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CONTROL TEMPORAL DEL METABOLISMO: INTERACCIÓN ENTRE GENES RELOJ Y GENES METABÓLICOS.

TESIS

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TEMPORAL CONTROL OF METABOLISM

Interaction between clock genes and metabolic genes.

Thesis

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RESUMEN

En mamíferos, el núcleo supraquiasmático (NSQ) del hipotálamo es el reloj circadiano maestro, el cual dirige todos los ritmos circadianos del organismo incluyendo los conductuales (como los ciclos de descanso/actividad y ayuno/alimentación), la temperatura, el latido cardiaco, las concentraciones plasmáticas de varios metabolitos y hormonas, la expresión de genes, etc. A nivel molecular, se considera que el mecanismo circadiano tiene su base en un grupo de reguladores transcripcionales codificados por el conjunto de genes denominados "genes reloj". Estos genes se expresan no solamente en el NSQ sino en prácticamente todos los tejidos y se cree que ellos son responsables en gran medida de dar orden temporal a las funciones específicas de cada órgano, participando de manera importante en la regulación de varias ramas del metabolismo. Por todo ello, se ha prestado gran atención al estudio de los factores que regulan a estos genes; se sabe, por ejemplo, que el ciclo de ayuno/alimentación es una de las señales con mayor influencia sobre los relojes de los tejidos periféricos. Cuando se desacopla el ciclo de alimentación de la fase normal establecida por el NSQ, por ejemplo cuando se cambia el consumo de alimento hacia la fase de descanso, el ritmo de los genes reloj en el hígado se invierte por completo, aún cuando la actividad del NSQ no es afectada por este cambio conductual. Curiosamente, estas condiciones experimentales provocan que algunos de los genes metabólicos considerados como parte importante de las asas de retroalimentación del reloj (como *PPARα* y *Nampt*) pierdan su ritmo o disminuyan considerablemente su amplitud, sugiriendo que la relación que se ha propuesto entre dichos genes metabólicos y los genes reloj probablemente no siempre se cumple. Así mismo, de estos antecedentes surge la hipótesis de que algunos de estos genes podrían estar siendo conducidos por los ciclos de alimentación-ayuno mientras que otros podrían estar controlados principalmente por otras señales dependientes del NSQ. El objetivo de esta tesis fue evaluar si un horario de alimentación similar al que ocurre en condiciones fisiológicas normales es capaz de inducir un ritmo circadiano en los genes reloj del hígado y en los genes metabólicos propuestos como asas accesorias a él (*PPARα*, *Rev-erbα*, *Nampt*, *Sirt1* e *Id2*) en animales carentes de alguna otra señal circadiana proveniente del NSQ. Con este fin, se hicieron lesiones electrolíticas del NSQ en ratas macho adultas a las que posteriormente se les organizó un horario de alimentación de 12h alimento/12 h ayuno por día, durante 3-4 semanas. Después de este periodo las ratas fueron sacrificadas en diferentes puntos temporales del día; a un grupo de ellas se le proporcionó la comida de manera habitual el día del sacrificio, mientras que a otro grupo no; esto se hizo para evaluar si los patrones de expresión en los genes reflejan tan sólo una respuesta aguda relacionada con la ingestión de alimento o si se ha inducido en ellos un mecanismo oscilatorio. Se obtuvieron los hígados de estas ratas para evaluar la expresión de los genes reloj y los genes metabólicos asociados. Nuestros resultados muestran que en animales con lesiones del NSQ este esquema de alimentación induce en el hígado oscilaciones circadianas persistentes en los genes reloj así como en *PPARα* y *Rev-erbα*. Por el contrario, la expresión de *Nampt*, *Sirt1* e *Id2* no pudo ser recuperada por el horario de alimentación en los animales lesionados. Estos resultados nos permiten concluir que la expresión hepática dependiente del horario del día (al menos para el grupo pequeño de genes que nosotros evaluamos) es controlada por diferentes factores: mientras

que algunos presentan oscilaciones auto-sostenidas en su expresión -las cuales pueden ser inducidas por el horario de alimentación de manera independiente al reloj central-, otros genes son regulados por los estados de ayuno-alimentación pero requieren del NSQ para presentar una respuesta acorde a dichos estados. Finalmente, el ritmo en la expresión de otro grupo de genes, representados por *Id2*, está regulado por señales independientes del horario de alimentación y de los genes reloj, dependiendo de manera importante de otros factores provenientes del NSQ aún por determinar. Este conocimiento busca contribuir a desentrañar los mecanismos por los cuales ciertas condiciones de desincronización circadiana tales como el comer durante el periodo de descanso provocan el desarrollo de desórdenes metabólicos.

SUMMARY

The suprachiasmatic nucleus (SCN) of the hypothalamus is the master circadian clock in mammals; it drives all known circadian rhythms, from behavior (rest/activity, feeding/fasting cycles), to hormonal secretion, body temperature, heart rate, metabolite levels in blood, gene expression, etc. At the molecular level, the circadian mechanism is based on a group of genes known as the "clock genes"; they are present not only in the SCN but in virtually all peripheral tissues. Here they are thought to drive the circadian rhythms of tissue specific gene expression, as well as to be involved in metabolic functions of the organs through a determined set of clock-controlled genes. The feeding schedule influences the rhythm of many genes in peripheral organs in intact animals; it imposes a specific pattern in clock genes even without changing the activity of the SCN. The forced dissociation of the time of feeding from the time marked by the SCN (e.g. when food intake is moved to the resting phase) causes an uncoupling between the clock genes and the metabolic genes. Hereby clock genes completely reverse their rhythm of expression; but some of the associated genes lose amplitude or become flattened. This suggests to us that probably some of these clock-metabolic gene relationships, which have been proposed largely based on in vitro or in transgenic models, may not be always accomplished in vivo. Furthermore, we hypothesized that some genes could be driven more by the feeding-fasting cycles, whereas others could be mostly driven by other signals from the SCN. This thesis aimed to evaluate in animals devoid of any circadian signal from the SCN whether a feeding schedule similar to that that occurs under normal physiological conditions is able to induce a circadian rhythm in liver clock genes and in clock-controlled metabolic genes. To this end, electrolytic lesions of the SCN were made to adult male rats, after which they were subjected to a 12h feeding:12h fasting schedule per day during 3-4 weeks. Rats then were sacrificed at different hours along the day; one group received their usual food on the day of sacrifice whereas other group did not, in order to evaluate whether the gene profiles reflect just an acute response to food intake or if they are able to persist independently of the external stimulus. Livers were obtained to assess the mRNA levels of clock genes and metabolic genes associated. Our results show that in animals with SCN lesions this feeding scheme induces robust and persistent circadian oscillations in the liver clock genes as well as in *PPARα* and *Rev-erbα*. Conversely, the diurnal expression of *Nampt*, *Sirt1* and *Id2* cannot be recovered by the feeding schedule in SCNxx animals. These results allow us to conclude that time-of-day dependent expression in the liver (at least for this small set of genes) is differentially controlled; i.e. some genes have self-sustained oscillations in their expression that can be induced by the feeding schedule. Other genes are responsive to the nutritional state but require the SCN to fully display their normal rhythm. Finally, other genes are neither dependent on the feeding-fasting cycles nor in the local clock but necessarily rely on other yet-to-know pathways involving the SCN.

We hope this knowledge may help to understand why certain conditions of circadian desynchronization such as eating during resting period promote the development of metabolic diseases.

ABBREVIATIONS

AL, ad libitum
ADRP / ADFP, adipose differentiation related protein
AMPK, AMP-activated protein kinase
ANS, autonomic nervous system
CB1, cannabinoid receptor 1
CCG, clock controlled genes
ChREBP, carbohydrate-responsive element-binding protein
Cpt1, carnitine palmitoyltransferase 1
Cry1, Cryptochrome
DMV, dorsal motor nucleus of the vagus
DNL, de novo lipogenesis
FFA, free fatty acids
Hdac3, histone deacetylase 3
HSF1, heat shock factor 1
IL-6, interleukin 6
IML, intermedio lateral column
Intact-RF, intact animals with food provided during 12 h/day ("restricted feeding")
LPL, lipoprotein lipase
LPS, lipopolysaccharide
NAD, nicotinamide adenine dinucleotide
NAFLD, nonalcoholic fatty liver disease
NASH, nonalcoholic steatohepatitis
Nampt, nicotinamide phosphoribosyltransferase
OEA, N-oleoylethanolamine
PARP, poly-(ADP-ribose) polymerase
PEA, N-palmitoylethanolamine
Per1, Period
PP, pancreatic polypeptide
PPAR, peroxisome proliferator activated receptor
PGC1α, peroxisome proliferator-activated receptor gamma coactivator 1 alpha
PVN, paraventricular nucleus
RF, restricted feeding
ROS, reactive oxygen species
SCN, suprachiasmatic nucleus
SCNxx, suprachiasmatic nucleus lesion
SCNxx-AL , suprachiasmatic nucleus lesion with ad libitum food intake
SCNx-RF, suprachiasmatic nucleus lesion with food provided during 12 h/day ("restricted feeding")
Sirt1, sirtuin 1

SNS, sympathetic nervous system
SREBP1c, sterol regulatory element-binding protein 1c
TAG, triglycerides
TNF-α, tumor necrosis factor alpha
VLDL, very low density lipoprotein
WAT, white adipose tissue

CHAPTER 1.

INTRODUCTION

Since the dawn of life, most Earth living organisms have been exposed to environmental diurnal cycles of temperature, food availability, light and darkness. These periodic external changes have promoted the development of endogenous timekeepers that allow the organism to reliably predict the time of day, organizing behavior and physiology in a proactive rather than in mere responsive manner, optimizing in this way energy harvesting and utilization across the day/night cycle.

The specific term 'circadian (from the Latin *circa*-around and *dies*-day) rhythm' has been coined for those biological oscillations that have near-to 24h periodicity. Circadian rhythms comprehend vital physiological aspects e.g. the rest-activity and feeding-fasting cycles, body temperature, metabolism, blood pressure, hormonal and neurotransmitter secretion, as well as several parameters at the cellular level. The proper temporal coordination within these cycles, as well as their alignment with the external light/darkness is essential for the maintenance of health (Hastings et al. 2003).

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In mammals, there is a cellular group in the brain which is instrumental in the control of circadian timekeeping; it is a hypothalamic bilateral nucleus located at each side of the third ventricle, and above the optic chiasm, hence its name: suprachiasmatic nucleus (SCN). The crucial role of the SCN in circadian physiology has been largely revealed by lesion studies in rodents, demonstrating that surgical ablation of the SCN abolishes circadian rhythms in body temperature, food intake, locomotor activity, endocrine and metabolic factors (Fig. 1) (O'Neill and Feeney 2014).

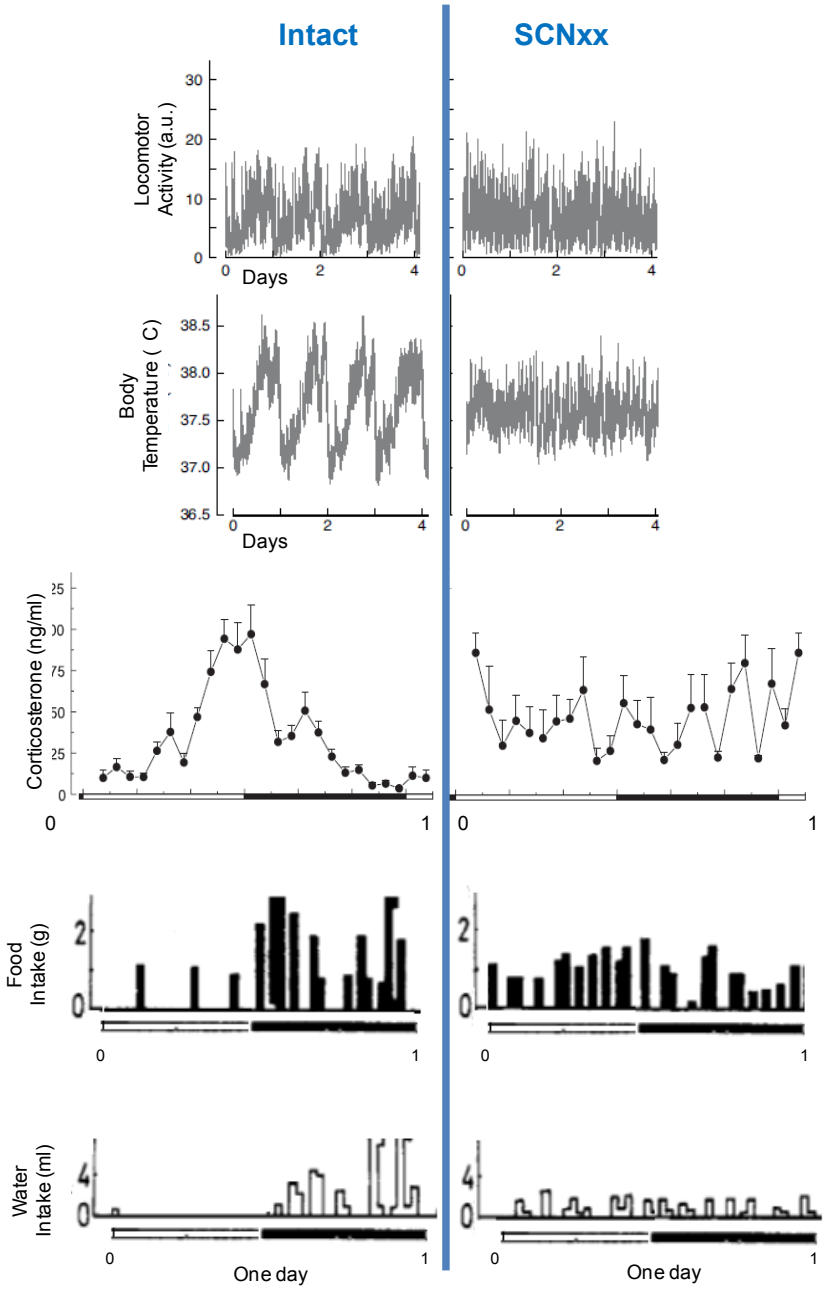

FIGURE 1: The SCN organizes circadian behavior and physiology in mammals. After bilateral lesion of the SCN (SCNxx), virtually all circadian rhythms (if there is no additional experimental manipulation) disappear. Here we can see that the normal pattern of locomotor activity, body temperature, corticosterone, and food and water intake (left panels), is lost after an SCN lesion (right panels). *Modified from: (Nagai et al. 1978, La Fleur et al. 1999, Scheer et al. 2005).*

Endogenous circadian cycles of high versus low neuronal activity, neurotransmitter release and gene expression are present in the SCN, being these intrinsic cycles a distinctive feature of this nucleus, as evinced by the fact that it is able to retain many of its circadian properties for several weeks even in *in vitro* slices (Herzog and Tosini 2001).

The endogenous near-24h (circadian) rhythm of the SCN is able to be adjusted (synchronized) to the 24h light-dark external cycle by means of a direct connection from the retina called the retinohypothalamic tract, whereby photic information is transduced and transmitted to the SCN, modifying its neuronal activity (Morin 2013), and allowing the downstream pathways from the SCN to be in correct consonance with the environment. The SCN further provides this temporal order to the body mainly by two pathways, one through connections with neurons that produce hypothalamic releasing factors (which in turn promote the secretion of hormones by the pituitary gland), and the other through hypothalamic and brainstem projections controlling the autonomic outputs to the periphery (Fig. 2) (Kalsbeek et al. 2011).

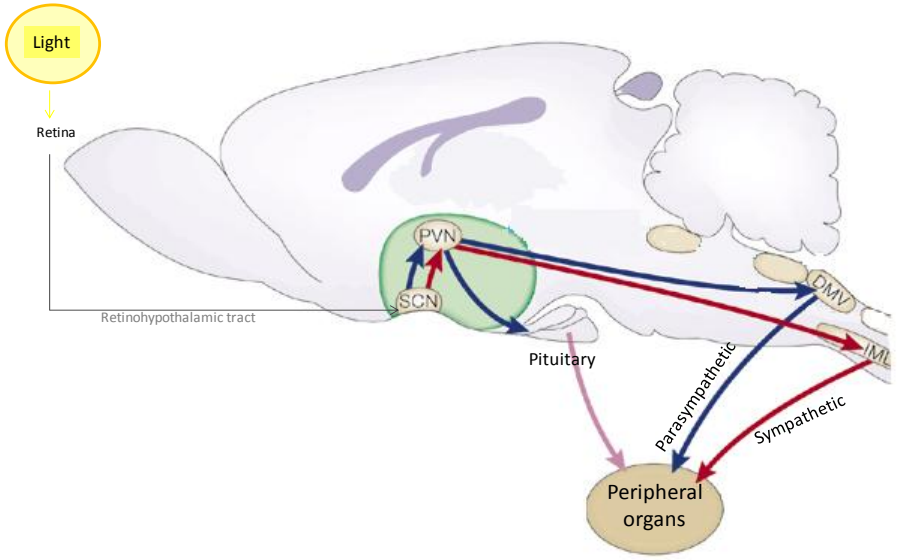

FIGURE 2: The SCN communicates circadian time to the body mainly through neuroendocrine and autonomic pathways. The hypothalamic paraventricular nucleus (PVN) is a prominent relay (though not the only) through which the SCN is able to influence the diurnal secretion of pituitary hormones and regulate the activity of parasympathetic and sympathetic nerves largely via the dorsal motor nucleus of the vagus (DMV) and the intermedio lateral column (IML) in the spinal cord. *Modified from: (Buijs and Kalsbeek 2001).*

A cell-autonomous circadian mechanism: the clock genes.

At the molecular level, the circadian clock mechanism is considered to be based on transcription-translation feedback loops within a group of genes highly conserved among animals, the so called 'clock genes' (Takahashi et al. 2008). The discovery of these circadian genes in mammals began in the late 90's (Tei et al. 1997), and among the characteristics that were proposed to be essential for candidate genes, apart from having an oscillating expression with a 24 hr rhythm, were that they should be expressed in the SCN, their circadian expression should be reset by changes in the oscillation of the environmental synchronizing cues (called entrainment) even though their rhythm must persist in the absence of those synchronizing cues; i.e., they should remain rhythmic under external constant conditions (Albrecht et al. 1997).

The set of genes that lie at the core of the circadian mechanism encode transcription factors that drive the expression of their own negative regulators. The basic helix-loop-helix transcription factors Clock (or its homologue Npas2) and Bmal1 heterodimerize and bind to E-box sequences within the promoters of *Period* (*Per1*, *Per2*, and *Per3*) and *Cryptochrome* (*Cry1* and *Cry2*) genes, inducing their transcription. Once translated, Per and Cry proteins form a complex that translocates to the nucleus and inhibits Clock(Npas2)/Bmal1-mediated gene expression, leading to a subsequent decrement in the expression of *Per* and *Cry* genes, which ultimately would relieve the inhibition on Clock(Npas2)/Bmal1, allowing the beginning of a new cycle (Fig. 3). These oscillations of expression-repression repeat with a periodicity of nearly 24h, leading to circadian variations on the transcript levels of the clock genes in the cell (Eckel-Mahan and Sassone-Corsi 2013, Gamble and Young 2013).

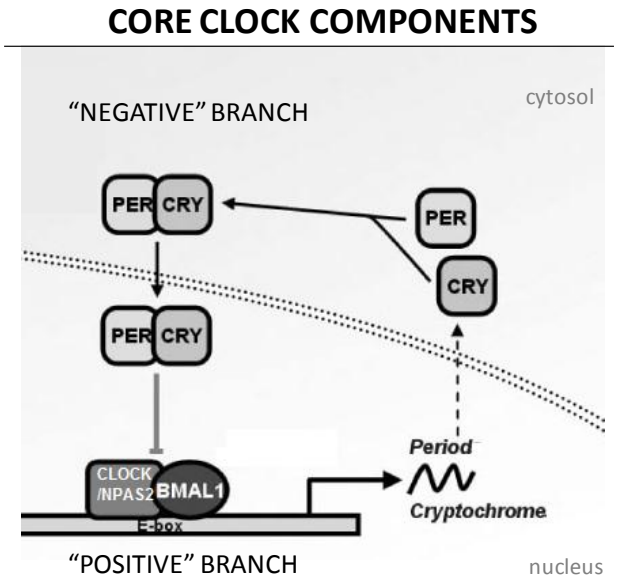

FIGURE 3: The basic clock mechanism lies in a transcription-translation negative-feedback loop where heterodimers of Clock(Npas2):Bmal1 drive the expression of their own negative regulators: Per and Cry. *Modified from: (Kwon et al. 2011).*

Besides the cycling genes clustered around the canonical core clock components, Ueda and colleagues (Ueda et al. 2002) identified in the liver a large cluster of circadian regulated transcripts with expression of *Id2* (inhibitor of DNA binding 2) as a prototype. *Id2* gene contains several E-box clusters in its regulatory region, and its expression may be regulated by a negative feedback self-regulatory loop (Neuman et al. 1995, Scobey et al. 2004). It is proposed that Id2 could have an important role as an output regulator of the clock due to its ability to interact with Clock:Bmal1 (Duffield et al. 2009).

Importantly, the clock genes have been found to be present (and oscillating) not only in the SCN, but also in virtually all mammalian tissues, including the liver, heart, lung, spleen, kidney, etc. (Yamamoto et al. 2004), raising questions about 1) the function of these genes in peripheral tissues, and 2) their reliance or independence from the central clock.

1) Physiological functions of peripheral clock genes.

Large-scale studies of the transcriptome have shown that nearly 10% of the genes expressed in a given tissue exhibit circadian fluctuations at the mRNA level (Storch et al. 2002, Vollmers et al. 2009). Many of these rhythmic genes are involved in diverse aspects of metabolism in a tissue-specific manner, including the regulation of carbohydrate and lipid metabolism, mitochondrial oxidative phosphorylation, adipocyte differentiation, etc. (Marcheva et al. 2013). Interestingly, when the molecular clock is disrupted in specific tissues, for instance in the liver (Lamia et al. 2008) or heart (Young et al. 2014), in those tissues most (though not all) genes with circadian oscillations lose their rhythm, suggesting that the local clock is in charge of giving temporal order to the majority of local circadian genes (Fig. 4), although caution must be taken when interpreting knockout- derived results because the clock network utilizes active compensatory mechanisms (Baggs et al. 2009).

