MODULATION OF CENTRAL AND PERIPHERAL MOLECULAR CLOCKS BY PROANTHOCYANIDINS

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MODULATION OF CENTRAL AND PERIPHERAL MOLECULAR CLOCKS BY PROANTHOCYANIDINS

PhD DOCTORAL THESIS

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FEM CONSTAR que aquest treball titulat "**Modulation of central and peripheral molecular clocks by proanthocyanidins**", que presenta Aleix Ribas Latre per l'obtenció del títol de Doctor, ha estat realitzat sota la nostra direcció al Departament de Bioquímica i Biotecnologia d'aquesta universitat i que compleix els requeriments per poder optar a Menció Internacional.

Tarragona, 3 de setembre de 2014

La directora de la tesi doctoral

Dra. M. Cinta Bladé Segarra

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AGRAÏMENTS

Fa uns anys, en un túnel d'una de les moltes mines subterrànies presents a l'Àfrica, sis miners van quedar atrapats sabent, per la seva experiència, que el temps per rescatar-los hauria de ser reduït, o bé acabarien tots morint d'asfíxia. Dels sis homes, només n'hi havia un que portava rellotge i a ell anaven totes les preguntes. Quanta estona ha passat? Quina hora és? etc. El que finalment va decidir fer l'home del rellotge, és enganyar els seus companys i dir que havia passat mitja hora per cada hora real que passava. D'aquesta manera, mentre per ell el pas del temps passava a una velocitat considerable, pels seus companys, la velocitat es reduïa a la meitat. Voleu que us digui com va acabar la història? Van salvar-se tots, excepte el que portava el rellotge. Sens dubte un clar exemple, amb el permís d'Einstein i la seva teoria de la relativitat, de com senyals exteriors ens estan influint constantment a nivell psíquic i mental i tal vegada, a nivell fisiològic.

Tot i sabent aquesta història, fa quatre anys vaig ser jo qui va decidir endinsar-me dins una mina totalment desconeguda per a mi, amb ganes de buscar algun tresor ocult. Passats aquests quatre anys, puc dir que el temps m'ha passat a la mateixa velocitat a la que li va passar al miner mort, però que per contra, va ser degut a les ganes d'aprendre, la intensitat del temps transcorregut i el gaudiment d'haver après coses tan importants com saber com funciona aquesta mina i buscar les eines necessàries per poder seguir explorant aquest camí, que pel fet de ser infinit, no ha acabat ni espero que ho faci mai.

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CONTENT

I. ABBREVIATIONS

AMP: adenosine monophosphate

AMPK: AMP-activated protein kinase

bHLH-PAS: basic helix-loop-helix; Per-Arnt-Single

BMAL1: brain and muscle ARNT like protein

CAR: constitutive androstane receptor

CK: casein kinase

CLOCK: circadian locomotor output cycles kaput

CRY: cryptochrome

DYRK1A: dual-specifity tyrosine-(Y)-phosphorylation regulated kinase 1A

FOXO1: forkhead box protein O1

GABA: γ-aminobutyric acid

GSH: glutathione

GSK-3B: F-box and leucine-rich repeat protein 3

GSPE: grape seed proanthocyanidin extract

HAT: histone acetyl transferasa

NAADP: nicotinate adenine dinucleotide phosphatase

NAD: nicotinamide adeninedinucleotide

NAM: nicotinamide

NHRs: nuclear hotmonal receptors

NMN: nicotinamide mononucleotide

I. ABBRVIATIONS

NR1D1: nuclear receptor subfamily 1, group D

OEA: oleylethanolamide

PAs: proanthocyanidins

PER: period

PGC-1α: peroxixome proliferator-activated receptor coactivator alpha

PPARα: peroxisome proliferator-activated receptor alpha

PPARγ: peroxixome proliferator-activated receptor gamma

REV-ERBα: reverse erythroblastosis virus alpha

RORα: retinoic acid-related orphan receptor alpha

ROS: reactive oxygen species

SCN: suprachiasmatic nucleus

SIRT1: sirtuine 1

SOD: superoxide dismutase

SREBP-1C: sterol regulatory element-binding protein 1C

STAT3: signal transducer and activator of transcription 3

TORC2: transducer of regulated CREB protein 2

TTFL: transcription-translation feedback loop

VIP: vasoactive intestinal peptide

βTrCP: β-transducing-repeat-containing protein

II. INTRODUCTION

1. Circadian rhythms: the output of the circadian system

1.1. From circannual to circadian rhythms

All organisms in Earth are organised towards diverse environmental influences that mediate transformation through biological signals, chemical processes, or various forms of energy. Among all these external cues, the sun is thought to be the most important factor in life, as without it, life on our planet would not be possible. As the case of a magnet, the sun exerts such a powerful effect, that even an enormous system as the planets that make up the solar system, react to the solar energy circling the sun. Our planet is not an exception and as cause of that factor, environmental changes occur yearly, generally called as the seasons. All kinds of challenges to the survival of organisms and their offspring arise with the beginning of every new season, reason why they have developed seasonally induced changes that adapt them to these predictable events. Winter, for instance, means in half of the world a decrease in ambient temperatures and therefore food availability, while energetic demands increase. Under this perspective, some species adapt themselves by doing hibernation, migration, huddling among them, increasing pelage, eating more calorically or allowing time for parturition, fattening process of flowering time in plants for another more appropriate season, among many others adaptations carried out year behind year [1]. Particularly, in human beings, the quality and quantity of food ingested flows along the different seasons, similar as, despite the globalisation, the type of fruits and vegetables do. In fact, weather and raw materials, therefore geography and social structure, traditionally have influenced the different world cuisines [2], as many phenotypic modifications [3]. Thereby, organisms are in constant interaction with the environment through time, which entrainmany behavioural, physiological, and metabolic aspects, suggesting the existence of an epigenetic fact that would explain how gene

regulation can modulate development, proposed by Waddington through his metaphorical epigenetic landscape [4].

Generally, if any event within a biological system occurs at approximately regular intervals, it is known as a biological rhythm which is a cyclical change in the biological or chemical function of body [5]. In the case of an annual biological rhythm, the mechanisms have not yet been clearly identified, although there is evidence for an annual clock, an endogenous calendar, that contributes recollecting the annual information in order to provide an advantage for survival expectations even when predictive cues are noisy or not immediately present, as the case of solstices, hibernators and species inhabiting high latitudes, where prolonged winters are associated with absence of an obvious external light-dark cue [6]. In addition to this endogenous calendar, a day length– measuring mechanism finally would conform the annual clock in order to adjust physiological state precisely to the seasons [7], although changes on the thyroid hormone signaling represent another important molecular mechanism, as this hormone is crucially required for the expression of seasonal rhythms [8] . Day length is the most accurate natural predictor of annual phase known as photoperiod, which drives seasonal physiology through the contraction of the melatonin pineal signal during short summer nights and its expansion in long winter nights, providing an accurate endocrine representation of day-night length. However some other non-photoperiodic seasonal factors like food, water, ambient temperature and social cues have all been shown to alter seasonal traits specifically among species [9]. Thus, a molecular system capable of measuring day length and controlling melatonin pineal signal, emerges as the key piece responsible for recollecting the required information for increasing survival expectations along the time. This system is called the circadian clock system.

1.2. Circadian clock system

Day length entails light-dark cycle, which is directly conditioned by the rotation of Earth to its own axis, implying daily biological rhythms with periods, the time interval after which the event recurs, of 24 hours. Then daily rhythms become termed circadian rhythms, coming from the Latin *circa*, meaning "around" / "about" or "approximately"; and *diem* or *dies*, meaning "day". In fact, internal circadian rhythms can be studied in isolated units, free from external signals and variations, such as bunkers, caves, suitable laboratories, etc., resulting still in periods slightly deviating from 24 h; or under normal environmental conditions where the internal rhythms of the organisms adapt their period, to the light regime of the sun, showing then a daily 24 h rhythm and underlining the importance of the daily Earth rotation, as organisms reside in it. In both cases, the presence of a circadian clock system that controls and emits rhythms is evident, as it is in the case of waking up at the same time spontaneously everyday even without an alarm [10]. In fact, the list of circadian physiological events is as long as complex are the different species living in this planet, but in general, their circadian cycle responds to a better predisposition for the next action, which is directly related to an external cue. For example, cycles in rest and activity, blood pressure, body temperature, alertness, coordination, reaction and brain activity, cardiovascular efficiency or muscle strength, hormonal secretion in blood and ions in urine, etc..; oscillate throughout the day, responding to an external demand in order to allow us a better adaptation. All these physiological circadian aspects are triggered by numberless molecular pathways, nonetheless, there is a circadian clock system which as a main function, allows us to prepare our physiology from a basic, original and universal mechanism among almost all species worldwide, from prokaryotes to higher organisms [11].

The hypothalamic suprachiasmatic nucleus (SCN) into the hypothalamus, that is found in nearly all living organisms, is capable of integrating direct photic input from the retina. No more regions thorough the body are able to accomplish that function, so that, SCN is also known as the master clock or oscillator in mammals, for synchronising our physiology or circadian rhythms, according to our main external cue or synchronizer coming from the sun, the light [12]. Nonetheless, organisms are surrounded by many other external synchronizers, therefore, other many peripheral and cerebral oscillators are present in cells throughout the body, sharing a similar structure at the molecular level and emitting rhythms in a self autonomous manner depending of the external cues [13]. Thus the circadian clock system is composed in one hand, by a master clock located in the SCN; and on the other hand, many others oscillators throughout the body which share a similar structure molecularly.

1.2.1. Molecular basis of the circadian clock system

For all organisms in which the molecular clock mechanism has been investigated, a common model has been observed: a transcription-translation feedback loop (TTFL). TTFL components are not, however, shared between organisms. For example, the cyanobacterial clock is modeled around three proteins: KaiA, B and C while the plant TTFL involves elements including TOC1 and CCA11 [14]. Furthermore, although some multicellular organisms such as *Drosophila*and mammals possess homologous components, their functions appear to differ between organisms, suggesting that daily timekeeping evolved independently within different lineages [15].

Focusing in the mammalian circadian clock system, it consists of a cycle with a periodicity of approximately 24 h, driving the positive limb of this loop by the transcriptional activators circadian locomotor output cycles kaput

(CLOCK) and brain and muscle ARNT like protein 1(BMAL1), which both encode bHLH-PAS (basic helix–loop–helix; Per-Arnt-Single) proteins that after their own heterodimeritation, initiate the transcription by binding to specific DNA elements like E-boxes (5'-CACGTG-3') and E'-boxes (5'-CACGTT-3') in the promoters of target genes. As a matter of fact, if CLOCK is removed from the system, the behavior of the animal remains perfectly rhythmic due to the gene *Npas2*, which acts as a surrogate for the loss of *Clock*, playing a role as transcriptional partner of *Bmal1* in the SCN [16].However, the loss of *Clock* abolishes the circadian rhythmicity of the molecular oscillations in peripheral clocks [17].

Among the CLOCK:BMAL1 target genes, Period (*Per*) and Cryptochrome (*Cry*) genes in turn inhibit the activity of CLOCK:BMAL1 heterodimer, after increasing to a critical concentrations that allow the PER and CRY proteins, dimerize and translocate to the nucleus, leading, therefore, to a decrease in *Per* and *Cry* expression [18] . Degradation of the negative limb proteins PER and CRY is required to terminate the repression phase and restart a new cycle of transcription, which is crucial in order to set the period of the clock. In line, casein kinase (CK)1ε and CK1δ target the PER proteins through phosphorylation, for ubiquitination and degradation by β-transducing-repeat-containing protein (βTrCP) and 26S proteasome respectively [20], while CRY1 is phosphorylated by 5' AMP-activated protein kinase 1 (AMPK1) [21] and CRY2 by a sequentialdual-specificity tyrosine- (Y)-phosphorylation regulated kinase 1A(DYRK1A) /glycogen synthase kinase 3beta (GSK-3β) cascade [22], being both targeted for ubiquitination and degradation byF-Box And Leucine-Rich Repeat Protein 3(FBXL3) [23].

In addition, the active CLOCK:BMAL1 heterodimer also promotes the transcription of the retinoic acid-related orphan receptor alpha (*Rorα*) and the nuclear receptor subfamily 1, group D (Nr1d1), also known asreverse erythroblastosis virus alpha(*Rev-erbα*), its own activator and repressor

respectively, competing both for a binding site within the response element (RORE) into the *Bmal1* promoter, generating another loop of regulation [18]. In addition to *Rorα,*peroxisome proliferator–activated receptoralpha (PPARα) is also a positive regulator of *Bmal1* expression, as *pparα*binds on a potential PPARα response element located in the *bmal1* promoter. BMAL1, in turn, is an upstream regulator of *pparα*gene expression, being this the reason why this gene can be considered aclock-controlled gene, which in turn suggest an additional regulatory feedback loop, involving BMAL1 and PPARα in peripheral clocks [24].

Therefore, CLOCK:BMAL1 heterodimer enhance the transcription of metabolic gens or clock controlled gens, as the case of *pparα*, which are implicated in manyaspects of metabolism and biochemical processes and, moreover, are often regulated through D-boxes elements in their promoters, revealing another potential transcription loop [19].

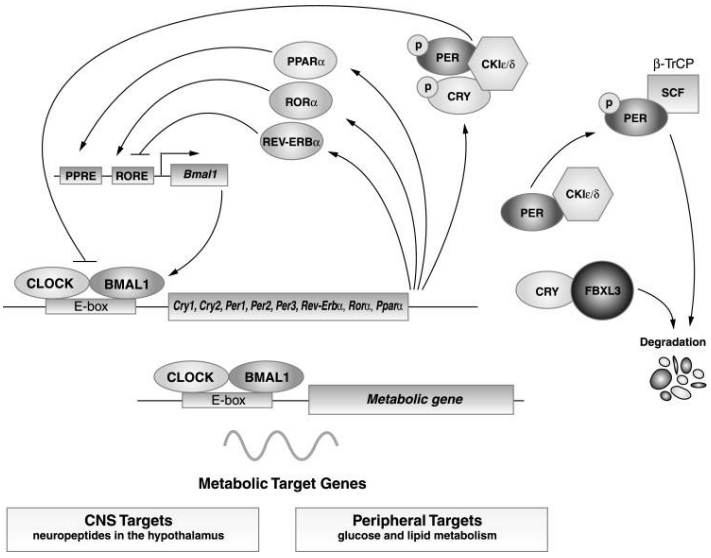

Figure 1. The molecular clock system, from Biliana Marcheva and colleagues[25]**.**

Hence, it has been proposed that the three known binding elements together provide the necessary delay to cycle about 24 h: E-box in the morning, D-box in the day, and RORE elements in the evening, which means maximum levels of *Bmal1* and *Clock* genes at night, while there are maximum levels of *Per2*, *Rev-erbα*, *Rorα*, …; in the morning [26].

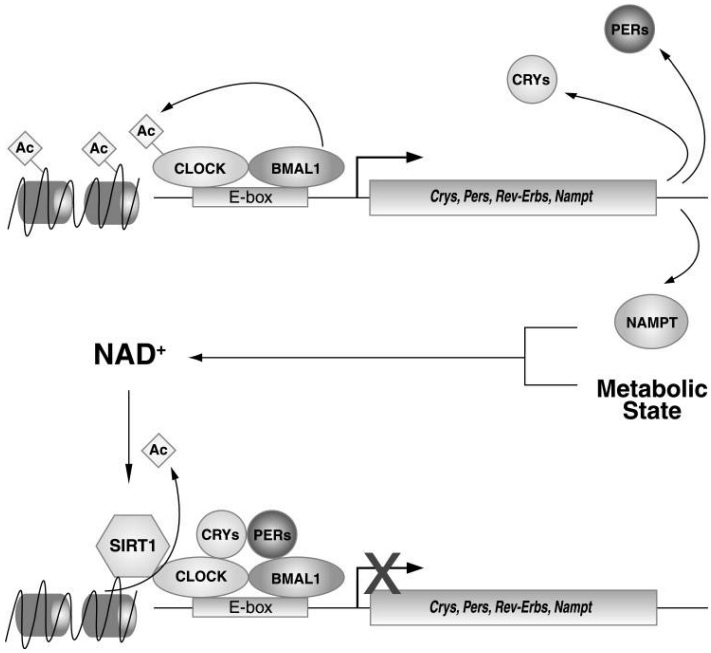

Figure 2. Acetylation and deacetylation of BMAL1, from Biliana Marcheva and colleagues [25].

In general, the chromatin remodeling necessary for this cyclic transcriptional activity, can be observed in the rhythmic acetylation/deacetylation of histones (H3 and H4) at multiple clock target genes, among other protein transformations as phosphorilations, etc..[27]. *Bmal1*, in this sense, is the angular stone of the cycle, as the robust transcriptional oscillations are lost in the absence of this core clock gene [28]. In fact, the acetylation of BMAL1 is carried out by its partner CLOCK, which possesses a histone acetyl

transferase (HAT) domain, while the rhythmic deacetylation of histone H3 at the promoters of circadian genes, such as BMAL1, is regulated by the deacetylase sirtuin 1 (SIRT1), which is sensitive to NAD+ levels (Figure 2) [29].

Related to this, the NAD+ to NADH ratio has been shown to regulate CLOCK/ BMAL1's ability to bind DNA in vitro [30], underlying the importance of the cellular metabolism towards the regulation of the transcriptional circadian state.

1.2.2. The master clock

The SCN appears to be highly conserved in its anatomical and physiological organization, containing approximately 20,000 neurons spread in two similar anatomic subdivisions: a ventral "core" region where light first induces immediate genes as retinal inputs are more dense in this subunit [31]; and a dorsal "shell" region, where circadian rhythms in gene expression occur after receiving projections from the "core" region [32]. Thus, compared to the whole human brain which is composed by 100,000,000,000 neurons, the SCN is a small area in the hypothalamus [33]. Taking into account the different functions carried out by the two SCN parts, it is essential that they stay well coupled in order to allow the SCN operates properly, as synchrony of these 20,000 neurons to each other, is vital for the robust generation of a coherent output signal. In this sense, three different mechanisms have been described for coupling: synaptic potentials, electrical synapses, and neuropeptidergic signalling [12]; being Vasoactive Intestinal Peptide (VIP) signalling a powerful mechanism in the latter, as mice laking VIP [34] or its receptor VPAC2 [35], lost rhythmicity. The robustness of the intact SCN is also important for its ability to remain in appropriate phase with the light–dark cycle. In the presence of rhythmic physiologic perturbations, as in cases

when food availability is restricted to a time of the day, when an animal is typically asleep and certain peripheral clocks shift their phase accordingly [36]; or under the presence of physiologic temperature fluctuations, being especially evident in cultured SCN, where the tissue becomes sensitive to physiologic temperature changes when communication between cells is lost because they are dissociated [37].

Therefore, for the propagation of the phase information to the rest of the brain and body, the SCN firstly has to be synchronised by photoreceptors found exclusively in the eyes under light cycles in the environment [38], so as it has been postulated before; and secondly, SCN cells consequently release and receive signals that allow them to synchronize to each other to integrate the incoming information from light and other peripheral clocks and, in turn, synchronising peripheral clocks and body rhythms, thereby, synchronising the individual cells of the body to a uniform internal time [32]. In other words, SCN has a pivotal role in maintaining circadian rhythms, as complete SCN lesions abolish circadian rhythmicity [39,40], while implantation of foetal SCN tissue can partially restore them [41], so as when isolated *in vitro*, the SCN continues to express circadian rhythms [42].

The SCN synchronizes peripheral clocks and body rhythms by neural connections and hormonal signals [13]. Beyond the brain, the autonomous nervous system plays a direct role in communicating circadian SCN timing signals to multiple tissues. For example, SCN signals travel via the autonomous nervous system to the liver in order to control the circadian glucose production [43], to the heart for cardiac rate regulation in circadian fashion [44], to the adrenal gland to regulate both circadian and light-dependent corticosterone production [45], or to the pineal gland to control circadian melatonin production [46], among others examples.

In total, sympathetic efferents have been documented for brown adipose tissue, thyroid gland, kidney, bladder, spleen, adrenal medulla, and adrenal cortex; while parasympathetic nervous system innervation of the thyroid, liver, pancreas, and submandibular gland has also been reported, being even innervated some tissues by both sympathetically and parasympathetically SNC innervations [47].

The case of driven melatonin production (Figure 3) is doubly important as the sympathetic innervations of the pineal gland, where melatonin is mostly synthesized during the dark phase, to connect the rhythmic activity of the SCN with the rhythmic release of melatonin, was the first output pathway which provided a circadian message to the organism. Melatonin is a robust signal that indicates the time of environmental darkness, being even secreted during

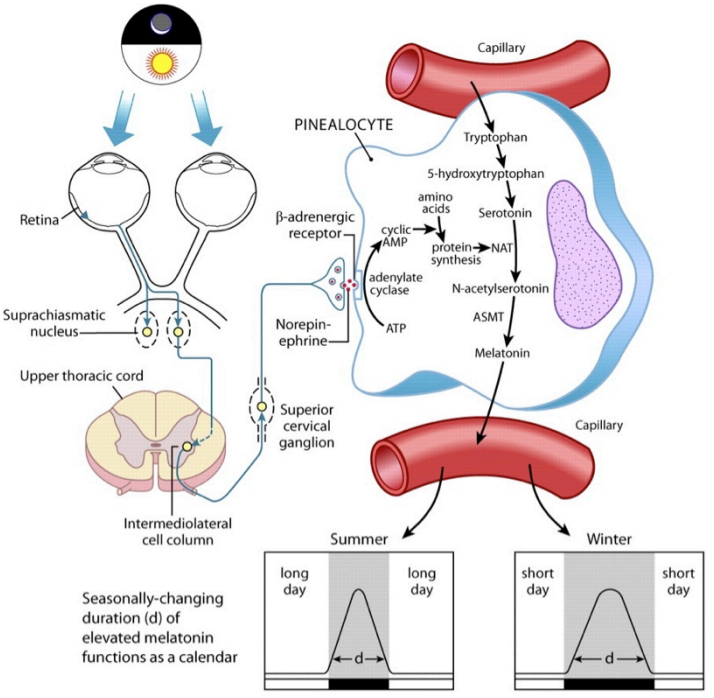

Figure 3. Circadian melatonin production through SCN signals, adapted from Reiter, Russel J and colleagues [48]

the dark phase of the circadian cycle in nocturnal animals, after direct control of SCN [49].Importantly, melatonin is not stored within pineal cells and is immediately released into the general circulation, underlying the fact that plasma melatonin concentrations reflect precisely its pineal synthesis, being in turn related with the better predisposition of its receptors spread throughout the body, as they are highly expressed at night, which explains the physiologic effects of melatonin [50].

In addition, although melatonin cannot be stored in pineal gland, it can do it in the gut and stomach at major concentrations than in plasma. In fact, it is now accepted that whereas night time levels of melatonin in blood are mostly from pineal origin, day time melatonin concentrations in blood are mostly produced in gut as its synthesizing enzymes; N-acetyltransferease and hydroxyindole-O-methyltransferase, were detected in this tissue [51]. Nonetheless, the implications of melatonin present in the gut go beyond its properties on the circadian system, as conversely to systemic melatonin levels, the gut melatonin levels are independent of light or the circadian rhythm, being more entrained its synthesis, as a result of food intake and digestion. For this reason, melatonin exerts in gut local effects regulating intestinal motility, the immune system, gut secretion, and the release of peptides involved in energy balance such as peptide YY; so as a protective effect against some disturbances, playing a role as antioxidant element [52].

1.2.3. The peripheral clocks

The circadian system can be observed not only in the SCN but also in nearly every mammalian tissue [53], including those essential for metabolic function, such as the pancreas [54], muscle [55], liver [53,55] and heart [53]; and other isolated brain regions apart from SCN [56], displaying circadian patterns of gene expression in a self autonomous form, as a mechanism of adaptation to local needs and better functional predisposition on time. In fact,

in SCN-lesioned animals, individual organs still maintain some degree of circadian synchrony in clock gene expression, although this varies among both animals and organs [57]. It is postulated that even at the single-cell level, the molecular clockwork of transcription and translation can be also observed working autonomously in every single cell [58], underlying that the number of oscillators might be as numerous as cells are. This cell-tissue-autonomous rhythms, can be translated in the fact that different mouse tissues show different circadian phases in tissue explants [53]. For example, a five-hour phase difference is observable between the liver and the spleen, and nearly eight hours between the liver and gonadal adipose tissue. Supporting a tissue-intrinsic mechanism for these phase differences, free-running period in tissue explants differed by 2–4 hours between liver and the other two tissues [59], again pointing to subtle tissue-specific differences in free-running clock mechanism, although alternatively, different entrainment signals might also play a role in these differences, as peripheral clocks respond to a complex and redundant combination of direct nervous stimuli, hormonal signals, and indirect activity-directed signals such as body temperature and the timing of food intake, among others, like it will be shown in the next section.

1.3. Clock system synchronisers

Any environmental factor that varies across the 24-h day can potentially serve as an entraining signal, *zeitgeber* (“time giver” in German). In this regard, light has a dominant role because light and therefore, darkness, are responsible for all other environmental rhythms, being the most reliable source of information about the time of day, so as the most important *zeitgeber* for the SCN. As a reminder, the SCN translates the information of light and darkness to the many other oscillators in the body, by providing endogenous *zeigebers*, through the control of the daily production of

melatonin, the hormonal alarm of night as it was described above. Nonetheless, in parallel, melatonin itself can exert an effect as a synchronizer, similar as light does, synchronizing SCN transcriptional loops with temporal organization, attending that melatonin receptors are present also in SCN [60]. In fact, melatonin administration several hours prior to the normal onset of secretion, causes a phase advance in the endogenous melatonin rhythm, which is particularly useful for the treatment of eastbound jetlag; while melatonin treatment following the endogenous onset of secretion, is often useful to improve westbound jetlag [61].

The rest-activity cycle, which is related to the light-dark cycle, play an important role in circadian physiology and gene expression in some tissues as, of 2,032 cortical transcripts under circadian control, only 391 remained rhythmic during sleep deprivation [62]. Thus, sleep itself can also be considered an important behavioral synchronizer, because, in addition, it influences the daily light profiles after closing the eyelids or retreating into a burrow or a dark room.

Temperature, both internal or external, entrain the cellular peripheral clocks in mammalian tissues cultures [63], although according to Takahashi and colleagues [37], due to the strong coupling of the SCN neurons, the central clock is resistant as it has been referenced formerly, so as in the case of food. In fact, until now, only external factors have affected the SCN when it has been presented in an uncoupled form. Besides temperature and food, daily administration of γ-aminobutyric acid (GABA) to cultured dissociated SCN neurons, can synchronize rhythms of spontaneous firing, shifting their phase under a single administration, as most neurons in the SCN produce this neurotransmitter [64]. In addition, caffeine adjusts circadian timing of electrical activity in the insolated SCN, the clock gene expression in cultured mammalian cells, and modestly lengthens the circadian period of locomotor activity in mice [65].

Food as a synchronizer has been studied amply. Particularly, it might be considered the oldest *zeitgeber* along with the light cycle, as the evolutionary oldest clocks known are those in cyanobacteria, photosynthesizing prokaryotes, in which light is both an energy resource and a *zeitgeber* [66]. In addition to its age, food is one of the most important synchronizers, as in the mouse liver, for example, only a small portion of transcripts displayed circadian expression patterns in the absence of food, being the circadian transcriptome restored, under temporal restricted feeding even in the absence of functional liver clocks [67]. In fact, on a molecular level, restricted feeding entrains circadian oscillations in peripheral tissues, such as the liver. In line with this, the speed as well as the degree of phase shift induced by inversed feeding, which inverses the timing of peripheral clocks, varies among different organs, without affecting the clock rhythms in the central pacemaker in the SCN, thereby uncoupling the phase of peripheral clocks from that of the SCN. For example, mRNA of the clock gene *Dbp* examined in mice fed only during the light phase shows a strong temporal difference in the liver, the kidneys, the heart, and the pancreas, whereas in mice fed during the dark phase, the accumulation of *Dbp* mRNA was around ZT14 to ZT18 in all analyzed tissues [36].

In addition to the timing of food availability affecting the circadian outputs of the clock, caloric restriction (i.e., restriction of the total number of calories consumed without malnutrition) induces phase advances in rat behavioral and physiological circadian rhythms, and alters expression of clock genes and neuropeptides in the mouse SCN [68], so as prolonged fasting, that also advances the phase of free-running rhythms of locomotor activity and temperature [69]. On the other hand, mice fed a high-fat diet have increased daytime activity, lengthened period of locomotor activity rhythms, and altered expression of clock and clock-controlled genes involved in fuel utilization [70]. These mice, by the way, consume nearly all of their extra

calories during the 12-h light phase, suggesting that feeding at the incorrect time in the light/dark cycle (i.e., their rest period) exacerbates the obesogenic effects of high caloric intake due to desynchronization of various behavioral, hormonal, and molecular rhythms involved in maintaining energy balance [70].

To date, from a more concrete point of view, some concrete dietary components as resveratrol [71], have shown a clear property as a signal for the clock machinery, although it will be studied more extensively in the second section.

1.3.1. Mechanisms for synchronisation

Apart from the mechanisms by which the central clock system entrains circadian rhythms in periphery, thereby exerting a role as a synchroniser through the autonomous system, as it has been commented in the 1.2.2. section, there are other mechanisms by which a synchroniser can synchronise the clock system.

On the one hand, three classes of signalling pathways have been identified as capable of independently phase-shifting peripheral circadian clocks: cAMP and MAP kinases, protein kinase C, and calcium signalling [72]. In fact, multiple signalling agents such as endothelin-1 [73], forskolin [74], fibroblast and epidermal growth factor [75], glucose [76] and prostaglandin E2 [77]; act through these pathways, inducing and synchronising circadian clocks *in vitro*, whereas prostaglandin E2 [77] and dexamethasone, a glucocorticoid analogue, have been shown to shift circadian clocks acutely in peripheral organs, when injected into mice. In the case of dexamethasone, the effect is conducted by other mechanism, the glucocorticoid signalling, wherein dexamethasone activates the glucocorticoid receptor, which can

independently control 60% of the circadian transciptome [78], reseting the peripheral clock in liver, heart, and kidney, presumably by direct glucocorticoid receptorregulation of *Per1*[79] and *Rev-erbα* [80] expression.

On the other hand, as sensors of metabolites including heme, fatty acids, and sterols; REV-ERBα/β and RORα integrate nutrient signals with transcriptional regulation of the clock, as they are the molecular *Bmal1* repressor or activator respectively, revealing another mechanism by which the clock system can be synchronized by a zeitgeber (in this case the heme metabolite or fatty acids and sterols). In relation, PPAR family of Nuclear Hormonal Receptors (NHRs) are also regulators of clock gene expression, as PPARα, which is a clock-controlled gene and a positive regulator of *Bmal1* expression, can be activated by various types of lipids, including the circulating gut metabolite oleylethanolamide (OEA) or pharmacologic doses or phenofibrate, all known as natural ligands [81], and therefore, exerting a role as a synchronizers through this alternative mechanism.

1.4. Circadian clock system and metabolism

Organisms are in constant interaction with the environment along the time, which entrain many behavioural, physiological, and metabolic aspects as for example, circadian cycles in rest and activity, blood pressure, body temperature, alertness, coordination, reaction and brain activity, cardiovascular efficiency or muscle strength, hormones secretion in blood and ions in urine, responding to an external demand in order to allow organisms a better adaptation [11].

In relation, physiologic disturbances curiously also cycletending to peak at particular times during the day, as for instance myocardial infarction [82] or asthma episodes [83] among others. The fact that glucose levels peak before the start of the active period, the glucose tolerance and insulin action are

known to vary throughout the day and oral glucose tolerance is impaired in the evening compared to morning hours, due to combined effects of reduced insulin sensitivity and diminished insulin secretion in the nighttime [84], can be considered other examples. Similar as the susceptibility to UV light-induced skin cancer [85] and that chemotherapy treatments varies greatly across the circadian cycle in mice [86], suggesting a better predisposition of cells in some concrete period of time.

In fact, transcriptional studies have revealed that approximately 10% of all mammalian genes across multiple tissues such as liver, muscle, heart, adipose tissue or the SCN [87–90] exhibit 24-h variations in mRNA levels, grouping many of these rhythmic genes within processes like mitochondrial oxidative phosphorylation, carbohydrate metabolism and transport, lipid biosynthesis, adipocyte differentiation, and cholesterol synthesis and degradation. From these processes, a small subset of these oscillating metabolic genes are direct target of the molecular clock, while many others encode transcription factors, transcription or translation modulators, or rate-limiting enzymes, which in turn impart rhythmicity on downstream metabolic genes and processes [19,89]. Among the direct target metabolic genes, *Nampt* is one of the most important because, a part of being a *Bmal1* target gene, it is also the rate-limiting enzyme that converts the Nicotinamide (NAM) to Nicotinamide Mononucleotide (NMN), which is a key reaction in the biosynthesis of Nicotinamide Adenine Dinucleotide (NAD) through its salvage pathway (Figure 4) [91].

NAD is a key molecule in metabolism, as it has been known to play a major role as a coenzyme in numerous oxidation-reduction reactions [92], being required in a number of important signaling pathways in mammalian cells, including poly(ADP-ribosyl)ation in DNA repair [93], mono-ADP-ribosylation in both the immune response and G protein-coupled signaling [94], and synthesis of cyclic ADP-ribose and nicotinate adenine dinucleotide phosphate (NAADP) in intracellular calcium signaling [95]. Furthermore,

NAD and its derivatives also play important roles in transcriptional regulation [96]. In fact, SIRT1 activity depends absolutely on the NAD levels (Figure 4).

SIRT1 itself, is another critical regulator of metabolic processes such as gluconeogenesis, lipid metabolism, and insulin sensitivity, as well as lifespan, as it targets several transcription factors involved in the maintenance of nutrient flux, including Peroxisome Proliferator-Activated Receptor gamma (PPARγ), Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-alpha (PGC-1α), Forkhead Box Protein O1 (FOXO1), Transducer of Regulated CREB Protein 2 (TORC2), Sterol Regulatory Element-Binding Protein 1c (SREBP-1c), and Signal Transducer and Activator of Transcription 3 (STAT3), among others [97,98]. In addition, SIRT1 also modulates CLOCK/BMAL1 activity, generating a negative feedback loop after deacetylating BMAL1 (Figure 4) [29].

Besides this, another molecular method of synchronizing circadian clocks to metabolism is probably mediated by cryptochrome clock proteins, which are phosphorylated and targeted for degradation by AMPK, an enzyme regulated by cellular ATP/ AMP balance [21].

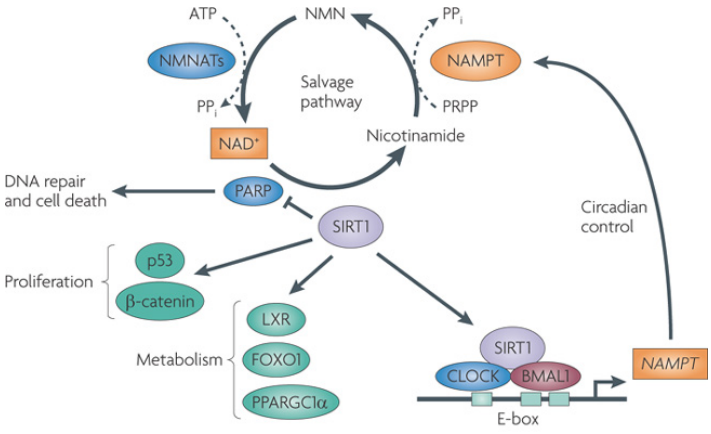

Figure 4. NAD salvage pathway and SIRT1 interelation, from Saurabh Sahar and Paolo Sassone-Corsi [99].

As it was postulated previously in the section of peripheral clocks, the phase of oscillation and the level of expression of each metabolic gene vary across different tissues, suggesting that the circadian system responds to both local and systemic cues, to control diverse metabolic processes in a physiologically autonomous manner [100]. Therefore, as a result, a large number of physiological processes are under circadian control:

- Xenobiotic detoxification: CLOCK and BMAL1 protein control the *Dbp* which binds to the constitutive androstane receptor (CAR) gene promoter, which in turn controls circadian expression of many cytochrome P450 isoforms that directly regulate metabolism of a wide variety of xenobiotics [101].

- Lipid metabolism: CLOCK and BMAL1 protein also control the expression of *peroxisome prolierator-activated receptor alpha* (PPARα), a key regulator of the lipid metabolism [102].

- Glucose metabolism: circadian clock ablation in pancreatic islets results in diabetes due to defects in coupling of beta cell stimulus to insulin secretion [54].

- Renal activity: mice lacking CLOCK show significant changes in renal expression of key regulators of water and sodium balance, as well as changes in sodium excretion itself [103].

- Immune function: circadian clocks in macrophages [104] and T cells [105]govern inflammatory immune responses, and the clock protein REV-ERBα appears to play a specific role in selectively regulating inflammatory cytokines [106].

