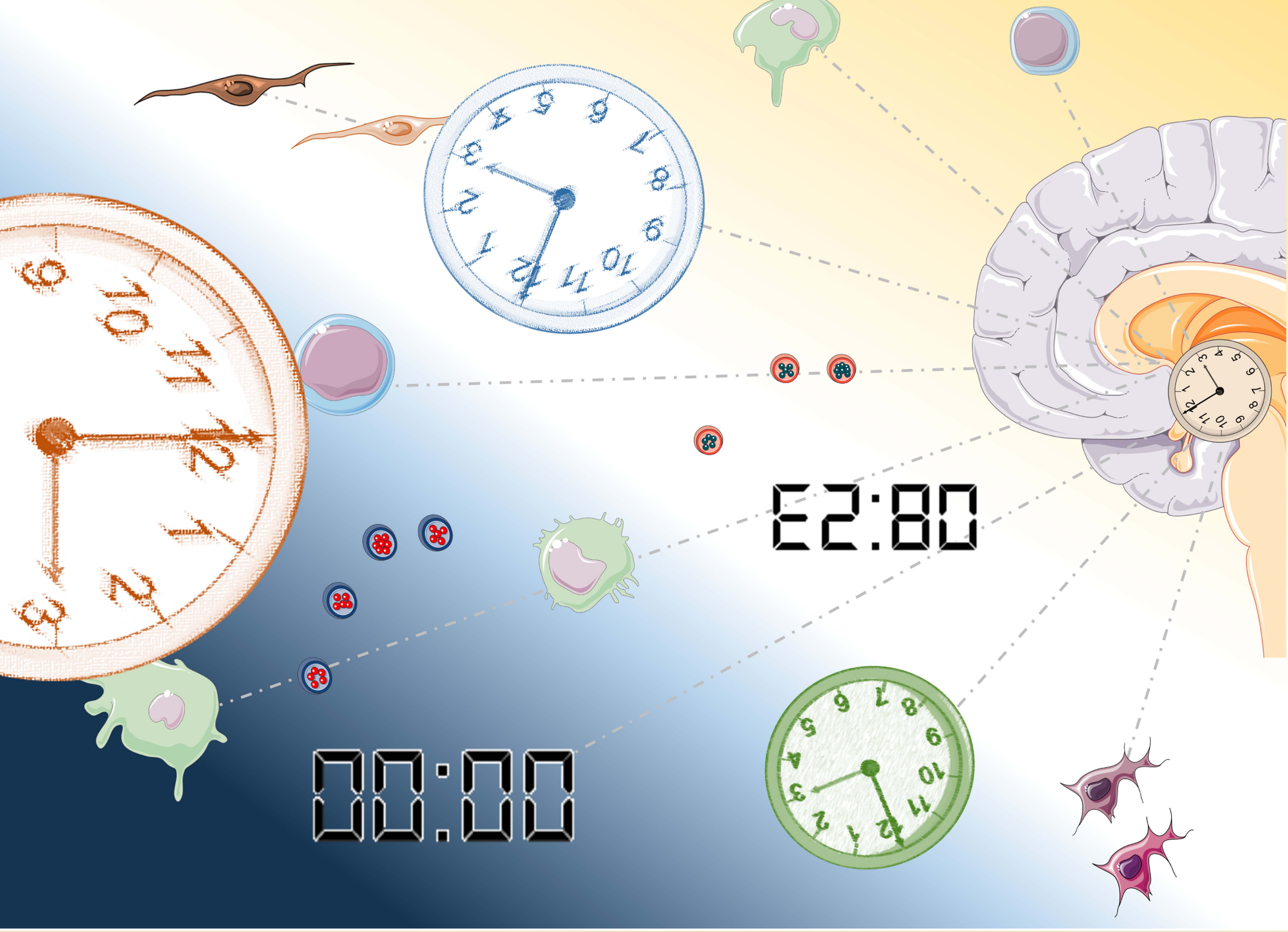

Laetitia da Silva Gaspar

CLOCK GENES PROFILE AS DISEASE BIOMARKERS

Dissertation in Cellular and Molecular Biology, under supervision of Ana Rita Álvaro and Professor Cláudia Cavadas presented to the Life Sciences Department of the Faculty of Sciences and Technology of the University of Coimbra.

July/2016

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UNIVERSIDADE DE COIMBRA

Laetitia da Silva Gaspar

CLOCK GENES PROFILE AS DISEASE BIOMARKERS

PERFIL DOS GENES RELÓGIO COMO BIOMARCADORES DE DOENÇA

Dissertation under the scientific supervision of Ana Rita Álvaro (PhD), Professor Cláudia Cavadas and Professor Emilia Duarte, presented to the Life Sciences Department of the Faculty of Sciences and Technology of the University of Coimbra, in fulfilment of the requirements for a Master degree in Cellular and Molecular Biology

Dissertação sob a orientação científica da Doutora Ana Rita Álvaro, Professora Doutora Cláudia Cavadas e Professora Doutora Emília Duarte, apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra para prestação de provas de Mestrado em Biologia Celular e Molecular.

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Cover note: Illustration of a dysregulated clock system, showing the central circadian clock, located in the hypothalamus, and its interaction with peripheral clocks located on different cells, among which peripheral blood mononucleate cells.

This work was performed in the “Neuroendocrinoly and Aging Group”, at the Center for Neuroscience and Cell Biology, University of Coimbra, Portugal, under the supervision of Ana Rita Costa Silva Álvaro, Professor Cláudia Margarida Gonçalves Cavadas and Professor Emília da Conceição Pedrosa Duarte.

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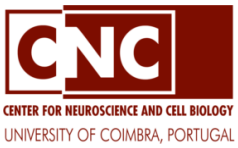

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"Para ser grande, sê inteiro: nada
Teu exagera ou exclui.
Sê todo em cada coisa. Põe quanto és
No mínimo que fazes…"
Fernando Pessoa

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AHI	Apnea-Hypopnea Index
ARC	Arcuate Nucleus
BiPAP	Bilateral Positive Airway Pressure
BMAL1	Brain and Muscle ARNT-Like 1
BMI	Body Mass Index
CK1	Casein Kinase 1
CLOCK	Circadian Locomotor Output Cycles Kaput
CNS	Central Nervous System
COPD	Chronic Obstructive Pulmonary Disease
CPAP	Continuous Positive Airway Pressure
CRE	cAMP Response Element
CRP	C-reactive protein
Cry	Chryptochrome
CT	Circadian Time
DBP	D-box Binding Protein
DEC	Differentially Expressed In Chondrocytes Protein
DMH	Dorsomedial Hypothalamus
EEGs	Electroencephalograms
E.g.	Example given
EKG	Electrocardiography
ER	Endoplasmic Reticulum
ERK	Extracellular signal-related Kinase
ERRα	Estrogen-Related Receptor alpha
GC	Golgi Complex
GHT	Geniculohypothalamic Tract
GPCR	G-Protein Coupled Receptors
GRE	Glucocorticoid Response Element
HIF-1	Hypoxia-Inducible Factor 1
HNS	Hypoglossal Nerve Stimulation
HPA	Hypothalamic-Pituitary-Adrenal axis
HRE	Hypoxia-Responsive Element
ICAM-1	Intercellular Adhesion Molecule-1
ICSD	International Classification of Sleep Disorders
IGL	Intergeniculate Leaflet
IH	Intermittent Hypoxia
IHR	Intermittent Hypoxia/Reoxygenation
IL	Interleukin
ipRGCs	Intrinsically photosensitive Retinal Ganglion Cells
MAPK	Mitogen-Activated Protein Kinase
MBH	Mediobasal Hypothalamic area
mRGCs	Intrinsically melanopsin-expressing Retinal Ganglion Cells
n.s.	Non significant
NAMPT	Nicotinamide Phosphoribosyltransferase
NHRs	Nuclear Hormone Receptors
NPAS2	Neuronal PAS Domain Protein 2
NPY	Neuropeptide Y
NREM	Non-Rapid Eye Movement

OPT	Oral Pressure Therapy
OSA	Obstructive Sleep Apnea
PAP	Positive Airway Pressure
PBMCs	Peripheral Blood Mononuclear Cells
Per	Period
PPAR	Peroxisome Proliferator-Activated Receptor
PP-fold	Pancreatic Polypeptide fold
PROX1	Prospero homeobox protein 1
PSG	Polysomnography
RDI	Respiratory Disturbance Index
REM	Rapid Eye Movement
RHT	Retinohypothalamic Tract
RORE	ROR Elements
RORα	Retinoic acid receptor-related Orphan Receptor
RRE	Rev Response Element
SCN	Suprachiasmatic Nuclei
TF	Transcription factor
TNFα	Tumour Necrosis Factor α
TTFL	Transcription and Translation Feedback Loop
VEGF	Vascular Endothelial Growth Factor
VMH	Ventromedial Hypothalamus

ABSTRACT

Circadian rhythms, defined by a set of biological clocks, are crucial to virtually all biological processes. A central clock is located in the hypothalamus and synchronises peripheral clocks, located in all cells. Disruptions in this complex circadian clock system, reflected by clock gene expression alterations, is associated with several diseases. Obstructive sleep apnea (OSA) is one of the most common sleep disorders. Untreated OSA has been implicated as risk factors for the development of several diseases, among which metabolic and cardiovascular disorders. However, OSA is still highly underdiagnosed, which limits its treatment. Consequently, it is urgent to find OSA biomarkers, molecular reflexes of its severity, and clock genes constitute promising candidates. Indeed, Several OSA molecular outcomes may contribute to clock genes expression alterations. In this context, the present work has three main aims: 1) to investigate the impact of OSA on peripheral clock genes, before and after treatment; 2) to find potential OSA biomarkers; 3) to evaluate the impact of neuropeptide Y (NPY) on hypothalamic clock genes.

We investigated the circadian rhythm integrity of an OSA cohort, by assessing a circadian rhythm marker (body temperature) and clock genes expression (*Per1-3; Cry1-2; BMAL1; CLOCK; CK1ε; DEC1-2*), at four time points (8 a.m.; 11 a.m.; 4.30 p.m. and 10.30 p.m.), in peripheral blood mononucleate cells (PBMCs), before and after OSA treatment by continuous positive airway pressure (CPAP). In parallel, using a mouse hypothalamic cell line (mHypoN42), we assessed the impact of NPY on clock genes levels, at different circadian times (CTs).

The results showed that OSA patients have disruptions in the typical oscillatory expression of several clock genes and abnormal clock genes messenger RNA levels. In addition, the expression of specific clock genes, at specific times, showed to correlate with OSA severity. We observed that the mRNA levels of the clock gene Cry1, at 8 a.m., have a negative correlation with OSA severity, which suggests that Cry1 has potential as a putative OSA biomarker. In hypothalamic cells, NPY changed clock genes expression, in a CT dependent manner. However, a short term constitutive expression of NPY is not sufficient to change clock genes modulation in hypothalamic cells.

Thus, the expression of specific clock genes, at specific times of the day, could be used as OSA biomarkers and reflect OSA severity. Further investigations may allow a more specific and early OSA diagnosis and, consequently, a more personalized and effective treatment.

Keywords: Circadian rhythms disruptions; Clock genes; OSA; NPY; Biomarkers; PBMCs

RESUMO

Os ritmos circadianos, definidos por um conjunto de relógios biológicos, são fundamentais para a maioria dos processos biológicos. O seu relógio central localiza-se no hipotálamo e é responsável pela sincronização dos restantes relógios periféricos, localizados em todas as células. Um funcionamento incorrecto destes relógios biológicos, demonstrado por alterações na expressão dos genes que os regulam, tem sido associado a várias doenças. A síndrome da apneia obstrutiva do sono (SAOS) é uma das perturbações do sono mais comuns. Não tratada, a SAOS constitui um factor de risco para o desenvolvimento de diversas doenças, nomeadamente doenças metabólicas e cardiovasculares. Contudo, esta é ainda uma doença altamente sub-diagnosticada, o que dificulta o seu tratamento. Desta forma, é crucial definir biomarcadores da SAOS, indicadores da sua severidade. Neste contexto, os genes reguladores dos ritmos circadianos surgem como potenciais biomarcadores uma vez que várias alterações moleculares decorrentes da SAOS poderão desencadear alterações na sua expressão. Assim, este trabalho tem três objectivos: 1) investigar o impacto da SAOS na expressão genética dos relógios periféricos, antes e depois do tratamento; 2) identificar potenciais biomarcadores da SAOS; e 3) investigar o papel do NPY na modulação dos genes relógio do hipotálamo.

Consequentemente analisámos os ritmos circadianos de um grupo de doentes diagnosticados com SAOS, através de um indicador do funcionamento dos seus relógios biológicos (a temperatura corporal) e da expressão dos genes que os regulam (*Per1-3; Cry1-2; BMAL1; CLOCK; CK1ε; DEC1-2)*, em quatro momentos do dia (8h, 11h, 16h30 e 22h30), em células mononucleadas do sangue, antes e depois do tratamento por pressão contínua positiva nas vias aérias. Paralelamente, recorrendo a uma linha celular de hipotálamo de murganho (mHypoN42), investigámos o papel do NPY na modulação da expressão dos genes do relógio central, em diferentes tempos circadianos.

Os resultados obtidos mostram que, de facto, doentes com SAOS podem ser caracterizados por alterações nos ritmos de expressão dos genes relógio, apresentando também níveis anormais de expressão. Além disso, a expressão de genes específicos, em certos momentos do dia, mostraram correlacionar-se com a severidade da SAOS. Observamos que os níveis de expressão do gene Cry1, as 8h, correlacionam-se negativamente com o índice de severidade da SAOS, podendo a expressão deste gene ser um potencial biomarcador da SAOS. Em células do hipotálamo, o NPY tem a capacidade de modular a expressão dos seus genes relógio, de uma forma dependente do tempo circadiano. No entanto, a sua expressão constitutiva, a curto prazo, não é suficiente interferir com a expressão dos principais genes reguladores do relógio central dos ritmos circadianos.

Portanto, as expressões de genes relógio específicos, em momentos do dia específicos, poderão ser usados como biomarcadores da SAOS e reflectir a sua severidade. Assim, investigações futuras poderão melhorar o diagnóstico desta perturbação do sono, tornando-o mais específico e precoce. Desta forma, um tratamento mais personalizado e eficaz poderá ser possível.

Palavras-chave: Ritmos circadianos; Genes relógio; SAOS; NPY; Biomarcadores; Células mononucleadas do sangue

CHAPTER I:

INTRODUCTION

I.1. CIRCADIAN RHYTHMS

Circadian rhythms are, approximately, 24 hours of biological oscillations that are crucial for virtually all living cells. This rhythmicity is generated by endogenous autonomous timers that act within each cell, regulating essentially all biological processes, including DNA synthesis, cell division, DNA damage repair and apoptosis (Oh *et al.*, 2010; Eckel-Mahan & Sassone-Corsi, 2013; Partch *et al.*, 2014). In multicellular organisms, clocks from different tissues are kept in phase-relationship with each other so the respective information is useful for the entire organism (Albrecht, 2012). This way, behavioural, physiological and metabolic processes are regulated, ensuring proper adaption to external daily rhythms (Reppert & Weaver, 2001; Gachon *et al.*, 2004). Such clock system is organized in a hierarchical manner, in which neurons at the suprachiasmatic nuclei (SCN), located at the anterior hypothalamus, are the central regulators (Dibner *et al.*, 2010) (figure I.1).

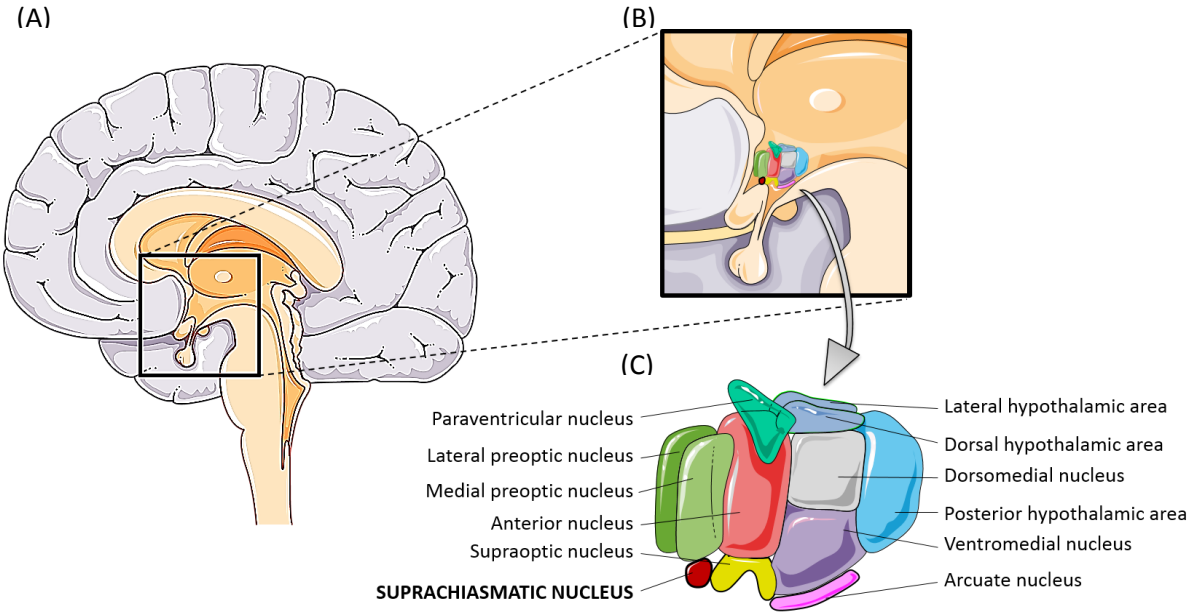


Figure I. 1: Anatomical localization of the hypothalamus and the suprachiasmatic nuclei in the human brain. (A) A sagittal section near the midline of the human brain shows the hypothalamus localization in the brain. (B) High-magnification of the hypothalamus. (C) High-magnification of the hypothalamic nuclei and anatomical localization of the suprachiasmatic nucleus. Adapted from Selvier Medical Art.

The timing phase of SCN neurons is adjusted by external timing cues, known as *zeitgebers* (or *time-givers*). These timing cues can shift the pre-existing hour (phase), either by advancing or delaying it, or can even reset the SCN at specific times. Such responses are dependent on the time of

exposure, consequently, the SCN may respond to the same stimulus differently. *Zeitgebers* can be photic (light/dark cycles) and non-photic (temperature, activity, food consumption, among others) and communicate with the SCN via different pathways and neurochemical signals (Yamazaki *et al.*, 2000; Piggins & Guilding, 2011; Mure & Panda, 2012). Light has been shown to be the most powerful external stimulus and it is sensed by retinal photoreceptors (intrinsically photosensitive and melanopsin-expressing retinal ganglion cells - ipRGCs and mRGCs) which can directly transmit the timing signal to the SCN, through the retinohypothalamic tract (RHT), or, indirectly, through the geniculohypothalamic tract (GHT), from the thalamus intergeniculate leaflet (IGL). Non-photic stimuli entrain the SCN via two main different pathways, directly or indirectly via the GHT and directly via the median raphe (MR) of the brain stem (Lamont, James, *et al.*, 2007; Piggins & Guilding, 2011; Mure & Panda, 2012). For example, if there is exposure to light in the early night, the SCN clock is delayed whereas if such exposure occurs in the late night, the SCN clock is advanced. By opposite, many non-photic *zeitgebers* have phase-shifting properties during the subjective day (Yannielli & Harrington, 2001). In addition, other timing cues than the external must also be considered. The SCN must also integrate the physiological state of the body, which information is provided by other brain regions such as other hypothalamic nuclei, forebrain and hindbrain up to the Raphe nuclei (Hastings *et al.*, 1997; Yannielli & Harrington, 2004).

According to the received timing cues, the around 20,000 SCN neurons phase align other brain centres clocks, responsible for important endogenous features, through a complex neural network. In addition, hormone cues allow the synchronization of clocks located at peripheral tissues, such as liver, kidneys, heart, pancreas, adipose tissue, peripheral blood mononucleate cells (PBMCs), among others (Ko & Takahashi, 2006; Videnovic *et al.*, 2014). Endogenous entraining signals, such as cortisol and melatonin, respond to SCN signals and are released accordingly, being of critical importance for the circadian rhythm dynamics (Huang *et al.*, 2011; Cuesta *et al.*, 2014). Cortisol is rhythmically secreted from the adrenal glands, with levels peaking in the early morning, and its release is regulated by the hypothalamic-pituitary-adrenal (HPA) axis (Debono *et al.*, 2009; Son *et al.*, 2011; Mavroudis *et al.*, 2012; Cuesta *et al.*, 2014). Melatonin, is also rhythmically secreted from the pineal gland but once light has suppressing effects on its release, melatonin levels begin to increase during the end of the day, peaking during the night and decrease to daytime levels after waking (Teclemariam-Mesbah *et al.*, 1999; Zeitzer *et al.*, 2000). By its turn, both cortisol and melatonin hormones can also act on the SCN, regulating the circadian phase and maintaining rhythm stability (Liu *et al.*, 1997; Pace-Schott & Hobson, 2002).

Although there is a phase relationship amongst central and peripheral clocks, communication to peripheral clocks requires more time and thus, peripheral clocks rhythm may be delayed by

several hours in relation to the SCN rhythm (Amir *et al.*, 2004; Lamont, James, *et al.*, 2007). This hierarchical link between the central and the peripheral clocks was demonstrated in studies in which the SCN was ablated. The result was a total desynchronization of peripheral tissues, which has suggested that the SCN maintains the phase alignment of tissue specific peripheral clocks (Balsalobre *et al.*, 1998; Vieira *et al.*, 2014).

Peripheral individual oscillators are predominantly more receptive to cues other than light. For example, nutrient input is a primary driver for clocks in specific tissues, such as in liver (Damiola *et al.*, 2000; Stokkan *et al.*, 2001). In addition, peripheral oscillators also respond to the metabolic status of the correspondent tissue. This way, cellular clocks are able to respond to both external and internal stimuli (input), to integrate the time-related information and to transmit the adjusted received cues to other cells (output). Such organization allows modulating metabolic and physiological processes such as glucose, lipids and drugs metabolism, hormonal regulation, sleep-wake cycles, memory consolidation, immunity, bone formation, body temperature, heart rate and blood pressure, among others (figure I.2).

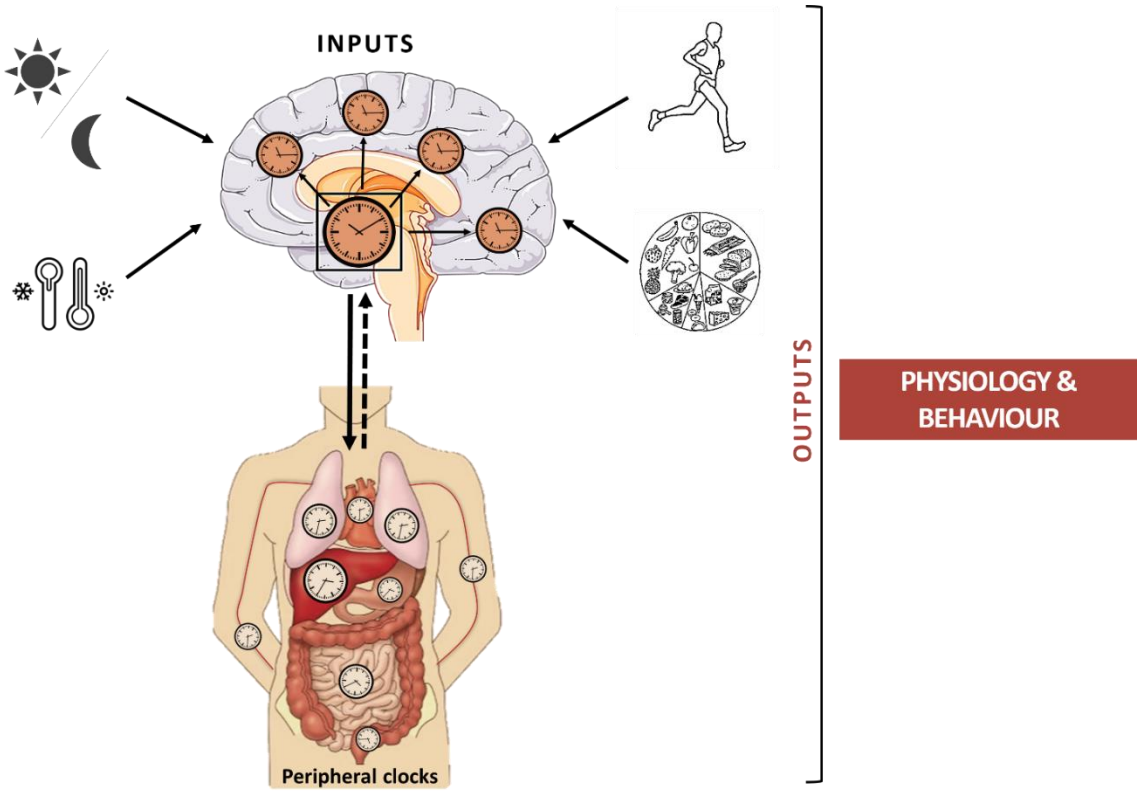


Figure I. 2: Simplified scheme of the circadian system in humans. The circadian system is organized in a 24h oscillating manner and is influenced by numerous external and internal time cues. Photic timing signals given by cycles of light and dark and non-photic cues derived from temperature, activity, food consumption, among others (inputs), are received by specialized brain regions, and transmitted to a master circadian clock, located at the suprachiasmatic nucleus (SCN) in the hypothalamus. This region is part of a complex neural network that projects to several other brain regions and synchronizes them according with the SCN received signals. In addition, the SCN is also responsible by the phase alignment of independent and self-sustained peripheral clocks located at peripheral tissues such as heart, lungs, liver, stomach, intestine, PBMCs, among others. The resultant outputs coordinate physiology and behaviour. Dashed arrows represent SCN indirect entrainers.

Rhythms similar behaviour in such divergent physiological processes and in virtually all organisms suggested that circadian clocks had its roots within DNA. Indeed, within cells, rhythmicity is assured by an autonomous molecular network that is common to all biological clocks, involving auto-regulatory transcriptional and translational feedback loops (TTFL) of clock genes (Okamura *et al.*, 2002; Ko & Takahashi, 2006). These genes are estimated to regulate about 10% of all the expressed genes (Videnovic et al., 2014).

I.1.1. OVERVIEW OF THE MOLECULAR CIRCADIAN CLOCKWORK

The molecular orchestrators of circadian rhythms were discovered around 1970 in the common fruit fly *Drosophila melanogaster* (Konopka & Benzer, 1971). Shortly thereafter, the molecular components of the mammalian biological clocks started to being discovered, using mice carrying mutations in one or more clock genes (reviewed in Ko & Takahashi, 2006). The core clock network comprises a positive and a negative limb that are interconnected. The positive limb involves the clock genes *Brain and Muscle ARNT-Like 1* (*BMAL1*/*ARNTL*) and *Circadian Locomotor Output Cycles Kaput* (*CLOCK*), or *Neuronal PAS Domain Protein 2* (*NPAS2*), a *CLOCK* isoform highly predominant in the mammalian forebrain. The clocks negative limb is composed by the clock genes *Period* (isoforms *Per1 and Per2 (*and *Per3* in humans)) and *Cryptochrome* (isoforms *Cry1* and *Cry2*) (Shearman *et al.*, 2000; Ko & Takahashi, 2006). This set of genes is designated as the core clock genes since they are necessary for rhythms generation, being active even in the absence of external inputs (Takahashi, 2004). For example, mice deficient for *BMAL1*, *CLOCK* and *NPAS2*, *Cry1* and *Cry2* or *Per1* and *Per2*, clearly lack a circadian organization of their behavioural rhythms, when kept under constant dark conditions (Lowrey & Takahashi, 2004).

BMAL1 codifies for a transcriptional activator protein that accumulates in the cytoplasm and dimerizes with CLOCK, a protein synthesized by the constitutively expressed *CLOCK* gene. Such heterodimers translocate into the nucleus and bind to E-box sequences, specific circadian DNA motifs present in promoters of clock and clock controlled genes (CCGs). This way, *Per* and *Cry* transcription and translation are activated. While Cry proteins accumulate in the cytoplasm, Per proteins are progressively phosphorylated at conserved residues by casein kinases (CK1δ and CK1ε). Such post-translational modifications promote Per degradation by the 26S proteasome, and delay its stability and accumulation in the cytoplasm. Consequently, Per acts as a rate limiting component, being crucial to the clocks period and phase determination and resetting. When its levels reach a critical concentration, Per forms heterodimers with Cry and translocate into the nucleus. There, these complexes act as negative transcription factors (TFs), binding to *BMAL1* and

CLOCK/NPAS2 promoters and inhibiting their transcription and, consequently *Per* and *Cry* own transcription, in negative feedback loop manner. The progressive degradation of the negative regulators allows *BMAL1* and *CLOCK* transcription and translation to occur, restarting the cycle in the same manner, in the following day (Shearman *et al.*, 2000; Reppert & Weaver, 2001; Pace-Schott & Hobson, 2002). This core molecular feedback loop is also influenced by additional clock regulators that regulate the localization and stability of the mentioned clock proteins, such as phosphatases (PP1; PP5) and kinases (CK1α; CK1δ; CK1ε; protein kinase A; extracellular signal-related kinase (ERK) and mitogen-activated protein kinase (MAPK) cascades) (Sehgal, 2008; Hansen *et al.*, 2011; Landgraf *et al.*, 2012; Partch *et al.*, 2014).

Additional feedback loops amongst core clock genes add further complexity and stability to this oscillatory network (Franken & Dijk, 2009). One of such auxiliary loops is the Rev Response Element (RRE)-mediated loop that comprises more two genes, whose expression is governed by BMAL1:CLOCK/NPAS2 activity. These genes are *REV-ERB* (also known as *nuclear receptor subfamily 1, group D, member 1 - Nr1d1*) and *ROR* (*Retinoic acid receptor-related Orphan Receptor*) and belong to the orphan Nuclear Hormone Receptors (NHRs) family. The resultant proteins (ROR isoforms α, β and γ and REV-ERB isoforms α and β) can also act as TFs, competing for ROR Elements (RORE) in gene promoters, such as in BMAL1 and CLOCK/NPAS2 promoters. REV-ERB proteins are able to negatively regulate BMAL1 and CLOCK/NPAS2 expression while ROR proteins can stimulate their expression (Preitner *et al.*, 2002; Landgraf *et al.*, 2012). This way, REV-ERB and ROR are able to regulate core clock genes expression amplitude and phasing. Consequently, *BMAL1*, *CLOCK*, *Per1-3*, *Cry1-2*, *RORα-γ* and *REV-ERBα-β* constitute the group of clock genes that coordinate TTFLs occurrence, although *ROR* and *REV-ERB* are dispensable for cellular rhythm generation (Sato *et al.*, 2004; Landgraf *et al.*, 2012).

In addition, the TFs DEC1 and DEC2 (Differentially Expressed In Chondrocytes Protein 1 and 2) are able to bind to E-box elements and to modulate *BMAL1* and *CLOCK/NPAS2* transcription, by inhibiting it (figure I.3) (Honma *et al.*, 2002; Landgraf *et al.*, 2012). Further auxiliary loops also involve metabolic TFs such as Peroxisome Proliferator-Activated Receptor alpha (PPARα), Nicotinamide Phosphoribosyltransferase (NAMPT) and Sirtuin 1 (SIRT1) or Estrogen-Related Receptor alpha (ERRα) a and Prospero homeobox protein 1 (PROX1) (Canaple *et al.*, 2006; Asher *et al.*, 2008; Nakahata *et al.*, 2008; Ramsey *et al.*, 2009; Dufour *et al.*, 2011). Finally, the promoters of *Per1-3*, *Rev-erbα*, *Rorα* and other clock controlled genes, have another circadian regulatory

motif (D-Boxes) to which other TFs are able to bind (Lopez-Molina *et al.*, 1997; Cowell, 2002; Zhang & Kay, 2010).

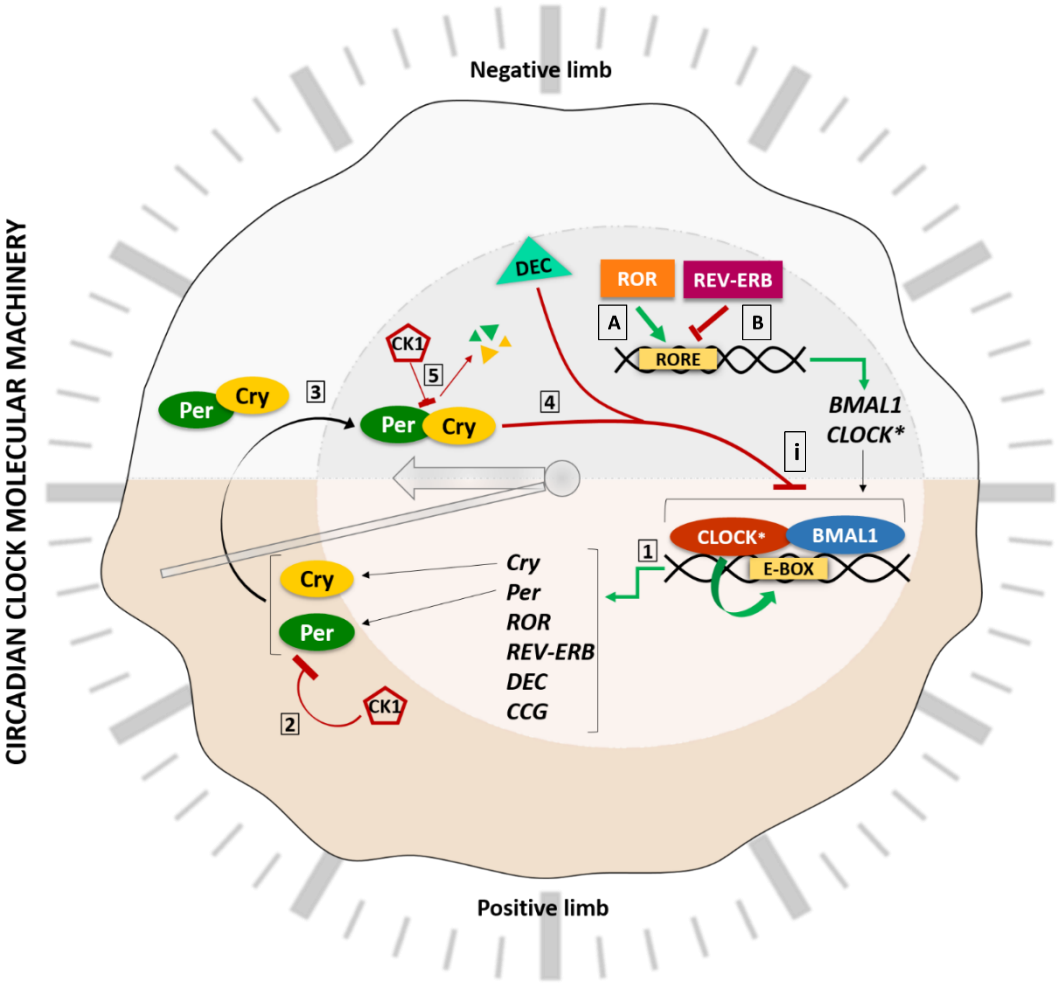


Figure I. 3: Clocks molecular machinery: transcriptional and translational feedback loops. Clocks rhythmicity is assured by transcriptional and translational auto regulatory feedback loops of a set of clock genes. The core loop is composed by CLOCK*, BMAL1, Cry and Per: [1] The positive limb is driven by heterodimers of the transcriptional activators BMAL1 and CLOCK which activate the transcription of *Per*, *Cry*, *ROR*, *REV-ERB* and *DEC* genes, among other clock controlled genes (CCGs), through binding to the E-box sequence at their promoters; [2] Per accumulation is delayed by casein kinases (CK1α; CK1δ; CK1ε) that progressively phosphorylate these proteins at specific residues, promoting their degradation; [3] After reaching a critical concentration, Per and Cry drive the negative loop, forming heterodimers and translocating into the nucleus; [4] Per/Cry inhibit *CLOCK* and *BMAL1* transcription, culminating into a decrease in their own transcription, in a feedback loop manner. [5] Along time, Per/Cry complexes end up being degraded, releasing the inhibitory action on *CLOCK/BMAL1* and allowing for a new cycle of transcription to restart. Additional loops add further complexity and stability to the molecular circadian network: [A] ROR (α; β; γ) and [B] REV-ERB (α; β) compete for ROR Elements (RORE) in *BMAL1* and *CLOCK* genes promoters - ROR proteins stimulate BMAL1 and CLOCK expression while REV-ERB proteins negatively regulate their expression; [i] DEC (1 and 2) bind to E-box elements and inhibit *BMAL1* and *CLOCK* transcription. *CLOCK or NPAS2 if in the forebrain.

In natural conditions, such TTFLs are aligned with the light-dark cycle. In SCN neurons from mammals with a regular circadian rhythm, it was shown that Per and Cry levels peak at roughly

midday, with Per being associated with an arousal state, whereas BMAL1, CLOCK and NPAS2 peak during the night (Morse & Sassone-Corsi, 2002). Thus, although the loop of the core clock genes is autonomous, its dynamics is affected by external stimuli. For example, previous studies showed that *Per1* and *Per2* mRNA levels increase upon further exposure to light (Albrecht *et al.*, 1997; Crosio *et al.*, 2000). Indeed, *Per1 and Per2* genes belong to the PAS-domain family, whose domain, at the cellular level, acts as a light and oxygen sensor (Taylor & Zhulin, 1999). This way, light speed up the clocks(s), shortening the rhythmicity period, whereas dark slows them down, lengthening the period. Additionally, the promoter of the clock gene *Per* has several enhancers. Among them are the cAMP response element (CRE) and the glucocorticoid response element (GRE). Consequently, Per expression can be induced by several other molecules, such as glucocorticoids (Balsalobre *et al.*, 2000). Other molecules, such as melatonin, may impact on the proteasome-regulated-transcription of the SCN clock genes, interfering with the negative feedback loops that regulate *BMAL1* gene transcription. In fact, BMAL1 protein levels are stabilized in the SCN particularly at night, when melatonin levels are naturally elevated (Poirel *et al.*, 2003).

A wide range of studies using global and tissue-specific knockout mice revealed that clock genes participate either directly or indirectly in several metabolic pathways, regulating metabolic homeostasis and energy balance. Indeed, molecular analysis revealed that genes involved in lipogenesis, lipid catabolism, gluconeogenesis and sterol metabolism have expressions varying along the 24h solar day (Panda *et al.*, 2002; Oishi *et al.*, 2003; Yang *et al.*, 2006; Zvonic *et al.*, 2006; Kohsaka *et al.*, 2007). Nevertheless, analyses of the mammalian cycling proteome has revealed that the portion of the clock cycling proteins is rather higher when in comparison with the proportion of the clock cycling circadian transcriptome, which in turn shows an higher abundance (Reddy *et al.*, 2006). This discrepancy suggests that additional transcriptional, post-transcriptional, translational and post-translational mechanisms are also important players in the circadian rhythmicity (Oishi *et al.*, 2003; McCarthy *et al.*, 2007; Miller *et al.*, 2007). For a rhythmic transcription and translation to occur, an efficient and temporal chromatin remodelling must also occur in order to modulate the circadian transcription/translation (Borrelli *et al.*, 2008). These additional mechanisms are the key to understand how molecular clocks, built upon the same molecular architecture, can regulate divergent gene expressions, in a tissue-dependent manner (Partch *et al.*, 2014). Cells from different tissues may have different regulatory mechanisms acting on clock genes, leading to cell type unique circadian responses according to tissue-specific metabolic cues (Albrecht, 2002). However, the complete regulatory system is not yet fully understood. In this context, contrasting with the circadian variation in the transcriptome, other circadian biomarkers have been used to assess the robustness of the human central pacemaker.

Such biomarkers include body temperature and melatonin and cortisol levels (Hida *et al.*, 2009; Möller-Levet *et al.*, 2013).

In order to better disassemble circadian clocks, reports exploiting cell lines as models of circadian clocks have exponentially increased. Indeed, rhythmic clock genes expression has already been demonstrated in several mammalian cell lines (Hurst *et al.*, 2002; Ramanathan *et al.*, 2012, 2014). However, *in vitro*, a temporal context does not exist, thus, artificial timing cues must be given to cells for them to express stable and robust circadian oscillations in clock genes transcription, as it occurs in the native SCN. Such stimulation sustains circadian oscillations for few circadian cycles and it is possible through a short treatment with substances such as forskolin, dexamethasone, 12-O-tetradecanoylphorbol-13-acetate and vasoconstricting endothelin-1 or through a serum shock, among others (Balsalobre *et al.*, 1998, 2000; Yagita & Okamura, 2000; Yagita *et al.*, 2001). These treatments induce a rapid and robust increase in *Per1* and *Per2* genes expression, followed by a sharp decay in corresponding mRNA levels, caused by their own repression, and subsequent synchronous transcription cycles of clock genes (Balsalobre *et al.*, 1998). This way, rhythmicity can be generated although in a much simpler version. In organisms, the crosstalk between clocks must be coordinated in order to allow general patterns, such as sleep and feeding. To achieve such complexity, several brain centres must collaborate.