A more accurate approximation to the knowledge of tissue specific transcriptional activities of clock genes has been done through chromatin immunoprecipitation assays; those studies have confirmed a daily rhythm in Bmal1 DNA binding to target genes, where metabolic genes and transcription factors, particularly nuclear receptors, predominate (Rey et al. 2011). Therefore, the transcriptional circadian control in a given tissue can be achieved either by directly controlling the transcription of target genes (these target genes are called 'Clock controlled genes', CCG) or secondary driven by transcription factors pertaining to the CCG group (Rey et al. 2011, Mazzoccoli et al. 2012).

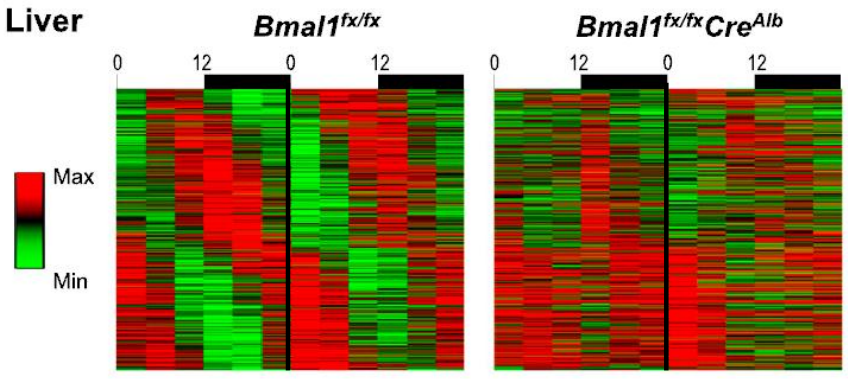

FIGURE 4: Circadian tissue-specific expression is largely dependent on the functionality of the local clock. Heatmaps of cycling transcripts in mice livers sampled over the course of two consecutive days show that the number and amplitude of rhythmic transcripts found in control animals (*Bmal1 fx/fx*; left panel, where each gene is represented by an horizontal line) is severely decreased when the clock gene *Bmal1* is specifically deleted in the hepatocyte lineage (*Bmal1 fx/fxCre-Alb*; right panel). The numbers at the top of heatmaps indicate the hours under the daily light-dark cycle, where 0 indicates lights-on, and 12 is the moment at which lights are turned off. *Modified from (Johnson et al. 2014).*

In several models of clock gene whole-body mutants and/or knockouts it has been found that many metabolic processes are deregulated, suggesting that besides their role in driving the local circadian expression, the molecular clock may have an important role in metabolism as well (Johnston 2014). Some examples are the following: a) *Bmal1* knockout mice are smaller and weigh less than wild-types, have an impaired gluconeogenesis, increased respiratory quotient, and reduced capacity of fat storage in adipose tissue, with a resultant increase in the circulating levels of triglycerides, free fatty acids and cholesterol, inducing the ectopic accumulation of fat in the liver and skeletal muscle (Rudic et al. 2004, Kondratov et al. 2006, Shimba et al. 2011); b) Mice knockout for *Npas2* have abnormal levels in the hepatic expression of several genes involved in fatty acid metabolism during the neonatal period; however, the majority are normalized in the adulthood probably due to a functional compensation with *Clock* (O'Neil et al. 2013); c) The absence of the clock gene *Per2* renders the animals more susceptible to cold temperatures due to the modification of essential genes in brown adipose tissue (Chappuis et al. 2013); d) Dysregulation on the circadian profile of several hepatic genes involved in lipid homeostasis is presented by *Id2-/-* mice (Hou et al. 2009, Mathew et al. 2013); e) Double knockout mice for *Cry1* and *Cry2* exhibit a marked reduction in size and weight, increased levels of liver triglycerides, and severely impaired glucose clearance in a glucose tolerance test, though they are normally responsive to insulin (Bur et al. 2009, Cretenet et al. 2010, Lamia et al. 2011); f) The last example mentioned here is about the clock gene *Clock*. Reports about *Clock* knockout mice phenotype are still scarce, yet we know that they are similar in weight to the wild-type but with notorious decrease in longevity (Dubrovsky et al. 2010). Most of our knowledge about functional implications of *Clock* came from mice homozygous for a dominant negative mutation of *Clock* in the C57BL/6J background (the *Clock delta19* mice), which are obese and develop hepatic steatosis, hyperlipidemia, hyperleptinemia and hyperglycemia (Turek et al. 2005). Interestingly and still under investigation of the causes, is the fact that this metabolic phenotype strongly depends on the genetic background; insomuch that homozygous for the same *Clock* mutation in another strain (the JcI:ICR) have undistinguished glucose, insulin and leptin levels, and even lower triglycerides and free-fatty acids that the wild-type (Oishi et al. 2006), showing the complexity of genetic interactions, and highlighting the care that should be taken when interpreting the function of a gene from whole body gene mutant/deletion experiments.

One thing that becomes evident when taken these data altogether is that there is not an identical phenotype resulting from the diverse knockout of the genes composing the clock machinery, what suggest that each of these genes may have additional clock-independent functions associated to specific metabolic pathways, as suggested by Dubrovsky et al. (Dubrovsky et al. 2010).
Gradually there have appeared animal models of tissue-specific deletion of some of the core clock genes, allowing a more accurate identification of the local role of the clock in specific metabolic pathways, and the consequences in whole body physiology. For instance, specific disruption of the clock mechanism in the liver decreases the ability to release glucose from the organ when it is needed, resulting in fasting hypoglycemia (Lamia et al. 2008), as well as in decreased hepatic lipogenic gene expression upon refeeding (Zhang et al. 2014). Pancreas-specific disruption impairs

glucose-stimulated insulin secretion (Sadacca et al. 2011). In skeletal muscle it leads to a fast to slow fiber-type shift and a more oxidative phenotype (Hodge et al. 2015), and adipose-specific disruption leads to obesity (Paschos et al. 2012); altogether showing an important participation of the local circadian clock in several aspects related with substrate utilization and energy metabolism.

The pathways that link the molecular clock with metabolism are currently a vast area of research, in search of enzymes, metabolites, and nuclear receptors that could serve as bridge to explain the many metabolic abnormalities found under conditions of disrupted clocks. Major attention has been paid to four of these "metabolic" genes: *Rev-erbα*, *PPARα*, *Nampt* (*nicotinamide phosphoribosyltransferase*) and *Sirt1*, which are particularly considered because of their reciprocal connection through feedback loops with the molecular clock, a property that additionally is thought may help to solve some questions regarding why circadian desynchronization is connected to metabolic abnormalities and why some metabolic abnormalities usually course with a certain degree of circadian disruption. In the next paragraphs the metabolic role and the form by which these four genes are linked to the clock will be briefly summarized.

Rev-erbα (NR1D1)

The nuclear receptor Rev-erbα was one of the first CCG described; its circadian expression was found to be inducible by serum shock in a cellular line of rat fibroblasts (Balsalobre et al. 1998). It is expressed in the SCN, liver, heart, white and brown adipose tissues, etc (Onishi et al. 2002, Triqueneaux et al. 2004, Yamamoto et al. 2004, Zvonic et al. 2006). *Rev-erbα* is positively regulated at the transcriptional level by the molecular clock through E-box sequences present in its promoter region (Triqueneaux et al. 2004), as well as by activation of PPARα (Gervois et al. 1999); conversely, its expression is downregulated by glucocorticoids (Torra et al. 2000).

Interestingly, Rev-erbα is an important repressor of *Bmal1*, *Clock* and *Npas2* expression, creating an accessory negative feedback loop in the clock mechanism (Fig. 5) (Preitner et al. 2002, Crumbley et al. 2010, Crumbley and Burris 2011). Rev-erbα represses *Bmal1* and several other genes by recruiting the nuclear receptor corepressor (N-CoR)/histone deacetylase 3 (Hdac3) complex (Yin and Lazar 2005).
When *Rev-erbα* is artificially overexpressed in the liver of adult mice, the rhythmic transcription of most hepatic circadian genes is abolished, a fact that although has been widely mentioned as a proof of the importance of the local clock mechanism for driving the circadian expression of local genes (Kornmann et al. 2007), is probably overstated because Rev-erbα is a wide transcriptional repressor besides its role in the clock mechanism.

The important involvement of Rev-erbα in metabolism is illustrated in *Rev-erbα*-deficient mice, which display increased plasma triglyceride-rich very low density lipoprotein (VLDL) particles, hepatic steatosis and enhanced the novo lipogenesis (Raspe et al. 2002, Sun et al. 2011). It is

known that genomic recruitment of Hdac3 by Rev-erbα is essential for maintaining the required gene expression of normal hepatic lipid homeostasis (Feng et al. 2011). Additionally, Rev-erbα is involved in the synthesis of bile acids (Duez et al. 2008), the establishment of daily temperature rhythm (Gerhart-Hines et al. 2013), the adipogenic process, and the production of cytokines ((Gibbs et al. 2012); Reviewed in (Duez and Staels 2008)).

PPAR (peroxisome proliferator activated receptor) α

PPARα is considered an important bidirectional link between circadian biology and metabolism. On the one hand, it positively influences the expression of *Bmal1* gene through a potential PPARα response element, with *PPARα* knockout mice presenting a severely blunted rhythm of *Bmal1* expression in the liver (Canaple et al. 2006). On the other, the *PPARα* gene contains several E-box motifs that allow the gene to be positively regulated by the molecular clock (Fig. 5) (Oishi et al. 2005); indeed, *PPARα* is circadianly expressed in liver (Lemberger et al. 1996), heart and soleus muscle (Stavinoha et al. 2004), white and brown adipose tissue (Yang et al. 2006).

In the liver, *PPARα* expression is also stimulated by glucocorticoids, inasmuch that stressing an experimental animal by immobilization during 4 h is able to induce the hepatic expression of *PPARα*, a fact that doesn't happen if it is administered a blocker of the glucocorticoid receptor (Lemberger et al. 1996). However, glucocorticoids seem to be not essential for the circadian rhythm of *PPARα* in the liver, since it is not affected by adrenalectomy in mice (Oishi et al. 2005).

During fasting, when there is an enhanced load of fatty acids from adipose tissue lipolysis to the liver, PPARα is in charge to stimulate hepatic fatty acid oxidation and ketogenesis (Desvergne et al. 2006); the crucial role of PPARα is revealed by the severe hypothermia, hypoketonemia, and increased accumulated lipids in the liver of the knockout mice under fasting (reviewed in (Desvergne et al. 2006)). PPARα is also implicated in other aspects of metabolism, for instance, it has been suggested as an important regulator of insulin sensitivity, although the downstream mechanisms are still not clear; and it also inhibits the production of some inflammatory cytokines by vascular cells (reviewed in (Desvergne and Wahli 1999, Lefebvre et al. 2006, Monsalve et al. 2013).

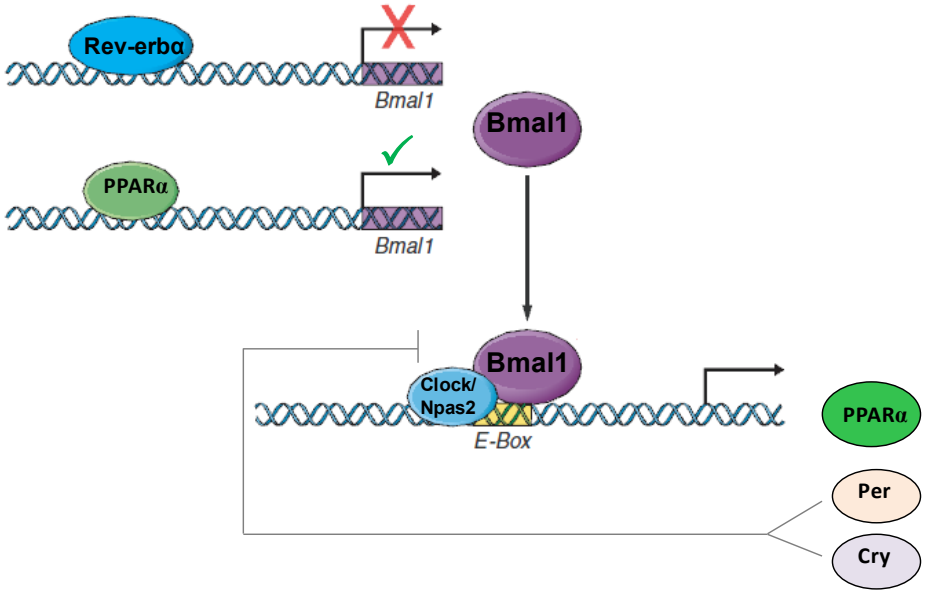

FIGURE 5: Rev-erbα and PPARα form an accessory loop of the clock. The heterodimer formed by Clock(Npas2):Bmal1 drives the expression of *Rev-erbα* and *PPARα*, which in turn are negative and positive regulators, respectively, of *Bmal1* gene transcription. *Modified from: (Eckel-Mahan and Sassone-Corsi 2013).*

Nampt (Nicotinamide phosphoribosyltransferase, also called visfatin and Pre-b-enhancing colony factor –Pbef)

Nampt catalyses the rate-limiting step in the biosynthesis of nicotinamide adenine dinucleotide (NAD^+) through the salvage pathway in mammals (Revollo et al. 2007).
The attention paid to Nampt has grown due to studies demonstrating that two particular enzymatic families, namely the NAD-dependent sirtuin family of deacetylases (involved in lifespan, metabolic diseases and inflammation), as well as the poly-(ADP-ribose) polymerases (PARPs, with a crucial role in DNA-repair), use NAD^+ not as a cofactor but as substrate for the reactions they catalize, requiring this molecule to be resynthesized in order to avoid its cellular depletion.

Nampt is present in several tissues, and the circadian nature of its expression has been confirmed in white adipose tissue, the pituitary gland, muscle, heart, and in the liver (Ando et al. 2005, Dupre et al. 2008, Nakahata et al. 2009, Ramsey et al. 2009, Um et al. 2011). The *Nampt* gene is thought to be target of the molecular clock through E-box sequences present in its regulatory region (Nakahata et al. 2009, Ramsey et al. 2009), suggesting that through this regulation the temporal information may be transmitted to several metabolic pathways that require NAD^+ in the cell (Eckel-Mahan and Sassone-Corsi 2013). This supposition has been enforced by the fact that mice

deficient or with dominant negative mutations of *Clock* and *Bmal1* exhibit constitutively low NAD^+ levels (Ramsey et al. 2009).

A bidirectional relationship of Nampt with the circadian clock was suspected when pharmacological inhibition of Nampt (in cultured cells) resulted in a significantly increased Clock:Bmal1-driven transcription of *Per2* (Ramsey et al. 2009), indicating an inhibitory control over the clock mechanisms exerted by Nampt. Indeed, Nampt was found to participate in a feedback loop to the clock implicating the action of Sirt1, as explained below.

Sirt1

Sirt1 is a NAD-dependent enzyme that deacetylates both histone and non-histone proteins. The non-histone group of substrates is so large that it has been divided in five subgroups: a)transcription factors, such as Foxo1, NF-κB, HIF-1α/HIF-2α, p53 and c-MYC; b) nuclear receptors and factors related with the circadian clock; e.g. Clock, Per2, Pgc1α, PPARγ, FXR, LXR; c) cell signaling molecules, such as STAT3, β-catenin and SMAD7; d) elements related with DNA repair; and e) histone-modifying enzymes (Roth and Chen 2014).

The growing importance of Sirt1 as a therapeutic target is largely based on experimental models in mice demonstrating that Sirt1 overexpression may contribute to mitigate diabetes, liver steatosis, inflammation, neurodegenerative diseases and bone loss (Chang and Guarente 2014). Under normal conditions, Sirt1 has a fundamental role in the orchestration of the physiological response to fasting; for instance, in the liver it interacts with PPARα and activates the coactivator PGC-1α (peroxisome proliferator-activated receptor gamma coactivator 1 alpha) through its deacetylation, resulting in the promotion of lipid catabolism, being these interactions so important that a specific deletion of *Sirt1* in hepatocytes results in the development of steatosis due to the decreased rate in fatty acid beta-oxidation (Purushotham et al. 2009, Wang et al. 2010). Additionally, liver-specific deletion of Sirt1 leads to glucose over-production even in the fed state associated to the impairment in hepatic insulin signaling (Wang et al. 2011).

It was recently proposed that *Sirt1* expression could be directly driven by the circadian clock because *Sirt1* gene promoter has two E-box regions to which Clock and Bmal1 bind, as demonstrated by chromatin immunoprecipitation essays. Additionally, ectopic expression of the clock components in primary hepatocytes increases *Sirt1* mRNA levels; and conversely, knockdown of clock components decreases *Sirt1* (Zhou et al. 2014). However, the circadian nature of *Sirt1* expression is still controversial, because other studies have not found a rhythm in hepatic *Sirt1* mRNA but find it only in its enzymatic activity –a rhythm attributed to circadian levels of NAD^+ by the rhythmic expression of Nampt (Nakahata et al. 2009, Ramsey et al. 2009).

Genetic ablation of *Sirt1* in cultured cells broadens the period of circadian cycles, i.e. it causes earlier onsets of increasing transcription and later decreases, suggesting that Sirt1, through its

histone deacetylase activity, has a role in the periodical silencing that follows a transcriptional peak (Nakahata et al. 2008). In this regard, it is thought that Sirt1 coexist with Clock:Bmal1 in a chromatin regulatory complex that operates on circadian promoters, regulating the sharpness of circadian gene expression. Since the activity of Sirt1 is regulated by NAD^+, and rhythmic NAD^+ levels are attributed to the circadian control of Nampt, this mechanism has been proposed as an important ancillary loop that connects circadian and metabolic pathways (Fig. 6) (Nakahata et al. 2008, Bellet and Sassone-Corsi 2010).

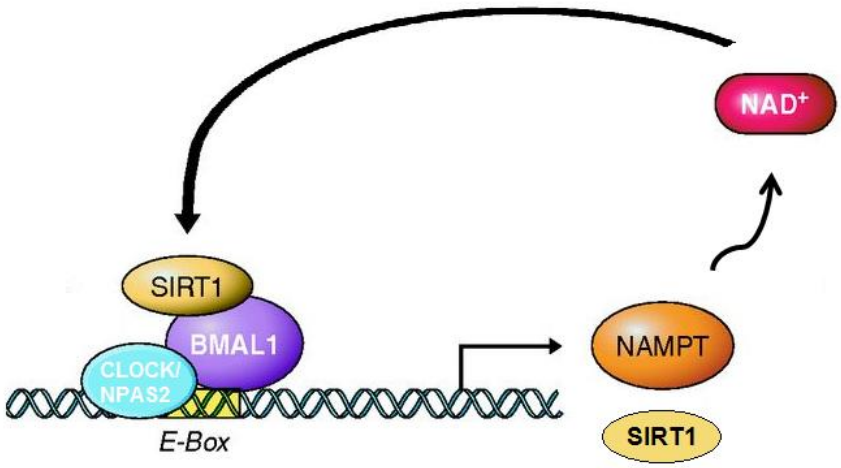

FIGURE 6: A second accessory loop thought to form an important bidirectional link between the circadian clock and metabolism involves Sirt1 and Nampt. The heterodimer formed by Clock(Npas2):Bmal1 drives the expression of *Nampt* and *Sirt*1; Nampt, in turn, favors the circadian production of NAD^+, which acts as a positive regulator of Sirt1 activity. Sirt1 sharpens the transcriptional circadian peaks by promoting the periodical silencing through its histone deacetylase activity. *Modified from: (Eckel-Mahan and Sassone-Corsi 2013).*

Up till now, only the connections between the molecular clock and these metabolic genes have been described, but one must come back to the aspect regarding whether the SCN is involved in the induction of these peripheral oscillators.

2) Reliance of peripheral clocks from the main clock.

After having discovered that a) clock genes are expressed in a circadian manner not only in the SCN but also in peripheral tissues, and b) that additionally a significant percentage of tissue-specific transcripts are also circadian and many of them depend on an integral local clock, it came into question whether the master pacemaker located in the brain, the SCN, has something to do in imposing a rhythm to those peripheral clocks, and how it could accomplish that.

It was found that bilateral lesions of the SCN completely destroyed or severely attenuated the cyclical expression of virtually all circadian genes in an organ taken as a sample, the liver (Fig. 7) (Sakamoto et al. 1998, Akhtar et al. 2002).

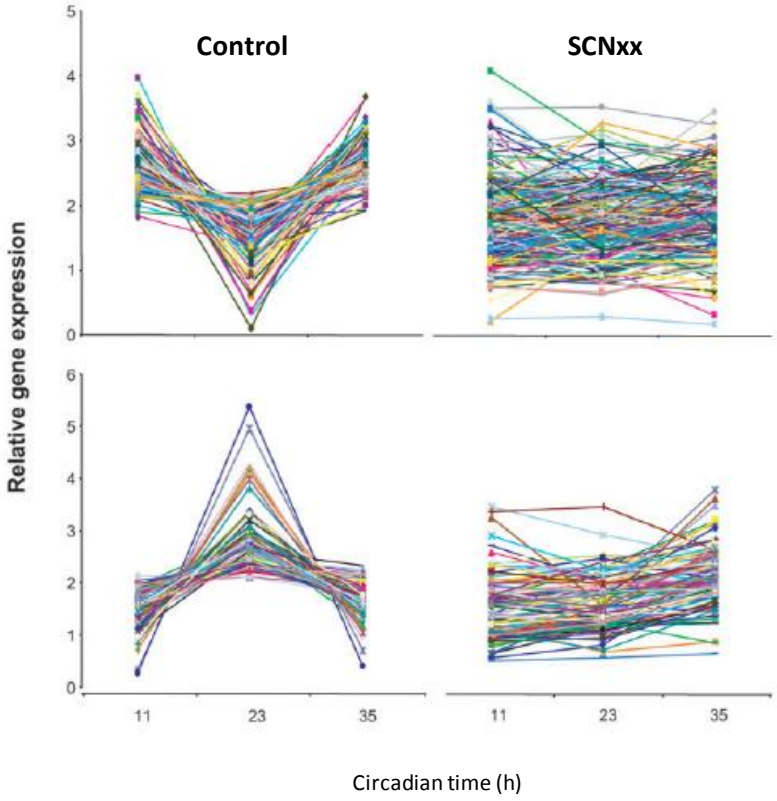

FIGURE 7: The SCN is largely responsible, either directly or indirectly, of driving circadian expression in the liver. Microarray analysis revealed that oscillations in transcript levels (samples taken at three temporal points; each gene represented by a different line color) found in the liver of intact animals (left panels) are lost in animals with bilateral SCN lesions (right panels). *Modified from: (Reddy et al. 2007).*

Besides that, and considering that tissue-specific disruption of the molecular clock leads to the loss of circadian oscillations in most local transcripts (Lamia et al. 2008, Young et al. 2014), then a model could be drawn where the SCN at the top, synchronizes organ/tissue clocks using endocrine

and/or neural pathways, and these local clocks in turn, are in charge of imposing a temporal order in the transcriptional profile of each tissue.