- Cardiovascular health: arterial transplants from animals lacking circadian clocks develop atherosclerosis in transplanted blood vessels [107]. In addition, circadian clock control of adrenal aldosterone production via the enzyme Hsd3b6 is an important regulator of blood pressure [108].

Thus, due to the vast amount of circadian biologic processes that the molecular clock system controls, the specific disruption of some clock gene can create, in turn, a wide range of pathologies, as it has been observed in some knockouts experiments in mice, reviewed [25,109] here:

- CLOCK knockout mice display increased blood levels of triglycerides, cholesterol, glucose, leptine and therefore, it presents obesity and metabolic syndrome, besides hyperfagia.

- BMAL1 knockout mice display impaired gluconeogenesis and adipogenesis, adipocyte differentiation, hyperlipidemia, glucose intolerance, hypoglycemia, reduced life span, premature aging, impaired steroidogenesis and reduced fertility.

- PER knockout mice display absent glucocorticoid rhythm and diurnal feeding rhythm, obesity, alternations in leptin-depenent bone density, cancer-prone, abnormal response to gamma irradiation, increased cellular proliferation, reduced muscular strength under stress, and less of food anticipatory activity.

- CRY knockout mice display impaired body growth, feminized patterns of growth hormone and metabolic genes in liver, impaired liver regeneration, reduced α-adrenoreceptor

responsiveness, salt sensitive hypertension, reduced tumor development and lowered incidence of cancer.

- REV-ERB knockout mice display elevated serum VLDL triglycerides, impaired Purkinje cells development, delayed proliferation and migration of granule cells, increased apoptosis of neurons in the internal granule cell layer and aberrant expression of myosin isoforms in skeletal muscles.

- ROR knockout mice display lower plasma triglycerides, abnormal lymphoid organ development, reduced survival rates of thymocytes, reduced susceptibility to autoimmune and inflammatory disorders, thin long bones, ataxia and severe cerebellar atrophy, fewer Purkinje cells and a loss of cerebellar granule cells.

Out of the laboratory, since the introduction of artificial light and nighttime work, serious health consequences have been reported for those who sleep less and/or routinely disconnect their working time from the light/dark cycle, as reduced sleep duration (both acute and chronic) and poor-quality sleep are linked with impaired glucose tolerance, reduced insulin responsiveness following glucose challenge, increased body mass index, decreased levels of leptin, and increased levels of ghrelin [110,111]; while association studies have further revealed that shift workers have increased risk of suffer some component of the metabolic syndrome [112–114]. In addition, social jetlag, the discrepancy between the circadian and social clock and then a small but chronic version of shift-work or circadian misalignment, results in chronic sleep loss and increased BMI [115]. In fact, one of the most compelling clinical studies to examine the role of circadian alignment on metabolic physiology comes from an experimental paradigm in which healthy

volunteers were placed on a 28-h "day" and scheduled to sleep at different phases throughout the circadian cycle. When the subjects were shifted 12 h from their normal sleep/wake cycle, they exhibited decreased leptin, increased glucose, and elevated blood pressure. In addition, their post-meal glucose response was similar to that seen in prediabetic patients [116]. Together, these studies highlight the detrimental health effects of disruption of the circadian system and the importance of synchronization of physiological systems with the light/dark cycle for maintenance of overall health. Nonetheless, is still not clear whether a misalignment of the clock firstly impaired metabolism and physiology, or contrary, is the presence of illness what disrupts the clock machinery in the first place, although this bidirectional crosstalk is clear.

2. Polyphenols

Polyphenols are secondary metabolites of plants, generally involved in defense against ultraviolet radiation, aggression by pathogens, reproduction, nutrition and growth [117].

2.1. Polyphenols classes and structures

Polyphenols are a diverse group of natural compounds present in a wide range of plants, about 8000 phenolic structures have been described within the plant kingdom, that contain multiple functionalities, in parallel with diverse structures, properties and sizes ranging from monomers to polymers, although they tend to share a similar basic structure formed by at least, one aromatic ring with one or more hydroxyl groups attached. Polyphenols can be classified into different groups based on the number of phenol rings that they contain, the number and disposition of their carbon atoms; and of the

structural elements that bind these rings to one another, such as sugars or organic acids [118–120]:

- Phenolic acids: two classes of phenolic acids can be distinguished: derivatives of benzoic acid (components of complex structures that have not been extensively studied, as they are found in only a few plants eaten by humans), and derivatives of cinnamic acid, which consist mainly of *p*-coumaric, caffeic, ferulic and sinapic acids, therefore, being more common its consumption, as caffeic acid is involved in the biosynthesis of chlorogenic acid, another acid found in coffee. These acids are usually bounded to glycosylated derivatives or esters of quinic acid, shikimic acid, and tartaric acid; except in processed food that has undergone freezing, sterilization, or fermentation; in which case they are found in the free form.

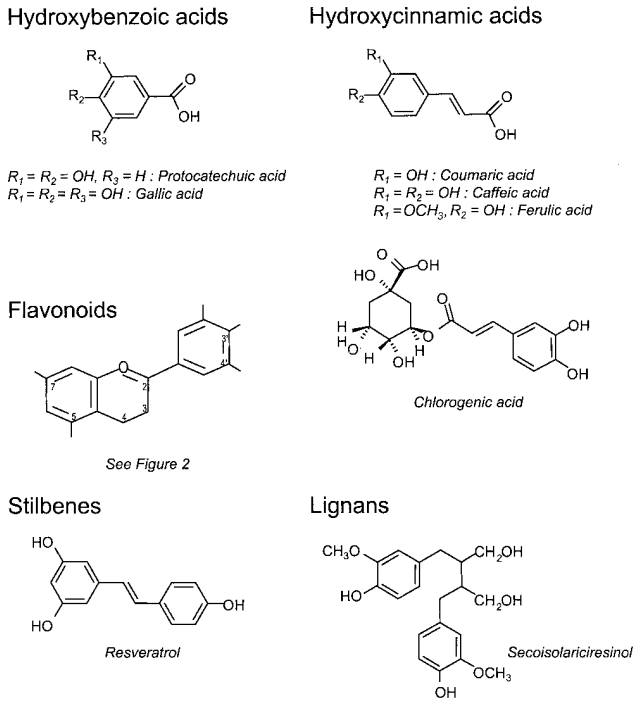

Figure 5. Polyphenol structures. Adaptation from Manach et al [118].

- Lignans: are formed of 2 phenylpropane units. The richest dietary source is linseed, which contains secoisolariciresinol and low quantities of matairesinol; while other cereals, grains, fruit, and certain vegetables contain minor traces. Lignans are metabolized to enterodiol and enterolactone by the intestinal microflora, which are known for its healthy properties.

- Stilbenes: are found in only low quantities in the human diet, being resveratrol the most representative constituent found in red wine and peanuts, and in lesser amounts in berries, red cabbage, spinach and certain herbs. Structurally, resveratrol can adopt a cis or trans conformation, being trans-resveratrol and trans-resvertrol-3-O-glucoside, which has been detected in pistachio nuts. This is important as these structures have presented widely, cellular healthy effects towards health.

- Flavonoids: found throughout the plant kingdom, is the largest group of polyphenols comprising generally 15 carbons with two aromatic rings connected by a three carbon bridge, being in turn able to bind to hydroxyl groups and sugars that increase their water solubility, as they use to appear naturally as a glycosides; or other substituents, such as methyl and isopentyl units, that make flavonoids lipophilic. Due to the amount of flavonoids existing, a subclassification is required [118–120]:

  - Flavonols such as quercetin and kaempferol as main representatives, are widely present at relatively low concentrations in fruits and vegetables, presented in glycosylated form, as they bound often to glucose or rhamnose and even, to galactose, arabinose, xylose and glucuronic acid.

  - Flavones are much less common than flavonols in fruit and vegetables but, similarly, consist mainly of glycosides, being luteolin and apigenin two representative examples of flavones, which are not distributed amply, as significant occurrences, to date, have been

reported in only parsley, celery and some herbs. In addition, the skin of citrus fruit contains large quantities of polymethoxylated flavones, such as tangeretin, nobiletin, and sinensetin; which are the most hydrophobic flavonoids.

- Flavanones are found in tomatoes and certain aromatic plants such as mint; and in higher concentrations only in citrus fruit. They are generally glycosylated by a disaccharide at position 7, being the main aglycones naringenin in grapefruit, hesperetin in oranges, and eriodictyol in lemons.

- Isoflavones are flavonoids with structural similarities to estrogens, which confer pseudohormonal properties such as the ability to bind to estrogen receptors, reason why they are classified as phytoestrogens. They are founded almost exclusively in leguminous plants, being soy and its processed products the main source in the human diet, where they are found in 4 forms: aglycone, 7-*O*-glucoside, 6''-*O*-acetyl- 7-*O*-glucoside, and 6''-*O*-malonyl-7-*O*-glucoside, with the most representative molecules being genistein, daidzein,and glycitein.

- Anthocyanidins are widely dispersed throughout the plant kingdom, being particularly evident in fruit and flower tissue where they are responsible for red, blue and purple colours. They are also found in leaves, stems, seeds and root tissue, being present in the human diet in red wine, certain varieties of cereals, and certain leafy and root vegetables such as aubergines, cabbage, beans, onions or radishes. The most common anthocyanidins in plant tissues are found as sugar conjugates that are known as anthocyanins, which may also be conjugated to hydroxycinnamates and organic acids such as acetic

acid. Some representative examples of anthocyanidins are pelargonidin, cyanidin, delphinidin, peonidin, petunidin or malvidin.

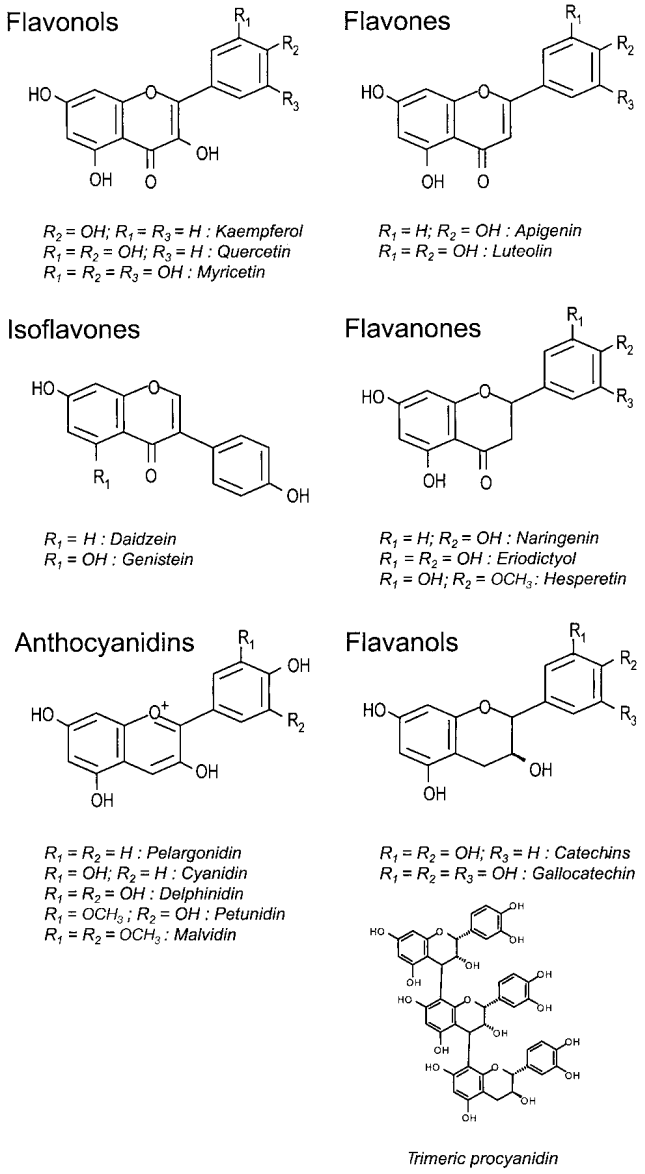

Figure 6. Flavonoids structures from Manach et al [118].

- Flavan-3-ols or flavanols are the most structurally complex subclass of flavonoids, ranging from the simple monomers (+)-catechin and its isomer (-)-epicatechin, which can be hydroxylated to form gallocatechins and also undergo esterification with gallic acid, to complex structures including oligomeric and polymeric proanthocyanidins, which are also known as condensed tannins. Catechins are found in many types of fruit (apricots are the richest source), beverages like red wine and highly in green tea and in significant quantities in chocolate. In contrast to other classes of flavonoids, flavanols are not glycosylated in foods.

2.2. Physiological functions, conformation, bioavailability and metabolism of grape seed proanthocyanidins extract

Procanthocyanidins (PAs) are the most consumed polyphenols in human diet for its widespread presence in vegetables, fruits, cacao, nuts and some beverages like red wine or tea [121].

The Grape Seed Proanthocyandin Extract (GSPE) experimentally used in this thesis, has been used previously in several studies in our group, using various *in vitro* and animal models, demonstrating that PAs have a vast range of health effects improving insulin resistance [122], inflammation [123], hypertension [124], oxidative stress [125] and lipid metabolism [126]. Therefore as other groups agree, PAs have protective properties against metabolic syndrome [127] and cardiovascular diseases [128]. In addition, PAs have also shown anticancer properties by inducing apoptosis or inhibiting cell proliferation but also neuroprotective effects by inversely regulating apoptotic mechanisms [138–140], as well as, antibacterial power as they are able to produce antiadhesive actions against bacteria in urinary

and dental infections, including *Escherichia coli* and *Streptococcus* mutants [127].

GSPE used in this thesis was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France), with the following GSPE composition [129]: catechin (58 μmol/g), epicatechin (52 μmol/g), epigallocatechin (5.50 μmol/g), epicatechingallate (89 μmol/g), epigallocatechingallate (1.40 μmol/g), dimeric procyanidins (250 μmol/g), trimeric procyanidins (1568 μmol/g), tetrameric procyanidins (8.8 μmol/g), pentameric procyanidins (0.73 μmol/g) and hexameric procyanidins (0.38 μmol/g); therefore the dimeric procyanidins and specially, the trimeric procyanidins are the main constituents of this extract. In fact, procyanidins are formed from the condensation of monomeric units, catechin and its isomer, epicathechin, which in turn can be gallated, as it was commented previously. Between two and five units of monomers conform oligomers while over five units of monomers, are polymers [130]. Therefore, our extract is rich in oligomers.

During digestion, the oligomers are fragmented into monomeric units of cathechin and epicathechin, which are absorbed in the small intestine, resulting in a wide range of conjugated metabolites, from the combination of sulphatation, glucuronidation and methylation, after its exit from the liver by means of the bloodstream. It has been postulated that large doses are metabolised mainly in the liver, and small doses may be metabolised by the intestinal mucosa, with the liver playing a secondary role to further modify the polyphenol conjugates from the small intestine, underlying the important role of liver exerting this chemical changes [131]. In this sense, in a collaboration with Aida Serra from the Universitat de Lleida [132], the same extract used in the experiments of this thesis, was administered to rats with an acute large dose of 1g/kg of body weight. This resulted in a maximum peak concentration of several metabolites in the plasma, such as catechin and epicatechin glucuronide and methyl-glucuronidated, two hours after GSPE ingestion; while free forms of dimmers and trimers reached one hour after

GSPE administration, displaying an important stability under gastric and duodenal digestion conditions although a limited absorption. In consonance, it has been observed in another previous study using a similar extract with the same dose [130], where the vast majority of components detected in the gastrointestinal tract, that remained in the stomach for 6 hours appearing in lesser quantities in the intestine 1 hour after ingestion; were the original flavan-3-ols extract forms, as oligomeric procyanidins were not depolymerised into monomeric forms to any extent of the gastrointestinal tract, so as other authors had also observed [133]. Conversely, the circulatory system only contained methylated and glucuronidated flavan-3-ol metabolites, so as in the case of liver and kidney, suggesting that these structures could be hydrolysed by the colonic microflora, prior to the return to the blood stream [130]. In line, in other study where rats were administered a hazelnut skin extract rich in procyanidin oligomers [134], similar to ours, a variety of simple aromatic acids, probably the products of the colonic fermentation of procyanidins, was detected in the intestine and tissues. Underlining the similarity between GSPE and this hazelnut skin extract, as they both are rich in oligomers, it was observed again, the presence of glucuronidated and methyl-glucuronidated conjugates in the plasma two hours after hazelnut skin extract, even in some organs like the thymus, lung, kidney, spleen or testicles, although, the free forms of catechin and epicatechin were not detected in either the plasma or tissues, except in the lungs. The free forms of procyanidin dimer and trimers were only quantified in the plasma, but not in tissues, confirming the finding of other studies [130,132]. The disposition of these structures in tissues, could be the crucial importance in order to understand their biological activities. The glucuronidated and methyl-glucuronidated conjugates, so as the simple aromatic acids converted by the colonic flora, would be the major functional candidates, as they were found in liver, kidney, heart, lung, testicles, thymus and spleen in other study focused in the distribution of procyanidins and their

metabolites in rat tissues, after the ingestion of procyanidin-enriched or procyanidin-rich cocoa creams [135]. However, in 2009, Gemma Montagut and collages [136], found that dimeric and trimeric oligomers reproduced the bioactivity in glucose metabolism, lipid metabolism and macrophage functionality; which globally suggest, that the dose and duration of the treatment with procyanidins, is what ultimately determine its accumulation in different tissues and functionality. In this sense, in a more recent experiment carried out by Anna Arola-Arnal and colleagues [137], an acute dose of a similar GSPE (1g/kg of body weight) was administered to rats. Surprisingly, in the brain, catechin, epicatechin, and dimeric procyanidin B2 were quantified at low levels (1.27–2.39 nmol/g tissue), and some glucuronidated forms of catechin and epicatechin such as catechin glucuronide, epicatechin glucuronide, and methyl-epicatechin glucuronide at 1.15–2.20 nmol/g tissue, between 1 and 2 hours after GSPE administration, confirming the findings of other study where catechin and epicathechin metabolites were found in the brain after three weeks of a cocoa diet [138], all together underlying the ability of these flavonoids to cross the blood-brain barrier, dependently of the dose and duration of the treatment. In fact, being more accurated, another experiment with a rat model of Parkinson's disease under chronic oral administration (10mg/kg/day for 28 days) of tangeretin, resulted with a significant level of this citrus flavonoid in the hypothalamus, even at higher concentrations than in liver and plasma [139], suggesting the ability of these molecules in being stored in different structures of the brain.

3. Healthy effects of plant polyphenols in other species: a xenohormetic event regulated by the circadian system?

3.1. Xenohormesis concept

Over the course of long-term evolution, as well as compulsory quick seasonal adjustments, plants have learnt to cope with changing environmental conditions and pressures due to the formidable chemical arsenal of secondary metabolites, which have been implicated in the plant resistance against microbial pathogens and herbivores, such as insects, exerting an antibiotic and antifeeding effect respectively, protection against solar radiation and consequently, a role in reproduction, nutrition and growth [117]. Therefore, polyphenols exert a crucial role in life expectancy, in other words, the lifespan of a plant. In fact, the synthesis of polyphenols is induced in plants by a variety of environmental stresses, providing a chemical signature of the state of the environment, as environmental extremes of temperature variation, predation and water or nutrient availability, must be endured in place because plants, in general, cannot physically move away from stressors. For instance, the synthesis of resveratrol is stimulated by UV light, ozone, or pathogen stress [140]. Therefore, when this chemical cocktail is ingested, it comes into intimate contact with the receptors and enzymes within the consumer, providing a wide range of beneficial effects towards health, so as it has been observed in the previous section. In fact, one third of the current top 20 drugs on the market are plant derived, hatching another healthy plant molecule, weekly. Salicylates are just one example of dozens of known plant bioactives that produce wide-ranging health benefits in humans by interacting with more than one endogenous protein, so as resveratrol and other polyphenols.

Thus, the fact that stress-induced plant compounds tend to up-regulate pathways that provide stress resistance (healthy benefits) in other organisms such as mammals, suggests that plant consumers may have mechanisms to

perceive these chemical cues and react to them in ways that are beneficial. This hypothesis in fact, was set up by Konrad T. Howitz and David A. Sinclair [141,142]by coining the term xenohormesis, the process by which one organism benefits from the stress response of another (from *xenos*, the Greek word for stranger, and *hormesis*, the term for health benefits provided by mild biological stress, such as cellular damage or a lack of nutrition).

3.2. Is the circadian system a possible mechanism by perceiving molecules from a xenohormetic point of view?

There are several possible explanations by which the animal's stress response should be activated by products of a plant's stress response, but basically, the main explanation could be the fact that as mammals do not have the ability to synthesize polyphenols, they have the ability to sense these plant molecules, as polyphenols provide a highly useful advance warning of a deteriorating environment and/or food supply [142], which in turn, could precede the polyphenol beneficial effects, being in fact, the main cause of them. In this sense, the conservative functionality of biochemical molecules between plants and mammals, could mean that common biosynthetic pathways existed before the separation of the plant and animal kingdoms, suggesting that evolution has simply used these ancient pathways to create similar-looking signaling chemicals [143], as the protective properties of polyphenols in plants, are translated in healthy effects in mammals. Therefore, the same molecules that are able to expand the life of plants, also expand the life of mammals.

Related to this and coming back to the first section of this introduction, there is a mechanism shared by almost all species worldwide, which is able to measure the day length, gathering the required information to increase the survival expectations over time, through the improvement of the life adaptation after first driving the physiology. It is the circadian system. In fact, beyond its function of generating daily rhythms, circadian clocks are

sensors for environmental information that allows them to remain entrained to the regular changes of day and night, of light and dark, of warm and cold, of humidity and of all the resources that depend on these environmental changes such as availability of food or presence of enemies and/or competitors, etc. Therefore, it is not strange that the circadian clock of an animal, can sense the information of the state of the environment incorporated in a polyphenol, such as resveratrol, attending that its synthesis has been induced by a variety of environmental stresses [140]. In fact, resveratrol has been the first polyphenol in synchronizing the molecular clock system in mammals to date, hence being sensed by other specie that have taken advance of it, extending its lifespan [71].

Furthermore, regular environment changes occurred daily, implies an early development of circadian clocks in evolution, extending this conservative feature of circadian clocks among species, to a conservation along the evolution, as it is known that some multicellular organisms such as *Drosophila* and mammals possess homologous components [15], unraveling that a common basic clock mechanism existed before the separation of insects and mammals more than 500 million years ago. Nonetheless, some argue that the relationship of basic clock mechanism and proteins may be extended to *Neurospora* [144], 2.5 billion years ago, attesting that, although there is no evident relationship between the circadian proteins of cyanobacteria and those of mammals, as they do not share the same clock components at the molecular level [14]; the mere fact of presenting a transcription-translation feedback loop for all organisms in which the molecular clock mechanism has been investigated, point to the suggestion that a basic metabolic rhythm-generator may be ancestral to all circadian clocks and that the specific transcriptional-translational mechanisms represent adaptations in the respective phyla. In fact, circadian rhythms are also important for the fitness of cyanobacteria, as many cyanobacterial

species organize a large fraction of their metabolism under circadian transcriptional control, implying for example a selective advantage to transcribing photosynthesis genes at the right time during a light–dark cycle [145]. Therefore, for its functions advancing physiologic events according to external cues in order to extend the lifespan and, in addition, its conservation among different phyla and, thereby, evolution; circadian system is thought to be a fit mechanism for sensing *xeno* cues from other organisms, such as ancient as secondary metabolites from plants. In this sense, this thesis tries to add another actor apart of resveratrol, a member of flavonoids amply known for its healthy properties, the procyanidins.

4. Polyphenols as synchronizers of the circadian system machinery

Polyphenols not only exert an effect in plants, but also in depredators like animals and humans, acting mainly as scavengers of free radicals and reactive oxygen species (ROS), which are overproduced under oxidative stress conditions and are unable to be subdued by the regular action of endogenous cellular antioxidants such as glutathione (GSH), glutathione peroxidase, or superoxide dismutase (SOD), or by dietary antioxidant vitamins like vitamins E and C. In addition to its redox properties, polyphenols exerts a related protective effect against cancer and neurodegenerative disease, as well as some key components of the metabolic syndrome like diabetes and cardiovascular disease, which could be mediated by a direct union to a target protein [146]. In this regard, dietary polyphenols such as resveratrol, curcumin, quercetin, and catechins, display anti-inflammatory properties via modulating different pathways, such as NF-kappaB [147].

In fact, more concretely, resveratrol has gained a lot of importance since it was postulated to be the first, and unique polyphenol to date, in activating

SIRT1 [141,148,149], although more recent studies has gone in the opposite direction, arguing that resveratrol is not a specific activator of SIRT1 [150,151], opening an interesting unfinished debate, which nowadays claims that resveratrol increases SIRT1 activity in some settings, through a direct allosteric activation mediated by an N-terminal activation domain in SIRT1 [152]. SIRT1 is implicated in the prevention of many age-related diseases such as cancer, Alzheimer's disease, and type 2 diabetes [153], controlling, at the cellular level, DNA repair and apoptosis, inflammatory pathways, insulin secretion, mitochondrial biogenesis and, in addition, the circadian clocks [154]. In this sense, SIRT1 is known to deacetylate BMAL1, playing in turn a critical role in metabolic processes such as gluconeogenesis, lipid metabolism and insulin sensitivity, as it has been mentioned in the first section.

Considering the implication of the resveratrol and SIRT1 interrelation in the clock machinery, it has been observed, additionally, as resveratrol adjusts the circadian rhythms of locomotor activity and body temperature in animals [155], regulates the expression of clock genes *Per1*, *Per2*, and *Bmal1*in cultured fibroblasts [71]and reverses, in rats, the change induced by high-fat feeding in the expression of *Rev-Erbα* in adipose tissue [156], confirming that circadian molecular system is a target for this polyphenol. To date, no more publications exist showing the modulation of some molecular component of the circadian system by other polyphenol, but resveratrol.

5. References

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III. HYPOTHESIS AND OBJECTIVES

The work performed in this Ph.D. thesis is part of a general research project developed by the Nutrigenomics Research Group of the Universitat Rovira i Virgili, which focus on the potential health effects of dietary proanthocyanidins and their mechanisms of action. There is particular interest in these polyphenols because they are the most consumed class of flavonoids due to their widespread presence in foods. In addition, these compounds have a wide range of beneficial effects, improving all the components of metabolic syndrome. Many of these effects have been studied in this Research Group.

In previous assays (unpublished), it was observed that GSPE was able to affect NAD levels in the liver and modulate RORα function; these important metabolic regulators are also two important components of the clock system. Nonetheless, the effects of GSPE on the clock system have not been studied to date, though other polyphenols such as resveratrol have been shown to modulate clock gene expression in cultured fibroblasts and in rat organs.

As a main function, the clock system allows the anticipation of environmental changes and adaptation to the time of day and food availability through the generation of circadian rhythms, which is intimately related to metabolic regulation and integration. This important function can be altered as a cause of a disrupted situation, thereby compromising health. Thus, we hypothesised that the molecular clock system could partially mediate the beneficial effects of proanthocyanidins on metabolism.

Therefore, the **main objective** of this thesis was to assess whether proanthocyanidins can modulate the central and peripheral molecular clock in rats and to identify the mechanism by which this flavonoid affects this system. To this end, six **specific objectives** were proposed:

1. To assess whether the chronic consumption of proanthocyanidins, at dietary doses, can modulate the peripheral clock system in the gut, the liver and the mesenteric adipose tissue of healthy (normal circadian rhythm state) and obese (disrupted circadian rhythm state) rats (manuscript 1).

2. To challenge whether proanthocyanidins can modulate the peripheral clock system in the liver after two different times of administration (manuscript 2).

3. To evaluate whether proanthocyanidins can modulate the clock system in HepG2 cells and to identify some of the potential mechanisms by which proanthocyanidins could affect the molecular clock system (manuscript 3).

4. To challenge whether proanthocyanidins can modulate the central clock system in the hypothalamus after two different times of administration (manuscript 4).

5. To determine whether proanthocyanidins can entrain internal body rhythms (manuscript 4).

6. To challenge whether proanthocyanidins can modulate the peripheral and central clock system in a disrupted situation such as jet lag simulation (manuscripts 2 and 4).

IV. RESULTS AND DISCUSSION

1. Chronic consumption of dietary proanthocyanidins entrains peripheral clocks in healthy and obese rats.

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Abstract: Circadian rhythm plays an important role in maintaining homeostasis, and its disruption increases the risk of developing metabolic syndrome. Circadian rhythm is maintained by a central clock in the hypothalamus that is entrained by light, but circadian clocks are also present in peripheral tissues. These peripheral clocks are trained by other cues, such as diet. The aim of this study was to determine whether proanthocyanidins, the most abundant polyphenols in the human diet, modulate the expression of clock and clock-controlled genes in the liver, gut and mesenteric white adipose tissue (mWAT) in healthy and obese rats. Grape seed proanthocyanidins (GSPE) were administered for 21 days at 5, 25 or 50 mg GSPE/Kg body weight in healthy rats and 25 mg GSPE/Kg body weight in rats with diet-induced obesity. In healthy animals, GSPE administration led to the overexpression of core clock genes in a positive dose-dependent manner. Moreover, the acetylated BMAL1 protein ratio increased with the same pattern in the liver and mWAT. With regards to clock-controlled genes, Per2 was also overexpressed, whereas Rev-erbα and RORα were repressed in a negative dose-dependent manner. Diet-induced obesity always resulted in the overexpression of some core clock and clock-related genes although the particular gene affected was tissue specific. GSPE administration counteracted disturbances in the clock genes in the liver and gut but was less effective in normalising the clock gene disruption in WAT. In conclusion, proanthocyanidins have the capacity to entrain peripheral molecular clocks in both healthy and obese states.

Chronic consumption of dietary proanthocyanidins entrains peripheral clocks in healthy and obese rats.

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Abstract

Circadian rhythm plays an important role in maintaining homeostasis, and its disruption increases the risk of developing metabolic syndrome. Circadian rhythm is maintained by a central clock in the hypothalamus that is entrained by light, but circadian clocks are also present in peripheral tissues. These peripheral clocks are trained by other cues, such as diet. The aim of this study was to determine whether proanthocyanidins, the most abundant polyphenols in the human diet, modulate the expression of clock and clock-controlled genes in the liver, gut and mesenteric white adipose tissue (mWAT) in healthy and obese rats. Grape seed proanthocyanidins (GSPE) were administered for 21 days at 5, 25 or 50 mg GSPE/Kg body weight in healthy rats and 25 mg GSPE/Kg body weight in rats with diet-induced obesity. In healthy animals, GSPE administration led to the overexpression of core clock genes in a positive dose-dependent manner. Moreover, the acetylated BMAL1 protein ratio increased with the same pattern in the liver and mWAT. With regards to clock-controlled genes, *Per2* was also overexpressed, whereas *Rev-erbα* and *RORα* were repressed in a negative dose-dependent manner. Diet-induced obesity always resulted in the overexpression of some core clock and clock-related genes although the particular gene affected was tissue specific. GSPE administration counteracted disturbances in the clock genes in the liver and gut but was less effective in normalising the clock gene disruption in WAT. In conclusion, proanthocyanidins have the capacity to entrain peripheral molecular clocks in both healthy and obese states.

Keywords: Flavonoids, *Bmal1, Clock, RORα, Per2, Rev-erbα.*

Introduction

Proanthocyanidins are a class of polyphenolic compounds in vegetables, fruits, cacao, nuts and some beverages such as red wine and tea; therefore, their presence in the human diet is considerably high [1]. Importantly, proanthocyanidins are considered to be bioactive compounds for their physiological and cellular processes, and several studies using various *in vitro* and animal models have elucidated a varied range of health effects in relation to metabolism, such as effects on insulin resistance [2], obesity [3], inflammation [4], cardiovascular disease [5], hypertension [6], oxidative stress [7] and lipid abnormalities. In fact, the effect of proanthocyanidins on lipid metabolism via correcting dyslipidemia in obese rats and reducing triglyceridemia and lipogenesis is due to some well-studied mechanisms in liver, such as the repression of lipogenic genes, transcriptional activation of the nuclear receptor FXR or even the modulation of miRNAs [8–11].

However, it is well-established that lipid and carbohydrate metabolism and the expression of their key genes exhibit circadian oscillation. The circadian rhythm of every organism is regulated by a central molecular clock localised in the hypothalamic suprachiasmatic nuclei (SCN). However, an equal core clock mechanism is also expressed in extra-SCN regions of the brain and nearly all peripheral tissues [12]. The molecular clock consists of a transcription–translation autoregulatory feedback loop that cycle with a periodicity of approximately 24 h. The positive limb of this loop is driven by the transcriptional activators circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT like protein 1 (BMAL1), which after their own heterodimerisation activate the transcription of the period (Per) and cryptochrome (Cry) genes, and once they reach a critical concentration, the PER and CRY proteins translocate into the nucleus and inhibit the activity of the CLOCK:BMAL1 heterodimer, thus leading to a decrease in Per and Cry expression. In addition, the active CLOCK:BMAL1 heterodimer also promotes the transcription of retinoic acid-related orphan receptor alpha (Rorα) and nuclear receptor subfamily 1, group D (Nr1d1, also known as Rev-erbα), its own activator and repressor, respectively, generating another loop of regulation. Finally, the CLOCK:BMAL1 heterodimer enhances the transcription of metabolic genes or clock-controlled genes (CCGs) e.g., nicotinamide phosphoribosyltransferase (Nampt) [13].

It has been well established that circadian rhythms play an important role in maintaining homeostasis and normal body function [14], and the disruption of circadian regulation affects normal physiological and biochemical functions, inducing diseases. Association studies have revealed that shift workers, night workers, and sleep-deprived individuals have an increased risk for developing metabolic syndrome symptoms [15,16]. Interestingly, whereas light is the major synchroniser of the central clock, peripheral clocks are entrained by other cues such as rhythmic access to food [17], diet composition [18] and food biocompounds [19].Therefore, the aim of this study was to determine whether proanthocyanidins can entrain the peripheral clock to identify novel cellular mechanisms by which proanthocyanidins can modulate lipid metabolism and cell functionality. The capacity of proanthocyandins to entrain peripheral clocks was evaluated in normal (healthy rats) and disrupted (obese rats) circadian rhythm states.

Materials and methods

Grape seed proanthocyanidin extract composition

Grape seed proanthocyandin extract (GSPE) was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). The following GSPE composition used in this study was previously analysed [20]: catechin (58 µmol/g), epicatechin (52 µmol/g), epigallocatechin (5.50 µmol/g), epicatechin gallate (89 µmol/g), epigallocatechin gallate (1.40 µmol/g), dimeric procyanidins (250 µmol/g), trimeric procyanidins (1,568 µmol/g), tetrameric procyanidins (8.8 µmol/g), pentameric procyanidins (0.73 µmol/g) and hexameric procyanidins (0.38 µmol/g).

Animals

All procedures involving the use and care of animals were reviewed and approved by The Animal Ethics Committee of the Universitat Rovira i Virgili (Permit number 4249 by Generalitat de Catalunya).

Forty-five male Wistar six-week-old rats (Crl: WI (Han)) were purchased from Charles River (Barcelona, Spain) for both experiments.

Healthy rats: Rats were singly caged in animal quarters at 22ºC with a 12 h light/dark cycle (light from 8:00 to 20:00 pm) and were fed ad libitum with a standard chow diet (STD, Panlab 04, Barcelona, Spain) and tap water. After one week of adaptation, the animals were randomly divided into four groups (n = 6) and supplemented with 0 (control group), 5, 25 or 50 mg GSPE/Kg body weight for 3 weeks. GSPE was dissolved in sugary milk (100 g: 8.9 g protein, 0.4 g fat, 60.5 g carbohydrates, 1175 kJ) at appropriate concentrations such that the same volume of milk (750 µL) was always administered to the animals. Before supplementation, all of the rats were trained to voluntarily lick the milk, and all groups were administered the same volume of sugary milk for 3 weeks. Treatment was administered every day at 9:00 am.

After 3 weeks of supplementation, the rats were fasted overnight. At 9:00 am, the rats were orally gavaged with lard oil (2.5 mL/kg of body weight) with or without (control groups) an adequate dose of GSPE (5, 25 or 50 mg/kg body weight). After 3 h, the rats were sedated using a combination of ketamine (70 mg/kg body weight, Parke-Davis, Grupo Pfizer, Madrid, Spain) and xylazine (5 mg/Kg body weight, Bayer, Barcelona, Spain). After anesthetisation, the rats were exsanguinated from the abdominal aorta. Blood was collected using heparin (Deltalab, Barcelona, Spain) as an anticoagulant. Liver, mesenteric white adipose tissue and the intestines were excised, immediately frozen in liquid nitrogen and then stored at -80ºC until RNA and protein extraction. Before freezing, duodenal mucosa was extracted by scraping with a small glass plate.

