon. 26th Conference of the ISC (International Society for Chronobiology). Vigo (Spain) 2010. Poster.

11. **Otalora BB**, Hagenauer MH, Rol MA, Madrid JA, Lee TM. *Period 1* gene expression in the brain of a dual phasing rodent, the *Octodon degus*. III World Congress of Chronobiology. Puebla (Mexico) 2011. Poster.

12. **Otalora BB**, Madrid JA, Rol MA. Cross-talk between environmental temperature and wheel running availability in the nocturnalism of the *Octodon degus*. XII Congress of the European Biological Rhythms Society: Oxford (UK) 2011. Poster.

13. **Otalora BB**, Rol MA, Madrid JA. Desynchronisation of the circadian system in *Octodon degus* subjected to LD cycles that simulate shift work conditions. Effects of melatonin. XII Congress of the European Biological Rhythms Society: Oxford (UK) 2011. Poster.

7.2. ANNEX II. Additional scientific production resulting from diverse collaborations and research projects

7.2.1. Publications

1. Popovic N, **Baño-Otálora B**, Rol MA, Caballero-Bleda M, Madrid JA, Popovic M. (2009). Aging and time of day effects on anxiety in female *Octodon degus*. Behav Brain Res. 200:117-121.

2. Vivanco P, **Otalora BB**, Rol MA, Madrid JA.(2010). Dissociation of the circadian system induced by T28 and T21 LD cycles in *Octodon degus*. Chronobiol Int. 27:1580-1595.

3. Van Groen T, Kadish I, Popovic N, Popovic M, Caballero-Bleda M, **Baño-Otálora B**, Vivanco P, Rol MA, Madrid JA. (2011). Age-related brain pathology in *Octodon degus*: Blood vessel, white matter and Alzheimer-like pathology. Neurobiol Aging. 32:1651-1661.

4. Escames G, Ozturk G, **Baño-Otálora B**, Pozo MJ, Madrid JA, Reiter RJ, Serrano E, Concepción M, Acuña-Castroviejo D. (2012). Exercise and melatonin in humans: reciprocal benefits. J Pineal Res. 52:1-11.

5. Lax P, Esquiva G, Esteve-Rudd J, **Otalora BB**, Madrid JA, Cuenca N. (2012). Circadian dysfunction in a rotenone-induced parkinsonian model. Chronobiol Int. 29:147-156.

7.2.2. Book chapters

1. Rol MA, Vivanco P, **Otalora B**, Sarabia, JA, Mondejar, MT, Madrid JA. Cronobiología del envejecimiento- «Chronobiology of aging»-. In, Actualizaciones en aspectos básicos y clínicos del envejecimiento y la flagilidad - «Basic and Clinical issues on aging and fragility» (2009), pp 167-186.

2. Madrid JA, **Otalora BB**, Rol MA. Cronobiología de la nutrición y metabolismo: efectos de la cronodisrupción -«Chronobiology of nutrition and metabolism: effects of chronodisruption»-. In, Del hambre a la saciedad. Contribuciones filosóficas, psicológicas, socioantropológicas y biológicas- «From hunger to satiety. Philosophical, psychological, social anthropological and biological contributions» (2011), pp 344-369.

7.2.3. Communications to national and international congresses.

1. **Otalora BB**, Popovic N, Rol MA, Caballero M, Madrid JA, Popovic M. Efecto de la edad y de la hora del día en la ansiedad de un roedor diurno *(Octodon degus).* III National Meeting SEMEG (Spanish Geriatrics Medicine Society). Oviedo (Spain) 2008. Poster.

2. Popovic N, **Baño-Otálora B**, Rol MA, Caballero-Bleda M, Madrid JA, Popovic M. Aging and time-of-day effects on anxiety in female *Octodon degus.* A Brain Research Meeting: Stress, Coping and Disease. VA (USA) 2008. Poster.

3. Sosa M, Sosa J, Martínez-Nicolas A, Ortiz-Tudela E, **Baño B**, Madrid JA, Rol MA, Campos M. Circadianware: a new informatic tool for the analysis of temperature, activity and

body position rhythms in humans. Annual Meeting of the Spanish Sleep Society. Alcoy (Spain) 2010. Poster.