Among the proposed pathways that can be used by the SCN for this synchronizing purpose are: hormones (glucocorticoids, in specific), the activity of autonomic branches, temperature, and behavior. In the next paragraphs the main findings regarding the association of these pathways with the tissue clocks will be summarized.

2.1) Glucocorticoids

Early studies that found circadian oscillations in clock gene expression induced by application of high concentrations of serum to cultured fibroblasts (Balsalobre et al. 1998), suggested that a humoral component secreted in a daily basis could be one of the means by which the SCN could 'tell the time" to peripheral organs. Strong candidates for this were the glucocorticoids because it is well known that corticosterone (in rodents, cortisol in humans) has a marked circadian rhythm that is dependent on the SCN (Moore and Eichler 1972).

The first experiments aimed to know whether glucocorticoids affected circadian clock gene expression were done, again, in cultured fibroblasts. Schibler and colleagues (Balsalobre et al. 2000, Balsalobre et al. 2000) noticed that 1h pulse of dexamethasone in the culture media induced circadian oscillations in the expression of *Per1*, *Per2*, *Cry1*, and *Rev-erbα* in those cells. After that, these researchers designed an *in vivo* experiment, intending to see the effects of dexamethasone when injected to mice at different hours of day; and found that it induced modest transient changes of phase in some circadian genes in liver, kidney, and heart. They repeated the experiment in transgenic animals devoid of the glucocorticoid receptor in the liver and compared the effects of dexamethasone with control vehicle injected animals. Unexpectedly, the more interesting results came from the vehicle injected receptor-deficient animals, given that the accumulation cycles of *Per1*, *Per2*, *Cry1*, and *Rev-erbα* in the liver of these animals were the same than in wild-type mice, leading to the conclusion that glucocorticoids cannot be the only signals setting the phase of these peripheral clocks under normal conditions, as stated as it by the authors (Balsalobre et al. 2000). Analogous findings were reported later in adrenalectomized mice, in which the hepatic expression of the core clock component *Per2* and of some clock-controlled output genes, such as *Dec1*, *Dbp*, and *E4bp4*, is not affected by the lack of adrenal hormones (Oishi et al. 2005), supporting the idea that endogenous glucocorticoids are not essential for the circadian expression of these clock-controlled genes. (Interestingly, despite adrenalectomy did not affect the expression of clock genes, it abolished the circadian expression of other clock-controlled genes, including *Nampt* (Oishi et al. 2005))

The modest effects of dexamethasone obtained when injected to mice, in contrast to the clear effects seen in vitro, were proposed to be caused by the existence of other opposing signals arising from the SCN. Therefore, the group of Hastings (Reddy et al. 2007) lesioned the SCN in a

group of mice and administered them dexamethasone, looking for its effects in the hepatic expression at 5, 17, and 29 h after the treatment. Although the authors state that the synthetic glucocorticoid synchronized the expression of the clock and other genes in the liver, a careful analysis of their results reveals that the drug increases the expression of *Per1* and *Bmal1* simultaneously (when they are supposed to be antiphasic due to the clock mechanism itself), raising the question about whether the effects represent a truly synchronizing effect of the glucocorticoid over the clock.

2.2) Autonomic nervous system

A multisynaptic autonomic pathway from the SCN to various peripheral organs such as the liver, heart, pancreas, and adrenal cortex has been demonstrated using viral tracing techniques. Thus there exist the anatomic substrate by which the SCN through the autonomic nervous system may regulate the circadian expression of genes in peripheral organs (Buijs et al. 2003).

The concentration of noradrenaline in liver exhibits a circadian rhythm; rhythm that is lost upon ablation of the SCN (a maneuver that also abolishes the rhythm of the hepatic clock genes (Terazono et al. 2003). Interestingly, daily injections of adrenaline in SCN-lesioned mice are able to induce oscillations (although smaller in amplitude than intact animals) in *Per2* and *Bmal1* expression (Terazono et al. 2003), suggesting that a rhythm in the local clock may be supported by the activity of the sympathetic nervous system.

The relation between sympathetic activity and clock gene expression was further supported by experiments in which electrical stimulation of the hepatic branch of the splanchnic nerve increases the expression of *Per1* in the liver, and conversely, mice treated with 6-hydroxydopamine for depletion of the sympathetic nerves have a severely attenuated rhythm in *Per1* and *Per2* (although the involvement of other factors cannot be ruled out, as these animals have reduced their food intake to half the normal, and also their locomotor activity is reduced to nearly one third of a normal animal) (Terazono et al. 2003).

However, there are some evidences that do not favor the involvement of the autonomic nervous system in establishing the circadian rhythm in peripheral clock genes. For example, hepatic specific sympathectomy modifies the rhythm of *Cry1*, especially during the light phase, but does not affect *Per1* rhythm and causes only minor phase changes in *Per2* (Cailotto et al. 2005). The same for other organs: sympathetic denervation of the spleen with guanethidine increases *Per2* expression but without affecting the overall rhythm of this gene (Logan et al. 2011). Additionally, mice under pharmacological blockade of both autonomic branches by administration of atropine plus propranolol, preserve the rhythmic expression of *Bmal1* and *Per2* in the heart, although it is significantly dampened, suggesting that the ANS is not completely responsible of establishing the timing of the clock (Tong et al. 2013).

2.3) Temperature

In mammals, body temperature exhibits circadian fluctuations that are under the control of the SCN (Morf and Schibler 2013).

Initial studies in a cellular line of fibroblasts (Brown et al. 2002) showed that although the normal physiological cycles in temperature are not able to engender spontaneous rhythmicity in the clock genes in these cells, these cycles can help to slow the dampening of already existing rhythms in the absence of any other rhythmic cue. Additional research showed that the circadian rhythm of *Per2* in cultured explanted tissues can be modified by different cycles of environmental temperature, and it was suggested that this effect could be mediated by the transcription factor Heat Shock Factor 1(HSF1), (which is often recognized for organizing the response to a heat stress but also can be influenced by other cues) through its action on heat-shock response elements present in the regulatory region of *Per2* (Kornmann et al. 2007, Buhr et al. 2010).

It is important to note that in response to a change in temperature cycles (in cultured fibroblasts), the expression of *Bmal1* is adapted within 3 to 4 days, while the phase of *Per2* occurs earlier, thus probably *Per2* may serve as a synchronizing gene for the rest of the clock machinery, given that after 3-4 days, the normal phase relationship between the clock genes is established and conserved thereafter (Saini et al. 2012).

2.4) Feeding-fasting cycles

In high order animals, the foresight of periods of fasting and foraging associated to the daily availability cycles in the environment probably favored the establishment and conservation of anticipatory mechanisms for a better use of exogenous and endogenous nutrient reserves. With this in mind, it was proposed that in mammals, the circadian clocks present in organs majorly involved in the metabolism of food components (such as the liver) may have a major role in the preparation of the organ for these processes; however, this usefulness of the clock should be also flexible to some extent in order to adapt to new circumstances of food accessibility. This rationale motivated Schibler and colleagues fifteen years ago to determine the changes in circadian expression of peripheral clock genes when animals are forced to change their feeding schedule by allowing the access to daily food only during the 12 hours of the resting phase (which in nocturnal rodents corresponds to the light phase). Those experiments demonstrated that whereas a number of clock genes completely reverse their phase in the liver -compared with animals fed during their active phase, the rhythm of clock genes in the SCN remains unchanged, indicating that the feeding schedule is such a powerful stimuli for peripheral oscillators that even can cause a decoupling of them with the main clock (Damiola et al. 2000); additionally, these findings suggested that the SCN may coordinate at least part of the peripheral rhythms through its role as the pacemaker for feeding behavior (Patton and Mistlberger 2013). This was also suggested in 2001 when Shibata's group reported that in animals devoid of SCN, the restriction of food intake (i.e. food available

only during 6 h/day) caused that the arrhythmic expression of liver *Per1* and *Per2* regained its usual daily pattern, as judged by the two temporal points sampled (Hara et al. 2001). However, the shorter the restriction the more other elements such as negative metabolic conditions start to play a role (see e.g. (Mendoza et al. 2005)).

With regard to the explanation of how feeding/fasting cycles could impact the molecular clock, it has been proposed that the feedback loops formed by the metabolic genes noted above may have an important role; in addition to other mechanisms such as the phosphorylation of some clock components by enzymes like the AMP-activated protein kinase (AMPK), changes in the redox state (that are suggested to affect Clock(Npas2)/Bmal1 binding to DNA, at least in vitro), and/or indirectly through changes in body temperature induced by the pattern of food intake (reviewed in (Asher and Schibler 2011).

DEFINITION OF THE PROBLEM

Changing the feeding schedule to the resting phase in experimental animal models causes several metabolic derangements, such as liver steatosis, glucose intolerance and obesity. The explanation of how this happens is still under investigation, but it is believed that it can be triggered by an internal state of desynchronization; in this vein, some groups have reported desynchronization between organs under these conditions because not all organs are equally responsive to the feeding-fasting associated cues (Bur et al. 2010, Bray et al. 2013, Reznick et al. 2013). Our group has further investigated the effects of changing the feeding schedule to the resting phase in the liver of rats and found that, even though the hepatic clock genes such as *Bmal1*, *Clock*, and *Per1* reverse their phase, the rhythm of some metabolic genes connected to the clock, namely *PPARα* and *Nampt*, is lost, leading to a state of desynchronization within the liver itself (Salgado-Delgado et al. 2013).

The fact that these CCG's, which are considered key bidirectional links between the circadian clock and metabolism, lose their rhythm or are severely blunted even though the core components of the clock remain rhythmic led us to hypothesize that there may be a 'confrontation' between pathways from the SCN and cues related to feeding and fasting cycles. This may influence the expression of these CCG in opposite directions (i.e. at different time of the day), leading to a reduction in the amplitude of their rhythmic expression. Moreover, it may be that some of the clock–metabolic interrelationships that are largely based on in vitro or in transgenic animal models are not adequate under certain conditions in vivo.

SCOPE

The purpose of this thesis was to evaluate whether a feeding schedule similar to that that occurs under physiological conditions is truly able to induce a rhythm in the hepatic clock and associated metabolic genes in animals devoid of any interfering signal coming from the SCN.
Additionally, in order to know whether the effects of the feeding-fasting cycle are 'acute and similar to an hourglass' or whether they promote an oscillatory process that could subsist at least for the next cycle, we examined the temporal expression of these genes in a parallel group of SCN-lesioned fed-scheduled animals that remained fasted during 24h before sacrifice.

Here it is important to point out why lesioning the SCN is the only method to completely eliminate the influence of the SCN. In the next paragraphs we discuss some alternative methods and explain why they were less appropriate to solve our task.
It is known that maintaining rats under constant light for 3-4 weeks induces a "functional lesion" of the SCN because it abolishes overt SCN-driven rhythms such as those of locomotor activity and food intake. However this condition does not disrupt the ability of SCN cells to oscillate; it desynchronizes these cells among each other, probably rendering the SCN unable to generate certain synchronized circadian signals to the rest of the body (Ohta et al. 2005, Polidarova et al. 2011). However, under these conditions the rhythm of some clock genes is still present in the liver, suggesting that constant light might "not compromise all SCN output pathways to the same degree and some of the remaining rhythmic cues may still be able to synchronize the peripheral clocks", as suggested by Polidarova and colleagues (Polidarova et al. 2011).

Other options include the pharmacological inactivation of the SCN by directly applying agents such as tetrodotoxin (which blocks most types of neuronal sodium channels) or a local anesthetic (such as procaine or lidocaine) in order to prevent initiation and transmission of action potentials (Ulbricht 2005). Tetrodotoxin indeed is a powerful tool in neurobiology to investigate circuits involved in determined task or behavior; however, its effects are reversible and last only from minutes to few hours depending on the dose (anesthetics usually are even of shorter duration (Gallo 2007)). As our feeding schedule protocol takes three to four weeks, we would require a specialized system in order to accomplish constant delivery of the drug during all this time; in addition, the longest (to our knowledge) chronic experiment of TTX infusion into the SCN, where the drug was continuously provided during 14 days, did not completely abolish the circadian rhythm that the authors tested (drinking behavior) under light-dark environmental conditions (Schwartz et al. 1987). Because in rodents the drinking and eating behaviors usually occur in parallel, this certainly could be a drawback for our protocol.

Finally, a chronic infusion of an inhibitory agent into the SCN can be used, either a GABAergic agonist or a glutamatergic antagonist; however, the chronic effects of these substances on the pacemaker and its downstream circadian functions are not fully described yet. While even there are papers suggesting that GABA has excitatory effects in the SCN at determined times of the day

due to changes in the intra-extracellular chloride gradient, an effect thought to be mediated by diurnal changes in cotransporter proteins (Choi et al. 2008).

Therefore, lesioning the SCN is the most secure method in order to remove all SCN-related influences, avoiding the risk of leaving a remaining circadian cue. This procedure including the use of a physiological feeding-fasting schedule would allow us to investigate the effect of food on metabolic and clock genes in the liver.

HYPOTHESIS

1) A 12h/day feeding schedule will be able to induce a circadian rhythm in the expression of clock genes *Per1*, *Per2*, *Cry1*, *Bmal1* and *Npas2*, as well as in their associated metabolic genes *Rev-erbα*, *PPARα*, N*ampt* and *Sirt1* in the liver of SCN lesioned rats.

2) Given the robust associations proposed for these clock-metabolic interlocked loops, we hypothesize that the induced rhythmic expression would at least be able to persist for a cycle under constant conditions (fasting) in these SCN lesioned animals.

In the next chapter is presented our article entitled “Food entrains clock genes but not metabolic genes in the liver of suprachiasmatic nucleus lesioned rats”, which includes the materials and methods utilized to solve our hypothesis, as well as the results and a brief discussion. An expanded discussion is presented in Chapter 3.

Because this thesis is part of a large body of research that aims to disentangle how circadian rhythms are established, and how their synchronization is important for the conservation of health, we included in Chapter 4 an essay about temporal control of metabolism at whole body level -with focus on lipid metabolism, describing how it is achieved through hormonal, neural and behavioral daily cycles organized by the SCN; there we do propose that circadian imbalance of those regulatory systems may lead to diseases such as non-alcoholic fatty liver.

Finally, our general conclusions are summarized in Chapter 5.

CHAPTER 2.

Food entrains clock genes but not metabolic genes in the liver of suprachiasmatic nucleus lesioned rats.

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INTRODUCTION

In mammals, the Suprachiasmatic Nucleus (SCN) synchronizes behavior and physiology to prepare for rest and activity associated with the light-dark cycle. The molecular machinery in charge resides in the so called "clock genes", whose protein products form intertwined feedback loops inducing mRNA and protein levels to oscillate with a near 24-hr period. At the core of this circadian oscillator in mammals are the *Period* (*Per*) and *Cryptochrome* (*Cry*) genes, whose transcription is favored by the heterodimer Clock (or Npas2): Bmal and negatively controlled by their own protein products, Per and Cry. Other important loops interacting with the core mechanism include the *Ror*, *Rev-erb* and *PPARα* genes, thought to give feedback and stabilize the core loop, but also to serve as major output regulators (Canaple et al. 2006, Stratmann and Schibler 2012). Clock genes also oscillate in peripheral tissues including the liver, heart, lung and skeletal muscle (Zylka et al. 1998, Yamazaki et al. 2000), and are proposed to drive oscillating functions within the cell. For example, nearly 90% of circadian transcripts in the liver lose their rhythm or are attenuated when the hepatocyte clocks are functionally inactivated (Kornmann et al. 2007). Moreover, it is known that also animals with SCN lesions lose the coordinated circadian rhythmicity of peripheral gene expression (Akhtar et al. 2002), leading to a hierarchical picture in which the SCN synchronizes via behavioral, humoral and/or autonomic pathways the peripheral clock genes, while these in turn sustain circadian physiology of each organ.

This view has been complemented by a number of studies directing attention to the importance of food for the rhythmicity of these peripheral clocks, particularly those of the liver. When the feeding schedule is inverted, *i.e.*, when animals are fed only during their resting period, the phase of the core clock genes in the liver follows food intake, even though the SCN does not change its activity pattern (Damiola et al. 2000). Mainly *in vitro* studies have demonstrated reciprocal connections between clock genes and many metabolic genes and proteins, including nuclear receptors and components of the redox system of the cell (reviewed in (Albrecht 2012)). These observations suggest that these interrelated loops could underlie the role of food as a main synchronizer for the liver, using the local clock genes to organize the rhythmicity of the metabolic profile.

Recent studies in our laboratory showed that feeding during the rest period indeed inverts the phase of *Per1*, *Clock* and *Bmal1* in the liver; however, surprisingly, the rhythmicity of some of the metabolic genes connected to the clock, namely that of *PPARα* and *Nampt*, was lost (Salgado-Delgado et al. 2013). These observations led us to hypothesize that some of the clock-metabolic interrelationships may not be adequate to drive the hepatic circadian motor under certain in vivo conditions.

In order to investigate the contribution of food for the rhythm of metabolic genes without the involvement of the SCN, we examined whether 24 hour cycles of feeding-fasting in SCN-lesioned animals are able to induce a rhythmic expression of clock genes and related metabolic genes in the liver, and whether this expression persists in fasting conditions.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (250–300 g) were housed individually in acrylic cages, under a 12:12 h light–dark cycle (lights on at 8:00 a.m.), with free access to water and constant conditions of humidity and temperature. Experiments were approved by the Ethics Committee of the Universidad Nacional Autónoma de México, in strict accordance with the Federal Regulations for Animal Care and Use (NOM-062-ZOO-1999).

Suprachiasmatic nucleus lesion

Bilateral electrolytic SCN lesions were performed stereotaxically under ketamine/xylazine (60:6 mg/kg body weight) intramuscular anesthesia. Constant current of 0.4 mA for 1 min was delivered through stainless steel electrodes into the SCN area. Coordinates from Bregma were as follows: 0.3 mm posterior, 8.0 mm ventral and 0.9 mm lateral, under an angle of 4°. After surgery, all animals had free access to water and food, and general activity was monitored as described below for at least 3 wk; after this time, only arrhythmic animals as determined by X2-periodogram analysis in the range of 15-30 h were subsequently used for the experimental procedures (Fig. S1). General activity was continuously recorded with movement sensors placed under individual cages. Behavioral events were collected at 1-min intervals with a digitized system and automatically stored. Analysis was carried out with data accumulated in 15-min intervals. For each rat, double-plotted actograms were obtained with SPAD9 analysis based on Matlab (Fig. S2). All experiments were done under 12:12h light-dark cycle (lights on at 8:00 a.m) to avoid possible metabolic changes associated with constant darkness conditions and known to affect circadian gene expression (Zhang et al. 2006, Zhao et al. 2011).

The completeness of the SCN-lesion was also evaluated by post-mortem analysis of vasoactive intestinal peptide (VIP) by immunohistochemistry. After being fixed by immersion in 4% paraformaldehyde at least for 72 hours, brains were transferred to a 30 % sucrose solution for 3 days for cryoprotection. Brains were frozen and the hypothalamic region was cut in 40 µm coronal

sections that were treated for 10 min in 0.5 % H2O2 and successively incubated with 1:2000 rabbit anti-VIP primary antibody dilution (Millipore), 1:200 biotinylated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch), avidin-biotin-peroxidase 1:500 complex (Vector laboratories), and the reaction was revealed with 3,3 diaminobenzidine tetrahydrochloride. Only the animals with complete absence of VIP immunoreactivity in the SCN area were selected for hepatic gene expression and posterior statistical analysis (Fig. S1).

Core body temperature recordings

At least five days before starting the feeding protocol, a series of rats (n=2-3 per group) underwent surgery to insert intra-abdominal temperature sensors (iButton Sensor-Temperature Logger; Maxim Integrated Products, Dallas Semiconductor, Dallas, TX). Sensors were programmed for collecting temperature data every 30 min for the total experimental interval. At the end of the protocol rats were overdosed with sodium pentobarbital, the iButtons were extracted and temperature data collected for analysis.

Data were organized by groups and represented as a daily average wave for each week of the experiment.

Restricted feeding

After verifying their arrhythmicity in locomotor activity, twenty five SCN-lesioned rats (SCNx) were randomly assigned to one of two groups: Ad libitum fed (SCNx-AL; n = 6) with free access to commercial standard chow food (Harlan Teklad); or Restricted Fed (SCNx-RF; n = 19), with food provided exclusively during 12 h/day either during the light or during the dark phase (see Temperature Results) for 3 weeks. Given that SCNx animals do not show any rhythmic behavior, we assumed the major timing cue to be the presence/absence of food. Therefore we named T0 (time zero) the moment at which food was delivered, and 12 h later T12 the moment when it was withdrawn. The intact rats were exposed to the same feeding schedule with access to food only during the 12-h of the dark phase for 3 weeks (Intact-RF; n = 25). The nomenclature for food was the same as for the SCN-lesioned rats.

Tissue collection

At the end of the third week of the restricted feeding protocol, intact rats were randomly assigned and sacrificed at 6-h intervals, beginning at time ‘T0’, i.e., immediately before food was given (n =3 per temporal point, except for T0 with n = 4). Half of the Intact-RF group received the 12 h food as usual while the other half did not receive it on the day of sacrifice in order to investigate persistence under fasting conditions. The SCNx-RF rats were also sacrificed at T0 (n = 6), T12 (n = 5) and T18 (n = 2), having received food on the day of sacrifice, and at T12 (n = 6) under fasting. The SCNx rats that were fed ad libitum since the beginning of the experiment (SCNx-AL) were sacrificed either in their fed state (n = 3) or after being fasted for 24 h (n = 3) for control purposes (Table S3). Rats were euthanized with an overdose of sodium pentobarbital (65 mg/ml) and part of the left lobule of the liver was quickly removed, frozen on dry-ice, and stored at _80 _C. The brain

was removed, placed in 4% paraformaldehyde, and processed for histological verification of the lesion.