Obese rats: Rats were housed in animal quarters at 22ºC with a 12 h light/dark cycle (light from 08:00 hours to 20:00 hours) and fed a standard chow diet (STD) ad libitum (Panlab, Barcelona, Spain). After one week, the rats were divided into 3 groups (n= 7): the STD control group in which rats were fed STD

ad libitum, and 2 other groups, which were fed a STD plus a cafeteria diet (CD) that comprised 23.4% lipids (0.05% cholesterol), 35.2% carbohydrates and 11.7% protein. The CD consisted of the following foods: cookies with foie-gras and cheese triangles, bacon, biscuits, carrots and sugary milk. After 10 weeks, rats feeding on the CD were trained to lick arabic gum (1 mL) (G9752, Sigma-Aldrich, Madrid, Spain), which was used as the vehicle, and they were randomly divided in two groups. One group was fed the CD plus 25 mg GSPE/kg bw dissolved in arabic gum for 3 more weeks. The second group was fed the CD plus the same volume of arabic gum (CD control group). All treatments were administered at the same time point (7 p.m.).

After 3 weeks of treatment, the rats were fasted overnight and killed at 9 a.m. by anesthetising them with 50 mg/kg bw sodium pentobarbital (0804118, Fagron Iberica, Terrasa, Spain), and they were sacrificed by bleeding. Blood was collected using heparin (Deltalab, Barcelona, Spain) as an anticoagulant. The livers, mesenteric adipose tissue and intestines were excised, immediately frozen in liquid nitrogen and stored at -80ºC until RNA could be extracted. The duodenal mucosa was equally extracted as described above.

RNA extraction and cDNA synthesis

Total RNA was extracted from liver, mesenteric white adipose tissue and intestinal mucosa using the TRIzol reagent and RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocols. RNA was quantified by spectrophotometry (Nanodrop 1000 Spectrophotometer, Thermo Scientific) at λ=260 nm and tested for purity (by A260/280 ratio) and integrity (by denaturing gel electrophoresis). Complementary DNA was generated using the High-Capacity complementary DNA Reverse Transcription Kit from Applied Biosystems (4368814).

mRNA quantification by real-time qRT-PCR

A total of 10 ng of cDNA was subjected to quantitative RT-PCR amplification using SYBR Green PCR Master Mix from Bio-Rad (172-5200). The forward and reverse primers for the rat genes analysed are listed in Table 1. Reactions were run with a quantitative real-time PCR system (Bio-Rad), and the thermal profile settings were 50ºC for 2 min, 95ºC for 2 min, and 40 cycles of 95ºC for 15 s and 60ºC for 2 min. Finally, statistical data were converted and normalised to the linear form using the $2^{-}CT$ ($\Delta\Delta C_T$) calculation [21]. The relative expression level of the clock genes *rora, rev-erbα, bmal1, clock, per2, nampt* and *hmgCoAR* was assessed for the liver, mesenteric adipose tissue and intestinal mucosa, which was normalised to the cyclophilin mRNA level.

Western Blot analyses

Protein was extracted from liver and white adipose tissue using RIPA (radio-immunoprecipitation assay) lysis buffer (15 mM Tris–HCl, 165 mM-NaCl, 0.5% Na-deoxycholate, 1% Triton X-100 and 0.1% SDS), containing a protease inhibitor cocktail (1:1000; Sigma-Aldrich) and 1 mM-PMSF (phenylmethanesulfonyl fluoride solution). The total protein levels of the lysates were determined using the BCA method from Thermo Scientific (23227). Then, the samples were placed in sample buffer (0.5 M Tris–HCl, pH 6.8, 10% glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 0.05% bromophenol blue). After boiling for 5 min, 50 µg of protein was loaded and separated in a 10% SDS-polyacrylamide gel. The samples were then transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories) using a transblot apparatus (Bio-Rad) and blocked at room temperature for 1 h with 5% (w/v) non-fat milk in TTBS buffer (Tris-buffered saline (TBS) plus 0·5% (v/v) Tween-20). The membranes were incubated overnight at 4ºC with primary monoclonal antibodies directed against Nampt (Imgenex), Bmal1 (LS-Bio), acetyl-Bmal1 (Millipore) and anti-β-actin (Sigma-Aldrich) at a 1:1000 dilution in blocking solution. After washing with TTBS, the blots were incubated with a peroxidase-conjugated monoclonal anti-rabbit secondary antibody (Sigma-Aldrich) at a 1:10000 dilution at room temperature for 1.5 h. The blots were then washed thoroughly in TTBS followed by TBS. Immunoreactive proteins were visualised with an enhanced chemiluminescence substrate kit (ECL plus; Amersham Biosciences, GE Healthcare) according to the manufacturer's instructions. Images were obtained with a GBOX Chemi XL 1.4 image system (Syngene, pais). Band quantification was performed with ImageJ software (NIH, USA). The results were expressed as relative intensity (Nampt/b-actin, Bmal1/b-actin and acetil-Bmal1/b-actin) and are relative to the loading control group.

Statistical analysis

The results are presented as the mean plus the associated standard error (SE). The data were analysed using one-way ANOVA to determine significant differences using SPSS statistical software (version 17.0 for Windows; SPSS, Inc.). p values <0.05 were considered statistically significant.

Results

Chronic administration of GSPE in healthy rats differentially modulates the peripheral clock in the liver, mesenteric white adipose tissue and intestinal mucosa

The capacity of GSPE to modify circadian rhythms was evaluated in peripheral clocks using three different organs including the liver, mesenteric white adipose tissue (mWAT) and duodenal mucosa (GUT) of healthy animals chronically treated with GSPE at doses of 5, 25 and 50 mg/Kg body weight. In these organs, we evaluated the gene expression of *Clock* and *Bmal1* (clock core genes), *Per2* (a component of the negative loop of the circadian clock), *Rorα* and *Rev-erbα* (nuclear receptors whose expression is regulated by CLOCK:BMAL1 and act as an activator or repressor of Bmal1 gene expression, respectively), *Nampt* (a metabolic gene whose expression is directly regulated by

CLOCK:BMAL1) and *HmgCoAR* (a metabolic gene with circadian rhythm expression that is not directly controlled by CLOCK:BMAL1).

In the liver (Fig. 1), GSPE administration increased the expression of *Clock* and *Per2* in a dose-dependent pattern with a significant increase at 25 and 50 mg GSPE/Kg body weight. In contrast, *Rev-erbα* was repressed by GSPE with a significant reduction at the lowest dose. The effect of GSPE on *Rorα* expression was bimodal with repression at the lowest dose and overexpression at the two higher doses. GSPE did not modify the *Bmal1*, *Nampt* or *HmgCoAR* expression in the liver at any dose.

In mWAT (Fig. 2), GSPE administration modulated the expression of all genes studied, except that of *HmgCoAR*. GSPE consumption increased significantly the expression of *Clock*, *Bmal1* and *Per2* at 50 mg GSPE/Kg body weight. On the other hand, the expression of genes modulated directly by CLOCK:BMAL1, i.e., *Rorα*, *Rev-erbα* and *Nampt*, was repressed significantly by GSPE treatment.

In the GUT (Fig. 3), only *Bmal1* and *HmgCoAR* were modified by GSPE administration. *Bmal1* was up-regulated by all GSPE doses studied, whereas *HmgCoAR* expression was increased only by the 25 GSPE/Kg body weight dose.

To confirm the ability of GSPE to entrain peripheral molecular clocks, we determined the BMAL1 and NAMPT protein level in livers and mWAT (Fig. 4). The protein levels demonstrated the same pattern as the gene expression levels in all cases, and the effects of GSPE were more evident at the protein level than at the mRNA level, thus confirming the results observed at transcriptional level. The active form of BMAL1, which turns on the clock machinery, is the acetylated form; thus, we also measured the percentage of acetylated protein versus total protein (Fig. 4). In both tissues, the ratio of acetylated BMAL1 was reduced in animals treated with 5 mg GSPE /kg body weight, whereas the ratio was increased at 25 and 50 mg GSPE /kg body weight, indicating that higher doses of GSPE increased the transactivation activity of CLOCK:BMAL1.

Together, these results indicate that chronic GSPE consumption differentially modulates the peripheral clock of each organ, particularly the core clock genes, including GSPE-modulated *Clock* in the liver, *Bmal1* in the GUT and both in mWAT. Moreover, mWAT was the organ most sensitive to GSPE.

Chronic administration of GSPE counteracts the disturbances in the clock genes induced by obesity mainly in the liver and GUT

After determining that chronic GSPE treatment was capable of entraining peripheral clocks in animals with normal circadian rhythms, we studied the capacity of GSPE to modulate peripheral clocks in rats with obesity, a state with disrupted circadian rhythms. The study was conducted in the liver, WAT and GUT by chronically treating obese rats with 25 mg GSPE /Kg body weight and quantifying the expression of the same core clock and related genes studied in healthy rats.

In the liver (Fig. 5), obesity resulted in the overexpression of *Rev-erbα* and *Bmal1* and *HmgCoAR* repression. GSPE treatment normalised the expression of these genes in the liver to values similar to that of lean rats, particularly for *Rev-erbα*. In contrast with healthy animals, chronic administration of 25 mg GSPE/kg body weight GSPE did not affect the expression of *Clock*, *Per2* or *Rorα* in livers of obese rats.

In mWAT (Fig. 6), *Rev-erbα* and *Clock* were overexpressed in obese rats. GSPE treatment normalised only the expression of *Rev-erbα* in the WAT of obese rats. Moreover, GSPE administration significantly increased the expression of *Per2* in the WAT of obese rats compared with obese and lean rats in a manner similar to GSPE treatment in the WAT of healthy rats at the higher dose. In contrast with healthy animals, chronic administration of 25 mg GSPE/kg body weight did not repress *Rorα* or *Nampt* in the WAT of obese rats.

In the GUT (Fig. 7), *Bmal1*, *Per2* and *HmgCoAR* were significantly overexpressed in obese rats. GSPE administration normalised the expression of *Per2* and *HmgCoAR* in obese rat guts. However, the *Bmal1* expression in obese rats treated with GSPE had values in between that of obese and lean animals. Moreover, GSPE exacerbated the obesity-induced *Rev-erbα* overexpression in the GUT. As in healthy rats, chronic administration of 25 mg GSPE/kg body weight did not affect the expression of *Clock* or *Nampt* in the GUT of obese rats.

Overall, diet-induced obesity always resulted in overexpression of some core and related clock genes although the particular gene affected was tissue specific. In particular, obesity affected *Bmal1* in the liver and GUT, whereas *Clock* was affected in the mWAT. GSPE administration counteracted disturbances in the clock genes induced by obesity in the liver and GUT, whereas it was less effective in normalising the clock gene disruption induced by obesity in mWAT.

Discussion

Digestion, absorption and metabolism follow circadian rhythms that are regulated by peripheral clocks [22]. While light entrains the central clock, food is a potent synchroniser for peripheral clocks [23]. The expression of clock genes in the liver [18,24], gut [17] and WAT [25] is entrained by the frequency and daytime meals and diet composition. Moreover, it has been described that bioactive food components also modify circadian rhythms. Specifically, resveratrol adjusts the circadian rhythms of locomotor activity and body temperature in animals [26,27] and alters clock gene expression in cultured fibroblasts [28] and rat organs [19]. Therefore, this study was designed to determine whether proanthocyanidins, which have a powerful hypolipidemic effect [29], modulate the expression of clock genes in tissues significant for lipid homeostasis i.e., the liver, gut and WAT, in healthy and obese rats.

In healthy animals, GSPE was administered once a day at 5, 25 or 50 mg GSPE/kg body weight to evaluate whether the clock genes responded in a dose-dependent manner. By extrapolating to human doses [30] and estimating the daily intake for a 70 kg human, these doses match an intake of 57, 284 and 560 mg of GSPE/day. As the estimated proanthocyanidin intake for humans ranges between 90 and 200

mg/day [31–33], the GSPE doses used in this study simulate low, similar or high human proanthocyanidin dietary intake.

This study shows that GSPE differentially modulates the peripheral clock in each organ studied. In healthy animals, the number of core clock and clock-controlled genes with altered expression in response to GSPE was high in mWAT, medium in the liver and low in the GUT. It has been described that different mouse tissues have different circadian phases, suggesting the existence of organ-specific circadian rhythms synchronisers at the cell and tissue level [34]. For instance, a nearly eight hour phase difference is observed between the liver and gonadal WAT [23]. Therefore, the fact that we quantified the expression of clock genes at different points of the circadian phase in each organ could make the effectiveness of GSPE modulating clock gene expression relatively evident. Alternatively, the absorption kinetics and tissue distribution of proanthocyanidins could also lead to dissimilar clock modulation by GSPE in each organ. Proanthocyanidins accumulate in adipose tissue [35]; thus, the constant presence of high levels of proanthocyandins in adipose cells can result in greater modification of the clock loops in mWAT than in the liver or GUT.

GSPE administration increased the expression of core clock genes (Clock in liver, Bmal1 in GUT and both in mWAT) in a dose-dependent mode, indicating that these genes are targets of proanthocyanidin. Interestingly, GSPE supplementation, at the same doses used in this experiment, improves lipid tolerance in healthy rats with a dose-dependent effect [36]. This fact indicates the involvement of the circadian clock in the hypolipidemic effects of proanthocyandin. CLOCK and BMAL1 knockouts have abnormalities in lipid homeostasis. For example, mice lacking *Bmal1* have hyperlipidemia and an elevated respiratory quotient value, indicating less utilisation of fat as an energy source [37], and *Clock* mutant mice have hypercholesterolemia, atherosclerosis and increased lipid absorption [38,39]. However, as gene expression was measured at only one point, it is not possible to infer whether the increased expression of core clock genes was due to a phase shift or amplitude intensification after GSPE chronic consumption. Nevertheless, by increasing core clock gene expression or shifting their phase, GSPE consumption could repress lipid absorption and increase fatty acid oxidation, inducing the hypolipidemic effects described for proanthocyanidins [29,36].

Despite the general overexpression of the core clock genes by GSPE, there were two different patterns in the expression of genes directly controlled by CLOCK:BMAL1 in the liver and mWAT. *Per2*, a component of the negative loop of the circadian clock, was overexpressed in a positive dose-dependent manner by GSPE similar to *Clock* and *Bmal1*. In contrast, *Rev-erbα* and *Rorα* were repressed by GSPE with a negative dose-dependent pattern. BMAL1 binding to the DNA of its target genes peaks around Zeitgeber time (ZT) 4 to ZT8, and the mRNA level of BMAL1 target genes peaks earlier or it is delayed [40]; thus, as in this study we only analysed expression at one time point, the *Per2* mRNA level could be in a different phase than that of *Rev-erbα*, *Rorα* and *Nampt*. It is important to note the negative dose-dependent behaviour of *Rev-erbα*, *Rorα* and *Nampt*, which suggests that low dietary proanthocyanidin consumption could be effective in repressing these genes and thus controlling circadian rhythms.

The protein levels of BMAL1 and NAMPT confirmed that chronic GSPE administration entrained the peripheral clock in the liver and mWAT. The active form of BMAL1 that turns on the clock machinery is its acetylated form; thus, the ratio of acetylated to total BMAL1 protein provides direct information about the transactivation activity of BMAL1:CLOCK. BMAL1 is acetylated by CLOCK [41] and deacetylated by sirtuin (SIRT) 1 [42]. SIRT1 activity depends absolutely on the NAD+ levels, thus directly connecting the circadian peripheral clock activity and BMAL1 deacetylation to metabolism using the level of NAD+ as a metabolic cue, which in turn is directly proportional to the level of NAMPT activation [43]. In mWAT, the activation of the clock machinery as measured as the percentage of acetylated BMAL1 was directly proportional to the GSPE dose with a positive dose response similar to that of Bmal1 mRNA. Moreover, the maximal BMAL1activation at 50 mg/Kg body weight matched the maximal NAMPT protein levels. These results confirmed that the mWAT molecular clock is a clear target for proanthocyanidins.

As GSPE chronic intake was capable of modifying circadian clock gene expression in healthy animals at doses that simulate regular proanthocyanidin intake in the Mediterranean diet [33] i.e., 25 mgGSPE/Kg body weight, we further determined the capacity of GSPE to entrain peripheral molecular clocks in rats with obesity, a pathological condition associated with disrupted circadian rhythms [14,44] . We used rats fed with the cafeteria diet (CD) as a nutritional model for obesity. Feeding rats with this diet results in over weight rats (20%) and dyslipidemia (increases in the triglyceride, total cholesterol and LDL-C levels in plasma of 60, 45 and 120%, respectively) [11]. GSPE administration to obese animals normalises the plasma triglyceride and C-LDL but not total cholesterol levels [11], whereas it has no effect on body weight. In parallel with this hypotriglyceridemic effect, GSPE administration also nearly corrected the all of the disruptions in the clock genes induced by obesity in the liver and GUT, the two organs that generate triglyceride-rich lipoproteins [45]. In contrast, GSPE administration was less effective in normalising the clock gene disruption in mWAT, which is in agreement with the ineffectiveness of GSPE in reducing body weight.

The availability of phenolic compounds to counteract the disruption of clock genes induced by obesity has also been evaluated with resveratrol in adipose tissue and livers [19]. Despite the difficulty in comparing our results with those in this study because diets and adipose tissue locations are different, resveratrol counteracts the increase in *Rev-erbα* expression induced by obesity in the liver and WAT similar to the proanthocyanidins in this study. Interestingly, *Rev-erbα* is essential for adipogenesis, and its overexpression results in the overexpression of adipogenic genes [46], thus suggesting that phenolic compounds can reduce adipogenesis in obesity. However, resveratrol is more powerful than GSPE in normalising *Rev-erbα* expression in WAT, which is in agreement with the mimetic effects of energy restriction described for resveratrol and not for proanthocyanidins.

In summary, proanthocyanidins have the capacity to entrain peripheral molecular clocks in healthy and obese states. Moreover, in healthy animals, GSPE administration leads to the overexpression of core clock genes and increases the percentage of acetylated BMAL1 in a positive dose-dependent manner similar to the hypolipidemic effects of GSPE. Therefore, the modulation of peripheral circadian clocks appears to be a novel molecular mechanism by which proanthocyanidins regulates metabolism and cell

functionality in peripheral organs, such as the liver and mWAT. However, as there is bidirectional cross talk between molecular clocks and metabolism through AMPK and sirtuins [47], it cannot be inferred whether proanthocyanidins alter clock genes first and then lipid metabolism or vice versa. Therefore, further research is needed to clarify whether proanthocyanidins can directly modulate the clock phase, amplitude and/or period of peripheral and central clocks.

Acknowledgments

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Fig. 1 Effect of different chronic doses of GSPE on the relative expression level of some clock and clock-controlled genes in the liver.

The mRNA level of some clock and clock-controlled genes in the livers of rats chronically supplemented with different doses of grape seed proanthocyanidin extract (GSPE) for 3 weeks. The mRNA levels were normalised to the PPIA endogenous gene and then to the control group without GSPE supplementation. White bars, control group; coloured bars, GSPE treated groups. The different letters indicate statistically significant differences as determined by the 1-way ANOVA test ($p<0.05$).

Fig. 2 Effect of different chronic doses of GSPE on the relative expression level of some clock and clock-controlled genes in white adipose tissue.

The mRNA level of some clock and clock-controlled genes in white adipose tissue from rats chronically supplemented with different doses of grape seed proanthocyanidin extract (GSPE) for 3 weeks. The mRNA levels were normalised to the PPIA endogenous gene and then to the control group without GSPE supplementation. White bars, control group; colour bars, GSPE treated groups. The different letters indicate statistically significant differences by the 1-way ANOVA test ($p<0.05$).

Fig. 3 Effect of different chronic doses of GSPE on the relative expression level of some clock and clock-controlled genes in the gut.

The mRNA level of some clock and clock-controlled genes in the guts of rats chronically supplemented with different doses of grape seed proanthocyanidin extract (GSPE) for 3 weeks. The mRNA levels were normalised to the PPIA endogenous gene and then to the control group without GSPE supplementation. White bars, control group; coloured bars, GSPE treated groups. The different letters indicate statistically significant differences by the 1-way ANOVA test ($p<0.05$).

Fig. 4 Effect of different chronic doses of GSPE on the relative protein expression level of BMAL1 and NAMPT in the liver and mWAT.

Total and acetylated levels of the BMAL1 protein and the protein level of NAMPT in the liver (a) and mWAT (b) of rats chronically supplemented with different doses of grape seed proanthocyanidin extract (GSPE) for 3 weeks. Proteins were extracted with radioimmunoprecipitation (RIPA) buffer and analysed via Western blot. Proteins were normalised with the endogenous protein β-actin. Relative intensity units were obtained by dividing the intensity of the protein band of interest by the intensity of the band of the endogenous protein. In the case of acetylated and total BMAL1 protein, both results were divided to

obtain the acetyl Bmal1/ total Bmal1 protein ratio, which is shown as a percentage. Different letters indicate statistically significant differences by the 1-way ANOVA test ($p<0.05$).

Fig. 5 Effect of chronic dose GSPE on the relative expression level of some clock and clock controlled-genes in the liver of obese rats.

The mRNA level of some clock and clock-controlled genes in livers of rats fed for 10 weeks on a cafeteria diet (CD) followed by vehicle or chronic supplementation with 25 mg/kg bw grape seed proanthocyanidin extract (GSPE) for 3 weeks. The mRNA levels were normalised to the PPIA endogenous gene and then to the STD group. White bars, control group; grated bars, vehicle group; brown bars, GSPE-treated groups. Different letters indicate statistically significant differences by the 1-way ANOVA test ($p<0.05$).

Fig. 6 Effect of chronic dose GSPE on the relative expression level of some clock and clock controlled-genes in the white adipose tissue of obese rats.

The mRNA level of some clock and clock-controlled genes in white adipose tissue from rats fed for 10 weeks with a cafeteria diet (CD) followed by vehicle administration or chronic supplementation with 25 mg/kg bw grape seed proanthocyanidin extract (GSPE) for 3 weeks. The mRNA levels were normalised to the PPIA endogenous gene and then to the STD group. White bars, control group; grated bars, vehicle group; brown bars, GSPE-treated groups. Different letters indicate statistically significant differences by the 1-way ANOVA test ($p<0.05$).

Fig. 7 Effect of chronic dose GSPE on the relative expression level of some clock and clock controlled genes in the gut of obese rats

The mRNA levels of some clock and clock-controlled genes in guts of rats fed for 10 weeks with a cafeteria diet (CD) followed by vehicle administration or chronic supplementation with 25 mg/kg bw grape seed proanthocyanidin extract (GSPE) for 3 weeks. The mRNA levels were normalised to the PPIA endogenous gene and then to the STD group. White bars, control group; grated bars, vehicle group; brown bars, GSPE-treated groups. Different letters indicate statistically significant differences by the 1-way ANOVA test ($p<0.05$).

Table 1. Rat-specific primer sequences.

Gene	Primer sequence
rorα	Fw: 5'-GAAGGCTGCAAGGGCTTTTTCAGGA-3'
	Rv: 5'-CCAAACTTGACAGCATCTCGA-3'
rev-erbα	Fw: 5'-CTGCTCGGTGCCTAGAATCC-3'
	Rv: 5'-GTCTTCACCAGCTGGAAAGCG-3'
bmal1	Fw: 5'-GTAGATCAGAGGGCGACGGCTA-3'
	Rv: 5'-CTTGTCTGTAAAACTTGCCTGTGAC-3'
clock	Fw: 5'-TGGGGTCTATGCTTCCTGGT-3'
	Rv: 5'-GTAGGTTTCCAGTCCTGTCG-3'
per2	Fw: 5'-CGGACCTGGCTTCAGTTCAT-3'
	Rv: 5'-AGGATCCAAGAACGGCACAG-3'
nampt	Fw: 5'-CTCTTCACAAGAGACTGCCG
	Rv: 5'-TTCATGGTCTTTCCCCCACG-3'
hmgcr	Fw: 5'- GAAACCCTCATGGAGACGCA-3'
	Rv: 5'- ACCTCTGCTGAGTCACAAGC-3'
ppia	Fw: 5'-CTTCGAGCTGTTTGCAGACAA-3'
	Rv: 5'-AAGTCACCACCCTGGCACATG-3'

Rorα, RAR-related orphan receptor A; Rev-erbα (also known as Nr1d1), nuclear receptor subfamily 1, group D, member 1; Bmal1 (also known as ARNTL), aryl hydrocarbon receptor nuclear translocator-like; Clock, circadian locomotor output cycles kaput; Per2, period circadian clock 2; Nampt, nicotinamide phosphoribosyltransferase; Hmgcr, 3-hydroxy-3-methylglutaryl-Coenzyme A reductase; Ppia, cyclophilin A. Fw, forward primer sequence; Rv, reverse primer sequence.

Fig.1

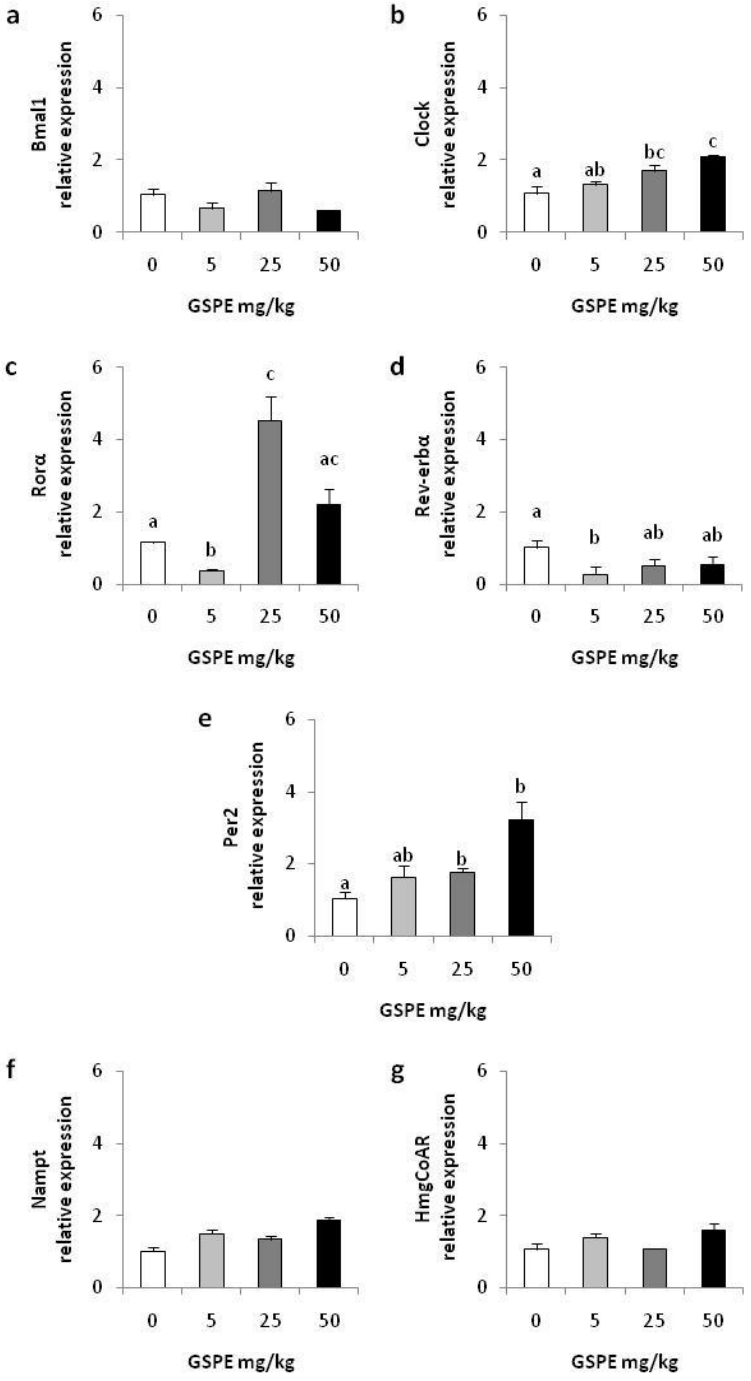

a
Bmal1 relative expression
b
Clock relative expression
a
ab
bc
c
c
Rorα relative expression
a
b
c
ac
d
Rev-erbα relative expression
a
b
ab
ab
e
Per2 relative expression
a
ab
b
b
f
Nampt relative expression
g
HmgCoAR relative expression
0
2
4
6
0
5
25
50
GSPE mg/kg