4. Vivanco P, **Otalora BB**, Rol MA, Madrid JA. Temporal expression of arginine vasopressin, vasoactive intestinal poplypeptide and Fos protein in the hypothalamus of diurnal and nocturnal *Octodon degus*. 26th Conference of the ISC (International Society for Chronobiology). Vigo (Spain) 2010. Poster.

5. **Otalora BB**, Popovic N, Popovic M, Venero C, Pereza-Pérez I, Madrid JA, Rol MA. Effects of isolation stress on the circadian system and cognitive performance in a social diurnal rodent, *Octodon degus*. 26th Conference of the ISC (International Society for Chronobiology). Vigo (Spain) 2010. Oral communication.

6. Bonmatí-Carrión MA, Rico F, Martínez-Nicolás A, Ortiz-Tudela E, Mondejar MT, **Otalora BB**, Madrid JA, Rol MA. Evaluation of the circadian system in blind people by monitoring wrist skin temperature rhythm. XX Annual Meeting of the Spanish Sleep Society. Sevilla (Spain) 2011. Poster.

7. Bonmatí-Carrión MA, Martinez-Nicolas A, **Bano-Otalora B**, Madrid-Perez JA, Rol-de Lama MA. Circadian system evaluation in blind people through wrist skin temperature rhythm. Should blind people be exposed to bright light? XII Congress of the European Biological Rhythms Society. Oxford (UK) 2011. Poster.

8. Madariaga A, Sebastián I, Martinez FJ, **Baño-Otalora B**, Vivanco P, Rol de Lama MA, Madrid JA. Octodon degus: un modelo para estudios de Cronobiología. Encuentro Científico Internacional para la Optimización de Modelos Murinos en Investigación. Valdivia (Chile) 2012. Poster.

7.3. ANNEX III. Stays in laboratories out of the University of Murcia during the realization of the PhD.

1. Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom.
   Responsible researchers: Professor Hugh Piggins and Dr. Maria Canal PhD.
   Duration: 7 months (02/06/08-31/08/08, 13/10/08- 21/12/08 and 12/01/09-15/02/09)

2. Department of Psychology, University of Michigan, Ann Arbor, MI, United States.
   Responsible researcher: Theresa M Lee.
   Duration: 4 months (01/09/10- 22/12/10)

7.4. ANNEX IV. Research projects supporting the experiments performed in the present PhD

1. Project Title: Chronodisruption and aging: animal models
   Funding Body: Fundación Séneca (05700/PI/07)
   Duration: 2007-2009

2. Project Title: Premature aging and disruption of the circadian system. Role of melatonin
   Funding Body: CICYT (BFU2007-60658)
   Duration: 2007-2010

3. Project Title: Research network on aging and fragility-RETICEF
   Funding Body: Instituto de Salud Carlos III (RD06/0013/0019)
   Duration: 2007-2012

4. Project Title: Exercise as a modulating factor of aging. Effect on oxidative stress
   Funding Body: Fundación Séneca (12005/PI/09)
   Duration: 2010-2011

5. Project Title: Preventing chronodisruption by circadian-healthy lighting. Relevance in aging and cancer
   Funding Body: CICYT (BFU2010-21945-C02-01)
   Duration: 2011-2013

7.5. ANNEX V.

Along the period of realization of this Doctoral Thesis, the author has been granted with a Spanish Ministry of Science PhD Scholarship AP2006-04117 (FPU: Formación de Profesorado Universitario), from February 2007 to February 2011.

8. RESUMEN EN ESPAÑOL

Los objetivos generales de la presente Tesis Doctoral fueron:

1. Investigar si existe una alteración en la organización temporal interna del sistema circadiano del *Octodon degus* cuando los animales invierten "espontáneamente" su fase de actividad del día a la noche.

1.1. Evaluar si el desplazamiento de la fase diurna de actividad hacia la noche también implica una inversión completa de las fases de los ritmos fisiológicos, bioquímicos y hematológicos en el degu.

1.2. Estudiar la expresión día-noche de los genes *Period* (*Per1* y *Per2*) en el núcleo supraquiasmático (NSQ) y en otras áreas del cerebro de degus diurnos, nocturnos enmascarados y nocturnos encarrilados.