RNA extraction and semi-quantitative RT-PCR

Total RNA was extracted from 50 – 100 mg of frozen liver samples with Trizol (Invitrogen) according to the manufacturer's instructions. RNA integrity was verified by visualization of the 28S and 18S bands with an approximate 2:1 ratio on agarose gel (1.25 %) electrophoresis stained with ethidium bromide. Purity was confirmed by measurement of the 260/280 nm absorbance ratio. All RNA samples used for subsequent analysis had an optic density >1.9 (Nanodrop ND-1000 Spectrophotometer, Thermo Scientific Inc). 4.5 µg of RNA in a final reaction volume of 20 µl were reverse transcribed with oligo(dT) primers using the SuperScript III First-strand synthesis kit (Life Technologies). 100 ng of cDNA were used as template for PCR in a 10 µl/reaction volume containing 1x PCR Buffer (Green GoTaq Flexi Buffer, Promega), 2.0 mM MgCl2, 0.2 mM dNTP mixture, 0.5 µM forward/reverse specific primers (Sigma-Aldrich, listed in Table S1) and 0.25 u of Taq DNA polymerase (GoTaq Flexi DNA Polymerase, Promega). PCR was performed on a T100 thermal cycler (Bio-Rad) using the following conditions: 94°C (5 min) followed by the number of cycles specified in Table S1 at 94°C (30 s), annealing temperature (45 s) and 72°C (60 s), concluding with 1 min at 72°C. PCR products were electrophoresed on ethidium bromide-stained 2 % agarose gel and analyzed on a Personal Molecular Imager FX system with Quantity One software (Bio-Rad); relative gene expression was determined by normalization to *β-actin.* The expression of each gene for the Intact-RF group sacrificed at T0 was set as one.

Statistical analysis

Data are expressed as means ± S.D. The gene expression temporal profiles were analyzed by one-way ANOVA; comparisons between the intact and the SCNx groups were done by two-way ANOVA with post hoc Holm–Sidak analysis. Differences between only two groups were analyzed by an unpaired two-tailed Student t-test. Results were considered statistically significant with $P < 0.05$.

RESULTS

Temperature fluctuations are induced by physiological feeding/fasting cycles in SCNx animals.

Body temperature is dramatically affected by daytime food schedule in intact rodents (Damiola et al. 2000) and is considered one of the means by which restricting feeding may influence peripheral clocks (Mohawk et al. 2012). Our recordings revealed that core body temperature oscillations with peak to trough differences of more than one centigrade were induced by the 12-h feeding scheduled in otherwise arrhythmic SCNx animals, resembling the profile seen in the pair-fed intact animals (Fig. 8). Feeding-time related increment in temperature seems to be anticipatory rather

than reactive, as it begins to rise nearly 2 h before food delivery. A concomitant daily low-amplitude oscillation in locomotor activity was also induced in the food-scheduled SCNx rats, although this activity did not anticipate the coming food as clearly as the temperature (Fig. S2).
Just as for temperature, the overall analysis of gene expression was also similar in the SCNx animals fed either during the light or the dark phase; thus they are grouped together in the gene analysis for simplification purposes.

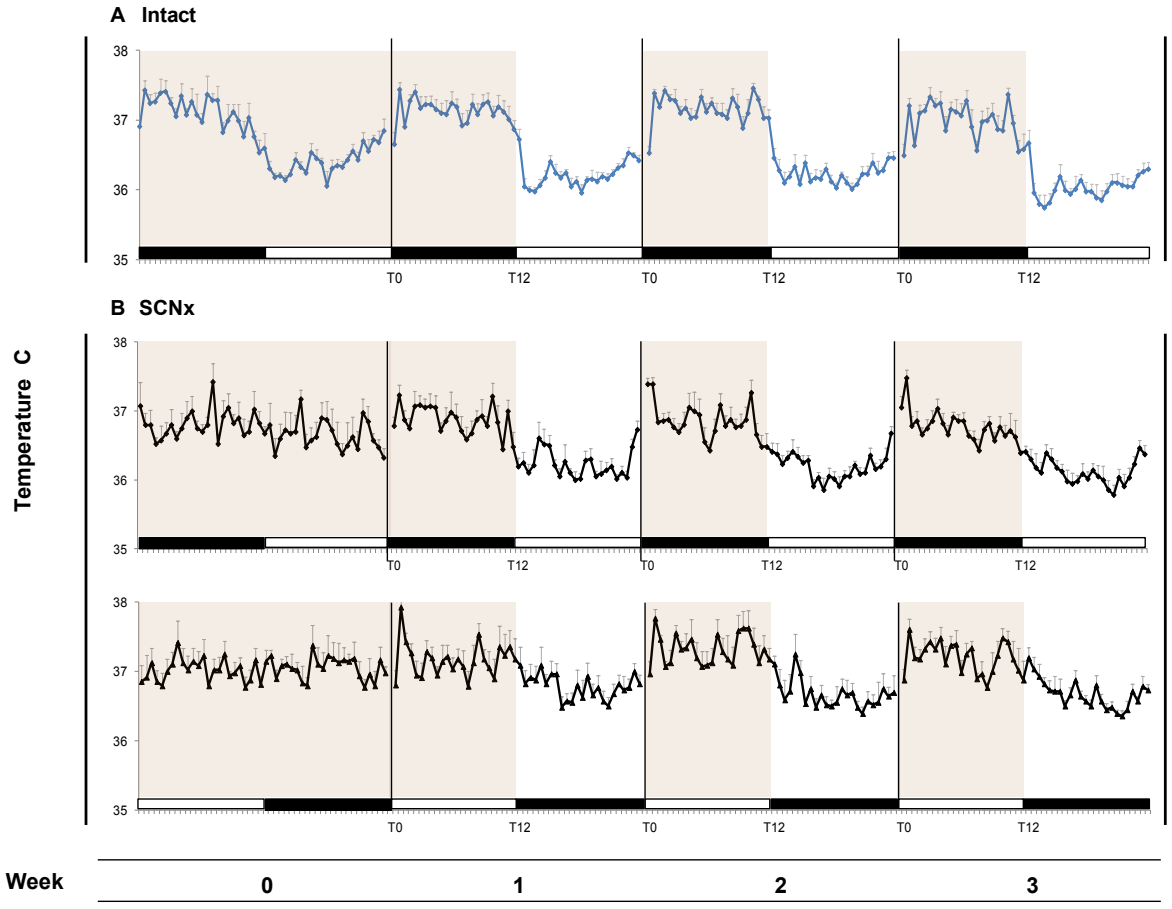
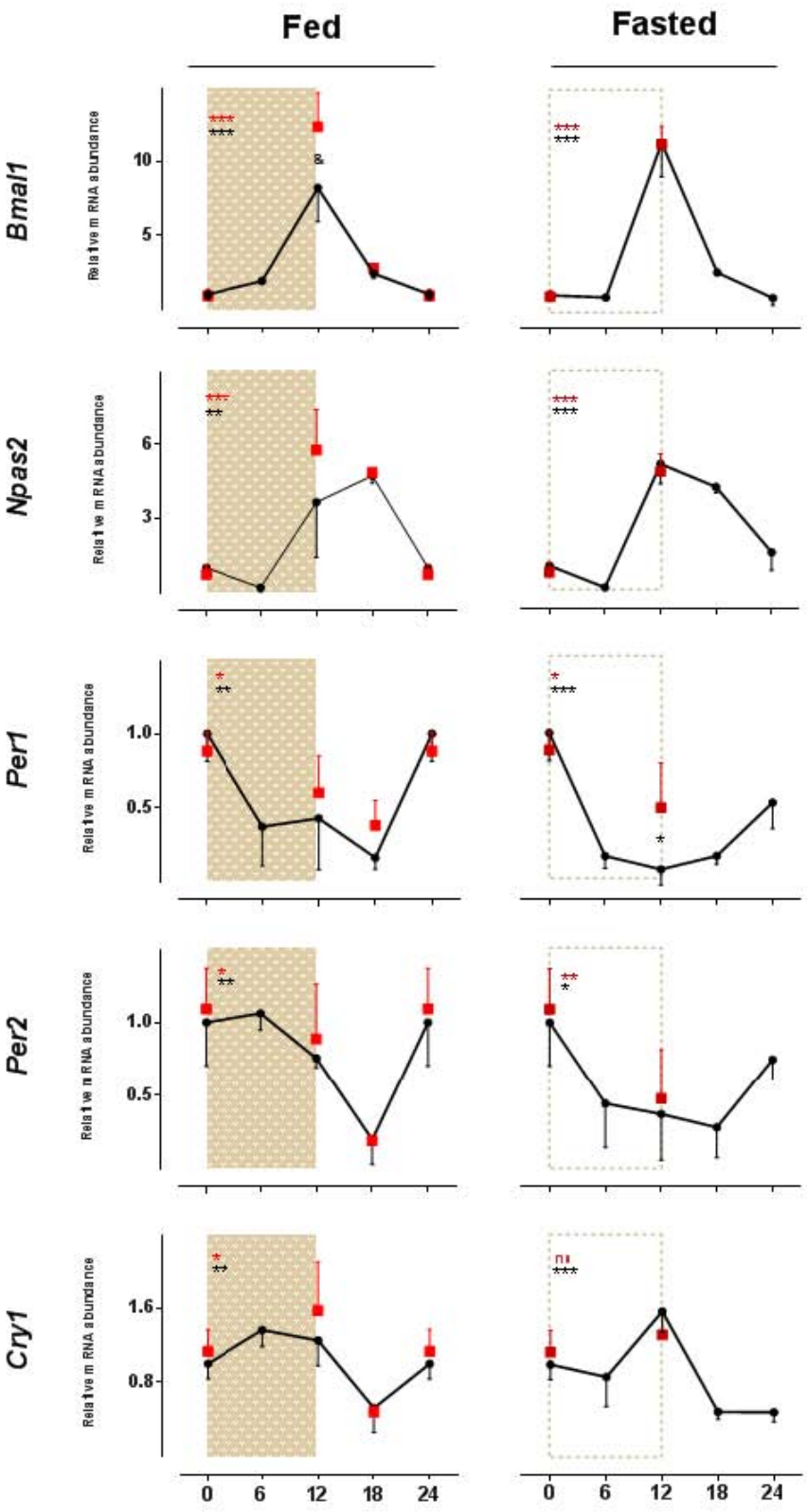


FIGURE 8: Daily core body temperature fluctuations are induced by a physiological feeding schedule in SCN-lesioned rats.
Daily temperature averages (± SD) per week are presented in each box for representative individual A) Intact, food restricted, B) SCNx, with food restricted to either the dark (top) or light (bottom) phase. Feeding periods are indicated by the shaded areas in each box and photoperiod is represented by the white/black bars in each panel. Number 0 represents the ad libitum baseline period, and 1, 2 and 3 the subsequent 12h/day food-scheduled weeks. The abscissa indicates the time at which food was delivered (T0) and when it was withdrawn (T12).

Clock genes in the liver of food-scheduled SCNx animals respond to food and show persistence.

In the intact-RF animals, the diurnal expression of *Bmal1, Npas2, Per1*, *Per2* and *Cry1* was confirmed both in the fed as well as in the fasting state. Significant temporal changes in these genes were also present in SCNx-RF rats sacrificed in the fed-state (Fig. 9). Importantly, also in the SCNx animals these differences were conserved under fasting conditions, at least for *Bmal1, Npas2, Per1*, and *Per2*, demonstrating that this similar-to-physiological feeding schedule in the absence of the master clock was able to induce a rhythm in the peripheral molecular clock. A lack of time-effect difference in the expression of *Cry1* in the SCNx-RF fasted animals could be due to limited sampling, which did not enable us to construct a complete profile (statistical results summarized in Table S2).

FIGURE 9 (next page)**: Rhythmic clock gene expression persists under fasting conditions in the liver of food-scheduled intact and SCN-lesioned rats.**
Mean +/- SD normalized expression of *Bmal1*, *Npas2, Per1*, *Per2* and *Cry1* in the liver of intact-RF (black circles with continuous line) and SCNx-RF rats (red squares). Feeding schedule is represented by the shaded areas. The abscissa indicates the time of day referred to food presentation (in hours), where 0 is the moment when food was delivered. Gene expression was evaluated either in rats which received their food on the day of sacrifice (Fed) or which were fasted that day (Fasted). In the fasted group, 0 indicates the normal time point where animals were fasted for 12 hours; consequently at 24 they were fasted for 36 hours. Each group was analyzed by one-way ANOVA for the effect of time followed by *post hoc* Holm-Sidak test, except for the SCNx-fasted groups that were analyzed by Student's t-test. Statistically significant differences within each group are indicated by the asterisks at the upper left of each graph; black for the intact group and red for the SCNx. **P*<0.05, ***P*<0.01 and ****P*<0.001. Differences between the intact and the SCNx groups were analyzed by two-way ANOVA, with **P*<0.05 and &*P*<0.01 at the specific time point. For clarity purposes, T0 values are re-plotted at T24 in the Fed graph.


Fed
Fasted
Bmal1
Npas2
Per1
Per2
Cry1
Relative mRNA abundance
10
5
6
3
1.0
0.5
1.6
0.8
0
6
12
18
24

SCN-independent expression of *Rev-erbα* and *PPARα* in the liver.

Rev-erbα and *PPARα* are considered important links between the circadian and metabolic systems. Both are clock output genes and provide feedback by regulating the expression of *Bmal1*; additionally, *Rev-erbα* could also block the action of transcriptional activators on *Npas2* (Crumbley et al. 2010). Our results show that *Rev-erbα* expression was strongly rhythmic and preserved the same profile under fasting conditions both in intact and SCNx animals (Fig. 10).

Likewise, *PPARα* exhibited significant diurnal fluctuations in both the intact and SCNx groups sacrificed under fed condition. However, although under fasting the differences over time were maintained, the expression pattern was moderately changed, probably indicating a competition between fasting-derived homeostatic signals and the liver clock ($F(4,23)=2.937$; $P=0.043$ for interaction between time and sacrifice condition by two-way ANOVA in the intact; $P<0.05$ by t-test for Fed-Fasted difference at T12 in SCNx).

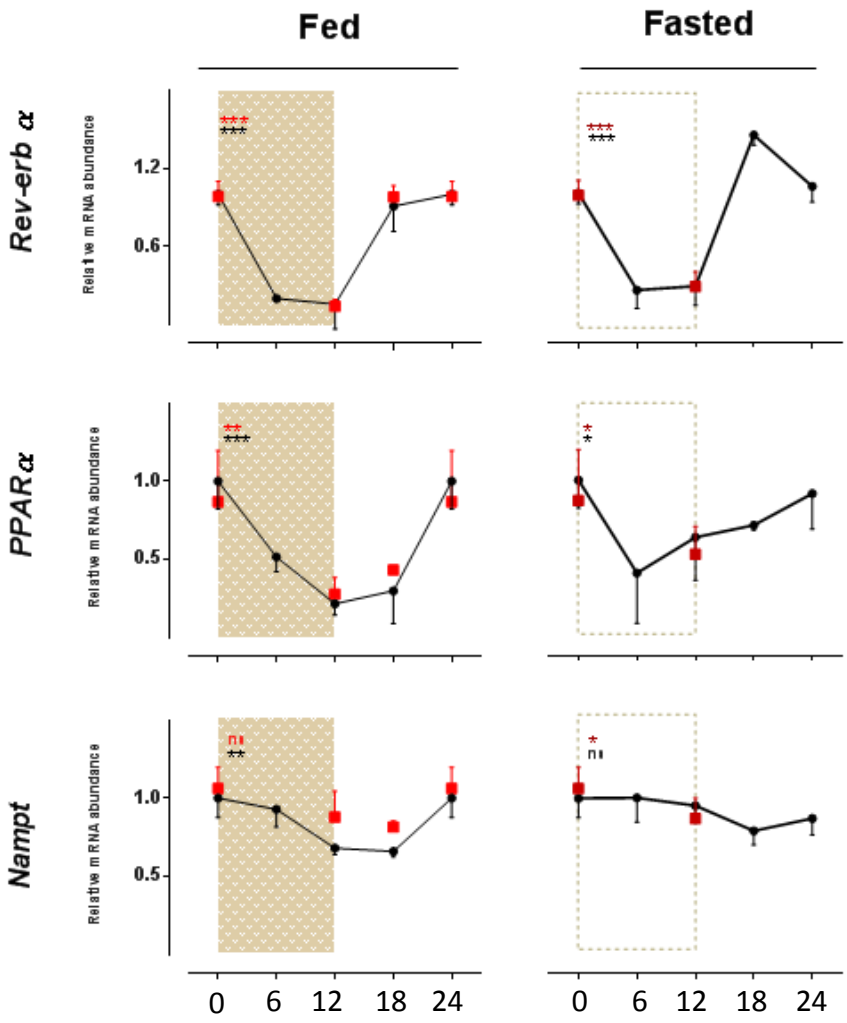


FIGURE 10: Clock-associated metabolic genes are differentially affected by the feeding status.
Mean +/- SD normalized expression of *Rev-erbα*, *PPARα* and *Nampt* in the liver of intact-RF (black symbols with continuous line) and SCNx-RF rats (red squares) See Figure 9 for details. Time-effects of each group on the fed or fasted state were evaluated by one-way ANOVA followed by post hoc Holm-Sidak test, except for the SCNx-fasted groups in which Student's t-test was used; **P*<0.05, ***P*<0.01 and ****P*<0.001.

Only together with the SCN can the metabolic state influence hepatic *Nampt* expression.

Nampt has been reported to be a direct target of the molecular clock via the E-boxes present on its promoter (Nakahata et al. 2009, Ramsey et al. 2009), while the encoded protein also gives feedback, mainly through its product of biosynthesis, the nicotinamide adenine dinucleotide (NAD). In our experiment, the expression of genes from the positive limb as well as some output genes was robust and persistent under fasting conditions; thus we expected *Nampt* to have the same behavior. However, the significant differences in *Nampt* expression in the intact-RF fed animals were not sustained under fasting conditions (*P*=0.0018 versus *P*=0.1879, respectively. Fig. 10). Moreover SCNx-RF animals exhibited small but not significant variations in the fed condition and a significant difference only when food-deprived (*P*=0.0324). An analysis of the data with respect to time under fasting (Fig. 11) revealed that indeed *Nampt* significantly increased after 12 and 24h of fasting but intriguingly, these differences are absent in the SCNx-RF animals.

To determine whether the lack of response in our lesioned animals reflects a loss of sensitivity and/or responsiveness to signals related to the feeding status, we measured *Sirt1* expression, which is known to be induced during fasting in the liver (Noriega et al. 2011). Our results revealed that while *Sirt1* gradually increased in the Intact-RF, reaching more than two-fold its basal levels at 24h of fasting, in SCNx-RF animals it remained at almost constant levels (Fig. 11), indicating a fundamental role of the SCN either for the sensing and/or manifestation of the metabolic state in the liver.

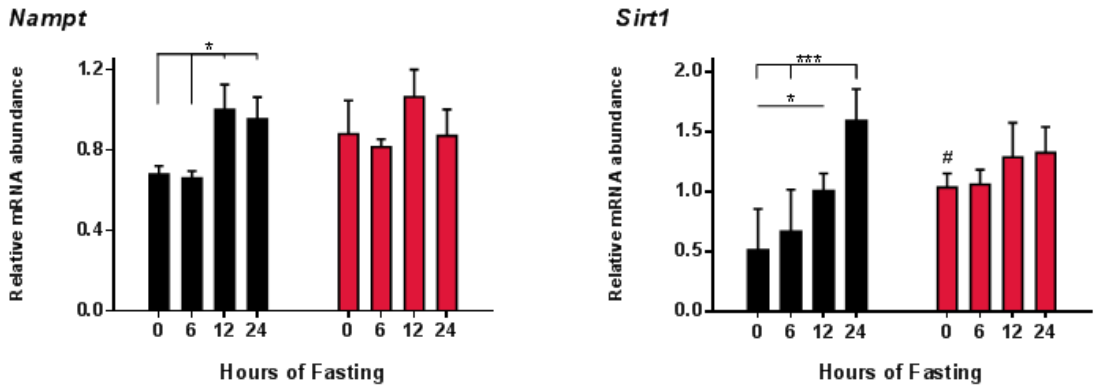


FIGURE 11: The transcriptional response to the fasting state is altered in SCNx animals.
Mean +/- SD normalized expression of *Nampt* and *Sirt1* in the liver of intact-RF (black) and SCNx-RF rats (red bars) sacrificed after the indicated hours of fasting. **P*<0.05 and ****P*<0.001 vs Time-0 by one-way ANOVA for each group. #<0.05 vs T0 expression in the intact-RF animals by two-way ANOVA for *Sirt1* comparisons, with an interaction effect of *P*=0.0219.

Signals from the SCN, other than food intake, are necessary for rhythmic *Id2* expression.

An analysis of cycling transcripts in the liver (Ueda et al. 2002) revealed a group of genes whose expression is aligned to the circadian phase of the small protein Inhibitor of DNA-binding 2 (Id2). We analyzed *Id2* expression in our intact-RF animals, and observed that it exhibited a diurnal expression in the fed state which persisted robustly under fasting (Fig. 12). Conversely, the SCNx-RF showed a severe dampening of these temporal differences, reaching significance only under fasting. It is tempting to speculate that *Id2* is part of a group of cycling genes importantly potentiated by outputs of the SCN independent of food intake.

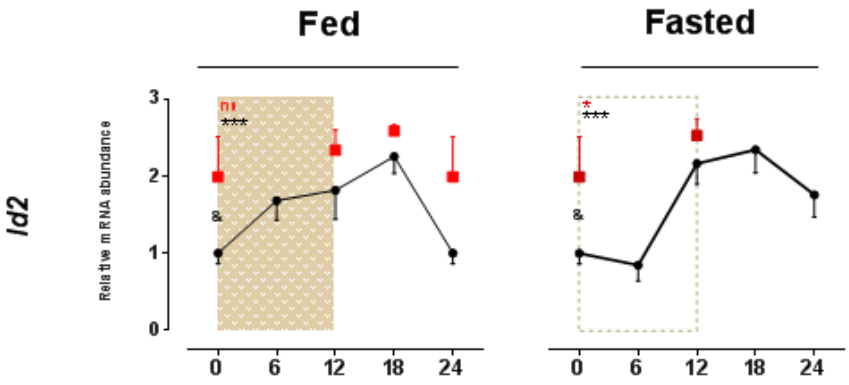


FIGURE 12: Expression of the clock-output regulator Id2 is affected by the SCN lesion.
Mean +/- SD normalized expression of *Id2* in the liver of intact-RF (black circles with continuous line) and SCNx-RF rats (red squares). The abscissa indicates the time of day referred to food presentation. See Figure 9 for details. **P*<0.05 and ****P*<0.001 by one-way ANOVA for the effect of time followed by *post hoc* Holm-Sidak test, except for the SCNx-fasted groups that were analyzed by Student's t-test. Differences between the intact and the SCNx groups were analyzed by two-way ANOVA, with & indicating *P*<0.01 at the specific time point.