Fig.2

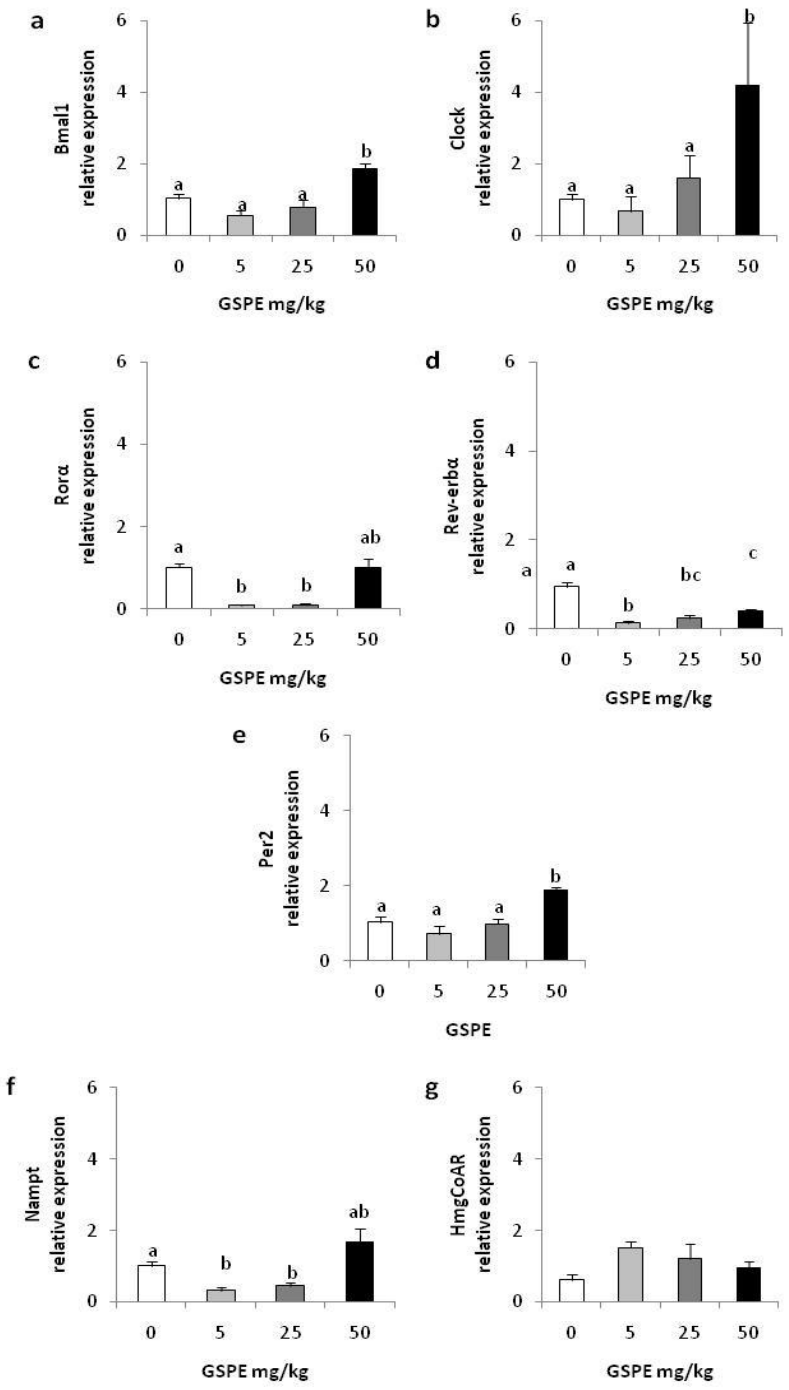


Fig.3

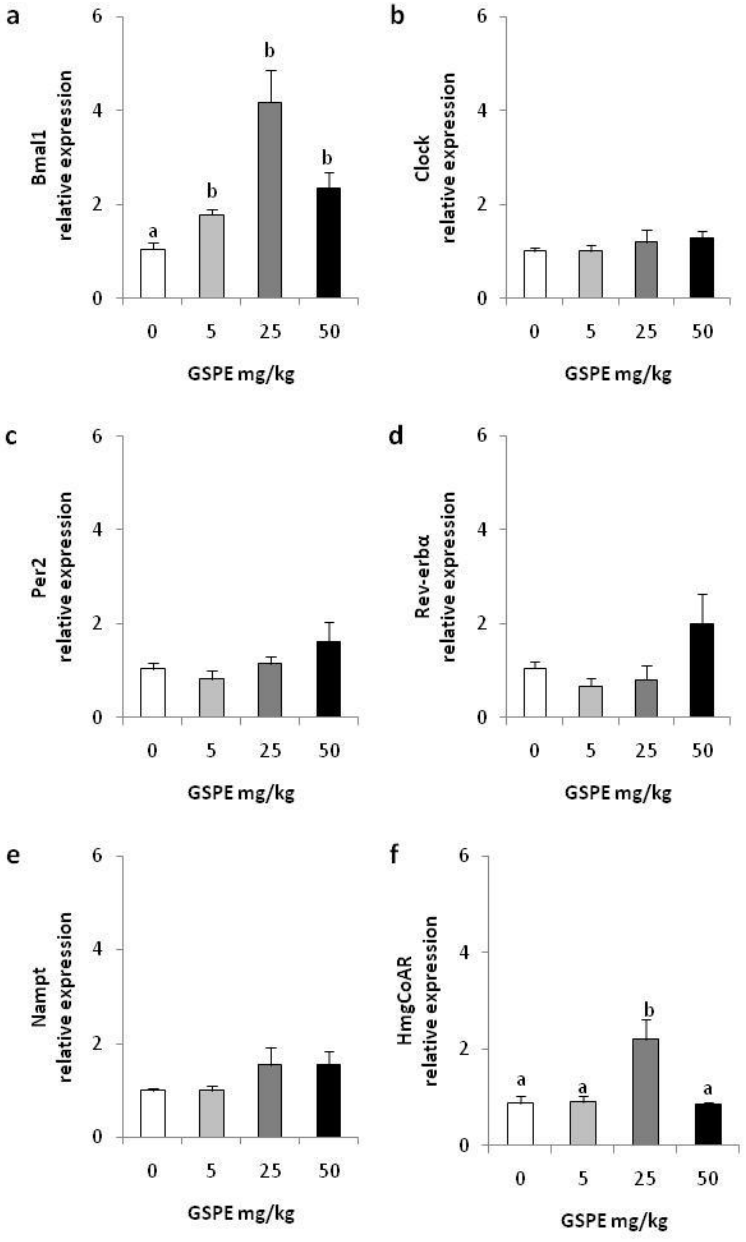


Fig.4

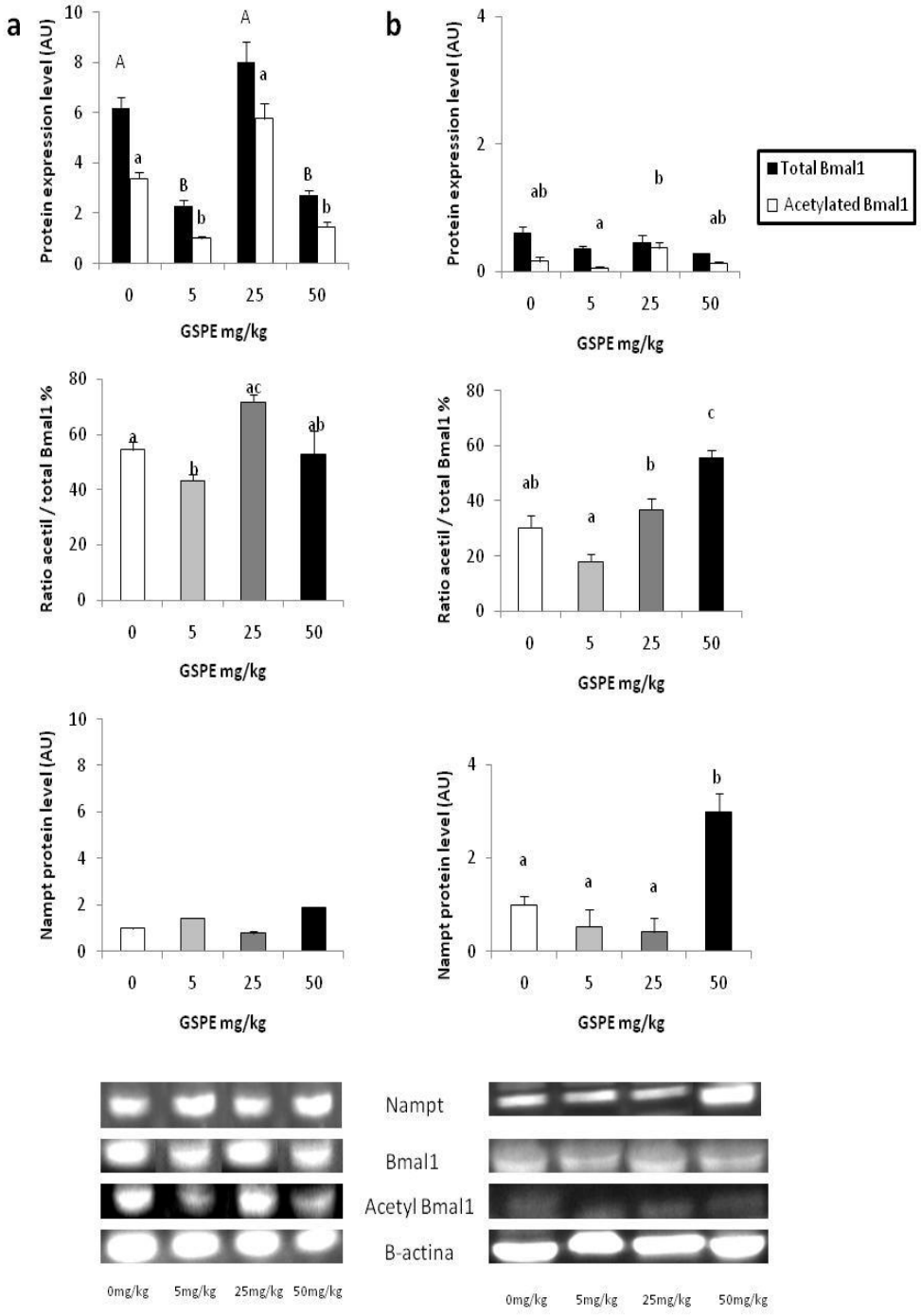


Fig.5

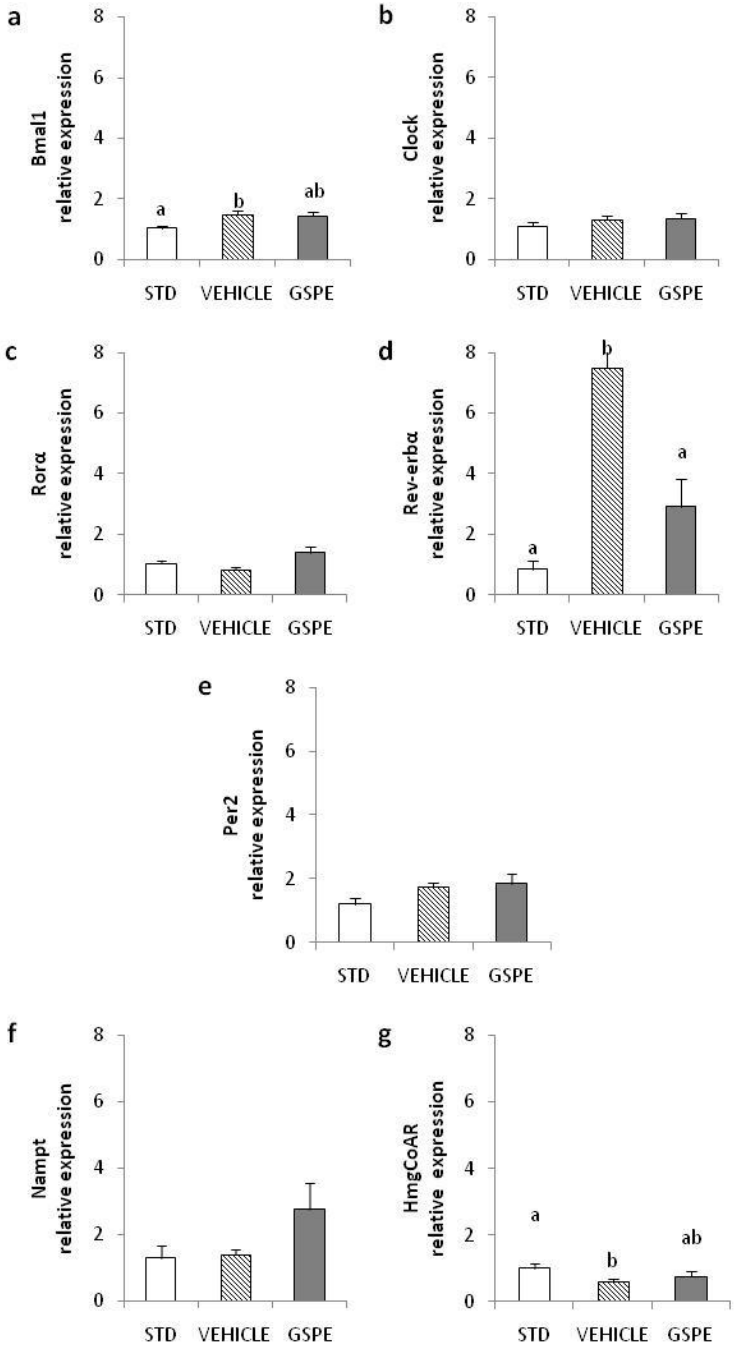


Fig. 6

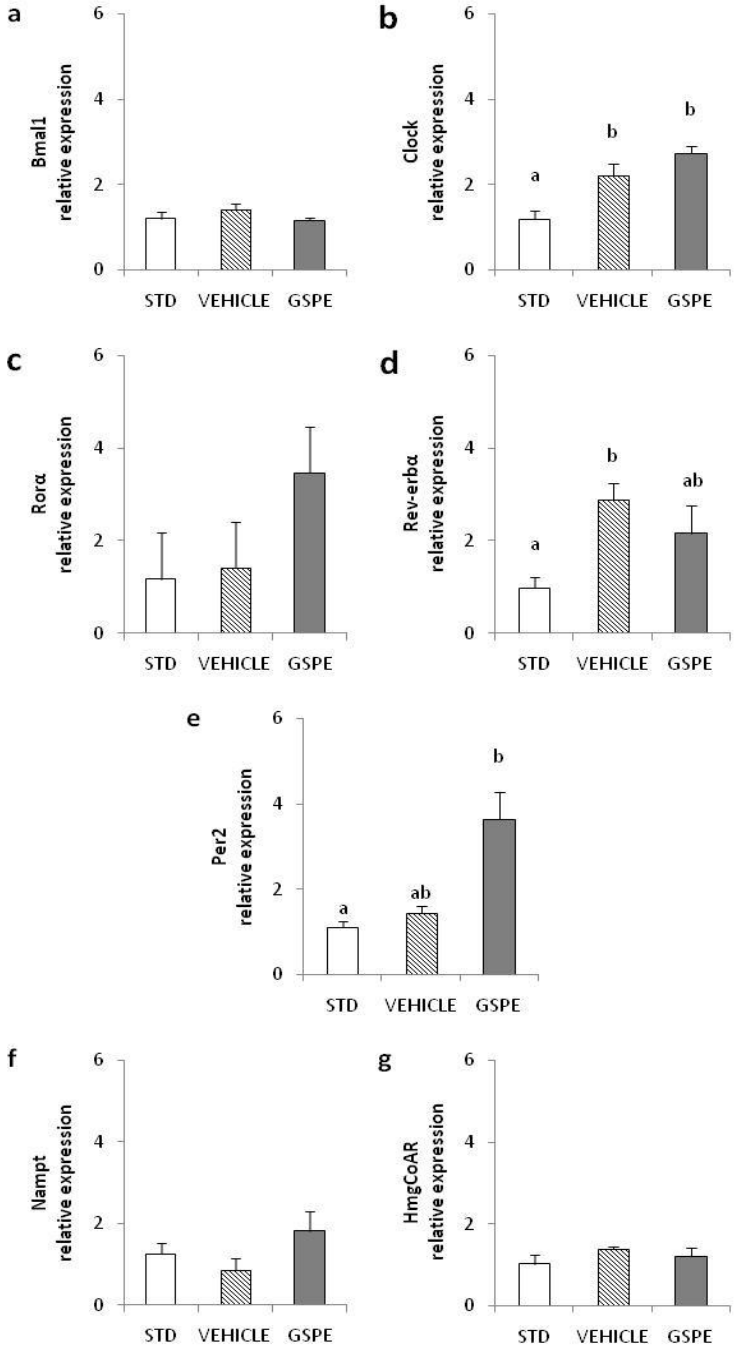
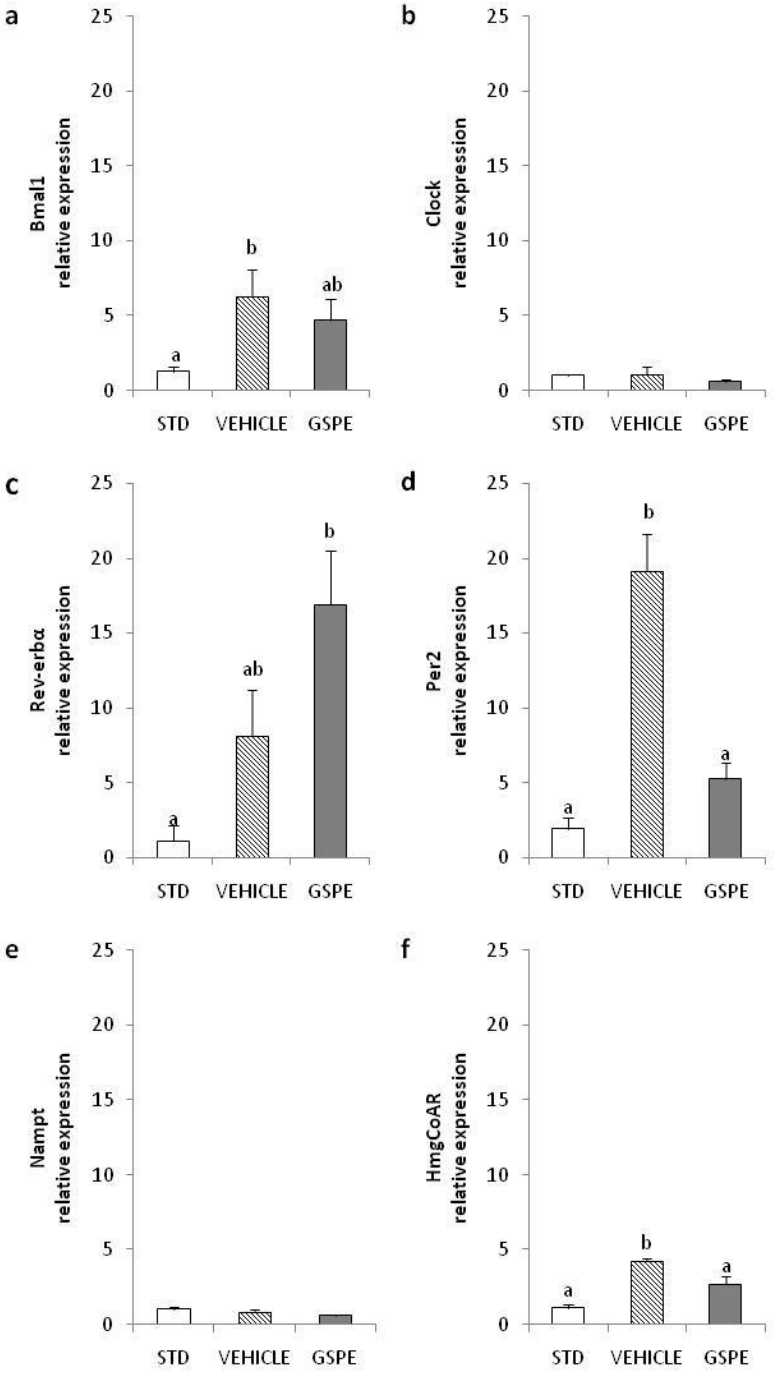


Fig.7



2. Dietary proanthocyanidins modulate BMAL1 acetylation, Nampt expression and NAD levels in rat liver.

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Dietary proanthocyanidins modulate BMAL1 acetylation, Nampt expression and NAD levels in rat liver

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Manuscript Keywords:	peripheral clocks, NAD, Nampt, Bmal1, flavonoids
Abstract:	Metabolism follows circadian rhythms, which are driven by peripheral clocks. The clock in the liver is one of the most important because this organ plays a central role in maintaining homeostasis. Clock genes in the liver are entrained by daytime meals and food components such as proanthocyanidins (PAs), which are the most abundant flavonoids in the human diet and serve to modulate lipid and glucose homeostasis. The aim of the current study was to determine whether PAs could entrain the peripheral clock system in the liver. Male Wistar rats were orally gavaged with 250 mg grape seed proanthocyanidin extract (GSPE)/kg body weight at zeitgeber time (ZT) 0, when the lights were turned on, or at ZT12, when the lights were turned off; these treatments were also given to rats with 6 hours of jet lag. The 24 hour rhythm of clock-core (Clock and Bmal1) and clock-controlled (Per2, Rorα, Rev-erbα and Nampt) gene expression indicated that Nampt was the most sensitive gene to GSPE. However, GSPE modulated Nampt expression in opposite ways when it was administered at ZT0 compared to administration at ZT12, reducing or increasing Nampt protein and mRNA levels when administered at ZT0 or ZT12, respectively. Accordingly, NAD levels, which also exhibit circadian rhythm, were significantly decreased or increased 6 hours after GSPE administration at ZT0 or ZT12, respectively. GSPE administered at either time increased both mRNA and protein levels of Bmal1 after 1 hour of treatment. Nonetheless, the ratio of acetylated Bmal1 only increased when GSPE was administered at ZT12, which was the same condition under which Nampt was overexpressed. Therefore, Bmal1acetylation, Nampt and NAD emerge as

	GSPE targets in the liver, indicating that PAs can modulate lipid and glucose metabolism in the liver by entraining the daily rhythm of some components of the clock system.

Dietary proanthocyanidins modulate BMAL1 acetylation, Nampt expression and NAD levels in rat liver

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Abstract

Metabolism follows circadian rhythms, which are driven by peripheral clocks. The clock in the liver is one of the most important because this organ plays a central role in maintaining homeostasis. Clock genes in the liver are entrained by daytime meals and food components such as proanthocyanidins (PAs), which are the most abundant flavonoids in the human diet and serve to modulate lipid and glucose homeostasis. The aim of the current study was to determine whether PAs could entrain the peripheral clock system in the liver. Male Wistar rats were orally gavaged with 250 mg grape seed proanthocyanidin extract (GSPE)/kg body weight at zeitgeber time (ZT) 0, when the lights were turned on, or at ZT12, when the lights were turned off; these treatments were also given to rats with 6 hours of jet lag. The 24 hour rhythm of clock-core (*Clock and Bmal1*) and clock-controlled (*Per2, Rorα, Rev-erbα* and *Nampt*) gene expression indicated that *Nampt* was the most sensitive gene to GSPE. However, GSPE modulated *Nampt* expression in opposite ways when it was administered at ZT0 compared to administration at ZT12, reducing or increasing *Nampt* protein and mRNA levels when administered at ZT0 or ZT12, respectively. Accordingly, NAD levels, which also exhibit circadian rhythm, were significantly decreased or increased 6 hours after GSPE administration at ZT0 or ZT12, respectively. GSPE administered at either time increased both mRNA and protein levels of *Bmal1* after 1 hour of treatment. Nonetheless, the ratio of acetylated *Bmal1* only increased when GSPE was administered at ZT12, which was the same condition under which *Nampt* was overexpressed. Therefore, *Bmal1*acetylation, *Nampt* and NAD emerge as GSPE targets in the liver, indicating that PAs can modulate lipid and glucose metabolism in the liver by entraining the daily rhythm of some components of the clock system.

Keywords: NAD, Nampt, Bmal1, flavonoids, circadian rhythm

Introduction

Digestion, absorption and metabolism follow circadian rhythms that are regulated by peripheral clocks (Tahara and Shibata 2013), and the disruption of the clock system triggers different types of illnesses, indicating that peripheral clocks play important roles in maintaining homeostasis and normal body function (Froy 2010). In this way, association studies have revealed that shift workers, night workers, and sleep-deprived individuals (clear examples of disrupted circadian rhythms) have an increased risk of developing symptoms of metabolic syndrome (Chaput et al. 2008; DiLorenzo et al. 2003).

Among peripheral clocks, the clock in the liver is one of the most important, because this organ plays a central role in metabolism and energy production, thus significantly affecting the physiological status of the whole organism. For instance, the liver is the major site of intermediate metabolism, including the synthesis and removal of cholesterol (Edwards et al. 1972), as well as the regulation of glucose homeostasis (Lamia et al. 2008). In fact, 10% of all transcripts, or 20% of all proteins, in mouse liver are under circadian regulation (Reddy et al. 2006), underscoring the importance of the clock present in this organ.

At the molecular level, the clock system consists of transcription–translation autoregulatory feedback loops. Driving the positive side of this loop are transcriptional activators circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-like protein 1 (BMAL1). After forming a heterodimer, these factors activate the transcription of the Period (*Per*) and Cryptochrome (*Cry*) genes. In turn, once they reach a critical concentration, PER and CRY proteins translocate to the nucleus and inhibit the activity of the CLOCK:BMAL1 heterodimer, thus leading to a decrease in *Per* and *Cry* expression. In addition, the active CLOCK:BMAL1 heterodimer also promotes the transcription of retinoic acid-related orphan receptor alpha (*Rorα*) and nuclear receptor subfamily 1, group D, member 1 (*Nr1d1*, also known as *Rev-erbα*), its own activator and repressor, respectively, generating another loop of regulation. Finally, the CLOCK:BMAL1 heterodimer enhances the transcription of metabolic genes, such as nicotinamide phosphoribosyltransferase (*Nampt*), which are implicated in many aspects of metabolism and biochemical processes, therefore supporting the tight relation between the clock system and metabolism or physiology (Green et al. 2008; Bass and Takahashi 2010). Interestingly, the expression of clock genes in the liver (Hirao et al. 2009; Reznick et al. 2013), and in turn, metabolic circadian rhythm, is entrained by the frequency of daytime meals as well as by diet composition.

Proanthocyanidins (PAs) are a class of polyphenols present in vegetables, fruits, cacao, nuts and beverages such as red wine or tea; therefore, their presence in the human diet is considerably high (Serrano et al. 2009). PAs are considered to be dietary bioactive compounds because their consumption exerts a varied range of healthy effects, including the reduction of cardiovascular diseases (Rasmussen et al. 2005) and improvement in insulin resistance (Montagut et al. 2010),

obesity (Montagut et al. 2007), inflammation (Terra et al. 2011), hypertension (Quiñones et al. 2013), oxidative stress (Puiggros et al. 2005) and dyslipidemia (Quesada et al. 2009). Interestingly, the liver is a key organ in which PAs are active, restoring lipid (Bladé et al. 2010) and glucose (Pinent et al. 2012) homoeostasis after a disruption.

Remarkably, resveratrol is a polyphenol that adjusts the circadian rhythms of locomotive activity and body temperature in animals (Pifferi et al. 2013, 2011) and alters clock gene expression in cultured fibroblasts (Oike and Kobori 2008) and rat organs, such as adipose tissue (Miranda et al. 2013). However, there is little information about the effect of polyphenol consumption on circadian rhythm in the liver. Therefore, the aim of the current study was to determine whether PAs could modulate the peripheral clock system in the liver to set a new cellular mechanism by which PAs can modulate cell functionality and improve some pathological conditions. To this end, we have measured the expression rhythm of clock-core and clock-controlled genes in the liver by administering PAs during the day, at night or in jet-lagged rats.

Materials and methods

Grape seed proanthocyanidin extract composition

Grape seed proanthocyanidin extract (GSPE) was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). Specifically, GSPE contains (Serra et al. 2010): catechin (58 µmol/g), epicatechin (52 µmol/g), epigallocatechin (5.50 µmol/g), epicatechingallate (89 µmol/g), epigallocatechingallate (1.40 µmol/g), dimericprocyanidins (250 µmol/g), trimericprocyanidins (1568 µmol/g), tetramericprocyanidins (8.8 µmol/g), pentamericprocyanidins (0.73 µmol/g) and hexamericprocyanidins (0.38 µmol/g).

Animals

All procedures involving the use and care of animals were reviewed and approved by The Animal Ethics Committee of the Universitat Rovira i Virgili (Permit number 4249 by Generalitat de Catalunya).

Eighty-four eight-week-old male Wistar rats (Crl: WI (Han)) were purchased from Charles River (Barcelona, Spain) and fed ad libitum with a standard chow diet (STD, Panlab 04, Barcelona, Spain) and tap water. Rats were divided into three groups according to the Zeitgeber time (ZT) when GSPE was administered.

Administration of GSPE at ZT0: Forty rats were singly caged in animal quarters at 22ºC with a 12 h light/dark cycle (light from 9:00 to 21:00 pm). After three weeks of adaptation, the rats were orally gavaged with tap water (control group) or 250 mg of GSPE /kg body weight

dissolved in tap water at ZT0 (9:00 am, light turned on). Rats were sacrificed by beheading at ZT0, ZT0.5, ZT1, ZT3, ZT6, ZT12 and ZT24 (n=3 for control and n=3 for GSPE-treated groups).

Administration of GSPE at ZT12: Twenty-two rats were singly caged in animal quarters at 22ºC with a 12 h light/dark cycle (light from 21:00 pm to 9:00 am). After three weeks of adaptation, the rats were orally gavaged with tap water (control group) or 250 mg of GSPE/kg body weight dissolved in tap water at ZT12 (9:00 am, light off). Rats were sacrificed by beheading at ZT12, ZT13, ZT15, and ZT18 (n=3 for control and n=3 for GSPE-treated groups).

Administration of GSPE to jet-lagged rats: Twenty-two rats were singly caged in animal quarters at 22ºC with a 12 h light/dark cycle (light from 15:00 pm to 03:00 am). After three weeks of adaptation, rats were orally gavaged with tap water (control group) or 250 mg of GSPE /kg body weight dissolved in tap water at ZT6 (9:00 am, middle of the light day) and immediately moved to a dusk room (ZT12), thus giving rats a jet lag of 6 hours. Rats were sacrificed by beheading at ZT12, ZT13, ZT15, and ZT18 (n=3 for control and n=3 for GSPE-treated groups).

For the three experiments, the liver was excised, frozen immediately in liquid nitrogen and stored at -80ºC until RNA and protein extraction.

RNA extraction and cDNA synthesis

Total RNA from liver was extracted using TRIzol reagent and an RNeasy Mini Kit (Qiagen, 74106, Barcelona, Spain) according to manufacturer protocols. RNA was quantified by spectrophotometry (Nanodrop 1000 Spectrophotometer, Thermo Scientific, Madrid, Spain) at λ=260 nm and tested for purity (by A260/280 ratio) and integrity (by denaturing gel electrophoresis). Complementary DNA was generated using the High-Capacity complementary DNA Reverse Transcription Kit from Applied Biosystems (4368814, Madrid, Spain).

mRNA quantification by real-time qRT-PCR

A total of 10 ng of cDNA was subjected to quantitative RT-PCR amplification using SYBR Green PCR Master Mix from Bio-Rad (172-5200, Barcelona, Spain). The forward and reverse primers of the genes analyzed are shown in Table 1. Reactions were run on a quantitative real-time PCR system (CFX96 touch of Bio-Rad, Barcelona, Spain); the thermal profile settings were 50ºC for 2 min, 95ºC for 2 min, and then 40 cycles at 95ºC for 15 s and 60ºC for 2 min. Finally, statistical data were converted and normalized to the linear form by the $2^{-}CT$ ($\Delta\Delta C_T$) calculation (Livak and Schmittgen 2001). The relative expression of the clock genes was normalized to cyclophilin mRNA levels.

Western blot analyses

Protein was extracted from liver using RIPA (radio-immunoprecipitation assay) lysis buffer (15 mM Tris–HCl, 165 mM NaCl, 0.5% Na-deoxycholate, 1% Triton X-100 and 0.1% SDS) containing a protease inhibitor cocktail (1:1000; Sigma-Aldrich P8340-1 mL, Madrid, Spain) and 1 mM PMSF (phenylmethanesulfonyl fluoride solution, Sigma-Aldrich 93482, Madrid, Spain). The total protein levels of the lysates were determined using the BCA method from Thermo Scientific (23227, Barcelona, Spain). The samples were then placed in sample buffer (0.5 M Tris–HCl, pH 6.8; 10% glycerol; 2% (w/v) SDS; 5% (v/v) β-mercaptoethanol; and 0.05% bromophenol blue). After boiling for 5 min, 50 µg of protein was loaded and separated on a 10% SDS-polyacrylamide gel. The samples were then transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories, 162-017, Barcelona, Spain) using a transblot apparatus (Bio-Rad, 16580229SP) and blocked at room temperature for 1 h with 5% (w/v) non-fat milk in TTBS buffer (Tris-buffered saline (TBS) plus 0.5% (v/v) Tween-20). The membranes were incubated overnight at 4ºC with primary monoclonal antibodies directed against Nampt (Imgenex, IMX-6096, Nanterre, France), Bmal1 (LS-Bio, LS-C16603, Vizcaya, Spain), acetyl-Bmal1 (Millipore, AB15396, Madrid, Spain), HmgcoAR (Santa Cruz, SC-33827, Nanterre, France) and anti-β-actin (Sigma-Aldrich, A2066-0.2 mL, Madrid, Spain) at a 1:1000 dilution in blocking solution. After washing with TTBS, the blots were incubated with a peroxidase-conjugated monoclonal anti-rabbit secondary antibody (Sigma-Aldrich, A1949, Madrid, Spain) at a 1:10,000 dilution at room temperature for 1.5 h. The blots were then washed thoroughly in TTBS followed by TBS. Immunoreactive proteins were visualized with an enhanced chemiluminescence substrate kit (ECL plus; Amersham Biosciences, GE Healthcare, RPN2132, Barcelona, Spain) according to the manufacturer's instructions. Images were obtained with a GBOX Chemi XL 1.4 image system (Syngene, UK). Band quantification was performed with ImageJ software (NIH, USA). The results were expressed as relative intensity (Nampt/ β -actin, Bmal1/ β -actin, HmgcoAR/ β -actin and acetyl-Bmal1/ β -actin) and are relative to the loading control group.

NAD quantification

NAD levels in the liver were quantified using an ELISA kit following the manufacturer's instructions (Sigma-Aldrich, MAK037-1KT, Madrid, Spain).

Data and statistical analysis

The mRNA of each gene was fitted by single cosinor analysis, as data formed a period curve, to determine whether significant circadian rhythms were present (Acro.exe, version 3.5; designed by Dr. Refinetti (Refinetti et al. 2007)). Cosinor analysis provides information on the rhythm through peak-to-trough amplitude; the time of the peak of the rhythm, or acrophase, with a confidence interval; and the middle value of the cosine wave or MESOR. The regression fitting

also produces an R-squared statistic which is then used to compute the percentage of variance, or rhythmicity, in an individual time-series data set that is accounted for by the fitted 24 h curve. In addition, the results are presented as the mean with the associated standard error (SE).The data were analyzed using a two-way ANOVA and Student t-test to determine the significant difference using SPSS statistical software (version 17.0 for Windows; SPSS, Inc.). P values < 0.05 were considered statistically significant.

Results

Acute administration of GSPE at ZT0 shifted the acrophase of Clock, Rorα, Per2 and Nampt in the liver.

The capacity of GSPE to modify the molecular clock in the liver was evaluated by measuring the 24h mRNA oscillation of *Clock* and *Bmal1* (clock core genes), *Per2* (a component of the negative loop of the circadian clock), *Rorα* and *Rev-erbα* (nuclear receptors whose expression is regulated by CLOCK:BMAL1 and which act as an activator or repressor, respectively, of Bmal1 gene expression), *Nampt* (a metabolic gene whose expression is directly regulated by CLOCK:BMAL1) and *HmgCoAR* (a metabolic gene that has circadian rhythm expression but that is not directly controlled by CLOCK:BMAL1). To determine whether GSPE significantly altered the circadian gene pattern, we applied the ANOVA and cosinor analyses to each mRNA curve.

Overall, the ANOVA test indicated that GSPE administered at ZT0 (light turned on) did not significantly affect the mRNA rhythm of any gene studied in the liver (Figure 1). Nonetheless, the cosinor analysis demonstrated that GSPE, administered at this ZT, modulated the wave parameters of some of these mRNAs. Specifically, GSPE delayed the *Clock* acrophase by two hours, from ZT20 to ZT22, and doubled its amplitude (Figure 1B), while not altering the profile of *Bmal1* (Figure 1A). The mRNA from the clock-controlled genes *Rorα* and *Per2* (Figure 1C and 1E, respectively) shared the same profile in control animals, peaking at ZT17. Remarkably, GSPE treatment delayed the mRNA acrophase of both genes by 3h, until ZT20. In the same way, the mRNA acrophase of *Nampt* (Figure 1F), which is also controlled by CLOCK-BMAL1, was delayed by 6h, shifting from ZT11 to ZT17. Nonetheless, GSPE did not affect the wave parameters of *Rev-erbα* (Figure 1D) or *HmgCoAR* (Figure 1G).

In view of the importance of these circadian waves over the course of 24h, the percentage of rhythm was also computed. In general, GSPE treatment slightly affected the wave rhythm of expression. Only *Per2* decreased its rhythmicity after GSPE treatment (approx. from 74% to 55%).

Altogether, these results indicate that PAs have the capacity to adjust the molecular clock in the liver by delaying the acrophase of *Clock*, *Rorα, Per2* and *Nampt* when GSPE was administered at ZT0. However, taking into account the confidence interval of the acrophase, the shift was only relevant for *Nampt*.

Acute administration of GSPE at ZT12 significantly affected the expression of Per2, Nampt and HmgCoAR in the liver.

Rats are nocturnal animals and eat mainly at night. Therefore, we next studied whether PAs can modulate the molecular clock in the liver when GSPE is administered at night, when the liver actively manages the ingested nutrients. GSPE was administered when the light was turned off (ZT12), and the expression of clock-core and clock-controlled genes in the liver was determined at four time points: ZT12, ZT13, ZT15 and ZT18. Nonetheless, to achieve a better visualization of the changes induced by GSPE administered at ZT12, we drew the figures (Figure 2) with a 24-hour curve for the control group by assembling the expression values of the control group from both this experiment and the former experiments. Because of the short period studied (6 hours) in this experiment, we analyzed the effects of GSPE by the ANOVA test and did not apply a cosinor analysis.

GSPE, administered at ZT12, induced slight effects on the mRNA levels of clock-core genes *Bmal1* (Figure 2A) and *Clock* (Figure 2B) as well as on the mRNA levels of the clock-controlled genes *Rorα* (Figure 2C) and *Rev-erbα* (Figure 2D). Nonetheless, the mRNA levels of *Per2* (Figure 4E) and *Nampt* (Figure 4F), two clock-controlled genes, were significantly affected by GSPE. Moreover, the mRNA levels of *HmgCoAR* (Figure 4G), a gene with circadian rhythm but not directly controlled by the clock-core genes, were also significantly increased by GSPE treatment.

Comparing the effects of GSPE administered at ZT12 (beginning of the night) or at ZT0 (beginning of the day), it is evident that *Nampt* was modified under both conditions, thus indicating that this gene is a target of GSPE. However, GSPE showed a powerful capacity to entrain *Nampt* when administered at ZT12.

Acute administration of GSPE modulated the peripheral clock in the liver of jet-lagged rats

The capacity of GSPE to modulate the peripheral clock was also evaluated in a situation where circadian rhythm was disrupted using rats subjected to a 6h jet-lag. Rats at ZT6 (middle of the light period) were administered GSPE and moved to ZT12 (light turned off). The capacity of GSPE to modulate clock-core and clock-controlled genes was evaluated at ZT12, ZT13, ZT15 and ZT18. Because of the short period studied (6h) in this experiment, we analyzed the effects of GSPE by the ANOVA test and did not apply a cosinor analysis.

In control animals, the jet lag induced a clear shift in the mRNA rhythmicity for all of the genes that were studied (Figure 3) when the rhythms were compared with the 24-hour control waves (built by assembling the expression values of the control groups, such as in the former experiment).

GSPE, administered at the beginning of jet lag, did not modulate *Clock* (Figure 3B) or *Per2* (Figure 3E), whereas it significantly altered the expression rhythm of *Rev-erbα* (Figure 3D), *Bmal1* (Figure 3A), *Nampt* (Figure 3F) and *HmgCoAR* (Figure 3G) when compared with the jet lag control group. Remarkably, *Nampt* was again one of the genes most sensitive to GSPE, as in when GSPE was administered at ZT0 and, in particular, at ZT12.

Acute administration of GSPE had opposite effects on both Nampt expression and NAD levels in the liver at different treatment times

Given that *Nampt* was the most susceptible gene to GSPE in the liver and that this protein is the rate-limiting enzyme of the NAD salvage pathway, we next quantified the oscillations of *Nampt* protein and NAD concentration to confirm *Nampt* as a target of GSPE in the liver.

Remarkably, GSPE induced opposite effects when it was administered at ZT0 or ZT12 (Figure 4). *Nampt* protein and mRNA levels were decreased 3h after GSPE administration at ZT0 (Figure 4A), while *Nampt* mRNA and protein levels were significantly elevated at 3 and 6h, respectively, after GSPE administration at ZT12 (Figure 4B). These modifications in *Nampt* expression agreed with the alterations in NAD levels that were induced by GSPE in each situation: NAD levels were significantly decreased 6h after GSPE administration at ZT0, whereas they were significantly increased 6h after GSPE administration at ZT12.

Altogether, these results implicate *Nampt* and NAD modulation as key factors in GSPE activity in the liver.

Acute administration of GSPE at ZT12 increased the ratio of acetylated Bmal1 in rat liver

Nampt is a direct target gene of CLOCK:BMAL1. However, GSPE did not induce a strong modification in the mRNA rhythms of *Clock* or *Bmal1* that could explain the observed alteration in *Nampt* expression. However, the transcriptional activity of CLOCK:BMAL1 is dependent on *Bmal1* acetylation. Thus, we focused further on the ratio of *Bmal1* acetylation by measuring *Bmal1* mRNA, protein and acetylated protein during the first six hours after GSPE administration at ZT0 or ZT12.

GSPE administered at either time increased mRNA and protein levels of *Bmal1* after 1 hour of treatment (Figure 5A and B; ZT1 and ZT13, respectively). Nonetheless, the ratio of acetylated *Bmal1* was only increased when GSPE was administered at night (ZT12); these are the same circumstances under which *Nampt* was overexpressed.

Acute administration of GSPE repressed the relative expression of HmgcoAR in rat liver.
Finally, the mRNA and protein levels of *HmgcoAR,* a metabolic gene not controlled by the clock molecular machinery, were determined according to the three experimental designs (day, night and jet lag) during only the first six hours after GSPE treatment (ZT1, ZT3 and ZT6 or ZT13, ZT15 and ZT18).

HmgcoAR expression was significantly repressed at ZT1 (mRNA) and ZT3 (protein) after GSPE treatment was administered at ZT0 (Figure 6A). While mRNA levels were increased at ZT15 after GSPE treatment was administered at ZT12, the protein levels did not reflect that fact (Figure 6B). Finally, in the jet-lagged rats, *HmgcoAR* protein and mRNA levels were decreased at ZT18 (Figure 6C).

Discussion

While light is the major synchronizer of the central clock in the suprachiasmatic nucleus (SCN), many other external cues such as temperature, social events or meal timing (Hirao et al. 2009) can entrain circadian rhythms in other cerebral regions or peripheral tissues. This phenomenon is especially the case in the liver, which is the most important metabolic organ due to its involvement in glucose (Lamia et al. 2008) and lipid (Edwards et al. 1972) metabolism, among other crucial physiological functions (Panda et al. 2002). Even specific components in foods could also be important synchronizers, such as dietary fat (Kohsaka et al. 2007) or phenolic compounds like resveratrol (Pifferi et al. 2013, 2011; Oike and Kobori 2008; Miranda et al. 2013). Therefore, the aim of this work was to determine the capacity of an acute dose of GSPE to act as a signal to entrain the molecular clock in the liver and as a global mechanism by which PAs can exert their beneficial metabolic effects in the liver.

To accomplish this outcome, three different experimental approaches were performed to determine whether PAs can modulate the liver clock: the administration of GSPE at ZT0, at the beginning of the light phase; at ZT12, at the beginning of the dusk phase; and to rats with 6h of jet lag. The data clearly show that the power of PAs to entrain the circadian rhythm of clock-core and clock-controlled genes in the liver depends on the time of their administration.