1.3. Establecer el patrón temporal y espacial de activación de las neuronas orexinérgicas en degus diurnos y nocturnos a lo largo del ciclo luz-oscuridad.

1.4. Investigar la inversión de fase de los ritmos fisiológicos y de comportamiento en respuesta a elevadas temperaturas ambientales y al acceso a rueda.

1.5. Estudiar la disrupción del sistema circadiano en degus sometidos a rotaciones del ciclo luz/oscuridad que simulan las condiciones de iluminación del trabajo a turnos en humanos y evaluar el efecto cronobiótico del tratamiento con melatonina.

2. Estudiar el funcionamiento del sistema circadiano en distintas patologías asociadas a la cronodisrupción y evaluar el papel cronopotenciador de la melatonina exógena en dichas patologías.

2.1. Caracterizar la disfunción del sistema circadiano en un modelo de retinosis pigmentaria (ratas transgénicas con rodopsina mutante P23H) así como los posibles efectos beneficiosos de la melatonina sobre el deterioro de dicho sistema.

2.2. Evaluar el funcionamiento del sistema circadiano y los niveles de estrés oxidativo en el hipocampo de un modelo transgénico de Alzheimer (ratón APPswe/PS1dE9), así como los efectos del tratamiento a largo plazo con melatonina o ramelteón.

2.3. Investigar cómo la progresión del melanoma afecta a la ritmicidad circadiana en el ratón, y determinar si la melatonina exógena mejora la funcionalidad del sistema circadiano, restringe el crecimiento tumoral y aumenta su supervivencia.

CAPÍTULO EXPERIMENTAL 1

Orden temporal interno en el sistema circadiano de una especie de roedor dual, el *Octodon degus*

Existen numerosos artículos científicos que describen los ritmos diarios de distintas variables bioquímicas y hematológicas tanto en especies diurnas como en nocturnas, pero hasta el momento no existen estudios de los ritmos de dichas variables en especies duales como el degu. El *Octodon degus* es un roedor capaz de invertir su fase diurna de actividad a la noche en respuesta al libre acceso a rueda en condiciones de laboratorio. Esta especie es de gran utilidad para investigar si existe una inversión completa del orden temporal interno en paralelo a su inversión del patrón de actividad. Así, el objetivo del presente estudio fue determinar la relación de fase día-noche entre 26 variables (conductuales, fisiológicas, bioquímicas y hematológicas) en degus diurnos y nocturnos con libre acceso a rueda.

Un total de 39 degus machos se alojaron individualmente en jaulas con acceso a rueda bajo un fotoperiodo LD 12:12. Durante todo el experimento se registraron los ritmos de actividad en rueda y de temperatura corporal. Cada 6 horas (ZT 1, ZT 7, ZT 13, y ZT 19) se tomaron muestras de sangre para cuantificar los niveles de melatonina plasmática y las variables hematológicas y bioquímicas.

Nuestros resultados mostraron que a pesar de las grandes diferencias en los ritmos de actividad y temperatura entre animales nocturnos y diurnos, los patrones temporales de la mayoría de variables biológicas analizadas fueron similares en ambos cronotipos; únicamente se encontraron diferencias en los niveles de urea plasmática y en el número de linfocitos, así como un ligero retraso de fase del ritmo de melatonina en los animales con carácter nocturno.

Este estudio demuestra que no existe una inversión completa del orden temporal interno en paralelo a la inversión del patrón de actividad y temperatura en esta especie dual. Estos datos sugieren que los mecanismos implicados en la inversión del degu estarían localizados aguas abajo del marcapasos.

CAPÍTULO EXPERIMENTAL 2

Expresión de los genes *Period* en el cerebro de un roedor dual, el *Octodon degus*

La expresión de los genes reloj no se limita solamente al marcapasos central localizado en el núcleo supraquiasmático (NSQ), sino que además se produce en otras muchas regiones cerebrales. Se ha propuesto que la relación de fase entre el NSQ y los osciladores fuera de él puede contribuir a las diferencias que existen entre los distintos cronotipos. El *Octodon degus* es un roedor diurno capaz de cambiar su fase de actividad de diurna a nocturna cuando tiene

libre acceso a rueda. Con el fin de comprender mejor la relación entre los genes reloj y el cronotipo de un animal, en este estudio comparamos la expresión diaria de los genes reloj *Period* (*Per1* y *Per2*) en el NSQ y en otras áreas cerebrales de degus diurnos y nocturnos. Dado que tanto el enmascaramiento negativo por la luz como el encarrilamiento a la fase de oscuridad están involucrados en el nocturnalismo del degu, también evaluamos, por primera vez, la expresión de genes reloj en degus nocturnos encarrilados y enmascarados.