Discussion

Our results show that a similar to physiological feeding schedule is sufficient to induce a seemingly complete functional clock in the liver of SCN-lesioned animals, as judged by the expression of positive and negative components of the clock and their persistence for at least a cycle under fasting conditions. Changes of hepatic *Bmal1* and *Per2* induced by 12h/day feeding in SCNx have been reported (Tahara et al. 2012, Saini et al. 2013). In fact, it was shown that inverted feeding cycles synchronize *Bmal1* oscillations even more rapidly in the hepatocytes of SCNx than in those of intact mice (Saini et al. 2013). The importance of the feeding schedule for the synchronization of the clock and CCG in the liver has also been demonstrated in animals that became arrhythmic due to experimental prolonged exposure to constant light (Polidarova et al. 2011). The mechanisms underlying the influence of feeding cycles on peripheral clocks may include direct cues interacting with nutrient and redox sensitive enzymes, hormones and/or transcription

factors, but could also comprise indirect ones such as temperature downstream pathways, which are also known to have an impact on some components of the clock (Albrecht 2012, Mohawk et al. 2012). Since we observed that a 12-h feeding schedule induced daily fluctuations of apparently anticipatory nature in locomotor activity and core body temperature in SCNx animals, this could be one of the pathways transmitting time to the liver clock.

We analyzed the expression of some metabolic genes known to be intertwined with the clock mechanism. Transactivation of the *Rev-erbα* and *PPARα* genes takes place via E-box regions located in their promoters, and they are circadian in the liver of intact animals (Torra et al. 2000, Triqueneaux et al. 2004, Oishi et al. 2005). Here we demonstrate that in SCNx-RF rats the expression of both genes exhibited robust diurnal fluctuations that persisted under fasting conditions; although *PPARα* showed modifications in its transcriptional profile probably due to an integration of the clockwork with the homeostatic information associated with the food deprivation state. This is clearly possible, considering that its expression is influenced by glucocorticoids (Lemberger et al. 1996) and the autonomic nervous system (Bernal-Mizrachi et al. 2007, Banni et al. 2012). It is important to note that the relative expression of *PPARα* and *Rev-erbα* in SCNx-RF animals was remarkably coincident with that in the intact animals at the sampled time points, suggesting independence from other SCN related pathways, at least under the conditions tested.

Providing food to intact rats only during their rest phase abolishes the circadian rhythm of the CCG *Nampt* in the liver, uncoupling it from the local clock (Salgado-Delgado et al. 2013); although other studies, in which food was provided for only 6 hours during the resting phase, showed that *Nampt* expression still had a rhythm (Polidarova et al. 2013). This apparent discrepancy could be related to the time of the experimental protocol (35 vs 10 days), with the longer leading to more severe consequences. Uncoupling of the rhythmic expression of *Nampt* and clock genes has also been found in other models, *e.g.*, in knockout mice for the catalytic subunit alpha2 of the AMP protein kinase (AMPK) the circadian profile of *Nampt* in epididymal fat is lost despite preserved cyclic expression of clock genes in that tissue (Um et al. 2011). Our present results revealed that fasting abolishes *Nampt* temporal fluctuations in the liver of intact-RF rats, although robust fluctuations in clock genes persist. Moreover in SCNx-RF sacrificed in fed condition, even though the expression of clock genes exhibited time-dependent significant changes, *Nampt* did not significantly change over time. The question remains as to what extent *Nampt* expression is influenced by nutrient sensors such as AMPK, PGC1*α* and/or the FoxO transcription factors under certain conditions (Canto et al. 2010, Tao et al. 2011), overriding the direct clock influence. As the homeostatic changes in *Nampt* impact a number of cellular processes, mainly through the NAD+ dependent activity of enzymes such as SIRT1 and the poly (ADP-ribose) polymerases (PARPs) (Belenky et al. 2007), it would be important to dissect the impact of the diverse transcriptional contributors, considering context dependent interactions.

The decreased response of *Sirt1* to the metabolic changes (in addition to a smoothed *AMPKα2* pattern of expression, Fig. S3) in our SCNx rats suggests the involvement of the SCN not only in circadian processes but also in sensing and organizing adequate responses to the metabolic state. This is supported by studies in which the metabolic effect of humoral factors is lost in animals with SCN lesions (Shen et al. 2007) and by evidence that the SCN is sensitive to the nutrient-related

signals, being illustrated also by changes in SCN activity after fasting and refeeding (Saderi et al. 2013, Yulyaningsih et al. 2014).

Besides the cycling genes clustered around the canonical core clock components, it has been identified a large cluster of circadian transcripts in the liver having *Id2* as a prototype (Ueda et al. 2002). Id2 could have an important role as an output regulator of the clock due to its ability to interact with DNA binding-driven transcriptional activation (Duffield et al. 2009). We confirmed the oscillatory nature of *Id2* transcript in the liver of food-scheduled intact animals and observed that this pattern persists under fasting conditions. Importantly, this differential expression is severely flattened in SCNx-RF rats, suggesting that *Id2* transcription is markedly influenced by non-food related signals from the SCN. For example *Id2* transcription is induced by stimulators of cyclic-AMP production, suggesting that it could be the target of some hormones (Scobey et al. 2004) as well as other transmitters influencing adenylate cyclase activity.

In conclusion, our results show that both intact-RF and SCNx-RF animals have a diurnal differential expression of the liver clock and of some of the important metabolic genes interconnected with it, *e.g.*, *Rev-erbα* and *PPARα*. These fluctuations persist under fasting conditions, indicating the functionality of the local clockwork and the strength of these interrelationships. The fact that the transcription of *Nampt* in the intact-RF animals reflects more the nutrient status than the persistent transcription by clock components, together with the decreased responsiveness in SCNx rats, suggests that the metabolic signals through yet to be determined SCN-related pathways have an important role for their adequate expression in the liver. The pronounced rhythmicity of *Id2* expression in the intact animals and the diminishment of this pattern in SCNx animals also indicate the importance of additional factors organized by the central clock and which are indispensable for the robust transcription of this gene in the periphery.

The fact that some genes completely follow the food schedule whilst others are aligned to signals coming from the SCN could be an important basis for the development of metabolic disorders associated with the misalignment of food consumption with SCN signals (reviewed in (Huang et al. 2011)).

SUPPLEMENTARY DATA

Supplementary Figures

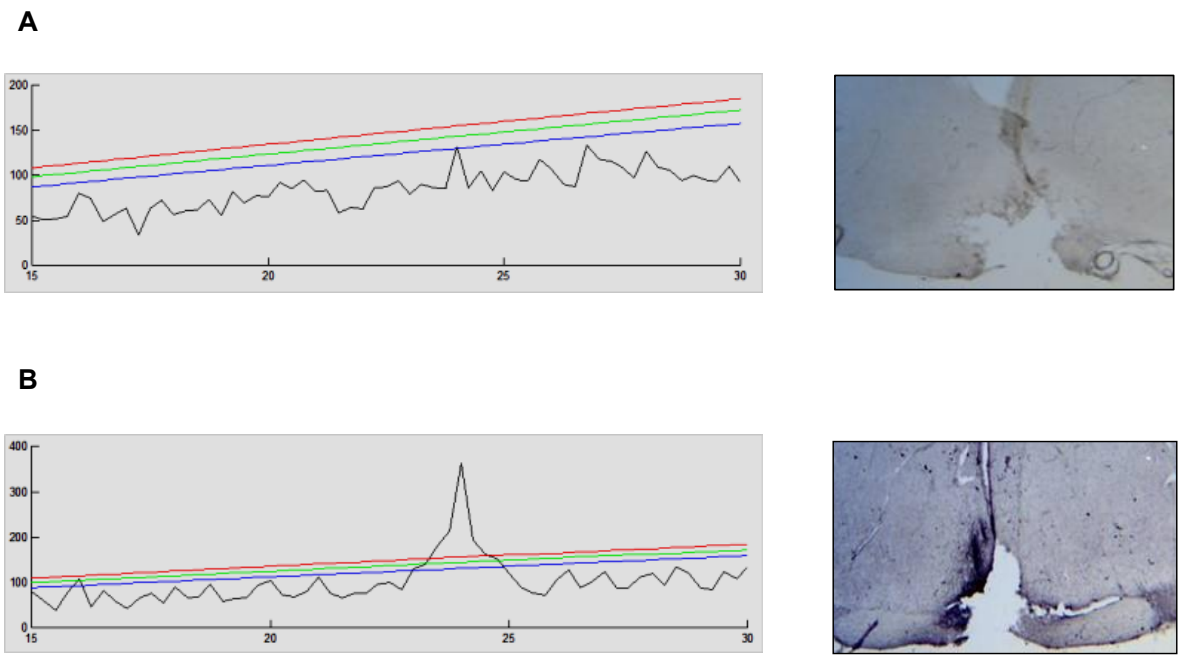
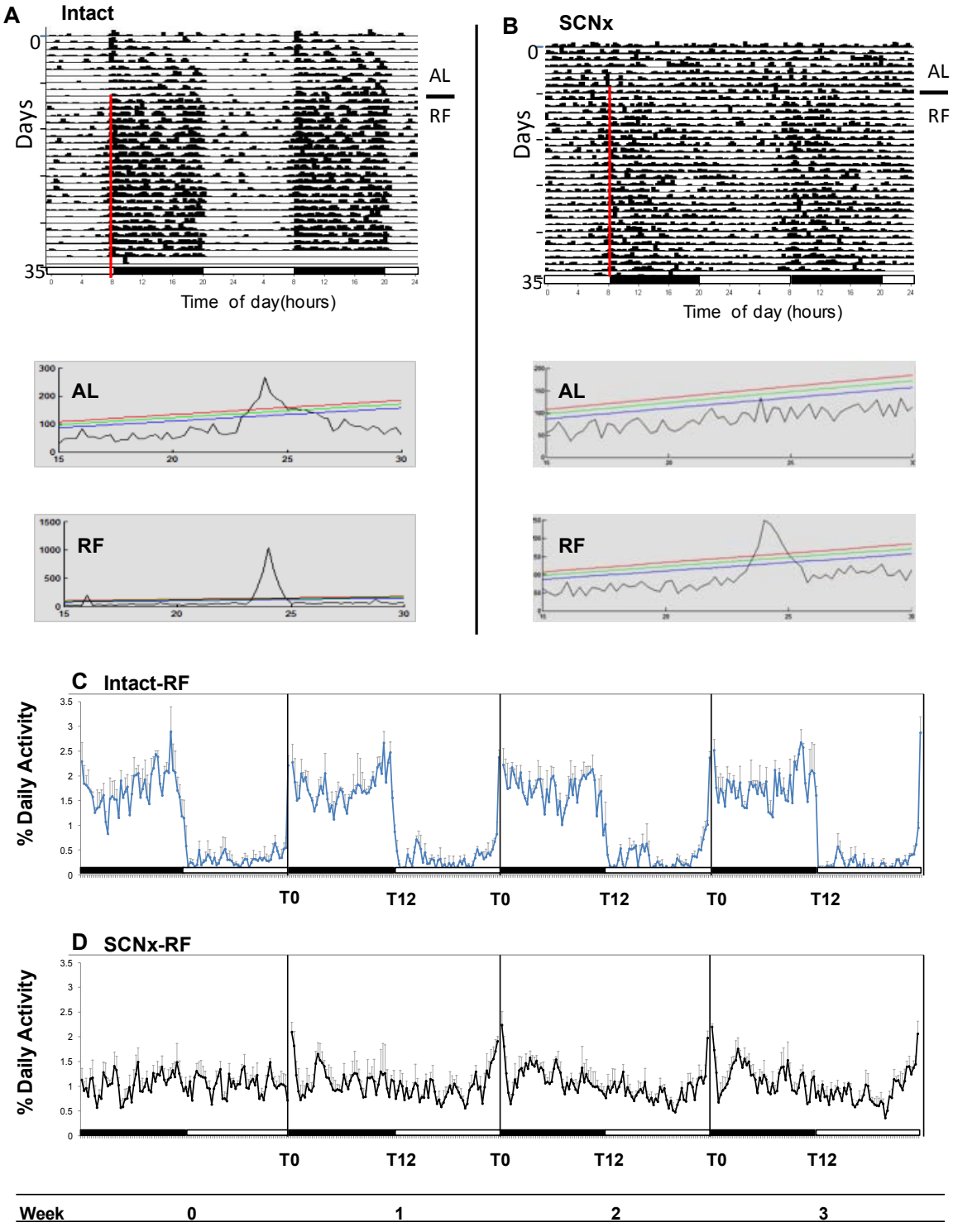


FIGURE S1: Representative χ^2-periodograms and VIP-immunohistochemistry in the SCN region, presented as examples of the criteria used for (A) acceptance or (B) rejection of SCN-lesioned animals.

FIGURE S2 (next page)**: Physiological feeding cycles organize locomotor activity of SCNx in a low-amplitude rhythmic pattern**.
Top: Representative double-plot actograms and χ^2-periodograms of individual intact (A) and SCNx (B) animals fed ad libitum (AL) subsequently fed during the 12h of the dark phase (RF). Photoperiod is indicated with black/white bars at the base of each figure. Bottom: Movement counts were normalized as percentage of total daily activity and presented as mean +/- SEM waveforms (C, intact-RF, n=3; D, SCNx-RF, n=4). Each box represents the average day per week, were 0 corresponds to the baseline ad libitum period and 1 to 3 the subsequent RF weeks. The abscissa indicates the time at which food was delivered (T0, red line in the actograms) and when it was withdrawn (T12). Please note in the wave forms the anticipatory activity of the SCNx animals before the food was given.


A
Intact
0
35
Days
AL
RF
Time of day(hours)
AL
RF
B
SCNx
0
35
Days
AL
RF
Time of day (hours)
AL
RF
C
Intact-RF
% Daily Activity
T0
T12
T0
T12
T0
T12
D
SCNx-RF
% Daily Activity
T0
T12
T0
T12
T0
T12
Week
0
1
2
3

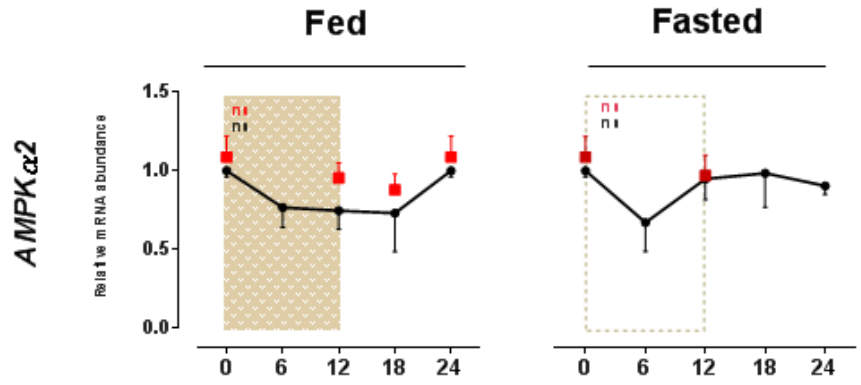


FIGURE S3: *AMPKα2* expression in the liver of food-scheduled intact and SCN-lesioned rats. Mean +/- SD normalized expression of *AMPKα2* in the liver of intact-RF (black circles with continuous line) and SCNx-RF rats (red squares). Feeding schedule is represented by the shaded areas. The abscissa indicates the time of day referred to food presentation (in hours). Gene expression was evaluated either in rats which received their food on the day of sacrifice (Fed) or which were maintained on fasting that day (Fasted). Each group was analyzed by one-way ANOVA for the effect of time, except for the SCNx-fasted groups that were analyzed by Student t-test. Differences between the intact and the SCNx groups were analyzed by two-way ANOVA. For clarity purposes, T0 values are re-plotted at T24 in the Fed graph.

Supplementary Tables

TABLE S1: Gene nomenclature, primer sequences, PCR conditions and product size.

Gene **GenBank accession number**	Primer sequences	Number of cycles; T_m	PCR product size, bp
Actin, β **NM_031144.3**	F 5'-ATCGTGGGCCGCCCTAGGCA-3' R 5'-ACGTACATGGCTGGGGTGTTG-3'	29, 63°C	302
Ampk α2 **NM_023991.1**	F 5'-CAAGTGATCAGCACTCCAACAGACT-3' R 5'-GCATCATAGGAGGGGTCTTCAGGA-3'	30, 66 °C	445
Bmal1 **NM_024362.2**	F 5'-ACTGCACCTCGGGAGCGACT-3' R 5'-CGCCCGATTGCAACGAGGCA-3'	30, 67.5°C	320
Cry1 **NM_198750.2**	F 5'-GCTGGCCACTGAGGCTGGTG-3' R 5'-GTTGGGCTTGCGAGCAGGGA-3'	29, 67.5°C	402
Id2 **NM_013060.3**	F 5'-CCGCTGACCACCCTGAACACG-3' R 5'-GCCGGAGACACCTGGGGAGAT-3'	27, 66°C	318
Nampt **NM_177928.3**	F 5'-GTGCTACTGGCTCACCAACTGGA-3' R 5'-GCTGACCACAGACACAGGCACT-3'	30, 67.5°C	415
Npas2 **NM_001108214.2**	F 5'-TGCCAGCCCAGCCCTGACTT-3' R 5'-GCGCCAAAGTCACACAACCGC-3'	32, 67 °C	583
Per1 **NM_001034125.1**	F 5'-TGTGTTTCGGGGTGCTCGCT-3' R 5'-CGTGCGCACTTTATGGCGGC-3'	30, 67.5°C	649
Per2 **NM_031678.1**	F 5´-TGCTGTGGCTGTGTCCCTGG R 5´-GGGACCGCCCTTTCGTCCAC	30, 67.5°C	477
Ppar α **NM_013196.1**	F 5'-GCGTAACTCACCGGGAGGCG-3' R 5'-GAGCCCTCCGAGCCTGGACA-3'	28, 67.0°C	376
Rev-erbα **NM_001113422.1**	F 5'-CCAGCAAGGGCACCAGCAACA-3' R 5'-CCCACCAAAGGTGTCGGGGC-3'	30, 67.5°C	451
Sirt1 **XM_006223877.1**	F 5´-TGACTGGACTCCAAGGCCACG R 5´-CCCACAGGAAACAGAAACCCCAGC	30, 67.0°C	286

TABLE S2: Statistical analysis of differences in liver gene expression (*P* values) within and between intact and SCNx food-restricted rats.
One-way ANOVA was applied for the evaluation of differences among time points within each group. Except for the SCNx-RF fasted group, in which a t-test was used. Comparisons between Intact-RF and SCNx-RF groups were done by two-way ANOVA of the temporal points shared.
Reference to Figures 9, 10, 12 and S3.

Gene	Group	Sacrifice condition	One-way ANOVA /t-test	Two-way ANOVA		
				Time (T)	Group (G)	Interaction (T x G)
Bmal1	Intact-RF	Fed	< 0.0001	< 0.0001	0.0304	0.0152
	SCN-RF		< 0.0001			
	Intact-RF	Fasted	< 0.0001	< 0.0001	0.8605	0.9824
	SCN-RF		< 0.0001			
Npas2	Intact-RF	Fed	0.0014	< 0.0001	0.2139	0.1128
	SCN-RF		< 0.0001			
	Intact-RF	Fasted	< 0.0001	< 0.0001	0.2950	0.9585
	SCN-RF		< 0.0001			
Per1	Intact-RF	Fed	0.0057	< 0.0001	0.3325	0.2457
	SCN-RF		0.0155			
	Intact-RF	Fasted	< 0.0001	< 0.0001	0.1510	0.0172
	SCN-RF		0.0147			
Per2	Intact-RF	Fed	0.0015	0.0001	0.5623	0.9072
	SCN-RF		0.0151			
	Intact-RF	Fasted	0.0219	0.0007	0.5095	0.9567
	SCN-RF		0.0065			
Cry1	Intact-RF	Fed	0.0048	0.0006	0.3487	0.6223
	SCN-RF		0.0162			
	Intact-RF	Fasted	0.0001	0.0028	0.5760	0.0911
	SCN-RF		0.2025			
Rev-erbα	Intact-RF	Fed	< 0.0001	< 0.0001	0.8292	0.7832
	SCN-RF		< 0.0001			
	Intact-RF	Fasted	< 0.0001	< 0.0001	0.8560	0.9003
	SCN-RF		< 0.0001			
PPARα	Intact-RF	Fed	0.0003	< 0.0001	0.8288	0.4827
	SCN-RF		0.0065			
	Intact-RF	Fasted	0.0418	0.0097	0.3327	0.9252
	SCN-RF		0.0474			

Nampt	Intact-RF	Fed	0.0018	0.0004	0.0212	0.5130
	SCN-RF		0.0772			
	Intact-RF	Fasted	0.1879	0.0713	0.8674	0.2599
	SCN-RF		0.0324			
Id2	Intact-RF	Fed	0.0007	0.0004	0.0010	0.2024
	SCN-RF		0.1872			
	Intact-RF	Fasted	< 0.0001	0.0001	0.0009	0.0762
	SCN-RF		0.0413			
AMPKα2	Intact-RF	Fed	0.0947	0.0053	0.0211	0.6369
	SCN-RF		0.0883			
	Intact-RF	Fasted	0.0665	0.1594	0.3619	0.5767
	SCN-RF		0.1549			

TABLE S3: Statistical analysis of differences in liver gene expression between fed and 24h-fasted SCNx-AL rats. *P* values were obtained by unpaired t-test analysis.