I.1.2. CLOCKS OUTSIDE THE HYPOTHALAMIC SCN

Neural tracing studies showed that several hypothalamic regions have reciprocal anatomical connections with the SCN, demonstrating an integrative function in generating rhythms (Watts & Swanson, 1987; Watts *et al.*, 1987; Yi *et al.*, 2006). Indeed, many of those hypothalamic regions, as the arcuate nucleus (ARC), the dorsomedial (DMH) and the ventromedial hypothalamus (VMH), in the mediobasal hypothalamic area (MBH), also house autonomous circadian clocks (Abe *et al.*, 2002; Orozco-Solis *et al.*, 2016). Such regions are known to be crucial for detection and integration of signals governing feeding behaviour and energy homeostasis (Williams & Elmquist, 2012). In fact, lesions in the MBH culminated into hyperphagia and disruption of the diurnal patterning of feeding and sleeping (King, 2006; Peterfi *et al.*, 2009). This way, integration of sleep-wake and feeding timing cues was suggested to also occur outside of the SCN, which would provide more flexibility to respond to varying challenges (Wiater *et al.*, 2011). In 2011, Wiater and its co-workers, showed that such extra-SCN hypothalamic integrative functions are orchestrated by key components, the Neuropeptide Y (NPY) receptor-expressing neurons.

I.1.2.1. Neuropeptide Y

In 1982, Tatemoto isolated NPY from extracts of porcine brain. Its sequencing, based on its physicochemical properties, revealed a linear polypeptide with 36 amino acid residues, with an α-amidated carboxyl terminus and a large number of tyrosine residues in both ends (Tatemoto, 1982; Tatemoto *et al.*, 1982). Due to its amino-acid constitution, this peptide adopts a specific three-dimensional structure, named pancreatic polypeptide fold (PP-fold), being part of the gastric peptide family (Tatemoto, 1982; Schwartz *et al.*, 1992). NPY is highly conserved across species, suggesting important physiological functions (Akabayashi *et al.*, 1994). Indeed, it plays numerous roles in several physiological processes that include control of the feeding behaviour, growth and development, memory and learning, proliferation modulation and neuroprotection, anxiety, locomotion and heart activity (Alvaro et al., 2008; Alvaro et al., 2008; Minor et al., 2011; Silva et al., 2005; Thorsell and Heilig, 2002).

This peptide is well described in rodents (Allen *et al.*, 1983) and humans (Adrian *et al.*, 1983). It is expressed both during development and adulthood (Allen *et al.*, 1984). As many other peptides and proteins, NPY is firstly synthesized as a precursor peptide after translation, that is directed into the Endoplasmic Reticulum (ER), where it undergoes several specifically regulated post-translational enzymatic events. After it, NPY is translocated to the Golgi Complex (GC), where several structural and functional adjustments take place, before being sorted and guided towards the secretory pathway (Silva *et al.*, 2002; Hörsten *et al.*, 2004). Its local action is dependent on its concentration, different receptor selectivity, local expression of the different NPY-receptors and different peptidases that influence its life-time and receptor selectivity (Hörsten *et al.*, 2004).

NPY receptors belong to the G-protein coupled receptors (GPCR) class, showing a heptahelical structure (Michel *et al.*, 1998; Fredriksson *et al.*, 2003). Such receptors are common to all members of the NPY family and are named as Y_1, Y_2, Y_4, Y_5 and y_6 (Larhammar *et al.*, 2015).

NPY and its receptors are expressed throughout the body, being one of the most abundant peptides not only in the central nervous system (CNS), but also in the peripheral nervous system as well as in other peripheral tissues (Allen *et al.*, 1987; Zukowska *et al.*, 2003). In the mammalian CNS, NPY has particularly high levels in the hypothalamus, where it plays relevant functions in several physiological processes such as in the regulation of food intake, energy homeostasis, reproduction, sexual behaviour and in circadian rhythms (Thorsell & Heilig, 2002; Kalra & Kalra, 2004; Silva *et al.*, 2005; Chee & Colmers, 2008).

The NPY role on the circadian rhythm started to be uncovered when neurons co-expressing NPY, in the arcuate nucleus (ARC), were discovered to project to the SCN (Yi *et al.*, 2006). In 2013, Saderi and co-workers showed that the thalamus IGL was the main source of the NPY innervation in the SCN. Consequently, NPY from the IGL, has been strongly implicated in the IGL transmission of both photic and non-photic signals to the SCN. This region, in thalamus, has widespread connections with several other brain regions, thus it has been suggested that the IGL is also responsible for processing metabolic information from those diverse sites into a NPY signal for the SCN clock regulation (Glass *et al.*, 2010). *In vivo* electrical stimulation of the IGL or microinjection of NPY into the SCN can cause phase shifts similar to those induced by non-photic stimuli and phase shifts caused by non-photic stimuli can be blocked by microinjection of antiserum to NPY, into the SCN area (Biello *et al.*, 1994; Harrington & Schak, 2000). That suggested that behaviours such as activity or food intake could promote NPY release to the SCN.

In the SCN, Fukuhara and co-workers (2001) showed that, at ZT (*zeitgeber* time) 6, NPY was able to induce advancing phase shifts by mechanisms involving or resulting in the reduction of both *Per1* and *Per2* clock genes expression levels, an effect opposite to light. Such action may be mediated by different receptor subtypes depending on the circadian time (CT), this is, the time of the circadian cycle. During the night, NPY effects are thought to be mediated by Y_5 receptors activation (Yannielli & Harrington, 2001). In accordance, deletion of NPY receptors Y_5 and also Y_1, in mice, altered the daily patterns of activity and feeding (Edelsbrunner *et al.*, 2009). However, there are no reports in the literature of the NPY effect on other circadian times neither on other clock genes.

NPY synthesis and consequent release are controlled by multiple neural and peripheral signals. Indeed, it is usually induced by hunger-signalling hormones such as ghrelin, and inhibited by satiety signals, given by hormones such as insulin and leptin (Näslund & Hellström, 2007). Those multiple signals result in NPY oscillations during the light-dark circadian cycle. In accordance, through microdialysis, Glass and colleagues (2010) measured the IGL-mediated NPY release to the SCN, in hamsters, and verified that the levels of this peptide rhythmically rise and fall throughout the day. Higher levels of this peptide were seen at transitions from light to dark and dark to light (Shinohara *et al.*, 1993; Shinohara & Inouye, 1995). Even in human, plasma levels of NPY oscillate with peaks every 6-8 hours and the largest peak at approximately 16 hours (Lockinger *et al.*, 2004). This way, the role that NPY plays in feeding behaviour may be linked to circadian rhythms regulation (Edelsbrunner *et al.*, 2009). Actually, in 2011, in our group (Sousa-Ferreira *et al.*, 2011) we showed that the up-regulation of NPY levels in the ARC of adult rats resulted in overfeeding but only during the day, the less active phase of these animals, disrupting their feeding patterns.

That has highlighted the importance of hypothalamic NPY oscillations during the circadian cycle to maintain feeding circadian rhythms and thus to preserve body weight and prevent obesity. Alterations in NPY levels have indeed been observed in several human diseases such as depression (Widdowson *et al.*, 1992; Heilig, 2004), neurodegenerative disorders (Davies *et al.*, 1990; Cannizzaro *et al.*, 2003; Botelho & Cavadas, 2015; Wagner *et al.*, 2016) and obesity (Stephens *et al.*, 1995; Kuo *et al.*, 2007). In addition, NPY levels have also been reported to change during the aging process, being suggested that this neuropeptide may also play a role in aging and life span determination (Gruenewald *et al.*, 1994; Vela *et al.*, 2003; Botelho & Cavadas, 2015). Consequently, it is expected that NPY level alterations may be accompanied by circadian rhythms disruptions due to this neuropeptide impact on circadian rhythms. As so, a better understanding of NPY role on hypothalamic circadian rhythms generation is crucial since hypothalamic NPY manipulations may ameliorate NPY associated disorders, based on circadian rhythms resynchronization.

I.1.3. CIRCADIAN CLOCK DISRUPTIONS

Due to the importance of the circadian clock system for almost all biological processes, it would be expected that disruptions in the clock itself or in its downstream components would be associated with pathology. Indeed, changes in circadian times cause desynchrony amongst clocks, the circadian network and the environment, which results into severe consequences. Common causes and consequences of circadian clock disruptions are pointed in table I.1.

Table I. 1: Causes and consequences of circadian rhythm disruptions.

<table>
<tr><th colspan="2">Circadian rhythm disruptions</th></tr>
<tr><th>Causes</th><th>Consequences</th></tr>
<tr><td rowspan="8">Electrical lightning
Continuous noise
Fatigue
Stress
Illness
Surgeries and sedation
Feeding at inactive phases
Night and rotational shift work
Jet lag
Sleep deprivation

(Barger et al., 2009; Archer et al., 2014; Brainard et al., 2015; Yasumoto et al., 2016)</td><td>Metabolic diseases
(reviewed in Yang & Shieh, 2009; Maury et al., 2010; Eckel-Mahan & Sassone-Corsi, 2013; Shanmugam et al., 2013)</td></tr>
<tr><td>Cardiovascular diseases
(reviewed in Dominguez-Rodriguez et al., 2009; Takeda & Maemura, 2011; Shanmugam et al., 2013)</td></tr>
<tr><td>Neurodegenerative diseases
(reviewed in Hastings & Goedert, 2013; Musiek, 2015; Mattis & Sehgal, 2016)</td></tr>
<tr><td>Cancer
(reviewed in Wood et al., 2009; Savvidis & Koutsilieris, 2012; Shanmugam et al., 2013)</td></tr>
<tr><td>Psychiatric disorders
(reviewed in Lamont et al., 2007; Wulff et al., 2010; Jagannath et al., 2013)</td></tr>
<tr><td>Sleep disorders
(reviewed in Wulff et al., 2009; Zhu & Zee, 2012)</td></tr>
<tr><td>Hypertension
(reviewed in Wu & Sigmund, 2016)</td></tr>
<tr><td>Aging
(reviewed in Kondratova & Kondratov, 2012; Costa & Ripperger, 2015)</td></tr>
</table>

Among the described causes of circadian rhythm disruptions, sleep deprivation is very common, mainly amongst people who suffer from sleep disorders. Such perturbations are extremely common, being estimated that approximately 30-40% of the world adult population is affected, an incidence expected to increase (Bent *et al.*, 2006).

I.2. SLEEP DISORDERS

Sleep is a fundamental phenomenon that is conserved among the most genetically diverse eukaryotes, from lower phyla, such as arthropods, to higher-order phyla, such as mammals (Crocker & Sehgal, 2010). It arises from interactions between multiple brain nuclei, neurotransmitter systems and modulatory hormones that either promote sleep or wakefulness (Cirelli, 2014). Along the years, its importance has been associated with several brain functions, such as protein synthesis, cellular and molecular aspects of the neural function or memory consolidation (Ramm & Smith, 1990; Stickgold *et al.*, 2001; Cirelli *et al.*, 2004). In humans, sleep quantity and quality can be measured by brain electrical activity with electroencephalograms (EEGs) (Cirelli, 2014). A typical sleep EEG consists of one to four non-rapid eye movement (NREM) stages, in which stage I and II are light sleep stages and stages III and IV are deeper sleep stages,

and a rapid eye movement stage (REM) (Dumermuth *et al.*, 1983; Crocker & Sehgal, 2010). Light sleep stages are easily disrupted by surrounding stimuli, opposing to the deepest sleep stages, in which there is a quiescent neural activity with slow and regular breathing. On the other hand, in REM, many brain areas are active and the breathing pattern is rapid with shallow respirations (Gupta *et al.*, 2010; Sehgal & Mignot, 2011). Still, the REM stage is accompanied by a considerable body muscle paralysis (Wulff *et al.*, 2010).In the end, a night of sleep is characterized by cycles between stages of NREM and REM throughout the night, in which NREM stages 3 and 4 may eventually disappear as the sleep episode progresses. In a normal adult, NREM constitutes about 75 to 80% of the total time spent in sleep, whereas REM only constitutes the remaining 20-25% (Colten & Altevogt, 2006).

Several characteristics of sleep are tightly regulated, including the timing of sleep, its length and quality (Borbély, 1982; Crocker & Sehgal, 2010). Circadian rhythms highly contribute for such regulation, in collaboration with a homeostatic regulatory process (Borbély, 1982). Opposite to circadian rhythms, the homeostatic process is not intrinsic and depends on previous wakefulness, tracking the need of sleep. These two processes are generated independently, however, it is their interaction that determines sleep characteristics (Franken & Dijk, 2009).

Disruptions in timing, duration and/or quality of sleep and wakefulness are common among sleep disorders. There are numerous disturbances affecting sleep, which, according with the International Classification of Sleep Disorders (ICSD) (2014), can be grouped into six major classes, as shown in the following table:

Table I. 2: Sleep disorders classification.

Sleep Disorders	
Category	**Sub-Category**
Insomnia	
Sleep Related Breathing Disorders	Obstructive Sleep Apnea
	Central Sleep Apnea
	Sleep Related Hypoventilation Disorders
	Sleep Related Hypoxemia Disorder
Central Disorders of Hypersomnolence	
Circadian Rhythm Sleep-Wake Disorders	
Parasomnias	NREM-Related Parasomnias
	REM-Related Parasomnias
	Other Parasomnias
Sleep Related Movement Disorders	
Other Sleep Disorders	

The table classifies sleep disorders into categories and correspondent sub-categories, according with the ICSD (2014).

Among sleep disorders, obstructive sleep apnea (OSA) constitutes one of the most common sleep disorders and its frequency is estimated to continuously increase (Xu, Zheng, *et al.*, 2015; Calik, 2016). This particular sleep disorder will be focused in the following sub-chapter.

I.2.1. OBSTRUCTIVE SLEEP APNEA

In general, sleep related breathing disorders are characterized by respiration abnormalities during sleep. OSA is an example of such disorders in which recurrent episodes of complete (apnea) or partial (hypopnea) upper airway obstruction occur during sleep, while respiratory efforts continues (at least during some portion of the event) (Darien, 2014; Greenstone & Hack, 2014). Characterized by apneas are two other sleep disorders, the central sleep apnea and the mixed sleep apnea. However, in central sleep apnea, respiratory efforts during sleep do not occur and the breathing cessation is caused by a defect in the ventilator control at the levels of the central nervous system. On the other hand, the mixed sleep apnea is characterized by episodes of both conditions. Among apneas, OSA is the most common disorder (Gupta *et al.*, 2010; Darien, 2014). OSA characteristic obstruction of the upper airway occurs mainly due to a reduced cross-sectional area of the upper airway lumen that is characteristic in OSA patients (figure I.4). Consequently, the negative pressure generated in this region, during inspiration, promotes the upper airway closure (Greenstone and Hack, 2014). Such events can occur in any sleep stage although it is more frequent in stages NREM1, NREM2 and REM than in NREM3 and NREM4 (Darien, 2014; Greenstone & Hack, 2014). Independently of the sleep stage, apneic and hypopneic events last a minimum of 10 seconds and, generally, have a duration of 10 to 30 seconds (Gupta *et al.*, 2010). Occasionally, such episodes may also persist for one minute or longer. Apnea and hypopneas are considered similar in terms of pathophysiology and consequences. On the other hand, OSA patients have also been reported to have variable amounts of central apnea (Darien, 2014).

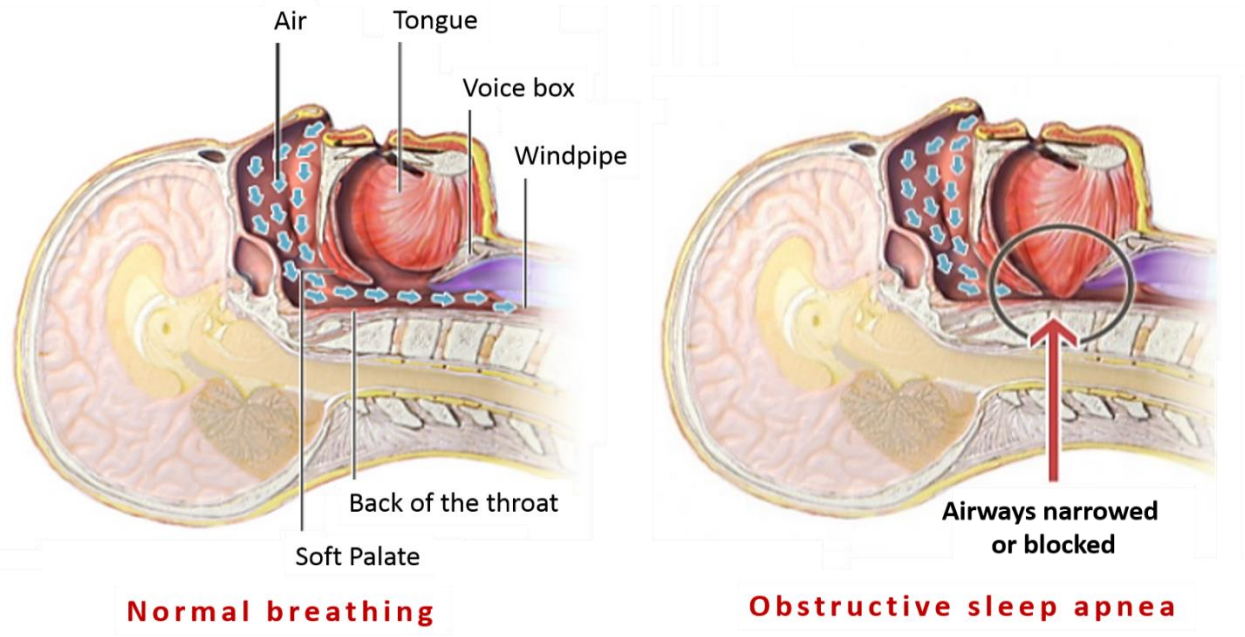


Figure I. 4: Illustration of normal breathing and OSA breathing during sleep. In normal conditions, the air is able to move through the upper airway whereas during an OSA, airways become narrowed or even blocked and the air cannot move through. Adapted from: U.S. National Library of Medicine, 2015.

Obstruction episodes are characterized by changes in the intrathoracic pressure and consequent reductions in blood oxygen saturation (increase of the oxygen levels following a respiratory event), ending in hypercapnia (abnormally elevated levels of carbon dioxide in blood) (Greenstone & Hack, 2014). Consequently, a series of obstructive events culminate into repeated changes in the intrathoracic pressure and cyclical deoxygenation/oxygenation (Caples, 2005; Pack, 2006; McNicholas *et al.*, 2007). The degree of oxygen saturation reduction and the correspondent period of time is variable, being dependent on the sleep stage, body position, duration of the event, baseline oxygen saturation, lung volume, and co-existence of comorbid lung conditions. When occurring in stage REM, or, when the individual is sleeping at a supine position (lying with the face upward), episodes are associated with more severe reductions in oxygen saturation (Darien, 2014). Usually, such reductions range from 4% up to 30-40% or even greater (Greenstone & Hack, 2014). The resumption of the normal breathing usually returns the oxygen saturation to baseline values however, if events are more frequent and prolonged or if there is an underlying pulmonary pathology, oxygen saturation may remain low (Darien, 2014). These episodes are usually terminated by brief arousals from sleep in which it is common to occur a burst in the sympathetic nervous system activity and in systemic blood and pulmonary arterial pressure (Greenstone & Hack, 2014). In addition to repetitive brief arousals, the sleep of OSA patients is frequently characterized by snores between apnea, witnessed episodes of gasping or chocking and body

movements that disrupts sleep (Darien, 2014). Such episodes are especially frequent on stage REM, subsequently, OSA patients usually spend insufficient time in the REM stage (Greenstone & Hack, 2014). Furthermore, OSA characteristics also impact at the molecular level.

I.2.1.1. OSA molecular pathophysiology

Over the last years, OSA has been associated with an increase of biochemical markers of inflammation. Although the exact causative mechanism is not known yet, sleep deprivation and OSA characteristic hypoxia are among the most likely contributors to such effect (Mullington *et al.*, 2009; Patel *et al.*, 2009). Indeed, OSA hypoxemia have several repercussions. It is known that sustained hypoxia leads to activation of the hypoxia-inducible factor 1 (HIF-1) transcription. HIF-1 acts as a regulator of oxygen homeostasis during hypoxia and it is involved in the regulation of the expression of many genes. Furthermore, once expressed, HIF induces the expression of the vascular endothelial growth factor (VEGF) (Manalo *et al.*, 2005). However, apnoeic episodes may not be long enough to lead to HIF activation. Still, reoxygenation after a brief period of hypoxia is also able to activate the inflammatory pathways of the nuclear factor-κB (NF-κB) (Ryan *et al.*, 2005). Thus, either one way or the other, OSA characteristic hypoxia culminates into inflammation (Montesi *et al.*, 2012). Indeed, along the years, several studies reported elevated levels of inflammatory factors in the blood of OSA patients (Steiropoulos *et al.*, 2010). Such inflammatory factors include cytokines such as tumour necrosis factor α (TNFα) and interleukins (IL) 6 and 8 and also other inflammation mediators such as the C-reactive protein (CRP). Inflammation culminates into endothelial dysfunction, by the overexpression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), E-selectin and L-selectin (Mills & Dimsdale, 2004; Kapur, 2010). Additionally, the adipose tissue can also produce pro-inflammatory markers and obesity is a common comorbidity with OSA (Mullington *et al.*, 2009). Thus, a certain subset of patients can be particularly susceptible to a pro-inflammatory state.

Associated to hypoxia events is also the formation of free radical species and the initiation of cascades of oxidative stress (Lavie, 2003). Oxidative stress has a strong impact on the cellular transcriptome, by activating several TFs (Ryan *et al.*, 2005). Consequently, 8-isoprostane and nitric oxide were also shown to be elevated in the serum of OSA patients (Carpagnano *et al.*, 2002; Alonso-Fernández *et al.*, 2009).

On the other hand, derived from such stress conditions and from abnormal sympathetic activation, certain hormones have also been reported to be elevated in the plasma of OSA patients such as noradrenaline, adrenaline and cortisol (Coy *et al.*, 1996; Parlapiano *et al.*, 2005).

Certain metabolic markers can also be associated with OSA. Namely, glucose impairment which has been associated with OSA severity. An increased OSA severity is accompanied by increased glucose levels and an increased pancreatic β-cells activity (Pallayova *et al.*, 2010).

1.2.1.2. OSA long-term effects

Due to the characteristic fragmented sleep, OSA patients are frequently reported to awaken feeling tired, independently of the time spent in bed, with most of them complaining about insomnias, poor sleep quality, fatigue, morning headaches, concentration problems, forgetfulness, depression and excessive sleepiness during day, mainly in relaxing or inactive situations (Caples, 2005; Pack, 2006). In this context, life quality is extremely affected, at several levels, including at job performance. In extreme sleepiness cases, sleep may even occur while eating, walking or driving. Indeed, the risk of motor vehicle accidents is significantly increased among those with OSA (George, 2007).

On the other hand, increasing evidence have also implicated untreated OSA as a risk factor for several diseases, including glucose impairment, type 2 diabetes mellitus independent of obesity, obesity and a variety of metabolic disorders, pulmonary hypertension, incident systemic hypertension, arrhythmias, coronary artery disease, atrial fibrillation, congestive heart failure, stroke and premature mortality (Park *et al.*, 2011; Darien, 2014; Weaver *et al.*, 2014). Such effects seem to be more evident in men and middle-aged individuals (Darien, 2014). In addition, the development of such disorders is also more common in patients with comorbid conditions such as morbid obesity or chronic obstructive pulmonary disease (COPD). Patients with COPD and OSA have greater nocturnal oxygen desaturation and daytime hypercapnia, which turns them more susceptible to develop pulmonary hypertension and heart failure (Darien, 2014). In a similar manner, OSA coexistence with cardiomyopathy or ischemic heart disease increase the severity of the underlying heart disease and predisposition to congestive heart failure (Park *et al.*, 2011). On the other hand, mood disturbances have also been reported in some patients with OSA, as well as an increase in depression severity (Darien, 2014) (Ejaz *et al.*, 2011).

I.2.1.3. OSA epidemiology and aetiology

OSA affects 6-13% of the world adult population and 1-4% of the pediatric population (Lumeng & Chervin, 2008; Lurie, 2011). In patients with cardiac or metabolic disorders, OSA prevalence sharply increases up to more than 50%, with prevalence rates estimated to increase due to the increasing rates of metabolic disorders (Lurie, 2011). Indeed, the major predisposing factor for OSA is excess body weight, being estimated that approximately 60-70% of the moderate to severe OSA patients are obese (Lin *et al.*, 2012; Feliciano *et al.*, 2015). That can be associated with an increased fat accumulation in the upper airway which facilitates its obstruction. Besides, increased neck circumferences predicts higher apnea and hypopnea indexes (Attal & Chanson, 2010; Lin *et al.*, 2012). Even so, OSA is still a highly underdiagnosed disorder since many individuals are not aware that they may suffer from OSA (Feliciano *et al.*, 2015). In addition, OSA prevalence also increases with age, although it appears to plateau in the elderly, after approximately the age of 65 (Palmer & Redline, 2003; Darien, 2014). This way, OSA can occur in any age group. Men are also more affected than women, with an estimated ratio of approximately two to one. Such gender difference has been attributed to a differential sleep structure and to a differential body fat distribution in which men have a more central body fat distribution, including in the neck (Gupta *et al.*, 2010; Camara *et al.*, 2013). That way, the risk for narrowing and closure of the upper airway increases. Nevertheless, this disparity tends to decline in middle to older age, since after menopause, women are more prone to suffer from OSA (Palmer & Redline, 2003; Darien, 2014). In addition, endocrine disorders such as acromegaly and hypothyroidism are also risk factors for OSA (Skjodt *et al.*, 1999).

Studies on familial clustering of OSA patients suggested that this disease has a heritable component (reviewed in Palmer & Redline, 2003). Indeed, first-degree relatives of OSA patients are twice as likely to suffer from OSA. Nonetheless, a gene or genes responsible for OSA heritability have not been identified (Greenstone & Hack, 2014). Thus, the expression of OSA is likely determined by multiple genetic and environmental factors and their interactions (Palmer & Redline, 2003). In this context, the apparent OSA heritability may arise from familiar obesity and familial environmental factors such as physical activity and eating habits or heritable craniofacial morphology. On the other hand, in patients with normal weight or underweight, upper airway obstruction is usually due to a localized structural abnormality. Various abnormalities of the head and neck bony and soft tissue structures may be familiar and predispose individuals to OSA (Madani & Madani, 2009; Lurie, 2011).

If untreated, OSA has been suggested to be a progressive disease in which the pathological evolution would come from snoring at first, then, developing upper airway resistance syndrome and, finally, OSA, progressively more severe (Calverley, 1997).

I.2.1.4. OSA diagnosis

For OSA diagnosis, individuals must first fulfil one of two criteria (Darien, 2014):

Table I. 3: Criteria that individuals must first fulfil for OSA diagnosis.

OSA diagnosis criteria		
1	Feeling an excessive daytime sleepiness that is not explained by any other factor	
2	Showing two or more of a set of symptoms	Choking or gasping during sleep
		Recurrent awakenings from sleep
		Un-refreshing sleep
		Daytime fatigue
		Impaired concentration

Such information is firstly obtained through appropriate questionnaires. However, apnea and hypopneas frequency during sleep correlates poorly with daytime symptoms severity and impact on life quality. For patients suffering from sleepiness, there are several methods to assess it, including self-reported severity of sleepiness, indices such as Epworth Sleepiness Scale and objective measures, however, such methods are not strongly correlated, which difficult its assessment and reliability. Besides, sleepiness has a wide range of possible aetiologies and of manifestations. On the other hand, patients that do not complain about feeling sleepy, may have adapted to their usual sleepiness and fail to report it as a problem. In addition, patients may not be aware of snoring or of having apneic episodes unless someone warns them (Park *et al.*, 2011).

In this context, this first approach only indicates if an individual is likely suffering from OSA. If that is the case, then the patient is recommended for OSA overnight monitoring, which can or cannot confirm the suspicion (Greenstone & Hack, 2014). OSA gold-standard diagnosis consists of an overnight laboratory-based polysomnography (PSG). This test allows to record several parameters, pointed in table I.4 (Gupta *et al.*, 2010):

Table I. 4; Parameters assessed by the PSG test.

Polysomnography test	
Assessed parameters	**Description**
Electroencephalogram	Monitors sleep time and stages
Electrooculogram	Monitors eye movements
Chin electromyogram	Determines the level of muscle tone
Electrocardiogram	Follows cardiac rhythm
Respiration	Measures airflow and thoracic and abdominal respiratory effort
Oximetry	Assess arterial oxygen saturation
Body position	Assesses body orientation during sleep
Leg electromyogram	Detects limb movements
Snoring	

In order to perform the PSG test, patients must spend the night at a sleep laboratory, assisted by a PSG technician with a minimum of six hours of monitoring. Consequently, its availability is restricted due to its high cost and it is cumbersome (Ross *et al.*, 2000; Kushida *et al.*, 2005; Darien, 2014). As an alternative, several portable sleep monitors have been developed so the patients' sleep can be evaluated at home. Still, devices measuring the same parameters as a PSG may require a technician to set up the equipment at the patient's residence. In turn, there are portable sleep monitors that does not require a professional set up but those devices will not be able to measure all the physiological parameters (Ferber *et al.*, 1994; Collop, 2008; Kuna, 2010). Additionally, portable sleep monitors will also not be able to determine the actual sleep time. Instead, results will be analysed per hour of recorded time. However, the recorded time does often exceeds the actual sleep time, underrepresenting OSA severity (Park *et al.*, 2011). Additionally, cases diagnosed with such devices may require further confirmation by PSG at the laboratory, especially to rule out false negatives when there is high disease probability and the portable sleep monitoring results are negative (Collop, 2008). In addition, without monitoring along the night, such devices may fail or sensors may become disconnected or lost. Besides, there is an increased variability of the equipment and the technologies associated (Kuna, 2010). Still, either using portable sleep monitors or undergoing the PSG test at the laboratory, variability between nights of sleep may require a second test (Ahmadi *et al.*, 2009). For example, apnea and hypopneas events may be intensified following alcohol ingestion, sedating medications prior to sleep, nocturnal nasal restriction or congestion and following an increase in body weight (Darien, 2014).

After sleep monitoring, the severity of the disease is determined by several factors, in which the frequency of apnea and hypopneas and the degree of oxygen desaturation constitute two preponderant factors. Accordingly, severity indexes can be generated, such as the apnea-hypopnea index (AHI) and the respiratory disturbance index (RDI) (Greenstone & Hack, 2014). The

RDI consists of the sum of apnea, hypopneas and respiratory effort related arousals per recorded hour. The AHI consists of the sum of apnea and hypopneas per hour of sleep and according to it, different OSA classifications can be obtained, as showed in the following table (Berry *et al.*, 2012; Darien, 2014).

Table I. 5: Classification of OSA severity, according to the apnea-hypopnea index (AHI).

Obstructive Sleep Apnea	
AHI	Classification
< 5/h	Disease free
≥ 5/h - < 15/h, with associated symptoms	Mild
≥ 15/h - < 30/h, regardless of associated symptoms	Moderate
≥ 30/h, regardless of associated symptoms	Severe

Nonetheless, hypopneas definition criteria is not consensual, resulting in highly variable AHI levels. Consequently, AHI have a non-linear correlation with OSA symptoms and there is not a standard AHI value that indicates the need for treatment (Greenstone & Hack, 2014). In this context, some OSA patients may be under or over diagnosed and may not undergo the most appropriate treatment (Berry *et al.*, 2012; BaHammam *et al.*, 2014).

I.2.1.5. OSA treatment

Regarding OSA treatment, there are several options, depending upon the severity of the correspondent cases and presence of associated co-morbidities or anatomical anomalies.

For patients with a mild OSA condition, certain lifestyle changes may significantly improve the disease symptoms and consequences. For example, avoiding certain substances, such as sedatives, tranquilizers, anti-histamines and alcohol was shown to decrease OSA episodes (Darien, 2014). On the other hand, patients with an excessive weigh may also observe significant improvements if they lose weight, due to ameliorations in the pharyngeal structure and function, neuromuscular control and functional residual capacity (Gupta *et al.*, 2010). Additionally, positional treatment may also be of benefit if most apnea events occur in a specific sleep position (usually supine). However, since this treatment limits sleep in any position, it is uncomfortable and it is not that effective (Greenstone & Hack, 2014). Still, such lifestyle changes will not be so efficient in more severe cases.

For patients with mild OSA with associated comorbidities such as hypertension or for moderate and severe OSA cases, the first line therapy consists on a positive airway pressure mask that supports breathing during sleep. This device blows compressed air into the airways, keeping the

upper airway free. The compressed air may be continuously released (CPAP) or two levels of pressure may exist (BiPAP), one that corresponds to the inhale and another that corresponds to the exhale, being adjustable to the person's breathing. In the last case, patients recommend for treatment must return to the laboratory for a new sleep monitoring, assisted by a technician, so that the ideal pressure produced by PAP can eliminate obstructive events. This way, sleep quality is improved, usually stopping the typical OSA snoring and decreasing the number of breathing pauses. Subsequently, oxygen levels in blood do also increase, improving long-term OSA consequences (Gupta *et al.*, 2010). Nevertheless, residual sleepiness may persist and most chronic consequences may not be fully reverted (Ballard, 2008; Sanchez *et al.*, 2009). In addition, the CPAP mask may be unpleasant and constrictive and patients may refuse to use it. Indeed, adherence was reported to be as low as 50% in certain populations (Weaver & Grunstein, 2008). More pleasant CPAP devices have been developed in order to increase adherence rates, but the success is limited. Consequently, for cases in which the CPAP treatment fails, there are other options that can be followed. Although the efficacy is not as great as with CPAP, adherence rates are higher and might be a better long-term option (Okuno *et al.*, 2014; Calik, 2016).

As an alternative, there are several nasal and oral appliances available that can be used during sleep (Weaver *et al.*, 2014). Amongst them are nasal devices that increase the positive pressure at the expiratory phase, preventing upper airway collapse; plastic mouth guards, that assure that the mandible is moved forward or stops the tongue for moving back and blocking the airway; and oral pressure therapy (OPT) in which a mouthpiece, connected to a small console, creates a vacuum in the oral cavity and applies a small negative pressure that prevents airway obstruction (Okuno *et al.*, 2014; Calik, 2016). Such appliances may also be recommended to patients whose OSA severity does not justify the use of the CPAP mask such as mild cases (Greenstone & Hack, 2014).

On the other hand, hypoglossal nerve stimulation (HNS) has also been implemented. This new method allows to electrically stimulate the hypoglossal nerve of the tongue, counteracting the negative pressure and collapsing forces of the upper airways during inspiration. For that, a silicone cuff with a breathing sensor, a small generator and stimulating electrodes is positioned in the specified nerve during a surgical procedure (Eastwood *et al.*, 2011; Weaver *et al.*, 2014; Calik, 2016). However, as all the other methods, it also has drawbacks. HNS does not have therapeutic effects in individuals with elevated BMIs, it is surgically invasive and some adverse events were already reported. Still, its long-term significant improvements turn it into a viable treatment over CPAP (Woodson *et al.*, 2014, 2016).

Nevertheless, all these OSA strategies only relief sleep apnea symptoms and once treatment is stopped, symptoms tend to reappear (Calik, 2016). Instead, there are different types of surgery that definitely improve breathing in OSA patients, reducing the mean AHI by correcting anatomical deformities or tithing or even removing tissue to widen the airway (Gupta *et al.*, 2010). Surgical procedures are usually recommended for cases in which the CPAP mask and oral appliances are not recommended or have failed (Greenstone & Hack, 2014). However, it has not been rigorously evaluated as a medical treatment, being still under investigation, and not all the patients submitted to the surgery have a successful treatment, some have associated pain, bleeding or trouble swallowing and effects on daytime sleepiness were not reported yet (Sundaram *et al.*, 2005; Browaldh *et al.*, 2013).

Table I.6 summaries the available types of OSA treatment (Gupta *et al.*, 2010; Weaver *et al.*, 2014; Calik, 2016):

Table I. 6: Treatment types, according to OSA severity, and correspondent limitations.

Obstructive Sleep Apnea			
Severity	**Treatment type**		**Limitations**
Mild cases	Avoidance of factors that aggravate OSA	Sedatives Tranquilizers Anti-histamines Alcohol Excessive weight Supine sleep position	• Uncomfortable; • In some patients it may not be efficient.
Mild cases with associated co-morbidities; Moderate and severe cases	Positive airway pressure mask	Continuous positive airway pressure mask (CPAP) Bi-level positive airway pressure mask (BiPAP)	• Residual sleepiness may persist; • Most chronic consequences may not be fully reverted; • Unpleasant and, consequently, low adherence.
Patients that did not adapt to PAP mask or such option was not recommended	Nasal and oral appliances	Nasal devices Plastic mouth guards Oral pressure therapy (OPT)	• May also be unpleasant; • Lower efficacy.
Mild patients with associated co-morbidities; Moderate and severe cases	Electrical stimulation	Hypoglossal nerve stimulation (HNS)	• No therapeutic effects in individuals with elevated BMIs; • Surgically invasive; • Reported adverse effects.
Patients that did not adapt to both CPAP mask and oral appliances or such options were not recommended	Surgery	Anatomical deformities correction Widen the airway by tithing or removing tissue	• Not all the patients submitted to the surgery have a successful treatment

Pharmacological treatments have also been investigated and developed to improve OSA symptoms. Clinical trials of various drugs have been performed, targeting drugs capable of increasing the respiratory control; increasing the upper airway tone; reducing the cross-sectional area or the surface tension of the upper airway; decreasing REM sleep; or increasing the arousal threshold (Mason *et al.*, 2013). However, it has not been proved that it actually improves OSA and, consequently, it was not approved for its treatment yet. Drugs with potential for improving OSA conditions have still to be studied in terms of efficacy, side-effects and long-term viability (Mason *et al.*, 2013; Greenstone & Hack, 2014; Calik, 2016).

In the end, combining two or more strategies may lead to greater OSA improvements. But for that, the specific pathophysiology of each patient has to be discerned which requires more simple and affordable screening tests (Deacon *et al.*, 2015; Calik, 2016). Early diagnosis would also significantly improve OSA treatment and avoid severity progression. In this context, identifying potential biomarkers for early diagnosis, severity, prognosis and response to OSA treatment are absolutely necessary to improve both OSA diagnosis and treatment.

I.2.1.6. OSA biomarkers

Along the years, several attempts have been made in order to find ideal OSA molecular biomarkers. An ideal biomarker or set of biomarkers should have use as a diagnostic measure, being highly sensitive and specific and should correlate with the severity of the disease. For that, it should be involved in the pathogenesis of the complications associated with the disease. If so, such biomarker/s would be useful for early diagnosis and for predicting outcome. In addition, its levels would change in response to a therapy, being dose-responsive, allowing to measure treatment response and reflecting the adequacy of a therapy (Shih & Malhotra, 2011; Montesi *et al.*, 2012). In this context, associated with OSA pathophysiology, different class biomarkers have been searched (table I.7).

Table I. 7: Types of OSA biomarkers assessed so far and correspondent samples.

OSA biomarkers		
Biomarker type	Sample	Reviewed in:
Proteomic patterns	Urine Serum Plasma	Montesi *et al.*, 2012; Archontogeorgis *et al.*, 2014; De Luca Canto *et al.*, 2015; De Luca Canto *et al.*, 2015; Feliciano *et al.*, 2015; Maeder *et al.*, 2015
Lipid profile	Serum	
Metabolic profile	Serum	
Inflammation markers	Urine Serum Plasma Exhaled breath condensate	
Oxidative stress markers	Urine Plasma Exhaled breath condensate	
Cardiovascular function markers	Urine Serum Plasma	
Endothelial function markers	Plasma Urine Exhaled breath condensate	
Hormones	Urine Saliva	
Uric acid	Serum	
Circulating microparticles	Blood	
Blood tension	Blood	

Nevertheless, no ideal OSA biomarker/s currently exist. Among OSA pathogenesis, intermittent hypoxia, sleep fragmentation, abnormal sympathetic activation during sleep and bursts in systemic blood and pulmonary arterial pressure are central to OSA pathogenesis. Curiously, all these events are strong contributors to circadian rhythm alterations. In this context, a cross-talk between OSA and clock dysfunctions have been suggested. Such cross-talk hold promising candidates for the first OSA biomarkers.

I.2.1.6.1. A biomarker system based on the clock genes and OSA cross-talk

Over the last decade, a cross-talk between hypoxia and circadian rhythms have been suggested. According to the literature, hypoxia is able to induce alterations on the circadian oscillations of several physiological processes, such as body temperature or metabolic processes, sleep-waking pattern of rats and humans, and on the circadian markers melatonin and cortisol (Chilov *et al.*, 2001; Egg *et al.*, 2013). Indeed, as previously referred, *Per1* also acts as an oxygen sensor, which means that its expression levels are affected by oxygen levels. Actually, *Per1* and HIF-1 belong to the same family of PAS-domain-positive proteins and the E-box element that controls *Per1* and many other clock genes is the core sequence of the hypoxia-responsive element (HRE) (Taylor &

Zhulin, 1999; Egg *et al.*, 2013). Subsequently, HIF-1 has also been shown to interact with CLOCK and BMAL1, *in vitro* (Ghorbel et al., 2003). Furthermore, the auxiliary clock gene RORα was also shown to play a role in hypoxia signalling pathway, inducing HIF-1 stabilization and transcriptional activation (Kim *et al.*, 2008). And, on the other hand, the expression of both *DEC1* and *DEC2* was also been shown to be upregulated under hypoxic conditions (Miyazaki *et al.*, 2002). Additionally, intermittent hypoxia (IH) leads to sleep fragmentation that, *per se*, also contributes to circadian rhythm disruptions. Consequently, these observations triggered the question of whether OSA characteristic IH would be causing, or not, clock genes dysfunctions in OSA patients.

On the other hand, OSA patients have an over activity of their sympathetic nervous systems. In healthy individuals, the activation of the sympathetic nervous system in the morning, following nocturnal restitution, acts as a powerful zeitberg. Therefore, abnormal sympathetic activation may be deregulating such alarm clock. In addition, heart rate and blood pressure also exhibit diurnal variations crucial for several physiological processes (Young, 2006). Subsequently, the characteristic OSA bursts in blood and pulmonary pressure interfere with the essential normal diurnal variations. Thus, OSA characteristics strongly suggest an impact on circadian rhythms. An abnormal clock function could be in the origin of the characteristic OSA excessive daytime sleepiness and mood disturbances.