Interestingly, *Nampt* and NAD emerge as molecular targets of PAs in the liver. *Nampt* is the rate-limiting enzyme in NAD biosynthesis through its salvage pathway (Magni et al. 1999). NAD plays a major role as a coenzyme in numerous oxidation-reduction reactions (Rongvaux et al. 2003) and is required in a number of important signaling pathways in mammalian cells, including poly(ADP-ribosyl)ation in DNA repair (Ménissier de Murcia et al. 2003), mono-ADP-ribosylation in both the immune response and G protein-coupled signaling (Corda and Di Girolamo 2003), and the synthesis of cyclic ADP-ribose and nicotinate adenine dinucleotide phosphate (NAADP) in intracellular calcium signaling (Lee 2001). Furthermore, NAD activates

several NAD(+)-dependent deacetylases (SIRT), such as SIRT1 and SIRT3, thus controlling the activity of many cellular proteins by cycling them between their acetylated and deacetylated forms. Specifically, the circadian oscillations of NAD levels have been shown to modulate mitochondrial respiration by controlling the activity of SIRT3, thus generating rhythms in the acetylation and activity of oxidative enzymes that synchronize mitochondrial oxidative functions across the daily cycles of fasting and feeding (Peek et al. 2013). Therefore, NAD is a key molecule in the synchronization of liver metabolism, and the modulation of its levels in the liver by PAs is an attractive candidate for the explanation of some of the metabolic effects of PAs. Although GSPE modulated *Nampt* and NAD levels in opposite ways 6h after its administration during the day versus at night, reducing or increasing their levels, respectively, it is noteworthy that the *Nampt* acrophase was delayed by 6 hours by GSPE administration during the day, suggesting that NAD also peaked at night. Therefore, in this sense, PAs could act as an element of adaptation in the liver, improving the energetic profile of rats and increasing mitochondrial function and oxidation at night, when rats are active. In keeping with the idea of an adaptation mechanism, as these animals are resting during the light phase, PA activity could be acting as an energy saver through the decreased levels of NAD after PA administration at ZT0.

NAD concentration oscillates in a circadian manner due to the circadian expression of *Nampt*, which in turn is mediated by the CLOCK:BMAL1 heterodimer (Nakahata et al. 2009). The rhythm of *Clock* and *Bmal1* expression was not altered by GSPE administration, either during the day or at night (Figures 1 and 2). However, when *Bmal1* expression was studied during the first six hours of GSPE administration (Figure 5), both mRNA and protein levels were always increased one hour after PA consumption, regardless of whether GSPE was administered diurnally, at night, or even under jet lag conditions, suggesting a robust relationship between *Bmal1* and PAs. However, to be active, BMAL1 should be acetylated by CLOCK, which is its own partner. Therefore, the ratio of acetylated to total BMAL1 protein provides direct information about the transactivation activity of BMAL1:CLOCK (Nakahata et al. 2009). We found that GSPE significantly increased the ratio of BMAL1 that was acetylated at ZT13, whereas this effect was not observed at ZT1. This differential pattern of BMAL1 acetylation, which depends on the time of GSPE administration, could explain the overexpression of NAMPT and therefore the peak in NAD levels in the liver only when GSPE was administered at night. Therefore, it is globally supposed in this work that NAD levels peaked 6 hours after GSPE consumption at ZT12 (thereby at ZT18) as a consequence of an increased BMAL1 acetylation ratio at ZT13 that, in turn, increased *Nampt* mRNA and protein levels at ZT15-ZT18.

PAs modulate lipid metabolism in the liver (Bladé et al. 2010). Therefore, we also analyzed the expression of *HmgcoAR*, the key enzyme in the cholesterol biosynthetic pathway, which has

circadian rhythm but is not directly controlled by the clock-core genes (Patel et al. 2001). As was expected, the *HmgcoAR* acrophase occurred at ZT17, and PAs did not modify the expression rhythm of this enzyme. However, GSPE had a dual effect on *HmgcoAR* expression depending on the time of its administration. GSPE repressed both *HmgcoAR* mRNA and protein levels at ZT0, whereas protein levels at ZT12 were not affected. Therefore, as in the case of BMAL1 acetylation, Nampt expression and NAD levels, the time of PA administration conditions the circadian adjustment outputs.

Overall, these findings agree with the Xenohormesis Hypothesis, which proposes that heterotrophs are able to sense chemical cues, such as polyphenols, that are synthesized by plants in response to stress (Howitz and Sinclair 2008). In fact, circadian rhythms allow the anticipation of environmental changes and adaptation to the time of day and food availability, which has been shown in this work through NAD, NAMPT and BMAL1 acetylation levels. Thus, PAs can advise animals about environmental conditions by entraining biological rhythms to obtain a better ability to adapt to changing conditions over the course of their lives.

In conclusion, PAs entrain the molecular clock in the liver even though their effectiveness depends largely on the time of administration. Specifically, *Bmal1* and *Nampt,* as well NAD, emerge as targets of GSPE in the liver.

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Figure legends

Fig. 1

Nampt is the most sensitive clock-controlled gene in the liver after an oral dose of a grape seed proanthocyanidin extract (GSPE) administered at Zeitgeber Time 0.

Rats were orally gavaged with tap water (control group) or 250 mg of GSPE /kg body weight dissolved in tap water at ZT0 (light turned on), and mRNA levels were measured at ZT0, ZT0.5, ZT1, ZT3, ZT6, ZT12 and ZT24. The capacity of GSPE to modify the peripheral clock was evaluated by measuring the oscillation of the levels of mRNA from the clock core genes (A) *Bmal1* and (B) *Clock* as well as the CLOCK:BMAL1-controlled genes, (C) *Rorα*, (D) *Rev-erbα,* (E) *Per2* and (F) *Nampt.* (G) The expression of *HmgCoAR*, a gene that has circadian rhythm expression but that is not directly controlled by CLOCK:BMAL1, was also evaluated. Each graph shows the mean ± s.e. for each data point (n=3). T, significant effect of proanthocyanidins; t, significant effect of Zeitgeber Time; T*t, interaction between the two variables by two-way ANOVA. For the cosinor analysis: %R, cosine wave rhythm percentage; M, mesor; AMP, amplitude of cosine wave; Acro, acrophase; CI Acro, confidence interval of acrophase.

Fig. 2

An oral dose of a grape seed proanthocyanidin extract (GSPE) administered at Zeitgeber Time 12 significantly affects the expression of Per2, Nampt and HmgCoAR in the liver.

Rats were orally gavaged with tap water (control group) or 250 mg of GSPE /kg body weight dissolved in tap water at ZT12 (light turned off), and mRNA levels were measured at ZT12, ZT13, ZT15 and ZT18. The capacity of GSPE to modify the peripheral clock was evaluated by measuring the oscillation of the levels of mRNA from the clock core genes (A) *Bmal1* and (B) *Clock* as well as the CLOCK:BMAL1-controlled genes (C) *Rorα*, (D) *Rev-erbα,* (E) *Per2*, and (F) *Nampt.* (G) The expression of *HmgCoAR*, a gene that has circadian rhythm expression but that is not directly controlled by CLOCK:BMAL1, was also evaluated. Each graph shows the mean ± s.e. for each data point (n=3). For the control group, a 24-hour curve was constructed by assembling the expression values of the control group from this experiment and those from Fig. 1. T, significant effect of proanthocyanidins; t, significant effect of Zeitgeber Time; T*t, interaction between the two variables by two-way ANOVA.

Fig. 3

An oral dose of a grape seed proanthocyanidin extract (GSPE) administered to jet-lagged rats significantly alters the expression rhythms of *Rev-erbα*, *Bmal1*, *Nampt* and *HmgCoAR*.

Rats were orally gavaged with tap water (control group) or 250 mg of GSPE /kg body weight dissolved in tap water at ZT6 (middle of the light period) and were moved to ZT12 (light turned off). mRNA levels were measured at ZT12, ZT13, ZT15 and ZT18. The capacity of GSPE to modify the peripheral clock was evaluated by measuring the oscillation of the levels of mRNA from the clock core genes (A) *Bmal1* and (B) *Clock* as well as the CLOCK:BMAL1 controlled genes (C) *Rorα*, (D) *Rev-erbα,* (E) *Per2* and (F) *Nampt.* (G) The expression of *HmgCoAR*, a gene that has circadian rhythm expression but that is not directly controlled by CLOCK:BMAL1, was also evaluated. Each graph shows the mean ± s.e. for each data point (n=3). For the control group with no jet lag, a 24-hour curve was constructed by assembling the expression values of the control groups from Fig. 1 and 2. T, significant effect of proanthocyanidins; t, significant effect of Zeitgeber Time; T*t, interaction between the two variables by two-way ANOVA.

Fig. 4

An oral dose of a grape seed proanthocyanidin extract (GSPE) administered at Zeitgeber Time 0 or 12 oppositely affects both Nampt expression and NAD levels in the liver.

Rats were orally gavaged with tap water (control group) or 250 mg of GSPE /kg body weight dissolved in tap water both at ZT0 (light turned on) and ZT12 (light turned off), and Nampt protein expression and NAD levels were measured at ZT1, ZT3, ZT6, ZT13, ZT15 and ZT18. Proteins were extracted by radioimmunoprecipitation (RIPA) buffer and analyzed by Western blot. Proteins were normalized to β-actin, an endogenous protein. Relative intensity units were obtained by dividing the band intensity of the protein of interest by the band intensity of the endogenous protein. NAD quantification was performed using an ELISA kit following the manufacturer's instructions. Each graph shows the mean ± s.e. for each data point (n=3). White bars, control group; colored bars, GSPE-treated groups. *Statically significant differences found by independent Student T-test ($p<0.05$) between the control group and GSPE-treated group for each ZT.

Fig. 5

An oral dose of a grape seed proanthocyanidin extract (GSPE) administered at Zeitgeber Time 12 increases the ratio of acetylated Bmal1 in rat liver.

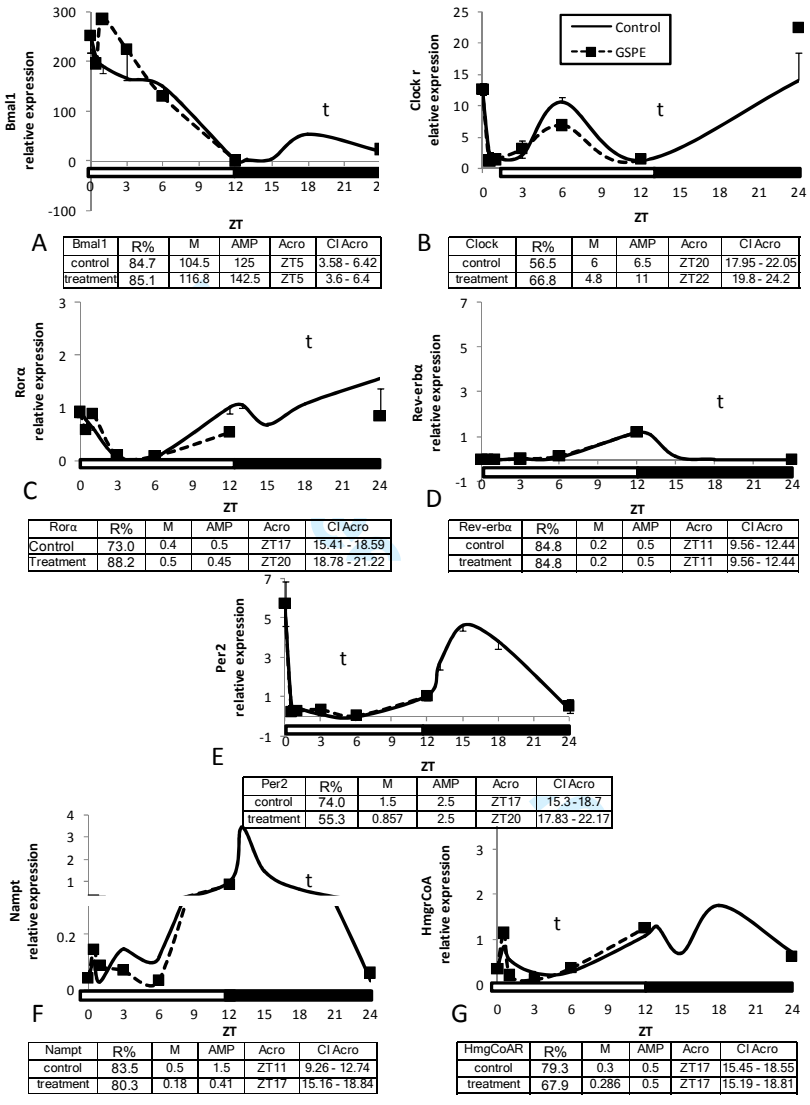
Rats were orally gavaged with tap water (control group) or 250 mg of GSPE /kg body weight dissolved in tap water both at ZT0 (light turned on) and ZT12 (light turned off), and Bmal1 total protein and acetylated protein were measured at ZT1, ZT3, ZT6, ZT13, ZT15 and ZT18. Proteins were extracted by radioimmunoprecipitation (RIPA) buffer and analyzed by Western blot. Proteins were normalized to β-actin, an endogenous protein. Relative intensity units were obtained by dividing the band intensity of the protein of interest by the band intensity of the endogenous protein. Acetylated and total BMAL1 protein samples were then divided to obtain the acetylated Bmal1/total Bmal1 protein ratio, shown as a percentage. Each graph shows the mean ± s.e. for each data point (n=3). White bars, control group; colored bars, GSPE-treated groups. *Statically significant differences found by independent Student T-test ($p<0.05$) between the control group and GSPE-treated group for each ZT.

Fig. 6

An oral dose of a grape seed proanthocyanidin extract (GSPE), administered at Zeitgeber Time 0, 12, or to jet-lagged rats, modulates HmgcoAR expression in rat liver.

Rats were orally gavaged with tap water (control group) or 250 mg of GSPE /kg body weight dissolved in tap water at ZT0 (light turned on), ZT12 (light turned off) or ZT6 (middle of the light period), in the case of the jet-lagged rats, which were moved to ZT12 (light turned off) after GSPE administration. HmgcoAR protein expression was then measured at ZT1, ZT3, ZT6, ZT13, ZT15 and ZT18. Proteins were extracted by radioimmunoprecipitation (RIPA) buffer and analyzed by Western blot. Proteins were normalized to β-actin, an endogenous protein. Relative intensity units were obtained by dividing the band intensity of the protein of interest by the band intensity of the endogenous protein. Each graph shows the mean ± s.e. for each data point (n=3). White bars, control group; colored bars, GSPE-treated groups. *Statically significant differences found by independent Student T-test ($p<0.05$), between the control group and GSPE-treated group for each ZT.

Figure 1



Bmal1	R%	M	AMP	Acro	CI Acro
control	84.7	104.5	125	ZT5	3.58 - 6.42
treatment	85.1	116.8	142.5	ZT5	3.6 - 6.4

Clock	R%	M	AMP	Acro	CI Acro
control	56.5	6	6.5	ZT20	17.95 - 22.05
treatment	66.8	4.8	11	ZT22	19.8 - 24.2

Rorα	R%	M	AMP	Acro	CI Acro
Control	73.0	0.4	0.5	ZT17	15.41 - 18.59
Treatment	88.2	0.5	0.45	ZT20	18.78 - 21.22

Rev-erbα	R%	M	AMP	Acro	CI Acro
control	84.8	0.2	0.5	ZT11	9.56 - 12.44
treatment	84.8	0.2	0.5	ZT11	9.56 - 12.44

Per2	R%	M	AMP	Acro	CI Acro
control	74.0	1.5	2.5	ZT17	15.3 - 18.7
treatment	55.3	0.857	2.5	ZT20	17.83 - 22.17

Nampt	R%	M	AMP	Acro	CI Acro
control	83.5	0.5	1.5	ZT11	9.26 - 12.74
treatment	80.3	0.18	0.41	ZT17	15.16 - 18.84

HmgCoAR	R%	M	AMP	Acro	CI Acro
control	79.3	0.3	0.5	ZT17	15.45 - 18.55
treatment	67.9	0.286	0.5	ZT17	15.19 - 18.81

Figure 2

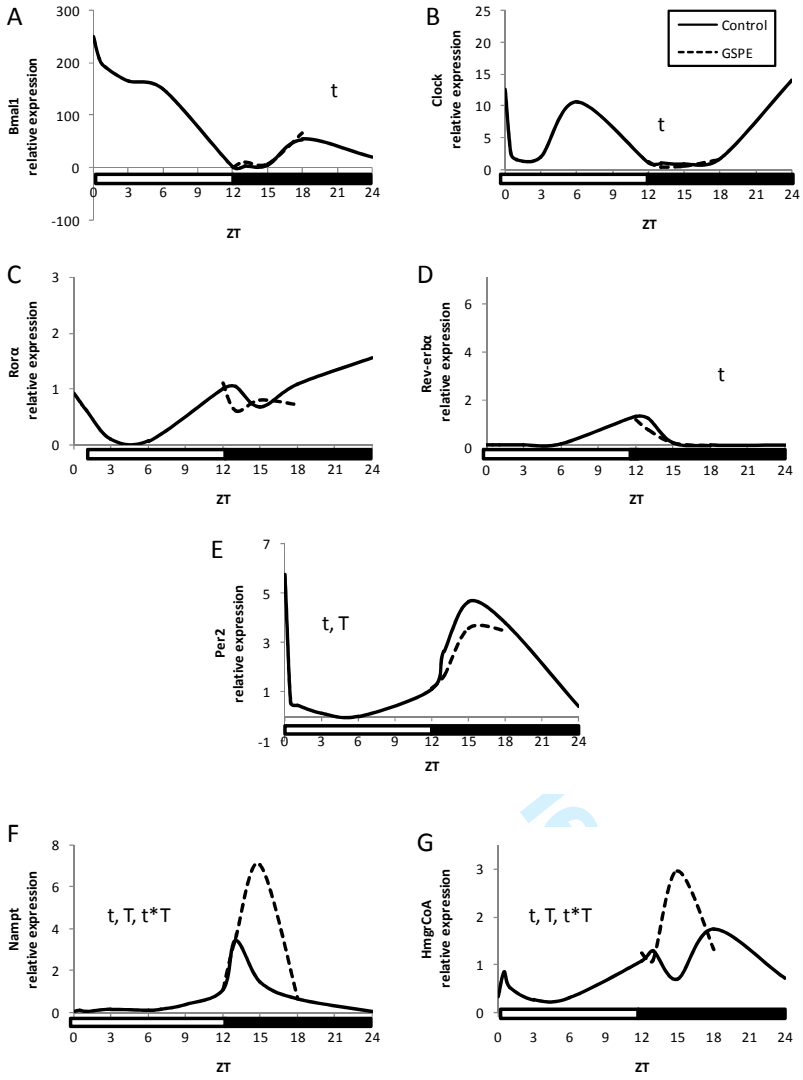


Figure 3

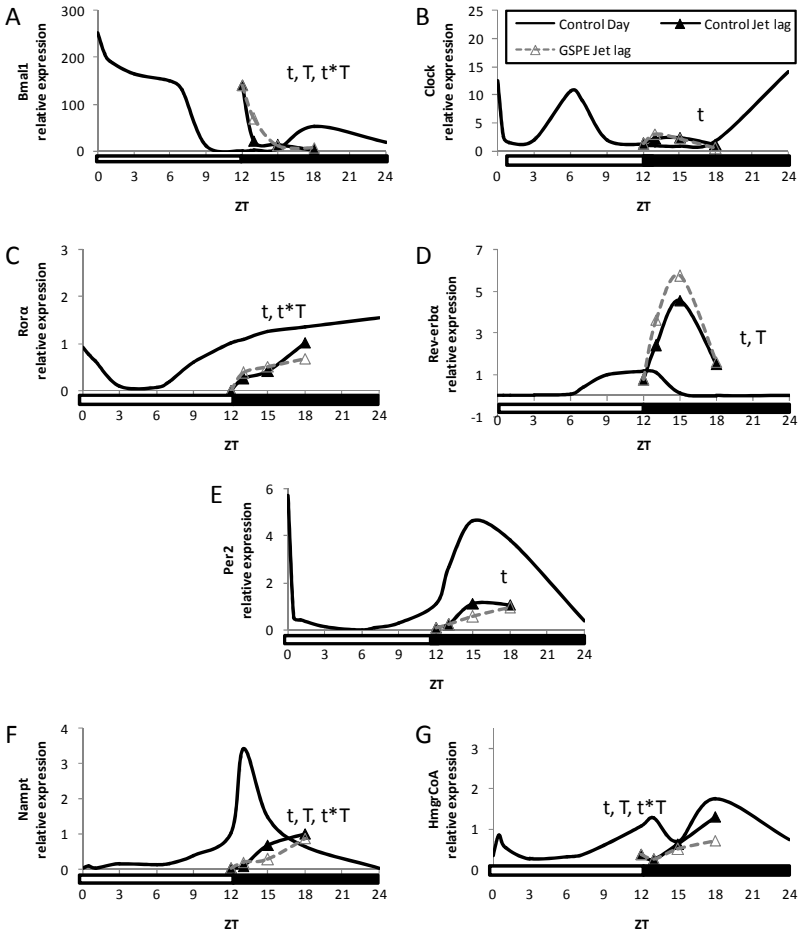


Figure 4

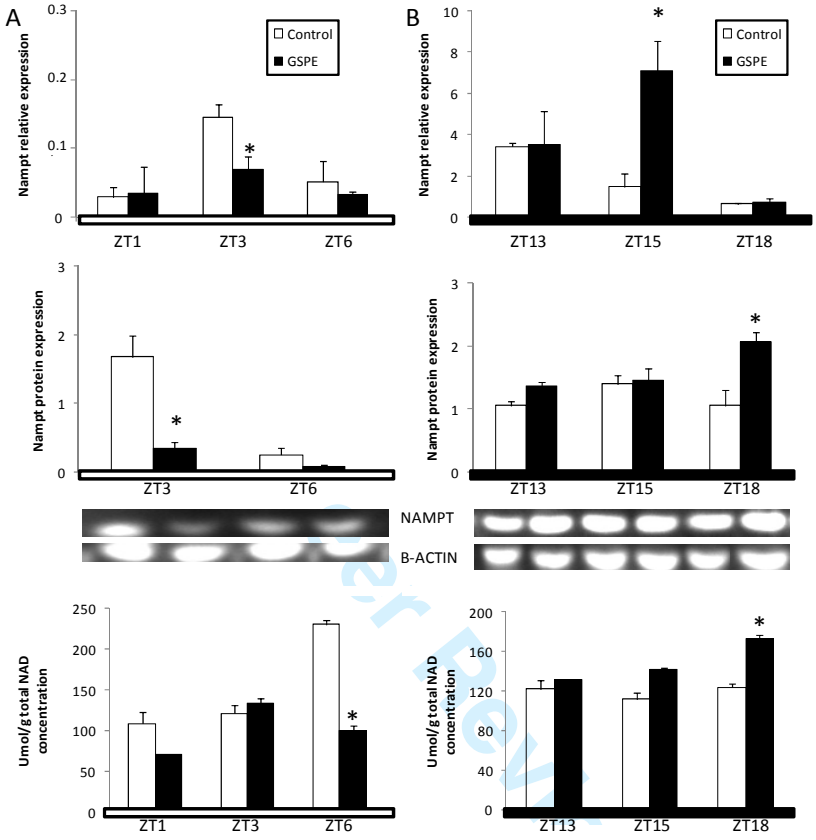


Figure 5

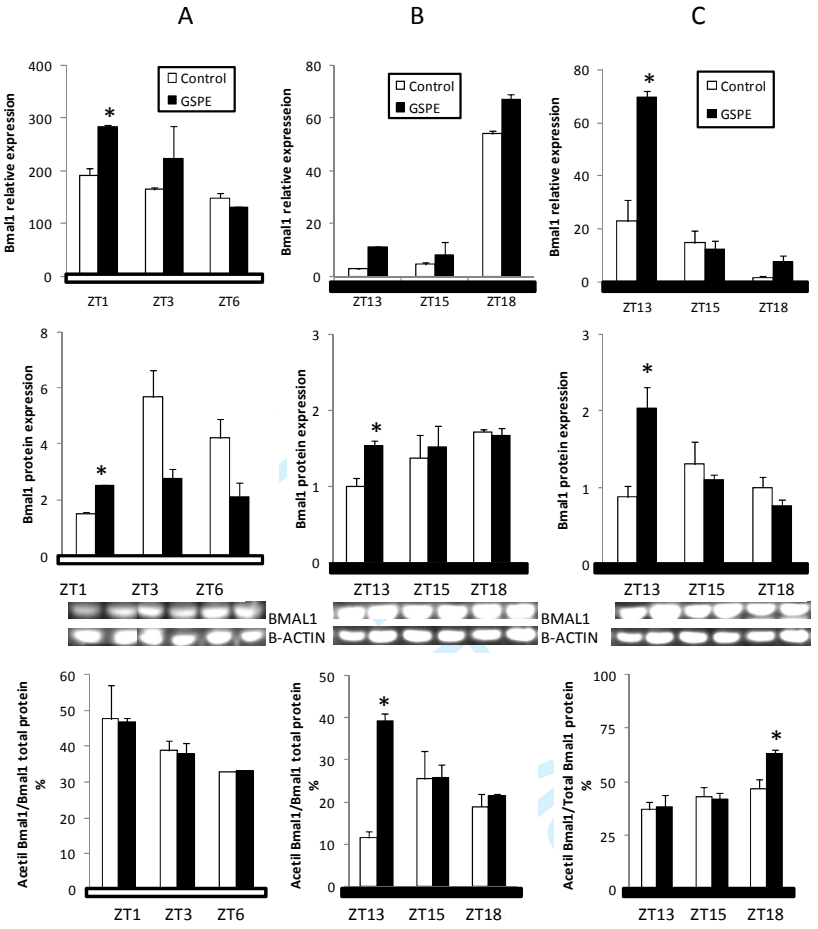


Figure 6

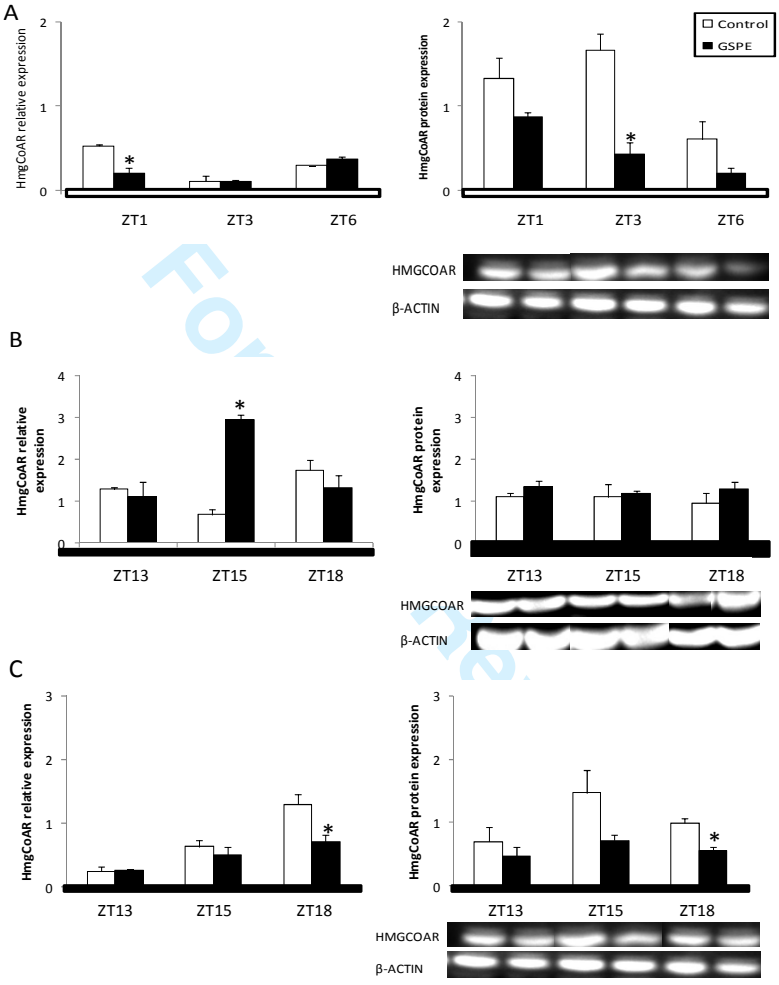


Table 1. Primer sequences

	Forward	***Reverse***
Bmal1	5′-GTAGATCAGAGGGCGACGGCTA-3′	5′-CTTGTCTGTAAAACTTGCCTGTGAC-3′
Clock	5′-TGGGGTCTATGCTTCCTGGT-3′	5′-GTAGGTTTCCAGTCCTGTCG-3′
Per2	5′-CGGACCTGGCTTCAGTTCAT-3′	5′-AGGATCCAAGAACGGCACAG-3′
Rorα	5′-GAAGGCTGCAAGGGCTTTTTCAGGA-3′	5′-CCAAACTTGACAGCATCTCGA-3′
Rev-erbα	5′-CTGCTCGGTGCCTAGAATCC-3′	5′-GTCTTCACCAGCTGGAAAGCG-3′
Nampt	5′-CTCTTCACAAGAGACTGCCG-3′	5′-TTCATGGTCTTTCCCCCACG-3′
HmgCoAR	5′- GAAACCCTCATGGAGACGCA-3′	5′- ACCTCTGCTGAGTCACAAGC-3′
Cyclophilin	5′-CTTCGAGCTGTTTGCAGACAA-3′	5′-AAGTCACCACCCTGGCACATG-3′

Bmal1 (also known as *ARNTL)*: aryl hydrocarbon receptor nuclear translocator-like; *Clock*: circadian locomotor output cycles kaput; Per2: period circadian clock 2; *Rorα*: RAR-related orphan receptor A; *Rev-erbα* (also known as *Nr1d1*): nuclear receptor subfamily 1, group D, member 1; *Nampt:* nicotinamide phosphoribosyl transferase; *HmgCoAR*: 3-hydroxy-3-methyl-glutaryl-CoA reductase.

3. Dietary proanthocyanidins modulate the rhythm of BMAL1 expression and induce RORα transactivation in HepG2 cells.

(Manuscript 3, submitted)

Dietary proanthocyanidins modulate the rhythm of *BMAL1* expression and induce RORα transactivation in HepG2 cells.

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Abstract

Proanthocyanidins (PAs), a flavonoid sub-class, alter the expression of clock genes in the liver of lean and obese rats. The present study aimed to determine whether PAs could modulate the 24-hour rhythmicity of clock gene expression and to identify the molecular mechanism through PAs could adjust the clock system in HepG2 cells. The 24-hour rhythmicity of core clock (*CLOCK* and *BMAL1*) and clock-controlled (*CRY, PER2, RORα, REV-ERBα*) gene expression indicated that a grape seed proanthocyanidin extract (GSPE) shifted the acrophase of nearly all of them, but *BMAL1* appeared as the most sensitive gene to GSPE. Specifically, GSPE increased *BMAL1* expression strongly and very quickly. This effect was also reproduced by melatonin. The overexpression of *BMAL1* was MT1 dependent for melatonin but MT1 independent for GSPE. However, GSPE increased the transcriptional activity of RORα, suggesting that this nuclear receptor could be responsible for the modulation of *BMAL1* by GSPE.

Keywords: Bmal1, melatonin, flavonoids, Rorα

1. Introduction

Many aspects of metabolism display circadian rhythms that are regulated by peripheral clocks in a tissue-autonomous manner, revealing a mechanism by which the organism can improve its adaptation in the face of external demands and integrating information from the environment (Tahara & Shibata, 2013). Disruption of the clock system triggers different types of illnesses, such as cancer and metabolic syndrome, indicating that peripheral clocks play an important role in maintaining homeostasis and normal body function (Froy, 2010). Among the peripheral clocks present throughout the body, the clock in the liver is one of the most important because this organ plays a central role in metabolism and energy production, significantly affecting the physiological status of the entire organism. For instance, the liver is the major site of intermediate metabolism, including the synthesis and removal of cholesterol (Edwards, Muroya, & Gould, 1972) and the regulation of glucose homeostasis (Lamia, Storch, & Weitz, 2008). In fact, 10% of all transcripts or 20% of all proteins in mouse liver are under circadian regulation (Reddy et al., 2006), emphasizing the importance of the clock present in this organ.

At the molecular level, the clock system consists of transcription–translation autoregulatory feedback loops, with the transcriptional activators circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-like protein 1 (BMAL1) driving the positive limb of this loop. After their heterodimerization, the complex activates transcription of the Period (*PER*) and Cryptochrome (*CRY*) genes; once reaching a critical concentration, the PER and CRY proteins translocate to the nucleus and inhibit the activity of the CLOCK:BMAL1 heterodimer, thus leading to a decrease in *PER* and *CRY* expression. In addition, the active CLOCK:BMAL1 complex also promotes transcription of the retinoic acid-related orphan receptor alpha (*RORα*) and nuclear receptor subfamily 1, group D (*Nr1d1*, also known as *REV-ERBα*), its own activator and repressor, respectively, thereby generating another loop of regulation (Green, Takahashi, & Bass, 2008).

Peripheral clocks are adjusted to a 24-hour circadian rhythm by the master clock in the suprachiasmatic nucleus (SCN) of the hypothalamus, which is entrained by the light/dark cycle, through the autonomic nervous system and hormonal signals such as melatonin and glucocorticoids (Dibner, Schibler, & Albrecht, 2010). Moreover, the molecular clock in the liver, and therefore the metabolic circadian rhythm, is also entrained by the frequency of daytime meals as well as by the composition of the diet (Hirao, Tahara, Kimura, & Shibata, 2009; Reznick et al., 2013).

Proanthocyanidins (PAs) are a sub-class of flavonoids that are present in vegetables, fruits, cacao, nuts and some beverages, such as red wine and tea (Serrano, Puupponen-Pimiä, Dauer, Aura, & Saura-Calixto, 2009). The consumption of PA extracts and PA-rich food has been associated with a variety of healthy effects, such as reduced cardiovascular disease (Rasmussen, Frederiksen, Struntze Krogholm, & Poulsen, 2005), improved insulin resistance (Montagut et

al., 2010), and decreased obesity (Montagut et al., 2007), inflammation (Terra et al., 2011), hypertension (Quiñones et al., 2013), oxidative stress (Fernández-Iglesias et al., 2014) and dyslipidemia (Quesada et al., 2009). The liver has been found to be a key organ in the mechanism of action by which PAs improve lipid (Bladé, Arola, & Salvadó, 2010) and glucose (Pinent, Cedó, Montagut, Blay, & Ardévol, 2012) homoeostasis in disrupted situations. Interestingly, PAs modulate the expression of clock genes in the liver of lean and obese rats (Ribas-Latre et al., 2014), suggesting that PAs can improve health by entraining biological rhythms. Therefore, the aim of this study was to determine whether PAs could modulate the 24-hour rhythmicity of clock gene expression in hepatocytes and to identify the molecular mechanism through PAs could adjust the clock system in the liver. To determine the direct effect of PAs in the liver clock and avoid synchronization by the SCN, this study was carried out using the human hepatocyte carcinoma cell line HepG2.

2. Materials and methods

2.1.Grape seed proanthocyanidin extract composition

The grape seed proanthocyandin extract (GSPE) was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). GSPE contains (Serra et al., 2010) catechin (58 μmol/g), epicatechin (52 μmol/g), epigallocatechin (5.50 μmol/g), epicatechin gallate (89 μmol/g), epigallocatechin gallate (1.40 μmol/g), dimeric procyanidins (250 μmol/g), trimeric procyanidins (1568 μmol/g), tetrameric procyanidins (8.8 μmol/g), pentameric procyanidins (0.73 μmol/g) and hexameric procyanidins (0.38 μmol/g).

2.2. Cell culture

HepG2 cells were used as the experimental model. The cells were routinely propagated in a 5% CO_2 humidified atmosphere at 37 °C in minimum essential medium (DMEN, BE12-917F, Lonza, Barcelona, Spain) supplemented with 10% (v/v) fetal bovine serum (DE14-801F, Lonza, Barcelona Spain), L-glutamine (2 mM) (BE17-605E, Lonza, Barcelona, Spain), N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES, 25 mM) (H-3375-500 g, Sigma, Madrid, Spain), non-essential amino acids (NEAA, 0.1 mM) (M7145, Sigma, Madrid, Spain) and penicillin (100 U/ml) / streptomycin (100 μg/ml) (DE17-602E, Lonza, Barcelona, Spain). The cells were passaged every 3 days, and the medium was changed every 2 days.

The day before the experiment, cells at approximately 80% of confluence were plated in six-wells plates (657160, Cellstar, Tarragona, Spain) at a density of 1,000,000 cells/well. After 24 hours, the cells were synchronized by serum shock using 50% horse serum (v/v) (H1138-500 mL, Sigma, Madrid, Spain) in DMEN for two hours. The medium was then replaced with DMEN, supplemented as indicated before, and the specific treatment was added. GSPE and melatonin were dissolved in ethanol at the appropriate concentration to attain a final

concentration of ethanol ≤0.1% in the medium. For each experiment, control cells were incubated with the same concentration of ethanol in the medium.

Experiment 1: HepG2 cells were incubated with GSPE at 100 mg/L and lysed at 0, 1, 3, 6, 9, 12, 15, 18, 21 or 24 hours after GSPE addition.