The net effects of feeding (n=3) and fasting (n=3) were evaluated in a group of SCNx rats fed ad libitum during the 3 weeks posterior to the lesion. The increase in *Nampt* and *Sirt1* in response to food deprivation is absent.
However among the genes that we tested this lack of responsiveness is specific, because *Per2* and *Id2* decreased under fasting in these SCNx-AL rats, suggesting that distinct mechanisms regulate the associated fasting response of these genes.
The decrease in hepatic *Per2* in response to starving has been reported before (Kawamoto et al. 2006).

Gene	***P***	***Gene***	***P***
Bmal1	0.1878	***Rev-erbα***	0.7354
Npas2	0.3913	***PPARα***	0.7039
Per1	0.7992	***Nampt***	0.5018
Per2	0.0050	***AMPKα2***	0.0550
Cry1	0.1447	***Sirt1***	0.1383
		Id2	0.0328

CHAPTER 3.

GENERAL DISCUSSION

The objective of this thesis was to determine whether a similar to physiological feeding schedule could be able to restore the circadian expression of clock and CCG in the liver of SCNxx animals, and if that is the case, whether the effect on the expression is either like an hourglass (acute response to fed and fasted conditions) or like an oscillatory clock.

In summary, our results (that we will discuss further below) show that:

- The feeding schedule is able to restore a rhythm in the expression of clock genes in the liver of SCNxx animals, as well as in two of the metabolic genes considered to be part of an important feedback accessory loop for the clock: *Rev-erbα* and *PPARα*. The induced daytime differential expression of this set of genes persisted even when the animals did not receive the expected food the day of sacrifice, suggesting that has taken place the induction of an endogenous oscillatory clock mechanism.

- With regard to the additional accessory loop that is supposed to connect the clock with metabolism: Although the signals related with feeding and fasting states influence the expression of *Nampt* and *Sirt1* in intact animals, in SCNxx rats the feeding schedule is not able to restore the rhythmic expression of *Nampt*; and we found no circadian rhythm in *Sirt1* neither in the intact nor in SCNxx animals that were fed scheduled.

- The circadian expression of *Id2*, prototype gene of a fifth class of circadian phases in the liver according to Ueda ((Ueda et al. 2002)), is also not restored in SCNxx animals and it is not affected by the extended fasting in intact rats.

Based on this small sample of genes chosen for being considered important parts of the interlocked loops connecting the circadian clock and metabolism, we can deduce that in the liver there are (at least) three classes of circadian genes with respect to their dependence on the integrity of the central clock (the SCN), the local clock and the feeding and fasting cycles. In the next paragraphs we will discuss this differential dependency and the possible mechanisms behind it.

a) Clock genes, *PPARα* and *Rev-erbα*.

These are genes whose oscillations can be induced by the feeding schedule but after this induction do not depend any more (at least for an additional cycle) on the presence of this synchronizing cue. Thus, are they dependent on food first and then no? How could this be accomplished?
At first, one could say that this is possible because the effect is like a pendulum, rather than a sand clock. Food is the impulse needed to make the pendulum oscillate after which it will slowly cancel out.
In the attempt to solve this, we have to consider first what the initial situation of SCNxx animals without such a feeding schedule is.

Previous studies have shown that under ad libitum conditions, SCNxx animals lose their circadian rhythms of rest and activity, and of food and water intake. They are active and eat a small portion for a short time, rest and stop eating, again active, and so forth (Chapter 1, fig. 1).

In addition to this loss of behavioral circadian rhythms, many studies have shown that in SCNxx animals (with ad libitum feeding) the circadian rhythm in liver gene expression is also lost (Fig. 7) (Hara et al. 2001, Terazono et al. 2003, Guo et al. 2006, Reddy et al. 2007). It has been proposed that the lack of rhythm reported in those studies may be either due to a constant expression in all cells of the tissue (the liver), or could be due to the presence of individual cells conserving their rhythms but that they oscillate out of phase with each other, leading to an apparent lack of rhythm when they are sampled at the tissue level (Fig. 13). Possibly, the mechanism by which the feeding schedule induces a rhythm could be different depending on which situation is true. Since (to the best of our knowledge) there is still no universal agreement on this theme, we will briefly discuss the picture derived from both options.

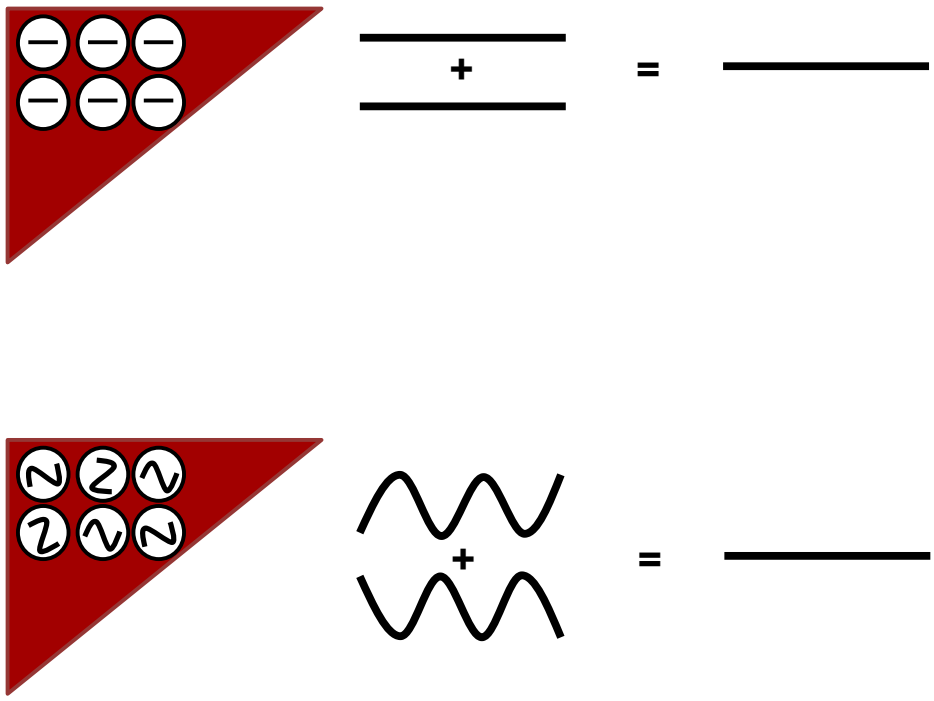


FIGURE 13: The apparent lack of transcriptional circadian rhythms in the liver throughout the day may reflect either a constant expression in each one of the cells that compose the tissue (top) or the presence of rhythms in individual cells that oscillate in disordered phases (bottom).

a.1) Constant expression among all tissue cells.

The isolation of tissues or cells in culture has been used as a model to understand the properties of peripheral oscillators without the influence of the SCN, similar to what is supposed to occur in SCNxx animals. This approach has been empowered by the use of genetic engineering tools for generating cellular lines and organisms expressing bioluminescent reporter genes or proteins fused to a determined component of the molecular clock, allowing to investigate the presence of circadian oscillations at the single-cell resolution level in order to (try to) solve conflicts such as the one posed in the above paragraph.

Using this technique, Per2 expression was monitored in isolated primary hepatocytes, and it was found that the oscillations in each hepatocyte nearly disappear after three days if the culture media is not changed, suggesting that the damping of rhythms observed at the whole culture/tissue level is due to a damping of the clock gene rhythms (reaching an almost constant expression) in each individual cell (Guenthner et al. 2014).

Then the next question is how is it possible to activate/repress the loops of transcription that compose the molecular clock from a nearly constant to a clear oscillation in the expressed genes?

Through mathematical modeling it has been proposed that several factors may be involved in this circadian induction; among them are: the existence of cooperation i.e. facilitation of transcription related with increments in the number of molecules present, the number of transcriptional events that occur within a determined timeframe, the stability and degradation rates of the transcribed proteins that in turn are going to act as activators or repressors in the system, etc. (Gonze et al. 2004, Brown et al. 2012). For instance, Gonze et al. (Gonze et al. 2004) have suggested that sustained oscillations can only emerge *above a critical value* in the rate of the repressors' binding to their target genes, which depends among other things on the number of repressor molecules in the cell. Although our experiment was not aimed to tackle the mechanisms that take place in our SCNxx animals, when we compare the expression levels reached in food restricted SCNxx animals (SCNxx-RF) with those from SCNxx animals that were fed ad libitum (SCNxx-AL**) (Fig. 14), it is noticeable that the mRNA relative quantities in SCNxx-AL always is lower than the one presented at the peaks in SCNxx-RF animals, and higher than the one presented at troughs, suggesting that the critical value in activator and repressor molecules is not reached by "feeding small amounts-fasting a short time" ultradian cycles such as those present in SCNxx-AL animals, but rather require a longer period of fasting followed by a considerable amount of food intake in a determined lapse.

In intact animals, for instance, it is known that the phase of the peripheral clocks is altered by the amount of food and the fasting interval (Hirao et al. 2010, Kuroda et al. 2012), with the larger meal after the longer fasting time determining the circadian phasing of the liver clock (Wu et al. 2011).

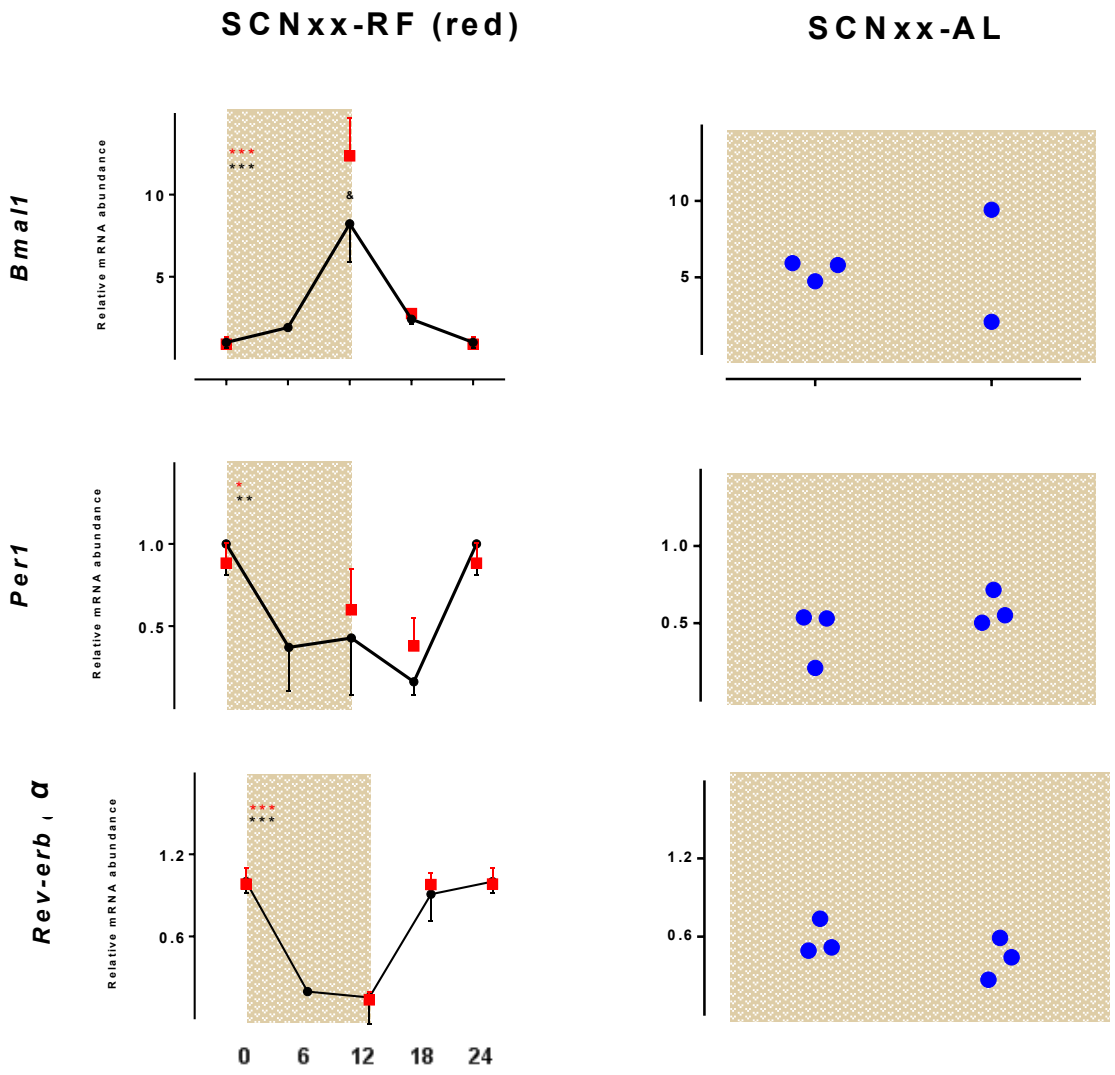


FIGURE 14: SCN lesioned animals that were under the 12h:12h feeding schedule (left panel) have both higher and lower relative transcript levels than SCNxx animals maintained under ad libitum feeding (right panel, SCNxx-AL). It is possible that in the food-scheduled animals the larger periods of fasting together with a concentrated time for food intake (more calories consumed in a given time, despite the overall caloric intake is virtually the same in both groups; data not shown), give the conditions needed for larger rates of transcriptional activation and repression that lead to this oscillatory pattern.
(**However it is important to consider that our results obtained from SCNxx-AL does not necessarily mean that they are the result of a constant expression in all liver cells, given that the presence of individual oscillators with high levels of expression may appear dampen when mixed with other individual not synchronized oscillators.)

What are the variables associated to the feeding schedule that impinge on the clock allowing it to reach those critical values? Some of the factors that may be involved are the ones listed below, here are included those affecting the transcription as well as those affecting other steps within the clock mechanism:

Fasting:

- The acylethanolamines N-palmitoylethanolamine (PEA) and N-oleoylethanolamine (OEA) are among the most potent endogenous ligands for PPARα. The levels of OEA and PEA increase in the liver after an overnight fast (and begin to decrease after refeeding; (Izzo et al. 2010)). As mentioned in the introduction, PPARα is able to induce the transcription of *Bmal1* (Canaple et al. 2006).

- Glucagon, a major fasting hormone, activates CREB/CRTC2 transcriptional complex that is recruited to *Bmal1* promoter and induces its expression in the liver (Sun et al. 2015).

- AMP-dependent protein kinase (AMPK) is a major sensor of the AMP/ATP ratio, a parameter that reflects the energetic state of a cell. AMPK can phosphorylate Cry, promoting its degradation and thereby shortens the half-life of this core clock repressor (Lamia et al. 2009). Additionally, it appears that AMPK promotes the phosphorylation and degradation of Per2 through an indirect mechanism involving the casein kinase 1 epsilon (Um et al. 2007).

Feeding:

- Insulin induces the expression of *Per1* and *Per2* in cultured hepatoma cells (Yamajuku et al. 2012).

- In the liver, the activation of HSF1 can be induced during food intake, probably functioning to reduce the oxidative stress caused by feeding (Katsuki et al. 2004). As mentioned in the introduction, it is thought that the expression of *Per2* may be influenced by HSF1 (Kornmann et al. 2007, Buhr et al. 2010).

- In vitro experiments have shown that glucose induces the expression of the transcriptional regulator *Tieg1*, which is able to bind to *Bmal1* gene promoter and repress its transcription (Hirota et al. 2010).

- The activity of PPARα (activator of *Bmal1* transcription) is inhibited by bile acids (Sinal et al. 2001, Okamura et al. 2014).

- In vitro experiments have demonstrated that the binding of Clock/Npas2:Bmal1 heterodimers to their E box target sequences is sensitive to the oxidated to reduced NAD(P)+/NAD(P)H ratio. An increase in the reduced forms stimulates the binding, while incrementing the oxidized forms strongly inhibits it (Rutter et al. 2001). The ratio of oxidized to reduced forms is higher in the fed state (compared to a condition of 48h of starvation; (Veech et al. 1969)), suggesting that it could contribute to a decrease in the activity of the positive components of the clock.

Altogether, these data suggest that the fasting state promotes the increase in the positive components of the clock (activators) as well as a decrease in the repressors; conversely, the fed state would favor the negative feedback loop of the clock (repressors) and decrease/inhibit the positive. The strength of these factors could possibly be determined by examining how many feeding fasting cycles would be necessary to induce a rhythm. In this regard, it was demonstrated that in SCNxx mice to whom a feeding schedule has been established, an abrupt change in the schedule adjusts the expression of *Bmal1* in the liver in only 2-3 days to the new phase (Saini et al. 2013). This observation shows that resetting indeed takes place quite fast, as can also be concluded from analogous behavioral studies in intact animals (Escobar et al. 2007).

Some effects of food intake in the expression of liver clock genes have been shown to be dependent on the type of nutrients; for example, none of the macronutrients alone is able to induce the same changes that a balanced diet, whereas e.g. the combination of glucose with determined aminoacids does (Hirao et al. 2009, Oike et al. 2011). Further studies are required to evaluate the effects of these combinations on SCNxx animals.

In this a.1 model in which we considered that the basal state of the clock in livers of SCNxx animals consists of individual non-oscillating cells, where a critical point in some of the clock components is reached by imposing a feeding schedule (Fig. 15), how could the persistence of oscillations during fasting be explained?

This can be explained when the interconnected loops between clock genes is robust; still, as occurs in explanted tissues, it is expected that these oscillations will eventually dampen after a few days in the absence of regular food intake.

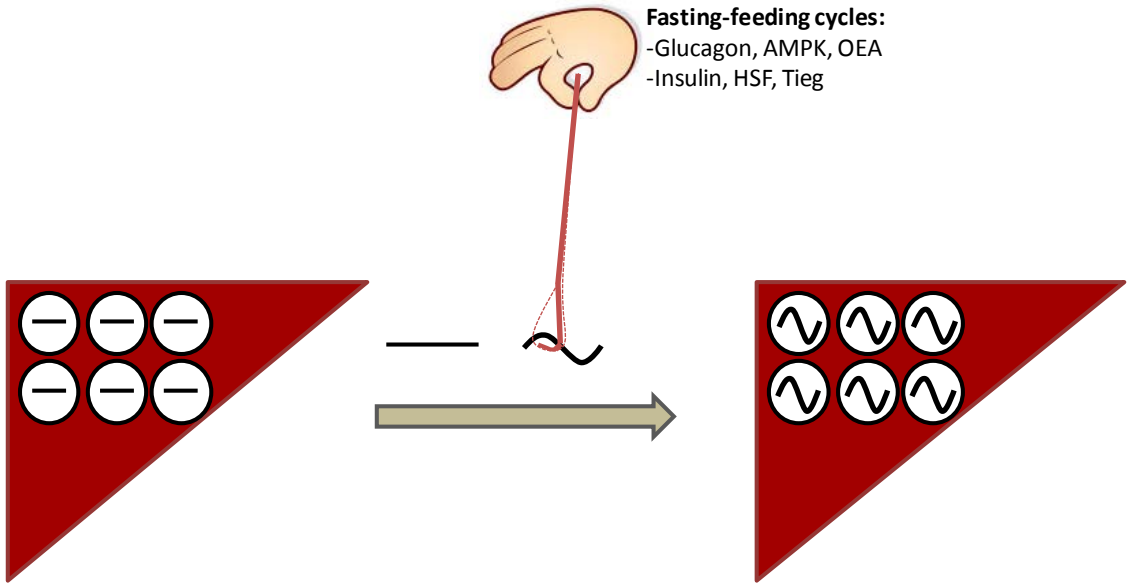


FIGURE 15: The larger periods of fasting and the feeding bursts associated with the feeding schedule induce higher rates of transcription and repression, thus inducing oscillations in otherwise arrhythmic liver individual cells found in SCNxx animals with food ad libitum.

a.2) The second alternative, clock genes oscillate in every cell of the liver but their phases are asynchronous from each other, thus when we make a pool of them at any given time, the result is an apparent lack of rhythmic expression.

This premise arose from the observation that clock genes in individual fibroblasts (either primary or from a cellular line) can oscillate robustly and independently with undiminished amplitude for up to two weeks in culture, and due to differences in their intrinsic periods and their lack of coupling there was a gradual loss of synchrony among cells and damping of the ensemble rhythm at the population level (Nagoshi et al. 2004, Welsh et al. 2004, Leise et al. 2012).
Based on these studies, it is widely accepted the idea that cellular clocks in peripheral tissues "are actually self-sustained and autonomous in nature failing to maintain coherence at the population level" (O'Neill and Feeney 2014, Tsang et al. 2014).

However, caution most be taken when making such generalizations, given that factors may influence the clock differently in hepatic and fibroblast lineages (Yamajuku et al. 2012). Fibroblasts in culture do not experience cellular coupling, being unable to unify their phases and periods, whereas hepatocytes indeed couple cellularly, they experience a localized, though weak, coupling in vitro and this is expected to be higher in the liver tissue (Yamajuku et al. 2012).

If one assume this second premise to be true, then the interpretation of our results would be that in SCNxx animals, the effect of the rhythmic cues related to the feeding schedule (those mentioned for a.1) is not to induce out of nothing an oscillation in each cell but rather to align the individual clocks to the same phase, in such a way that at a population level now we can see a clear maximum and a minimum in the expression of those clock genes (Fig. 16).

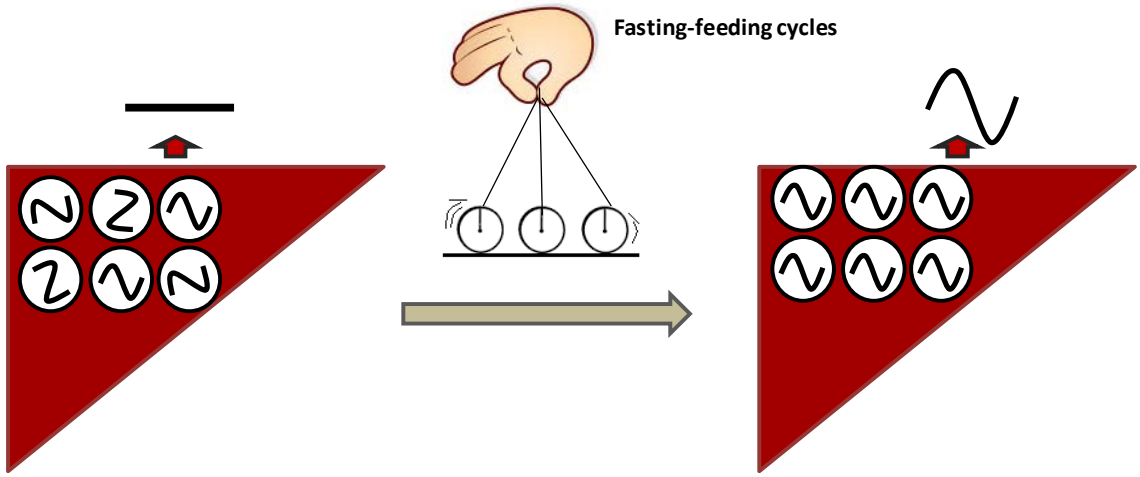


FIGURE 16: Oscillations in disordered phases in gene expression between individual cells in the liver of SCNxx animals are adjusted to a same phase by the feeding schedule, so that when expression is now evaluated at the whole liver level, it changes from an apparent arrhythmic to a robustly oscillatory pattern.