Indeed, in 2008, Burioka and its co-workers showed for the first time, a link between OSA and circadian rhythm alterations. The expression of the clock gene *Per1* was quantified in peripheral blood cells of eight healthy individuals and eight OSA patients, at different time points over 24 hours, by real time PCR analysis. As expected, heathy individuals showed daily variations in *Per1* expression however, by opposite, OSA patients did not show those daily expression variations. After 3 months of CPAP treatment, the diurnal variation of *Per1* mRNA expression was significantly improved, which has suggested that OSA causes clocks dysfunction. However, the *Per1* gene was the only gene whose expression was assessed in OSA patients. Therefore, it is crucial to investigate whether other clock genes, either from the central loop or from auxiliary loops, are also affected by OSA.

To understand the underlying clock gene dysfunction in OSA patients, Burioka and colleagues performed further experiments. Consequently, these researchers assessed whether the levels of the clock genes *Per1*, *Per2*, *Cry*, *CLOCK*, *BMAL1* and *REV-ERBα* were affected in a lung adenocarcinoma cell line treated with intermittent hypoxia/reoxygenation (IHR) and no significant alterations were found. Additionally, in vitro experiments in mouse embryonic fibroblasts (NIH3T3) showed that inflammatory mediators such as IL-6, did not impact on *Per1* mRNA

expression (Burioka *et al.*, 2008). By opposite, treatment of the same cells with noradrenaline induced *Per1* mRNA levels in a concentration-dependent manner. In the same context, *in vivo*, the administration of noradrenaline for 6 days, in three mice, induced *Per1* gene expression in the cerebral cortex and other tissues, when mice were asleep. Thus, OSA intermittent hypoxia per se may not be sufficient to induce alterations in the circadian rhythm but the abnormal sympathetic activation occurring during sleep may be one of the factors involved in *Per1* mRNA expression dysfunction. Indeed, an adrenaline β_2-adrenoceptor agonist was shown to induce *Per1* gene expression both *in vitro* and *in vivo*, namely in humans (Burioka *et al.*, 2007). Thus, the authors suggested that changed levels of circulating factors, either derived from the abnormal sympathetic activity or from inflammation or oxidative stress, may be inducing alterations in patients' circadian clocks.

On the other hand, it is known that OSA prevalence considerably increases in individuals with excessive weight and with aging (Gruenewald *et al.*, 1994; Stephens *et al.*, 1995; Vela *et al.*, 2003; Kuo *et al.*, 2007; Botelho & Cavadas, 2015). Interestingly, NPY levels were reported to be abnormal in these particular population groups, as previously described. Given NPY involvement in the circadian rhythm modulation, changes in its levels are also strong contributors to the OSA patients' apparent abnormal clocks (figure I.5). Understanding how the circadian rhythm molecular machinery is affected by abnormal NPY levels and whether it can be contributing to clocks disruption, in OSA, may be fundamental for OSA diagnosis improvement.

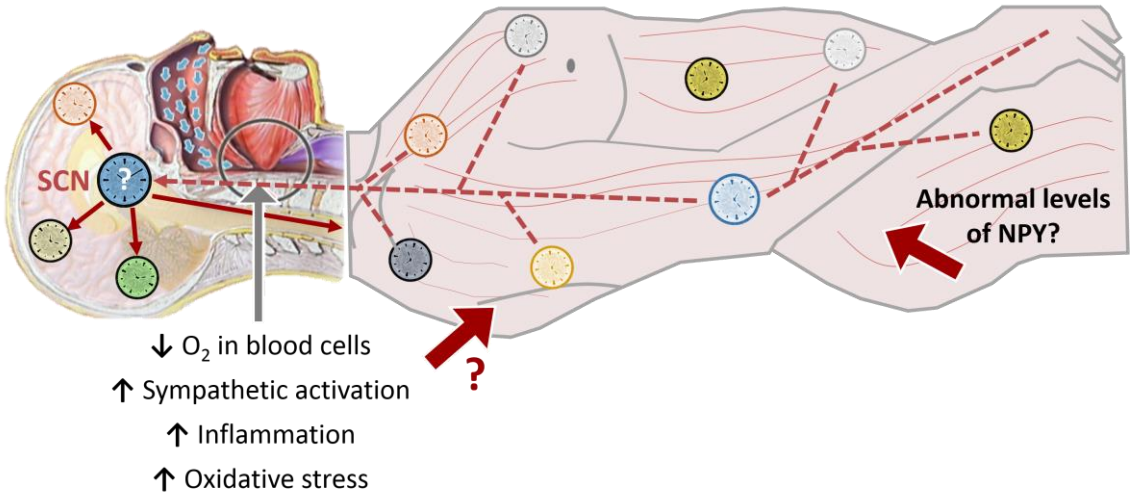


Figure I. 5: Illustration of circadian rhythms and OSA cross-talk. The figure shows a summary of the possible contributors to OSA clock dysfunction. On the one hand, OSA episodes cause a decrease of the blood oxygen levels, an abnormal sympathetic activation, inflammation and oxidative stress which may change the levels of circulating factors and lead to the misalignment of the biological clocks. But, on the other hand, abnormal levels of NPY, characteristic of obese and elderly patients, may also be in the origin of such clock alterations. (Based on Stephens *et al.*, 1995; Burioka *et al.*, 2008; Darien, 2014; Botelho & Cavadas, 2015)

In fact, in response to physiological and pathological stimuli, RNA molecules are continually transcribed, translated and turned over. Therefore, if interpreted appropriately, the RNA profile of a cell can be used as a reflection of its current functional status (Rao *et al.*, 2013). In this context, clock genes RNA levels could be used as reliable biomarkers. However, initially, the sources used for RNA analysis were invasive, being mainly at the tissue level. Along time, minimal invasive or even non-invasive strategies have been developed for its assessment (Leidinger *et al.*, 2014). One of the most attractive alternatives is to measure the RNA expression from blood. In blood, PBMCs are a heterogeneous population of lymphocytes and monocytes (mononuclear cells) that are the most transcriptionally active cells in blood due to their continuous survey of body signs of infection or other threats. Thus, their gene expression responses can potentially provide an early warning of pathological conditions, including cancer, auto-immune, metabolic and genetic disorders. This way, PBMCs can be used as viable tools to identify expression variations between patients and healthy individuals and to characterize underlying mechanisms of diseases (Affymetrix, 2003; Whitney *et al.*, 2003). In addition, previous studies have shown that inter individual variations of gene expression patterns in PBMCs are strikingly smaller than differences between blood samples, highlighting its potential use for diagnosing blood-related diseases and monitoring effects of drugs and therapies (Debey *et al.*, 2004). Consequently, since these cells were reported to also have a rhythmic expression of clock-related molecules, they could act as reliable sources to assess the expression levels of such genes (Teboul, Barrat-Petit, *et al.*, 2005; Boivin *et al.*, 2015; Xu, Xu, *et al.*, 2015).

Thus, since an abnormal clock genes expression may be involved in the pathogenesis of the complications associated with OSA, clock genes expression in OSA patients' PBMCs should be investigated. The expression of specific clock genes, even in association with other molecules, may allow a highly sensitive and specific OSA diagnosis, acting as an index for OSA severity. Such approach would contribute for a personalized medicine in which treatment response could also be assessed, by the improvement of the abnormal clock gene expression levels.

CHAPTER II:

OBJECTIVES

In the last decades, the study of biological rhythms has boomed. The perception that disruptions in the temporal system have severe consequences has turned the circadian rhythm system into a recent field of extensive research. Indeed, an abnormal function of the circadian clock system has been associated with several diseases, including OSA. There are several possible contributors to OSA clock dysfunction, however abnormal NPY levels may be a possible and promising connecting link between this sleep disorder and circadian rhythms disruptions. A better understanding of the biological clock architecture, going from the central to peripheral clocks, and how such clocks may become damaged in OSA may provide important clues for a better diagnosis and correspondent treatment. In this context, this project has three specific aims:

1) **To evaluate the impact of OSA on a circadian rhythm marker and peripheral clock genes expression**

   To do so, we intend to assess the circadian rhythm of OSA patients, before (T0) and after treatment (T1), through a circadian rhythm marker (body temperature) and through the clock genes expression (*Cry1-2; Per1-3; BMAL1; CLOCK; CK1ε; DEC1-2*) in PBMCs.

2) **To find a putative OSA biomarker**

   By establishing correlations between clock genes expression, at different times of the day, and OSA severity index.

3) **To investigate the role of NPY in central clock genes expression**

   For that, we aim to evaluate the effect of NPY, at different CTs, on clock genes mRNA levels, using a hypothalamic cell line.

At the end of this project we expect to unravel the NPY impact on clock genes expression modulation and to contribute to an earlier and more specific OSA diagnosis in order to develop a more effective and personalized treatment.

CHAPTER III:

MATERIALS AND METHODS

III.1. CIRCADIAN RHYTHM PROFILE OF OSA PATIENTS

III.1.1. STUDY DESIGN

The present study was carried out with the collaboration of *Centro de Medicina do Sono*, of *Centro Hospitalar Universitário de Coimbra* (CHUC), in Coimbra, Portugal, with the approval of the ethical committee of the Faculty of Medicine of Coimbra, Portugal. Before starting, all the conditions were set up with the responsible physicians and nurses. Accordingly, the medical team selected from their cases list twenty male patients to the study, with ages between 37 and 75, due to strong suspects of suffering OSA. Consequently, these patients undergone the polysomnography test in the medical clinic. During their scheduled hospital stay (T0), they were informed about the study and signed an informed consent form if interested in participate. If so, patients were also asked to fill a questionnaire about their daily routine, addressing their sleep habits, physical and psychiatric state, medication need, physical exercise, meals, night shifts and travels across time zones in the past 6 months (form in appendice 1). In addition, at previously optimized time points (8 a.m., 11 a.m., 4.30 p.m. and 10.30 p.m.), the body temperature of each patient was measured and blood was collected (figure III.1). After the polysomnography test, a report was generated, by the medical team, addressing patients' demographics and medical history and the sleep, EKG, respiration, oxygenation and movement summaries along the night.

After confirmation of each patient condition, by the polysomnography test results, patients were suggested to undergo the CPAP therapy. After 4 months of therapy, a new medical appointment was scheduled for each patient (T1), in order to assess the therapy outcome. During that day, the patients went through the same protocol for their circadian rhythms profile - at the same time points, their body temperatures were measured and blood was collected. The therapy adherence was taken into consideration by accessing to the CPAP chip data. This chip records the percentage of days in which CPAP was used more than 4h and also apnea and hypopnea episodes per hour.

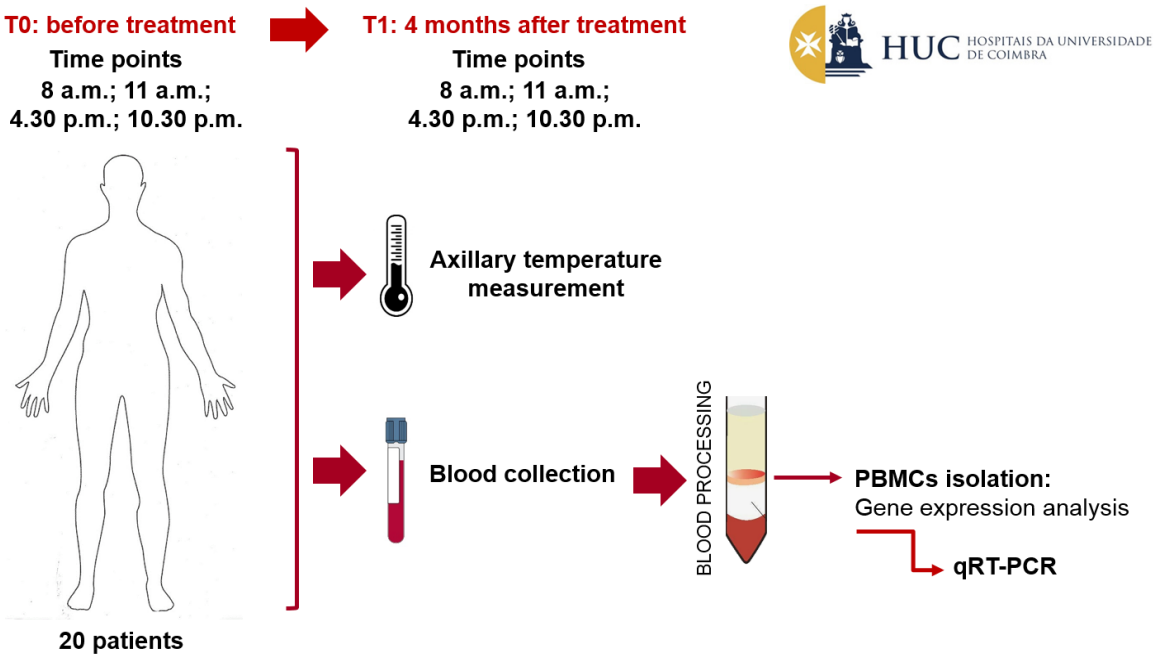


Figure III. 1: Protocol for the circadian rhythm profile of healthy and OSA patients. At previously optimized time points (8a.m.; 11a.m.; 4.30 p.m. and 10.30 p.m.), body temperature was measured and blood was collected from each individual. As soon as possible, blood was processed in order to isolate plasma, to analyse hormone and peptide levels, and PBMCs to analyse gene expression.

III.1.2. BODY TEMPERATURE MEASUREMENT

The axillary temperature was measured twice in each patient, at the previously stated time points, both at T0 and T1, through an electronic thermometer, left over approximately 5 minutes in the armpit. The average between the two measures, was used as the body temperature at each time point. The body temperature of patients 1-6 was not measured at T0.

III.1.3. SAMPLE COLLECTION

At the four stated time points, peripheral blood (16-20 mL) was harvested from the forearm of each patient, by syringe, and immediately transferred into blood collection tubes coated by K_2EDTA (BD Vacutainer CPT glass tubes), stored at 4°C until further processing.

III.1.3.1. Plasma and PBMCs isolation from peripheral blood

To avoid gene expression alterations, peripheral blood was processed as soon as possible after its collection, never beyond 4h after blood collection. The total volume harvested at each time point

was diluted in 25 mL of PBS 1x and was slowly added on the top of 10 mL of histopaque. A centrifugation of 20 mins at 800g, at 20^{o}C and with the brake off was then performed in order to separate blood cells. After it, 4.5 mL of plasma were stored at -80^{o}C and the interface band (mononuclear cells) was aspirated, along with no more than 5 mL of fluid above the pellet, into a new tube. These cells were then purified with PBS 1x, followed by a centrifugation of 10 mins, at 600xg, at 20^{o}C, with the brake on. The supernatant was aspirated and the pellet was resuspended with 3 mL of PBS. Three aliquots of PBMCs were prepared, with 1mL each, and centrifuged at 15 mins at 300xg, at 20^{o}C, with the brake on. The supernatant was aspirated and the aliquots were stored at -80^{o}C.

III.1.3.1.1. PBMCs RNA extraction

Total RNA, including miRNAs, was extracted from PBMCs using the miRCURY RNA Isolation kit for animal cells (Exiqon), according to the manufacturer's instructions. DNAse digestion was performed during the process to exclude any contamination with genomic DNA. Total RNA was quantified by optical density (OD) measurements using a ND-1000 Nanodrop Spectrophotometer (Thermo Scientific) and the purity was assessed with the ratio of OD at 260 and 280 nm. Total RNA samples were kept at -80^{o}C.

III.1.3.1.1.1. DNAse treatment in solution

In order to avoid genomic DNA contamination and co-amplification, samples were treated with DNAse (Qiagen, Hilden, Germany) in solution. Briefly, the reaction final volume was adjusted to 10 µL, containing 1 µL of DNase buffer, 0.5 µL of DNase and 1000 ng of mRNA. After a 30 min incubation at 37^{o}C, 1 µL of 20 mM EDTA, at pH=8, was added to the solution to stop the reaction. The final step was a 65^{o}C incubation for 10 min.

III.1.3.1.1.2. cDNA synthesis and quantitative real time PCR (qRT-PCR)

The mRNA levels of the clock genes *Per1-3*; *Cry1-2*; *BMAL1*; *CLOCK; DEC1-2* and *CK1*ε were assessed by qRT-PCR. Briefly, cDNA was obtained following DNAse treatment in solution, using the iScript cDNA Synthesis Kit (Bio-Rad), according to the manufacturer's instructions. Prior to use, samples were diluted to 1:12.5 (for *Cry2*, *Per2*, *Per3* and *CK1*ε genes expression analysis) or 1:25 (for *Cry1*, *Per1*, *BMAL1*, *CLOCK* and *DEC1-2* genes expression analysis), in RNase-free water. For

mRNA quantification, the iScript SYBR Green (BioRad) was used in combination with each gene respective primers (table III.1). The genes *β-actin*, *β-2-microglobulin* (*β-2M*), *18S ribosomal RNA* (*18S*), *Hypoxanthine-guanine phosphoribosyltransferase* (*HPRT*) and *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) were tested as housekeeping genes, using the softwares GeNorm and NormFinder. The *β-2M* gene showed to be the most stable and was selected as housekeeping gene.

Table III. 1: Clock genes evaluated in PBMCs and experimental PCR conditions.

Gene	Transcript accession number	Primers design	Primers sequence (5'-3')	Annealing temperature	Primers concentration	cDNA amount
Per1	NM_002616	Sigma	Fw:ACACTTCAGAACCAGGATAC Rev:AGTGGAACCATAGAAGACTC	60 ºC	0.5 µM	8 ng
Per2	NM_022817	Sigma	Fw:GCCAATGAAGAGTATTACCAG Rev:ATTCTTCACAATGTGCTCAG	55.5 ºC	0.5 µM	16 ng
Per3	NM_001289861 NM_001289862 NM_001289863 NM_001289864 NM_016831	Sigma	Fw:AAGTTTTGAAGTATGCAGGG Rev:TCCAGTATGATGTAGTCTCC	60 ºC	0.5 µM	16 ng
Cry1	NM_004075.4	Sigma	Fw:CCCAATGGAGACTATATCAGG Rev:ACCTTTTGGATACCTTCTGG	60 ºC	0.5 µM	8 ng
Cry2	NM_001127457 NM_021117.3	Sigma	Fw:CTTTCTTCCAGCAGTTCTTC Rev:CTCATAGATGTATCGAGAGGG	60 ºC	0.5 µM	16 ng
BMAL1	NM_001030272 NM_001030273 NM_001178 NM_001297719 NM_001297722 NM_001297724	Sigma	Fw:AGATGAATTGGCTTCTTTGG Rev:TGGCACCTCTTAATGTTTTC	60 ºC	1 µM	8 ng
CLOCK	NM_001267843 NM_004898	Sigma	Fw:ACTACAAGACGAAAACGTAG Rev:CATCTCTGTCAACAATCGAG	60 ºC	0.5 µM	8 ng
DEC1	NM_003670	Sigma	Fw:CAAGTGTACAAGTCAAGACG Rev:GTCTCTTTTTCTCGATGAGC	58 ºC	0.5 µM	8 ng
DEC2	NM_030762	Sigma	Fw:CAAGGATACCTACAAATTACCG Rev:AACACTCAGCTTTCTCCAG	58 ºC	0.5 µM	8 ng
CK1ε	NM_001894 NM_152221	Sigma	Fw:CTCCGAATTCTCAACATACC Rev:AATTTCAGCATGTCCCAGTC	60 ºC	0.5 µM	16 ng
β-2M	NM_004048	Designed by Magda Santana	Fw: GCCGTCTTCCCCTCCATCGTG Rev:GGAGCCACACGCAGCTCATTGTAGA	55.5 ºC	0.25 µM	8 ng; 16 ng

Fw, forward; Rev, reverse

III.2. CIRCADIAN RHYTHM *IN VITRO* EXPERIMENTS

III.2.1. CELLULAR MODEL

The adherent cell line mHypoE-N42 (CELLutions Biosystems Inc./Cederlane, Ontario, Canada) was obtained from immortalized hypothalamic primary cultures, derived from mouse embryonic day 15, 17, 18 (E15, 17, 18), through retroviral transfer of Simian Vacuolating Virus 40 TAntigen (SV40 TAg). According to the cell line screen profile[1], mHypoE-N42 neurons express the predominant

[1] https://www.cedarlanelabs.com/Products/Listing/Embryonic_Mouse_Hypothalamic?lob=Cellutions

neuropeptides responsible for rhythm generation, markers of the central clock: Arginine Vasopressin (AVP) and Vasoactive Intestinal Polypeptide (VIP). In this context, these neurons could be used as a reliable cellular model for circadian rhythm experiments. In addition, mHypoE-N42 neurons do not express NPY and express biologically active NPY receptors, among an array of other neuropeptides, receptors and enzymatic markers. mHypoE-N42 neurons were maintained in high glucose Dulbecco's Modified Eagle Medium (DMEM; 4.5 g/L/D-glucose, Sigma-Aldrich), supplemented with 10% of heat inactivated fetal bovine serum (FBS), 100 U/mL of penicillin and 100 µg/mL of streptomycin (all from Invitrogen, Carlsbad, CA, USA), at 37°C, in a humidified atmosphere with 95% of air and 5% of CO_2, in 75 cm^2 tissue culture flasks. Cells doubling time is approximately 24 h. When cells reached approximately 90% confluence, they were washed twice with pre-warmed PBS 1x and detached from the flask by trypsinization, for 3-5 min, at 37°C. Trypsin (Life Technologies, from Invitrogen) action was then inhibited by adding growth medium. The cells were then sedimented by centrifugation, at 800 rotations per minute (rpm), for 5 min. The supernatant was discarded and cells were ressuspended in pre-warmed fresh growth medium. Cell density was determined with a hemocytometer, by direct counting and cells were seeded in a new tissue culture flask, for passaging. For RNA extraction purposes, cells were plated in uncoated 35 mm plates. mHypoE-N42 neurons were used at passages 27 to 36.

III.2.2. N42 MOLECULAR CLOCKS SYNCHRONIZATION AND CIRCADIAN RHYTHM CHARACTERIZATION

The molecular oscillators of mHypoE-N42 neurons were synchronized through serum shock. When reaching 100% confluence, at 37°C with 95% of air and 5% of CO_2, cells were washed with pre-warmed PBS 1x and cultured in serum-free medium overnight. After it, cells were subjected to a serum shock by replacing the culture medium with a serum-free medium supplemented with 50% horse serum (Gibco®, Life Technologies), for two hours. After washing with pre-warmed PBS 1x, the medium was then removed and replaced with regular culture medium. This was considered circadian time (CT) 0. In order to track mHypoE-N42 neurons circadian rhythm, cells were washed two times with pre-warmed PBS 1x, one time with PBS 1x at 4 ^{o}C, and frozen at -80^{o}C, at CT0, 4, 8, 12, 20, 24, 28, 32, 36, 44, 48, 52, 56, 60, 68, and 72, until further RNA extraction, cDNA synthesis and qRT-PCR for expression analysis.

III.2.2.1. Hypothalamic cells (mHypoE-N42) incubation with exogenous NPY

To evaluate the effect of abnormal NPY levels on the clock genes expression modulation, in the hypothalamus, at different CTs, synchronized mHypoE-N42 neurons were treated with a previously optimized concentration of NPY (Phoenix Pharmaceuticals, Inc) - 100 nm - at different circadian time points – CT0, 4, 8, 12, 20, 24, 28, 32, 36, 44 and 48. After 24h of each treatment, namely at CT24, 28, 32, 36, 44, 48, 52, 56, 60, 68 and 72, respectively, cells were washed two times with pre-warmed PBS 1x, one time with PBS 1x at 4ºC, and frozen at -80ºC until further RNA extraction, cDNA synthesis and qRT-PCR analysis. Non-treated cells, at the same circadian times, were used as controls (figure III.2).

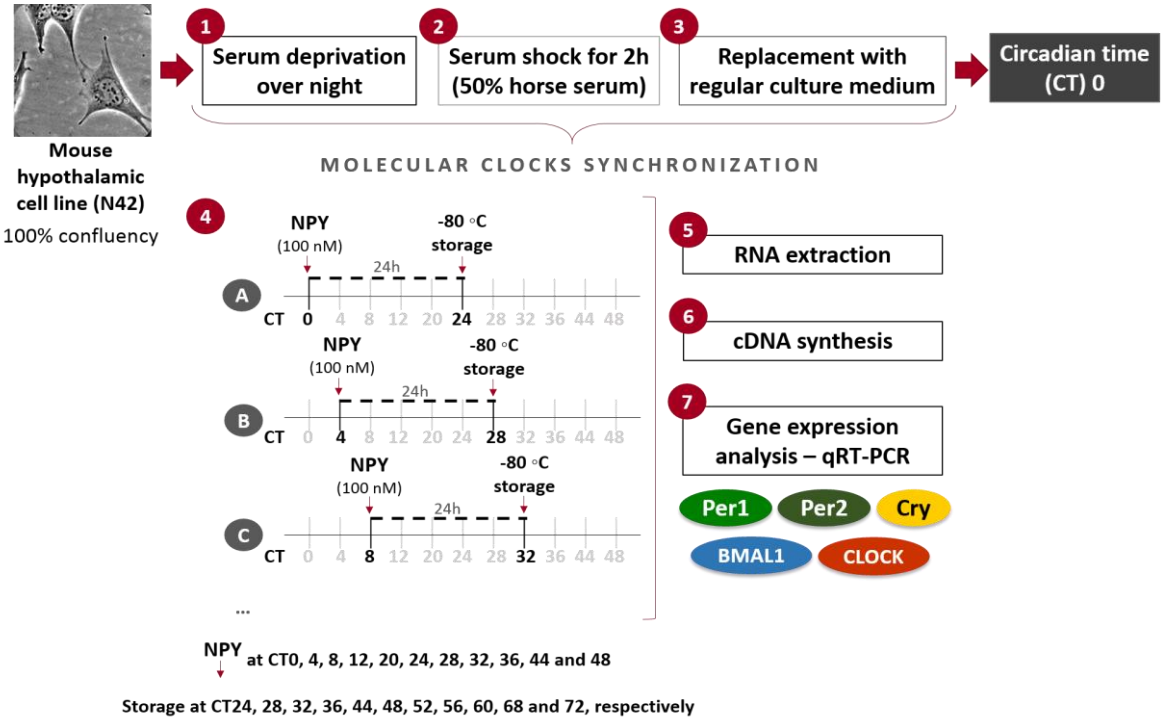


Figure III. 2: Scheme representing the workflow followed to analyse whether NPY affects mHypoE-N42 neurons clock genes expression at specific CTs. When mHypoE-N42 neurons reached 100% confluency, their molecular clocks were synchronized by a three-step protocol requiring serum deprivation overnight, a serum shock composed of 50% of horse serum for 2 and replacement with regular culture medium. At this point, cells CT was defined as CT0. Then cells were treated with NPY (100nM) at each time point from CT0 to CT48 and after 24h of each incubation, RNA was extracted, cDNA was synthesized and clock genes (*Per1*, *Per2*, *Cry*, *BMAL1* and *CLOCK*) expression was analysed.

III.2.2.2. Transfection of the mHypoE-N42 with NPY plasmids

III.2.2.2.1. NPY vector construction and amplification

The NPY vector was previously constructed and optimized in the *Vectors and Gene Therapy Group* of Center for Neuroscience and Cell Biology of University of Coimbra. Briefly, rat NPY cDNA from p46F06444D-NPY (RZPD) was cloned into an AAV plasmid vector back-bone, under the control of

the human *synapsin 1* gene promoter, a neuronal specific promoter (figure III.3) (Sousa-Ferreira *et al.*, 2011). NPY plasmids were then incorporated, through temperature shock, in Escherichia Coli bacteria and amplified. The amplified NPY plasmids were then purified using NucleoBond Xtra Midi/Maxi kit (MACHEREY-NAGEL), according to the manufacturer's instructions. Plasmids integrity was confirmed through restriction enzymes digestion and agarose gel electrophoresis.

Figure III. 3: Schematic representation of the NPY AAV vector. The NPY expression vector contains one cassette to express NPY under a neuronal specific promoter (human synapsin 1 gene promoter). ITR, inverted terminal repeats; hSyn, human Synapsin 1 promoter; WPRE, woodchuck hepatitis virus posttranslational control element; PolyA, polyadenosine sequence.

III.2.2.2.2. Transfection assay

In order to evaluate the effect of a constitutive expression of NPY on hypothalamic clock genes profile modulation, mHypoN42 neurons were grown to 80% confluency, at 37°C with 95% air and 5% CO_2. Cells were then transfected with 0.5 µg of NPY plasmid DNA using Torpedo ^{DNA} Transfection Reagent (Ibidi) for 48 h, according to the manufacturer's specifications. After 48h, cells were subjected to circadian rhythm synchronization, as described above. At the circadian times 0, 4, 8, 12, 20, 24, 28, 32, 36, 44 and 48, mHypoN42 neurons were washed two times with pre-warmed PBS 1x, one time with PBS 1x at 4^{o}C, and frozen at -80^{o}C until RNA extraction, cDNA synthesis and qRT-PCR analysis. GFP-transfected cells were used as transfection controls in order to validate whether differences were indeed due to the effect of NPY or due to the transfection protocol and non-transfected cells were used as non-transfection controls (figure III.4).

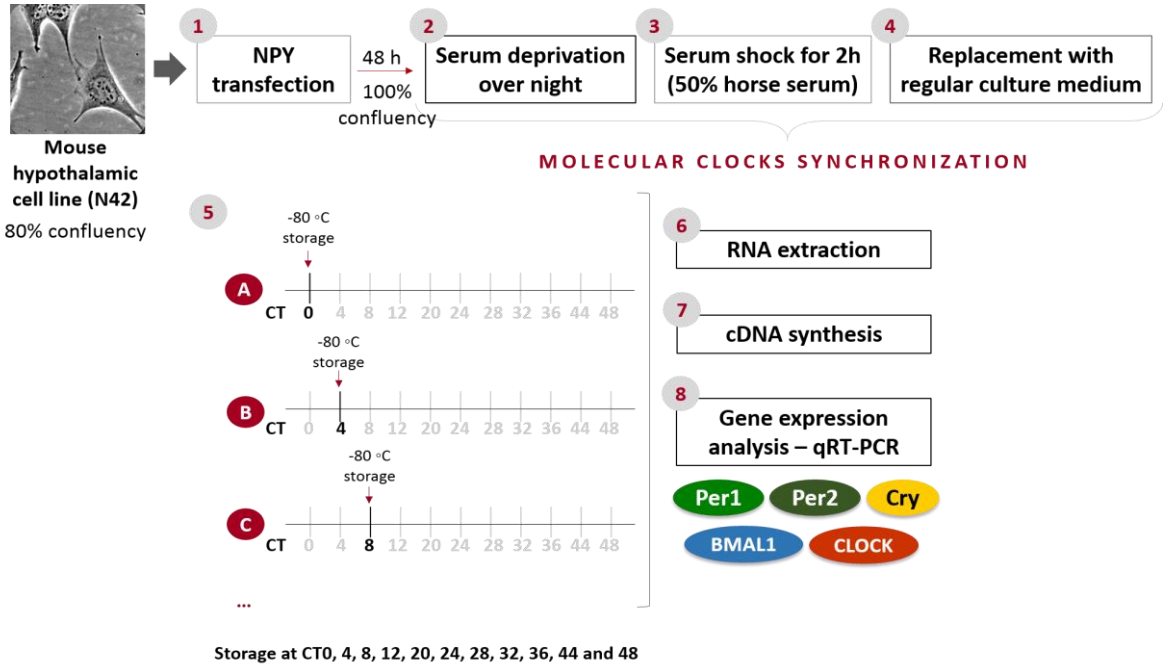


Figure III. 4: Scheme of the workflow followed to analyse whether a constitutive expression of NPY alter the clock genes expression profile of the mHypoE-N42 neurons. When mHypoE-N42 neurons reached 80% confluency, cells were transfected with NPY plasmids. After 48h, when mHypoE-N42 neurons reached 100% confluency, cells molecular clocks were synchronized by a three-step protocol requiring serum deprivation overnight, a serum shock composed of 50% of horse serum for 2 and replacement with regular culture medium. At this point, cells CT was defined as CT0. Then cells were harvested at each time point from CT0 to CT48 until further RNA extraction, cDNA synthesis and clock genes (*Per1*, *Per2*, *Cry*, *BMAL1* and *CLOCK*) expression analysis.

III.2.3. VALIDATION OF THE CIRCADIAN RHYTHM *IN VITRO* EXPERIMENTS

To confirm whether the circadian rhythm of mHypoE-N42 neurons is, indeed, being affected by external factors, a positive control was performed. According to the literature, SIRT1 is required for the transcription of several core clock genes, including *Bmal1* and *Per2* (Asher *et al.*, 2008; Bellet *et al.*, 2013). In 2013, Bellet and its co-workers showed that, in fact, pharmacological manipulation of SIRT1 activity affects the molecular clock activity in mouse embryonic fibroblasts. Consequently, we used a SIRT-1 activator, Resveratrol, to interfere with SIRT-1 levels, in mHypoE-N42 neurons, and confirm whether *BMAL1* and *Per2* genes transcription was affected. For that, synchronized mHypoE-N42 neurons in culture, at CT0, were treated with an optimized concentration of Resveratrol (Sigma) - 10 µM. After 24h of incubation, cells were harvested at CT24, 28, 32, 36, 44 and 48, and washed two times with pre-warmed PBS 1x, one time with PBS 1x at 4°C, and frozen at -80°C until further RNA extraction, cDNA synthesis and qRT-PCR analysis (figure III.5). Vehicle-treated (DMSO) cells were used as controls.

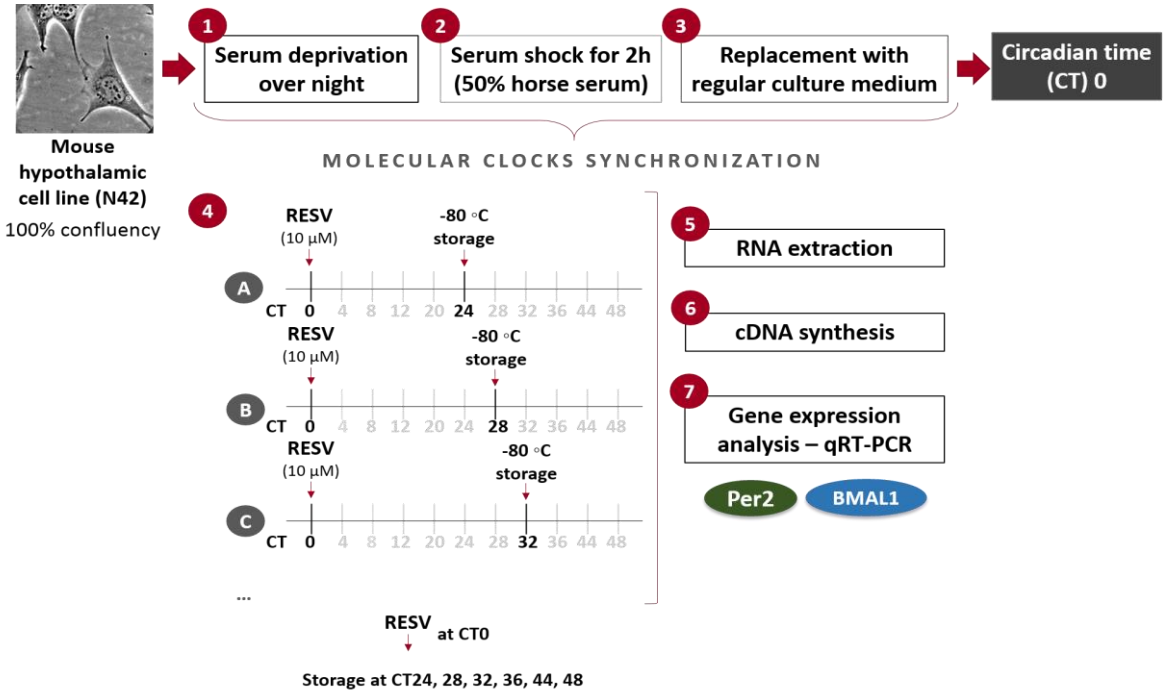


Figure III. 5: Scheme representing the workflow followed to confirm whether the circadian rhythm of mHypoE-N42 neurons could be affected by external factors. When mHypoE-N42 neurons reached 100% confluency, their molecular clocks were synchronized by a three-step protocol requiring serum deprivation overnight, a serum shock composed of 50% of horse serum for 2 and replacement with regular culture medium. At this point, the CT was defined as CT0 and cells were treated with Resveratrol (10 µM), an activator of SIRT-1. SIRT-1 is required for the transcription of several clock genes, including BMAL1 and Per2. After 24h, cells were harvested from CT0 to CT24, until further RNA extraction, cDNA synthesis and *Per2* and *BMAL1* gene expression analysis.

III.2.4. RNA EXTRACTION

Messenger RNA was extracted from mHypoE-N42 using the NucleoSpin RNA Isolation kit from cells and tissue (MACHEREY-NAGEL), according to the manufacturer's instructions. DNAse digestion was performed during the process to avoid contamination with genomic DNA. The extracted RNA was quantified by optical density (OD) measurements using a ND-1000 Nanodrop Spectrophotometer (Thermo Scientific) and the purity was assessed with the ratio of OD at 260 and 280 nm. Finally, RNA samples were stored at -80°C.

III.2.5. cDNA SYNTHESIS AND SEMI-QUANTITATIVE REAL TIME PCR (QRT-PCR)

The mRNA levels of the core clock genes (Per1-2; Cry1-2; BMAL1 and CLOCK) on mHypoE-N42 neurons were assessed by semi-quantitative real time PCR (qRT-PCR). Briefly, cDNA was obtained from the conversion of 1000 ng of mRNA, using the iScript cDNA Synthesis Kit (Bio-Rad), according to the manufacturer's instructions. Prior to use, samples were diluted 1:50, in RNase-free water.

For mRNA quantification, the iScript SYBR Green (BioRad) was used in combination with each gene respective primers (table 1). The genes *β-actin*, *β-2M*, *β-glucoronidase*, *HPRT* and *GAPDH* were tested as housekeeping genes, using the softwares GeNorm and NormFinder. HPRT and GAPDH showed to be the most stable and both were selected as housekeeping genes.

Table III. 2: Clock genes assessed on mHypoE-N42 neurons and PCR experimental conditions.

Gene	Transcript accession number	Primers design	Primers sequence (5'-3')	Annealing temperature	Primers concentration	cDNA amount
Per1	NM_011065 NM_001159367	Sigma	Fw:GTTCTCATAGTTCCTCTTCTG Rev:GTGAGTTTGTACTCTTGCTG	57.5 ºC	0.3 µM	4 ng
Per2	NM_011066	Sigma	Fw:CTTTCACTGTAAGAAGGACG Rev:CTGAGTGAAAGAATCTAAGCC	57.5 ºC	0.5 µM	4 ng
Cry1	NM_007771	Sigma	Fw:AGAAGGGATGAAGGTCTTTG Rev:CTCTTAGGACAGGTAAATAACG	58 ºC	0.3 µM	4 ng
BMAL1	NM_007489 NM_001243048	Sigma	Fw:AAATCCACAGGATAAGAGGG Rev:ATAGTCCAGTGGAAGGAATG	60 ºC	0.5 µM	4 ng
CLOCK	NM_007715 NM_001289826 NM_001305222	Sigma	Fw:AAGTGACTCATTAACCCCT Rev:CTATGTGTGCGTTGTATAGTTC	58 ºC	0.3 µM	4 ng

Fw, forward; Rev, reverse

III.3. PCR CONDITIONS AND ANALYSIS

For each reaction, to 4 µL of template cDNA were added 6 µL of master mix (5 µL of iScript SYBR Green and 1 µL of the respective forward and reverse primers). All reactions were performed in duplicate (two cDNA reactions per RNA sample). PCR was performed in a thermocycler (BIORAD) and the cycling conditions are presented below, in table 3. In this protocol the melting curve protocol started immediately after amplification. PCR runs with RNA samples (no reverse transcriptase samples - NRT) and with no sample (no template control -NTC) were used as negative controls to monitor sample or reagent contaminations.

Table III. 3: PCR cycling conditions.

Cycle	Repeats	Step	Temperature	Duration	Temperature change
1	1x	Initial denaturation	95ºC	3 mins	
2	45x	Denaturation	95ºC	15 secs	
		Annealing	Primers specific	30 secs	
		Extension	72ºC	30 secs	
3	80x	Melting curve	Primers specific	10 secs	0.5ºC up to 95ºC

The amplification efficiency for each target and the values for threshold cycle determination (Ct) was determined automatically by the iQ5 software (BIORAD). Relative mRNA quantification was performed using the ΔΔCt method for targets with the same amplification efficiency, as stated in

the formula bellow. Sample A refers to any analysed sample whereas sample B refers to the average of all the controls values.

$$\frac{\text{Sample A}}{\text{Sample B}} = \frac{2^{CtA_{Target} - CtA_{Reference}}}{2^{CtB_{Target} - CtB_{Reference}}} = 2^{(CtA_{Target} - CtA_{Reference}) - (CtB_{Target} - CtB_{Reference})} = 2^{\Delta\Delta Ct}$$

III.4. AGAROSE GEL ELECTROPHORESIS

The size of the expected product of each gene qRT-PCR was obtained through the NCBI tool *Primer-BLAST* (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) that uses each gene primers' sequences to search for predicted amplified targets, according to selected databases (NCBI genome (reference assembly only) and Refseq mRNA databases). According to it, and in order to confirm that the products being amplified by qRT-PCR were the expected, several agarose gel electrophoresis were performed. Agarose gels [1.5% (w/v)] were prepared by boiling a solution of agarose (Sigma) in 1xTris-acetate-EDTA buffer (TAE). After cooling enough, greensafe (3 µL/100mL, NZYTech) was added and the solution was homogenized. Then it was poured inside sealed gel trays with gel combs, and left at RT to solidify. DNA samples (7 µL) were mixed with an appropriate amount (1.4 µL) of gel loading buffer (Orange loading dye, Thermo Fisher Scientific) and a DNA ladder with known concentrations was loaded aside (Thermo Fisher Scientific). Gels were run at 90 volts, for 30-45 mins and visualized with Image Lab software (Gel Doc™ EZ imager, BIORAD).