Para ello, se obtuvieron cerebros de degus diurnos y nocturnos durante la fase de luz (ZT4) y la fase de oscuridad (ZT16) para posteriormente analizar los niveles de RNAm de *Per1* y *Per2* mediante hibridación *in situ*.

En el NSQ, la expresión de *Per1* y *Per2* fue mayor en ZT4 independientemente del cronotipo. Sin embargo, en el hipocampo de los nocturnos encarrilados, la expresión de *Per1* mostró una inversión de fase con respecto al NSQ, dado que los valores fueron más altos en ZT16. En los nocturnos enmascarados se observó una tendencia similar aunque la diferencia día-noche en la expresión de *Per1* no llegó a ser significativa. Curiosamente, en el hipocampo de los degus diurnos no se observaron diferencias diarias en los niveles de *Per1*. En el resto de áreas cerebrales analizadas (corteza, estriado, núcleo arcuato del hipotálamo e hipotálamo ventromedial), no se encontraron diferencias entre cronotipos en los niveles de *Per1*. Por otro lado, la expresión de *Per2* en el hipocampo y en el córtex cingulado y piriforme estaba en fase con los ritmos actividad de los animales. Así, los degus diurnos mostraron mayores niveles de *Per2* a ZT4, mientras que en los dos tipos de degus nocturnos, la expresión de *Per2* fue máxima a ZT16.

En resumen, el presente estudio apoya las evidencias que indican que los mecanismos que determinan el nicho temporal diurno o nocturno de los mamíferos se encuentran aguas abajo del NSQ. Además, nuestros datos indican que existen ciertas diferencias entre los degus nocturnos enmascarados y los encarrilados.

CAPÍTULO EXPERIMENTAL 3

Patrón temporal y espacial de activación de las neuronas orexinérgicas en cronotipos diurnos y nocturnos de Oc*todon degus*

El neuropéptido orexina desempeña un papel clave en la regulación del sueño, la vigilia y el nivel de activación. Las neuronas orexinérgicas del hipotálamo lateral interaccionan recíprocamente con el sistema circadiano para orquestar el ritmo diario de sueño-vigilia. El *Octodon degus* es un roedor que puede invertir su patrón de actividad de diurno a nocturno cuando tiene libre acceso a rueda. Dado que ambos cronotipos están presentes en esta

especie, el degu constituye un modelo animal ideal para estudiar los sistemas implicados en el nivel de activación y la vigilia al permitir comparar animales diurnos y nocturnos de la misma especie.

En este capítulo, hemos estudiado los cambios en el patrón de activación de las neuronas orexinérgicas a lo largo del ciclo de luz-oscuridad en degus diurnos y nocturnos, utilizando técnicas de inmunohistoquímica de doble marcaje para orexina y c-Fos (un marcador de la actividad neural) en secciones hipotalámicas obtenidas de degus nocturnos y diurnos, sacrificados a distintas horas del día.

Nuestros resultados mostraron que el patrón inverso de actividad en rueda que existía entre animales diurnos y nocturnos, también se observó en la activación del sistema orexinérgico. Así, la expresión de Fos en neuronas con orexina fue mayor durante el día en animales diurnos y durante la noche en los animales con actividad nocturna. No se detectaron diferencias espaciales en los patrones de activación de las neuronas con orexina a lo largo de los ejes rostro-caudal o medio-lateral para ninguno de los cronotipos. La expresión de Fos en las neuronas hipotalámicas negativas para orexina también fue mayor en la fase en la que los animales estaban activos.

En resumen, nuestros resultados en el degu proporcionan una nueva evidencia de que los patrones diarios de activación de las neuronas con orexina están relacionados con el estado de actividad de los individuos. Así pues, proponemos a los investigadores que utilicen especies duales como el degu para profundizar en el conocimiento de cómo tiene lugar el acoplamiento entre el marcapasos central y los sistemas que promueven el estado de activación o *arousal* para controlar los ritmos diarios de comportamiento.