Experiment 2: HepG2 cells were incubated with GSPE at 100 mg/L or with melatonin (M5250-250MG, Sigma, Madrid, Spain) at 0.1, 10 or 100 μM and lysed after 1 hour of treatment.

Experiment 3: HepG2 cells were incubated with GSPE at 100 mg/L or with melatonin (M5250-250MG, Sigma, Madrid, Spain) at 10 μM and lysed after 0,1, 3, 6, 9 and 15 hours of treatment.

Experiment 4: HepG2 cells were incubated with luzindole (0877, Tocris, Madrid, Spain) at 1 μM for 1 hour and then with GSPE at 100 mg/L or melatonin at 10 μM. In parallel, another set of cells was cultured with GSPE or melatonin, but without luzindole, at the same doses and times. The cells were lysed after 1, 6, 9 and 15 hours of GSPE or melatonin treatment.

For the four experiments, three independent experiments were run in duplicate, and the cells were lysed with the lysis buffer RLT (Qiagen, 74106, Barcelona, Spain) and stored at -80ºC until RNA extraction.

2.3. RNA extraction and cDNA synthesis

Total RNA from cells was extracted using the TRIzol reagent and an RNeasy Mini Kit (Qiagen, 74106, Barcelona, Spain) according to the protocols of both manufacturers. The RNA was quantified by spectrophotometry (Nanodrop 1000 Spectrophotometer, Thermo Scientific, Madrid, Spain) at λ=260 nm and tested for purity (by A260/280 ratio) and integrity (by denaturing gel electrophoresis). Complementary DNA was generated using the High-Capacity complementary DNA Reverse Transcription Kit from Applied Biosystems (4368814, Madrid, Spain). The cDNA was subsequently amplified by PCR using specific TaqMan Assay-on-Demand Probes from Applied Biosystems (Madrid, Spain) for circadian locomotor output cycles kaput (*CLOCK*) (Hs00231857_m1), brain and muscle ARNT-like protein 1 (*BMAL1*) (Hs00154147_m1), Period 2 (*PER 2*) (Hs00256143_m1), Cryptochrome1 (*CRY 1*) (Hs01565974_m1), retinoic acid-related orphan receptor alpha (*ROR*α) (Hs00536543_m1), nuclear receptor subfamily 1, group D (NR1D1, also known as *REV-ERB*α) (Hs00253876_m1) and cyclophilin peptidylprolyl isomerase A (*PPIA*) (Hs99999904_m1).

2.4. mRNA quantification by real-time qRT-PCR

A total of 10 ng of cDNA was subjected to quantitative RT-PCR amplification using the TaqMan PCR Core Reagent Kit, according to the manufacturer's protocol, and analyzed using a Real-Time 7300 PCR System, both from Applied Biosystems (Madrid, Spain). The thermal cycling comprised an initial step at 50 °C for 2 min, followed by a polymerase activation step at 95 °C for 10 min and a cycling step under the following conditions: 40 cycles of denaturation at

95 °C for 15 s and annealing at 60 °C for 1 min. Finally, the statistical data were converted and normalized to the linear form by the $2^{-}CT$ ($\Delta\Delta C_T$) calculation (Livak & Schmittgen, 2001). The relative expression of the clock genes was normalized to PPIA mRNA levels.

2.5. Luciferase-based reporter system for Rorα transcriptional activity assays

A luciferase reporter system was obtained by cloning the ligand-binding domain (LBD) and the hinge region of RORα in the pCMV-BD plasmid (Stratagene, USA) using the primers (forward) AAAAGGATCCTATGCAGAAGTACAGAAACAC and (reverse) AAAACTGCAGTTACCCATCAATTTGCATTGC, which also contain restriction sites for BamH1 and Pst1 digestion; HepG2 cDNA was used as the template, and a previously described protocol was followed (Josep M del Bas, Laos, Caimari, Crescenti, & Arola, 2012). The resulting construct coding for an RORα-LBD:Gal4 DNA-binding domain fusion protein was delivered into HepG2 cells cultured in 48-well plates using linear polyethylenimine (Sigma, Madrid, Spain) as the transfection agent (Reed, Staley, Mayginnes, Pintel, & Tullis, 2006) together with pFRLuc (Stratagene, USA) as the reporter plasmid. The pRL-TK vector expressing Renilla luciferase (Promega) was also co-transfected as the transfection efficiency control. For ligand activity assays, the complete medium was replaced with fresh DMEM medium supplemented with 10% charcoal-stripped FBS (Life Technologies, USA), 1% NEAA and 1% L-glutamine containing either 10 μM melatonin (Sigma, Spain), 100 mg/L GSPE or both, with DMSO as the vehicle. After 24 hours, the cells were washed with PBS, and the Renilla and firefly luciferase activities were assayed with the Dual-Glo luciferase assay system (Promega, Spain) following the manufacturer's instructions.

2.6. Data and statistical analysis

The results are presented as the mean with the associated standard error (SE) of three independent experiments. The expression of clock genes was fitted by the method of single cosinor analysis, as the data formed a period curve, to determine whether significant circadian rhythms were present (Acro.exe, version 3.5; designed by Dr. Refinetti (Refinetti, Lissen, & Halberg, 2007)). The cosinor analysis provides information of the rhythm through the peak-to-trough amplitude and time of the peak of the rhythm, or acrophase, with a confidence interval and the middle value of the cosine wave or MESOR. The regression fitting also produces an R-squared statistic, which is then used to compute the percentage of variance or rhythmicity in individual time-series data accounted for by the fitted 24-h curve.

In addition, data were analyzed using a two-way ANOVA and *Student t-test* to determine significant differences with SPSS statistical software (version17.0 for Windows; SPSS, Inc.). P values <0.05 were considered statistically significant.

3. Results

3.1. GSPE shifts the mRNA acrophases of core clock and clock-controlled genes and targets BMAL1.

The capacity of GSPE to modify the molecular clock in HepG2 cells was evaluated by measuring the mRNA oscillations of *CLOCK* and *BMAL1* (core clock genes), *PER2* and *CRY1* (components of the negative loop of the circadian clock), and *RORα* and *REV-ERBα* (nuclear receptors with expression regulated by CLOCK:BMAL1 and that act as an activator or a repressor of *BMAL1* gene expression, respectively).

The HepG2 cells exhibited a circadian expression of core clock and clock-controlled genes, as indicated by the cosinor values obtained from the 24-hour mRNA levels of each gene in the control cells (Figures 1A to 1F).

GSPE treatment did not affect the percentage of rhythmicity of any gene studied but did increase the amplitude of *REV-ERBα* (Figures 1A to 1F). In contrast, GSPE treatment remarkably shifted the acrophase of both core clock and clock-controlled genes, except *PER2* (Figure 1E). Specifically, GSPE treatment advanced the mRNA acrophase of *RORα* (Figure 1C) by 15 hours (shifting from T23 to T8) while delaying the mRNA acrophase of *BMAL1* (Figure 1A) by 6 hours (shifting from T14 to T20), *CLOCK* (Figure 1B) and *CRY1* (Figure 1F) by 18 hours (shifting from T5 to T23) and *REV-ERBα* (Figure 1D) by 3 hours (shifting from T2 to T5).

The effect of GSPE on the mRNA levels of core clock and clock-controlled genes was also evaluated by ANOVA (Figures 1A to 1F), with only *BMAL1* and *RORα* expression being significantly affected by the treatment. Therefore, *BMAL1* and *RORα* emerged as the genes most sensitive to GSPE.

3.2. GSPE mimics the modulation of BMAL1 expression induced by melatonin in HepG2 cells.

As BMAL1 is essential for maintaining rhythmicity in cells, we next focused on *BMAL1* modulation by GSPE. Melatonin is one of the most important synchronizers of peripheral clocks; therefore, we determined whether GSPE could modulate *BMAL1* expression by simulating melatonin.

Comparing *BMAL1* mRNA levels in cells treated with GSPE with those of the control at each time point independently, it became evident that GSPE increased *BMAL1* expression at 1 and 15 hours after treatment (Figure 2A). Thus, the cells were cultured with melatonin, or GSPE, for 1, 3, 6, 9 or 15 hours. To define the suitable concentration of melatonin, cells were first cultured with three different levels of melatonin for 1 hour (Figure 2B); as 10 μM melatonin induced the highest level of *BMAL1* overexpression, this concentration was selected for the experiment.

Cells cultured with 10 μM melatonin significantly overexpressed *BMAL1* at 1 hour, whereas the control values were observed at 6, 9 and 15 hours (Figure 2C). Remarkably, the overexpression of *BMAL1* induced by melatonin matched completely with that induced by GSPE at 1 hour: both GSPE and melatonin increased the *BMAL1* mRNA abundance by 6 times when compared with the levels of the control cells. However, melatonin did not reproduce the overexpression of *BMAL1* induced by GSPE at 9 and 15 hours: at these times, GSPE was little effective in increasing the *BMAL1* mRNA (1.5 times the control value). Thus, the melatonin curve overlapped with the GSPE curve for the first 6 hours and thereafter with the control curve.
BMAL1 controls the expression of RORα and *REV-ERBα*, two genes that shifted their acrophase by GSPE treatment. Therefore, we also determined whether melatonin reproduced this effect by measuring the mRNA levels of *REV-ERBα* and *RORα* at their shifted acrophase, i.e., 3 and 9 hours, respectively (Figure 2D). The results shown that melatonin did not reproduce the overexpression of *REV-ERBα* and *RORα* induced by GSPE.
Overall, these results indicate that GSPE mimicked the action of melatonin for the first 6 hours of treatment only with regard to *BMAL1* modulation.

3.3. Overexpression of BMAL1 induced by GSPE is not mediated by the MT1 melatonin receptor.

Some of the effects of melatonin are mediated by its interaction with membrane receptors. Thus, the next step was to evaluate the contribution of the MT1 melatonin membrane receptor on the impact of GSPE on *BMAL1* expression. To accomplish this, HepG2 cells were pre-incubated with luzindole, a competitive antagonist of MT1.
Figure 3 shows that luzindole significantly blocked the overexpression of *BMAL1* induced by melatonin at 1 hour. In contrast, luzindole did not block the overexpression of *BMAL1* induced by GSPE at any time studied, though a non-statistically significant slight inhibition was observed at 1 hour. Altogether, these results indicated that the molecular mechanisms by which melatonin and GSPE induce the overexpression of *BMAL1* are different, with MT1 dependence for melatonin and MT1 independence for GSPE.

3.4. GSPE modulates the transcriptional activity of the **RORα** *LBD*

BMAL1 expression is activated by RORα (Teboul, Gréchez-Cassiau, Guillaumond, & Delaunay, 2009). Thus, we evaluated whether GSPE could affect the transcriptional activity of this nuclear receptor in the absence and presence of melatonin because this hormone has been shown to affect the activity of RORα in other cell lines (Dai, Ram, Yuan, Spriggs, & Hill, 2001). The results (Figure 4) show that incubation with GSPE caused an increase in RORα activity in both situations, reaching statistically significant activations of 50% and 100% with respect to the vehicle-treated cells. Despite the quantitatively low increase, it is notable that our system

displays a high basal transcriptional activity, a phenomenon that has been described previously for RORα activity assays (Dai et al., 2001). Therefore, slight effects might be expected for RORα activators when using these systems because the firefly luciferase measurements were high even for the vehicle-treated cells. As a result, the additional luminescence induced by activators was expected to be limited. Moreover, melatonin did not affect the activity of our RORα system or the effects of GSPE.

4. Discussion

While light is the major synchronizer of the central clock in the SCN, many other external cues, such as temperature, social events or meal timing, can entrain circadian rhythms in peripheral tissues (Hirao et al., 2009). Even specific components in foods could also be important signals for peripheral clocks, such as dietary fat (Kohsaka et al., 2007) or phenolic compounds such as resveratrol (Miranda et al., 2013) and PAs (Ribas-Latre et al., 2014). However, the molecular mechanisms by which polyphenols modulate peripheral clocks are poorly understood, though some studies implicate Sirtuine 1 (SIRT1) as the mechanism used by resveratrol to modulate the clock system (Hubbard et al., 2013). Therefore, the aim of this work was to investigate in depth the potential molecular mechanism by which PAs could adjust the clock system in the liver.

As the SCN adjusts peripheral clocks via the autonomic nervous system and hormones (Dibner et al., 2010), this study was carried out *in vitro* to circumvent hormonal and neuronal signals, thereby allowing the characterization of the direct effect of PAs in hepatocytes. HepG2 cells have been proven to be sensitive to GSPE and reproduce the metabolic effects induced by GSPE *in vivo* (Guerrero et al., 2013; Puiggros et al., 2005; Zhang et al., 2009); therefore, this cell line was chosen as the experimental model.

The results of this work clearly demonstrate that GSPE modulates the circadian rhythm of clock genes in HepG2 cells, indicating that PAs are able to directly modulate the clock system in the liver. Interestingly, some studies using the same dose of GSPE (100 mg/L) have already demonstrated in HepG2 cells a reduction in triglyceride secretion (Josep Maria Del Bas et al., 2008) and modulation of the expression of the microRNA miR-122 (Arola-Arnal & Bladé, 2011), two processes that exhibit circadian rhythm.

Among all the genes of the clock system, *BMAL1* emerges as a clear target of PAs in hepatocytes. Interestingly, *bmal1* is the only single gene in the circadian network for which knock out results in arrhythmicity (Hirayama et al., 2007). GSPE increased *BMAL1* expression robustly and very rapidly. Therefore, GSPE, through the overexpression of *BMAL1*, can drive a powerful rhythmicity to the core components of the clock network at the gene expression level, including *CLOCK*, *RORα*, *REV-ERBα* or *CRY1*.

Melatonin is secreted by the pineal gland during the dark phase and regulates circadian rhythms in humans and animals (Kalsbeek et al., 2000). At pharmacological concentrations, melatonin

improves immune function (Macchi & Bruce, 2004), acts as an effective antioxidant (Reiter et al., 2003) and exerts beneficial effects in cancer and some metabolic disorders, such as diabetes and obesity, and therefore in cardiac disorders (Singh & Jadhav, 2014). Clearly, melatonin and PAs exert similar healthy effects. Interestingly, both GSPE and melatonin resulted in *BMAL1* overexpression in the same pattern, with a rapid and sharp increase after 1 hour of treatment, indicating that GSPE mimics, at least with regard to BMAL1, the melatonin-induced modulation of the clock system in hepatocytes.

Melatonin actions in the liver are mediated by its membrane receptor MT1 (Naji, Carrillo-Vico, Guerrero, & Calvo, 2004). Therefore, because GSPE mimicked the action of melatonin on *BMAL1* expression, we studied whether melatonin and PAs share a molecular mechanism. The results showed that MT1 mediates the overexpression of BMAL1 induced by melatonin, whereas this receptor was not implicated in GSPE actions. In addition to the BMAL:CLOCK system, the molecular clock can also be regulated by another feedback loop driven by nuclear receptors RORα and REV-ERBα, which activate and repress, respectively, the expression of *BMAL1* by binding to a retinoic acid-related orphan receptor response element (RORE) in its promoter (Schmutz, Albrecht, & Ripperger, 2012). The possibility of PAs acting on this system was also explored by using classical nuclear receptor activity reporter assays. The observed enhancement of RORα activity by GSPE was consistent with the effects on *BMAL1* gene expression, which was found to be rapidly induced at 1 hour after GSPE treatment and at later time points. We also tested GSPE on a REV-ERBα reporter system using an identical experimental approach as that used for RORα and did not observe differences between the vehicle- and GSPE-treated cells (data not shown). Moreover, it has been suggested that melatonin can modulate RORα activity by interacting with the Ca^{2+}/CaM signaling pathway in human breast cancer cells (Dai et al., 2001). Our results show that melatonin does not affect our reporter system when used in the HepG2 model. Altogether, these results suggest that under our experimental conditions, RORα could be responsible for the direct modulation of *BMAL1* by GSPE but not by melatonin, though indirect actions of melatonin cannot be discounted. Therefore, PAs could modulate the clock system acting by on the clock network itself.

4. Conclusions

In conclusion, PAs entrain the molecular clock system in HepG2 cells. Specifically, *BMAL1* and *RORα* emerge as targets of GSPE in these cells. Based on our results, GSPE could modulate the clock system through the transactivation of *RORα*, resulting in the overexpression of *BMAL1*. Because the liver is the most important metabolic organ due to its role in glucose (Lamia et al., 2008) and lipid (Edwards et al., 1972) homeostasis, this results open a new door for further

research when considering the modulation of the clock system in the liver as a global mechanism by which PAs can exert their beneficial metabolic effects.

Conflict of interest

The authors have declared no conflicts of interest.

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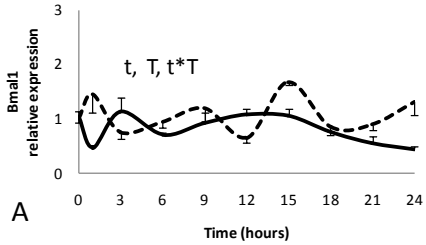
Figure 1. GSPE shifts the mRNA acrophases of core clock and clock-controlled genes and targets *BMAL1*. HepG2 cells were incubated with either vehicle (ethanol ≤0.1% in the medium) or GSPE at 100 mg/L. The cells were lysed at 0, 1, 3, 6, 9, 12, 15, 18, 21 or 24 hours after GSPE addition. Three independent experiments were run in duplicate. The capacity of GSPE to modify the peripheral clock was evaluated by measuring the oscillation of mRNA of the core clock genes (A) *BMAL1* and (B) *CLOCK* as well as the CLOCK:BMAL1-controlled genes (C) *RORA*, (D) *REV-ERBA*, (E) *PER2* and (F) *CRY1*. Each graph shows the mean±s.e. for each data point. T, significant effect of proanthocyanidins; t, significant effect of time; T*t interaction between the two variables by two-factor ANOVA ($p<0.05$). For the cosinor analysis: %R, cosine wave rhythm percentage; M, mesor; AMP, amplitude of cosine wave; Acro, acrophase; CI Acro, confidence interval of the acrophase.

Figure 2. GSPE mimics the modulation of *BMAL1* expression induced by melatonin in HepG2 cells. (A) HepG2 cells were incubated with either vehicle (ethanol ≤0.1% in the medium) or GSPE at 100 mg/L. The cells were lysed at 0, 1, 3, 6, 9, 12, 15, 18, 21 or 24 hours after GSPE addition. The graph shows the *BMAL1* mRNA levels of cells treated with GSPE compared to those of the control at each time point independently. * denotes statistical significance by an individual 1-way ANOVA ($p<0.05$) with respect to its independent vehicle-treated cells. (B) HepG2 cells were incubated with either vehicle (ethanol ≤0.1% in the medium) or melatonin at different doses (0.1, 10 and 100 μM). The cells were lysed at 1 hour after treatment, and the *BMAL1* mRNA levels were determined. Three independent experiments were run in duplicate. The graph shows the mean±s.e. for each data point, and the different letters indicate statically significant differences by an individual 1-way ANOVA test ($p<0.05$). (C) HepG2 cells were incubated with either vehicle (ethanol ≤0.1% in the medium), GSPE at 100 mg/L or melatonin at 10 μM. The cells were lysed at 0, 1, 3, 6, 9 and 15 hours after GSPE addition, and the *BMAL1* mRNA levels were determined. Three independent experiments were run in duplicate. (D) Additionally, *REV-ERBα* and *RORα* mRNA levels were determined at 3 and 9 hours, respectively, after treatment. The graph shows the mean±s.e. for each data point, and the different letters indicate statically significant differences by an individual 1-way ANOVA test ($p<0.05$).

Figure 3. Overexpression of *BMAL1* induced by GSPE is not mediated by the MT1 melatonin receptor. HepG2 cells were incubated with luzindole at 1 μM for 1 hour and then with GSPE at 100 mg/L or melatonin at 10 μM. In parallel, another set of cells was cultured with vehicle (ethanol ≤0.1% in the medium), GSPE or melatonin, but without luzindole, at the same doses and times. The cells were lysed after 1, 6, 9 and 15 hours of the GSPE or melatonin treatment. Three independent experiments were run in duplicate. The graph shows the mean±s.e. for each data point, and the different letters indicate statically significant differences by an individual 1-way ANOVA test ($p<0.05$).

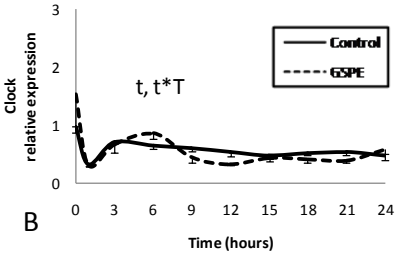
Figure 4. GSPE modulates the activity of an RORα-LBD:Gal4-DBD reporter system. HepG2 cells were seeded in 48-well plates and co-transfected with an RORα-LBD:Gal4-DBD fusion protein expression vector together with a reporter plasmid, with the luciferase gene controlled by 5 Gal4 binding sites. The cells were treated with either vehicle (DMSO), GSPE (100 mg/L), melatonin (10μM) or both for 24 hours. Three independent experiments were run in duplicate. * denotes statistical significance ($p<0.05$) with respect to the vehicle-treated cells by Student's t-test.

Figure 1



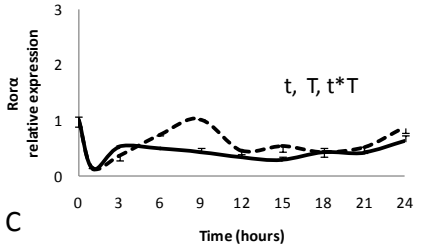
A

Bmal1	R%	M	AMP	Acro (h)	CI acro
Control	34	0.4	0.5	14	11.78 - 16.22
GSPE	47	0.5	0.5	20	17.73 - 22.27



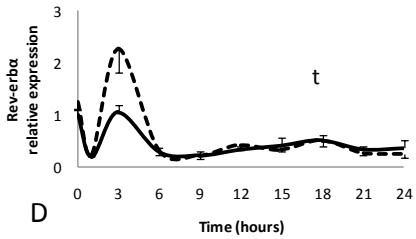
B

Clock	R%	M	AMP	Acro (h)	CI acro
Control	53.4	0.57	0.32	5	2.34 - 7.66
GSPE	50	0.1	0.5	23	20.42 - 25.58



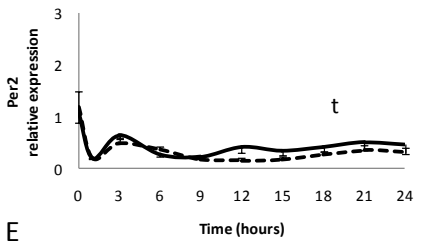
C

Rorα	R%	M	AMP	Acro (h)	CI acro
Control	50	0.1	0.5	23	20.42 - 25.58
GSPE	71.1	0.1	0.5	8	5.96 - 10.04



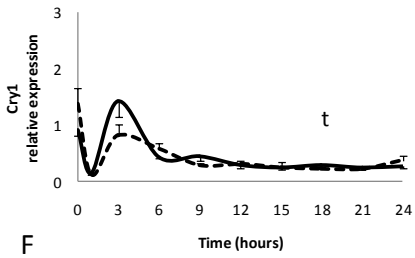
D

Rev-erbα	R%	M	AMP	Acro (h)	CI acro
Control	67.2	0.2	0.5	2	0.13 - 3.87
GSPE	67	0.3	1	5	2.91 - 7.09



E

Per2	R%	M	AMP	Acro (h)	CI acro
Control	50	0.1	0.5	23	20.42 - 25.58
GSPE	50	0.1	0.5	23	20.42 - 25.58



F

Cry1	R%	M	AMP	Acro (h)	CI acro
Control	62.5	0.1	0.5	5	2.79 - 7.21
GSPE	50	0.1	0.5	23	20.42 - 25.58

Figure 2

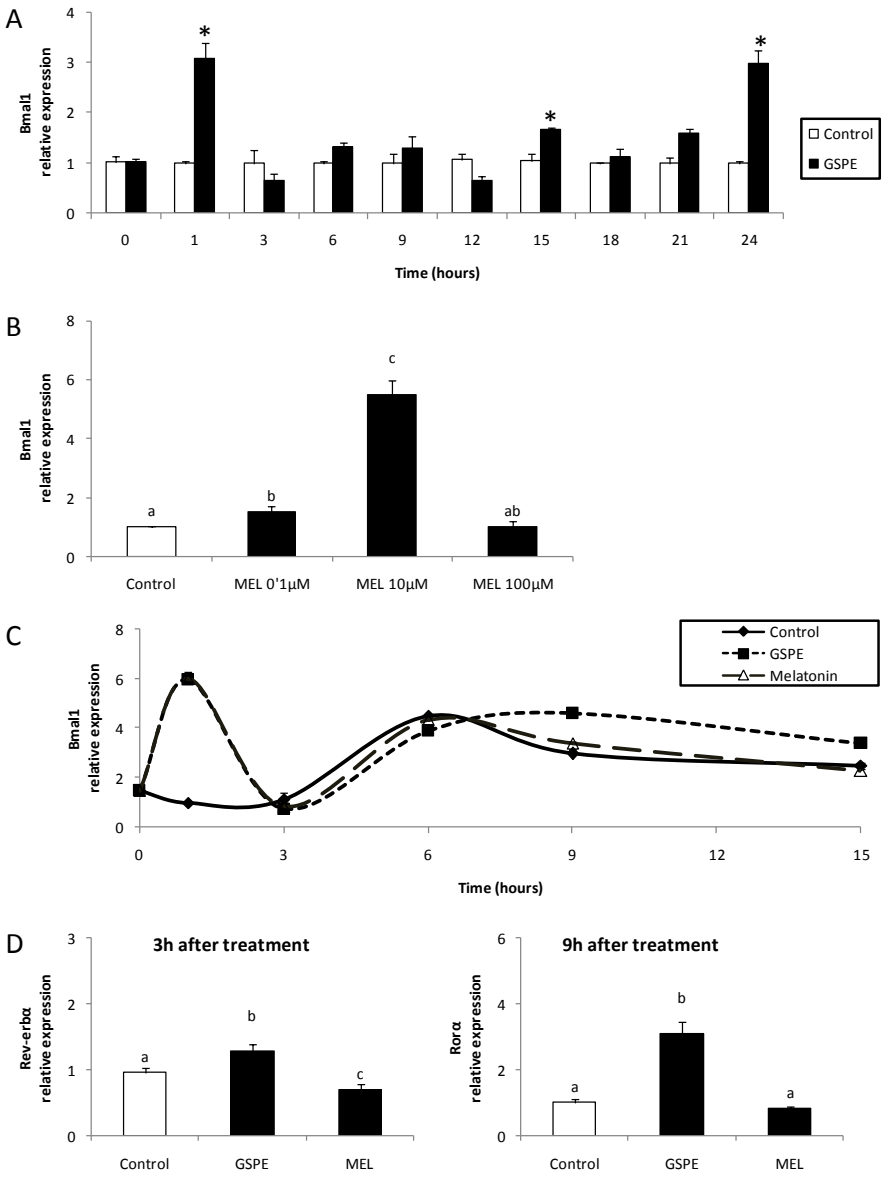

A
Bmal1 relative expression
Control
GSPE
Time (hours)
B
Bmal1 relative expression
Control
MEL 0'1µM
MEL 10µM
MEL 100µM
C
Bmal1 relative expression
Control
GSPE
Melatonin
Time (hours)
D
3h after treatment
Rev-erbα relative expression
9h after treatment
Rorα relative expression
Control
GSPE
MEL

Figure 3

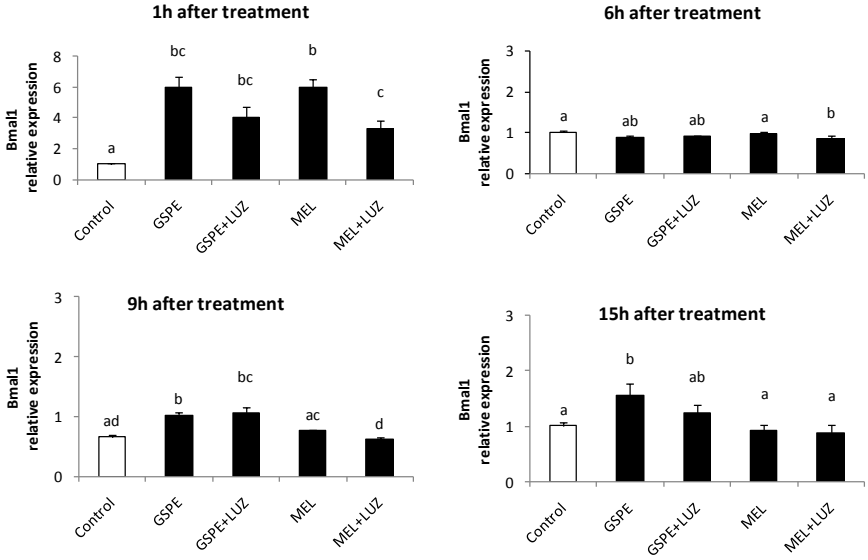
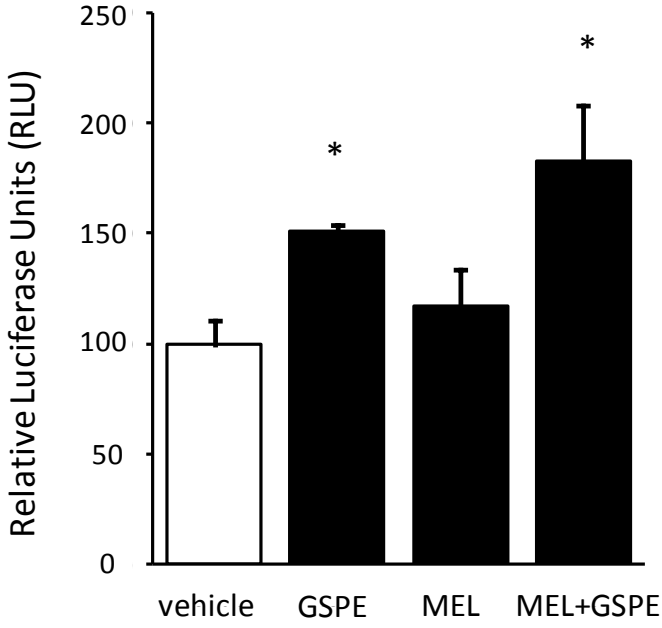


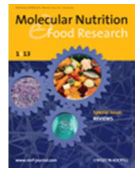
Figure 4.



4. Dietary Proanthocyanidins Entrain Melatonin Circadian Rhythm and Adjust the Central Clock in Rats.

(Manuscript 4, submitted)

Molecular Nutrition and Food Research



Dietary Proanthocyanidins Entrain Melatonin Circadian Rhythm and Adjust the Central Clock in Rats

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Keywords:	BMAL1, flavonoids, melatonin, metabolomics, xenohormesis

Dietary Proanthocyanidins Entrain Melatonin Circadian Rhythm and Adjust the Central Clock in Rats

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Keywords: BMAL1, flavonoids, melatonin, metabolomics, xenohormesis

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Abbreviations: all-trans retinoic acid (ARA), brain and muscle ARNT-like protein-1(BMAL1), circadian locomotor output cycles kaput (CLOCK), clock controlled gens (CCGs), cryptochrome (CRY), grape seed proanthocyanidin extract (GSPE), 3-hydroxy-3-methyl-glutaryl CoA reductase (HMGCOAR) monounsaturated fatty acids (MUFA), nicotinamide phosphoribosyltransferase (NAMPT), period (PER), polyunsaturated fatty acids (PUFA), proanthocyanidins (PAs), nuclear Overhauser effect spectroscopy (NOESY), principal component analysis (PCA), nuclear receptor subfamily 1 group D (Nr1d1, also known as REV-ERBα), retinoic acid-related orphan receptor alpha (RORα), suprachiasmatic nucleus (SCN), tetramethylsilane (TMS), zeitgeber time (ZT).

Abstract

Scope: Circadian rhythms allow organisms to anticipate and adapt to environmental changes; food components can adjust internal rhythms. Proanthocyanidins (PAs), improve cardiovascular risk factors that exhibit circadian oscillations. Therefore, the aim of the current study was to determine whether PAs modulate body rhythms.

Methods and results: Male Wistar rats were orally gavaged with 250 mg grape seed proanthocyanidin extract (GSPE)/kg body weight at zeitgeber time (ZT) 0, light on. Phenotypic biorhythm was evaluated by measuring a 24 hour rhythm of melatonin and plasma metabolites using MNR-metabolomics. GSPE treatment maintained nocturnal melatonin levels until ZT 6 (middle light day) and shifted the rhythm of 28 plasma metabolites. Quantification of expression of clock-core (*Clock and Bmal1*) and clock-controlled (*Per2, Rorα, Rev-erbα and Nampt*) genes in the hypothalamus by RT-PCR showed that this shift of phenotypic rhythm was concomitant with modulation of the central clock. GSPE administration shifted the rhythms of *Bmal1* and clock-controlled genes in the hypothalamus. However, GSPE did not modulate the rhythm of clock genes when administered at ZT 12 (light off). Furthermore, GSPE administered to jet-lagged rats improved night adaptation of *Bmal1* and *Nampt.*

Conclusions: PAs have chronobiological properties, although their activity depends largely on the time of administration.

Introduction

Circadian rhythms are the approximately 24-hour endogenous oscillations of most biological processes that are entrained to the environment by external cues called zeitgebers, thus allowing organisms to anticipate environmental changes and to adapt the time of day and food availability [1]. The most important zeitgeber is light. The hypothalamic suprachiasmatic nucleus (SCN) integrates direct photic input from the retina. No other region in the body is able to accomplish that function; thus, SCN is considered the central and master clock [2]. Nonetheless, many other peripheral and cerebral oscillators (peripheral clocks) that emit rhythms in a self-autonomous manner but are synchronized by outputs from the central clock and by external cues such as fasting-feeding time or temperature cycling are present throughout the body [3]. The SCN contains approximately 10,000 neurons in two anatomic subdivisions: a ventral "core" region and a dorsal "shell" region, which must be well coupled to integrate incoming information from light and other peripheral clocks [4]. In fact, complete SCN lesions abolish circadian rhythmicity [5,6], but implantation of fetal SCN tissue can partially restore them [7], thus demonstrating an essential role of SCN in maintaining circadian rhythms.

The SCN synchronizes peripheral clocks and body rhythms by hormonal signals and neural connections [3]. In this regard, melatonin receives special attention as it is a robust hormonal signal that indicates the time of environmental darkness and is even secreted during the dark phase of the circadian cycle in nocturnal animals, when these animals are also increasing their activity [8]. In fact, the sympathetic innervations of the pineal gland, where melatonin is primarily synthesized, that connect the rhythmic activity of the SCN with the rhythmic release of melatonin is the first output pathway that provides a circadian message to the organism through general circulation at night that is driven by the SCN master clock, therefore, making the SCN an anatomical target for chronotherapeutic studies [9].

At the molecular level, the master clock consists of an autoregulatory transcription–translation feedback loop cycling with a periodicity of approximately 24 h, driving the positive branch of this loop by the transcriptional activators circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-like protein-1(BMAL1), which activate the transcription of the Period (*Per*) and cryptochrome (*Cry*) genes after their own heterodimerization. After reaching a critical concentration, their protein products, PER and CRY, translocate to the nucleus and inhibit the activity of the CLOCK:BMAL1 heterodimer, thus leading to a decrease in *Per* and *Cry* expression. Additionally, the active CLOCK:BMAL1 heterodimer also promotes the transcription of the retinoic acid-related orphan receptor alpha (Rorα) and the nuclear receptor subfamily 1, group D (Nr1d1, also known as Rev-erbα), its activator and repressor, respectively, generating another regulatory loop. Finally, the CLOCK:BMAL1 heterodimer enhances the transcription of metabolic genes or clock controlled gens (CCGs), such as nicotinamide

phosphoribosyltransferase (*Nampt*), which has been implicated in many metabolic and biochemical processes. Here lies the tight relationship among light, SCN and metabolism or physiology [1,10].

Proanthocyanidins (PAs) are a class of polyphenols present in vegetables, fruits, cacao, nuts and some beverages such as red wine or tea; therefore, its presence in the human diet is considerably high [11]. Several studies that used various *in vitro* and animal models demonstrate that PAs have a vast range of health effects, such as improving insulin resistance [12] and decreasing inflammation [13], hypertension [14], oxidative stress [15] and lipid abnormalities [16], thus reducing metabolic syndrome [17] and cardiovascular diseases [18]. Interestingly, all these processes exhibit circadian rhythms [19] and humans with disrupted circadian rhythms have increased risk of developing symptoms of metabolic syndrome [20,21].

Although light is considered the master zeitgeber, the feeding-fasting cycle [22] as well as dietary components, such as dietary fat [23] and resveratrol [24], also work as external cues that synchronize biological rhythms. Therefore, the aim of the current study was to determine whether PAs can entrain internal body rhythms and modulate the central clock. Biological rhythms have been evaluated by analyzing plasma melatonin and metabolite oscillation during a 24-hour cycle. The capacity of PAs to adjust the SCN has been determined by measuring the expression rhythm of clock-core and clock-controlled genes in the hypothalamus while administering PAs diurnally or at night or even in concert with circadian disruption using jet-lagged rats.