III.5. STATISTICAL ANALYSIS

Results are expressed as mean ± standard error of the mean (SEM), or mean ± standard error (SD) when less than three experiments were performed. Likely outliers were identified by using the ROUT method, through a Q-test of 10%. Differences between two groups such as treated and non-treated cells or OSA patients before and after treatment, at each time point, were analysed using Student's unpaired t-test with two-tailed p value. On the other hand, differences along time, either in the expression profiles of treated or non-treated cells or in the expression profiles of OSA patients before or after treatment, were analysed through one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test, to determine differences between the different defined time points. For all the statistical tests, a value of $p<0.05$ was considered significant.

Correlation analysis were performed through Pearson correlation coefficient with two-tailed p value. The software GraphPad Prism 6.0 was used for all statistical analysis.

CHAPTER IV:

RESULTS DISCUSSION

IV.1. CIRCADIAN RHYTHM PROFILE OF OSA PATIENTS

The first part of this work was to analyse the circadian rhythm of OSA patients. Firstly, the OSA cohort was characterized regarding their medical history, sleep characteristics and other relevant information for the study. Then, at T0 (before treatment), each patient circadian rhythm was assessed through a circadian rhythm biomarker (body temperature) and clock genes expression. Finally, at T1 (4 months after treatment), the data from the CPAP chip was assessed and the same parameters (body temperature and clock genes expression) as in T0, were evaluated once again, through the same procedure, in order to detect whether treatment resulted in changes in such parameters. For the purpose of this study and, in a first analysis, we considered the average values of the OSA cohort as a whole. Still, in appendice 2, the same parameters are presented for each patient individually.

IV.1.1. OSA COHORT CHARACTERIZATION

The OSA cohort used in this study was composed of 20 patients with 37 to 75 years old (figure IV.1-A), with an average of 52.3±2.3 years (table IV.1-A). Only 5% of the patients had normal weight, 95% had excessive weight (BMI average = 30.7±1, table IV.1-A), comprising the four excessive weight BMI categories: overweight (25<BMI<29,9) and obese class I (30<BMI<34,9), II (35<BMI<39,9) and III (BMI>40) (figure IV.1-B). Still, 57% of these patients did not have any diagnosed disorder. On the other hand, 11% were diagnosed with metabolic disorders (diabetes), 11% with cardiovascular disorders (hypertension), 11% with both disorders, one patient suffered from a psychiatric disorder (depression) and another was diagnosed with both cardiovascular and renal disorders (renal insufficiency) (figure IV.1-C). Regarding sleep, 16% of the patients feel difficulty in falling asleep so, usually, these patients take benzodiazepines to induce sleep. Still, 84% fall asleep without drugs (figure IV.1-D). During the night, 79% of the patients are aware that they awake several times and complain about not sleeping well or enough (figure IV.1-E). In this context, 58% of them feel sleepy during the day (figure IV.1-F). Regarding circadian rhythms, 16% of these patients regularly practice sports (figure IV.1-G), 84% consider to have a healthy eating and 63% of the patients eat at regular hours (figure IV.1-H and -I). However, 63% of the patients already worked in nightshifts (IV.1-J). Regarding travels across time zones in the past 6 months, 21% suffered from jet lag in a recent period (IV.1-K).

About the PSG test (table IV.1-A), its results revealed that these patients had on average 31.8±4 arousal events per hour and 8.2±1.2 awakening events per hour. Such events were associated with

severe apnea and hypopnea episodes, an average of 47.5±5.6 per hour. Thus, the majority of the patients suffered from severe OSA (16 severe cases, 1 moderate case and 3 mild cases) (figure IV.1-L). Their blood oxygenation capacity was also affected, these patients showed, on average, 91.4%±0.6 of arterial oxygen saturation and 26±6.8 desaturation events per hour. Finally, the electrocardiography (EKG) statistics showed that the patients' heart rate when awake was, on average, 80.7±4.6 beats per minute (bpm) and their heart rate when steady sleep was around 69.8±2.7 bpm.

After four months of treatment (T1) (table IV.1-B), 9 of the 20 patients were already assessed. To significantly improve OSA, it is recommended to use the CPAP mask more than 4 hours per night. In this context, the adhesion percentage among the 9 patients was, in average, 84.8%±8.1. The CPAP chip results showed that patients' AHI significantly decreased, changing from an average index of 47.5±5.6 to 1.2±0.32, an AHI that turned these patients into disease-free individuals (figure IV.2).

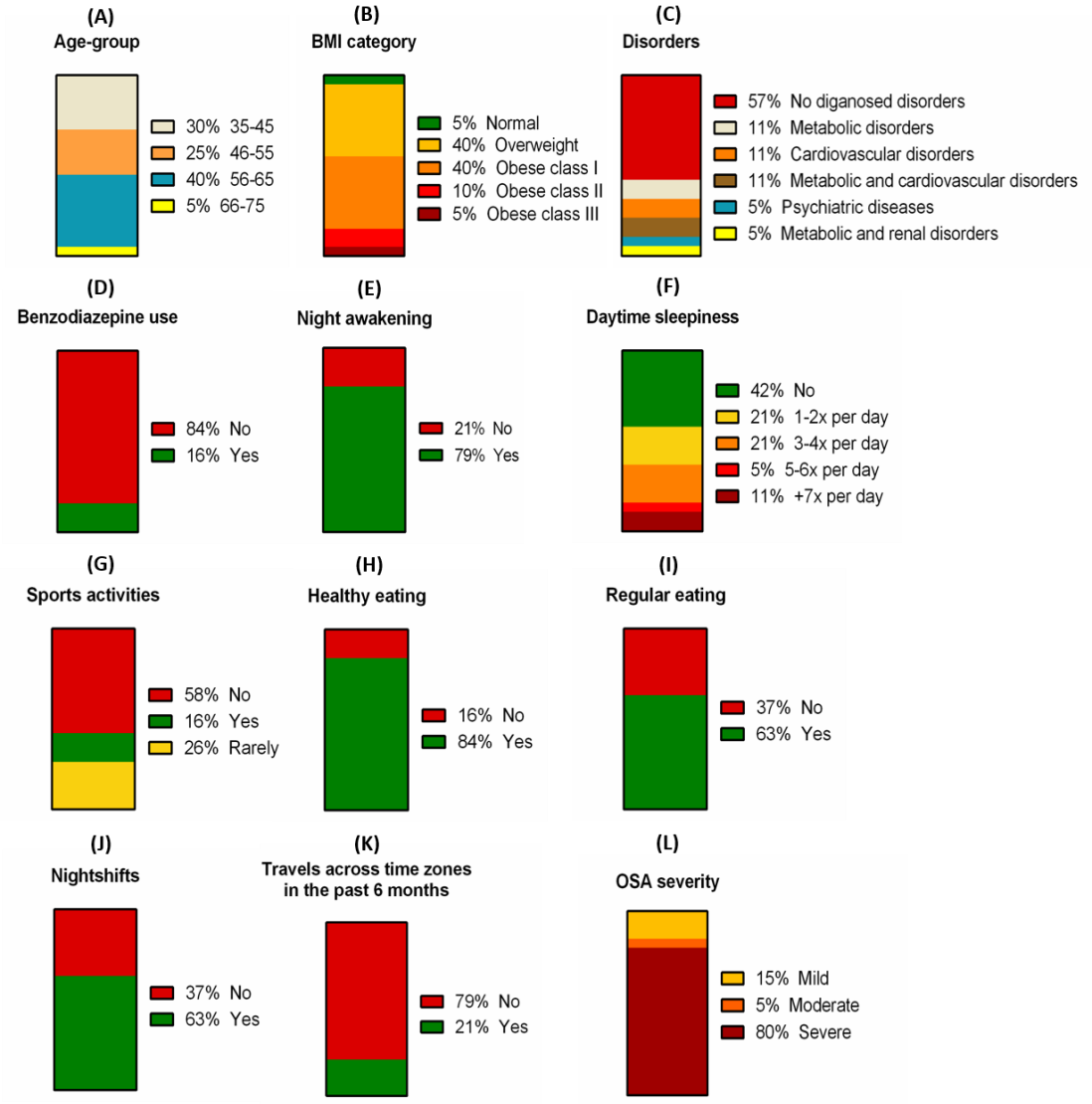


Figure IV. 1: OSA cohort characterization. The figure shows the OSA cohort characterization, including the results obtained from the circadian rhythm questionnaire filled by the selected OSA patients. (A) Age-group. 40% of the selected OSA patients have ages between 56 and 65, 30% have 35-45 years, 25% have ages between 46 and 55 and 5% have 66 - 75 years. (B) BMI category. Only 3% of the patients have normal weight, the majority (41%) are overweight, 38% have obesity class I, 14% are obese class II and 3% are obese class III. (C) Disorders. 58% of the selected patients do not suffer from any disorder, 11% suffer from metabolic disorders, 11% have cardiovascular diseases, 5% have both metabolic and cardiovascular diseases and 5% have psychiatric disorders. (D) Benzodiazepine use. Only 16% consume benzodiazepines to sleep, 84% fall asleep without drugs. (E) Night awakening. 79% of the patients complained about awaking during sleep nights whereas 21% do not awake or are not aware of it. (F) Daytime sleepiness. 42% of the patients do not fell sleepy along the day however 54% complained about it (21% feel sleepy 1-2 times Per day; 21% have daytime sleepiness 3-4 times Per day; 5% feel sleepy 5-6 times Per day and 11% have daytime sleepiness more than 7 times Per day. (G) Sports activities. The majority of the patients do not practice sports (58%), whereas 26% rarely practice and 16% usually do sports at least once per week. (H) Healthy eating. 84% of the patients have healthy eating habits whereas 16% do not. (I) Regular eating. The majority of the patients (63%) have meals at regular hours, whereas 37% do not. (J) Nightshifts. 63% of the patients work or already worked by nightshifts, whereas 37% never did it. (K) Travels across time zones in the past 6 months. 79% of the patients did not suffer from jetlag in the past 6 months, opposing to 21% who had travelled across time zones in a recent Period. (L) OSA severity. The majority of the patients have severe OSA (80%), 5% are moderate and 15% are mild cases.

Table IV. 1: Main OSA parameters assessed in all OSA patients before (T0) and after treatment (T1).

(A)

		T0
Medical history	Age (years)	52.3±2.3
	BMI	30.7±1
Sleep disruption events	Arousal events/h	31.8±4
	Awakening events/h	8.2±1.2
Respiration summary	**AHI**	**47.5±5.6**
Oxygenation summary	SpO_2 mean (%)	91.4±0.6
	Desaturation events/h	26±6.8
EKG statistics	Heart rate, wake (bpm)	80.7±4.6
	Heart rate, steady sleep (bpm)	69.8±2.7

(B)

		T1
Adherence	Days with more than 4h of use (%)	84.8±8.1
Respiration summary	**AHI**	**1.2±0.32**

(A) Parameters assessed before treatment. Information about the age and bmi of each patient were obtained by the medical team. Data about sleep disruption events (arousal and awakening events per hour), respiration (apnea and hypopnea index), oxygenation (percentage of arterial oxygen saturation and desaturation events per hour) and EKG statistics (heart rate when awake and when steady sleep) were measured, in each patient, by the PSG test. Data are presented as mean±SEM of 20 patients. (B) Parameters assessed after treatment. Both the adherence (percentage of days with more than 4h of use) and respiration (apnea and hypopnea events per hour) were recorded by the CPAP chip. Data are presented as mean±SEM of 9 patients. A+H events/h are highlighted in bold.

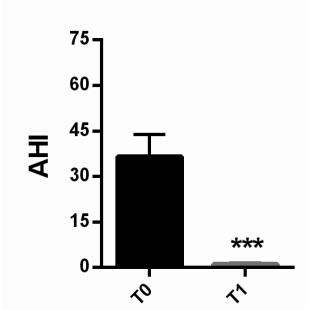


Figure IV. 2: OSA patients' AHI before and after treatment. The graph shows the AHI of OSA patients at T0 and T1. At T1, AHI significantly decreased. Data are presented as mean±SEM with n=20 (T0) and n=9 (T1). ***p<0.001, Student's t-test.

IV.1.3. EVALUATION OF A CIRCADIAN RHYTHM MARKER IN OSA PATIENTS

In order to assess OSA patients' circadian rhythms, we started by analysing patients' body temperature along the different time points, at T0 and T1, as body temperature acts as a circadian rhythm biomarker. Consequently, evidences of disruptions in OSA patients' circadian rhythms could be detected. Firstly, we assessed the axillary body temperature of all the evaluated patients, 14 out of 20 at T0 and 9 at T1, so far. In this context, at T0, the minimum temperature was observed at 8 a.m. (35.6±0.1°C) and then it significantly increased up to 4.30 p.m. (36±0.1 °C),

remaining stable up to 10.30 p.m. At T1, the minimum temperature was reached at 11 a.m. (35.7±0.1 ºC) and the maximum temperature was verified at 10.30 p.m. (36±0.2 ºC), although no significant temperature variations were found along time. When comparing both profiles, a statistical difference was observed at the first time point (8 a.m.), in which T1 temperature was considerably higher (0.44ºC) than the temperature observed at T0 (figure IV.3-A). Lastly, we compared the axillary body temperature profiles, of the patients whose data is completed so far (both measurements at T0 and T1 - 3 patients so far). No significant oscillations in the T0 and T1 body temperature profiles were found and no significant differences between T0 and T1 temperature values were detected (figure IV.3-B).

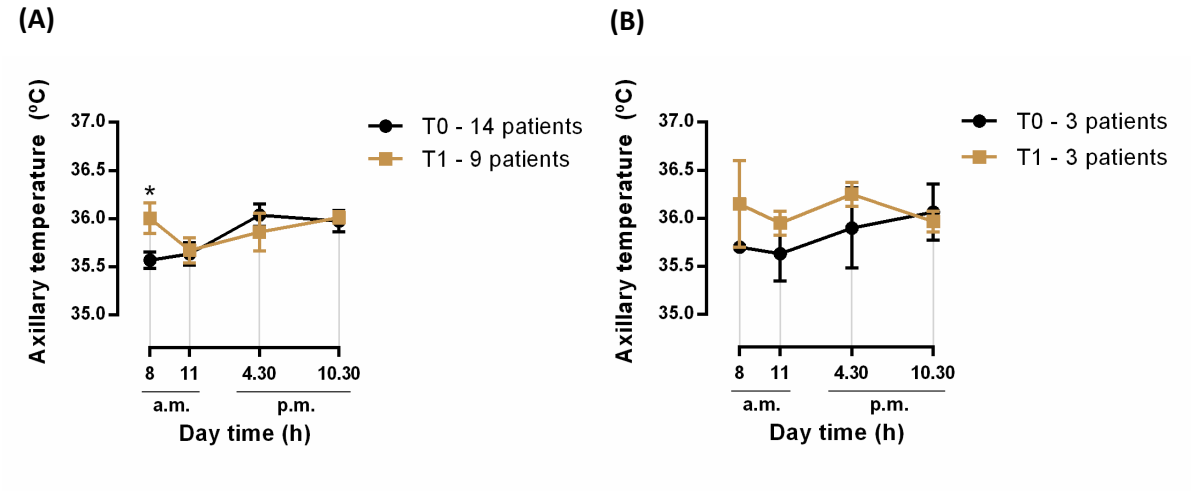


Figure IV. 3: Axillary body temperature measurements before and after treatment. (A) Axillary body temperature values of all the assessed patients, at T0 (14 patients out of 20) and T1 (9 patients so far). A significant variation was observed at 8 a.m., in which patients' temperature, after treatment, increased. ***p<0.05, Student's t-test.** (B) Axillary body temperature profiles, at T0 and T1, of the patients whose data is already completed (both T0 and T1 measurements, 3 patients). No variations were found. Data are presented as mean±SEM.

After body temperature assessment, the expression of several clock genes in OSA patients was also evaluated, at T0 and T1.

IV.1.4. TREATMENT CHANGES CLOCK GENES EXPRESSION PROFILES IN PBMCS OF OSA PATIENTS

Nine clock genes (*Per*1-3; *Cry*1-2; *BMAL1*; *CLOCK*; *CK1ε;* and *DEC1*) were screened in the PBMCs of each patient. *DEC2* showed a low expression in the used conditions, being its mRNA molecules not detectable in the majority of the samples. By opposite, all the other genes expression were detected by qRT-PCR. The results, before and after treatment, are shown in figure IV.4-6.

Regarding *Per*1-3 genes, the three isoforms showed a very similar expression profile, characterized by a marked oscillation, with higher mRNA levels in the morning (8 a.m.), lower mRNA levels at 4.30 p.m. and the stabilization of the mRNA levels up to 10.30 p.m. (figure IV.4, A-C, T0). At T1, 4 months after CPAP treatment, *Per*1 and *Per*3 showed a similar but more pronounced expression pattern (figure IV.4-A and C, T1) however the expression profile of *Per2* changed. *Per*2 mRNA levels did not showed to oscillate along time, being only visible a tendency to decrease in the last time point (10.30 p.m.) (figure IV.4-B, T1). When comparing gene mRNA levels of the same patients at T0 and T1, no differences were observed regarding *Per1* (figure IV.4-A, T0 vs T1). By opposite, at T1, *Per*2 mRNA levels became lower at 10.30 p.m. (decreased 27%±5) (figure IV.4-B, T0 vs T1) and *Per*3 transcripts decreased 32.8%±12.9 at 11 a.m. and 40.4%±16.9 at 10.30 p.m. (figure IV.4-C, T0 vs T1). The expression profiles of the 9 patients out of 20, assessed at T0, did not changed significantly from the expression profiles of all the 20 patients at T0 (both are showed in T0 vs T1 graphs).

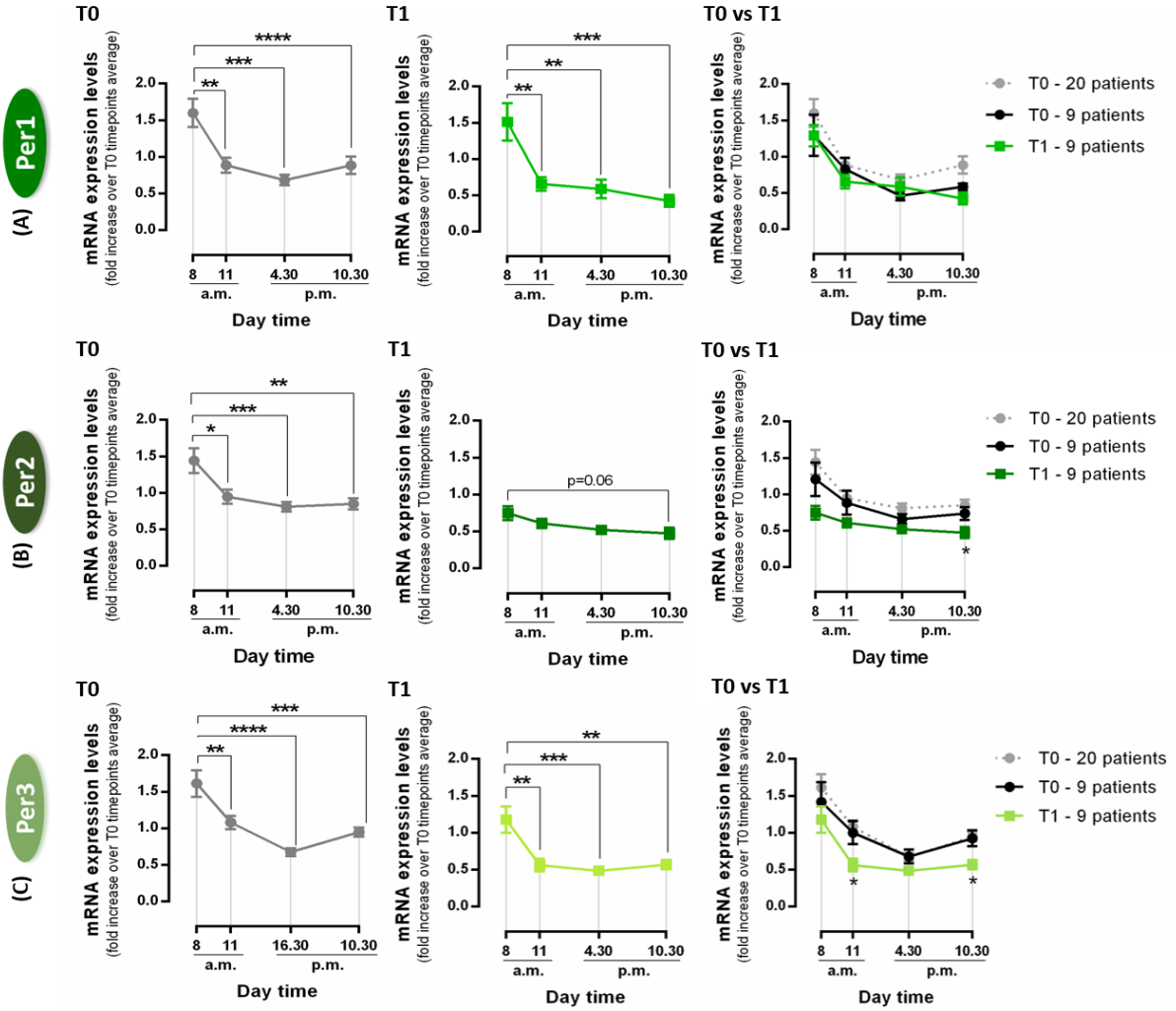


Figure IV. 4: Expression profiles of *Per1*-3 isoforms, in OSA patients, before (T0) and after treatment (T1). The figure shows the expression profiles of the three *Per* isoforms in OSA patients, before treatment (T0 – 20 patients), after treatment (T1 – 9 patients) and a comparison between both (T0 vs T1 – 9 patients, with the average of the 20 patients assessed at T0 dashed). (A) *Per1*. At T0, *Per1* expression profile showed a marked oscillation, with higher mRNA levels in the morning (8 a.m.), a lower mRNA levels at 4.30 p.m, and the stabilization of the mRNA levels up to 10.30 p.m. After treatment, the same pattern and levels were maintained, comparing the same 9 patients, consequently, no differences were observed between the expression profiles before and after treatment. (B) *Per2*. At T0, *Per2* mRNA levels showed a marked oscillation, being higher in the morning (8 a.m.), lower at 4.30 p.m. and stabilizing until 10.30 p.m. At T1, *Per2* mRNA levels loss the oscillation, being detected only a tendency to decrease at 10.30 p.m. When comparing T0 and T1 profiles, differences in mRNA levels were observed at 10.30 p.m., in the expression profiles of the same 9 patients (C) *Per3*. At T0, *Per3* expression profile showed a marked oscillation, with higher mRNA levels in the morning (8 a.m.), a lower mRNA levels at 4.30 p.m. and the stabilization of the mRNA levels up to 10.30 p.m. After treatment, the same pattern was maintained however the levels of *Per3* significantly decreased at 11 a.m. and at 10.30 p.m, comparing the same 9 patients. In all genes, the expression profiles of the 9 patients out of 20, assessed at T0, did not changed significantly from the expression profiles of all the 20 patients**.** *B-2M* was used as housekeeping gene. Data is presented as mean ± SEM from 20 individuals (T0) and 9 individuals (T1; T0 vs T1), *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, compared to T0 time points average. One-way ANOVA was used to evaluate differences over time (at T0 and T1, respectively) and student's t-test to assess differences at specific time points, from the same 9 patients (T0 vs T1).

On the other hand, at T0, the mRNA levels of *Cry1* and *BMAL1* did not change along the evaluated time points. By opposite, the mRNA levels of *Cry2* oscillated (figure IV.5, T0). *Cry2* mRNA levels were higher in the morning (8 a.m.) but decreased up to 10.30 p.m. (figure IV.5-B, T0). At T1, *Cry*1 (figure IV.5-A, T1), *Cry2* (figure IV.5-B, T1) and *BMAL1* mRNA levels were higher at 8 a.m. and decreased at 11 a.m., remaining constant up to 10.30 p.m. (figure IV.5-C, T1). Cry2 mRNA levels did not change between T0 and T1 (figure IV.5-B, T0 vs T1). By opposite, at T1, Cry1 mRNA levels significantly increased 50.7%±10.8 at 8 a.m. and 56.8%±23.5 at 11 a.m., in comparison to T0 (figure IV.5-A, T0 vs T1). On the other hand, *BMAL1* mRNA levels decreased 44.7%±5.7 at 10.30 p.m. (figure IV.5-C, T0 vs T1). The expression profiles of the 9 patients out of 20, assessed at T0, did not changed significantly from the expression profiles of all the 20 patients also assessed at T0 (both are showed in T0 vs T1 graphs), except in the Cry2 gene, where the cohort average expression levels are higher in comparison to only 9 patients.

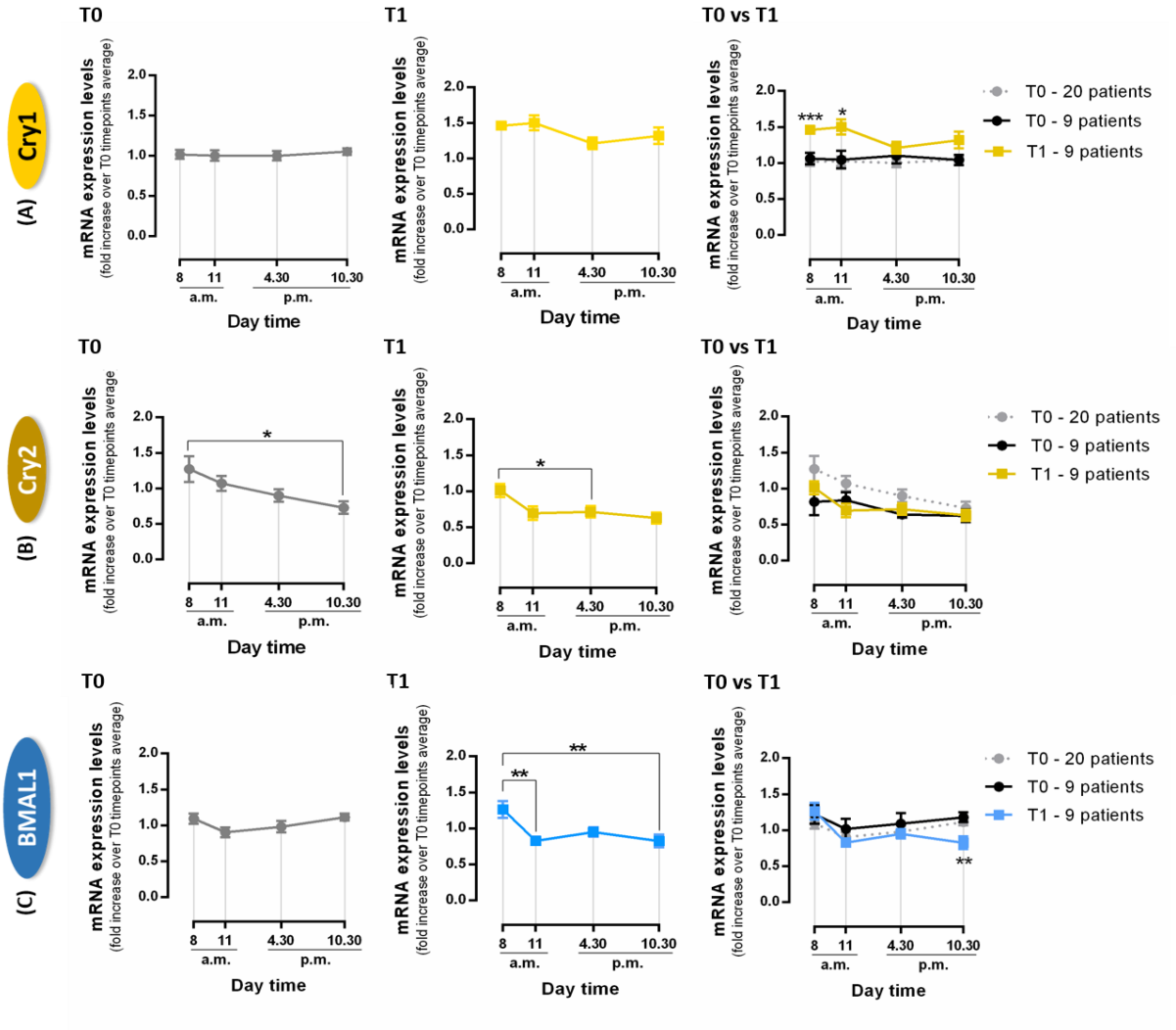


Figure IV. 5: Expression profiles of Cry1-2 isoforms and BMAL1, in OSA patients, before (T0) and after treatment (T1). The figure shows the expression profiles of the two Cry isoforms and of BMAL1 in OSA patients, before treatment (T0 – 20 patients), after treatment (T1 – 9 patients) and a comparison between both (T0 vs T1 – 9 patients, with the average of the 20 patients assessed at T0 dashed). (A) Cry1. At T0, no oscillations were detected in Cry2 mRNA levels. After treatment, the expression profile was maintained but a tendency of oscillatory mRNA levels became evident. When comparing both profiles (T0 vs T1), an increase of Cry1 mRNA levels was observed after treatment, at 8 a.m. and at 11 a.m. (B) Cry2. At T0, Cry mRNA levels showed to be higher in the morning (8 a.m.) but from then, its mRNA levels decreased up to 10.30 p.m. At T1, the higher Cry2 mRNA levels were also observed at 8 a.m. but its decrease was observed earlier, at 4.30 p.m. When comparing T0 and T1 profiles, no differences in mRNA levels were observed. (C) BMAL1. At T0, no oscillations were observed in BMAL1 mRNA levels. After treatment, BMAL1 mRNA levels showed to be higher at 8 a.m., and to decrease at 10.30 p.m. Lower values of BMAL1 transcripts were observed at 10.30 p.m., after treatment. The expression profiles of the 9 patients out of 20, assessed at T0, did not changed significantly from the expression profiles of all the 20 patients, except in the Cry2 gene, where the cohort average expression levels are higher in comparison to only 9 patients. B-2M was used as housekeeping gene. Data is presented as mean ± SEM from 20 individuals (T0) and 9 individuals (T1; T0 vs T1), *p<0.05, **p<0.01, ***p<0.001, compared to T0 time points average. One-way ANOVA was used to evaluate differences over time (at T0 and T1, respectively) and student's t-test to assess differences at specific time points (T0 vs T1).

At T0, DEC1 mRNA levels also oscillated along the evaluated time points (figure IV.6, T0), opposing to CLOCK and CK1ε. *DEC1* showed a similar profile as *Per* genes, characterized by higher mRNA levels in the morning (8 a.m.), lower mRNA levels at 4.30 p.m. and the stabilization of the mRNA levels up to 10.30 p.m., however with much less marked oscillations (figure IV.6-C, T0). At T1, *CLOCK* (figure IV.6-A, T1) and *CK1ε* (figure IV.6-B, T1) showed the same expression pattern. On the other hand, the expression profile of *DEC1* at T1 (figure IV.6-C, T1) turned to be more oscillatory, with higher mRNA levels at 8 a.m that decreased immediately at 11 a.m., remaining constant up to 10.30 p.m. When comparing mRNA levels at each time point, we found reductions in T1 expressions in the three genes. *CLOCK* mRNA levels decreased in all the time points (38.7%±10.9 at 8 a.m.; 46.8%±14.8 at 11 a.m.; 48.5%±16.6 at 4.30 p.m and 62.7%±11.4 at 10.30 p.m.) (figure IV.6-A, T0 vs T1). Regarding CK1ε, a reduction of 27.3%±14.5 in its mRNA levels was observed at 11 a.m. and, at 10.30 p.m., a decrease of 32.9%±9.9 was observed (figure IV.6-B, T0 vs T1). *DEC1* transcripts also decreased 22.3%±18.9 at 11 a.m. and 22.2%±3.8 at 10.30 p.m. (figure IV.6-C, T0 vs T1). The expression profiles of the 9 patients out of 20, assessed at T0, did not changed significantly from the expression profiles of all the 20 patients also assessed at T0 (both are showed in T0 vs T1 graphs).

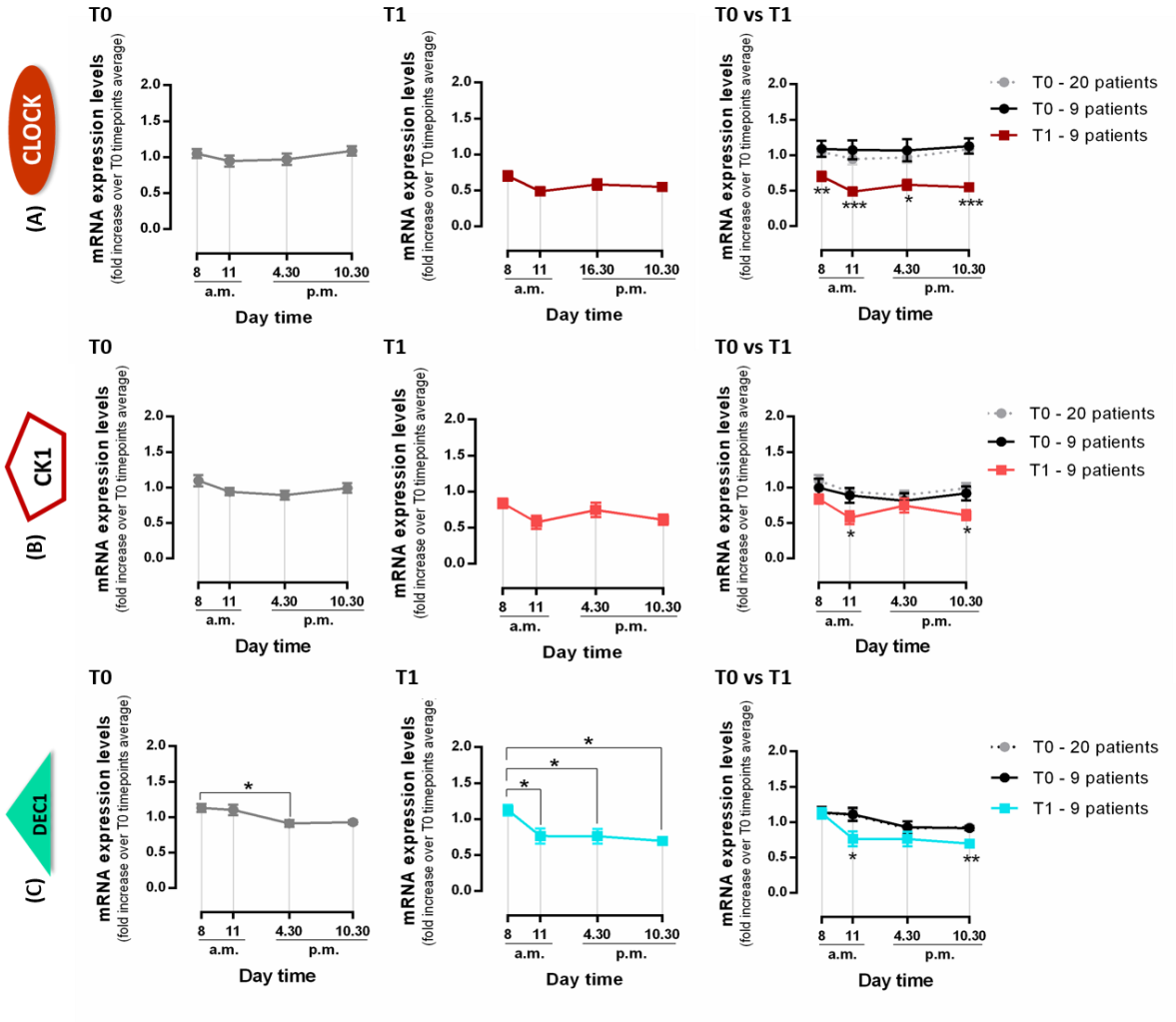
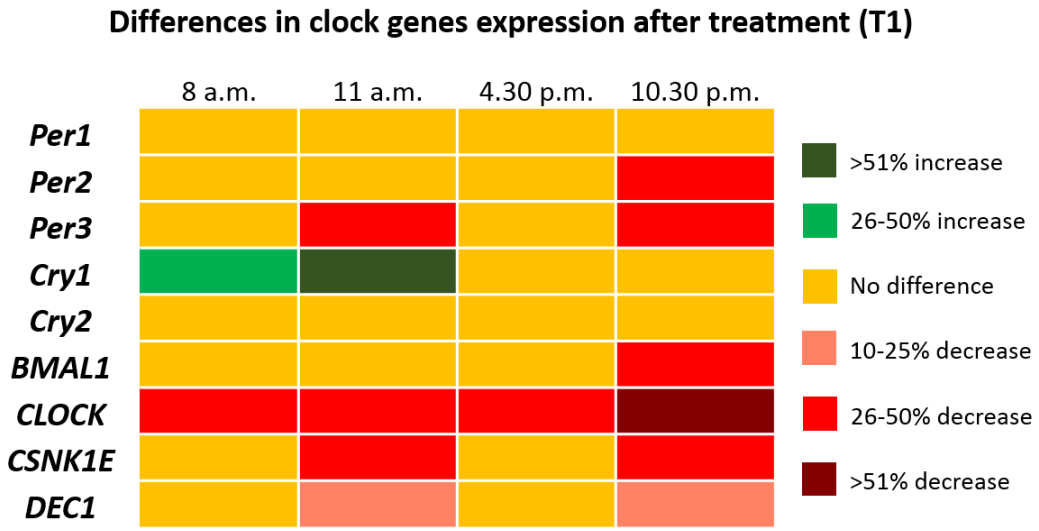


Figure IV. 6: Expression profiles of CLOCK, CK1ε and DEC1 in OSA patients, before (T0) and after treatment (T1). The figure shows the expression profiles of CLOCK, CK1**ε** and DEC1 in OSA patients, before treatment (T0 – 20 patients), after treatment (T1 – 9 patients) and a comparison between both (T0 vs T1 – 9 patients, with the average of the 20 patients assessed at T0 dashed). (A) CLOCK. At T0, no oscillations were detected in CLOCK mRNA levels. After treatment, the expression profile was maintained however CLOCK mRNA levels decreased in all the evaluated time points. (B) CK1**ε**. At T0, no oscillations were detected in CK1**ε** mRNA levels. After treatment, the expression profile was maintained but a tendency of oscillatory mRNA levels became evident. When comparing both profiles (T0 vs T1), lower CK1**ε** mRNA levels were observed t 11a.m. and 10.30 p.m. (C) DEC1. At T0, higher DEC1 mRNA levels were observed in the morning (8 a.m.) and lower mRNA levels were observed 4.30 p.m., stabilizing from then. After treatment, DEC1 mRNA levels showed to be higher also at 8 a.m., but decreased immediately at 11 a.m., from which no differences were observed from then. When comparing T0 and T1 expression profiles, lower DEC1 mRNA levels were observed at 11 a.m. and at 10.30 p.m. In all genes, the expression profiles of the 9 patients out of 20, assessed at T0, did not changed significantly from the expression profiles of all the 20 patients. B-2M was used as housekeeping gene. Data is presented as mean ± SEM from 20 individuals (T0) and 9 individuals (T1; T0 vs T1), *p<0.05, **p<0.01, ***p<0.001, compared to T0 time points average. One-way ANOVA was used to evaluate differences over time (at T0 and T1, respectively) and student's t-test to assess differences at specific time points (T0 vs T1).

The table IV.2 represents a summary of the differences observed at T1, in each time point evaluated. The different colours represent different difference percentages, either relative to the increases or decreases observed in clock genes expression at T1.

Table IV. 2: Summary table showing the differences in clock genes mRNA levels observed in each time point, before and after treatment.



IV.1.5. THE EXPRESSION OF SPECIFIC CLOCK GENES AT SPECIFIC TIMES COORELATES WITH OSA SEVERITY

After OSA patients' genetic circadian rhythms characterization, we analysed whether some of the evaluated parameters could be related with OSA severity, as putative biomarkers. For that we performed correlation analyses to measure the strength of the association between each two variables. The strength of a linear relationships is commonly reflected by the correlation coefficient (r) which range between 1 and –1. The closer to -1, the stronger a negative linear relationship will be, namely, two variables will be inversely related. By opposite, the closer to 1, the stronger a positive linear relationship will be and the variables will be directly related. Finally, the closer to 0, the weaker the linear relationship between two variables will be.

In this context, we found that the expression of some clock genes, at specific time points could be related, either inversely or directly, with OSA AHI (table IV.3). The strongest correlation observed ($p<0.0001$) was between *Cry*1 mRNA levels, at 8 a.m., and AHI ($r = -0.71$) which suggested that the higher the expression of *Cry1*, at 8 a.m., the lower the OSA severity will be. Such relation is clear in the linear regression model shown in figure IV.7. An inverse correlation was also observed for *Cry1* mRNA levels at 11 a.m., 4.30 p.m. and 10.30 p.m., *BMAL1* mRNA levels at 8 a.m. and *CLOCK* mRNA levels at 10.30 p.m. By opposite, *Cry*2 mRNA levels at 11 a.m., 4.30 p.m. and 10.30 p.m., *Per*1 mRNA levels at 8 a.m. and 10.30 p.m., *Per*2 mRNA levels at 10.30 p.m. and *CK1ε* transcripts at 10.30 p.m. appear to increase with OSA severity.