If this were the case, under the extending fasting it would be expected that the individual oscillators in liver cells of SCNxx animals would become again desynchronized from each other; however, this may take place not immediately but after a number of cycles. One may assume that with robust individual oscillations it may take longer for the cells to synchronize and desynchronize than when the oscillations are absent in SCNxx animals. What has been seen in food-restricted SCNxx mice is that when the feeding schedule is discontinued and food becomes available ad libitum, the oscillations that had been induced decrease in two to three times its amplitude already in the first day (Saini et al. 2013), suggesting the absence of robust individual oscillators in these animals.

Now we continue with the second group of genes:

b) Metabolic genes that present diurnal fluctuations comprising those mainly driven by feeding-fasting associated cues (in the intact), and barely influenced by the local clock machinery, at least under the conditions we tested. To this group belong *Nampt* and *Sirt1*.

Nampt

Intact animals under extended fasting lose their rhythm in *Nampt*, in spite of the positive arm of the clock and its other output genes retain a robust differential expression along the day (see Chapter 2, figures 9 and 10). This indicates that the fasted state is probably more relevant for the induction of *Nampt* expression in the liver than the local clock. This dissociation of *Nampt* from the clock has also been found in other studies (Um et al. 2011). The factors that are able to regulate *Nampt* expression are multiple; for instance, there have been described glucocorticoid response elements, heat shock response elements, cAMP response elements, AP-1 and SP1 sites in its regulatory region, besides the E-boxes (Nakahata et al. 2009); therefore, it is possible that in vivo there are conditions where the regulation of *Nampt* by the clock genes may be overcomed by other factors.

Although signals related with the nutritional state radically influence the diurnal expression of *Nampt* in intact animals, in SCNxx rats the feeding schedule is not able to restore this diurnal expression because the change in *Nampt* levels in response to the fasting and feeding states is not fully displayed in these animals, as can be seen when one re-orders the data according to the hours on fasting (Fig. 11, Chapter 2).
In SCNxx animals *Nampt* remains almost at constant levels, they have an already elevated expression of *Nampt* even by the time of 6 hours of fasting, even more, they have constitutively elevated *Nampt* even under the fed condition (time 0 of fasting), suggesting that the SCN is involved either in the sensing and/or the response to the nutritional state.
Indeed, there are some findings supporting this idea. First, there is evidence that the SCN is sensitive to the nutritional status as illustrated by changes in SCN activity after fasting and refeeding (Yi et al. 2006, Yi et al. 2008, Saderi et al. 2013). Second, the metabolic effects of humoral factors (such as leptin) are lost in animals with SCN lesions (Shen et al. 2007). And finally, the actions of the pancreatic polypeptide (PP;, (Banks et al. 1995, Zhao et al. 2013)), whose levels depend on the fasted-fed states and that is able to cross the blood brain barrier, have also been suggested to take place through a pathway that involves the SCN, which is rich in a specific type of receptor for PP (Yulyaningsih et al. 2014).

Sirt1

We found no circadian rhythm in *Sirt1*, neither in the intact nor in food restricted SCNxx animals, in spite of the rhythm present in the clock genes suggesting that there is not a tight link with the clock at least under the conditions we tested. There is debate regarding the circadian rhythmicity in Sirt1 expression both at the mRNA as well as at the protein level (Asher et al. 2008, Nakahata et al. 2008, Salgado-Delgado et al. 2013, Zhou et al. 2014). Some studies have found no circadian rhythm in Sirt1 mRNA whereas others indeed have found it in isolated primary hepatocytes, moreover, detecting binding of Clock/Bmal1 to the E-box region present in the promoter region of *Sirt1* (Nakahata et al. 2008, Zhou et al. 2014). Although the reason for this discrepancy is not clear, is possible that the amplitude of the oscillations induced in *in vitro* systems is enhanced due to the resetting imposed by the serum shock, it is also possible that *Sirt1* is differently expressed in other cellular populations of the liver, thus when taken as a whole tissue the oscillations in *Sirt1* expression may appear attenuated; however, this is less probable since it is known that hepatocytes account for nearly 80% of total parenchymal volume (Blouin et al. 1977).

Sirt1 is affected by the feeding-fasting conditions **in intact animals**, as shown in Fig.11 Chapter 2, and as shown before by Noriega et al. who demonstrated that the transcription factors CREB and ChREBP are able to regulate *Sirt1* expression differentially in response to energy availability (Noriega et al. 2011). Thus *Sirt1* (and *Nampt*) may be part of a group of genes that are mainly driven by homeostatic signals related with the nutrient status or with the energetic state. This group has been described by Vollmers et al. (Vollmers et al. 2009); they use a model where the molecular clock is disrupted in the whole body by means of deleting *Cry1* and *Cry2*. When the authors reinstate a similar to ours feeding schedule, a vast number of liver genes (including, for instance, several related with metabolic pathways e.g. the sterol regulatory element binding factor 1, the ATP citrate lyase, the low density lipoprotein receptor; but also with immune function such as the STATs 1 and 2; cell cycle regulation such as the cyclin-dependent kinase 2 (CDK2)-associated protein; and membrane traffic such as the light chain B of clathrin) recover their rhythms, indicating that the oscillations they present are mainly driven by feeding/fasting associated cues rather than by the molecular clock mechanism.

Interestingly, **in SCNxx animals** *Sirt1* expression does not reflect the energetic state, because the levels remain almost constant regardless the time of fasting (Fig.11 Chapter 2). As for *Nampt*, it seems that for *Sirt1* to display the levels according to this energetic status it needs the SCN. It would be interesting to know whether other genes thought to be mainly driven by homeostatic signals (Vollmers et al. 2009) are also so dependent on the integrity of the SCN.

c) SCN dependent genes prototyped by *Id2*.

Id2 is neither affected, nor induced by the feeding-fasting cycles, as judged by its persistence in the intact animals under extended fasting and by its lack of a robust rhythmic pattern in SCNxx animals under the feeding schedule. Given that clock genes are rhythmic in these rats, it indicates that *Id2* also cannot be driven by the molecular clock. Therefore, its rhythm in the liver strictly depends on other signals provided by the SCN.

Id2 expression is higher in SCNxx rats as compared to the intact at all temporal points, which can be due to an excess of activators or to a deficit in repressors of its transcription.

Among the factors that induce *Id2*, there are some members of the Bone Morphogenetic Protein (BMP) family and the RFX1 (regulatory factor for X-box1) transcription factor (Kinoshita et al. 2007, Wang et al. 2007, Nakahiro et al. 2010, Kurooka et al. 2012). In some cellular types, its transcription is also induced by stimulators of cyclic-AMP production suggesting *Id2* as a possible target of some hormones (Scobey et al. 2004) as well as other systems influencing adenylate cyclase activity, such as beta-adrenergic receptors activated by the sympathetic (although this option seems improbable, since after an SCN lesion the content of noradrenaline in the liver, assumed to reflect sympathetic activation, remains constant at lower levels; (Terazono et al. 2003)).

On the other hand, among the repressors of *Id2* are the Transforming Growth Factor beta (TGF-b) (Cao et al. 2009), the tumor suppressor p53 (Paolella et al. 2011), and the oncogene Myc (Kurland and Tansey 2008). It would be interesting to evaluate which of these factors is/are responsible of Id2 remaining at high constant levels; a research that also would shed light in the importance of other communication pathways from the SCN in order to establish peripheral rhythms.

The physiological consequences of this increased *Id2* expression still need further research. However, by studies of whole body deletion of the gene, we know that in the liver it has an important role in the regulation of the circadian profile of several genes involved in lipid and carbohydrate metabolism (Hou et al. 2009, Mathew et al. 2013). Besides that, Id2 also participates in the orchestration of the innate and adaptive immune responses, endocrine, regenerative and tumoral processes (Lasorella et al. 2001, Yokota et al. 2001, Zebedee and Hara 2001, Sikder et al. 2003, Damdinsuren et al. 2005, Rodriguez et al. 2006, Rankin and Belz 2011, Tsunedomi et al. 2013).

Why is important to know that the diurnal rhythms of gene expression are differentially driven by certain circadian signals or by others?

Experimental conditions where food is provided to rodents only during the 12h of their resting phase for a few weeks (3 to 4) induce several metabolic abnormalities such as 1) obesity, 2) glucose intolerance, and 3) ectopic accumulation of fat in the liver. A molecular signature in those animals is the desynchronization in gene expression between organs (Bur et al. 2010, Bray et al. 2013, Reznick et al. 2013) as well as within each organ (Bray et al. 2013, Reznick et al. 2013, Salgado-Delgado et al. 2013); this is comprehensible given that, as we have shown, these oscillations depend in certain cases on the feeding schedule, whereas others mostly rely on other signals from the SCN.

The case of *Nampt*, for instance, which becomes arrhythmic in those animals, may be explained by the fact that it usually is expressed according to the feeding-fasting status but requires the SCN to fully display the expression level according to these states. The problem is that the activity of the SCN is not changed by this specific feeding schedule but rather conserves its usual pattern of activation anchored to the light-dark cycle, then probably the sensing/response to the nutrient status will become defective (as indeed is). Importantly, *Nampt* and its ultimate reaction product, NAD, are intimately related with the management of lipids in the liver (Tao et al. 2011).

In the next Chapter, we present an analysis of how many metabolic pathways (we selected those involved in lipid metabolism) are regulated by the interconnected action of hormones and the autonomic nervous system but also are largely influenced by feeding/fasting associated cues. As those processes follow a circadian rhythm, there we provide a basis of how an imbalance in the diurnal activity between the components sets the conditions for the development of metabolic diseases; in particular here we will refer to non-alcoholic fatty liver.

CHAPTER 4.

Non-alcoholic fatty liver disease as a consequence of autonomic imbalance and circadian desynchronization.

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Elizabeth Sabath, Adrián Báez-Ruiz, Ruud M. Buijs.

Abstract

The circadian system, headed by the suprachiasmatic nucleus (SCN), synchronizes behavior and metabolism according to the external light-dark cycle through neuroendocrine and autonomic signals. Metabolic diseases such as steatosis, obesity, and glucose intolerance, have been associated with conditions of circadian misalignment wherein the feeding schedule has been moved to the resting phase. Here we describe the physiological processes involved in liver lipid accumulation, and show how they follow a circadian pattern importantly regulated by both the autonomic nervous system and the feeding-fasting cycle. We propose that an unbalanced activity of the sympathetic-parasympathetic branches between organs induced by circadian misalignment provides the conditions for the development and progression of nonalcoholic fatty liver disease.

3.1 INTRODUCTION.

Nearly all organisms experience marked diurnal fluctuations such as changes in temperature and food availability associated with the photoperiodic cycle in the environment. Therefore, it is not surprising that many oscillatory physiological processes have evolved that are dependent on the time of day, conferring to the organism the advantage to anticipate the rest or activity phase. These oscillations of nearly 24-h periodicity are referred to as circadian.

In mammals, the Suprachiasmatic Nucleus (SCN) of the hypothalamus is in charge of the synchronization of circadian behavior and metabolism to the external light-dark cycle (Kalsbeek et al. 2011). Besides the photoperiod, the schedule of food intake is also an important synchronizer especially for the peripheral organs. This is illustrated by experiments in rodents that are allowed to eat exclusively during their resting phase, which result in that a large number of genes in the liver completely reverse their rhythm, as compared to animals receiving food during their usual activity period (Vollmers et al. 2009). Interestingly the rhythmicity of clock genes in the SCN is not changed, suggesting that the feeding time is able to override the central

signals to the periphery, causing a decoupling from the main clock (Damiola et al. 2000). In these decoupled animals also ensues a state of inter-organ misalignment, with uncoordinated diurnal profiles of substrate utilization and energy expenditure (Bur et al. 2010, Bray et al. 2013). Moreover, desynchrony occurs within the liver itself, characterized by misaligned temporal patterns among genes; i.e. whereas many of them indeed reverse their phase, others have an intermediate phase or lose completely their rhythm (Salgado-Delgado et al. 2013). Importantly, these desynchronized animals develop steatosis, glucose intolerance as well as adipose and whole body weight gain (Arble et al. 2009, Bray et al. 2013, Salgado-Delgado et al. 2013). In humans, a relationship between the circadian system and metabolic features has also been found at both genomic and behavioral levels; for instance, certain polymorphisms in the clock genes have been associated with the susceptibility to obesity, non-alcoholic fatty liver disease (NAFLD), type 2 diabetes, and hypertension (Sookoian et al. 2007, Woon et al. 2007, Sookoian et al. 2008). Moreover, weight gain and obesity have been associated with shifted meal patterns to late in the day and evening in humans (Bo et al. 2014).

Our proposal is that conflicting information from the feeding schedule and the SCN may cause disturbances in the autonomic output to the organs, eventually changing the coordinated function within and between them.

In the next section we will describe the confluence of circadian and autonomic aspects of the metabolic physiology whose disruption or imbalance may lead to accumulation of fat in the liver and set the inflammatory and pro-oxidative conditions for its progression to nonalcoholic steatohepatitis (NASH).

3.2 LIVER STEATOSIS: CIRCADIAN AND AUTONOMIC PATHWAYS.

NAFLD comprises a clinically heterogeneous group of liver diseases that share the infiltration of fat in the liver parenchyma; encompassing from “simple steatosis” to NASH with significant necroinflammation and progressive fibrosis (Stickel and Hellerbrand 2010). Simple steatosis is defined by the presence of $\geq$ 5.5% intra hepatocellular lipid content (Cuthbertson et al. 2014) with no evidence of hepatocellular injury in the form of ballooning of the hepatocytes, and without a previous history of significant alcohol consumption (Chalasani et al. 2012). NAFLD is the most frequent hepatic disease worldwide; being present in about 20-30% of individuals in Western countries, with the highest incidence in males between 40 and 65 years (Bedogni et al. 2014). Obesity, insulin resistance and dyslipidemia are potent risk factors associated with fatty liver, and it has even been called the “hepatic manifestation of metabolic syndrome”; however, it can also be the result of other events (Chalasani et al. 2012, Yilmaz 2012).

The excessive accumulation of lipids in the liver may be seen as an imbalance between the supply of fatty acids to the tissue at the one hand, and their utilization or export, at the other (Postic and Girard 2008, Kawano and Cohen 2013). These processes are regulated on a daily basis in such a way that during the resting period (fasting), there is an enhanced lipolysis from white adipose tissue (WAT), increased fatty acid oxidation, and very low density lipoprotein (VLDL) secretion

from the liver. Meanwhile in the fed state, WAT is in charge of sequestering a major part of dietary fat, and carbohydrates will be taken by the tissues with the excess being re-routed to triglyceride (TAG) synthesis for storage (Figure 17). In the next sections we will briefly describe the manner in which many of the pathways involved are regulated by the autonomic nervous system (ANS) and by signals related to the feeding/fasting state, so to picture a framework in which the rupture of the balance among the participants may lead to liver steatosis.

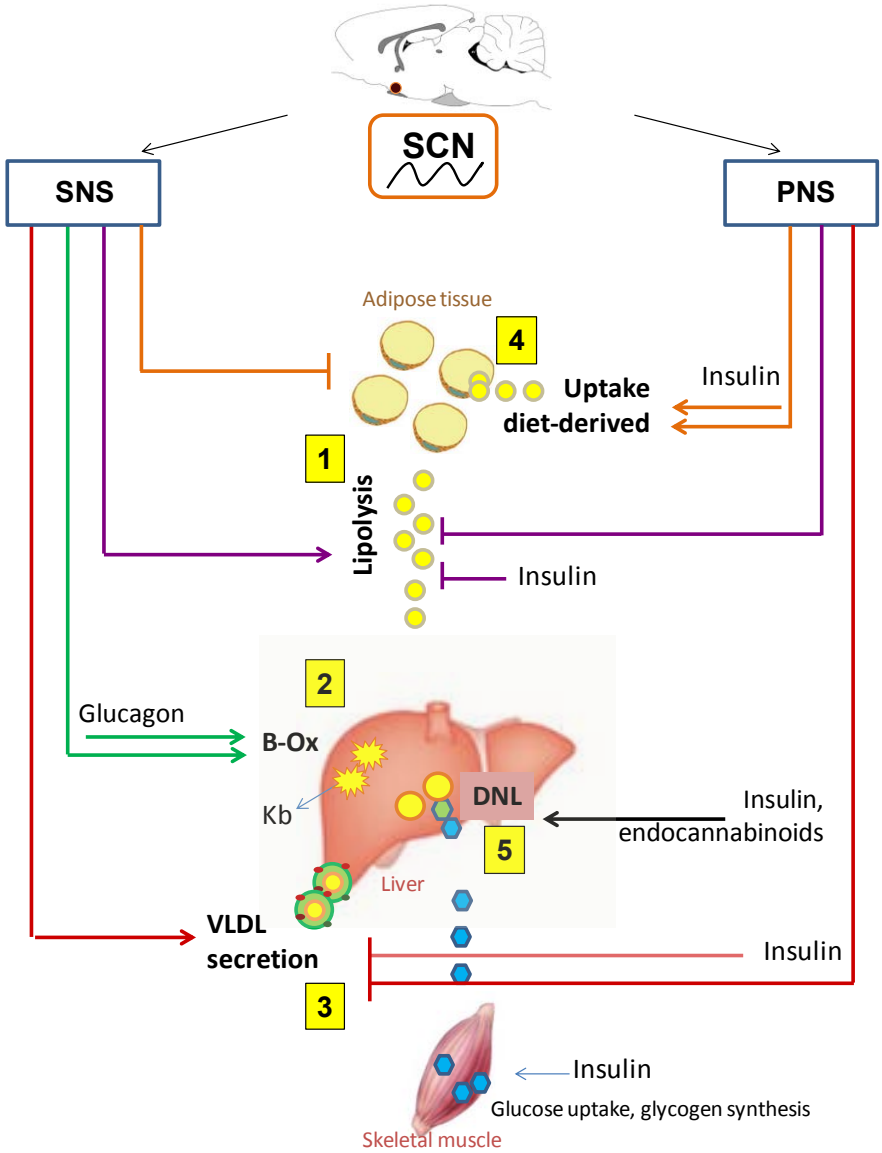

FIGURE 17 (previous page)**:** The SCN plays an essential role in coordinating and synchronizing the autonomic activity with the daily feeding and fasting cycle. During the day (the fasting period in rodents), [1] adipose tissue undergoes an increased rate of lipolysis, and liver lipids are dispatched by routes that include [2] the oxidation of fatty acids and [3] VLDL secretion. On the other hand, during the night, i.e. the prandial and postprandial states (in rodents), [4] the adipose tissue retains an important amount of diet-derived TAG, and excess of carbohydrate goes to TAG synthesis through the DNL pathway [5]. The SCN must be able to balance the activity of the sympathetic SNS and parasympathetic (PNS) branches to the different organs along the day according to the feedback information about the nutrient/metabolic status that is given to it. Internal desynchronization such as food intake during the resting phase decouples certain functions of the peripheral organs from SCN signals and induces decoupling among peripheral organs that also are not equally responsive to food-related signals. This may lead to a conflict between the autonomic and humoral/local signals within and between the organs. This desynchronization leads to steatosis, glucose intolerance, and adipose and whole body weight gain. The drawing shows the points of autonomic regulation and some of the humoral factors involved.

3.2.1 Increased Free Fatty Acids arising from WAT Lipolysis.

A decreased insulin sensitivity and increased sympathetic input to WAT lead to increased free fatty acids (FFA) from WAT lipolysis arriving to the liver.

WAT plays a major role in regulating the lipid flux in the body providing a long-term fuel reserve by the storage of fatty acids in form of TAGs. The process by which these TAG are hydrolyzed and released into the blood as glycerol and non-esterified fatty acids (mostly known as FFA) is termed lipolysis, and presents diurnal fluctuations under normal conditions (Sukumaran et al. 2010, Shostak et al. 2013). FFA release is stimulated by the sympathetic activation of WAT during fasting, whilst in the fed state insulin acts as a potent suppressor (Frayn et al. 1995, Trayhurn and Beattie 2001). People with NAFLD have increased levels of FFA in plasma (Diraison et al. 2003), an effect usually attributed to adipose tissue insulin resistance (Bugianesi et al. 2005). The relevance of this pathway for NAFLD is illustrated by the fact that nearly 60% of liver TAGs in these patients are derived from this lipolytic pool (Donnelly et al. 2005).

Although insulin and the sympathetic system are fundamental for the lipolytic control, there are several other known modulators (reviewed in (Wang et al. 2008)). For example, parasympathetic denervation of WAT increases the activity of the lipolytic enzyme hormone sensitive lipase by nearly 50% (Kreier et al. 2002), suggesting that a decrease in the parasympathetic tone to WAT can also result in the release of FFA.

One of the physiological processes that trigger the lipolytic process in the fasted state is the decrease in liver glycogen, communicated via hepatic vagal afferents to the brain resulting in sympathetic-mediated lipolysis of visceral adipose tissue. The response of subcutaneous WAT seems to be independent of this neural pathway (Izumida et al. 2013). In this regard, it is noteworthy that the autonomic innervation of different fat depots; i.e., subcutaneous vs. visceral, is controlled by a distinct subset of neural networks (Kreier et al. 2002), and this differential organization is also found in pre-autonomic neurons of the hypothalamus, as well as in the SCN (Kreier et al. 2006); thus this data raises the possibility that an autonomic and/or circadian impairment may contribute to the excessive FFA found in NAFLD.

3.2.2 Decreased Hepatic Beta Oxidation.

A decreased response/sensitivity to the fasting state, as well as a reduction in the sympathetic input to the liver at the appropriate time of day, may contribute to lipid accumulation by reducing the oxidation of liver fat.