CAPÍTULO EXPERIMENTAL 4

Temperatura ambiental, termorregulación y disponibilidad de rueda en el nocturnalismo del *Octodon degus*

El *Octodon degus* es principalmente un animal diurno, aunque en condiciones de laboratorio puede invertir su fase de actividad del día a la noche en respuesta a la disponibilidad de rueda. Se ha propuesto que esta inversión del patrón de actividad obedece a procesos de termorregulación relacionados con el ejercicio físico intenso. Así, los animales desplazan su actividad hacia la noche donde la temperatura ambiental es menor.

Para evaluar la relación entre la termorregulación y la inversión de la fase de actividad en el degu en respuesta al libre acceso a rueda giratoria, primero sometimos degus diurnos, sin experiencia previa en rueda, a incrementos progresivos de la

temperatura ambiental desde los 26ºC a 32ºC. Una vez que los animales se hallaban a la temperatura ambiental más alta de este rango, introdujimos ruedas giratorias en sus jaulas. Dado que ambos procesos, enmascaramiento negativo por la luz y el encarrilamiento a la fase de oscuridad están involucrados en el nocturnalismo del degu, en este capítulo también estudiamos si las respuestas termorreguladoras de nocturnos encarrilados y enmascarados eran diferentes, así como las relaciones de fase entre sus variables hematológicas y bioquímicas.

Nuestros resultados mostraron que los degus sin acceso a rueda giratoria, no invirtieron su patrón diurno de actividad y temperatura corporal a la fase de oscuridad cuando se sometieron a incrementos secuenciales de la temperatura ambiental. Sin embargo, cuando se les proporcionaron las ruedas mientras la temperatura ambiental era elevada, todos los animales invirtieron su fase de actividad diurna a la noche y surgieron dos cronotipos diferentes de animales nocturnos, encarrilados y enmascarados. La respuesta termorreguladora al ejercicio en rueda durante las fases de luz y oscuridad fue significativamente diferente entre ambos cronotipos. En las variables bioquímicas y hematológicas también se detectaron pequeñas diferencias entre ambos grupos de nocturnos.

Estos datos ponen de manifiesto la importancia que tiene la interacción entre la disponibilidad de rueda y la temperatura ambiental en el nocturnalismo del degu. Además, estos resultados apoyan la idea de que la termorregulación relacionada con el ejercicio físico intenso en rueda puede ser el mecanismo por el cual los degus adoptan un patrón de actividad nocturna sin grandes modificaciones en la fase de sus variables hematológicas y bioquímicas. Así, los mecanismos implicados en la diferenciación de los distintos cronotipos en el degu parecen estar localizados aguas abajo del marcapasos central.

CAPÍTULO EXPERIMENTAL 5

Disrupción del sistema circadiano del *Octodon degus* sometido a rotaciones del ciclo luz/oscuridad que simulan las condiciones de iluminación del trabajo a turnos en humanos. Efecto de la melatonina exógena

Los objetivos de este estudio fueron, en primer lugar, evaluar la disrupción del sistema circadiano en degus sometidos a rotaciones del ciclo luz/oscuridad (LD) simulando las condiciones de iluminación que experimentan los seres humanos durante el trabajo a turnos (5 "días hábiles" + 2 días "fines de semana"); en segundo lugar, evaluar el potencial terapéutico del tratamiento con melatonina para la mejora de la ritmicidad circadiana bajo estas

condiciones de trabajo a turnos.

Un total de 18 degus fueron sometidos a turnos rotatorios del tipo "5 +2" durante 19 semanas. Este protocolo consistía en un retraso semanal de 8h en el ciclo LD durante los 5 días "de trabajo" (Lunes-Viernes), lo que determinaba 3 turnos de trabajo: turno de mañana, tarde y noche (luces encendidas de 08:00-20:00, 16:00-04:00 y 00:00-12:00 h, respectivamente); mientras que durante los fines de semana (2 días), el régimen de luz siempre volvía al turno de mañana. A las 9 semanas de experimento, la disponibilidad de agua de bebida se restringió a un periodo de 6h por día (18:00-00:00 h). Durante este tiempo, la mitad de los animales se trataron con melatonina, mientras que la otra mitad recibió el correspondiente vehículo. Como grupos controles de los animales sometidos a la rotación de turnos, 17 degus se mantuvieron bajo un turno no rotatorio de mañana (luces encendidas de 08:00-20:00h) a lo largo de todo el experimento.