Materials and methods

Grape seed proanthocyanidin extract composition

Grape seed proanthocyanidin extract (GSPE) was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). The composition of GSPE is as follows [25]: catechin (58 μmol/g), epicatechin (52 μmol/g), epigallocatechin (5.50 μmol/g), epicatechingallate (89 μmol/g), epigallocatechingallate (1.40 μmol/g), dimeric procyanidins (250 μmol/g), trimeric procyanidins (1568 μmol/g), tetrameric procyanidins (8.8 μmol/g), pentameric procyanidins (0.73 μmol/g) and hexameric procyanidins (0.38 μmol/g).

Animals

All procedures involving the care and use of animals were reviewed and approved by The Animal Ethics Committee from the Universitat Rovira i Virgili (Permit number 4249 by Generalitat de Catalunya).

Eighty-four eight-week-old male Wistar rats (Crl: WI (Han)) were purchased from Charles River (Barcelona, Spain) and fed a standard chow diet (STD, Panlab 04, Barcelona, Spain) and

tap water ad libitum. Rats were divided in three groups, according to the zeitgeber time (ZT) when GSPE was administered.

Administration of GSPE at ZT0: Forty rats were singly caged in animal quarters at 22ºC with a 12-h light/dark cycle (light from 9:00 to 21:00 P.M.). After three weeks of adaptation, rats were orally gavaged with tap water (control group) or 250 mg of GSPE /kg body weight dissolved in tap water at ZT0 (9:00 am, light on). Rats were sacrificed by decapitation at ZT0, ZT0.5, ZT1, ZT3, ZT6, ZT12 and ZT24 (n=3 for control and n=3 for GSPE treated groups).

Administration of GSPE at ZT12: Twenty-two rats were singly caged in animal quarters at 22ºC with a 12-h light/dark cycle (light from 21:00 pm to 9:00 am). After three weeks of adaptation, the rats were orally gavaged with tap water (control group) or 250 mg of GSPE /kg body weight dissolved in tap water at ZT12 (9:0 am, light off). Rats were sacrificed by decapitation at ZT12, ZT13, ZT15, ZT18 (n=3 for control and n=3 for GSPE treated groups).

Administration of GSPE to Jet-lagged rats: Twenty-two rats were singly caged in animal quarters at 22ºC with a 12-h light/dark cycle (light from 15:00 pm to 03:00 am). After three weeks of adaptation, rats were orally gavaged with tap water (control group) or 250 mg of GSPE /kg body weight dissolved in tap water at ZT6 (9:00 am, middle of light day) and immediately moved to a dusk room (ZT12), thus rats received a jet lag of 6 hours. Rats were sacrificed by decapitation at ZT12, ZT13, ZT15, ZT18 (n=3 for control and n=3 for GSPE treated groups).

For the three experiments, blood was collected using heparin (Deltalab, Barcelona, Spain) as an anticoagulant and plasma was obtained by centrifugation. Plasma was frozen at -80 ºC until melatonin and metabolomic analysis. The hypothalamus was excised and frozen immediately in liquid nitrogen and stored at -80ºC until RNA extraction.

RNA extraction and cDNA synthesis

Total RNA from hypothalamus was extracted using TRIzol reagent and an RNeasy Lipid Tissue Mini Kit (Qiagen, 74804, Barcelona, Spain) according to both manufacturer protocols. RNA was quantified using spectrophotometry (Nanodrop 1000 Spectrophotometer, Thermo Scientific) at λ=260 nm and tested for purity (by A260/280 ratio) and integrity (by denaturing gel electrophoresis). Complementary DNA was generated using the High-Capacity complementary DNA Reverse Transcription Kit from Applied Biosystems (4368814, Madrid, Spain)

mRNA quantification by real-time qRT-PCR

A total of 10 ng cDNA was subjected to quantitative RT-PCR amplification using SYBR Green PCR Master Mix from Bio-Rad (172-5200, Barcelona, Spain). The forward and reverse primers

of the analyzed genes are shown in Table 1. Reactions were run on a quantitative real-time PCR system (CFX96 touch of Bio-Rad, Barcelona, Spain); the thermal profile settings were 50ºC for 2 min and 95ºC for 2 min and then 40 cycles at 95ºC for 15 s and 60ºC for 2 min. Finally, statistical data were converted and normalized to the linear form using the 2^{-}CT ($\Delta\Delta C_T$) calculation [26]. The relative expression of the clock genes was normalized to cyclophilin mRNA levels.

Melatonin measurement

Melatonin plasma levels were assayed using an ELISA method following the manufacturer's instructions (RE54021 IBL international, Hamburg, Germany).

MRN analysis and sample preparation

MRN analysis and sample preparation was performed according to the method described by Vinaixa et al [27] for untargeted metabolomics. From 400 µL of the plasma sample, 200 µL were placed in a tube with 1800 µL of a methanol:water mixture (8:1) for an aqueous extraction (methanol from Panreac Química S.A. Barcelona, Spain). The other 200 µL were placed in a tube with 3 mL of a chloroform:methanol mixture (2:1) for lipid extraction (chloroform from Panreac Química S.A. Barcelona, Spain). The mixtures were vortexed vigorously and centrifuged for 10 min at 4500 rpm (4ºC). For the aqueous extraction, the pellet was washed twice with additional methanol:water (8:1), vortexed and centrifuged, combining the liquid phases. Finally, the upper aqueous phases were partially dried in a nitrogen stream to remove methanol and quickly frozen. The lipid extraction was completely dried in a nitrogen stream. For NMR measurements, the hydrophilic extracts were reconstituted in 600 µl of D_2O containing 0.67 mM trimethylsilyl propionic acid (TSP). The lipophilic extracts were subsequently extracted in 700 µl of $CDCl_3/CD_3OD$ (2:1) containing 1.18 mM tetramethylsilane (TMS). Samples were then vortexed, homogenized for 20 min, and centrifuged for 15 min at 6000 × g at 4 °C. Finally, redissolved samples were placed into 5 mm NMR tubes. ^{1}H NMR spectra were recorded at 300 K on an Avance III 600 spectrometer (Bruker, Germany) operating at a proton frequency of 600.20 MHz using a 5 mm CPTCI triple resonance (^{1}H, ^{13}C, ^{31}P) gradient cryoprobe. One-dimensional ^{1}H pulse experiments were carried out using the nuclear Overhauser effect spectroscopy (NOESY) presaturation sequence (RD–90°–t1–90°–tm–90° ACQ) to suppress the residual water peak, and the mixing time was set at 100 ms. Solvent presaturation with irradiation power of 75 Hz was applied during recycling delay (RD = 5 s) and mixing time. The 90° pulse length was calibrated for each sample and varied from 6.57 to 6.99 ms. The spectral width was 12 kHz (20 ppm), and a total of 256 transients were collected into 64 k data points for each ^{1}H spectrum. The exponential line broadening applied before Fourier

transformation was of 0.3 Hz. The frequency domain spectra were phased and baseline-corrected using TopSpin software (version 2.1, Bruker).

The acquired ^{1}H NMR spectra were phased, baseline-corrected, and referenced to the chemical shift of residual A signal at B ppm. Pure compounds from the metabolic profiling AMIX spectra database (Bruker), HMDB, and Chenomx databases were used as references for metabolite identification. In addition, we assigned metabolites by ^{1}H–^{1}H homonuclear correlation (COSY and TOCSY) and ^{1}H–^{13}C heteronuclear (HSQC) 2D NMR experiments and by correlation with pure compounds run in-house. After baseline correction, specific ^{1}H NMR regions identified in the spectra were integrated using the AMIX 3.9 software package.

Data (pre-) processing, data analysis, and statistical calculations were performed in R.

Data and statistical analysis

Metabolite and melatonin concentrations and clock genes expression were fitted using single cosinor analysis because the data formed a period curve, to determine whether significant circadian rhythms were present (Acro.exe, version 3.5; designed by Dr. Refinetti [28]). Cosinor analysis provides information about the rhythm through peak-to-trough amplitude, time of the peak of the rhythm or acrophase with a confidence interval and the middle value of the cosine wave or MESOR. The regression fitting also produces an R-squared statistic, which is then used to compute the percentage of variance or rhythmicity, in an individual time-series data that is accounted by the fitted 24-h curve.

Results are presented as the mean with the associated standard error (SE). The data were also analyzed using a two-way ANOVA to determine the significant difference using SPSS statistical software (version17.0 for Windows; SPSS, Inc.). P values<0.05 were considered statistically significant.

Multivariate analysis was performed using R software for Windows [29]. The full set of NMR metabolomics data were subjected to principal component analysis (PCA) using the *pca* function of the mixOmics package [30] with three principal components using the centering and scaling options. The rest of the options were left at default. Visual 3D representations were constructed with the Rcmdr R package [31].

Results

Acute administration of GSPE at Zeitgeber Time 0 entrains the internal body rhythm.

The capacity of PAs to entrain circadian rhythms was evaluated by measuring the circadian oscillation of several metabolites and melatonin in the plasma of the rats maintained on a 12-h light/12-hdark cycle and treated with an acute dose of 250 mg GSPE /kg body weight at zeitgeber time (ZT) 0, when the light was turned on.

Melatonin levels in plasma were measured at ZT0, ZT0.5, ZT1, ZT3, ZT6, ZT12 and ZT24 (Fig. 1). ANOVA analysis shows that GSPE treatment significantly altered the melatonin rhythm in plasma, resulting in high levels of melatonin until ZT3 and ZT6, when control animals had the lowest levels. However, despite this significant difference, GSPE did not modify melatonin's acrophase, which was maintained at ZT20 like in the control animals.

Plasma metabolomics, carried out using RMN, was performed at ZT0, ZT6, ZT12 and ZT24. Forty-nine metabolites were identified, including amino acids, glucose, lactate, 3-hydroxybutyrate, glycerol, triglycerides, cholesterol and some phospholipids and fatty acids (Tables 2, 3 and 4). To determine whether GSPE significantly altered the circadian metabolite pattern, we applied the ANOVA and cosinor analysis to each metabolite's circadian rhythm (Tables 2, 3 and 4). GSPE treatment significantly affected the circadian oscillations of tyrosine, serine, glycerolphosphocholine, oleic acid and monounsaturated fatty acids (MUFA). Moreover, the circadian rhythms of glucose, pyruvate, citrate, valine, leucine, lysine, tyrosine, glutamine, isoleucine, histidine, serine, cholesterol, esterified cholesterol, phosphatidylcholine, linoleic acid, MUFA, polyunsaturated fatty acids (PUFA) and *all-trans* retinoic acid (ARA) showed a significant interaction between time and GSPE. Additionally, some metabolites showed a significant modification of their acrophase time. Specifically, GSPE treatment shifted the acrophases of glucose, pyruvate, lactate, 3-hydroxybutyrate, glutamine, isoleucine and histidine from ZT0 (ZT11 for glucose) to ZT23 and the acrophase of alanine, serine and threonine from ZT11 to ZT0. GSPE treatment also shifted the acrophases of some lipid metabolites, such as cholesterol, phosphatidylcholines, MUFAs and PUFAs.

We subjected the full set of NMR data from samples at ZT0, ZT6, ZT12 and ZT24 to PCA analysis (Fig. 2). Because the value at each ZT for GSPE- or vehicle-treated animals is derived from material pooled from three animals and clustering techniques are not recommended in such small groups, we did not use this multivariate analysis for classification. Instead, we assessed the separation between the projection of the data for each animal (scores) as a measure of whole metabolic variability through the 24-hour period. Remarkably, the scores for GSPE-treated animals were clearly condensed when compared with the control animals (Fig. 2A). The differences were not due to increased variability within a given ZT but to increased variability between ZTs, as observed in fig. 2B when the scores for ZT6 and ZT12 animals, as representative groups, are plotted for both GSPE and control conditions.

Taken together, these results indicate that GSPE treatment profoundly alters melatonin and metabolite oscillation in plasma and that GSPE entrains internal body circadian rhythms.

Acute administration of GSPE at ZT0 shifted the mRNA acrophases of Bmal1 and Bmal1-controlled clock genes in the hypothalamus.

Because the central clock is the master regulator of body circadian rhythms, we further studied whether this modulation of phenotypic rhythm by GSPE was associated with adjustment of the central clock. The capacity of GSPE to modify the central clock was evaluated by measuring the oscillations of *Clock* and *Bmal1* (clock core genes), *Per2* (component of the negative loop of the circadian clock), *Rorα* and *Rev-erbα* (nuclear receptors, the expression of which is regulated by CLOCK:BMAL1 and that act as activator or repressor of Bmal1 gene expression, respectively), *Nampt* (metabolic gene that its expression is regulated directly by CLOCK:BMAL1) and *HmgCoAR* (metabolic gene that has circadian rhythm expression but not directly controlled by CLOCK:BMAL1) mRNA in the hypothalamus. As was the case with metabolites, we also applied the ANOVA and the cosinor analysis to each mRNA curve.

Of the two clock core genes, only *Bmal1* (Fig. 3A) responded significantly to GSPE whereas *Clock* (Fig. 3B) remained similar to that of the control animals. ANOVA showed a significant effect of GSPE treatment on the 24-hour rhythmicity of *Bmal1* mRNA. Moreover, cosinor analysis showed that GSPE administered at ZT0 accelerated the onset of *Bmal1* acrophase by three hours, from ZT8 to ZT5.

The mRNAs of the *Bmal1* targeted genes, *Rorα, Rev-erbα, Per2, Nampt* (Fig. 3C-F, respectively) shared the same profile in the control animals, peaking at ZT5. Remarkably, GSPE treatment delayed the mRNA acrophase of all these genes. In particular, the mRNA acrophase of *Rev-erbα, Per2* and *Nampt* was delayed by 15 hours, shifting it to ZT20, whereas the mRNA acrophase of *Rorα* was delayed by 3 hours, shifting it to ZT8. Despite GSPE modulating the acrophase of all these genes, only the circadian rhythm of *Nampt* was significantly altered by GSPE. On the contrary, the mRNA rhythm of *HmgCoAR* (Figure 3G), a gene not directly controlled by *Bmal1,* was not affected by the administration of GSPE, indicating a different pattern of those of *Bmal1*-controlled genes.

Taking into account the importance of these circadian waves over 24 h, the percentage of rhythm was also computed (Fig. 3). In general, small differences were observed between the control and GSPE rhythms, but only *Rev-erbα* decreased its rhythmicity after GSPE treatment (from 76% to 47% approx.).

Altogether, these results indicate that GSPE can adjust the central clock and that *Bmal1*and *Nampt* were the most significant clock genes involved in the entrainment of internal body rhythm by GSPE.

Acute administration of GSPE at ZT12 affected the expression of only Rev-erbα

Next, we studied whether PAs could modulate the central clock independently of the time during the circadian cycle they are administered. Thus, GSPE was administered at ZT12 (light turned off) and the expression of clock and clock-controlled genes was determined at four time points, ZT12, ZT13, ZT15 and ZT18. To achieve a better visualization of the changes induced by GSPE administered at ZT12, we drew the figures with a 24-hour curve for the control group (Fig. 4) by assembling the expression values of the control group from this and the former experiments. Because of the short period studied (6 hours) in this experiment, we analyzed the effects of GSPE by ANOVA and did not apply the cosinor analysis.

GSPE, administered at ZT12, induced slight effects on the mRNA levels of the clock core genes *Bmal1* (Fig. 4A) and *Clock* (Fig. 4B) as well as the mRNA levels of the *Bmal1*-controlled clock genes *Rorα* (Fig. 4C) and *Per2* (Fig. 4E). However, GSPE significantly affected the mRNA levels of *Rev-erbα* (Fig. 4D), and the expression of *Nampt* and *Rev-erbα* showed a significant interaction between time and GSPE. GSPE treatment at ZT12 did not affect the expression of *HmgCoAR* (Fig. 4G)

Plasma metabolites were analyzed at ZT12 and ZT15 (Table 5). No significant differences were observed in any metabolite after GSPE administration at ZT12, which is in accordance with the few effects of GSPE on the central clock when it was administered at ZT12.

Despite the fact that we did not analyze the effects of GSPE on the 24-h cycle in this experiment, these results indicate that GSPE entrained the circadian rhythms more strongly when it was administered at ZT0 (starting of day) than at ZT12 (starting of night).

Acute administration of GSPE modulated the central clock in jet-lagged rats

Finally, the capacity of GSPE to modulate the central clock was evaluated in a simulation of circadian disruption using rats subjected to a 6 hours jet lag. GSPE was administered to rats at ZT6 (middle of light period), and the rats were then moved to ZT12 (light off). The capacity of GSPE to modulate core and related clock genes, was evaluated at ZT12, ZT13, ZT15 and ZT18. Because of the short period studied (6 hours) in this experiment, we analyze the effects of GSPE using ANOVA and did not apply cosinor analysis.

In the control animals, six hours of jet lag induced a clear shift of the mRNA rhythmicity of all the genes studied (Fig. 5) when they were compared with the 24-hour control rhythms (built by assembling the expression values of the control groups as in the former experiment) at ZT12, ZT13, ZT15 and ZT18.

GSPE, administered at the beginning of jet lag, did not modulate *clock* (Fig. 5B), *Rev-erbα* (Fig. 5D) or *HmgCoAR* (Fig. 5G), whereas the expression rhythms of *Bmal1* (Fig. 5A), *Rorα* (Fig. 5C), *Per2* (Fig. 5E) and *Nampt* (Fig. 5F) were altered significantly when compared with the jet-

lag control group. Remarkably, *Bmal, Rora* and *Nampt* were again the most sensitive genes to GSPE, similar to when GSPE was administered at ZT0. Both when GSPE was administered at ZT0 and in the jet-lag situation, the mRNA levels of *Bmal1* and *Nampt* decreased significantly after GSPE treatment. This decrease of *Bmal1* and *Nampt* mRNA levels induced by GSPE in the jet-lag situation adjusted the expression of both genes to the 24-hour control rhythm at ZT15. Therefore, GSPE treatment partially counteracted the jet-lag disruption of *Bmal1* and *Nampt*.

Discussion

Light and meal timing entrain circadian rhythms, but specific components in foods could also be important signals. For instance, resveratrol adjusts the circadian rhythms of locomotor activity and body temperature in animals [32,33] and alters clock gene expression in cultured fibroblast [24] and rat organs [34]. Therefore, the aim of this work was to determine the capacity of an acute dose of GSPE to entrain biological rhythms and to adjust the central clock.

Melatonin is a robust indicator of the internal body time [9]. Additionally, plasma metabolites exhibit circadian oscillations, and blood metabolomics has been proposed as a method to analyze internal body time [35]. Remarkably, GSPE administered at ZT0 strongly increased plasma melatonin levels in the middle of the light period, maintaining similar levels as at dusk, and shifted the acrophase of many important plasma metabolites, such as amino acids, glucose and cholesterol among others. Moreover, GSPE treatment masked changes in metabolite concentration that were very evident in the control animals at the ZT studied. Therefore, acute GSPE treatment at ZT0 actually affected biological rhythms in the rats.

To study the relationship between PAs and the molecular clock, we focused on the hypothalamus, where the SCN integrates direct input from light and information from other oscillators present throughout the body, thus acting as a master synchronizer [3]. The SCN contains approximately 10,000 neurons in two anatomic subdivisions, which must be well coupled as demonstrated by the fact that the period of an intact SCN is more precise than the period of independently oscillating SCN neurons [4]. Therefore, assaying the whole hypothalamus allows the study of the intact SCN despite increased noise.

Three different experimental approaches were used to determine whether PAs can modulate the central clock: GSPE treatment at ZT0, at the beginning of the light phase; at ZT12, at the beginning of the dusk phase; and at ZT6 with a jet lag of 6 hours. The data clearly show that GSPE adjusted the circadian rhythms of clock-core and clock-controlled genes in the hypothalamus when was administered at ZT0 or in jet-lagged rats, whereas the administration at ZT12 caused minimal effects. Thus, GSPE is primarily active when administered during the day

period, indicating that the effectiveness of PAs to adjust the central clock depends largely on the time of administration. In fact, SCN cells have been established to be extensively coupled during the day, when the cells exhibit synchronous neural activity, but to be minimally coupled during the night, when the cells are electrically silent [36]. So, this discrepant functionality of SCN cells between day and night could determine the effectiveness of PAs. Moreover, exposure to light has been observed to cause shifts in the phase of the SCN clock primarily during the subjective night in nocturnal rodents, whereas non-photic cues trigger these shifts mainly during the subjective day [3]. Therefore, GSPE could act as a non-photic cue, triggering the central clock system during the light period. Nonetheless, no studies have determined whether PAs reach the hypothalamus, although some studies in Wistar rats have elucidated the distribution of flavonols and their metabolites to different tissues, e.g., the brain [37,38] and ruled out the capacity of flavonols to cross the blood-brain barrier. Even, an experiment with a rat model of Parkinson's disease with chronic oral tangeretin administration (10 mg/kg/day for 28 days) confirmed a significant level of this citrus flavonoid in the hypothalamus, even at higher concentrations than in liver and plasma [39]. Therefore, PAs could act as direct non-photic cues in the SCN. Alternatively, PAs could adjust the central clock by acting at intestinal levels through the brain-gut axis, which send gut cues to the brain by neuronal and hormonal mechanisms [40]. Thus, more studies are needed to define the molecular mechanism by which PAs adjust the central clock.

Among the observed effects of GSPE treatment at ZT0 on the circadian rhythm expression of clock-core and clock-controlled genes in the hypothalamus, special attention should be paid to *Bmal1*, which showed a significant advanced shift in its phase. Because *clock* was not affected by GSPE, which is consistent with its constitutive expression in the SCN [41], such a dramatic shift in *Bmal1* rhythm could explain the delayed phase of the clock-controlled gens *Rorα, Rev-erbα, Per2* and *Nampt*, according to the defined role of *Bmal1* as master regulator of the molecular clock system [42]. Interestingly, *Bmal1* was also targeted by GSPE in jet-lagged rats. In this situation, GSPE counteracted the jet-lag effect and allowed *Bmal1* to recover its normal rhythm.

SCN synchronizes circadian rhythm of the whole body by hormonal signals and neural connections [3]. In particular, nocturnal secretion of melatonin by the pineal gland is directly controlled by the SCN [43]. Remarkably, GSPE administered at ZT0 triggered high plasma melatonin levels at middle light day. GSPE extract does not contain melatonin; thus the adjustment of the melatonin rhythm could be due to the adjustments induced by GSPE in the SCN. However, the SCN expresses melatonin receptors [44]. Thus, the inverse mechanism, including an initial effect of GSPE on melatonin secretion followed by melatonin action on SCN, cannot be ruled out.

Because GSPE maintained night levels of melatonin during the light period, entrained clock genes when it was administered at ZT0 and not a ZT12, and partially counteracted the jet-lag disruption, these data strongly suggest that PAs could be a non-photic cue of night. These findings agree with the xenohormesis hypothesis, which proposes that heterotrophs are able to sense chemical cues synthesized by plants, such as polyphenols, in response to stress [45]. In fact, circadian rhythms allow anticipation of environmental changes and adaptation to the time of day and food availability. Thus, PAs can advise animals about environmental conditions by entraining biological rhythms.

In conclusion, GSPE treatment clearly entrained biological rhythms by maintaining high levels of melatonin during the light period. Moreover, GSPE adjusted the circadian rhythms of clock genes in the hypothalamus when it was administered during the light period but not at dusk, indicating that the ability of PAs to adjust the central clock depends largely on the time of administration. Specifically, *Bmal1* emerges as a target of GSPE. Therefore, PAs have chronobiologic properties. However, as rats are nocturnal animals, assessing the ZT when PA-rich foods can entrain circadian rhythms in humans will be necessary.

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Conflict of Interest

The authors have declared no conflicts of interest.

Author Contribution

Aleix Ribas-Latre, Anna Arola-Arnal, M Josepa Salvadó, Lluís Arola and Cinta Bladé designed the experiments. Aleix Ribas-Latre, Laura Baselga-Escudero and Ester Casanova performed the experimental procedures and the molecular analysis. Josep M. Del Bas performed the PCA analysis, and Aleix Ribas-Latre performed the statistical and cosinor analyses. All the authors participated in the interpretation and discussion of data. Aleix Ribas-Latre, Josep M. Del Bas, Lluís Arola and Cinta Bladé wrote the manuscript. All the authors contributed to and approved the final manuscript.

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Figure legends

Fig. 1

An oral dose of a grape seed proanthocyanidin extract (GSPE) administered at zeitgeber time 0 entrains melatonin circadian rhythm in rats. Rats were orally gavaged with tap water (control group) or 250 mg of GSPE /kg body weight dissolved in tap water at ZT0 (9:00 am, light on), and plasma melatonin was measured at ZT0, ZT0.5, ZT1, ZT3, ZT6, ZT12 and ZT24. The graph shows the mean±s.e. for each data point (n=3). T, significant effect of proanthocyanidins; t, significant effect of zeitgeber time; T*t interaction between the two variables by two factors ANOVA. For the cosinor analysis: %R, cosine wave rhythm percentage; M, mesor; AMP, amplitude of cosine wave; Acro, acrophase; CI Acro, confidence interval of acrophase.

Fig. 2

An oral dose of a grape seed proanthocyanidin extract (GSPE) administered at zeitgeber time 0 entrains metabolite circadian rhythms in rats. Rats were orally gavaged with tap water (control group) or 250 mg of GSPE /kg body weight dissolved in tap water at ZT0 (9:00 am, light on) and plasma metabolites were measured by NMR metabolomics at ZT0, ZT6, ZT12 and ZT24. (A) The values of abundance of all metabolites were subjected to a principal component analysis. Scores for each animal are represented in the three dimensions defined by PC1, PC2 and PC3 (explaining a 72%, 8% and 6% of the variance respectively) as blue dots for the control group and green dots for the GSPE-treated group. Ellipsoids containing the 50% of the scores are represented for each group. (B) Heat map depicting temporal patterns of changing metabolites. The fold-change respect the ZT0 of each metabolite (rows) is represented as explained in the color key insert for each ZT (columns). Metabolites patterns are clustered by euclidean distance as depicted in the dendogram.

Fig. 3

An oral dose of grape seed proanthocyanidin extract (GSPE) administered at zeitgeber time 0 adjusts the rhythm expression of clock-core and clock-controlled genes in the hypothalamus. Rats were orally gavaged with tap water (control group) or 250 mg of GSPE /kg body weight dissolved in tap water at ZT0 (light on), and mRNA levels were measured at ZT0, ZT0.5, ZT1, ZT3, ZT6, ZT12 and ZT24. The capacity of GSPE to modify the central clock was evaluated by measuring the oscillation of mRNA of the clock core genes (A) *Bmal1* and (B) *Clock* as well as the CLOCK:BMAL1 controlled genes (C) *Per2*, (D) *Rorα*, (D) *Rev-erbα* and (E) *Nampt*. (G) The expression of *HmgCoAR*, a gene that has circadian rhythm expression but that is not

directly controlled by CLOCK:BMAL, has also been evaluated. Each graph shows the mean±s.e. for each data point (n=3). T, significant effect of proanthocyanidins; t, significant effect of zeitgeber time; T*t interaction between the two variables by two factors ANOVA. For the cosinor analysis: %R, cosine wave rhythm percentage; M, mesor; AMP, amplitude of cosine wave; Acro, acrophase; CI Acro, confidence interval of acrophase.

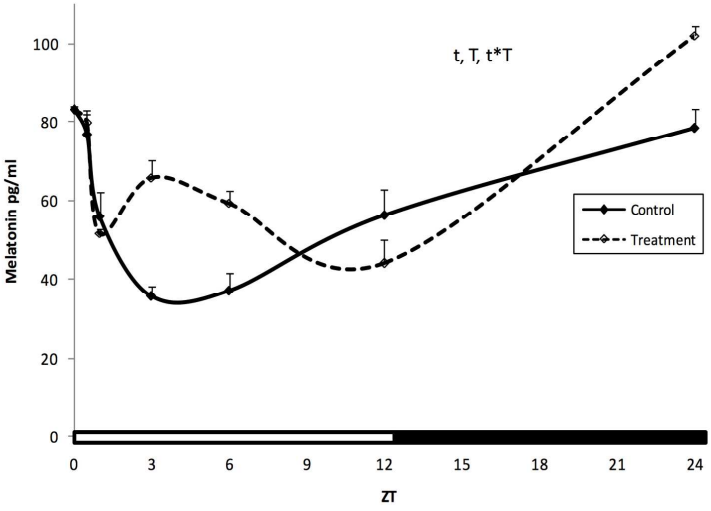
Fig. 4

An oral dose of a grape seed proanthocyanidin extract (GSPE) administered at zeitgeber time 12 slightly affects the rhythm of clock-core and clock-controlled gene expression in the hypothalamus. Rats were orally gavaged with tap water (control group) or 250 mg of GSPE /kg body weight dissolved in tap water at ZT12 (light off), and mRNA levels were measured at ZT12, ZT13, ZT15 and ZT18. The capacity of GSPE to modify the central clock was evaluated by measuring the oscillation of mRNA of the clock core genes (A) *Bmal1* and (B) *Clock* as well as the CLOCK:BMAL1 controlled genes (C) *Per2*, (D) *Rorα*, (D) *Rev-erbα* and (E) *Nampt.* (G) The expression of *HmgCoAR*, a gene which has circadian rhythm expression but that is not directly controlled by CLOCK:BMAL, has also been evaluated. Each graph shows the mean±s.e. for each data point (n=3). For the control group, a 24-hour curve was constructed by assembling the expression values of the control group from this experiment and those of Fig. 3. T, significant effect of proanthocyanidins; t, significant effect of Zeitgeber Time; T*t interaction between the two variables by two factors ANOVA.

Fig. 5

An oral dose of a grape seed proanthocyanidin extract (GSPE) administered to jet-lagged rats entrains the rhythm expression of clock-core and clock-controlled genes in the hypothalamus to the new zeitgeber time. Rats were orally gavaged with tap water (control group) or 250 mg GSPE /kg body weight dissolved in tap water at ZT6 (middle of light period) and moved to ZT12 (light turn off). mRNA levels were measured at ZT12, ZT13, ZT15 and ZT18. The capacity of GSPE to modify the central clock was evaluated by measuring the oscillation of mRNA of the clock core genes (A) *Bmal1* and (B) *Clock* as well as the CLOCK:BMAL1 controlled genes (C) *Per2*, (D) *Rorα*, (D) *Rev-erbα* and (E) *Nampt.* (G) The expression of *HmgCoAR*, a gene which has circadian rhythm expression but that is not directly controlled by CLOCK:BMAL, was also evaluated. Each graph shows

the mean±s.e. for each data point (n=3). For the non-jet-lagged control group, a 24-hour curve was constructed by assembling the expression values of the control group from Fig. 3 and 4. T, significant effect of proanthocyanidins; t, significant effect of zeitgeber time; T*t interaction between the two variables by two factors ANOVA.



	%R	M	AMP	Acro	CI Acro
Control	90.8	59.85	24	ZT20	18.94 - 21.06
GSPE	88.6	69	29	ZT20	18.64 - 29.36

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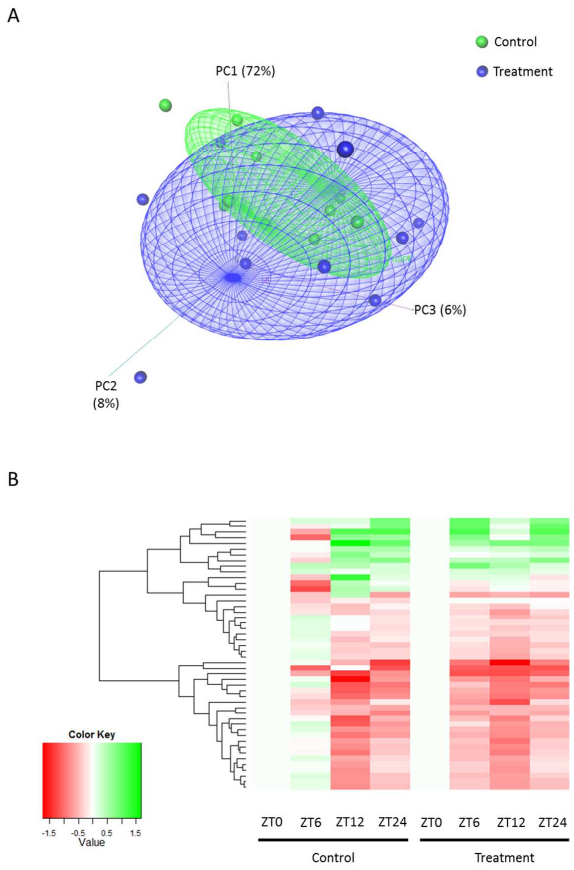

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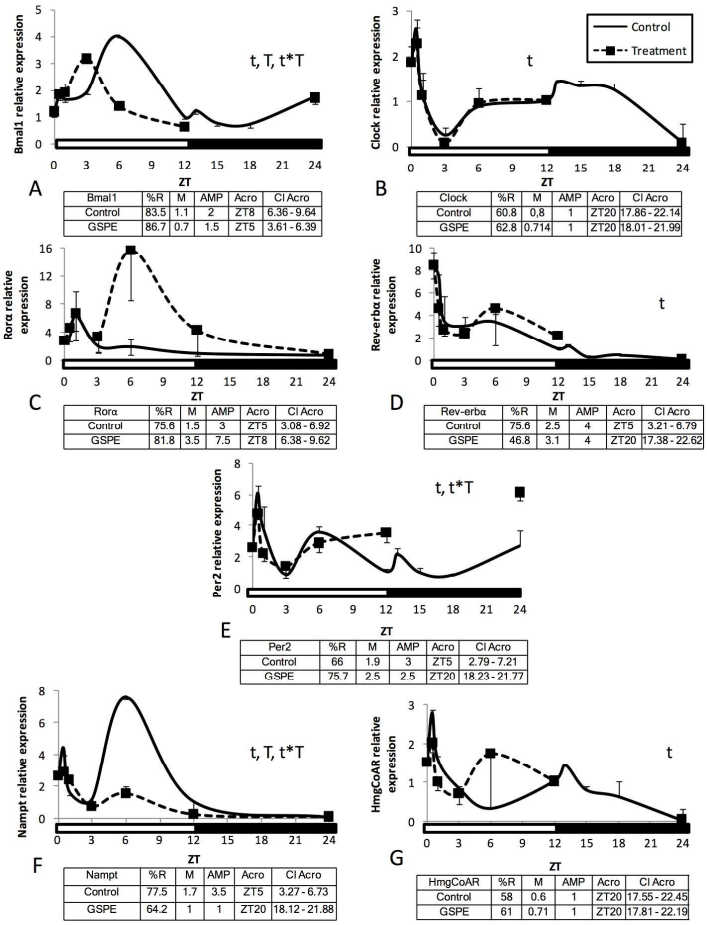


Bmal1	%R	M	AMP	Acro	CI Acro
Control	83.5	1.1	2	ZT8	6.36 - 9.64
GSPE	86.7	0.7	1.5	ZT5	3.61 - 6.39

Clock	%R	M	AMP	Acro	CI Acro
Control	60.8	0,8	1	ZT20	17.86 - 22.14
GSPE	62.8	0.714	1	ZT20	18.01 - 21.99

Rorα	%R	M	AMP	Acro	CI Acro
Control	75.6	1.5	3	ZT5	3.08 - 6.92
GSPE	81.8	3.5	7.5	ZT8	6.38 - 9.62

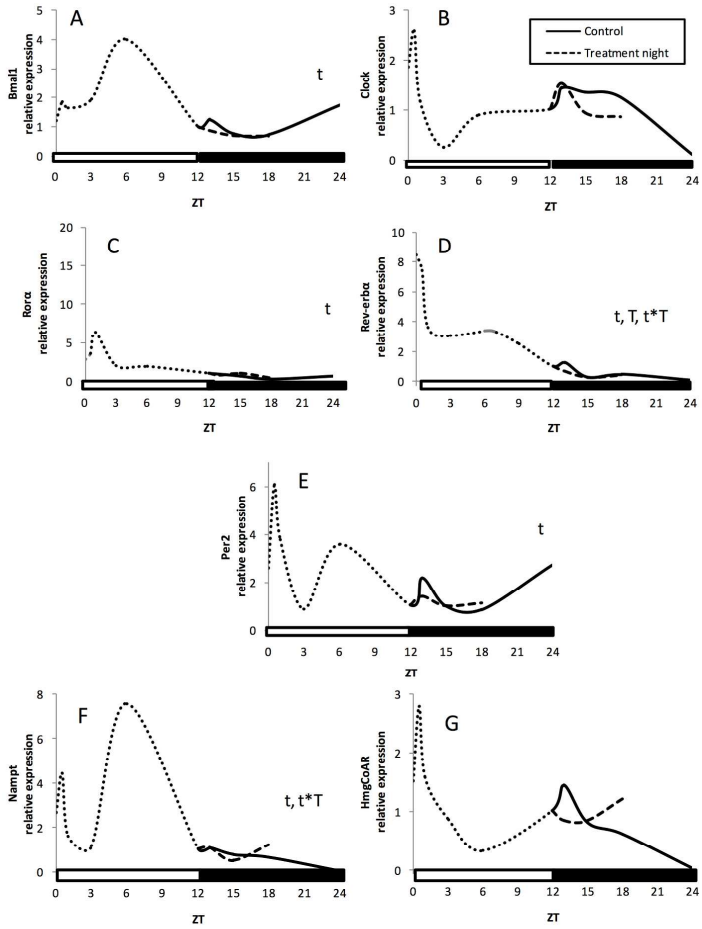
Rev-erbα	%R	M	AMP	Acro	CI Acro
Control	75.6	2.5	4	ZT5	3.21 - 6.79
GSPE	46.8	3.1	4	ZT20	17.38 - 22.62

Per2	%R	M	AMP	Acro	CI Acro
Control	66	1.9	3	ZT5	2.79 - 7.21
GSPE	75.7	2.5	2.5	ZT20	18.23 - 21.77

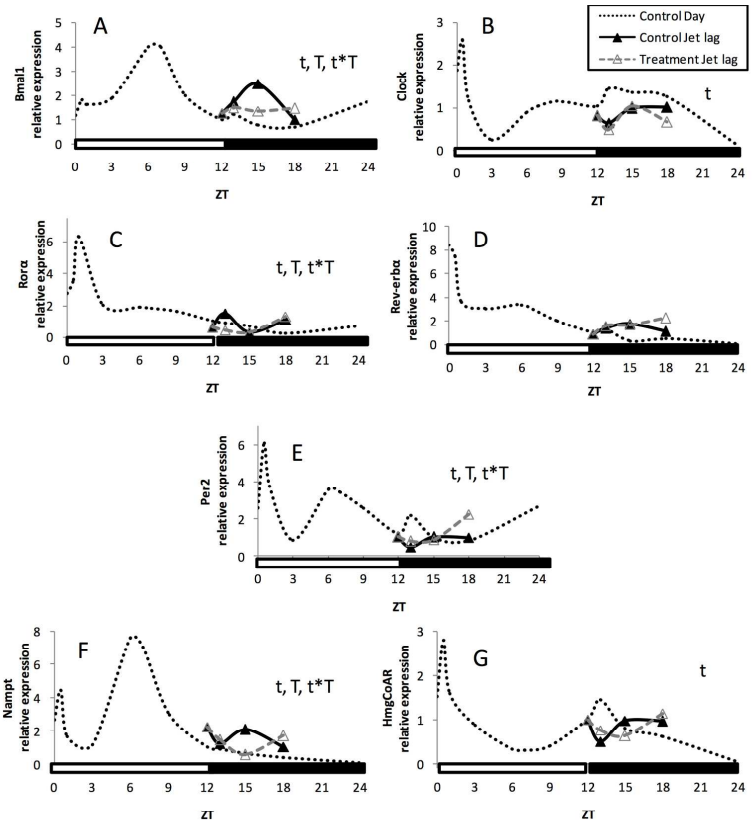
Nampt	%R	M	AMP	Acro	CI Acro
Control	77.5	1.7	3.5	ZT5	3.27 - 6.73
GSPE	64.2	1	1	ZT20	18.12 - 21.88

HmgCoAR	%R	M	AMP	Acro	CI Acro
Control	58	0.6	1	ZT20	17.55 - 22.45
GSPE	61	0.71	1	ZT20	17.81 - 22.19

1454x1886mm (40 x 40 DPI)



1430x1890mm (40 x 40 DPI)



1462x1674mm (40 x 40 DPI)

Table 1. Primer sequences

	Forward	***Reverse***
Bmal1	5′-GTAGATCAGAGGGCGACGGCTA-3′	5′-CTTGTCTGTAAAACTTGCCTGTGAC-3′
Clock	5′-TGGGGTCTATGCTTCCTGGT-3′	5′-GTAGGTTTCCAGTCCTGTCG-3′
Per2	5′-CGGACCTGGCTTCAGTTCAT-3′	5′-AGGATCCAAGAACGGCACAG-3′
Rorα	5′-GAAGGCTGCAAGGGCTTTTTCAGGA-3′	5′-CCAAACTTGACAGCATCTCGA-3′
Rev-erbα	5′-CTGCTCGGTGCCTAGAATCC-3′	5′-GTCTTCACCAGCTGGAAAGCG-3′
Nampt	5′-CTCTTCACAAGAGACTGCCG-3′	5′-TTCATGGTCTTTCCCCCACG-3′
HmgCoAR	5′- GAAACCCTCATGGAGACGCA-3′	5′- ACCTCTGCTGAGTCACAAGC-3′
Cyclophilin	5′-CTTCGAGCTGTTTGCAGACAA-3′	5′-AAGTCACCACCCTGGCACATG-3′

Bmal1 (also known as *ARNTL)*: aryl hydrocarbon receptor nuclear translocator-like; *Clock*: circadian locomotor output cycles kaput; Per2: period circadian clock 2; *Rorα*: RAR-related orphan receptor A; *Rev-erbα* (also known as *Nr1d1*): nuclear receptor subfamily 1, group D, member 1; *Nampt:* nicotinamide phosphoribosyl transferase; *HmgCoAR*: 3-hydroxy-3-methyl-glutaryl-CoA reductase.