Table IV. 3: Correlations found between the expression of some clock genes, at specific times, and OSA severity.

Clock gene	Time point	Correlation coefficient (r) with AHI	
*Cry*1	8 a.m.	-0,71	****
	11 a.m.	-0,62	***
	4.30 p.m.	-0,53	**
	10.30 p.m	-0.54	**
***Cry*2**	11 a.m.	0.53	**
	4.30 p.m.	0.45	*
	10.30 p.m	0.55	**
*Per*1	8 a.m.	0.42	*
	10.30 p.m	0.40	*
*Per***2**	10.30 p.m	0.41	*
BMAL1	8 a.m.	-0.39	*
CLOCK	10.30 p.m	-0,48	*
*CK1*ε	10.30 p.m	0.42	*

*p<0.05; **p<0.01, *** p<0.001, **** p<0.0001, Pearson correlation.

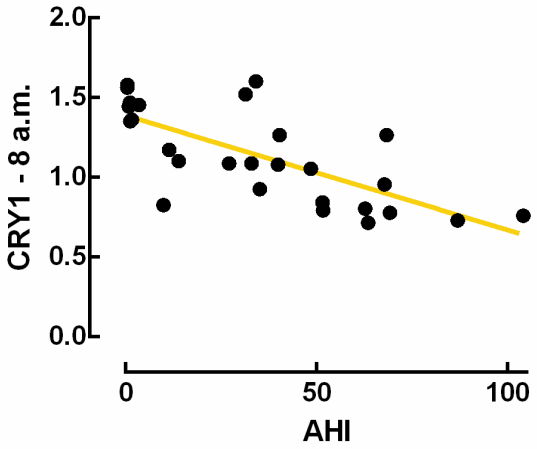


Figure IV. 7: Linear regression between Cry1 mRNA levels at 8 a.m. and OSA AHI. The figure shows the dispersion of *Cry*1 mRNA levels and correspondent OSA AHIs. A linear regression suggests that *CRY*1 mRNA levels, at this time point, decrease with the increase of OSA severity.

The correlation between *Cry*1 mRNA levels at 8 a.m. and OSA AHI is sustained by the correlation of *Cry*1 mRNA levels with other OSA relevant parameters, namely with the arousals index ($p<0.05$, $r=-0.54$) and the desaturation index ($p<0.05$, $r=-0.59$). Consequently, the higher the mRNA levels of *Cry*1, the lower the arousals index, the desaturation index and OSA severity.

In the second part of this study, we aimed to understand whether abnormal values of NPY could be contributing to the observed circadian rhythm molecular machinery alterations. According to the literature, NPY levels are reported to be systemically abnormal in individuals with excessive weight and in the elderly (Kuo et al., 2007; Vela et al., 2003). Since having excessive weight or being elderly constitute risk factors for developing OSA, and NPY has already been reported to impact on clock genes expression (Fukuhara *et al.*, 2001), its role clock genes modulation was further investigated in the following sub-chapter.

IV.2. CIRCADIAN RHYTHM *IN VITRO* EXPERIMENTS

IV.2.1. CIRCADIAN EXPRESSION PROFILE OF mHypoE-N42 NEURONS CLOCK GENES

Before starting any of the experiments, the circadian rhythmicity of our cellular model, mHypoE-N42 neurons, was firstly investigated. After cells synchronization, the expression of the core clock genes (*Per1-2*; *Cry*; *CLOCK* and *BMAL1*) was assessed from CT0 to CT72. The results (figure IV.7) showed rhythmic oscillations in the expression of the clock genes *Per1*, *Per2* and *BMAL1.* On the other hand, *Cry* and *CLOCK* mRNA levels did not rhythmically oscillate over time, in mHypoE-N42 neurons.

The profiles of both *Per1* and *Per2* isoforms mRNAs were similar, with the difference of *Per*2 expression variations being considerably more marked (figure IV.8-A and IV.8-B). Rhythmicity is very clear among these isoforms expression, with maximal levels of both *Per* isoform mRNA molecules observed at CT0, 24, 48 and 72 while minimum mRNA levels were detected at CT12, 32 and 52. *BMAL1* showed an opposite expression profile (figure IV.8-D). Consequently, higher levels of *Per* isoforms mRNAs (CT20-24; CT48) were associated with a decrease in *BMAL1* transcript levels whereas lower mRNA levels of *Per* isoforms (CT32; CT52-60) were coincident with peak *BMAL1* transcript levels. An exception to such pattern was observed from CT0-CT20, period in which *BMAL1* mRNA levels did not oscillated. *Cry* mRNA levels did not change overtime (figure IV.8-C). Finally, *CLOCK* showed constitutive levels of mRNA molecules, except up to CT20, period in which its mRNA levels were considerably lower (figure IV.8-E). The CTs at which the highest and the lowest mRNA expression levels were observed are summarized in the following table (table IV.4).

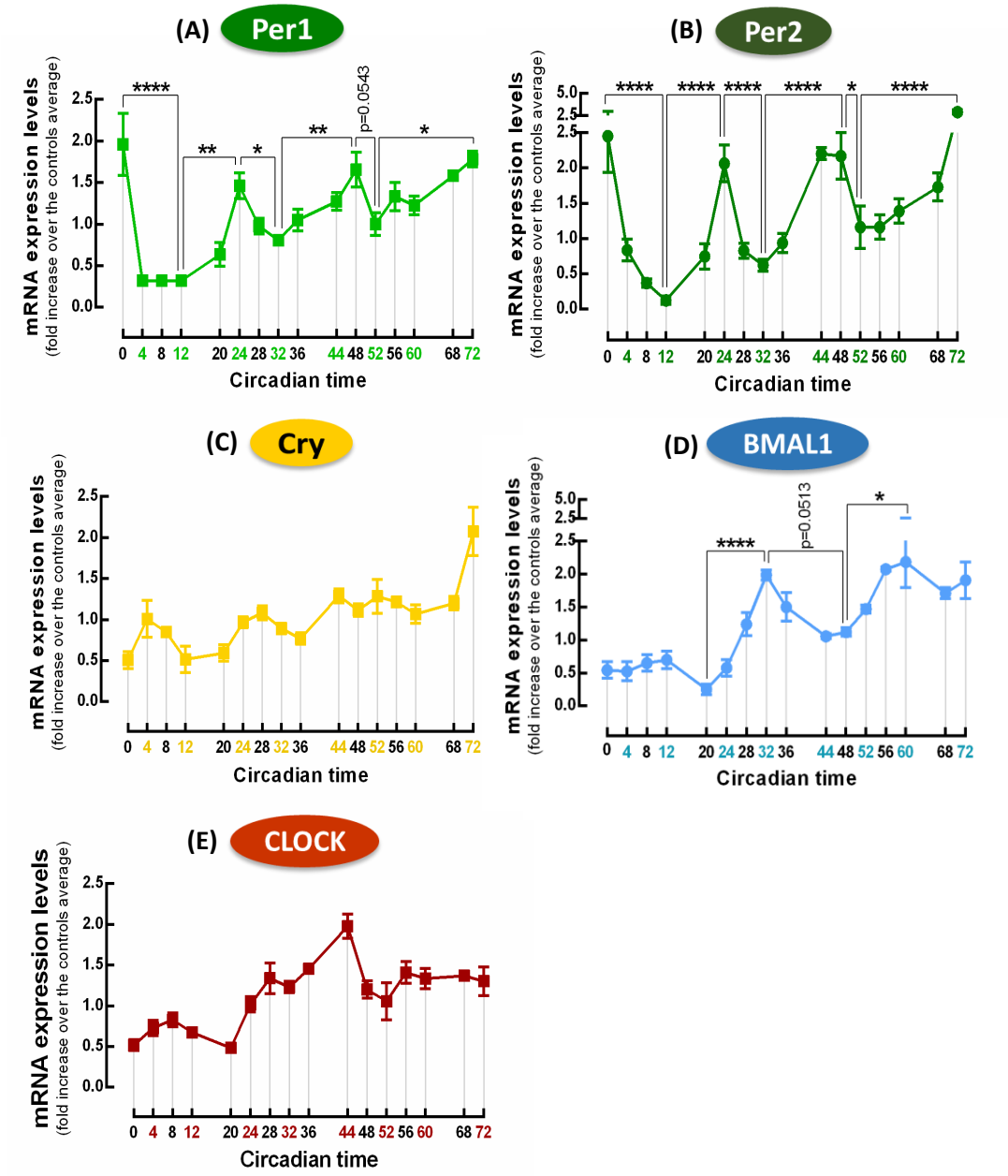


Figure IV. 8: mHypoE-N42 core clock genes expression profile. The figure shows the mRNA expression levels profile of the hypothalamic cell line core clock genes from CT0 to CT72, and the statistical significance between lower and higher mRNA levels. (A) Per*1*. Per1 mRNA levels showed to rhythmically oscillate, showing four peaks of mRNA levels, at CT0, 24, 48 and 72. On the other hand, Per*1* minimal mRNA levels were found at CT12, 32 and 52. (B) Per*2*. The mRNA levels of Per2 rhythmically oscillated, showing to be higher at CT0, 24, 48 and 72 and lower at CT 12, 32 and 52. (C) *Cry*. Slight rhythmic oscillations were observed up to CT44, however, such oscillations did not show to be significant. (D) *BMAL1*. An oscillatory variation in its mRNA levels was observed from CT20. Lower mRNA levels were detected at CT20 and 48 whereas at CT32, 60 higher mRNA levels were found. (E) *CLOCK*. No rhythmic oscillations were detected, however, minimal mRNA levels were observed from CT0 to 20. *GAPDH* and *HPRT* were used as housekeeping genes. Data is presented as mean ± SEM from 3-6 independent experiments and in relation to the controls average. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, compared to the controls average. One-way ANOVA.

Table IV. 4: Circadian times at which the highest and lowest mRNA levels of the core clock genes were detected in mHypoE-N42 neurons.

	CT															
Gene	0	4	8	12	20	24	28	32	36	44	48	52	56	60	68	72
Per1																
Per2																
Cry																
BMAL1																
CLOCK																

Higher RNA levels

Lower RNA levels

Once mHypoE-N42 neurons were characterized for core clock genes rhythmicity, the role of NPY in the modulation of the same genes, in the mHypoE-N42 neurons, was then investigated. To start, we screened the NPY effect on each clock gene modulation, from 4 to 4 hours.

IV.2.2. NPY CHANGES mHypoE-N42 NEURONS CLOCK GENES EXPRESSION IN A TIME DEPENDENT MANNER

Firstly, we assessed whether NPY impacts on the clock gene expression modulation of mHypoE-N42 neurons and, if so, whether that effect is dependent upon the CT. Consequently, cells were treated with NPY (100 nM) from CT0 to CT48 and 24h later, the clock genes mRNA levels were evaluated. The results showed that NPY affects the clock molecular machinery of the hypothalamic neurons, in a time dependent manner (figure IV.9). When cells were treated with NPY at CT4, NPY caused an increase of 34.0%±12.0 in *BMAL1* mRNA levels (CT28, $p<0.01$) (figure IV.9-D); at CT12, a decrease of 21.9%±11.4 in *Per1* mRNA levels was observed (CT36, $p<0.05$) (figure IV.9-A); at CT20, *CLOCK* mRNA levels increased 57.5%±20.4 (CT44, $p<0.01$) (figure IV.9-E); at CT24, *CRY* mRNA levels decreased 15.4%±8 (CT48, $p<0.05$) (figure IV.9-C); and, at CT48, a reduction of 57.3%±32.3, on *Per*2 expression, was observed (CT72, $p<0.05$) (figure IV.9-B). No other changes in mHypoE-N42 clock genes expression were observed in any of the other CTs. Table IV.5 summarizes the alterations observed, depending upon the CT at which cells were treated with NPY. The different colours represent different difference percentages, either relative to the increases or decreases observed in clock genes expression 24h after the NPY treatment.

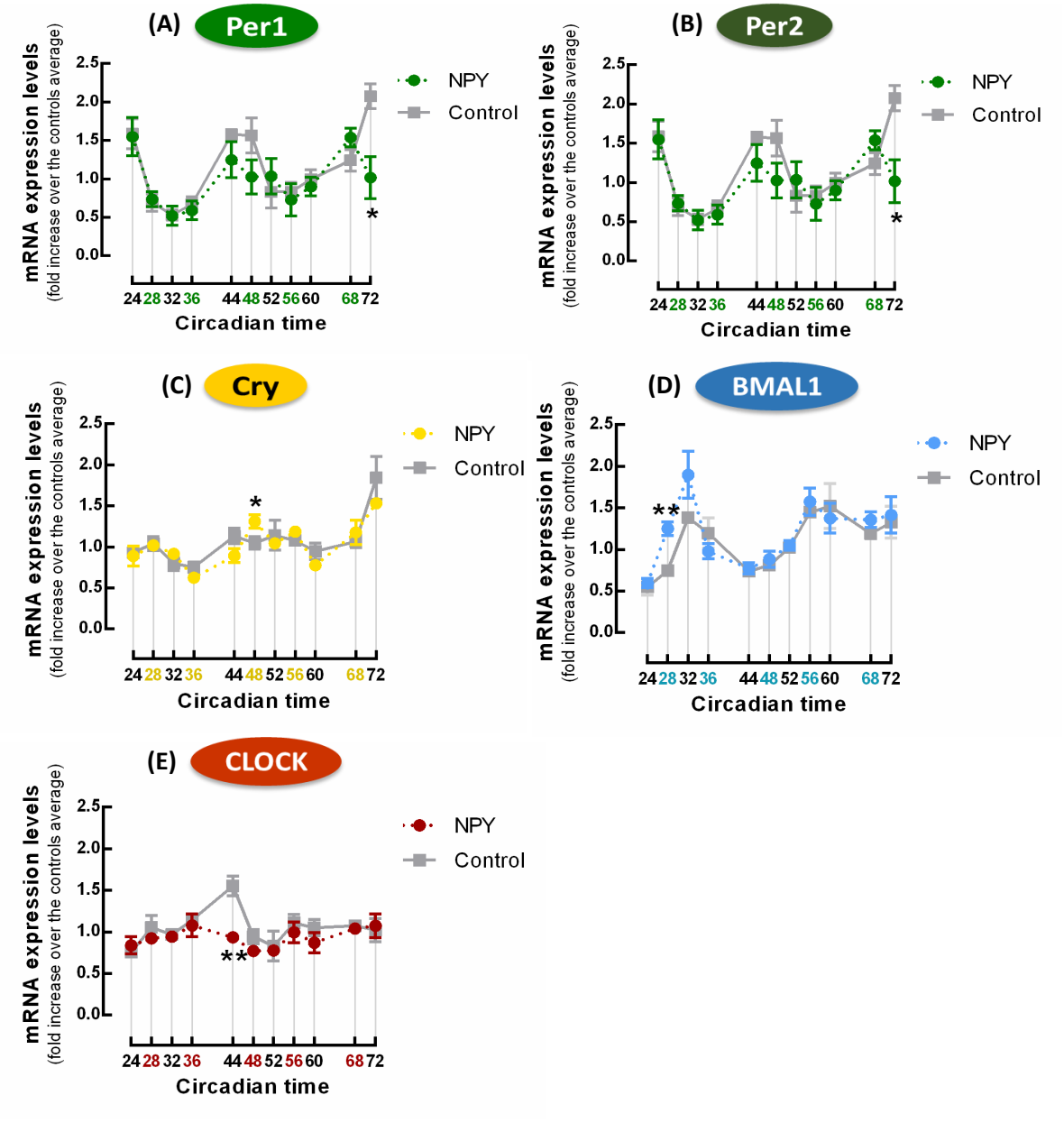
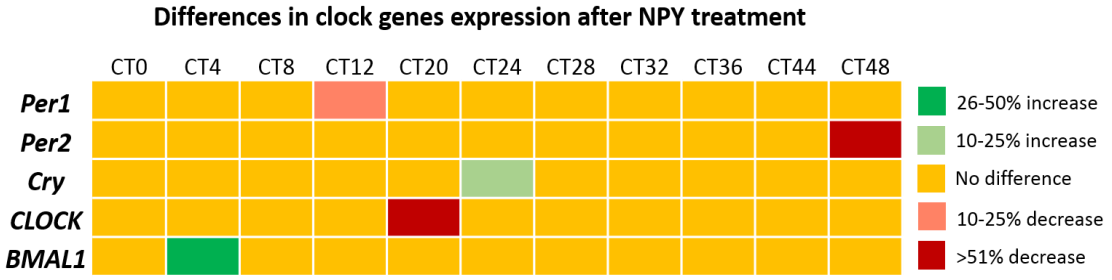


Figure IV. 9: NPY effect, at different CTs, on the mHypoE-N42 clock genes expression. The figure shows the effect of NPY, at different CTs (from CT0 to CT48), on the clock genes expression of the hypothalamic cell line (from CT24 to CT72) in comparison with cells non-treated with NPY. NPY modulates the clock genes expression in a time dependent manner. (A) Per*1*. An underexpression of Per*1* was observed at CT36. (B) Per*2*. Per*2* mRNA levels decreased at CT72. (C) *Cry*. An overexpression of *Cry* was observed at CT48. (D) *BMAL1*. At CT4, the mRNA levels of *BMAL1* increased. (E) *CLOCK*. *Clock* mRNA levels decreased at CT44. *GAPDH* and *HPRT* were used as housekeeping genes. Data is presented as mean ± SEM from at least 5-6 independent experiments and in relation to the controls average. *p<0.05, **p<0.01, compared to the respective control. Student's t-test.

Table IV. 5: Summary table showing the differences in clock genes mRNA levels after 24h of NPY treatment, at different CTs, on mHypoE-N42 neurons.



The represented CTs are correspondent to the CTs at which cells were treated with NPY. The correspondent effects were observed 24h after NPY treatment.

Once the NPY effect was characterized at different times, the effects of a NPY constitutive expression on the mHypoE-N42 circadian clock genes profile were then investigated.

IV.2.3. A CONSTITUTIVE EXPRESSION OF *NPY* DID NOT CHANGE CLOCK GENES PROFILE IN THE mHypoE-N42 NEURONS

NPY constitutive expression in mHypoE-N42 neurons was induced through transfection of a *NPY* plasmid. Transfection efficiency was evaluated through qRT-PCR assessment of the *NPY* mRNA levels in the transfected cells, relative to non-transfected cells. The results show, as expected, that *NPY*-transfected cells have the highest levels of *NPY* mRNA molecules (figure IV.10-B), clearly demonstrated by the fastest increase of fluorescence (figure IV.10-A) in comparison with *GFP*-transfected and non-transfected cells. Thus, as expected, *NPY* expression is highly increased in the cells transfected with the *NPY* plasmid.

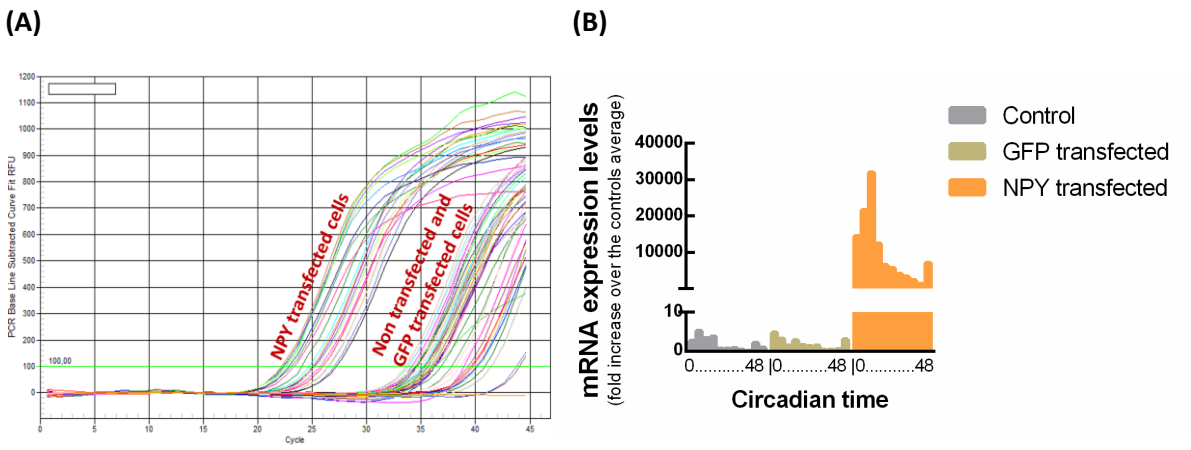


Figure IV. 10: Validation of the NPY gene transfection of mHypoE-N42 neurons. The figure shows the efficiency of the NPY gene transfection of the hypothalamic cell line. (A) Amplification plot of a qRT-PCR experiment for NPY mRNA levels quantification of NPY transfected cells, *GFP* transfected cells and non-transfected cells. NPY transfected cells showed the fastest increase of fluorescence, and consequently, of NPY mRNA levels, in comparison with GPF transfected and non-transfected cells. (B) Quantification graphic of the NPY mRNA levels of in control, *GFP* transfected and NPY transfected cells.

With a constitutive *NPY* expression, up to CT48, there was no significant changes in the clock genes expression profile of mHypoE-N42 neurons (figure IV.11). The mRNA levels of all clock genes, in *NPY*-transfected cells, did not suffered significant alterations in any of the evaluated CTs, except for the *Per*1 isoform, at CT32 (figure IV.11-A). At this CT, the mRNA levels of *Per*1 decreased in comparison with the control cells (41.3%±3.6, *p<0.05). Such effect was confirmed by *GFP*-transfected cells, in which *Per1* mRNA levels were not different from control cells. However, no other significant differences, in *Per1* expression, were observed from then. In the conditions tested, at least up to CT48, no changes in Per2, *Cry*, *BMAL1* and *CLOCK* mRNA levels were observed upon *NPY* constitutive expression (figure IV.11-C, D, E).

In order to validate the observed effects of *NPY* on mHypoE-N42 neurons clock genes expression, a positive control was performed, as described below.

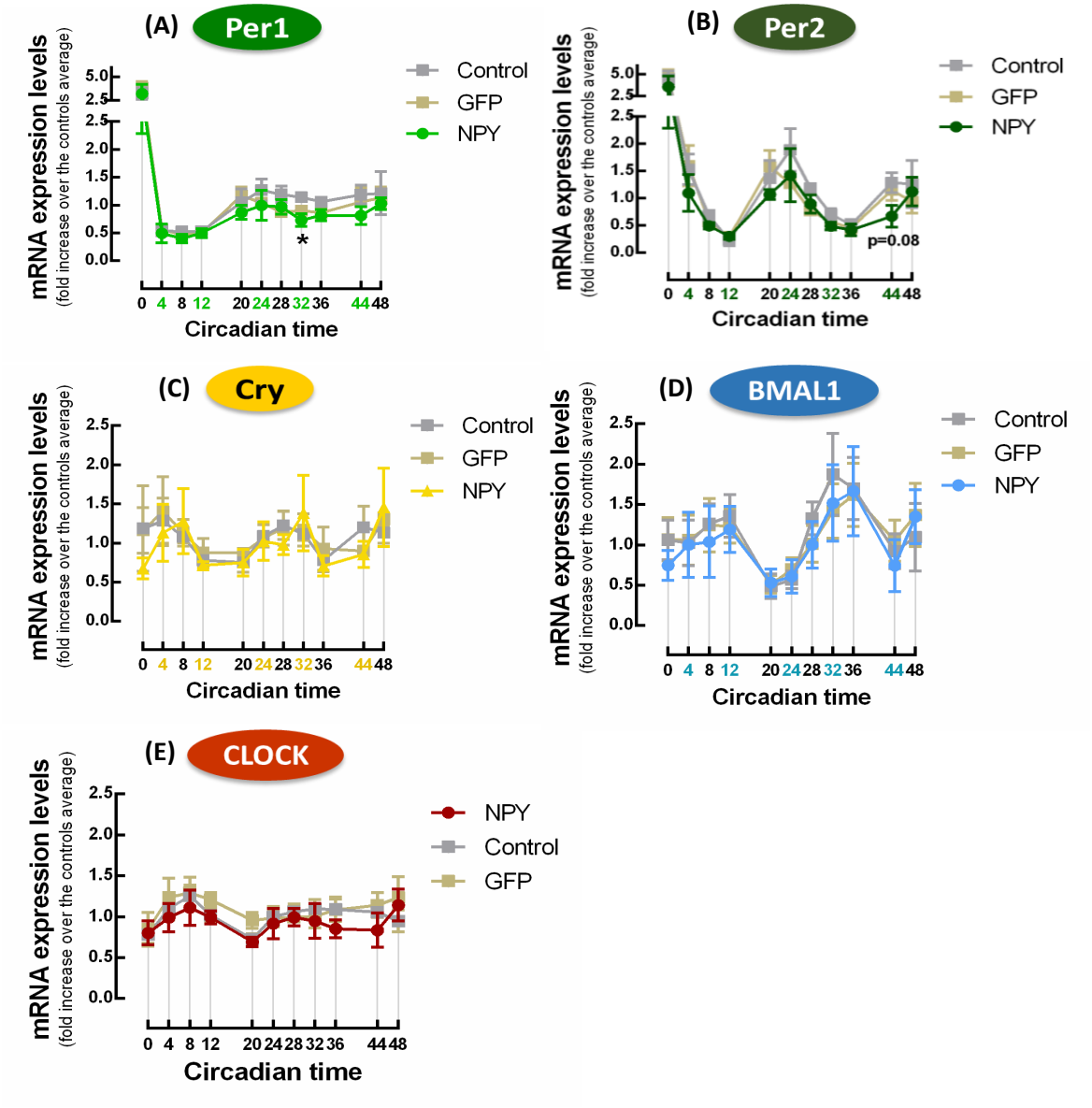


Figure IV. 11: Effect of NPY constitutive expression, up to CT48, on mHypoE-N42 clock genes expression profile. The figure shows the expression profiles of the core clock genes of mHypoE-N42 neurons (grey), neurons transfected with GFP (beige) and neurons transfected with the NPY plasmid. (A) Per1. No alterations were observed in Per1 mRNA levels of NPY transfected cells except at CT32, in which the mRNA levels decreased in comparison with the control cells. By opposite, GFP cells did not show differences in Per1 mRNA levels, at this CT, in relation to control cells. (B) Per2. NPY constitutive expression did not caused alterations in Per2 mRNA levels. GFP cells did not show variations in Per2 mRNA levels, at this point, in comparison to control cells. (C) Cry. No alterations were found in the Cry mRNA levels of NPY transfected cells. (D) BMAL1. NPY constitutive expression did not impacted on BMAL1 mRNA levels. (E) CLOCK. No alterations were observed in CLOCK mRNA levels of NPY transfected cells. GAPDH and HPRT were used as housekeeping genes. Data is presented as mean ± SEM from 3 independent experiments and in relation to the controls average. *p<0.05, compared to the respective control. Student's t-test.

IV.2.4. THE CIRCADIAN RHYTHM OF mHypoE-N42 NEURONS IS AFFECTED BY EXTERNAL FACTORS KNOWN TO MODULATE CLOCK GENES EXPRESSION

To confirm whether the circadian rhythm of mHypoE-N42 neurons is, indeed, being affected by external factors, a positive control was performed. According to the literature (Asher *et al.*, 2008; Bellet *et al.*, 2013), SIRT-1 is required for the transcription of several clock genes, among which *Per2* and *BMAL1.* Since resveratrol is known to act as a SIRT-1 activator, mHypoE-N42 neurons were treated, at CT0, with this polyphenolic compound (10 µM), and the clock genes mRNA levels were evaluated.

Although only two experiments were performed the results were the expected, in both experiments. Resveratrol induced an increase in *Per2* mRNA levels at CT32, CT36 and CT44 (figure IV.12-A). In addition, resveratrol also increased the mRNA levels of *BMAL1* at CT44 and CT48 (figure IV.12-B). These results show that clock genes in hypothalamic neurons may change due to external factors.

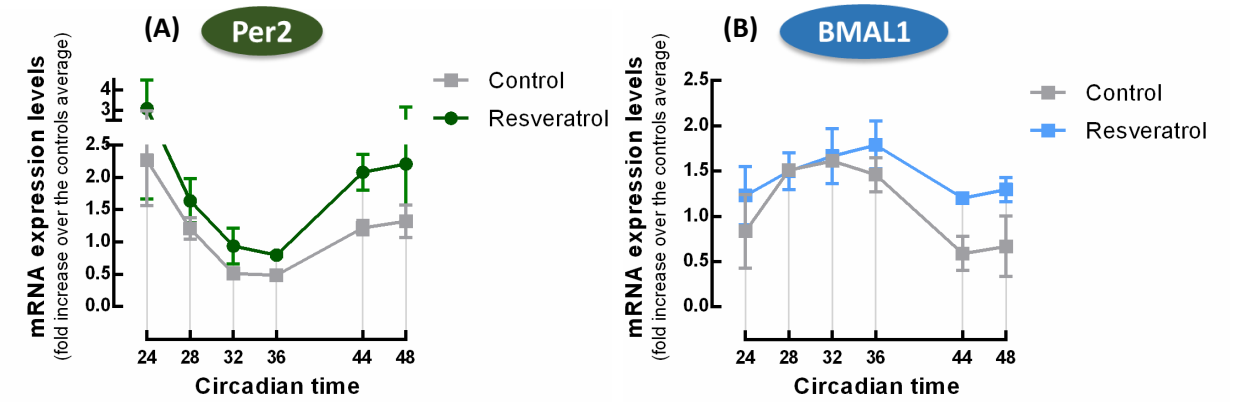


Figure IV. 12: Effect of resveratrol on Per2 and BMAL1 clock genes transcription. (A) Per2. Per2 mRNA levels increased at all the evaluated CTs, in comparison with non-treated cells. (B) BMAL1. Resveratrol caused an increase in BMAL1 mRNA levels at CT44 and CT48. Data is presented as mean ± SD from 2 independent experiments and in relation to the controls average.

CHAPTER V:

DISCUSSION

Circadian rhythms are defined by a system of circadian clocks, powerful endogenous timing commanders that are crucial to drive numerous physiological and behavioural processes. Consequently, disruptions in its correct functioning have been associated with several diseases which has drawn the attention of the research community (Toh *et al.*, 2001; Fu *et al.*, 2002; Takahashi *et al.*, 2008; Shanmugam *et al.*, 2013; Landry & Liu-Ambrose, 2014; Zelinski *et al.*, 2014; Brainard *et al.*, 2015; Sharma *et al.*, 2016). Among the factors that highly contribute to circadian rhythms misalignment is sleep deprivation, a common characteristic of sleep disorders (Archer *et al.*, 2014).

OSA is estimated to be one of the most common sleep disorders and its prevalence is expected to continuously increase due to the highly increasing rates of metabolic disorders (Lurie, 2011; Xu, Zheng, *et al.*, 2015). Untreated OSA has been associated with cyclical deoxygenation/oxygenation, fragmented sleep and an abnormal sympathetic activation, outcomes that have been implicated as risk factors for the development of several diseases, ranging from metabolic disorders to cardiovascular diseases (Darien, 2014). Despite its limitations, CPAP treatment has been show to considerably improve OSA outcomes (Gupta *et al.*, 2010). Nevertheless, for a good treatment, a good diagnosis is demanded. However, there are several limitations associated with OSA diagnosis, including its high cost, symptoms variability over different sleep nights, the considerable patients' discomfort and the fact that people are not aware that they may suffer from OSA (Ross *et al.*, 2000; Kushida *et al.*, 2005; Ahmadi *et al.*, 2009; Darien, 2014). In this context, OSA is still highly underdiagnosed, being urgent to improve its diagnosis. Several attempts to find ideal OSA biomarkers, molecular reflexes of its severity, have been made, however without success.

Recently, an association between OSA and circadian rhythm disruptions was found. OSA patients were suggested to have a disrupted circadian rhythm, reflected by alterations in the expression of one of the core genes involved in its regulation (*Per1*) (Burioka *et al.*, 2008). Such disruption could justify some OSA complications, mainly its poor sleep quality, fatigue and excessive sleepiness during the day and also OSA implication as a risk factor for the development of metabolic and cardiovascular diseases (Darien, 2014). However, the cross-talk between OSA and circadian rhythm disruptions is not clear. A better understanding of such association may provide important cues for the establishment of the first OSA biomarkers.

In this context, in the present work, we intend to investigate the circadian rhythm of OSA patients, by assessing a circadian rhythm marker (body temperature) and clock genes expression (*Cry1-2; Per1-3; BMAL1; CLOCK; CK1ε; DEC1-2*), at four time points (8 a.m.; 11 a.m.; 4.30 p.m. and 10.30

p.m.), before and after CPAP treatment, in PBMCs. PBMCs constitute a minimal invasive and easy source to assess the circadian rhythm molecular machinery once this population of cells are reported to act as peripheral clocks, showing rhythmic expression of clock-related molecules (Teboul, Barrat-Petit, *et al.*, 2005; Boivin *et al.*, 2015; Xu, Xu, *et al.*, 2015).

Consequently, we assessed the circadian rhythm of 20 men with OSA, the major cohort studied so far in the field, before treatment and 9 of them, so far, after CPAP treatment. Only male patients were selected for this study not only to avoid gender differences and, thus, to decrease variability but also because clock genes expression profiles, in PBMCs, were reported to be influenced by the menstrual cycle (Xu, *et al.*, 2015). In order to evaluate whether there were disruptions in the circadian rhythms of the selected OSA patients, we considered the same individuals, after treatment, as controls. Healthy individuals without OSA could also be used as controls, however, such individuals may not be aware that they may suffer from OSA or other sleep disorder, which would interfere with the obtained results. In addition, problems of sleep deprivation are common among society and thus, no individual would act as an ideal control. By using this approach, we limited patients' variability and differences in circadian rhythms, caused by OSA, would be clearer.

The selected cohort can be considered representative of OSA affected individuals, once 95% of the patients have excessive weight and 70% are older than 45 years. In addition, some were previously diagnosed with metabolic (diabetes), cardiovascular (hypertension) and psychiatric disorders (depression), associated long-term consequences of untreated OSA (Park *et al.*, 2011; Darien, 2014; Weaver *et al.*, 2014). On the other hand, 79% of the selected patients awake several times during sleep nights, complaining about a disrupted sleep, and consequently fall asleep during the day. These are common symptom among OSA patients, although OSA severity correlates poorly with daytime symptoms (Park *et al.*, 2011; Darien, 2014). Concerning the patients' routine, although only 16% of the patients regularly practice sports, 84% has healthy eating habits and 63% eat at regular hours, which helps generating rhythmicity. However, the majority of the cohort patients already worked on nightshifts and some of them travelled across time zones in the past 6 months, actions known to contribute to circadian rhythms disruptions (Archer et al., 2014). The PSG results, obtained by the technicians of *Clínica do Sono* of *Centro Hospitalar da Universidade de Coimbra (CHUC)*, confirmed that the great majority of the cohort patients suffered from severe OSA; one patient was diagnosed with moderate OSA and three with mild OSA. In accordance, these patients underwent several arousals and awakenings during sleep and their blood oxygenation capacity along the night was affected, showing a reduced arterial oxygen saturation (91.4±0.6%), comparing to the normal standard values (97-99%, Schutz, 2001)

(figure IV.1; table IV.1). The average wake heart rate was also higher in these patients (80.7±4.6 bpm) in comparison with the standard reference values (75 bpm) (Fox, 2014).

Taking this into account, OSA patients' circadian rhythms were evaluated. We started by evaluating it through the axillary body temperature throughout the defined time points. According to the literature, body temperature reflects clocks functioning by providing relevant information about the phase and amplitude of circadian clocks timing. In addition, body temperature was reported to play an essential role in the molecular clocks coordination, subsequently, alterations in its profile could reflect alterations in clock genes expression (Scully *et al.*, 2011). Previous reports (Giuffre *et al.*, 1990) showed that the axillary temperature can be used as a reflection of core body temperature, and can be opted when small differences in temperature are clinically meaningful. In this context, since for the purpose of this study the body temperature profile along the selected time points would be more important than the values per se, we opted for assessing body temperature through this method, once it would be easier and more comfortable for patients to measure.

According to the literature, the highest value in core body temperature is exhibited in the evening and the lower in the middle of the night (Kryger *et al.*, 2010). At T0, our results, in both groups (20 patients or 3 patients) are consistent with what is described in the literature, higher temperatures were measured in the evening and early night (4.30 p.m. and 10.30 p.m.) comparing to the lower temperatures measured in the morning (8 a.m.), probably derived from a lower temperature in the middle of the night. However, at T1, the axillary body temperature profile was not the same. The assessed patients, in both groups (9 patients or 3 patients), showed higher body temperatures in the morning (8 a.m.) (figure IV.3-A and B). Such change may be related with different protocol conditions at T0 and T1. At T0, patients slept the night in the medical clinic, in order to do the PSG test, consequently their body temperature was measured after wake up. On the other hand, at T1, patients did not slept the night before in the medical clinic, which demanded their dislocation to the clinic and only then body temperature was assessed. Consequently, patients were probably more active at 8 a.m. of T1 than at the same time point of T0. Such difference could be enough to change body temperature since this parameter is easily affected by factors such as hormones (cortisol and the thyrotropin releasing hormone), stress, carbohydrate intake, sleep and activity (Lewy *et al.*, 1999; Kräuchi *et al.*, 2002). Thus, a more feasible analysis would require other circadian rhythm markers, such as plasma cortisol and melatonin, once such circulating factors are less affected by external factors, reproducing better possible circadian rhythm misalignments that may occur in OSA patients (Lewy, 1999; Lewy *et al.*, 1999; Kräuchi *et al.*, 2002).

Even so, the hypothesis of a disrupted clock existence in OSA patients, was reinforced by the obtained results regarding clock genes expression in OSA patients. As cortisol and melatonin, molecular components such as clock genes are less affected by the pointed external factors, which is supported by the obtained results. In this study, aside from *Per1*, we showed, for the first time, the expression profile of eight clock genes (*Per2-3*, *Cry1-2*, *BMAL1*, *CLOCK*, *CK1ε* and *DEC1*) in OSA patients PBMCs. The obtained results showed disruptions in OSA patients 'circadian rhythms, reflected by both loss of the oscillatory expression characteristic of some clock genes and abnormal mRNA levels in practically all the assessed genes before treatment.

Regarding clock genes profiles, according to the literature, these genes, except *CLOCK*, show daily variations in their expression levels in PBMCs of healthy individuals (Boivin et al, 2003; Teboul et al, 2005; Xu et al, 2015). However, in the OSA patients analysed in this study, only Per isoforms showed marked oscillations in their diurnal expression, with the highest mRNA levels detected in the early morning and lower Per transcription detected along the afternoon and evening (figure IV.4). Indeed, the highest Per transcription, in peripheral blood, was previously observed at times experienced as morning and lower Per transcription was detected along the afternoon and evening, to allow *BMAL1* transcription (Boivin *et al.*, 2003; Teboul, Barrat-Petiti, *et al.*, 2005; Xu, Xu, *et al.*, 2015). In accordance, after treatment, the suspicions of a correct profile were confirmed and the more pronounced oscillations were maintained, except for *Per2*. The *Per2* expression profile of the 9 patients, after treatment, did not show a so marked oscillation as in T0 or as it occurred for *Per1* and *Per3* expressions. That apparent loss of rhythmicity may be erroneous and likely attributed to the lower number of individuals assessed in comparison to T0. The results obtained for *Per1* contradict the only results, in the field, reported by Burioka and colleagues (2008). The authors analysed the expression of this clock gene in eight healthy individuals and in eight patients with severe OSA (AHI 53.9±6.2), before and after CPAP treatment, at 2 a.m., 6 a.m., 2 p.m. and 6 p.m. According to these authors, no significant diurnal variations were found in the expression profile of *Per1* mRNA levels in OSA PBMCs. *Per1* cyclical expression was only observed in healthy individuals and OSA patients that underwent CPAP therapy for three months. A likely reason that may underlie the difference between the results that we obtained for *Per1* gene expression and the results of Burioka and its co-workers may be the size of the cohort that they analysed, that was smaller, or the different times of the day evaluated. The major differences observed in OSA patients Per1 expression profiles were at 6 p.m. and at 2 a.m. In our cohort, at the evaluated times of the day, our results suggest that Per genes expression, in PBMCs, is not affected by OSA.

On the other hand, *Cry*2 and *DEC1* genes showed discreet variations along the day and *BMAL1*, *Cry*1 and *CK1ε* showed no variations at all in OSA patients PBMCs (figure IV.5-6). By opposite, after treatment, the oscillatory expression profiles of these genes considerably changed, approaching the oscillations described in the literature, for healthy individuals PBMCs (Takata *et al.*, 2002; Boivin *et al.*, 2003; Teboul, Barrat-Petit, *et al.*, 2005; Xu, Xu, *et al.*, 2015). *Cry*2 expression turned to be more phase related with Per isoforms, which is consistent with the correspondent protein interactions. *DEC1* showed a more obvious oscillation in comparison with T0, with a decrease of its mRNA levels as night arose, so *BMAL1* transcription can occur. In accordance, *BMAL1* mRNA levels turned to oscillate with CPAP treatment, showing higher levels at 8h that may be derived from its higher mRNA levels during the night, and low levels during the day. *Cry*1 and CNSK1E expression profiles did not showed significant oscillations after treatment, but a tendency was observed. Indeed, *Cry*1 mRNA levels tended to be higher in the morning, coincident with *Cry*2 and *Per1*-3 and lower during the afternoon. By opposite, *CK1ε* mRNA levels tended to be higher in the morning and in the afternoon, period when Per proteins should be degraded. The evaluation of the remaining patients may turn such oscillations more clear. Regarding *CLOCK*, no oscillations in its expression were found before and after treatment, which is consistent once this gene is reported to have a constitutive expression (Takata *et al.*, 2002). To sum up, the loss of the diurnal variations in the expression profiles of several clock genes, in OSA PMBCs, suggests that the circadian clock is, indeed, disrupted in OSA patients.