Nuestros resultados mostraron que durante el protocolo de turnos rotatorios "5+2", los patrones de actividad en rueda y temperatura corporal de los degus se disociaron hasta en 3 componentes circadianos distintos: uno de más de 24 h (~25 h) que siguió a los retrasos de 8h semanales; uno más corto de 24h (~22 h) relacionado con los avances de fase de los fines de semana, y por último, un componente de 24h. La cronodisrupción fue más evidente en los degus nocturnos, especialmente durante el turno de tarde donde se observó una desincronización entre las acrofases de los ritmos de actividad en rueda y temperatura corporal. El tratamiento con melatonina exógena y, en menor medida, la restricción de agua mejoraron la potencia del componente 24h y atenuaron la potencia de los otros dos componentes (~25 h y ~22 h).

En resumen, el protocolo de turnos rotatorios "5+2" representa una condición experimental que simula las condiciones de iluminación que experimentan los trabajadores a turnos. Este régimen causa una disrupción de los ritmos de actividad en rueda y temperatura corporal de los degus. El tratamiento con melatonina constituye una estrategia terapéutica eficaz para reforzar la periodicidad 24h y así mejorar los efectos perjudiciales del trabajo a turnos.

CAPÍTULO EXPERIMENTAL 6

Disfunción del sistema circadiano en un modelo de retinosis pigmentaria (ratas transgénicas con rodopsina mutante P23H): Efectos de la melatonina exógena

Este capítulo se centra en el estudio de los efectos de la degeneración retiniana sobre los patrones circadianos de las ratas transgénicas P23H, así como del efecto de la

administración exógena de melatonina. Para ello, se registró de forma continua la temperatura corporal de ratas P23H y Sprague-Dawley (SD) y sus retinas se examinaron en distintas etapas de degeneración. A lo largo de todo el experimento, un subgrupo de animales fue tratado con melatonina *ad libitum* (2 mg/ kg de peso corporal/ día).

No se encontraron diferencias significativas entre los periodogramas y las ondas medias de temperatura corporal de las ratas SD y P23H. Sin embargo, en las ratas P23H se produjo una disminución progresiva de la amplitud relativa (RA) del ritmo junto con una disminución de la regularidad (estabilidad inter-diaria, IS) y un incremento de la fragmentación (variabilidad intra-diaria, IV). Además, los animales P23H mostraron una disminución progresiva de la respuesta de la retina a la luz hasta los 18 meses de edad. A partir de este momento, los fotorreceptores ya habían desaparecido, y los electrorretinogramas no mostraron ninguna respuesta.

La administración exógena de melatonina mejoró la respuesta visual de las ratas P23H. De hecho, el máximo de la onda b registrado a los 14 meses de edad fue significativamente mayor en las ratas P23H tratadas con melatonina que en los animales control.

En resumen, la pérdida de visión en las ratas P23H se correlaciona con una fragmentación progresiva de su patrón circadiano. Ambos efectos se pueden revertir parcialmente por la administración de melatonina.

CAPÍTULO EXPERIMENTAL 7

Funcionamiento del sistema circadiano, estrés oxidativo en el hipocampo y memoria espacial en un modelo transgénico de la enfermedad de Alzheimer (ratón APPswe/PS1dE9). Efectos de la melatonina y el ramelteón

La enfermedad de Alzheimer (EA) es una enfermedad neurodegenerativa en la que se produce una acumulación de la proteína β-amiloide en el cerebro, dando lugar a déficits cognitivos y conductuales. Los pacientes con EA, también sufren graves trastornos de sus ritmos circadianos, aunque las causas subyacentes todavía no se conocen completamente. Estos pacientes, además, muestran niveles reducidos de melatonina lo que puede contribuir a sus síntomas, ya que la melatonina es un agente cronobiótico, con propiedades antioxidantes y neuroprotectoras.