Table 2. Circadian rhythm parameters of glucidic plasma metabolites in rats treated with an oral dose of a grape seed proanthocyanidin extract (250 mg /kg body weight) at zeitgeber time 0

Metabolite		%R	MESOR	AMP	Acro	CI Acro	Annova	P-value
Glucose	C	70,4	6212.5	787.5	CT11	9.01-12.99	T	0.146
	GSPE	97,8	6412.5	347.5	CT23	22.41-23.59	T*t	0.001*
Lactate	C	45,6	5011.25	1360	CT0	± 2.33	T	0.811
	GSPE	95,7	5159.25	1580	CT23	22.14-23.86	T*t	0.208
Pyruvate	C	38,9	171.5	77.5	CT0	± 2.42	T	0.632
	GSPE	92,9	165.5	36.6	CT23	21.29-24.08	T*t	0.005*
Citrate	C	37	373.75	170	CT0	± 2.43	T	0.713
	GSPE	59	362.75	75	CT0	± 2.11	T*t	0.044*
Formate	C	71,9	63.5	23	CT0	± 1.88	T	0.484
	GSPE	70,5	68.5	16.5	CT0	± 1.93	T*t	0.881
Methylsuccinate	C	21,7	214	97.5	CT0	± 2.72	T	0.661
	GSPE	34,3	222.5	97.5	CT11	8.36-13.64	T*t	0.055
Dihydroxyacetone	C	67,3	755.25	187.5	CT11	8.48- 13.16	T	0.291
	GSPE	93,3	680.75	61.5	CT23	21.98-24.02	T*t	0.117
Mannose	C	55,3	102	23.5	CT0	± 2.19	T	0.768
	GSPE	59,2	97	23.5	CT23	20.84-25.16	T*t	0.934

Rats were gavaged at ZT0. Cosinor analysis and two factors ANOVA have been applied to plasma metabolite concentration at ZT0, ZT6, ZT12 and ZT24. %R , cosine wave rhythm percentage; AMP, amplitude of cosine wave; Acro, acrophase; CI Acro, confidence interval of acrophase; T, Effect of proanthocyanidins; T*t, interaction between proanthocyanidins and Zeitgeber Time; C, control animals, GSPE, group treated with a grape seed proanthocynidin extract.

Table 3. Circadian rhythm parameters of nitrogen plasma metabolites in rats treated with an oral dose of a grape seed proanthocyanidin extract (250 mg /kg body weight) at zeitgeber time 0

Metabolite		%R	MESOR	AMP	Acro	CI Acro	Annova	P-value
Alanine	C	32	905.25	500	CT11	8.35-13.65	T	0.526
	GSPE	87,9	980.5	173	CT0	± 1.4	T*t	0.143
Serine	C	65,5	1131	337.5	CT11	8.8-13.2	T	0.028*
	GSPE	93,2	965.25	183	CT0	± 1.08	T*t	0.09*
Threonina	C	87,6	1001.5	270	CT11	9.63-12.37	T	0.154
	GSPE	89	802	227.5	CT0	± 1.35	T*t	0.175
Dimethyl-glycine	C	59,6	7.25	4	CT0	± 2.18	T	0.330
	GSPE	80,8	6.5	2	CT0	± 1.69	T*t	0.019*
Tyrosine	C	54,6	158.75	47.5	CT0	± 2.25	T	0.024*
	GSPE	91	137	43.5	CT0	± 1.24	T*t	0.06*
Glutamine	C	28,6	1602.75	432.5	CT0	± 2.61	T	0.222
	GSPE	87,7	1488.5	213	CT23	21-59-24.41	T*t	0.026*
Glutamate	C	60,1	1072.5	331	CT0	± 2.21	T	0.077
	GSPE	85,2	934.25	225	CT0	± 1.54	T*t	0.172
Leucine	C	47,2	418	134	CT0	± 2.31	T	0.285
	GSPE	94	393.75	96.5	CT0	± 1.02	T*t	0.016*
Isoleucine	C	24,2	531.25	157.5	CT0	± 2.66	T	0.312
	GSPE	94,3	497.5	85	CT23	22.02 - 23.98	T*t	0.015*
Valine	C	52,6	478.75	162,5	CT0	± 2.18	T	0.135
	GSPE	88,3	445	135	CT0	± 1.4	T*t	0.007 *
Lysine	C	35,9	621.25	282.5	CT0	± 2.47	T	0.357
	GSPE	49,4	577.5	132.5	CT0	± 2.42	T*t	0.016*
Histidine	C	19,2	145.75	67.5	CT11	8.24-13.16	T	0.629
	GSPE	71,4	135.25	26.5	CT23	21.12-24.88	T*t	0.006*
Phenylalanine	C	24,8	99	25	CT0	± 2.71	T	0.06
	GSPE	89,4	86	16.5	CT0	± 1.34	T*t	0.095
Tryptophan	C	95,1	176.25	18	CT23	22.11-23.89	T	0.740
	GSPE	97,5	173.25	25	CT23	22.35-23.65	T*t	0.915
Methionine	C	40	341	135.5	CT0	± 2.2	T	0.868
	GSPE	60,2	336.25	111.5	CT0	± 2.11	T*t	0.237
Taurine	C	85,3	1469.75	266.5	CT0	± 1.53	T	0.294
	GSPE	19,9	1243.25	491.5	CT0	± 2.77	T*t	0.833
Methylhistidines + xanthine	C	31,6	44.25	20	CT17	14.31-19.69	T	0.687
	GSPE	84,9	39.25	23	CT0	± 1.55	T*t	0.828
Urea	C	88,2	448.25	102.5	CT17	15.96-18.04	T	0.818
	GSPE	83,3	456.25	75	CT17	15.44-18.56	T*t	0.575
Creatine	C	79,8	842.5	432.5	CT0	± 1.73	T	0.766

	GSPE	74,6	762.25	373.5	CT23	21.21-24.79	T*t	0.939
Cadaverine	C	38,3	816.25	367.5	CT0	± 2.44	T	0.275
	GSPE	57,8	756.25	165	CT0	± 2.23	T*t	0.008*
Tymidine	C	70,9	27	4	CT23	21.15-24.85	T	0.897
	GSPE	76,7	27.5	2.5	CT17	15.58-18.42	T*t	0.793
Cytosine	C	91,2	26.5	10	CT17	16.01-17.99	T	0.822
	GSPE	93,3	27.25	3.5	CT11	10.01-11.99	T*t	0.236
Allantoin	C	86,4	152.75	26	CT11	9.53-12.47	T	0.825
	GSPE	62,8	139	8.5	CT0	± 2.07	T*t	0.989

Rats were gavaged at ZT0. Cosinor analysis and two factors ANOVA have been applied to plasma metabolite concentration at ZT0, ZT6, ZT12 and ZT24. %R , cosine wave rhythm percentage; AMP, amplitude of cosine wave; Acro, acrophase; CI Acro, confidence interval of acrophase; T, Effect of proanthocyanidins; T*t, interaction between proanthocyanidins and Zeitgeber Time; C, control animals, GSPE, group treated with a grape seed proanthocynidin extract.

Table 4. Circadian rhythm parameters of lipidic plasma metabolites in rats treated with an oral dose of a grape seed proanthocyanidin extract (250 mg /kg body weight) at zeitgeber time 0

Metabolite		%R	MESOR	AMP	Acro	CI Acro	Annova	P-value
Triglycerides	C	66,2	288	59	CT0	± 2.05	T	0.826
	GSPE	40,2	271.5	110	CT0	± 2.56	T*t	0.846
Linoleicacid	C	86,6	146.25	88	CT17	15.84-18.16	T	0.293
	GSPE	57,9	161.25	46	CT23	20.79-25.21	T*t	0.004*
Oleicacid	C	84,8	195.25	43	CT17	15.64-18.36	T	0.033*
	GSPE	79,9	224.25	3	CT11	9.37-12.63	T*t	0.153
MUFA	C	84,1	178.5	126	CT23	21.43-24.57	T	0.030*
	GSPE	76,7	256.75	127.5	CT0	± 1.76	T*t	0.026*
PUFA	C	84,2	392.25	213.5	CT17	15.49-18.51	T	0.167
	GSPE	89,7	351	123.5	CT11	9.7-12.3	T*t	0.028*
omega-3 fattyacids	C	76,6	57.75	11	CT17	15.58-18.42	T	0.730
	GSPE	63,3	55.75	8.5	CT17	15.04-18.96	T*t	0.924
ARA+EPA	C	84,8	73.25	41.5	CT17	15.71-18.29	T	0.155
	GSPE	85,4	62.25	15.5	CT11	9.54-12.46	T*t	0.010*
Freeglycerol	C	31	1319.75	267	CT0	± 2.4	T	0.140
	GSPE	74,3	1183.5	283.5	CT0	± 1.87	T*t	0.411
3-hydroxy-butyrate	C	18,7	527	214	CT0	± 2.71	T	0.817
	GSPE	61,6	549.75	136.5	CT23	20.85-25.15	T*t	0.669
Acetates	C	56,1	570	234	CT0	± 2.06	T	0.838
	GSPE	61,3	544.5	186.5	CT0	± 2.14	T*t	0.789
Total cholesterol	C	81,2	372.5	168.5	CT17	15.7-18.3	T	0.549
	GSPE	23,4	368.75	107	CT11	8.32-13.68	T*t	0.007*
Esterifiedcholesterol	C	83	253.75	127.5	CT17	15.68-18.32	T	0.332
	GSPE	24,2	271.75	99.5	CT17	14.4-19.6	T*t	0.037*
Cholate	C	24,6	181	77.5	CT0	± 2.66	T	0.219
	GSPE	88,9	162.25	38	CT23	21.65-24.35	T*t	0.013*
Phosphocholines	C	81,5	457.75	327	CT17	15.72-18.28	T	0.963
	GSPE	52,1	455	226	CT0	± 2.14	T*t	0.002*
Glycerol-phosphocholine	C	69,2	880.25	218.5	CT11	8.94-13.06	T	0.025*
	GSPE	88,3	692.75	130	CT0	± 1.38	T*t	0.155
Choline	C	72,9	70.75	25	CT0	± 1.79	T	0.948
	GSPE	92,8	69.5	20	CT0	± 1.12	T*t	0.309
Plasmalogen	C	21,5	15.25	1.5	CT0	± 2.52	T	0.831
	GSPE	95,4	15.5	1.5	CT11	10.52-11.88	T*t	0.504
Sphingomyelin	C	72,8	43.25	6.5	CT11	9.15-12.65	T	0.437
	GSPE	79,4	46.75	15	CT11	9.27-12.73	T*t	0.325

Rats were gavaged at ZT0. Cosinor analysis and two factors ANOVA have been applied to plasma metabolite concentration at ZT0, ZT6, ZT12 and ZT24. %R , cosine wave rhythm percentage; AMP,

amplitude of cosine wave; Acro, acrophase; CI Acro, confidence interval of acrophase; T, Effect of proanthocyanidins; T*t, interaction between proanthocyanidins and Zeitgeber Time; C, control animals, GSPE, group treated with a grape seed proanthocynidin extract; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ARA, all-trans retinoic acid; EPA, Eicosapentaenoic acid.

Table 5. Plasma metabolite levels of rats treated with an oral dose of a grape seed proanthocyanidin extract (GSPE, 250 mg /kg body weight) at zeitgeber time 12 at 3 hours post treatment

Metabolite	Control	GSPE	ANOVA p-value
Glucose	6.65	6.71	0.92
Lactate	5.16	6.35	0.62
Pyruvate	0.16	0.16	1.00
Citrate	0.36	0.34	0.50
Methylsuccinate	0.15	0.15	1.00
Dihydroxy acetone	0.62	0.64	0.60
Mannose	0.07	0.05	0.45
Alanine	1.00	0.95	0.60
Serine	0.98	0.93	0.34
Threonina	0.78	0.75	0.58
Dimethyl-glycine	0.06	0.05	0.20
Tyrosine	0.12	0.11	0.65
Glutamine	1.49	1.50	0.66
Glutamate	0.91	0.80	0.17
Leucine	0.37	0.34	0.42
Isoleucine	0.43	0.38	0.23
Valine	0.43	0.38	0.30
Lysine	0.47	0.40	0.50
Histidine	0.01	0.01	0.71
Phenylalanine	0.09	0.07	0.26
Tryptophan	0.15	0.16	0.54
Methionine	0.25	0.23	0.51
Taurine	1.41	1.22	0.38
Methylhistidines + xanthine	0.03	0.04	0.63
Urea	0.34	0.31	0.46
Creatine	0.05	0.05	0.25
Cadaverine	0.65	0.60	0.41
Triglycerides	0.52	0.52	0.98
Linoleic acid	23	25	0.77
Oleic acid	20	21	0.81
MUFA	35	35	0.78
PUFA	51	55	0.73

omega-3 fatty acids	7	7	0.81
ARA+EPA	11	12	0.74
Free glycerol	0.99	0.94	0.41
3-hydroxy-butyrate	0.39	0.39	0.90
Acetates	0.41	0.43	0.74
Total cholesterol	0.60	0.67	0.80
Esterified cholesterol	0.45	0.43	0.95
Cholate	0.13	0.12	0.62
Phosphocholines	0.91	0.90	0.97
Glycerol-phosphocholine	0.55	0.55	0.95
Choline	0.06	0.05	0.47
Plasmalogen	0.02	0.02	0.90
Sphingomyelin	0.05	0.06	0.90
Tymidine	0.02	0.04	0.18
Cytosine	0.02	0.04	0.21
Formate	0.05	0.04	0.45
Allantoin	0.12	0.12	0.94

Metabolite concentration is expressed as mM except for linoleic acid, oleic acid, MUFA, PUFA, omega-3 fatty acids and ARA+EPA that are expressed as percentage of molar chain. GSPE, group treated with a grape seed proanthocynidin extract; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ARA, all-trans retinoic acid; EPA, Eicosapentaenoic acid.

V. GENERAL DISCUSSION

Since resveratrol was found to modulate clock gene expression in cultured fibroblasts [1] and rat white adipose tissue and liver [2], no further studies have been performed with other groups of polyphenols. In the latter study with resveratrol, the aim was to analyse the potential effects of this polyphenol on changes induced by a high-fat diet on the expression of core clock genes and clock-controlled genes [2]. As mentioned in the introduction of this thesis, it is known that the clock machinery is affected by some stress situations, such as the stress induced by a high-fat diet, as mice fed this diet display altered expression of core clock and clock-controlled genes involved in fuel utilisation and other circadian rhythm disturbances [3]. Therefore, based on the close relationship between the circadian system and metabolism and, in turn, the beneficial effects of this polyphenol on metabolic disturbances, it is possible that correction of these disruptions by resveratrol can be considered another mechanism by which the compound exerts its healthy effects. In fact, as a most important effect on the molecular clock, resveratrol counteracted the obesity-induced increase in liver and WAT expression of *Rev-erbα* [2], a gene that is essential for adipogenesis and for which overexpression results in the overexpression of adipogenic genes [4].

Accordingly, considering that PAs are the most consumed flavonoid due to their widespread presence in foods [5] and have a vast range of health effects, improving all the components of metabolic syndrome [6], we speculated whether PAs, similar to resveratrol, would be able to modulate the clock machinery. Thus, we decided to initially demonstrate in a similar condition as in the case of resveratrol, i.e., under a high-fat diet, whether PAs are capable of exerting a similar effect. We first performed another experiment without a pathological situation with different doses of PAs, at 5, 25 or 50 mg GSPE/kg body weight, to evaluate whether the clock genes responded in a dose-dependent manner. By extrapolating to human doses [7] and estimating the daily intake for a 70 kg human, these doses match an intake of 57, 284 and

560 mg of GSPE/day. As the estimated proanthocyanidin intake for humans ranges between 90 and 200 mg/day [8–10], the GSPE doses used in this study simulate low, similar and high human proanthocyanidin dietary intake.

For these two experiments (under or not stress), the peripheral oscillators in the liver, gut and mWAT were analysed, taking into account that the expression of clock genes in the liver [11,12], gut [13] and WAT [14] are entrained by the frequency of daytime meals and diet composition. As a result, these two experiments showed that GSPE differentially modulates the peripheral clock in each organ studied.

In healthy animals, the number of core clock and clock-controlled genes with altered expression in response to GSPE was high in mWAT, medium in the liver and low in the gut, with a general overexpression of core clock genes and two different patterns in the expression of genes directly controlled by CLOCK:BMAL1 in the liver and mWAT. *Per2* was overexpressed in a positive dose-dependent manner, similar to *Clock* and *Bmal1*; in contrast, *Rev-erbα* and *Rorα* were repressed, showing a negative dose-dependent pattern. As chronic GSPE intake was capable of modifying circadian clock gene expression in healthy animals at doses that simulate regular proanthocyanidin intake in the Mediterranean diet [10], i.e., 25 mg GSPE/kg body weight, we determined the capacity of GSPE to modulate peripheral molecular clocks in obese rats under this standard dose. GSPE administration nearly corrected all of the disruptions in the clock genes induced by obesity in the liver and gut yet was less effective in normalising clock gene disruption in mWAT. However, as gene expression was measured at only one point in both experiments, it is not possible to infer whether the effects induced by PAs on the molecular clock were due to a phase shift or amplitude intensification after chronic GSPE consumption.

Therefore, we next designed an experiment in which gene expression was measured at several points, shaping a curve so that it was possible to

determine the amplitude and acrophase for each gene. For this experiment, PAs administration was supplied acutely at 250 mg of GSPE /kg body weight to affect the clock system in a dose that, in previous experiments by our group, was found to be effective for modulating lipid and glucose metabolism [15–18]. PAs were administered at ZT0, when the light was turned on, and at ZT12, when the light was turned off. Despite mWAT being the tissue most affected by PAs in healthy rats, as indicated in the previous experiment, the effects of GSPE in the liver were not negligible. In addition, although comparisons are difficult because the experimental conditions were not equal, the liver was the organ that better matched the effects exerted by resveratrol in a similar study [2]. Therefore, we focused on the liver in the ensuing experiments as the tissue most closely related to rhythms. In fact, liver is the most important metabolic organ due to its implications in glucose [19] and lipid [20] metabolism, among other crucial physiological functions [21], and is thus a good candidate for studying the effects of PAs on the peripheral clock.

The data clearly showed that the ability of PAs to entrain the circadian rhythm of core clock and clock-controlled genes in the liver depends on the time of administration. Specifically, PAs administered at the beginning of the dusk phase (ZT12) were much more effective affecting the clock machinery. *Bmal1* mRNA and protein levels were consistently increased one hour after PAs consumption, independently of whether administration occurred diurnally or at night. However, only the acetylated form of BMAL1 is active, and GSPE significantly increased the ratio of acetylated BMAL1 only at ZT13, with no effect at ZT1. The acetylation of BMAL1 is carried out by its partner CLOCK, which possesses a histone acetyl transferase (HAT) domain; the rhythmic deacetylation of BMAL1 is regulated by the deacetylase SIRT1, which is sensitive to NAD+ levels [22]. Therefore, the ratio of acetylated to total BMAL1 protein provides direct information about the transactivation activity of both SIRT1 and BMAL1:CLOCK [22]. The different pattern of

BAML1 acetylation induced by GSPE administered in the day or at night, in turn, explains the overexpression of *Nampt*, a BMAL target gene [22]. Hence, the peak of NAD levels in liver was found only when GSPE was administered at night. In fact, although GSPE modulated NAMPT, the rate-limiting enzyme in the biosynthesis of NAD [23], and NAD levels in an opposite manner after 6 hours of its administration (day or night), reducing or increasing the levels, respectively, it is interesting that the *Nampt* acrophase was delayed by 6 hours when GSPE was administered in the day, suggesting that NAD picked also at night. Therefore, in addition to *Bmal1*, *Nampt* and NAD emerge as molecular targets of PAs in the liver; due to its importance in metabolism [24–28], the modulation of NAD levels by PAs, through the BMAL1 acetylation, is an attractive candidate for explaining some of the metabolic effects of PAs. In this sense, PAs could act as an element of adaptation in the liver by improving the energetic profile of rats and increasing mitochondrial function and oxidation at night, a time when rats are active. Conversely, during the light phase, when these animals are resting, PAs could be acting as an energy saver through decreased levels of NAD after PAs administration at ZT0. A systemic regulatory network for metabolic regulation in mammals, named “NAD World”, has been described that functions by orchestrating metabolic responses to a variety of nutritional and environmental cues, contributing to the maintenance of the robustness of metabolic regulation at a systemic level [29]. In other words, circadian rhythms allow anticipation of environmental changes and adaptation to the time of day and food availability, as has been shown in this study through NAD, NAMPT and BMAL1 acetylation levels modulated by PAs. These cues advise animals about environmental conditions by entraining biological rhythms to obtain better perspectives regarding lifespan, in agreement with the Xenohormesis hypothesis, which proposes that heterotrophs are able to sense chemical cues synthesised by plants, such as polyphenols, in response to stress [30].

As PAs modulate lipid metabolism in the liver [15], we also analysed the expression of *HmgcoAR*, the key enzyme of the cholesterol biosynthetic pathway, which shows a circadian rhythm but is not directly controlled by the core clock genes [31]. As expected, the *HmgcoAR* acrophase was at ZT17, and PAs did not modify the expression rhythm of this enzyme. However, GSPE had a dual effect on *HmgcoAR* expression depending on the time of its administration, repressing *HmgcoAR*, both at the mRNA and protein levels, at ZT0; in contrast, its expression was not affected at the protein level at ZT12, underlining this dual effect of PAs on liver metabolism as a function of the time of administration.

Still focusing on the liver, we attempted to unravel some of the possible mechanisms by which PAs are able to modulate the molecular clock in the liver through other experiments *in vitro*, specifically with HepG2 cells, as related to BMAL1 acetylation. For these experiments, we employed a dose, 100 mg/L in the medium, previously shown be active for lowering triglyceride levels [32] and modulating microRNA expression [33] in HepG2 cells. GSPE produced clear effects in the clock machinery in these cells, largely mediated through *Bmal1* and its activator *Rorα*. *Rorα* showed an advanced acrophase from T23 to T8, possibly explaining the delayed shift of the *Bmal1* acrophase from T14 to T20.

Then, we next attempted to ascertain whether melatonin and GSPE induced a similar effect, taking into account that at pharmacological doses, melatonin and PAs have similar effects on certain metabolic processes [34] and other physiological events [35,36]. RORα acts as a nuclear receptor of melatonin [37], and melatonin itself has been linked to the core circadian machinery genes [38]. We found that the *BMAL1* mRNA levels were increased to the same intensity at 1 h after treatment when the cells were treated with 10 μM and 100 mg/L of melatonin and GSPE, respectively. However, in the case of melatonin, the overexpression of *BMAL1* is mediated by the MT1 receptor, as

MT2 is not located in the liver [39], whereas the effect exerted by GSPE was mediated by another receptor. Taking into account that the effects of melatonin in the liver could also be mediated by its nuclear receptor RORα, which is also a *BMAL1* activator, we sought to determine whether PAs could act as a natural ligand of RORα. Although the results were inconclusive, they point to the idea that the effects of PAs on the molecular clock in the liver could be mediated through RORα. This activation of RORα would explain the rapid *BMAL1* overexpression induced, either *in vivo* or *in vitro*, within 1 h after GSPE treatment. Although it is not the subject of this thesis, the increase in *BMAL1* expression induced by GSPE could be interesting because a down-regulation of *BMAL1* accelerates the development of tumours and may influence the response to anti-cancer drugs [40]. In addition, the CLOCK:BMAL1 transactivation explained by the percentage of acetylation of BMAL1 would be more related to the time of PAs administration.

After these striking results in the modulation of the peripheral clock in the liver by PAs, using the same *in vivo* experiment, we evaluated whether PAs were able to entrain biological rhythms and to adjust the central clock system in the SCN.

To study the ability of PAs to entrain biological rhythms, the measurement of plasma metabolites, as blood metabolomics, has been proposed as a method to analyse internal body time [41], and the concentrations of melatonin, a robust indicator of the internal body time [42], were determined. As a result, GSPE administered at ZT0 strongly increased plasma melatonin levels in the middle of the light period, maintaining levels similar to those at dusk. Additionally, GSPE administered at ZT0 shifted the acrophase of the concentration of many important plasma metabolites, such as amino acids, glucose and cholesterol. Moreover, GSPE treatment masked the circadian rhythms of some metabolites that were very evident in the control animals at

the ZTs studied. Therefore, acute GSPE treatment at ZT0 actually affected biological rhythms in rats.

Furthermore, to study the capacity of PAs to adjust the central clock system, core clock and clock-controlled genes were analysed in the hypothalamus, as in the liver. The data clearly showed that GSPE adjusted the circadian rhythms of core clock and clock-controlled genes in the hypothalamus when administered at ZT0. This modulation was concomitant with the modification to the biological rhythms of melatonin and plasma metabolites. Conversely, the administration of GSPE at ZT12 caused minimal effects on the molecular clock of the hypothalamus. This pattern is contrary to those in the liver, which displayed exactly an opposite arrangement. In fact, it has been described that different mouse tissues have different circadian phases, suggesting the existence of organ-specific circadian rhythms synchronisers at the cell and tissue levels [43]. In other words, circadian gene expression is tissue specific and optimised in each tissue to best accommodate the respective functions of each throughout the circadian cycle, which, in turn, is dependent on external synchronisers, as described in the introduction of this thesis. For instance, a nearly eight hour phase difference has been observed between the liver and gonadal WAT [44]. In addition, it has been established that SCN cells are extensively coupled during the day, when the cells exhibit synchronous neural activity, but are minimally coupled during the night, when the cells are electrically silent [45]. Therefore, this discrepant functionality of SCN cells between day and night could determine the effectiveness of PAs. Accordingly, exposure to light has been observed to cause shifts in the phase of the SCN clock primarily during the subjective night in nocturnal rodents, whereas non-photic cues trigger these shifts mainly during the subjective day [46]. Therefore, GSPE could act as a non-photic cue, triggering the central clock system during the light period. Among the observed effects of GSPE treatment at ZT0 on the circadian rhythm expression of core clock and clock-controlled genes in the

hypothalamus, special attention should be paid to *Bmal1*, which showed a significant advanced shift in its phase. Because *clock* was not affected by GSPE, which is consistent with its constitutive expression in the SCN [47], such a dramatic shift in the *Bmal1* rhythm induced by GSPE could explain the delayed phase of the clock-controlled genes *Rorα, Rev-erbα, Per2* and *Nampt*. In addition, according to the defined role of *Bmal1* as a master regulator of the molecular clock system [48] and its importance in controlling metabolic processes, this finding could also explain the observed shifted acrophase of many important plasma metabolites [49,50]. Nonetheless, no studies have determined whether PAs reach the hypothalamus, though some studies in Wistar rats have elucidated the distribution of flavonols and their metabolites to different tissues, e.g., the brain [51,52], ruling out the capacity of flavonols to cross the blood-brain barrier. In addition, an experiment with a rat model of Parkinson's disease with chronic oral tangeretin administration (10 mg/kg/day for 28 days) confirmed a significant level of this citrus flavonoid in the hypothalamus, even at higher concentrations than in the liver or plasma [53]. Alternatively, PAs could adjust the central clock by acting at intestinal levels through the brain-gut axis, which sends gut cues to the brain via neuronal and hormonal mechanisms [54]. Thus, more studies are needed to define the molecular mechanism by which PAs adjust the central clock.

Because an effect of PAs under the standard condition was observed in the central and liver clocks, the capacity of GSPE to modulate the central and liver clocks was also evaluated in a circadian-disrupted situation using rats subjected to a 6 hour "jet-lag". Rats at ZT6 (middle of light period) were acutely administered GSPE at 250 mg of GSPE/kg body weight and moved to ZT12 (light off). Then, the capacity of GSPE to modulate core clock and clock-controlled genes was evaluated at ZT12, ZT13, ZT15 and ZT18 to establish whether PAs could exert some effect under this situation. In both tissues, "jet-lag" induced a clear shift in the rhythmicity of the mRNA levels

of all genes studied; indeed, GSPE administration significantly modulated the expression rhythm of most of them. Only *Clock*, *Rev-erbα* and *HmgCoAR* in the hypothalamus and *Clock* and *Per2* in the liver were not modulated by GSPE at the beginning of this jet lag simulation. Interestingly, even under this situation, *Bmal1* and *Nampt* were again modified, reinforcing the fact that they are targets of PAs. The effect of GSPE on *Bmal1* expression in the hypothalamus was the most important, as GSPE counteracted the jet-lag effect and allowed *Bmal1* to recover its normal rhythm. In the liver, the *Bmal1* mRNA and protein levels were curiously increased at one hour after PAs consumption, as in the previous experiment in which *Bmal1* was increased 1 h after GSPE treatment at ZT0 or ZT12. However, the percentage of acetylation was higher at six hours after GSPE administration, which could be related to the 6 hour delayed phase triggered by the jet lag situation. Altogether, these results suggest that PAs were not able to adapt to the jet lag situation in the liver until 6 h had elapsed; conversely, the adjustment of the molecular clock appeared to be immediate in the hypothalamus. This contradictory behaviour between the liver and hypothalamus could be related to the time of GSPE administration, i.e., ZT6, as that the major effects of GSPE in the central clock were observed during the light phase while the major effects were observed during the dark phase in the liver. In addition, another possible cause of this difference could lie in the coupling between cells. Peripheral cells oscillate largely independently of one another, whereas SCN neurons possess specific mechanisms, as explained in the introduction of this thesis, to maintain coupled neurons as a population such that they are able to oscillate robustly. These findings suggest that SCN cells are more prepared than peripheral cells to recover after a circadian-disrupted situation such as jet lag.

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VI. CONCLUSIONS

1. Chronic consumption of proanthocyanidins at different doses modulates the peripheral clock in the gut, liver and mWAT of rats under a standard condition.

2. Chronic consumption of proanthocyanidins nearly corrects the disruptions in clock genes induced by obesity in the liver and gut of obese rats.

3. *Bmal1* in the liver is a target of proanthocyanidins, independent of the time of administration or the situation (jet lag), as its expression is consistently increased 1 h after acute GSPE treatment. This effect is confirmed in HepG2 cells, in which proanthocyanidins could be acting as a natural ligand of RORα.

4. The acetylation of BMAL1 in the liver induced by proanthocyanidins depends on the time of administration, displaying a higher level of acetylation after acute GSPE treatment at ZT12 (start of the dark phase).

5. In addition to BMAL1, NAMPT and NAD emerge as molecular targets of proanthocyanidins in the liver, which could represent a mechanism of adaptation entrained by proanthocyanidins.

6. Melatonin plasma levels are increased in the middle of the light period and, in parallel, several plasma metabolites such as amino acids, glucose and cholesterol shift their acrophase after GSPE administration at ZT0.

7. GSPE affects biological rhythms and adjusts the circadian rhythms of core clock and clock-controlled genes in the hypothalamus when administered at ZT0, causing minimal effects after administration at ZT12

and displaying an opposite pattern compared to the liver, where GSPE exerts major effects after administration at ZT12. Therefore, proanthocyanidins could act as a non-photic cue in the hypothalamus and as a mechanism of adaptation in the liver during the night period.

8. *Bmal1* in the hypothalamus is also a target during the light phase, which could explain all the effects observed.

9. GSPE significantly alters the expression rhythm of most clock genes in the central and peripheral clock in the liver; these genes show altered rhythms after a circadian-disrupted situation such as jet lag. Furthermore, *Bmal1* is also targeted in the hypothalamus, as GSPE counteracts the jet-lag effect and allows this gene to recover its normal rhythm.