Such disruptions are reinforced when the mRNA levels of the evaluated clock genes are compared for each time point, before and after treatment. Statistically significant differences were detected in all genes except in *Per1* and *Cry2*, which transcription did not suffered alterations in OSA patients. By opposite, the mRNA levels of all the other genes were altered, in some time point, as it is clear in table IV.2. The expression of these genes generally decreased after treatment, which suggest that in a context of OSA, the transcription of these genes is induced, and consequently, over expressed at the correspondent times of the day. Indeed, previous reports have showed that *Per1*, *CLOCK* and *DEC1* are upregulated under hypoxic conditions (Chilov *et al.*, 2001; Miyazaki *et al.*, 2002). However, Burioka and its colleagues (2009) ruled out the hypothesis of OSA IH being in the origin of clock genes alterations, since, per se, OSA IH would not be sufficient to cause clock gene expression alterations. In this context, the same authors suggested that the change of circulating factors caused by an abnormal OSA sympathetic activity could be the cause of such disruptions. Indeed, an adrenaline β_2-adrenoceptor agonist is able to induce *Per1* gene expression both *in vitro* and *in vivo*. However, there are no reports in the literature regarding the other clock genes. Thus, the molecular mechanism/s that may underlie such alterations is/are not known. By

opposite, the only gene which expression increased, after treatment, was *Cry*1, indicating that the transcription of this gene is somehow under expressed in OSA patients, at the correspondent times. Again, the mechanism underlying is not known.

This observation that some clock genes mRNA levels, at specific time points, are abnormal in OSA is supported by the correlations found between the expressions of specific clock genes at specific times of the day and OSA severity. The stronger correlations with OSA severity were observed for *Cry*1 expression, in each of the evaluated time points. Consequently, the obtained results (a negative r) induce that the lower the expression of this gene, in each of the evaluated time points, the highest the OSA severity, reinforcing that the expression of this gene is somehow affected in OSA patients. Among the different time points, the strongest correlation for *Cry1* expression and OSA severity was found at 8 a.m. (r=-0.71) (table IV.3 and figure IV.7). Curiously, Doi and its co-workers (2010) observed that *Cry* knockout (KO) mice had hypertension, an abnormal blood pressure circadian rhythm and were hyperglycemic. In accordance, *Cry*1 overexpression in diabetic mice showed to decrease blood glucose and increased insulin sensitivity (Zhang *et al.*, 2010). In this context, *Cry*1 appears to have an important role in glucose control, diabetes and hypertension (Richards *et al.*, 2014). Interestingly, untreated OSA is also associated with those impairments (Darien, 2014). In this context, OSA molecular outcomes may be in the origin of the under expression of *Cry*1 and, consequently, *Cry*1 abnormal expression may be underlying OSA abnormal glucose, diabetes and hypertension. Such hypothesis was never investigated.

In addition, other correlations between clock gene expressions, at specific time points, and OSA severity were observed, although less strong. Such correlations involve the clock genes *Cry2*, *Per1*, *Per2*, *BMAL1*, *CLOCK* and *CK1*ε (table IV.3). *BMAL1*, at 8 a.m., and *CLOCK*, at 10.30 p.m., also showed a negative correlation with OSA severity. Both *BMAL1* and *CLOCK* genes have also been associated with diabetes, obesity and metabolic syndrome. Mutant mice for these genes show impaired glucose tolerance, hypoinsulinaemia and defects in size and proliferation of pancreatic islets (Marcheva *et al.*, 2010; Brainard *et al.*, 2015). By opposite, the correlations found, for the respective time points, between *Cry2*, *Per1*, *Per2* and *CK1*ε and OSA severity were positive (table IV.3). According to the literature, sleep deprivation is accompanied by an increase of *Cry2* mRNA levels. Thus, since OSA causes sleep deprivation, it would make sense that, in OSA patients, *Cry2* mRNA levels would be increased. A dysregulation of *Cry2* expression has also been associated with a higher vulnerability for depression, however depressed bipolar patients show lower *Cry2* mRNA levels (Lavebratt *et al.*, 2010). Regarding *Per1* and *Per2*, abnormal expressions of these gene have been associated with carcinogenesis and the development of malignant tumours (Yang *et al.*, 2009; Chen *et al.*, 2012; Zhao *et al.*, 2014). Concerning *CK1*ε, there are no reports in the literature

regarding the impact of its abnormal expression. Thus, considering that OSA molecular outcomes lead to clock dysfunction, the consequent clock genes expression alterations may be in the origin of OSA long-term consequences.

By increasing the cohort of assessed individuals, specific correlations may become stronger. Analysing the expression of other important related clock genes may also unravel important OSA severity correlations. ROR and REV-ERB genes are examples. In addition, analysing sub groups of OSA severity may help to better dissect such correlations. In the end, a deeper analysis may reveal a set of clock genes, which expression at specific time points may reliably reflect OSA severity.

As already mentioned, the contributors behind clock genes alterations observed in OSA patients are unknown. Evaluating the mechanisms underlying the observed results may provide new insights into the OSA cross talk with circadian rhythms. Having OSA molecular outcomes into consideration, clock genes alterations could be derived from IH mediators, abnormal circulating factors resulting from sympathetic nervous system over activity, inflammatory factors and oxidative stress resultant abnormal TFs. On the other hand, changes in NPY levels could also be contributing to clock genes alterations. NPY levels were reported to be abnormal in the elderly and in individuals with excessive weight, the great majority of the OSA global cohort. Indeed, this neuropeptide was reported to reduce the *Per1* and *Per2* mRNA levels clock genes. (Fukuhara *et al.*, 2001; Glass *et al.*, 2010). However, there are no reports in the literature of the NPY effect on other circadian times neither on other clock genes. Consequently, in the second part of this study, we aimed to investigate whether NPY could be involved in the core clock genes expression modulation and, consequently, if it could be related with OSA molecular clock outcomes.

In order to do so, we opted to use a mouse hypothalamic cell line model (mHYpoE-N42) so that we could evaluate NPY effects directly on the central circadian clock. Although a suprachiasmatic cell line would be more specific, we wanted to have into consideration the communication with other hypothalamic central clocks, due to their critical contribution to clock ticking (Williams & Elmquist, 2012). On the other hand, a cell line would allow for multiple tests and experiments which is essential for a pilot study. In addition, since NPY is not expressed in the opted cell line, it would be easier to evaluate its effect, by adding exogenous NPY. The mHYpoE-N42 neurons circadian rhythm was firstly characterized in order to validate the cellular model. The obtained results showed consistent core clock genes rhythmicity, except for *Cry*. *Per1* and 2 had similar expression profiles and *BMAL1* showed to be downregulated by higher levels of *Per1* and 2 mRNAs and upregulated when *Per1* and 2 gene expressions were decreased. *CLOCK* demonstrated to have a constitutive expression, as expected (figure IV.8 and table IV.5). On the other hand, *Cry* gene

expression oscillations were not evident in mHypo-N42 neurons, possibly a characteristic of the cell line model.

Regarding the NPY role on clock genes expression, we used two approaches to investigate NPY impact on clock genes modulation. Firstly, we tested the effect of exogenous NPY (100 nM), a previously optimized concentration in our laboratory, on clock genes modulation, at several CTs. The obtained results showed that the NPY effect is dependent upon the CT, namely, depending on the CT at which cells were treated with NPY, different clock genes expression changes were observed. After 24h, slight alterations were observed in *Per1* mRNA levels at CT36 and in *Cry*1 mRNA levels at CT48, whereas more evident alterations were observed for *BMAL1* mRNA levels at CT28, *CLOCK* at CT44 and *Per2* mRNA levels at CT72 (figure IV.9 and table IV.5). However, since we assessed the NPY effect, in the same manner, along two cycles of 24h (CT24-48 and CT48-72), it would be expected that the same results obtained from CT24-48 would be obtained from CT48-72, which did not occur. A possible explanation could be the possible loss of synchronization of mHypoE-N42 neurons along time. On the other hand, NPY effect on clock genes may be immediate, as reflected by Fukuhara and colleague's work (Fukuhara *et al.*, 2001). Indeed, these authors observed a decrease of *Per1* gene expression after 30 mins but after 2 hours that effect was lost and normal *Per1* mRNA levels were re-established. Thus, in 24h, circadian clocks may have re-established its normal rhythm and no differences may be observed.

Furthermore, we assessed the effect of a NPY constitutive expression, up to CT48, simulating the loss of NPY levels oscillations, in mHypoE-N42 neurons, so that the impact of disrupted NPY levels could be assessed on clock genes expression profiles. In 2011, Sousa-Ferreira and colleagues observed that six weeks of NPY constitutive expression, in rats, caused altered food intake circadian rhythms, however circadian clock genes expression was not assessed (Sousa-Ferreira *et al.*, 2011). In this context, there are no reports of a similar experiment in the literature. Surprisingly, the obtained results did not showed deep consequences in the clock genes expression profiles. The *Per1* gene was the only gene in which we found a statistically significant difference (at CT 32), in the presence of a NPY constitutive expression (figure IV.11). However such alteration was not sufficient to interfere with the expression profile of this gene and mRNA levels were re-established in the following CT. Thus, the impact of NPY oscillations loss may have effect on circadian rhythms at a longer term, which would not be viable in the context of a cellular model.

In order to confirm the obtained results regarding the NPY effect on clock genes modulation, we assessed whether the circadian rhythm of mHypoE-N42 neurons was indeed being affected by external factors. Consequently, we performed a positive control experiment by using resveratrol

(10 µM), an activator of SIRT-1 which is essential for *BMAL1* and *Per2* gene transcription (Asher *et al.*, 2008; Bellet *et al.*, 2013). The obtained results were consistent with the reported in the literature (Asher *et al.*, 2008; Bellet *et al.*, 2013), and *BMAL1* and *Per2* mRNA levels increased, validating the ability of external factors to interfere with mHypoE-N42 neurons circadian rhythms (figure IV.12).

Thus, in the context of our work, we suggest that NPY is able to modulate clock genes expression, in a CT dependent manner. However, its constitutive expression and consequent arrhythmia, at short-term, may not be sufficient to induce clock gene expression alterations. Further experiments must be performed in order to evaluate NPY long-term impact on circadian rhythm molecular machinery, so that, the possible involvement of NPY in OSA clock genes dysfunction may be elucidated.

CHAPTER VI:

CONCLUSION

Through the present study, we were able to conclude that:

1) **OSA causes clock dysfunction, which is clearly reflected by disruptions in the expression of several clock genes before treatment.**
   Such disruptions affect not only the typical oscillatory expression profiles but also clock genes mRNA levels.

2) **The expression of specific clock genes, at specific times of the day (e.g. Cry1 at 8 a.m.), may be used as OSA biomarkers and reflect OSA severity.**
   Several correlations between clock genes expression and OSA severity were found. Among them, Cry1, at 8 a.m., showed to be highly correlated with OSA AHI: the lower the expression of Cry1, at this specific time of the day, the higher OSA AHI and, subsequently, OSA severity. Thus, Cry1, at 8h, may be a putative OSA biomarker. Since clock genes are implicated in a broad range of physiological functions, disruptions in their mRNA levels, caused by OSA consequences, may be in the origin of OSA implication as a risk factor for metabolic and cardiovascular disorders.

3) **NPY is able to modulate clock genes expression, in a CT dependent manner, and a short constitutive expression of NPY, in hypothalamic neurons, is not sufficient to impact on clock genes modulation.**

Figure VI.1 resumes the main conclusions of the present work.

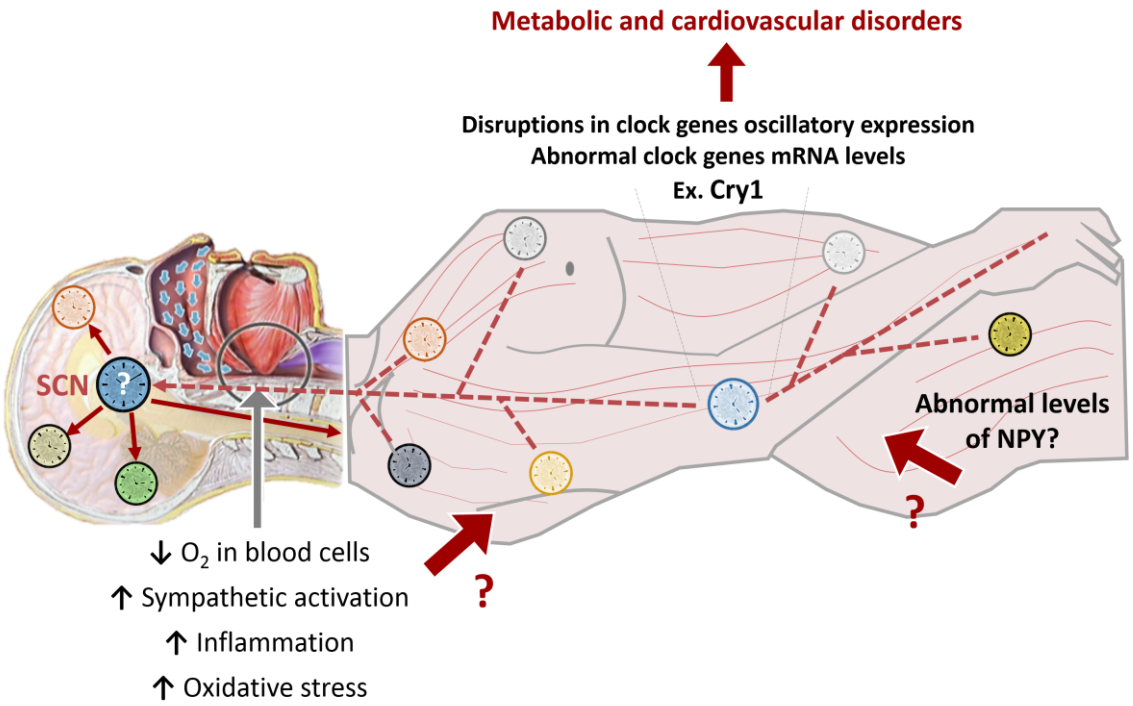


Figure VI. 1: Graphical abstract of OSA and circadian rhythms cross-talk. OSA characteristic apnea and hypopnea events lead to a decrease in blood cells oxygenation, an over activation of the sympathetic nervous system, inflammation and oxidative stress. These factors, together with abnormal NPY levels, characteristic of obese and elderly individuals, may underlie OSA clock dysfunction. Such dysfunction is characterized by disruptions in the clock genes oscillatory expressions and abnormal clock genes mRNA levels (ex.Cry1). Consequently, such alterations may be in the origin of OSA long-term outcomes such as metabolic and cardiovascular disorders.

FUTURE PERSPECTIVES

In order to complement this study, it would be important to show OSA clock dysfunction through other circadian rhythm markers, such as plasma cortisol and melatonin. In addition, the expression of other clock associated genes (e.g. ROR and REV-ERB) could also be assessed so that a higher spectrum of clock gene expression changes could be obtained. Such information would be helpful not only to understand the causes behind clock dysfunction but also to detect other possible correlations with OSA severity. On the other hand, increasing the number of assessed OSA patients, especially of different severity subgroups, through epidemiologic studies, would be essential to validate the obtained results. In addition, it would be interesting to compare clock gene expression profiles between OSA patients and sex and age-matched healthy individuals. Although we did not consider other individuals as adequate controls, due to possible circadian rhythm disruptions, using a greater cohort of healthy individuals could support that OSA patients' clock dysfunction is indeed caused by OSA. In the future, it would also be interesting to perform the same study in women and children. OSA varies among these groups, thus different clock

alterations may be involved. A validated system of OSA clock biomarkers may provide potential early diagnostic tools and lead to more personalized treatments.

On the other hand, further experiments should be performed in order to understand the mechanisms responsible for the observed disruptions in OSA patient's clock genes expression. In this context, it would be interesting to assess clock protein levels and to understand whether a decrease/increase of the genetic expression is indeed accompanied by a decrease/increase in the correspondent protein levels. Post-transcriptional, translational and post-translational mechanisms are also important players in the circadian rhythmicity and must be taken into consideration (Oishi *et al.*, 2003; McCarthy *et al.*, 2007; Miller *et al.*, 2007). For example, microRNAs, non-coding regulatory transcripts, have also been suggested as potential players in the circadian clock, regulating clock genes expression (Hansen *et al.*, 2011). Consequently, it would also be important to address this question and clarify whether the alterations in OSA patients' clock genes are directly caused by OSA molecular outcomes or indirectly by altered miRNA levels, other potential OSA biomarkers.

To elucidate how OSA outcomes lead to such dysregulation, it would be interesting to assess key biochemical parameters in OSA patient's plasma, among which NPY levels. The long-term NPY impact on the central clock genes modulation should also be further investigated. In the future it would also be interesting to investigate the NPY receptors through which NPY is modulating clock genes expression. Different receptors may be underlying the different NPY effects on clock genes expression. Understanding how NPY modulates the clock molecular machinery may provide valuable insights not only in the OSA context but also for obesity. NPY manipulation, through possible pharmacological therapies, may ameliorate such disorders, based on circadian rhythm resynchronization.

CHAPTER VII:

REFERENCES

Abe, M., Herzog, E.D., Yamazaki, S., Straume, M., Tei, H., Sakaki, Y., Menaker, M., & Block, G.D. (2002) Circadian rhythms in isolated brain regions. *J. Neurosci.*, **22**, 350–356.

Adrian, T.E., Allen, J.M., Bloom, S.R., Ghatei, M.A., Rossor, M.N., Roberts, G.W., Crow, T.J., Tatemoto, K., & Polak, J.M. (1983) Neuropeptide Y distribution in human brain. *Nature*, **306**, 584–586.

Affymetrix (2003) An Analysis of Blood Processing Methods to Prepare Samples for GeneChip® Expression Profiling\n. *Techical note RNA arrays reagents*, 1–16.

Ahmadi, N., Shapiro, G.K., Chung, S.A., & Shapiro, C.M. (2009) Clinical diagnosis of sleep apnea based on single night of polysomnography vs. two nights of polysomnography. *Sleep Breath.*, **13**, 221–226.

Akabayashi, A., Levin, N., Paez, X., Alexander, J.T., & Leibowitz, S.F. (1994) Hypothalamic neuropeptide Y and its gene expression: relation to light/dark cycle and circulating corticosterone. *Mol. Cell. Neurosci.*, **5**, 210–218.

Albrecht, U. (2002) Functional Genomics of Sleep and Circadian Rhythm: Invited Review: Regulation of mammalian circadian clock genes. *J. Appl. Physiol.*, **92**, 1348–1355.

Albrecht, U. (2012) Timing to Perfection: The Biology of Central and Peripheral Circadian Clocks. *Neuron*, **74**, 246–260.

Albrecht, U., Sun, Z.S., Eichele, G., & Lee, C.C. (1997) A differential response of two putative mammalian circadian regulators, mper1 and mper2, to light. *Cell*, **91**, 1055–1064.

Allen, J.M., Hughes, J., & Bloom, S.R. (1987) Presence, distribution, and pharmacological effects of neuropeptide Y in mammalian gastrointestinal tract. *Dig. Dis. Sci.*, **32**, 506–512.

Allen, J.M., McGregor, G.P., Woodhams, P.L., Polak, J.M., & Bloom, S.R. (1984) Ontogeny of a novel peptide, neuropeptide Y (NPY) in rat brain. *Brain Res.*, **303**, 197–200.

Allen, Y.S., Adrian, T.E., Allen, J.M., Tatemoto, K., Crow, T.J., Bloom, S.R., & Polak, J.M. (1983) Neuropeptide Y distribution in the rat brain. *Science (80-.).*, **221**, 877–879.

Alonso-Fernández, A., García-Río, F., Arias, M.A., Hernanz, A., de la Peña, M., Piérola, J., Barceló, A., López-Collazo, E., & Agustí, A. (2009) Effects of CPAP on oxidative stress and nitrate efficiency in sleep apnoea: a randomised trial. *Thorax*, **64**, 581–586.

Alvaro, A., Martins, J., Costa, A., Fernandes, E., Carvalho, F., Ambrósio, A., & Cavadas, C. (2008) Neuropeptide Y protects retinal neural cells against cell death induced by ecstasy. *Neuroscience*, **152**, 97–105.

Alvaro, A.R., Martins, J., Araújo, I.M., Rosmaninho-Salgado, J., Ambrósio, A.F., & Cavadas, C. (2008) Neuropeptide Y stimulates retinal neural cell proliferation--involvement of nitric oxide. *J. Neurochem.*, **105**, 2501–2510.

Amir, S., Lamont, E.W., Robinson, B., & Stewart, J. (2004) A circadian rhythm in the expression of PERIOD2 protein reveals a novel SCN-controlled oscillator in the oval nucleus of the bed nucleus of the stria terminalis. *J. Neurosci.*, **24**, 781–790.

Archer, S.N., Laing, E.E., Möller-levet, C.S., Veen, D.R. Van Der, Bucca, G., Lazar, A.S., Santhi, N., Slak, A., Kabiljo, R., Schantz, M. Von, Smith, C.P., & Dijk, D. (2014) Mistimed sleep disrupts circadian regulation of the human transcriptome. *Proc Natl Acad Sci U S A.*, **111**, E682–E691.

Archontogeorgis, K., Nena, E., Papanas, N., & Steiropoulos, P. (2014) Biomarkers to improve diagnosis and monitoring of obstructive sleep apnea syndrome: Current status and future perspectives. *Pulm. Med.*, **2014**.

Asher, G., Gatfield, D., Stratmann, M., Reinke, H., Dibner, C., Kreppel, F., Mostoslavsky, R., Alt, F.W., & Schibler, U. (2008) SIRT1 Regulates Circadian Clock Gene Expression through PER2 Deacetylation. *Cell*, **134**, 317–328.

Attal, P. & Chanson, P. (2010) Endocrine aspects of obstructive sleep apnea. *J. Clin. Endocrinol. Metab.*, **95**, 483–495.

BaHammam, A.S., Obeidat, A., Barataman, K., Bahammam, S.A., Olaish, A.H., & Sharif, M.M. (2014) A comparison between the AASM 2012 and 2007 definitions for detecting hypopnea. *Sleep Breath.*, 1–7.

Ballard, R. (2008) Management of patients with obstructive sleep apnea. *J Fam Pr.*, **57**, 24–30.

Balsalobre, A., Brown, S.A., Marcacci, L., Tronche, F., Kellendonk, C., Reichardt, H.M., Schütz, G., & Schibler, U. (2000) Resetting of circadian time in peripheral tissues by glucocorticoid signaling. *Science*, **289**, 2344–2347.

Balsalobre, A., Damiola, F., & Schibler, U. (1998) A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell*, **93**, 929–937.

Barger, L.K., Lockley, S.W., Rajaratnam, S.M.W., & Landrigan, C.P. (2009) Neurobehavioral, health, and safety consequences associated with shift work in safety-sensitive professions. *Curr. Neurol. Neurosci. Rep.*, **9**, 155–164.

Bellet, M.M., Nakahata, Y., Boudjelal, M., Watts, E., Mossakowska, D.E., Edwards, K. a, Cervantes, M., Astarita, G., Loh, C., Ellis, J.L., Vlasuk, G.P., & Sassone-Corsi, P. (2013) Pharmacological modulation of circadian rhythms by synthetic activators of the deacetylase SIRT1. *Proc. Natl. Acad. Sci. U. S. A.*, **110**, 3333–3338.

Bent, S., Padula, A., Moore, D., Patterson, M., & Mehling, W. (2006) Valerian for Sleep: A Systematic Review and Meta-Analysis. *Am. J. Med.*, **119**, 1005–1012.

Berry, R.B., Budhiraja, R., Gottlieb, D.J., Gozal, D., Iber, C., Kapur, V.K., Marcus, C.L., Mehra, R., Parthasarathy, S., Quan, S.F., Redline,

S., Strohl, K.P., Ward, S.L.D., & Tangredi, M.M. (2012) Rules for scoring respiratory events in sleep: Update of the 2007 AASM manual for the scoring of sleep and associated events. *J. Clin. Sleep Med.*, **8**, 597–619.

Biello, S.M., Janik, D., & Mrosovsky, N. (1994) Neuropeptide y and behaviorally induced phase shifts. *Neuroscience*, **62**, 273–279.

Boivin, D.B., James, F.O., Wu, A., Cho-Park, P.F., Xiong, H., & Sun, Z.S. (2003) Circadian clock genes oscillate in human peripheral blood mononuclear cells. *Blood*, **102**, 4143–4145.

Boivin, D.B., James, F.O., Wu, A., Cho-park, P.F., Xiong, H., & Sun, Z.S. (2015) Brief report Circadian clock genes oscillate in human peripheral blood mononuclear cells **102**, 4143–4146.

Borbély, A.A. (1982) A two process model of sleep regulation. *Hum. Neurobiol.*, **1**, 195–204.

Borrelli, E., Nestler, E.J., Allis, C.D., & Sassone-Corsi, P. (2008) Decoding the Epigenetic Language of Neuronal Plasticity. *Neuron*, **60**, 961–974.

Botelho, M. & Cavadas, C. (2015) Neuropeptide Y: An Anti-Aging Player? *Trends Neurosci.*, **38**, 701–711.

Brainard, J., Gobel, M., Scott, B., Koeppen, M., & Eckle, T. (2015) Health Implications of Disrupted Circadian Rhythms and the Potential for Daylight as Therapy. *Anesthesiology*, **122**, 1170–1175.

Browaldh, N., Nerfeldt, P., Lysdahl, M., Bring, J., & Friberg, D. (2013) SKUP3 randomised controlled trial: polysomnographic results after uvulopalatopharyngoplasty in selected patients with obstructive sleep apnoea. *Thorax*, **68**, 846–853.

Burioka, N., Fukuoka, Y., Takata, M., Endo, M., Miyata, M., Chikumi, H., Tomita, K., Kodani, M., Touge, H., Takeda, K., Sumikawa, T., Yamaguchi, K., Ueda, Y., Nakazaki, H., Suyama, H., Yamasaki, A., Sano, H., Igishi, T., & Shimizu, E. (2007) Circadian rhythms in the CNS and peripheral clock disorders: function of clock genes: influence of medication for bronchial asthma on circadian gene. *J. Pharmacol. Sci.*, **103**, 144–149.

Burioka, N., Koyanagi, S., Endo, M., Takata, M., Fukuoka, Y., Miyata, M., Takeda, K., Chikumi, H., Ohdo, S., & Shimizu, E. (2008) Clock gene dysfunction in patients with obstructive sleep apnoea syndrome. *Eur. Respir. J.*, **32**, 105–112.

Calik, M. (2016) Treatments for Obstructive Sleep Apnea. *JCOM*, **23**, 181–192.

Calverley, P. (1997) Obstructive sleep apnoea: a progressive disorder? *Thorax*, **52**, 843–844.

Camara, F.J., Rivera, C.G., Barreto, D.B., Meneses, P.L., Baena, E.M., & Calvo, R.E. (2013) Gender differences in obstructive sleep apnea syndrome. *Sleep Med.*, **14**, e85.

Canaple, L., Rambaud, J., Dkhissi-Benyahya, O., Rayet, B., Tan, N.S., Michalik, L., Delaunay, F., Wahli, W., & Laudet, V. (2006) Reciprocal regulation of brain and muscle Arnt-like protein 1 and peroxisome proliferator-activated receptor alpha defines a novel positive feedback loop in the rodent liver circadian clock. *Mol. Endocrinol.*, **20**, 1715–1727.

Cannizzaro, C., Tel, B.C., Rose, S., Zeng, B.Y., & Jenner, P. (2003) Increased neuropeptide Y mRNA expression in striatum in Parkinson's disease. *Mol. Brain Res.*, **110**, 169–176.

Caples, S.M. (2005) Obstructive Sleep Apnea. *Ann. Intern. Med.*, **142**, 187–197.

Carpagnano, G., Kharitonov, S., Resta, O., Foschino-Barbaro, M., Gramiccioni, E., & Barnes, P. (2002) Increased 8-isoprostane and interleukin-6 in breath condensate of obstructive sleep apnea patients. *Chest*, **122**, 1162–1167.

Chee, M.J.S. & Colmers, W.F. (2008) Y eat? *Nutrition*, **24**, 869–877.

Chen, R., Yang, K., Zhao, N.-B., Zhao, D., Chen, D., Zhao, C.-R., & Tang, H. (2012) Abnormal expression of PER1 circadian-clock gene in oral squamous cell carcinoma. *Onco. Targets. Ther.*, **5**, 403–407.

Chilov, D., Hofer, T., Bauer, C., Wenger, R.H., & Gassmann, M. (2001) Hypoxia affects expression of circadian genes PER1 and CLOCK in mouse brain. *FASEB J.*, **15**, 2613–2622.

Cirelli, C. (2014) The genetics and molecular regulation of sleep: from fruit flies to humans. *Am. J. Psychother.*, **68**, 277–286.

Cirelli, C., Gutierrez, C.M., & Tononi, G. (2004) Extensive and Divergent Effects of Sleep and Wakefulness on Brain Gene Expression. *Neuron*, **41**, 35–43.

Collop, N. a (2008) Portable monitoring for the diagnosis of obstructive sleep apnea. *Curr. Opin. Pulm. Med.*, **14**, 525–529.

Colten, H.R. & Altevogt, B.M. (2006) Sleep Physiology. In *Sleep Disorders and Sleep Deprivation: An Unmet Public Health Problem*. pp. 33–54.

Costa, S.S.F. & Ripperger, J.A. (2015) Impact of the circadian clock on the aging process. *Front. Neurol.*, **6**.

Cowell, I.G. (2002) E4BP4/NFIL3, a PAR-related bZIP factor with many roles. *BioEssays*, **24**, 1023–1029.

Coy, T. V, Dimsdale, J.E., Ancoliisrael, S., & Clausen, J. (1996) Sleep apnoea and sympathetic nervous system activity: a review. *J Sleep Res*, **5**, 42–50.

Crocker, A. & Sehgal, A. (2010) Genetic analysis of sleep. *Genes Dev.*, **24**, 1220–1235.

Crosio, C., Cermakian, N., Allis, C.D., & Sassone-Corsi, P. (2000) Light induces chromatin modification in cells of the mammalian

circadian clock. *Nat. Neurosci.*, **3**, 1241–1247.

Cuesta, M., Cermakian, N., & Boivin, D.B. (2014) Glucocorticoids entrain molecular clock components in human peripheral cells. *FASEB J.*, **29**, 1360–1370.

Damiola, F., Le Minli, N., Preitner, N., Kornmann, B., Fleury-Olela, F., & Schibler, U. (2000) Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes Dev.*, **14**, 2950–2961.

Darien, I. (2014) *International Classification of Sleep Disorders: Diagnostic and Coding Manual*, 3rd Edition, American Academy of Sleep Medicine.

Davies, C., Morroll, D., Prinja, D., Mann, D., & Gibbs, A. (1990) A quantitative assessment of somatostatin-like and neuropeptide Y-like immunostained cells in the frontal and temporal cortex of patients with Alzheimer's disease. *J Neurol Sci.*, **96**, 59–73.

De Luca Canto, G., Pacheco-Pereira, C., Aydinoz, S., Major, P.W., Flores-Mir, C., & Gozal, D. (2015) Diagnostic capability of biological markers in assessment of obstructive sleep apnea: A systematic review and meta-analysis. *J. Clin. Sleep Med.*, **11**, 27–36.

De Luca Canto, G., Pachêco-Pereira, C., Aydinoz, S., Major, P.W., Flores-Mir, C., & Gozal, D. (2015) Biomarkers associated with obstructive sleep apnea: A scoping review. *Sleep Med. Rev.*, **23**, 28–45.

Deacon, N.L., Jen, R., Li, Y., & Malhotra, A. (2015) Treatment of Obstructive Sleep Apnea: Prospects for Personalized Combined Modality Therapy. *Ann. Am. Thorac. Soc.*, **13**, 101–108.

Debey, S., Schoenbeck, U., Hellmich, M., Gathof, B.S., Pillai, R., Zander, T., & Schultze, J.L. (2004) Comparison of different isolation techniques prior gene expression profiling of blood derived cells: impact on physiological responses, on overall expression and the role of different cell types. *Pharmacogenomics J.*, **4**, 193–207.

Debono, M., Ghobadi, C., Rostami-Hodjegan, A., Huatan, H., Campbell, M.J., Newell-Price, J., Darzy, K., Merke, D.P., Arlt, W., & Ross, R.J. (2009) Modified-release hydrocortisone to provide circadian cortisol profiles. *J. Clin. Endocrinol. Metab.*, **94**, 1548–1554.

Dibner, C., Schibler, U., & Albrecht, U. (2010) *The Mammalian Circadian Timing System: Organization and Coordination of Central and Peripheral Clocks.*, Annual review of physiology.

Doi, M., Takahashi, Y., Komatsu, R., Yamazaki, F., Yamada, H., Haraguchi, S., Emoto, N., Okuno, Y., Tsujimoto, G., Kanematsu, A., Ogawa, O., Todo, T., Tsutsui, K., van der Horst, G.T.J., & Okamura, H. (2010) Salt-sensitive hypertension in circadian clock-deficient Cry-null mice involves dysregulated adrenal Hsd3b6. *Nat. Med.*, **16**, 67–74.

Dominguez-Rodriguez, A., Abreu-Gonzalez, P., & Kaski, J.C. (2009) Disruption of normal circadian rhythms and cardiovascular events. *Hear. Metab.*, **44**, 11–15.

Dufour, C.R., Levasseur, M.P., Pham, N.H.H., Eichner, L.J., Wilson, B.J., Charest-Marcotte, A., Duguay, D., Poirier-Héon, J.-F., Cermakian, N., & Giguère, V. (2011) Genomic Convergence among ERRα, PROX1, and BMAL1 in the Control of Metabolic Clock Outputs. *PLoS Genet.*, **7**, e1002143.

Dumermuth, G., Lange, B., Lehmann, D., Meier, C.A., Dinkelmann, R., & Molinari, L. (1983) Spectral analysis of all-night sleep EEG in healthy adults. *Eur. Neurol.*, **22**, 322–339.

Eastwood, P.R., Barnes, M., Walsh, J.H., Maddison, K.J., Hee, G., Schwartz, A.R., Smith, P.L., Malhotra, A., McEvoy, R.D., Wheatley, J.R., O'Donoghue, F.J., Rochford, P.D., Churchward, T., Campbell, M.C., Palme, C.E., Robinson, S., Goding, G.S., Eckert, D.J., Jordan, A.S., Catcheside, P.G., Tyler, L., Antic, N.A., Worsnop, C.J., Kezirian, E.J., & Hillman, D.R. (2011) Treating obstructive sleep apnea with hypoglossal nerve stimulation. *Sleep*, **34**, 1479–1486.

Eckel-Mahan, K. & Sassone-Corsi, P. (2013) Metabolism and the circadian clock converge. *Physiol. Rev.*, **93**, 107–135.

Edelsbrunner, M., Painsipp, E., Herzog, H., & Holzer, P. (2009) Evidence from knockout mice for distinct implications of neuropeptide-Y Y2 and Y4 receptors in the circadian control of locomotion, exploration, water and food intake. *Neuropeptides*, **43**, 491–497.

Egg, M., Köblitz, L., Hirayama, J., Schwerte, T., Folterbauer, C., Kurz, A., Fiechtner, B., Möst, M., Salvenmoser, W., Sassone-Corsi, P., & Pelster, B. (2013) Linking oxygen to time: the bidirectional interaction between the hypoxic signaling pathway and the circadian clock. *Chronobiol. Int.*, **30**, 510–529.

Ejaz, S.M., Khawaja, I.S., Bhatia, S., & Hurwitz, T.D. (2011) Obstructive sleep apnea and depression: A review. *Innov. Clin. Neurosci.*, **8**, 17–25.

Feliciano, A., Torres, V.M., Vaz, F., Carvalho, A.S., Matthiesen, R., Pinto, P., Malhotra, A., Bárbara, C., & Penque, D. (2015) Overview of proteomics studies in obstructive sleep apnea. *Sleep Med.*, **16**, 437–445.

Ferber, R., Millman, R., Coppola, M., Fleetham, J., Murray, C.F., Iber, C., McCall, V., Nino-Murcia, G., Pressman, M., & Sanders, M. (1994) Portable recording in the assessment of obstructive sleep apnea. ASDA standards of practice. *Sleep*, **17**, 378–392.

Fox, S. I (2014) *Human Physiology*, 13th Edition, The McGraw−Hill Companies.

Franken, P. & Dijk, D. (2009) Circadian clock genes and sleep homeostasis. *Eur. J. Neurosci.*, **29**, 1820–1829.

Fredriksson, R., Lagerström, M.C., Lundin, L.-G., & Schiöth, H.B. (2003) The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol. Pharmacol.*, **63**, 1256–1272.

Fu, L., Pelicano, H., Liu, J., Huang, P., & Lee, C.C. (2002) The circadian gene Period2 plays an important role in tumor suppression and

DNA damage response in vivo. *Cell*, **111**, 41–50.

Fukuhara, C., Brewer, J.M., Dirden, J.C., Bittman, E.L., Tosini, G., & Harrington, M.E. (2001) Neuropeptide Y rapidly reduces Period1 and Period2 mRNA levels in the hamster suprachiasmatic nucleus. *Neurosci. Lett.*, **314**, 119–122.

Gachon, F., Nagoshi, E., Brown, S.A., Ripperger, J., & Schibler, U. (2004) The mammalian circadian timing system: from gene expression to physiology. *Chromosoma*, **113**, 103–112.

George, C.F.P. (2007) Sleep apnea, alertness, and motor vehicle crashes. *Am. J. Respir. Crit. Care Med.*, **176**, 954–956.

Ghorbel, M.T., Coulson, J.M., & Murphy, D. (2003) Cross-talk between hypoxic and circadian pathways: cooperative roles for hypoxia-inducible factor 1alpha and CLOCK in transcriptional activation of the vasopressin gene. *Mol. Cell. Neurosci.*, **22**, 396–404.

Giuffre, M., Heidenreich, T., Carney-Gersten, P., Dorsch, J. a, & Heidenreich, E. (1990) The relationship between axillary and core body temperature measurements. *Appl. Nurs. Res.*, **3**, 52–55.

Glass, J.D., Guinn, J., Kaur, G., & Francl, J.M. (2010) On the intrinsic regulation of neuropeptide y release in the mammalian suprachiasmatic nucleus circadian clock. *Eur. J. Neurosci.*, **31**, 1117–1126.

Greenstone, M. & Hack, M. (2014) Obstructive sleep apnoea. *BMJ*, **348**, g3745–g3745.

Gruenewald, D.A., Naai, M.A., Marck, B.T., & Matsumoto, A.M. (1994) Age-related decrease in neuropeptide-Y gene expression in the arcuate nucleus of the male rat brain is independent of testicular feedback. *Endocrinology*, **134**, 2383–2389.

Gupta, R.K., Chandra, a, Verm, a K., & Kumar, S. (2010) Obstructive sleep apnoea: a clinical review. *J. Assoc. Physicians India*, **58**, 438–441.

Hansen, K.F., Sakamoto, K., & Obrietan, K. (2011) MicroRNAs: a potential interface between the circadian clock and human health. *Genome Med.*, **3**, 10.

Harrington, M.E. & Schak, K.M. (2000) Neuropeptide Y phase advances the in vitro hamster circadian clock during the subjective day with no effect on phase during the subjective night. *Can. J. Physiol. Pharmacol.*, **78**, 87–92.

Hastings, M.H., Duffield, G.E., Ebling, F.J.P., Kidd, A., Maywood, E.S., & Schurov, I. (1997) Non-photic signalling in the suprachiasmatic nucleus. *Biol. Cell*, **89**, 495–503.

Hastings, M.H. & Goedert, M. (2013) Circadian clocks and neurodegenerative diseases: Time to aggregate? *Curr. Opin. Neurobiol.*, **23**(5), 880-7.

Heilig, M. (2004) The NPY system in stress, anxiety and depression. *Neuropeptides*, **38**, 213–224.

Hida, A., Kusanagi, H., Satoh, K., Kato, T., & Matsumoto, Y. (2009) Expression profiles of PERIOD1 , 2 , and 3 in peripheral blood mononuclear cells from older subjects. *Life Sci.*, **84**, 33–37.

Honma, S., Kawamoto, T., Takagi, Y., Fujimoto, K., Sato, F., Noshiro, M., Kato, Y., & Honma, K. (2002) Dec1 and Dec2 are regulators of the mammalian molecular clock. *Nature*, **419**, 841–844.

Hörsten, S. von, Hoffmann, T., Alfalah, M., Wrann, C.D., Karl, T., Pabst, R., & Bedoui, S. (2004) *Neuropeptide Y and Related Peptides*, Handbook of Experimental Pharmacology. Springer Berlin Heidelberg.

Huang, W., Ramsey, K.M., Marcheva, B., & Bass, J. (2011) Circadian rhythms, sleep, and metabolism. *J. Clin. Invest.*, **121**, 2133–2141.

Hurst, W.J., Earnest, D., & Gillette, M.U. (2002) Immortalized suprachiasmatic nucleus cells express components of multiple circadian regulatory pathways. *Biochem Biophys Res Commun*, **292**, 20–30.

Jagannath, A., Peirson, S.N., & Foster, R.G. (2013) Sleep and circadian rhythm disruption in neuropsychiatric illness. *Curr. Opin. Neurobiol.*, **23**, 888–894.

Kalra, S.P. & Kalra, P.S. (2004) NPY and cohorts in regulating appetite, obesity and metabolic syndrome: Beneficial effects of gene therapy. *Neuropeptides*, **38**, 201–211.

Kapur, V.K. (2010) Obstructive sleep apnea: diagnosis, epidemiology, and economics. *Respir. Care*, **55**, 1155–1167.