En este capítulo estudiamos los efectos del tratamiento con melatonina a largo plazo sobre el funcionamiento del sistema circadiano, los niveles de estrés oxidativo en el hipocampo y la memoria espacial en un modelo transgénico de EA (ratón APPswe/PS1dE9).

Para estudiar la implicación de los receptores MT1/MT2 de melatonina en estos efectos, un subgrupo de ratones fueron tratados crónicamente con ramelteón, un agonista selectivo de los receptores MT1/MT2.

Nuestros resultados indican que muchos de los parámetros circadianos y de comportamiento analizados, además de los marcadores de estrés oxidativo en el hipocampo, no se vieron afectados significativamente en estos animales. Sin embargo, el grupo de animales transgénicos controles (Tg-CON), mostró niveles de actividad locomotora (LA) y temperatura corporal (Tb) más elevados que los del grupo de animales silvestres (WT, *wild type*). En general, la amplitud del ritmo de Tb fue significativamente menor en Tg que en los ratones WT. Aunque el tratamiento con melatonina no tuvo ningún efecto, el ramelteón redujo significativamente la amplitud del ritmo Tb en los ratones Tg. Al final del experimento, los ratones Tg tratados con ramelteon (Tg-RAM) mostraron una mayor fragmentación y menor potencia del ritmo de Tb que los controles. El período de curso libre (τ) de los ritmos de Tb y LA fue <24h en el grupo Tg-CON. Sin embargo, el tratamiento con melatonina mantuvo τ en 24h en animales de ambos genotipos, mientras que el tratamiento con ramelteon no tuvo ningún efecto. En las pruebas de comportamiento, el número de visitas y el tiempo dedicado a explorar nuevos objetos fueron significativamente mayores en Tg-CON que en los ratones WT. Los análisis del tejido cerebral revelaron una reducción significativa en la oxidación de las proteínas en el hipocampo de los grupos Tg-MEL y Tg-RAM con respecto a los animales Tg-CON.

En resumen, nuestros resultados sugieren que la alteración del sistema circadiano en los ratones APPswe/PS1 es mínima. Por lo tanto, se ha de tener cuidado a la hora de extrapolar los resultados obtenidos en este modelo transgénico para desarrollar nuevas terapias para pacientes con EA. Este estudio también reveló la complejidad de las acciones terapéuticas de la melatonina y ramelteon en este modelo de ratón.

CAPÍTULO EXPERIMENTAL 8

Efectos de la melatonina exógena y la sincronización circadiana en el desarrollo del melanoma implantado en ratones C57BL/6

A lo largo de la progresión tumoral se produce un deterioro de la ritmicidad circadiana, y a su vez, la disrupción del sistema circadiano puede resultar en una mayor incidencia de cáncer. La melatonina posee efectos oncostáticos en varios tipos de cáncer (cáncer de mama, próstata y colorrectal), mientras que puede ser contraproducente en otros, como es el caso del linfoma. El melanoma es uno de los cánceres más agresivos en

humanos, sin embargo, parece responder positivamente a la melatonina *in vitro*.

En el presente trabajo estudiamos cómo se ve afectado el ritmo de temperatura corporal (Tb) por la progresión del melanoma, y si la melatonina exógena es capaz de limitar el crecimiento tumoral, restaurar la ritmicidad circadiana y mejorar la supervivencia de los animales.

Para ello, implantamos sensores de temperatura intraperitonealmente en ratones de la cepa C57BL/6. Posteriormente, estos animales se inocularon subcutáneamente con células de melanoma. Los ratones se mantuvieron en condiciones de LD 12:12 o bajo luz continua (LL), con o sin la administración de melatonina.

En los animales mantenidos en LD 12:12, se observó una reducción de la amplitud del primer armónico y un incremento de la inestabilidad de fase (vector Rayleigh) del ritmo de Tb a medida que progresaba el desarrollo tumoral. El tratamiento con melatonina (2 mg/ kg de peso corporal/ día), incrementó la amplitud y la estabilidad de fase del ritmo de Tb, redujo el peso del tumor y previno la diseminación intraperitoneal del cáncer.