Kim, E.J., Yoo, Y.G., Yang, W.K., Lim, Y.S., Na, T.Y., Lee, I.K., & Lee, M.O. (2008) Transcriptional activation of HIF-1 by RORalpha and its role in hypoxia signaling. *Arterioscler. Thromb. Vasc. Biol.*, **28**, 1796–1802.

King, B.M. (2006) The rise, fall, and resurrection of the ventromedial hypothalamus in the regulation of feeding behavior and body weight. *Physiol. Behav.*, **87**, 221–244.

Ko, C.H. & Takahashi, J.S. (2006) Molecular components of the mammalian circadian clock. *Hum Mol Genet*, **15**, 1–7.

Kohsaka, A., Laposky, A.D., Ramsey, K.M., Estrada, C., Joshu, C., Kobayashi, Y., Turek, F.W., & Bass, J. (2007) High-Fat Diet Disrupts Behavioral and Molecular Circadian Rhythms in Mice. *Cell Metab.*, **6**, 414–421.

Kondratova, A. a & Kondratov, R. V (2012) The circadian clock and pathology of the ageing brain. *Nat. Rev. Neurosci.*, **13**, 325–335.

Konopka, R.J. & Benzer, S. (1971) Clock mutants of Drosophila melanogaster. *Proc. Natl. Acad. Sci. U. S. A.*, **68**, 2112–2116.

Kräuchi, K., Cajochen, C., Werth, E., & Wirz-Justice, A. (2002) Alteration of Internal Circadian Phase Relationships after Morning versus

Evening Carbohydrate-Rich Meals in Humans. *J. Biol. Rhythms*, **17**, 364–376.

Kryger, M.H., Roth, T., & Dement, W.C. (2010) Principles and Practice of Sleep Medicine. In *Melatonin and the Regulation of Sleep and Circadian Rhythms*, 5th edn. Saunders.

Kuna, S.T. (2010) Portable-monitor testing: an alternative strategy for managing patients with obstructive sleep apnea. *Respir. Care*, **55**, 1196–1215.

Kuo, L.E., Kitlinska, J.B., Tilan, J.U., Li, L., Baker, S.B., Johnson, M.D., Lee, E.W., Burnett, M.S., Fricke, S.T., Kvetnansky, R., Herzog, H., & Zukowska, Z. (2007) Neuropeptide Y acts directly in the periphery on fat tissue and mediates stress-induced obesity and metabolic syndrome. *Nat. Med.*, **13**, 803–811.

Kushida, C. a, Littner, M.R., Morgenthaler, T., Alessi, C. a, Bailey, D., Coleman, J., Friedman, L., Hirshkowitz, M., Kapen, S., Kramer, M., Lee-Chiong, T., Loube, D.L., Owens, J., Pancer, J.P., & Wise, M. (2005) Practice parameters for the indications for polysomnography and related procedures: an update for 2005. *Sleep*, **28**, 499–521.

Lamont, E.W., James, F.O., Boivin, D.B., & Cermakian, N. (2007) From circadian clock gene expression to pathologies **8**, 547–556.

Lamont, E.W., Legault-Coutu, D., Cermakian, N., & Boivin, D.B. (2007) The role of circadian clock genes in mental disorders. *Dialogues Clin. Neurosci.*, **9**, 333–342.

Landgraf, D., Shostak, A., & Oster, H. (2012) Clock genes and sleep. *Pflugers Arch. Eur. J. Physiol.*, **463**, 3–14.

Landry, G.J. & Liu-Ambrose, T. (2014) Buying time: A rationale for examining the use of circadian rhythm and sleep interventions to delay progression of mild cognitive impairment to Alzheimer's disease. *Front. Aging Neurosci.*, **6**, 1–21.

Larhammar, D., Beck-Sickinger, A., Colmers, W.F., Cox, H.M., Doods, H.N., Herzog, H., Michel, M.C., Quirion, R., Schwartz, T., & Westfall, T. (2015) Neuropeptide Y receptors [WWW Document]. *IUPHAR/BPS Guid. to Pharmacol.,*. URL http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=46

Lavebratt, C., Sjöholm, L.K., Soronen, P., Paunio, T., Vawter, M.P., Bunney, W.E., Adolfsson, R., Forsell, Y., Wu, J.C., Kelsoe, J.R., Partonen, T., & Schalling, M. (2010) CRY2 is associated with depression. *PLoS One*, **5**, e9407.

Lavie, L. (2003) Obstructive sleep apnoea syndrome - An oxidative stress disorder. *Sleep Med. Rev.*, **7**, 35–51.

Leidinger, P., Backes, C., Meder, B., Meese, E., & Keller, A. (2014) The human miRNA repertoire of different blood compounds **15**, 1–8.

Lewy, a J. (1999) The dim light melatonin onset, melatonin assays and biological rhythm research in humans. *Biol. Signals Recept.*, **8**, 79–83.

Lewy, a J., Cutler, N.L., & Sack, R.L. (1999) The endogenous melatonin profile as a marker for circadian phase position. *J. Biol. Rhythms*, **14**, 227–236.

Lin, Q.-C., Zhang, X.-B., Chen, G.-P., Huang, D.-Y., Din, H.-B., & Tang, A.-Z. (2012) Obstructive sleep apnea syndrome is associated with some components of metabolic syndrome in nonobese adults. *Sleep Breath.*, **16**, 571–578.

Liu, C., Weaver, D.R., Jin, X., Shearman, L.P., Pieschl, R.L., Gribkoff, V.K., & Reppert, S.M. (1997) Molecular dissection of two distinct actions of melatonin on the suprachiasmatic circadian clock. *Neuron*, **19**, 91–102.

Lockinger, A., Köberle, D., König, P.S., Saria, A., Herold, M., Corn??lissen, G., & Halberg, F. (2004) Neuropeptide chronomics in clinically healthy young adults: Circaoctohoran and circadian patterns. *Peptides*, **25**, 533–542.

Lopez-Molina, L., Conquet, F., Dubois-Dauphin, M., & Schibler, U. (1997) The DBP gene is expressed according to a circadian rhythm in the suprachiasmatic nucleus and influences circadian behavior. *EMBO J.*, **16**, 6762–6771.

Lowrey, P.L. & Takahashi, J.S. (2004) Mammalian circadian biology: elucidating genome-wide levels of temporal organization. *Annu. Rev. Genomics Hum. Genet.*, **5**, 407–441.

Lumeng, J.C. & Chervin, R.D. (2008) Epidemiology of pediatric obstructive sleep apnea. *Proc. Am. Thorac. Soc.*, **5**, 242–252.

Lurie, A. (2011) Obstructive sleep apnea in adults: epidemiology, clinical presentation, and treatment options. *Adv. Cardiol.*, **46**, 1–42.

Madani, M. & Madani, F. (2009) Epidemiology, Pathophysiology, and Clinical Features of Obstructive Sleep Apnea. *Oral Maxillofac. Surg. Clin. North Am.*, **21**, 369–375.

Maeder, M.T., Strobel, W., Christ, M., Todd, J., Estis, J., Wildi, K., Thalmann, G., Hilti, J., Brutsche, M., Twerenbold, R., Rickli, H., & Mueller, C. (2015) Comprehensive biomarker pro fi ling in patients with obstructive sleep apnea. *Clin. Biochem.*, **48**, 340–346.

Manalo, D.J., Rowan, A., Lavoie, T., Natarajan, L., Kelly, B.D., Ye, S.Q., Garcia, J.G.N., & Semenza, G.L. (2005) Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1. *Blood*, **105**, 659–669.

Marcheva, B., Ramsey, K.M., Buhr, E.D., Kobayashi, Y., Su, H., Ko, C.H., Ivanova, G., Omura, C., Mo, S., Vitaterna, M.H., Lopez, J.P., Philipson, L.H., Bradfield, C.A., Crosby, S.D., JeBailey, L., Wang, X., Takahashi, J.S., & Bass, J. (2010) Disruption of the clock components CLOCK and BMAL1 leads to hypoinsulinaemia and diabetes. *Nature*, **466**, 627–631.

Mason, M., Welsh, E.J., & Smith, I. (2013) Drug therapy for obstructive sleep apnoea in adults. *Cochrane database Syst. Rev.*, **5**, CD003002.

Mattis, J. & Sehgal, A. (2016) Circadian Rhythms, Sleep, and Disorders of Aging. *Trends Endocrinol. Metab.*, **27**, 192–203.

Maury, E., Ramsey, K.M., & Bass, J. (2010) Circadian rhythms and metabolic syndrome: From experimental genetics to human disease. *Circ. Res.* **106**(3), 447-62.

Mavroudis, P.D., Scheff, J.D., Calvano, S.E., Lowry, S.F., & Androulakis, I.P. (2012) Entrainment of peripheral clock genes by cortisol. *Physiol. Genomics*, **44**, 607–621.

McCarthy, J.J., Andrews, J.L., McDearmon, E.L., Campbell, K.S., Barber, B.K., Miller, B.H., Walker, J.R., Hogenesch, J.B., Takahashi, J.S., & Esser, K.A. (2007) Identification of the circadian transcriptome in adult mouse skeletal muscle. *Physiol. Genomics*, **31**, 86–95.

McNicholas, W.T., Bonsigore, M.R., & Bonsignore, M.R. (2007) Sleep apnoea as an independent risk factor for cardiovascular disease: current evidence, basic mechanisms and research priorities. *Eur. Respir. J.*, **29**, 156–178.

Michel, M.C., Beck-Sickinger, a, Cox, H., Doods, H.N., Herzog, H., Larhammar, D., Quirion, R., Schwartz, T., & Westfall, T. (1998) XVI. International Union of Pharmacology recommendations for the nomenclature of neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. *Pharmacol. Rev.*, **50**, 143–150.

Miller, B.H., McDearmon, E.L., Panda, S., Hayes, K.R., Zhang, J., Andrews, J.L., Antoch, M.P., Walker, J.R., Esser, K.A., Hogenesch, J.B., & Takahashi, J.S. (2007) Circadian and CLOCK-controlled regulation of the mouse transcriptome and cell proliferation. *Proc. Natl. Acad. Sci. U. S. A.*, **104**, 3342–3347.

Mills, P.J. & Dimsdale, J.E. (2004) Sleep apnea: A model for studying cytokines, sleep, and sleep disruption. *Brain. Behav. Immun.*, **18**, 298–303.

Minor, R.K., López, M., Younts, C.M., Jones, B., Pearson, K.J., Michael Anson, R., Di??guez, C., & de Cabo, R. (2011) The arcuate nucleus and neuropeptide Y contribute to the antitumorigenic effect of calorie restriction. *Aging Cell*, **10**, 483–492.

Miyazaki, K., Kawamoto, T., Tanimoto, K., Nishiyama, M., Honda, H., & Kato, Y. (2002) Identification of functional hypoxia response elements in the promoter region of the DEC1 and DEC2 genes. *J. Biol. Chem.*, **277**, 47014–47021.

Möller-Levet, C.S., Archer, S.N., Bucca, G., Laing, E.E., Slak, A., Kabiljo, R., Lo, J.C.Y., Santhi, N., Schantz, M. Von, Smith, C.P., & Dijk, D. (2013) Effects of insufficient sleep on circadian rhythmicity and expression amplitude of the human blood transcriptome. *Proc. Natl. Acad. Sci.*, **110**, E1132–E1141.

Montesi, S.B., Bajwa, E.K., & Malhotra, A. (2012) Biomarkers of sleep apnea. *Chest*, **142**, 239–245.

Morse, D. & Sassone-Corsi, P. (2002) Time after time: Inputs to and outputs from the mammalian circadian oscillators. *Trends Neurosci.*, **25**, 632–637.

Mullington, J.M., Haack, M., Toth, M., Serrador, J.M., & Meier-Ewert, H.K. (2009) Cardiovascular, Inflammatory, and Metabolic Consequences of Sleep Deprivation. *Prog. Cardiovasc. Dis.*, **51**, 294–302.

Mure, L.S. & Panda, S. (2012) Fear of the Light or Need for Action: The IGL Will Judge. *Neuron*, **75**, 546–548.

Musiek, E.S. (2015) Circadian clock disruption in neurodegenerative diseases: Cause and effect? *Front. Pharmacol.*, **6**.

Nakahata, Y., Kaluzova, M., Grimaldi, B., Sahar, S., Chen, D., Guarente, L.P., & Sassone-corsi, P. (2008) The NAD+-Dependent Deacetylase SIRT1 Modulates CLOCK-Mediated Chromatin Remodeling and Circadian Control. *Cell*, **134**, 329–340.

Näslund, E. & Hellström, P.M. (2007) Appetite signaling: From gut peptides and enteric nerves to brain. *Physiol. Behav.*, **92**, 256–262.

Oh, E.Y., Yang, X., Friedman, A., Ansell, C.M., Du-Quiton, J., Quiton, D.F., Wood, P.A., & Hrushesky, W.J.M. (2010) Circadian transcription profile of mouse breast cancer under light-dark and dark-dark conditions. *Cancer Genomics and Proteomics*, **7**, 311–322.

Oishi, K., Miyazaki, K., Kadota, K., Kikuno, R., Nagase, T., Atsumi, G.I., Ohkura, N., Azama, T., Mesaki, M., Yukimasa, S., Kobayashi, H., Iitaka, C., Umehara, T., Horikoshi, M., Kudo, T., Shimizu, Y., Yano, M., Monden, M., Machida, K., Matsuda, J., Horie, S., Todo, T., & Ishida, N. (2003) Genome-wide Expression Analysis of Mouse Liver Reveals CLOCK-regulated Circadian Output Genes. *J. Biol. Chem.*, **278**, 41519–41527.

Okamura, H., Yamaguchi, S., & Yagita, K. (2002) Molecular machinery of the circadian clock in mammals. *Cell Tissue Res.*, **309**, 47–56.

Okuno, K., Sato, K., Arisaka, T., Hosohama, K., Gotoh, M., Taga, H., Sasao, Y., & Hamada, S. (2014) The effect of oral appliances that advanced the mandible forward and limited mouth opening in patients with obstructive sleep apnea: A systematic review and meta-analysis of randomised controlled trials. *J. Oral Rehabil.*, **41**, 542–554.

Orozco-Solis, R., Aguilar-Arnal, L., Murakami, M., Peruquetti, R., Ramadori, G., Coppari, R., & Sassone-Corsi, P. (2016) The Circadian Clock in the Ventromedial Hypothalamus Controls Cyclic Energy Expenditure. *Cell Metab.*, **23**, 467–478.

Pace-Schott, E.F. & Hobson, J.A. (2002) The neurobiology of sleep: genetics, cellular physiology and subcortical networks. *Nat. Rev. Neurosci.*, **3**, 591–605.

Pack, A.I. (2006) Advances in sleep-disordered breathing. *Am. J. Respir. Crit. Care Med.*, **173**, 7–15.

Pallayova, M., Steele, K., Magnuson, T., Schweitzer, M., Hill, N., Bevans-Fonti, S., & Schwartz, A. (2010) Sleep apnea predicts distinct alterations in glucose homeostasis and biomarkers in obese adults with normal and impaired glucose metabolism. *Cardiovasc Diabetol.*, **9**, 1–11.

Palmer, L.J. & Redline, S. (2003) Genomic approaches to understanding obstructive sleep apnea. *Respir. Physiol. Neurobiol.*, **135**, 187–205.

Panda, S., Antoch, M.P., Miller, B.H., Su, A.I., Schook, A.B., Straume, M., Schultz, P.G., Kay, S.A., Takahashi, J.S., & Hogenesch, J.B. (2002) Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell*, **109**, 307–320.

Park, J.G., Ramar, K., & Olson, E.J. (2011) Updates on definition, consequences, and management of obstructive sleep apnea. *Mayo Clin. Proc.*, **86**, 549–554.

Parlapiano, C., Borgia, M.C., Minni, a, Alessandri, N., Basal, I., & Saponara, M. (2005) Cortisol circadian rhythm and 24-hour Holter arterial pressure in OSAS patients. *Endocr. Res.*, **31**, 371–374.

Partch, C.L., Green, C.B., & Takahashi, J.S. (2014) Molecular Architecture of the Mammalian Circadian Clock. *Trends Cell Biol.*, **24**, 90–99.

Patel, S.R., Zhu, X., Storfer-Isser, A., Mehra, R., Jenny, N.S., Tracy, R., & Redline, S. (2009) Sleep duration and biomarkers of inflammation. *Sleep*, **32**, 200–204.

Peterfi, Z., Makara, G.B., Obál, F., & Krueger, J.M. (2009) The anterolateral projections of the medial basal hypothalamus affect sleep. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **296**, R1228–R1238.

Piggins, H.D. & Guilding, C. (2011) The neural circadian system of mammals. *Essays Biochem.*, **49**, 1–17.

Poirel, V.J., Boggio, V., Dardente, H., Pevet, P., Masson-Pevet, M., & Gauer, F. (2003) Contrary to other non-photic cues, acute melatonin injection does not induce immediate changes of clock gene mRNA expression in the rat suprachiasmatic nuclei. *Neuroscience*, **120**, 745–755.

Preitner, N., Damiola, F., Luis-Lopez-Molina, Zakany, J., Duboule, D., Albrecht, U., & Schibler, U. (2002) The Orphan Nuclear Receptor REV-ERBα Controls Circadian Transcription within the Positive Limb of the Mammalian Circadian Oscillator. *Cell*, **110**, 251–260.

Ramanathan, C., Khan, S.K., Kathale, N.D., Xu, H., & Liu, A.C. (2012) Monitoring Cell-autonomous Circadian Clock Rhythms of Gene Expression Using Luciferase Bioluminescence Reporters. *J. Vis. Exp.*, **27**, 1–9.

Ramanathan, C., Xu, H., Khan, S.K., Shen, Y., Gitis, P.J., Welsh, D.K., Hogenesch, J.B., & Liu, A.C. (2014) Cell Type-Specific Functions of Period Genes Revealed by Novel Adipocyte and Hepatocyte Circadian Clock Models. *PLoS Genet.*, **10**.

Ramm, P. & Smith, C.T. (1990) Rates of cerebral protein synthesis are linked to slow wave sleep in the rat. *Physiol. Behav.*, **48**, 749–753.

Ramsey, K.M., Yoshino, J., Brace, C.S., Abrassart, D., Kobayashi, Y., Marcheva, B., Hong, H.-K., Chong, J.L., Buhr, E.D., Lee, C., Takahashi, J.S., Imai, S., & Bass, J. (2009) Circadian clock feedback cycle through NAMPT-mediated NAD+ biosynthesis. *Science*, **324**, 651–654.

Rao, P., Benito, E., & Fischer, A. (2013) MicroRNAs as biomarkers for CNS disease. *Front. Mol. Neurosci.*, **6**, 1–13.

Reddy, A.B., Karp, N.A., Maywood, E.S., Sage, E.A., Deery, M., O'Neill, J.S., Wong, G.K.Y., Chesham, J., Odell, M., Lilley, K.S., Kyriacou, C.P., & Hastings, M.H. (2006) Circadian Orchestration of the Hepatic Proteome. *Curr. Biol.*, **16**, 1107–1115.

Reppert, S.M. & Weaver, D.R. (2001) Molecular analysis of mammalian circadian rhythms. *Annu. Rev. Physiol.*, **63**, 647–676.

Richards, J., Diaz, A.N., & Gumz, M.L. (2014) Clock genes in hypertension: novel insights from rodent models. *Blood Press. Monit.*, **19**, 249–254.

Ross, S.D., Sheinhait, I. a, Harrison, K.J., Kvasz, M., Connelly, J.E., Shea, S. a, & Allen, I.E. (2000) Systematic review and meta-analysis of the literature regarding the diagnosis of sleep apnea. *Sleep*, **23**, 519–532.

Ryan, S., Taylor, C.T., & McNicholas, W.T. (2005) Selective activation of inflammatory pathways by intermittent hypoxia in obstructive sleep apnea syndrome. *Circulation*, **112**, 2660–2667.

Saderi, N., Cazarez-Márquez, F., Buijs, F.N., Salgado-Delgado, R.C., Guzman-Ruiz, M.A., del Carmen Basualdo, M., Escobar, C., & Buijs, R.M. (2013) The NPY intergeniculate leaflet projections to the suprachiasmatic nucleus transmit metabolic conditions. *Neuroscience*, **246**, 291–300.

Sanchez, A., Martinez, P., Miro, E., Bardwell, W., & Buela-Casal, G. (2009) CPAP and behavioral therapies in patients with obstructive sleep apnea: effects on daytime sleepiness, mood, and cognitive function. *Sleep Med Rev.*, **13**, 223–233.

Sateia, M.J. (2014) International classification of sleep disorders-third edition highlights and modifications. *Chest*, **146**, 1387–1394.

Sato, T.K., Panda, S., Miraglia, L.J., Reyes, T.M., Rudic, R.D., McNamara, P., Naik, K.A., Fitzgerald, G.A., Kay, S.A., & Hogenesch, J.B. (2004) A functional genomics strategy reveals rora as a component of the mammalian circadian clock. *Neuron*, **43**, 527–537.

Savvidis, C. & Koutsilieris, M. (2012) Circadian rhythm disruption in cancer biology. *Mol. Med.*, **18**, 1249–1260.

Schutz, S.L. (2001) Oxygen Saturation Monitoring by Pulse Oximetry. In Lynn-McHale, D.J. & Carlson, K.K. (eds), *AACN Procedure Manual for Critical Care*, Fourth Edi. edn.

Schwartz, M.W., Sipols, A.J., Marks, J.L., Sanacora, G., White, J.D., Scheurink, A., Kahn, S.E., Baskin, D.G., Woods, S.C., Figlewicz, D.P., & Porte, D. (1992) Inhibition of hypothalamic neuropeptide Y gene expression by insulin. *Endocrinology*, **130**, 3608–3616.

Scully, C.G., Karaboue, a., Liu, W.-M., Meyer, J., Innominato, P.F., Chon, K.H., Gorbach, a. M., & Levi, F. (2011) Skin surface temperature rhythms as potential circadian biomarkers for personalized chronotherapeutics in cancer patients. *Interface Focus*, **1**, 48–60.

Sehgal, A. (2008) Ac-ing the Clock. *Neuron*, **57**, 8–10.

Sehgal, A. & Mignot, E. (2011) Review Genetics of Sleep and Sleep Disorders. *Cell*, **146**, 194–207.

Shanmugam, V., Wafi, A., Al-Taweel, N., & Büsselberg, D. (2013) Disruption of circadian rhythm increases the risk of cancer, metabolic syndrome and cardiovascular disease. *J. Local Glob. Heal. Sci.*, **3**, 1–42.

Sharma, A., Tiwari, S., & Singaravel, M. (2016) Circadian rhythm disruption: health consequences. *Biol. Rhythm Res.*, **47**, 191–213.

Shearman, L.P., Sriram, S., Weaver, D.R., Maywood, E.S., Chaves, I., Zheng, B., Kume, K., Lee, C.C., van der Horst, G.T., Hastings, M.H., & Reppert, S.M. (2000) Interacting molecular loops in the mammalian circadian clock. *Science*, **288**, 1013–1019.

Shih, J. & Malhotra, A. (2011) Could vitamins be helpful to patients with sleep apnea? *Chest*, **139**, 237–238.

Shinohara, K. & Inouye, S.I.T. (1995) Photic information coded by vasoactive intestinal polypeptide and neuropeptide Y. *Neurosci. Biobehav. Rev.*, **19**, 349–352.

Shinohara, K., Tominaga, K., Isobe, Y., & Inouye, S.T. (1993) Photic regulation of peptides located in the ventrolateral subdivision of the suprachiasmatic nucleus of the rat: daily variations of vasoactive intestinal polypeptide, gastrin-releasing peptide, and neuropeptide Y. *J. Neurosci.*, **13**, 793–800.

Silva, A., Xapelli, S., Grouzmann, E., & Cavadas, C. (2005) he putative neuroprotective role of neuropeptide Y in the central nervous system. *Curr Drug Targets CNS Neurol Disord*, **4**, 331–347.

Silva, A.P., Cavadas, C., & Grouzmann, E. (2002) Neuropeptide Y and its receptors as potential therapeutic drug targets. *Clin. Chim. Acta.*, **326**, 3–25.

Skjodt, N.M., Atkar, R., & Easton, P.A. (1999) Screening for hypothyroidism in sleep apnea. *Am. J. Respir. Crit. Care Med.*, **160**, 732–735.

Son, G.H., Chung, S., & Kim, K. (2011) The adrenal peripheral clock: Glucocorticoid and the circadian timing system. *Front. Neuroendocrinol.*, **32**, 451–465.

Sousa-Ferreira, L., Garrido, M., Nascimento-Ferreira, I., Nobrega, C., Santos-Carvalho, A., Álvaro, A.R., Rosmaninho-Salgado, J., Kaster, M., Kügler, S., de Almeida, P.L., & Cavadas, C. (2011) Moderate Long-Term modulation of Neuropeptide Y in hypothalamic arcuate nucleus induces energy balance alterations in adult rats. *PLoS One*, **6**, e22333.

Steiropoulos, P., Papanas, N., Nena, E., Antoniadou, M., Serasli, E., Papoti, S., Hatzizisi, O., Kyriazis, G., Tzouvelekis, A., Maltezos, E., Tsara, V., & Bouros, D. (2010) Inflammatory markers in middle-aged obese subjects: Does obstructive sleep apnea syndrome play a role? *Mediators Inflamm.*, **2010**, 1–6.

Stephens, T.W., Basinski, M., Bristow, P.K., Bue-Valleskey, J.M., Burgett, S.G., Craft, L., Hale, J., Hoffmann, J., Hsiung, H.M., & Kriauciunas, A. (1995) The role of neuropeptide Y in the antiobesity action of the obese gene product. *Nature*, **377**, 530–532.

Stickgold, R., Hobson, J.A., Fosse, R., & Fosse, M. (2001) Sleep, learning, and dreams: off-line memory reprocessing. *Science*, **294**, 1052–1057.

Stokkan, K., Yamazaki, S., Tei, H., Sakaki, Y., & Menaker, M. (2001) Entrainment of the Circadian Clock in the Liver by Feeding. *Science (80-.).*, **291**, 490–493.

Sundaram, S., Bridgman, S. a, Lim, J., Lasserson, T.J., TJ, L., Bridgman, S. a, Lim, J., & Lasserson, T.J. (2005) Surgery for obstructive sleep apnoea. *Cochrane Database Syst. Rev.*, **19**, CD001004.

Takahashi, J.S. (2004) Finding new clock components: past and future. *J. Biol. Rhythms*, **19**, 339–347.

Takahashi, J.S., Takahashi, J.S., Hong, H.-K., Hong, H.-K., Ko, C.H., Ko, C.H., McDearmon, E.L., & McDearmon, E.L. (2008) The genetics of mammalian circadian order and disorder: implications for physiology and disease. *Nat. Rev. Genet.*, **9**, 764–775.

Takata, M., Burioka, N., Ohdo, S., Takane, H., Terazono, H., Miyata, M., Sako, T., Suyama, H., Fukuoka, Y., Tomita, K., & Shimizu, E. (2002) Daily expression of mRNAs for the mammalian Clock genes Per2 and clock in mouse suprachiasmatic nuclei and liver and human peripheral blood mononuclear cells. *Jpn. J. Pharmacol.*, **90**, 263–269.

Takeda, N. & Maemura, K. (2011) Circadian clock and cardiovascular disease. *J. Cardiol.*, **57**, 249–256.

Tatemoto, K. (1982) Neuropeptide Y: complete amino acid sequence of the brain peptide. *Proc. Natl. Acad. Sci. U. S. A.*, **79**, 5485–5489.

Tatemoto, K., Carlquist, M., & Mutt, V. (1982) Neuropeptide Y--a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. *Nature*, **296**, 659–660.

Taylor, B.L. & Zhulin, I.B. (1999) PAS Domains: Internal Sensors of Oxygen, Redox Potential, and Light. *Microbiol. Mol. Biol. Rev.*, **63**, 479–506.

Teboul, M., Barrat-Petit, M.A., Li, X.M., Claustrat, B., Formento, J.L., Delaunay, F., Levi, F., & Milano, G. (2005) Atypical patterns of circadian clock gene expression in human peripheral blood mononuclear cells. *J Mol Med*, **83**, 693–699.

Teboul, M., Barrat-Petiti, M., Li, X.M., Claustrat, B., Formento, J.-L., Delaunay, F., Lévi, F., & Milano, G. (2005) Atypical patterns of circadian clock gene expression in human peripheral blood mononuclear cells. *J Mol Med*, **83**, 693–699.

Teclemariam-Mesbah, R., Ter Horst, G.J., Postema, F., Wortel, J., & Buijs, R.M. (1999) Anatomical demonstration of the suprachiasmatic

nucleus-pineal pathway. *J. Comp. Neurol.*, **406**, 171–182.

Thorsell, A. & Heilig, M. (2002) Diverse functions of neuropeptide Y revealed using genetically modified animals **36**, 182–193.

Toh, K.L., Jones, C.R., He, Y., Eide, E.J., Hinz, W.A., Virshup, D.M., Ptácek, L.J., & Fu, Y.H. (2001) An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. *Science*, **291**, 1040–1043.

Vela, J., Gutierrez, A., Vitorica, J., & Ruano, D. (2003) Rat hippocampal GABAergic molecular markers are differentially affected by ageing. *J. Neurochem.*, **85**, 368–377.

Videnovic, A., Lazar, A.S., Barker, R.A., & Overeem, S. (2014) The clocks that time us - circadian rhythms in neurodegenerative disorders. *Nat Rev Neurol.*, **10**, 683–693.

Vieira, E., Burris, T.P., & Quesada, I. (2014) Clock genes, pancreatic function, and diabetes. *Trends Mol. Med.*, **20**, 685–693.

Wagner, L., Björkqvist, M., Hult Lundh, S., Wolf, R., Börgel, A., Schlenzig, D., Ludwig, H., Rahfeld, J., Leavitt, B., Demuth, H., Petersén, Å., & von Hörsten, S. (2016) Neuropeptide Y (NPY) in cerebrospinal fluid from patients with Huntington's Disease: increased NPY levels and differential degradation of the NPY1-30 fragment. *J Neurochem.*, **137**, 820–837.

Watts, a G., Swanson, L.W., & Sanchez-Watts, G. (1987) Efferent projections of the suprachiasmatic nucleus: I. Studies using anterograde transport of Phaseolus vulgaris leucoagglutinin in the rat. *J. Comp. Neurol.*, **258**, 204–229.

Watts, A.G. & Swanson, L.W. (1987) Efferent projections of the suprachiasmatic nucleus: II. Studies using retrograde transport of fluorescent dyes and simultaneous peptide immunohistochemistry in the rat. *J Comp Neurol*, **258**, 230–252.

Weaver, T.E., Calik, M.W., Farabi, S.S., Fink, A.M., Galang-Boquiren, M.T., Kapella, M.C., Prasad, B., & Carley, D.W. (2014) Innovative treatments for adults with obstructive sleep apnea. *Nat. Sci. Sleep*, **6**, 137–147.

Weaver, T.E. & Grunstein, R.R. (2008) Adherence to continuous positive airway pressure therapy: the challenge to effective treatment. *Proc. Am. Thorac. Soc.*, **5**, 173–178.

Whitney, A.R., Diehn, M., Popper, S.J., Alizadeh, A.A., Boldrick, J.C., Relman, D.A., & Brown, P.O. (2003) Individuality and variation in gene expression patterns in human blood. *Proc. Natl. Acad. Sci. U. S. A.*, **100**, 1896–1901.

Wiater, M.F., Mukherjee, S., Li, A.J., Dinh, T.T., Rooney, E.M., Simasko, S.M., & Ritter, S. (2011) Circadian integration of sleep-wake and feeding requires NPY receptor-expressing neurons in the mediobasal hypothalamus. *AJP Regul. Integr. Comp. Physiol.*, **301**, R1569–R1583.

Widdowson, P.S., Ordway, G.A., & Halaris, A.E. (1992) Reduced Neuropeptide Y Concentrations in Suicide Brain. *J. Neurochem.*, **59**, 73–80.

Williams, K.W. & Elmquist, J.K. (2012) From neuroanatomy to behavior: central integration of peripheral signals regulating feeding behavior. *Nat. Neurosci.*, **15**, 1350–1355.

Wood, P. a, Yang, X., & Hrushesky, W.J.M. (2009) Clock genes and cancer. *Integr. Cancer Ther.*, **8**, 303–308.

Woodson, B.T., Gillespie, M.B., Soose, R.J., Maurer, J.T., De Vries, N., Steward, D.L., Baskin, J.Z., Padhya, T.A., Lin, H.-S., Mickelson, S., Badr, S.M., Strohl, K.P., & Strollo, P.J. (2014) Randomized Controlled Withdrawal Study of Upper Airway Stimulation on OSA: Short-and Long-term Effect. *Otolaryngol. Head Neck Surg.*, **151**, 880–887.

Woodson, B.T., Soose, R.J., Gillespie, M.B., Strohl, K.P., Maurer, J.T., de Vries, N., Steward, D.L., Baskin, J.Z., Badr, M.S., Lin, H. -s., Padhya, T.A., Mickelson, S., Anderson, W.M., Vanderveken, O.M., & Strollo, P.J. (2016) Three-Year Outcomes of Cranial Nerve Stimulation for Obstructive Sleep Apnea: The STAR Trial. *Otolaryngol Head Neck Surg.*, **154**, 181–188.

Wright, M.C., Phillips-Bute, B., Mark, J.B., Stafford-Smith, M., Grichnik, K.P., Andregg, B.C., & Taekman, J.M. (2006) Time of day effects on the incidence of anesthetic adverse events. *Qual. Saf. Health Care*, **15**, 258–263.

Wu, J. & Sigmund, C.D. (2016) Hypertension A Disease That Strikes Around the Clock. *Hypertension*, **67**, 493–495.

Wulff, K., Gatti, S., Wettstein, J.G., & Foster, R.G. (2010a) Sleep and circadian rhythm disruption in psychiatric and neurodegenerative disease. *Nat. Rev. Neurosci.*, **11**, 589–599.

Wulff, K., Gatti, S., Wettstein, J.G., & Foster, R.G. (2010b) Sleep and circadian rhythm disruption in psychiatric and neurodegenerative disease. *Nat. Rev. Neurosci.*, **11**, 589–599.

Wulff, K., Porcheret, K., Cussans, E., & Foster, R.G. (2009) Sleep and circadian rhythm disturbances: multiple genes and multiple phenotypes. *Curr. Opin. Genet. Dev.*, **19**, 237–246.

Xu, H., Zheng, X., Jia, W., & Yin, S. (2015) Chromatography/Mass Spectrometry-Based Biomarkers in the Field of Obstructive Sleep Apnea. *Medicine (Baltimore).*, **94**, e1541.

Xu, J., Xu, Y., Miao, B., Deng, M., & Wang, Y. (2015) Influence of menstrual cycle on the expression of clock genes in peripheral blood mononuclear cells in Macaca fascicularis. *Eur. J. Obstet. Gynecol.*, **186**, 54–58.

Yagita, K. & Okamura, H. (2000) Forskolin induces circadian gene expression of rPer1, rPer2 and dbp in mammalian rat-1 fibroblasts. *FEBS Lett.*, **465**, 79–82.

Yagita, K., Tamanini, F., Van Der Horst, G.T., & Okamura, H. (2001) Molecular mechanisms of the biological clock in cultured fibroblasts.

Science, **292**, 278–281.

Yamazaki, S., Numano, R., Abe, M., Hida, A., Takahashi, R., Ueda, M., Block, G.D., Sakaki, Y., Menaker, M., & Tei, H. (2000) Resetting central and peripheral circadian oscillators in transgenic rats. *Science*, **288**, 682–685.

Yang, S.C. & Shieh, K.R. (2009) Implications of Circadian Rhythms on Metabolic Disorders. *Tzu Chi Med. J.*, **21**, 285–288.

Yang, X., Downes, M., Yu, R.T., Bookout, A.L., He, W., Straume, M., Mangelsdorf, D.J., & Evans, R.M. (2006) Nuclear Receptor Expression Links the Circadian Clock to Metabolism. *Cell*, **126**, 801–810.

Yang, X., Wood, P. a, Ansell, C.M., Quiton, D.F.T., Oh, E.-Y., Du-Quiton, J., & Hrushesky, W.J.M. (2009) The circadian clock gene Per1 suppresses cancer cell proliferation and tumor growth at specific times of day. *Chronobiol. Int.*, **26**, 1323–1339.

Yannielli, M.E. & Harrington, P.C. (2001) Neuropeptide Y in the mammalian circadian system : effects on light- induced circadian responses **22**, 547–556.

Yannielli, P. & Harrington, M.E. (2004) Let there be "more" light: Enhancement of light actions on the circadian system through non-photic pathways. *Prog. Neurobiol.*, **74**, 59–76.

Yasumoto, Y., Hashimoto, C., Nakao, R., Yamazaki, H., Hiroyama, H., Nemoto, T., Yamamoto, S., Sakurai, M., Oike, H., Wada, N., Yoshida-Noro, C., & Oishi, K. (2016) Short-term feeding at the wrong time is sufficient to desynchronize peripheral clocks and induce obesity with hyperphagia, physical inactivity and metabolic disorders in mice. *Metabolism*, **65**, 714–727.

Yi, C.X., Van Der Vliet, J., Dai, J., Yin, G., Ru, L., & Buijs, R.M. (2006) Ventromedial arcuate nucleus communicates peripheral metabolic information to the suprachiasmatic nucleus. *Endocrinology*, **147**, 283–294.

Young, M.E. (2006) The circadian clock within the heart: potential influence on myocardial gene expression, metabolism, and function. *Am. J. Physiol. Heart Circ. Physiol.*, **290**, H1–H16.

Zeitzer, J.M., Dijk, D.J., Kronauer, R., Brown, E., & Czeisler, C. (2000) Sensitivity of the human circadian pacemaker to nocturnal light: melatonin phase resetting and suppression. *J. Physiol.*, **526**, 695–702.

Zelinski, E.L., Deibel, S.H., & McDonald, R.J. (2014) The trouble with circadian clock dysfunction: Multiple deleterious effects on the brain and body. *Neurosci. Biobehav. Rev.*, **40**, 80–101.

Zhang, E.E. & Kay, S.A. (2010) Clocks not winding down: unravelling circadian networks. *Nat. Rev. Mol. Cell Biol.*, **11**, 764–776.

Zhang, E.E., Liu, Y., Dentin, R., Pongsawakul, P.Y., Liu, A.C., Hirota, T., Nusinow, D.A., Sun, X., Landais, S., Kodama, Y., Brenner, D.A., Montminy, M., & Kay, S.A. (2010) Cryptochrome mediates circadian regulation of cAMP signaling and hepatic gluconeogenesis. *Nat. Med.*, **16**, 1152–1156.

Zhao, H., Zeng, Z.L., Yang, J., Jin, Y., Qiu, M.Z., Hu, X.Y., Han, J., Liu, K.Y., Liao, J.W., Xu, R.H., & Zou, Q.F. (2014) Prognostic relevance of Period1 (Per1) and Period2 (Per2) expression in human gastric cancer. *Int. J. Clin. Exp. Pathol.*, **7**, 619–630.

Zhu, L. & Zee, P.C. (2012) Circadian Rhythm Sleep Disorders. *Neurol. Clin.*, **30**, 1167–1191.

Zukowska, Z., Pons, J., Lee, E.W., & Li, L. (2003) Neuropeptide Y: a new mediator linking sympathetic nerves, blood vessels and immune system? *Can. J. Physiol. Pharmacol.*, **81**, 89–94.

Zvonic, S., Ptitsyn, A.A., Conrad, S.A., Scott, L.K., Floyd, Z.E., Kilroy, G., Wu, X., Goh, B.C., Mynatt, R.L., & Gimble, J.M. (2006) Characterization of peripheral circadian clocks in adipose tissues. *Diabetes*, **55**, 962–970.

APPENDICES

APPENDIX A - QUESTIONNAIRE

1. **NOME:**
2. **Idade:** ___ anos
3. **Peso:** ____ kg
4. **Altura:** ____ cm

5. **Tem dificuldades em adormecer?**

( ) Não.
( ) Sim.

6. **Geralmente, quanto tempo demora a adormecer?**

1-5 min ( )
5-15 min ( )
15-30 min ( )
30-60 min ( )
mais de 60 min ( )

7. **Costuma acordar durante a noite?**

( ) Não.
( ) Sim.

8. **Durante o dia, sente sono?**

( ) Não.
( ) Sim.
( ) 1-2 vezes /dia
( ) 3-4 vezes /dia
( ) 5-6 vezes /dia
( ) mais do 7 vezes /dia

9. **Costuma tomar medicamentos para o/a ajudar a dormir? Se sim, que medicamentos e com que frequência?**

( ) Não.
( ) Sim.
Medicamentos: _____
Frequência:
( ) Raramente.
( ) Uma ou duas vezes por semana.
( ) Três ou mais vezes por semana.

10. **Sofre de alguma doença, seja ela física ou psiquiátrica? Se sim, qual?**

( ) Não.
( ) Sim. Doença: _____

11. **Nos últimos 6 meses, tem tomado algum tipo de medicação? Se sim, qual?**

12. **Trabalha, ou, já alguma vez trabalhou em turnos de noite?**

( ) Sim.
( ) Não.

13. Fez alguma viagem de longo curso, para um país com um fuso horário diferente, nos últimos 6 meses?

( ) Sim.
( ) Não.

14. Costuma praticar atividade física? Se sim, com que frequência?

( ) Não.
( ) Sim.
Frequência:
( ) Raramente.
( ) Uma ou duas vezes por semana.
( ) Três ou mais vezes por semana.

Tem por hábito fazer desporto depois da hora de jantar?

( ) Sim.
( ) Não.

15. Considera que tem uma alimentação saudável?

( ) Não.
( ) Sim.