Bajo condiciones de LL se observó un ritmo de curso libre de 1500 min, y tuvo lugar un aumento significativo de la malignidad del tumor, que resultó en una reducción de la supervivencia. Sorprendentemente, los tumores de mayor peso y la mayor morbilidad por metástasis se observaron en el grupo de animales mantenidos en LL y tratados con melatonina. Probablemente, debido a que la melatonina se administraba a distintas horas subjetivas a animales que se encontraban en curso libre.

En resumen, la ritmicidad circadiana puede utilizarse como un marcador de la progresión tumoral, dado que con la malignidad del tumor se produce un deterioro de los ritmos. El tratamiento con melatonina mejora la ritmicidad y aumenta la supervivencia cuando se administra a animales mantenidos en condiciones LD, mientras que los resultados son contraproducentes cuando la administración tiene lugar en animales mantenidos en LL. Esto pone de manifiesto la importancia de tener en cuenta la ritmicidad endógena y la necesidad de limitar la administración de melatonina a la noche subjetiva con el fin de restringir la progresión del melanoma.

CONCLUSIONES

1. Cuando el degu invierte su patrón de actividad en rueda, paralelamente no se produce una inversión completa del orden temporal interno de los ritmos de variables fisiológicas, bioquímicas y hematológicas.

2. La expresión diaria de los genes reloj *Period* en el NSQ de los degus nocturnos y diurnos es similar en los distintos cronotipos, mientras que en otras áreas del cerebro los patrones de expresión ocurren en antifase. Existen ciertas diferencias en la expresión de *Per* entre degus nocturnos enmascarados y encarrilados.

3. El patrón inverso de actividad en rueda que existe entre animales diurnos y nocturnos también se observa en la activación del sistema orexinérgico. Los patrones de activación de las neuronas con orexina son similares a lo largo de los ejes rostro-caudal y medio-lateral para cada uno de los cronotipos.

4. La exposición a elevadas temperaturas ambientales por si misma es incapaz de inducir nocturnalismo en el degus. Sin embargo, estas temperaturas facilitan la inversión del ritmo de actividad motora cuando a los degus se les permite el acceso a rueda.

5. El protocolo de turnos rotatorios "5+2", una situación experimental que simula las condiciones de iluminación que experimentan los trabajadores a turnos, causa una disrupción de los ritmos de actividad en rueda y temperatura corporal de los degus, especialmente en los animales nocturnos y durante el turno de tarde. El tratamiento con melatonina constituye una estrategia terapéutica eficaz para reforzar la robustez de la periodicidad de 24h.

6. La degeneración de la retina en un modelo de retinosis pigmentaria, las ratas transgénicas P23H, se correlaciona positivamente con una disfunción del sistema circadiano. El tratamiento con melatonina reduce tanto la degeneración visual como el deterioro de la ritmicidad circadiana en este modelo.

7. Sorprendentemente, los ratones transgénicos APPswe/PS1dE9 no muestran la mayoría de los trastornos circadianos y de comportamiento presentes en pacientes con enfermedad de Alzheimer. Así pues, no se obtiene ningún beneficio adicional por el tratamiento con melatonina o ramelteón en este modelo animal.

8. La progresión del melanoma lleva asociada un deterioro del ritmo circadiano de Tb. La ritmicidad circadiana endógena ha de tenerse en cuenta en el tratamiento con melatonina ya que su administración mejora la ritmicidad y aumenta la supervivencia bajo condiciones LD, pero el resultado es contraproducente cuando no se administra considerando la hora interna.

CONCLUSIONES GENERALES

El degu constituye un modelo excelente para estudiar la cronodisrupción. Esta especie muestra una disrupción "espontánea" de su organización temporal interna en respuesta al acceso a rueda, dado que su fase de actividad se desplaza a la noche, pero sin que todos los ritmos biológicos inviertan en paralelo. Esto probablemente se deba a la participación de distintos relés cerebrales, localizados aguas abajo del NSQ, en la transmisión de las señales temporales al organismo. Además del degus, hay otros modelos animales que son útiles para estudiar patologías relacionadas con la cronodisrupción tales como la degeneración de la retina, la enfermedad de Alzheimer y el cáncer.

El tratamiento con melatonina, a través de sus propiedades cronobióticas, contribuye a mejorar la funcionalidad del sistema circadiano en los modelos de cronodisrupción analizados, excepto cuando el momento de la administración de melatonina constituye un *zeitgeber* conflictivo.