16. Segue uma alimentação regular, com refeições a horas certas?

( ) Não.
( ) Sim.

17. Informações adicionais:

Se tiver informações adicionais que considere relevante para o estudo em questão, pode escrever aqui.

APPENDIX B – PATIENTS INDIVIDUAL DATA

PATIENT 1:

Note: The patient did not filled the circadian rhythms questionnaire and his body temperature was not assessed at T0.

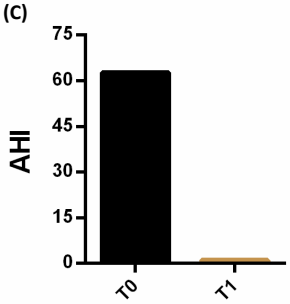
(A)

		T0
Medical history	Age	62
	BMI	25
	BMI category	Overweigh
Sleep disruption events	Arousal events/h	31,1
	Awakening events/h	16,8
Respiration summary	AHI	62.7
	OSA severity	Severe
Oxygenation summary	SpO_2 mean (%)	90
	Desaturation events/h	---
EKG statistics	Heart rate, wake	79
	Heart rate, steady sleep	66

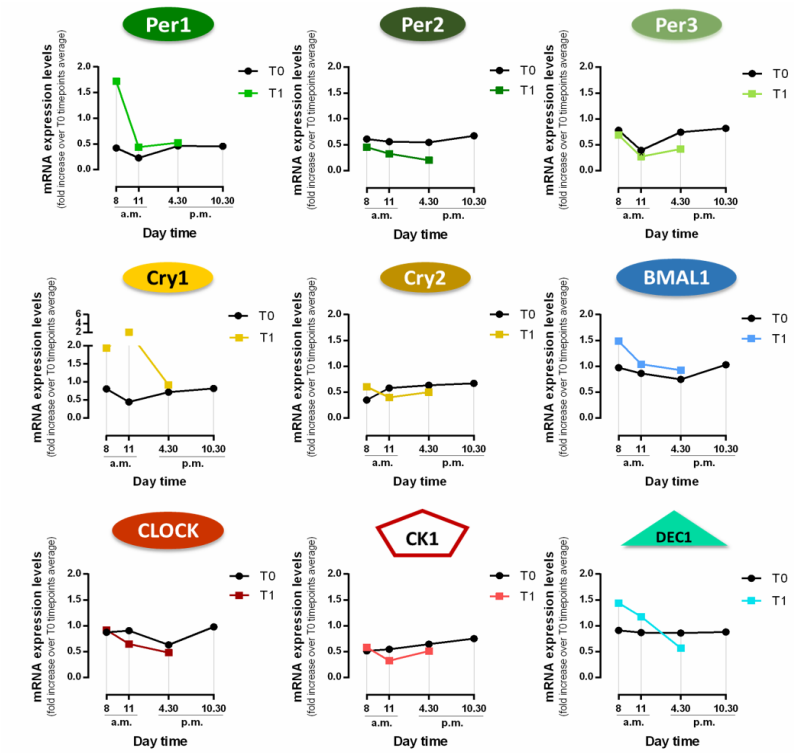
(B)

		T1
Adherence	Days with more than 4h of use (%)	97
Respiration summary	AHI	1

(C)



Panel 1: Main OSA parameters of patient 1 before (T0) and after treatment (T1). (A) Parameters assessed before treatment. Information about the age and bmi were obtained by the medical team. Data about sleep disruption events (arousal and awakening events per hour), respiration (apnea and hypopnea events per hour), oxygenation (percentage of arterial oxygen saturation and desaturation events per hour) and EKG statistics (heart rate when awake and when steady sleep) were measured, in each patient, by the PSG test. (B) Parameters assessed after treatment. Both the adherence (percentage of days with more than 4h of use) and respiration (apnea and hypopnea events per hour) were recorded by the CPAP chip. (C) **Patient AHI at T0 and T1.**



Panel 2: Clock genes expression profile, at T0 and T1, of patient 1. Expression profiles of *Per1, Per2, Per3, Cry1, Cry2, BMAL1, CLOCK, CK1ε* and *DEC1* clock genes, at T0 and T1. At T1, the 10.30 p.m. time point was not assessed.

PATIENT 2:

Note: Body temperature was not assessed at T0.

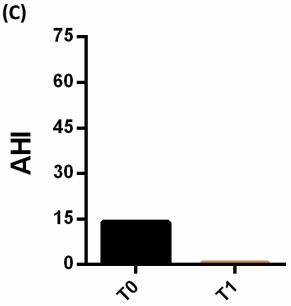
(A)

		T0
Medical history	Age	44
	BMI	28,1
	BMI category	Overweigh
Sleep disruption events	Arousal events/h	18
	Awakening events/h	3,8
Respiration summary	AHI	9,9
	OSA severity	Mild
Oxygenation summary	SpO_2 mean (%)	95
	Desaturation events/h	7,9
EKG statistics	Heart rate, wake	64
	Heart rate, steady sleep	60

(B)

		T1
Adherence	Days with more than 4h of use (%)	100
Respiration summary	AHI	**1,2**

(C)

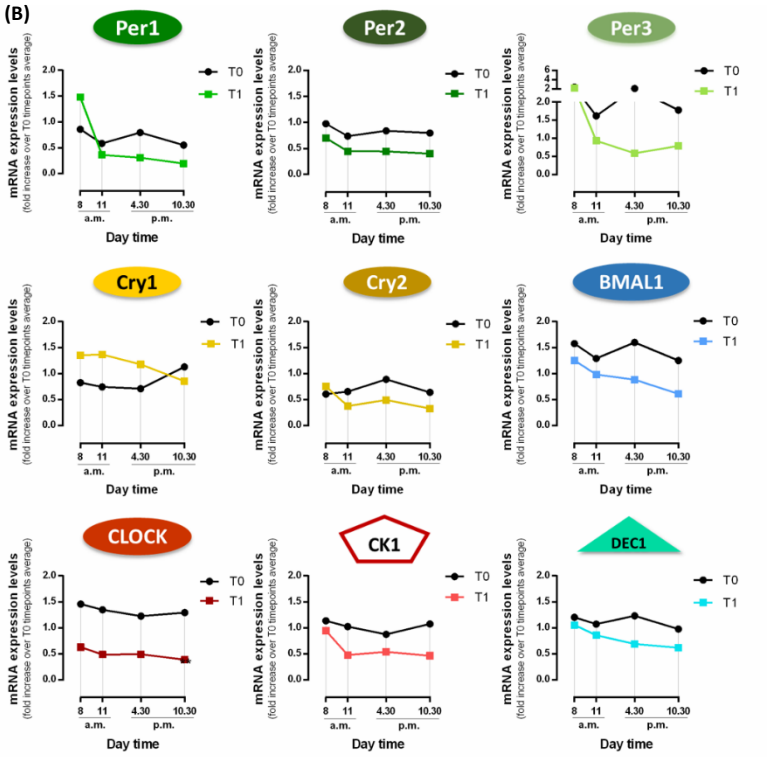


Panel 3: Main OSA parameters of patient 2 before (T0) and after treatment (T1). (A) Parameters assessed before treatment. Information about the age and bmi were obtained by the medical team. Data about sleep disruption events (arousal and awakening events per hour), respiration (apnea and hypopnea events per hour), oxygenation (percentage of arterial oxygen saturation and desaturation events per hour) and EKG statistics (heart rate when awake and when steady sleep) were measured, in each patient, by the PSG test. (B) Parameters assessed after treatment. Both the adherence (percentage of days with more than 4h of use) and respiration (apnea and hypopnea events per hour) were recorded by the CPAP chip. (C) **Patient AHI at T0 and T1.**

(A)

Disorders	---
Benzodiazepine use	---
Night awakening	Yes
Daytime sleepiness	Yes, 5-6x per day
Sports activities	No
Healthy eating	Yes
Regular eating	No
Nightshifts	Yes
Jetlag	No

(B)



Panel 4: Circadian rhythm questionnaire results and clock genes expression profile, at T0 and T1, of patient 2. (A) The figure shows the results obtained from the circadian rhythm questionnaire, which assessed diagnosed disorders, use of benzodiazepines to sleep, night awakening, daytime sleepiness, sports activities, healthy eating, regular eating, nightshifts and travels across time zones in the past 6 months (jetlag). (B) Expression profiles of *Per1, Per2, Per3, Cry1, Cry2, BMAL1, CLOCK, CK1ε* and *DEC1* clock genes, at T0 and T1.

PATIENT 3:

Note: Body temperature was not assessed at T0.

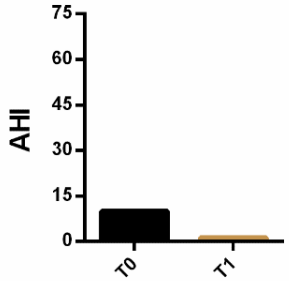
(A)

		T0
Medical history	Age	48
	BMI	30,7
	BMI category	Obese class I
Sleep disruption events	Arousal events/h	24,6
	Awakening events/h	3,8
Respiration summary	AHI	13,9
	OSA severity	Mild
Oxygenation summary	SpO_2 mean (%)	93
	Desaturation events/h	2,9
EKG statistics	Heart rate, wake	57
	Heart rate, steady sleep	59

(B)

		T1
Adherence	Days with more than 4h of use (%)	100
Respiration summary	AHI	**0,5**

(C)

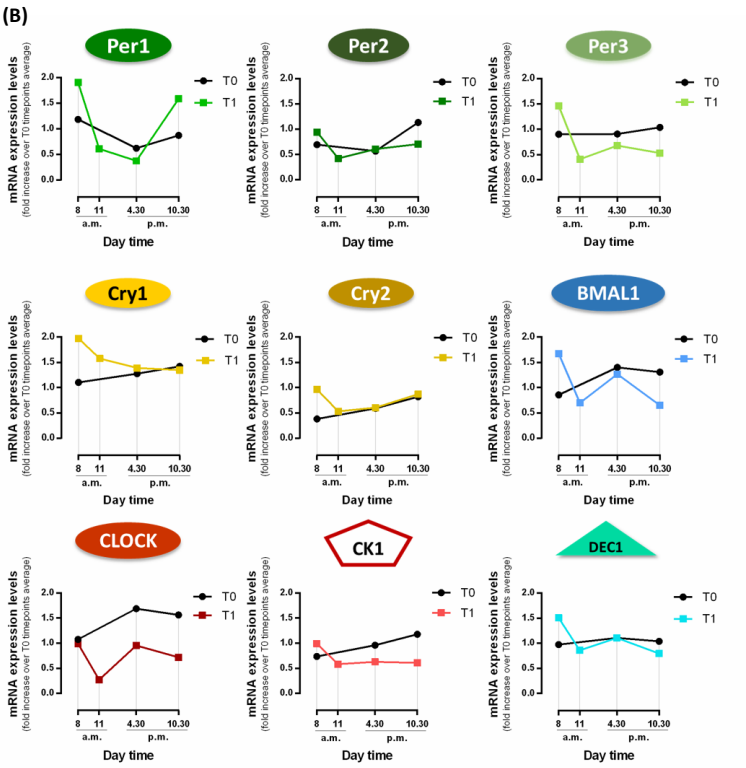


Panel 5: Main OSA parameters of patient 3 before (T0) and after treatment (T1). (A) Parameters assessed before treatment. Information about the age and bmi were obtained by the medical team. Data about sleep disruption events (arousal and awakening events per hour), respiration (apnea and hypopnea events per hour), oxygenation (percentage of arterial oxygen saturation and desaturation events per hour) and EKG statistics (heart rate when awake and when steady sleep) were measured, in each patient, by the PSG test. (B) Parameters assessed after treatment. Both the adherence (percentage of days with more than 4h of use) and respiration (apnea and hypopnea events per hour) were recorded by the CPAP chip. (C) **Patient AHI at T0 and T1.**

(A)

Disorders	---
Benzodiazepine use	---
Night awakening	No
Daytime sleepiness	No
Sports activities	No
Healthy eating	Yes
Regular eating	Yes
Nightshifts	No
Jetlag	No

(B)



Panel 6: Circadian rhythm questionnaire results and clock genes expression profile, at T0 and T1, of patient 3. (A) The figure shows the results obtained from the circadian rhythm questionnaire, which assessed diagnosed disorders, use of benzodiazepines to sleep, night awakening, daytime sleepiness, sports activities, healthy eating, regular eating, nightshifts and travels across time zones in the past 6 months (jetlag). (B) Expression profiles of *Per1, Per2, Per3, Cry1, Cry2, BMAL1, CLOCK, CK1ε* and *DEC1* clock genes, at T0 and T1. At T0, the 11 a.m. time point was not assessed.

PATIENT 4:

Note: Body temperature was not assessed at T0.

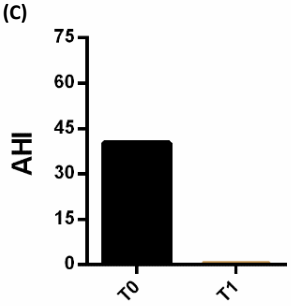
(A)

		T0
Medical history	Age	61
	BMI	29,8
	BMI category	Overweigh
Sleep disruption events	Arousal events/h	34,1
	Awakening events/h	4,3
Respiration summary	AHI	40,3
	OSA severity	Severe
Oxygenation summary	SpO_2 mean (%)	90
	Desaturation events/h	29,8
EKG statistics	Heart rate, wake	75
	Heart rate, steady sleep	73

(B)

		T1
Adherence	Days with more than 4h of use (%)	100
Respiration summary	AHI	**0,4**

(C)

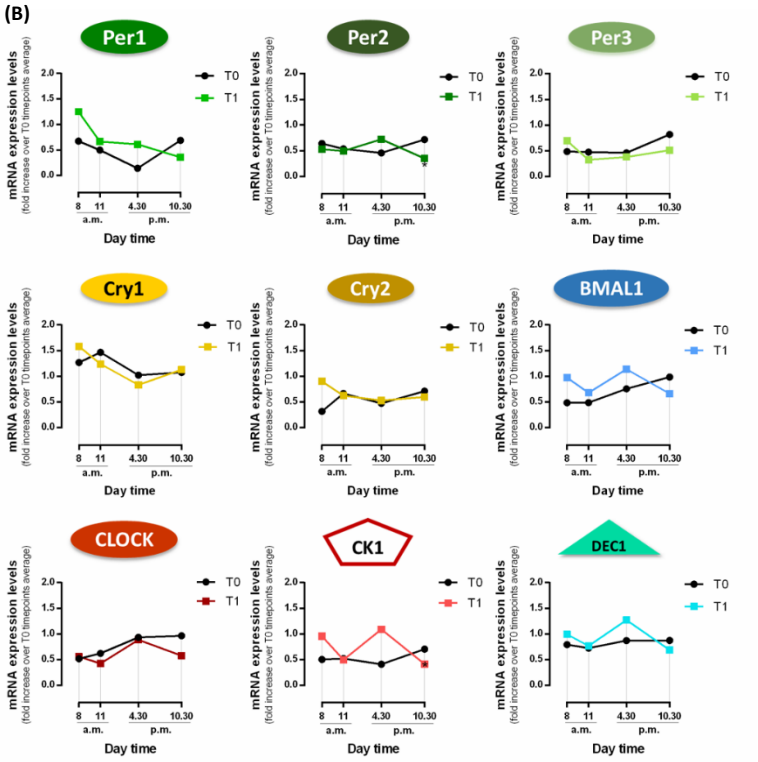


Panel 7: Main OSA parameters of patient 4 before (T0) and after treatment (T1). (A) Parameters assessed before treatment. Information about the age and bmi were obtained by the medical team. Data about sleep disruption events (arousal and awakening events per hour), respiration (apnea and hypopnea events per hour), oxygenation (percentage of arterial oxygen saturation and desaturation events per hour) and EKG statistics (heart rate when awake and when steady sleep) were measured, in each patient, by the PSG test. (B) Parameters assessed after treatment. Both the adherence (percentage of days with more than 4h of use) and respiration (apnea and hypopnea events per hour) were recorded by the CPAP chip. (C) **Patient AHI at T0 and T1.**

(A)

Disorders	Diabetes
Benzodiazepine use	No
Night awakening	Yes
Daytime sleepiness	Yes, +7x per day
Sports activities	No
Healthy eating	Yes
Regular eating	Yes
Nightshifts	Yes
Jetlag	No

(B)



Panel 8: Circadian rhythm questionnaire results and clock genes expression profile, at T0 and T1, of patient 4. (A) The figure shows the results obtained from the circadian rhythm questionnaire, which assessed diagnosed disorders, use of benzodiazepines to sleep, night awakening, daytime sleepiness, sports activities, healthy eating, regular eating, nightshifts and travels across time zones in the past 6 months (jetlag). (B) Expression profiles of *Per1, Per2, Per3, Cry1, Cry2, BMAL1, CLOCK, CK1ε* and *DEC1* clock genes, at T0 and T1.

PATIENT 5:

Note: Body temperature was not assessed at T0.

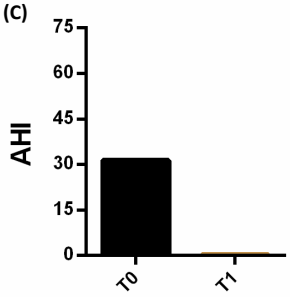
(A)

		T0
Medical history	Age	41
	BMI	32,4
	BMI category	Obese class I
Sleep disruption events	Arousal events/h	10,8
	Awakening events/h	2,1
Respiration summary	AHI	31,4
	OSA severity	Severe
Oxygenation summary	SpO_2 mean (%)	93
	Desaturation events/h	---
EKG statistics	Heart rate, wake	81
	Heart rate, steady sleep	75

(B)

		T1
Adherence	Days with more than 4h of use (%)	84
Respiration summary	AHI	**0,4**

(C)

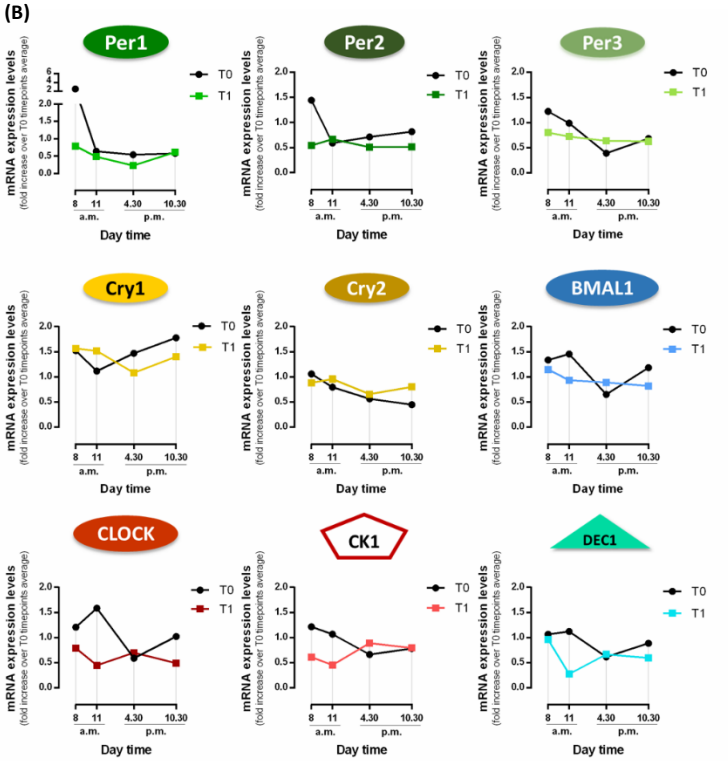


Panel 9: Main OSA parameters of patient 5 before (T0) and after treatment (T1). (A) Parameters assessed before treatment. Information about the age and bmi were obtained by the medical team. Data about sleep disruption events (arousal and awakening events per hour), respiration (apnea and hypopnea events per hour), oxygenation (percentage of arterial oxygen saturation and desaturation events per hour) and EKG statistics (heart rate when awake and when steady sleep) were measured, in each patient, by the PSG test. (B) Parameters assessed after treatment. Both the adherence (percentage of days with more than 4h of use) and respiration (apnea and hypopnea events per hour) were recorded by the CPAP chip. (C) **Patient AHI at T0 and T1.**

(A)

Disorders	No
Benzodiazepine use	No
Night awakening	Yes
Daytime sleepiness	No
Sports activities	No
Healthy eating	Yes
Regular eating	Yes
Nightshifts	Yes
Jetlag	No

(B)



Panel 10: Circadian rhythm questionnaire results and clock genes expression profile, at T0 and T1, of patient 5. (A) The figure shows the results obtained from the circadian rhythm questionnaire, which assessed diagnosed disorders, use of benzodiazepines to sleep, night awakening, daytime sleepiness, sports activities, healthy eating, regular eating, nightshifts and travels across time zones in the past 6 months (jetlag). (B) Expression profiles of *Per1, Per2, Per3, Cry1, Cry2, BMAL1, CLOCK, CK1***ε** and *DEC1* clock genes, at T0 and T1.

PATIENT 6:

Note: Body temperature was not assessed at T0.

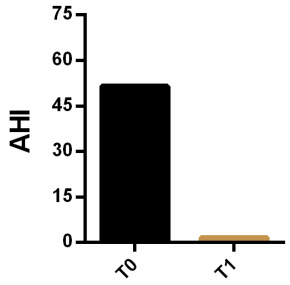
(A)

		T0
Medical history	Age	75
	BMI	25,7
	BMI category	Overweigh
Sleep disruption events	Arousal events/h	44,8
	Awakening events/h	5,5
Respiration summary	AHI	51,5
	OSA severity	Severe
Oxygenation summary	SpO_2 mean (%)	92
	Desaturation events/h	28,5
EKG statistics	Heart rate, wake	66
	Heart rate, steady sleep	61

(B)

		T1
Adherence	Days with more than 4h of use (%)	43
Respiration summary	AHI	**1,6**

(C)

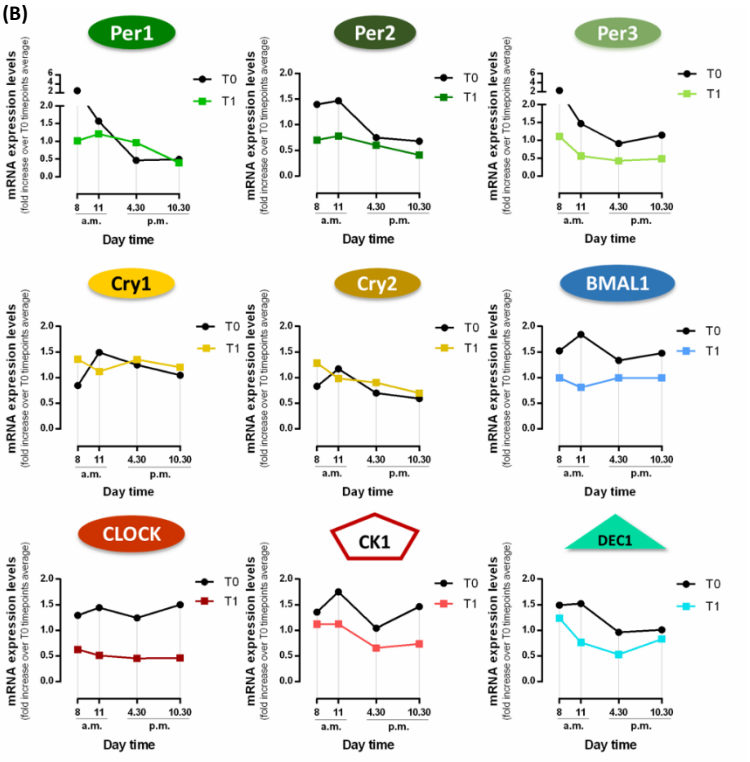


Panel 11: Main OSA parameters of patient 6 before (T0) and after treatment (T1). (A) Parameters assessed before treatment. Information about the age and bmi were obtained by the medical team. Data about sleep disruption events (arousal and awakening events per hour), respiration (apnea and hypopnea events per hour), oxygenation (percentage of arterial oxygen saturation and desaturation events per hour) and EKG statistics (heart rate when awake and when steady sleep) were measured, in each patient, by the PSG test. (B) Parameters assessed after treatment. Both the adherence (percentage of days with more than 4h of use) and respiration (apnea and hypopnea events per hour) were recorded by the CPAP chip. (C) **Patient AHI at T0 and T1.**

(A)

Disorders	No
Benzodiazepine use	No
Night awakening	Yes
Daytime sleepiness	No
Sports activities	No
Healthy eating	Yes
Regular eating	Yes
Nightshifts	Yes
Jetlag	No

(B)



Panel 12: Circadian rhythm questionnaire results and clock genes expression profile, at T0 and T1, of patient 6. (A) The figure shows the results obtained from the circadian rhythm questionnaire, which assessed diagnosed disorders, use of benzodiazepines to sleep, night awakening, daytime sleepiness, sports activities, healthy eating, regular eating, nightshifts and travels across time zones in the past 6 months (jetlag). (B) Expression profiles of *Per1, Per2, Per3, Cry1, Cry2, BMAL1, CLOCK, CK1ε* and *DEC1* clock genes, at T0 and T1.

PATIENT 7:

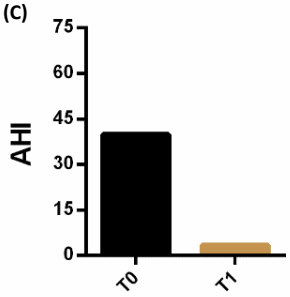
(A)

		T0
Medical history	Age	57
	BMI	34,7
	BMI category	Obese class I
Sleep disruption events	Arousal events/h	19,8
	Awakening events/h	12,2
Respiration summary	AHI	39,9
	OSA severity	Severe
Oxygenation summary	SpO_2 mean (%)	91
	Desaturation events/h	0,2
EKG statistics	Heart rate, wake	97
	Heart rate, steady sleep	74

(B)

		T1
Adherence	Days with more than 4h of use (%)	100
Respiration summary	AHI	**3,5**

(C)

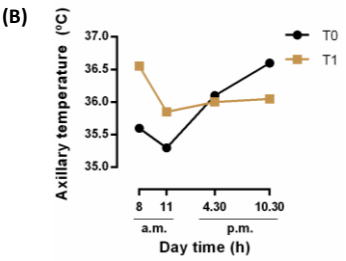


Panel 13: Main OSA parameters of patient 7 before (T0) and after treatment (T1). (A) Parameters assessed before treatment. Information about the age and bmi were obtained by the medical team. Data about sleep disruption events (arousal and awakening events per hour), respiration (apnea and hypopnea events per hour), oxygenation (percentage of arterial oxygen saturation and desaturation events per hour) and EKG statistics (heart rate when awake and when steady sleep) were measured, in each patient, by the PSG test. (B) Parameters assessed after treatment. Both the adherence (percentage of days with more than 4h of use) and respiration (apnea and hypopnea events per hour) were recorded by the CPAP chip. (C) **Patient AHI at T0 and T1.**

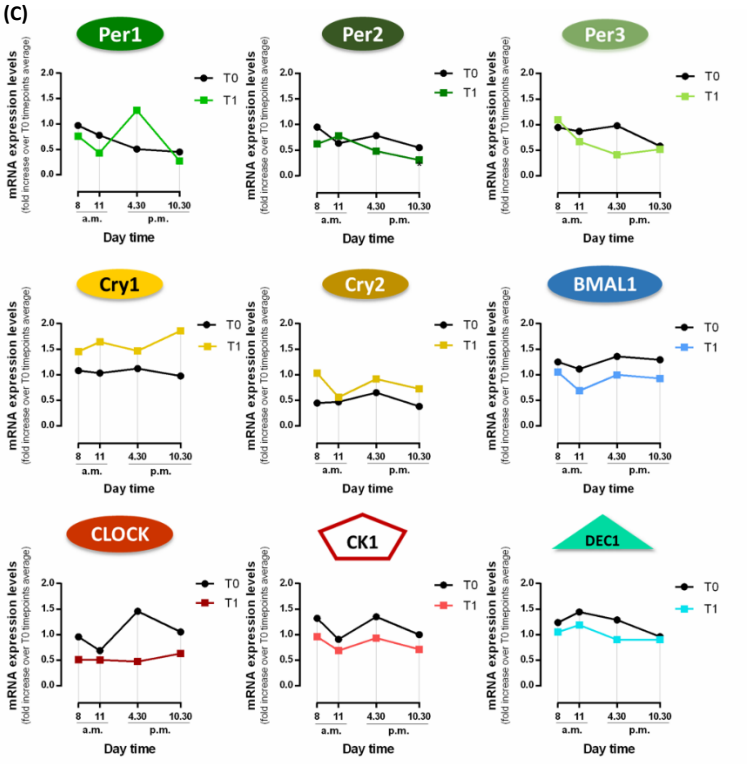
(A)

Disorders	No
Benzodiazepine use	No
Night awakening	Yes
Daytime sleepiness	Yes, 1-2x per day
Sports activities	No
Healthy eating	Yes
Regular eating	Yes
Nightshifts	Yes
Jetlag	No

(B)



(C)



Panel 14: Circadian rhythm questionnaire results and body temperature and clock genes expression profile, at T0 and T1, of patient 7. (A) The figure shows the results obtained from the circadian rhythm questionnaire, which assessed diagnosed disorders, use of benzodiazepines to sleep, night awakening, daytime sleepiness, sports activities, healthy eating, regular eating, nightshifts and travels across time zones in the past 6 months (jetlag). (B) Body temperature profile, at T0 and T1. (C) Expression profiles of *Per1, Per2, Per3, Cry1, Cry2, BMAL1, CLOCK, CK1*ε and *DEC1* clock genes, at T0 and T1.

PATIENT 8:

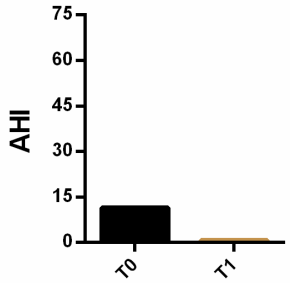
(A)

		T0
Medical history	Age	68
	BMI	23,1
	BMI category	Normal
Sleep disruption events	Arousal events/h	14,4
	Awakening events/h	4,8
Respiration summary	AHI	**11,4**
	OSA severity	Mild
Oxygenation summary	SpO_2 mean (%)	92
	Desaturation events/h	5,9
EKG statistics	Heart rate, wake	73
	Heart rate, steady sleep	67

(B)

		T1
Adherence	Days with more than 4h of use (%)	43
Respiration summary	AHI	**0,8**

(C)

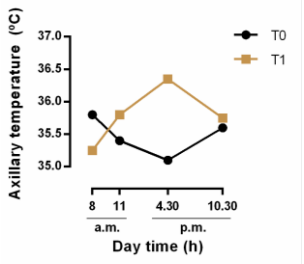


Panel 15: Main OSA parameters of patient 8 before (T0) and after treatment (T1). (A) Parameters assessed before treatment. Information about the age and bmi were obtained by the medical team. Data about sleep disruption events (arousal and awakening events per hour), respiration (apnea and hypopnea events per hour), oxygenation (percentage of arterial oxygen saturation and desaturation events per hour) and EKG statistics (heart rate when awake and when steady sleep) were measured, in each patient, by the PSG test. (B) Parameters assessed after treatment. Both the adherence (percentage of days with more than 4h of use) and respiration (apnea and hypopnea events per hour) were recorded by the CPAP chip. (C) **Patient AHI at T0 and T1.**

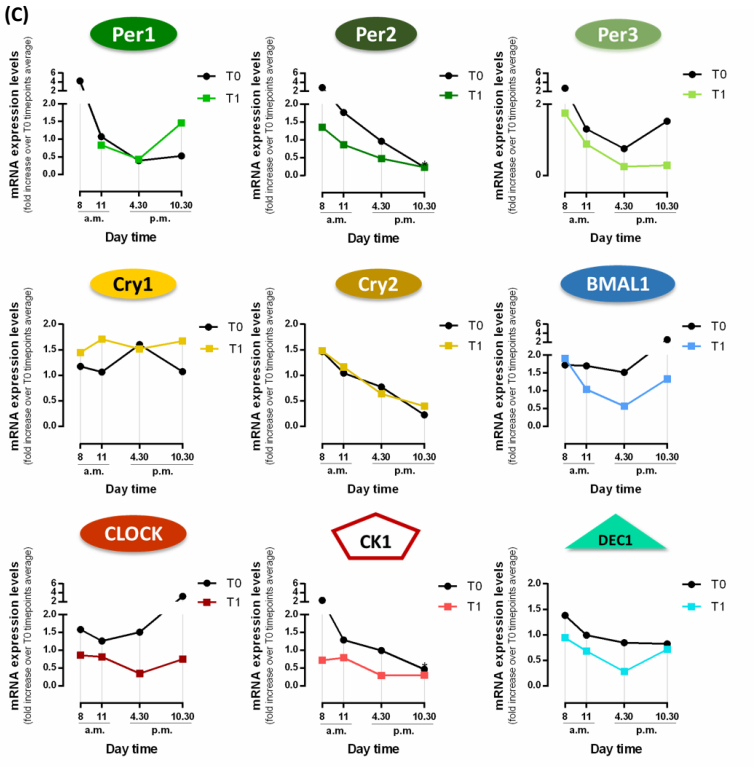
(A)

Disorders	Diabetes and renal insufficiency
Benzodiazepine use	Yes
Night awakening	Yes
Daytime sleepiness	Yes, 1-2x per day
Sports activities	Rarely
Healthy eating	Yes
Regular eating	Yes
Nightshifts	Yes
Jetlag	Yes

(B)



(C)



Panel 16: Circadian rhythm questionnaire results and body temperature and clock genes expression profile, at T0 and T1, of patient 8. (A) The figure shows the results obtained from the circadian rhythm questionnaire, which assessed diagnosed disorders, use of benzodiazepines to sleep, night awakening, daytime sleepiness, sports activities, healthy eating, regular eating, nightshifts and travels across time zones in the past 6 months (jetlag). (B) Body temperature profile, at T0 and T1. (C) Expression profiles of *Per1, Per2, Per3, Cry1, Cry2, BMAL1, CLOCK, CK1***ε** and *DEC1* clock genes, at T0 and T1.

PATIENT 9:

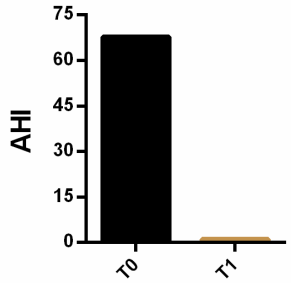
(A)

		T0
Medical history	Age	54
	BMI	33,6
	BMI category	Obese class I
Sleep disruption events	Arousal events/h	82
	Awakening events/h	8,1
Respiration summary	AHI	**67,8**
	OSA severity	Severe
Oxygenation summary	SpO_2 mean (%)	90
	Desaturation events/h	---
EKG statistics	Heart rate, wake	73
	Heart rate, steady sleep	72

(B)

		T1
Adherence	Days with more than 4h of use (%)	96
Respiration summary	AHI	**1,1**

(C)

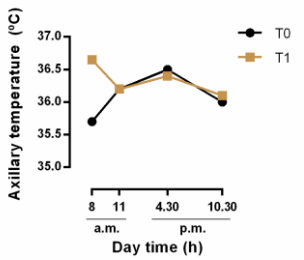


Panel 17: Main OSA parameters of patient 9 before (T0) and after treatment (T1). (A) Parameters assessed before treatment. Information about the age and bmi were obtained by the medical team. Data about sleep disruption events (arousal and awakening events per hour), respiration (apnea and hypopnea events per hour), oxygenation (percentage of arterial oxygen saturation and desaturation events per hour) and EKG statistics (heart rate when awake and when steady sleep) were measured, in each patient, by the PSG test. (B) Parameters assessed after treatment. Both the adherence (percentage of days with more than 4h of use) and respiration (apnea and hypopnea events per hour) were recorded by the CPAP chip. (C) **Patient AHI at T0 and T1.**

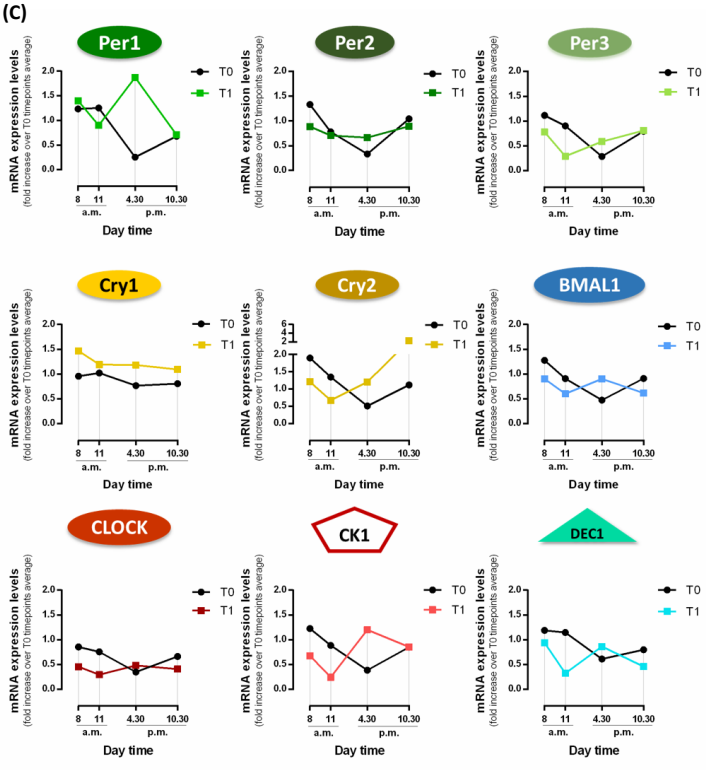
(A)

Disorders	Diabetes and renal insufficiency
Benzodiazepine use	No
Night awakening	No
Daytime sleepiness	Yes, 3-4x per day
Sports activities	No
Healthy eating	Yes
Regular eating	Yes
Nightshifts	Yes
Jetlag	No

(B)



(C)



Panel 18: Circadian rhythm questionnaire results and body temperature and clock genes expression profile, at T0 and T1, of patient 9. (A) The figure shows the results obtained from the circadian rhythm questionnaire, which assessed diagnosed disorders, use of benzodiazepines to sleep, night awakening, daytime sleepiness, sports activities, healthy eating, regular eating, nightshifts and travels across time zones in the past 6 months (jetlag). (B) Body temperature profile, at T0 and T1. (C) Expression profiles of *Per1, Per2, Per3, Cry1, Cry2, BMAL1, CLOCK, CK1***ε** and *DEC1* clock genes, at T0 and T1.

PATIENTS WHOSE DATA IS NOT COMPLETED (MISSING T1):

Table 1: Main OSA parameters of the remaining 11 patients whose data is not completed (missing T1). Parameters assessed before treatment. Information about the age and bmi were obtained by the medical team. Data about sleep disruption events (arousal and awakening events per hour), respiration (apnea and hypopnea events per hour), oxygenation (percentage of arterial oxygen saturation and desaturation events per hour) and EKG statistics (heart rate when awake and when steady sleep) were measured, in each patient, by the PSG test. *P* means patient.

		T0										
		P10	**P11**	**P12**	**P13**	**P14**	**P15**	**P16**	**P17**	**P18**	**P19**	**P20**
Medical history	Age	64	41	50	51	59	41	37	57	59	41	47
	BMI	35,5	30,7	25,5	32,7	28	42,4	26,5	32,8	28,4	33	35,4
	BMI Category	Obese class II	Obese class I	Overweight	Obese class I	Overweight	Obese class III	Overweight	Obese class I	Overweight	Obese class I	Obese class II
Sleep disruption events	Arousal events/h	36,2	33,3	53,4	15,5	52,1	82,9	15	43,8	17,6	43,1	16,8
	Awakening events/h	4,9	3,1	6,5	1,4	8	8,9	18,3	20	10,6	15,3	5,1
Respiration summary	AHI Severity	32,9 Severe	35,1 Severe	69,2 Severe	27,1 Moderate	86,9 Severe	104,1 Severe	34,1 Severe	63,5 Severe	51,7 Severe	68,3 Severe	48,5 Severe
Oxygenation summary	SpO_2 mean (%)	94	95	91	91	94	82	90	92	92	90	92
	Desaturation events/h	15,8	11,6	45,4	18,2	35,7	89,4	---	46,2	---	---	---
EKG statistics	Heart rate, wake	106	86	77	70	72	87	154	68	78	62	80
	Heart rate, steady sleep	111	68	80	56	69	75	56	60	75	69	71

Table 2: Circadian rhythm questionnaire results of the remaining 11 patients whose data is not completed (missing T1). (A) The table shows the results obtained from the circadian rhythm questionnaire, which assessed diagnosed disorders, use of benzodiazepines to sleep, night awakening, daytime sleepiness, sports activities, healthy eating, regular eating, nightshifts and travels across time zones in the past 6 months (jetlag). *P* means patient.

	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20
Disorders	Hypertension	No	No	No	Cardiovascular disorder; Diabetes	Depression	Hypertension	No	No	Diabetes	No
Benzodiazepine use	Yes	No	Yes	No	No	No	No	No	No	No	No
Night awakening	Yes	No	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No
Daytime sleepiness	Yes, 1-2x per day	No	No	No	No	Yes, 3-4x per day	Yes, 3-4x per day	Yes, 1-2x per day	No	Yes, +7x per day	Yes, 3-4x per day
Sports activities	Yes	Yes	Yes	Rarely	No	Yes	No	Rarely	Rarely	No	No
Healthy eating	Yes	No	Yes	Yes	Yes	No	Yes	Yes	Yes	No	Yes
Regular eating	Yes	No	No	Yes	Yes	No	Yes	Yes	No	No	No
Nightshifts	Yes	No	Yes	No	Yes	Yes	Yes	No	No	No	No
Jetlag	No	Yes	Yes	No	No	No	No	No	Yes	No	No