Liver TAG can be secreted into the blood as VLDL, or be hydrolyzed and the fatty acids channeled towards the β-oxidation pathway. It is known that a moderate experimental increase in beta-oxidation is sufficient to reduce the hepatic accumulation of TAG (Stefanovic-Racic et al. 2008); conversely, a reduction in hepatic and whole body oxidation of fat is associated with the severity of steatosis in overweight/obese NAFLD patients (Croci et al. 2013). However, there are also many reports about an increased rather than decreased mitochondrial fatty acid oxidation in people with NAFLD, suggesting that the excessive mitochondrial oxidative and anaplerotic fluxes found in these patients may lead to oxidative stress and eventually to mitochondrial impairment, resulting in a degenerative spiral favoring the progression to NASH (Cortez-Pinto et al. 1999, Sunny et al. 2011, Begriche et al. 2013).

In the liver, beta-oxidation presents diurnal fluctuations, with the highest levels reached in the second half of the resting phase, whereas the lowest are found at the end of the activity period in mice (Peek et al. 2013). The process is regulated at multiple levels including, for instance, transcriptional activation by fasting and glucagon stimulation (Wolfrum and Stoffel 2006, von Meyenn et al. 2013), posttranslational modifications by AMPK and Sirt1 enzymes (Fulco and Sartorelli 2008), as well as allosteric modulation by malonyl-CoA over Cpt1, a key enzyme that exerts flux control over the entry of fatty acids to mitochondria, where the oxidation takes place (Saggerson 2008). Several regulatory factors (e.g. PPARα, Pgc-1 α and beta, and NAD^+ bioavailability) show clear diurnal cycles whose phase and amplitude are influenced by the timing of food intake (Vollmers et al. 2009, Fuse et al. 2012, Peek et al. 2013, Salgado-Delgado et al. 2013).

Liver fatty acids are stored as TAG in lipid droplets, which interact physically and functionally with mitochondria. Some of the proteins that coat the lipid droplets, for instance Perilipins 1 and 5, function as inhibitors of the hydrolysis and stabilizers of the droplet, reducing the utilization of fatty acids by the mitochondria in a basal state. However, under stimulation of protein kinase A pathway (as can occur by sympathetic activation) the inhibition by these proteins is lifted (Wang et al. 2011), allowing fatty acids to be available for their oxidation.
In addition, the sympathetic nervous system (SNS) may have an important role in influencing the circadian nature of this process, as suggested by the following data: a) Cpt1 activity decreases after phenol-induced hepatic denervation (Carreno and Seelaender 2004). (Phenol denervation has no major effect on parasympathetic fibers (Olgin et al. 1998)); b) Pgc1α expression and activity are regulated by beta adrenergic/sympathetic pathways (Finck and Kelly 2006, Gerhart-Hines et al. 2011); and c) early studies indeed demonstrated that noradrenaline enhances the oxidation of

long-chain fatty acids in isolated rat hepatocytes (Oberhaensli et al. 1985, Nomura et al. 1991). Since circadian sympathetic input to the liver is controlled by the SCN (Buijs et al. 2003, Terazono et al. 2003), it is possible that via this autonomic branch the SCN could promote the oxidation of fatty acids at specific times of day.

3.2.3 Decreased Hepatic VLDL Secretion.

Decreased sympathetic stimulation and/or an enhancement of the parasympathetic input to the liver would reduce VLDL secretion, increasing the amount of stored TAG.

The liver secretes TAG in the form of VLDL for delivery to peripheral tissues. VLDL production and secretion requires several steps involving the regulation of lipid substrate and co-factors availability, and the secretory vesicle trafficking; the reader is referred to excellent reviews that address the theme in depth (Choi and Ginsberg 2011, Olofsson and Boren 2012).

VLDL levels in plasma present discrete diurnal fluctuations (Marrino et al. 1987, Mondola et al. 1995). Whereas fasting favors VLDL secretion mainly through stimulation of the SNS (Bruinstroop et al. 2012), during the fed state insulin acts as a potent suppressor (Choi and Ginsberg 2011); additionally, recent studies suggest that VLDL secretion is restrained by the hepatic parasympathetic branch (Bruinstroop et al. 2013). It has been proposed that decreased secretion of VLDL may contribute to steatosis mainly at the early stages of NAFLD; however in the progression of the disease, and probably supported by a state of insulin resistance, VLDL secretion increases, contributing to the pro-atherogenic dyslipidemia profile (Cano et al. 2009, Cohen and Fisher 2013). As a whole, the situation likely reflects an initial enhancement in the parasympathetic drive to the liver, which would block VLDL secretion, increasing the storage of lipids. But at later stages, the delicate balance between the autonomic branches probably is broken, resulting in excessive VLDL secretion and dyslipidemia.

3.2.4 Increased Uptake of Dietary Fat by the Liver.

In the fed state, insulin resistance and an increased ratio of sympathetic to parasympathetic input to WAT could promote a reduced extraction of diet-derived TAG/FFA in WAT, favoring their ectopic deposition.

It is estimated that nearly 15 % of accumulated TAG in the liver of NAFLD patients arise directly from diet sources (Donnelly et al. 2005). The amount of diet-derived TAG/fatty acids that finally reaches the liver after a meal depends on several factors, mainly: the amount absorbed by the intestine, uptake by other tissues, and the ‘spillover’ of fatty acids into the circulation.

Many enzymes and proteins involved in TAG processing and transport in the intestine are primarily influenced by the timing of food intake (Pan and Hussain 2009). When chylomicron-TAGs reach the capillary beds of peripheral tissues, the locally present lipoprotein lipase (LPL) hydrolyzes them into fatty acids to deliver lipid calories for local use or storage (Williams 2008). This hydrolysis of TAG from circulating TAG-rich lipoproteins also follows a diurnal rhythm, as illustrated by the fluctuations in LPL activity in white and brown adipose tissue, heart and skeletal muscle (Benavides et al. 1998). LPL expression and activity is complexly regulated; being tissue-specific and dependent on the nutritional status and whole body requirements (Klingenspor et al. 1996, Kersten 2014).

WAT has a pivotal role in the uptake of postprandial fatty acids (Ruge et al. 2009), and indeed it has been proposed that a reduced and maladaptive extraction of postprandial chylomicron-TAG by adipose tissue may contribute to the pathophysiological diversion to other organs not dedicated for fat storage (McQuaid et al. 2011). The activity of LPL in WAT is low during fasting (Kersten 2014), probably largely controlled by the sympathetic nervous system (Raynolds et al. 1990); conversely, in the postprandial period LPL activity is high under positive regulation by insulin (Kersten 2014). FFA uptake is additionally favored by the parasympathetic nerves (Kreier et al. 2002), suggesting that insulin in synergy with the parasympathetic input promotes the efficient clearance of circulating diet-derived triglycerides by WAT.

Not all fatty acids liberated from chylomicron-TAG hydrolysis by LPL are picked up by the tissues but rather "spillover" into the plasma FFA pool. The relative sensitivity of the adipose towards increased local FFA generated by LPL decides how much of the newly formed FFA will be extracted by the tissue and how much will end up in venous plasma (Teusink et al. 2003). Importantly, there are daily fluctuations in this sensitivity/efficiency of lipid uptake by WAT (Ruge et al. 2009), wherein the intrinsic intracellular pathways related with esterification and beta-oxidation may have a relevant role. Thereby, the correct synchrony between the specific local processes and the autonomic pathways would be essential in the decision of fat storage into WAT or its delivery for oxidation/storage by other tissues, e.g. the liver.

3.2.5 Increased Hepatic De Novo Lipogenesis (DNL).

Insufficient uptake of excess dietary carbohydrate by the tissues either through insulin-dependent or independent pathways, leads to increased lipogenic substrate towards liver DNL.

DNL refers to the process by which excess of carbohydrate from the diet is re-routed to lipid synthesis, since the amount of glucose that can be stored as glycogen is limited (Chong et al. 2007). The relevance of this pathway for people with NAFLD is illustrated by the fact that nearly 25% of their liver TAGs arise from DNL (Donnelly et al. 2005). Hepatic DNL exhibits diurnal fluctuations associated with the feeding and fasting cycle, being several fold higher during the fed state in both humans and rodents (Hems et al. 1975, Horton et al. 1998, Timlin and Parks 2005,

Feng et al. 2011). After food intake, the rise in blood glucose and insulin levels induce the activation of the transcription factors Carbohydrate-Responsive Element-Binding Protein (ChREBP) and Sterol Regulatory Element-Binding Protein 1c (SREBP1c) in the liver, which are master regulators of the lipogenic program (Postic and Girard 2008). SREBP1c is significantly increased in the livers of NAFLD patients (Kohjima et al. 2008, Mitsuyoshi et al. 2009), and experiments in animal models have demonstrated that activation of SREBP is essential for the development of steatosis induced by diet (Moon et al. 2012). Under normal conditions, SREBP expression and maturation follow a diurnal rhythm in the liver, which is markedly regulated by both food intake and the circadian clock genes (Cretenet et al. 2010, Matsumoto et al. 2010, Shen et al. 2014). Interestingly, in people with NAFLD the lipogenic process remains constant throughout the day, even when fasted, which probably reflects an inability of the liver to regulate the changes that normally occur during the transition between the nutritional states (Donnelly et al. 2005), possible associated with a weakness in the circadian control mechanisms.
A relevant local target for NAFLD is the endocannabinoid system, since activation of hepatic CB1 receptors increases the expression of several genes involved in DNL, including SREBP1c and its target enzymes (Osei-Hyiaman et al. 2005, Mallat et al. 2013). Given that the circulating levels of the endocannabinoids anandamide and 2-arachidonoylglycerol show circadian fluctuations in humans (Vaughn et al. 2010, Hanlon et al. 2015), this raises the question whether an imbalanced control of this system due to circadian desynchronization (Vaughn et al. 2010) could contribute to aberrant lipogenesis suggesting that a chronobiological approach would improve the therapeutic efficacy.

As mentioned before, the right functioning and interrelation between organs is crucial in determining the amount of substrate for steatosis. DNL is not an exception. In this regard, skeletal muscle was recently recognized as one of the major tissues responsible for the increase in hepatic DNL: as a result of muscle insulin resistance, the decreased postprandial uptake of glucose for muscle glycogen synthesis leads to an increment in the availability of the lipogenic substrate, glucose, towards DNL (Samuel and Shulman 2012). Indeed, muscle insulin resistance has been proposed as an early therapeutic target for the prevention and treatment of NAFLD (Jornayvaz et al. 2010, Rabol et al. 2011, Flannery et al. 2012). Importantly, muscle insulin sensitivity for glucose utilization follows diurnal fluctuations (Leighton et al. 1988); in humans, glucose tolerance is lower at night (Van Cauter et al. 1997), suggesting that carbohydrate intake during this period may result in enhanced availability of glucose for the DNL in the liver.

3.2.6 Circadian misalignment results in aberrant NAFLD-related pathways.

From the points presented above it is clear that each of the physiological pathways that may induce NAFLD is under circadian control at the systems level. Even beyond that, they include several points of transcriptional, post-translational, and epigenetic regulation that are bidirectionally communicated with the molecular clock in each tissue (for a comprehensive review of these molecular inter-relationships, see (Mazzoccoli et al. 2014)), illustrating why circadian

desynchronization may result in NAFLD. Circadian desynchronization due to eating during the resting phase results in alterations that are directly related with the pathophysiology of the disease, such as the increase in visceral fat, and glucose intolerance (Salgado-Delgado et al. 2010, Salgado-Delgado et al. 2013). It also deregulates the expression of many metabolic genes such as *Sirt1*, *PPARα*, *PGC1-α*, and *Nampt* (Salgado-Delgado et al. 2013), either inducing a down-regulation or changing the phase-relationship between them. An example of how this defective coordination may result in steatosis is given by one of the proteins that coat the lipid droplets where TAG are stored, the Adipose Differentiation Related Protein (ADRP or ADFP). When the feeding schedule is changed to the resting phase, ADRP conserves the same pattern as *ad libitum* fed animals, whereas a significant number of hepatic genes reversed their diurnal rhythm of expression (Gene Expression Omnibus database, accession no. GSE13093, from (Vollmers et al. 2009)). This is noteworthy because ADRP, like other family members, reduces lipid hydrolysis by excluding the presence of certain lipases from the droplet (Listenberger et al. 2007). In addition, ADRP has been proposed to play an opposing role with MTP (enzyme of the VLDL pathway), since the two proteins sequester the intracellular TAG in separate compartments for storage and secretion, respectively (Chang and Chan 2007). If protein levels reflect what has been found in ADRP mRNA, the aberrant pattern of expression in the desynchronized animals may contribute to a defective access to the fatty acids within the lipid droplet for its posterior oxidation or secretion (through their package into VLDL), at the time were it is required. ADRP levels are up-regulated in steatosis both in humans and mice. And conversely, ADRP-deficient mice are resistant to diet-induced fatty liver (Chang et al. 2006, Motomura et al. 2006).

Although we have centered our attention on local and autonomic mechanisms, it is important to note that hormonal influences can contribute to NAFLD development as well. Among them, cortisol may have a prominent role, since circadian disruption in humans and in murine models leads to increased glucocorticoid levels (Birketvedt et al. 1999), and/or changes in their circulating diurnal pattern (Salgado-Delgado et al. 2010, Bray et al. 2013). Glucocorticoids are involved in carbohydrate and lipid homeostasis; and their excess and/or chronic exposure can cause disruptions in lipid metabolism (reviewed in (Wang et al. 2012)). For instance, glucocorticoids increase the capacity of the liver for storing fatty acids as TAGs (Manmontri et al. 2008), decrease medium- and short-chain fatty acid oxidation (Letteron et al. 1997), and may even impair lipid export from the liver (Harris et al. 2013). Interestingly, some glucocorticoid actions could be associated with changes in the autonomic balance (Bell et al. 2002), increasing the level of complexity at which disturbances induced by circadian disruption may take place.

3.3 Expressway from simple steatosis to NASH. Contributions of circadian misalignment and autonomic imbalance.

Up till now we have focused on the causes that lead to “simple steatosis”, an entity that follows a relatively benign course (though not totally devoid of histological changes) (Vuppalanchi and Chalasani 2009). However in about 10-25% of the cases it may evolve into NASH (Vanni et al. 2010,

Wong et al. 2010), a more progressive disease with higher risk for cirrhosis and hepatocellular carcinoma (Wong et al. 2010, Masuoka and Chalasani 2013, Bedogni et al. 2014). The reasons why only a number of patients with simple steatosis advance to NASH are still not completely understood. A first suggestion was that accumulation of lipids in the liver constitutes a "first hit", needing a stimulus acting as a "second hit" for the induction of inflammation, necrosis, and fibrosis (Day and James 1998). However, certain cases indicate that simple steatosis and NASH could be twin but separate entities with "multiple and parallel" hits contributing to NASH development (Tilg and Moschen 2010, Bedogni et al. 2014). Interestingly, it was recently suggested that a disruption in the clock genes may be associated with the development of a similar to NASH pathology in the pancreas (Carter et al. 2014), raising the question whether the conditions needed for the development of more severe stages of NAFLD could be set by states of circadian and autonomic desynchronization.

The vast theme of NASH pathogenesis is beyond the scope of the present work, hence the reader is referred to expert reviews (Farrell et al. 2012, Wree et al. 2013). In the next section we will briefly describe some factors associated with NASH, such as inflammation, adiponectin levels, and oxidative stress, with emphasis on the circadian and autonomic nodes of control, and whose derangement may contribute to NASH progression.

3.3.1 Chronic exposure to endotoxin.

Endotoxin (lipopolysaccharide [LPS]), a constituent of gram-negative bacteria, is considered a possible agent for the ''second hit'' in NASH models (Tilg and Moschen 2010), and several findings point towards a relation between chronic endotoxin exposure and the severity of the disease (Verdam et al. 2011, Compare et al. 2012). Patients with NAFLD have an increased gut permeability, which may provide a continuous source of endotoxin (Miele et al. 2009).

Experimental data supports the idea that autonomic disturbance may lead to enhanced leakage from the gut. For instance, under situations in which gut integrity has been compromised, stimulation of the vagus nerve returns gut permeability almost to the normal levels (Costantini et al. 2010). Conversely, an increased sympathetic input may result in enhanced endotoxemia, as suggested by studies in which a reduction in cardiac and splanchnic sympathetic activity due to thoracic epidural anesthesia results in decreased endotoxin-induced epithelial permeability (Schaper et al. 2013).

LPS stimulates inflammatory cytokine production and accumulation of inflammatory cells (Compare et al. 2012); interestingly, the magnitude of the cytokine response to LPS challenge show circadian fluctuations that are critically influenced by the integrity of the central clock (the SCN) (Guerrero-Vargas et al. 2014). Since NASH implicates, by definition, an inflammatory component (Tilg and Moschen 2010), the relevance of these circadian-immune interactions become clear by the fact that circadian misalignment increases the expression of both basal and stimulated inflammatory profiles (Castanon-Cervantes et al. 2010, Leproult et al. 2014).

Hence, we conclude that patients with simple steatosis, when subjected to situations of circadian disruption and/or autonomic imbalance may be more prone to increased endotoxin exposure and inflammatory environment, favoring the progression to NASH.

3.3.2 Low adiponectin levels.

Adiponectin improves insulin sensitivity and reduces hepatic and body fat, in part mediated by activation of the AMPK and PPARα pathways. It also suppresses TNF-α as well as IL-6 in LPS-stimulated macrophages, and decreases the activation of Kupffer and hepatic stellate cells, protecting the liver from inflammation and fibrosis (Czaja 2004, Finelli and Tarantino 2013); indeed, adiponectin is able to prevent steatohepatitis progression in murine models (Fukushima et al. 2009). Importantly, in patients with NASH (especially at the first stages), adiponectin blood levels are significantly reduced in comparison with those with simple steatosis (Musso et al. 2005, Vuppalanchi et al. 2005).
The circulating levels of high-molecular-weight adiponectin in healthy humans follow circadian variations that are not driven by the feeding-fasting cycle (Scheer et al. 2010). This rhythm may be based on the activity of the autonomic branches, since in vitro and in vivo studies have shown that sympathetic/beta-adrenergic stimulation of adipose tissue inhibits the production and secretion of adiponectin (Delporte et al. 2002, Iwen et al. 2011), whereas parasympathetic activation increases it (Suzuki et al. 2014). Thus it is possible that in the course of NAFLD, an enhanced sympathetic and/or decreased parasympathetic input to adipose tissue, may result in decreased adiponectin levels, contributing to disease progression.

Moreover, as mentioned before, the autonomic imbalance and insulin resistance in WAT may lead to constant FFA efflux into the circulation, which is associated with the activation of hepatic macrophage residents (Kupffer cells), further promoting a pro-inflammatory environment (Cusi 2012).

3.3.3 Oxidative Stress.

Oxidants, such as reactive oxygen / nitrogen species, are formed as a normal product of aerobic metabolism and many of them are known to be involved in several physiological processes and specific signaling cascades. However, when there is an imbalance and the levels of oxidants surpass the capacity of the antioxidant response it can lead to increased lipid peroxidation and damage of cellular enzymes, DNA and organelles (Hybertson et al. 2011, Wilking et al. 2013). This state is usually termed ‘oxidative stress’, and is considered an important mediator for NASH progression, favoring a pro-inflamatory environment and fibrogenesis (Anderson and Borlak 2008, Farrell et al. 2012).
Several oxidant and antioxidant systems present day-night variations (Hardeland et al. 2003, Xu et al. 2012, Wilking et al. 2013). The importance of these oscillations is illustrated by experiments with carbon tetrachloride- induced liver damage and fibrosis (Bruckner et al. 2002), as well as in other models for fibrosis in other organs (Pekovic-Vaughan et al. 2014), where the higher

susceptibility to damage is associated with relative lower levels of certain antioxidants at the time of day at which the toxic agent is administrated.
The redox environment in a given organ may result from the interplay between the sympathetic and parasympathetic systems, and even circadian variations in both branches of the ANS could serve to restore the normal antioxidant reserves (Vecoli and Paolocci 2008). For example, stimulation of beta-adrenergic receptors increases reactive oxygen species (ROS) production and lipid peroxidation products in cardiac tissue, which can be partially antagonized by acetylcholine (Tsutsumi et al. 2008, Andersson et al. 2011); indeed vagal nerve stimulation suppresses free radical generation in the failing heart (Tsutsumi et al. 2008). In addition, adequate ROS levels can be optimally adjusted by feedback communication, since ROS can activate certain sensory afferent fibers via a mechanism involving the capsaicin receptors (Schultz and Ustinova 1998, Taylor-Clark and Undem 2011).
Thereby, circadian misalignment and/or an unbalanced activity of the autonomic branches, may lead to abnormal antioxidant responses in the liver with respect to specific threats, promoting an increase in oxidative stress that could favor NASH.

A note at margin is made here about a group of highly conserved proteins that are important regulators of the redox status, and may even function as ROS sensors, the peroxiredoxins (Rhee et al. 2012). It was recently found that these proteins present robust circadian oscillations in their redox status, a rhythm that persist even in the absence of the transcription-translation loops of canonical clock mechanisms (Edgar et al. 2012, Hoyle and O'Neill 2015). Since ROS are normal byproduct of aerobic metabolism, the question remains whether under conditions of circadian misalignment such as changing the feeding schedule (which implicate a strong metabolic cue for the liver), this could promote a dissociation between ROS production, peroxiredoxin levels and activity and the transcriptional anti-oxidant response (which is controlled by the canonical clock (Lee et al. 2013, Pekovic-Vaughan et al. 2014)). This may create conditions that promote oxidative stress. Interestingly, mutant mice lacking Peroxiredoxin 6 spontaneously develop insulin resistance and hepatic injury resembling NASH (Pacifici et al. 2014).

3.4 Conclusions.

NAFLD is much more than simply a “hepatic disease”; here we have seen that liver lipid accumulation is influenced by the function of other organs e.g., brain, adipose, skeletal muscle, etc., and how the autonomic and circadian systems affect the metabolic processes involved in the development and progression of the disease.

Circadian disruption either by the presence of light or food (or both) at the wrong time, causes derangements at many levels of the whole body system, finally leading to steatosis and associated pathologies. The influence of hormones such as cortisol and melatonin may also contribute, since they are known to participate in certain NAFLD related pathways (Reiter et al. 2000, Tarantino and Finelli 2013), and their levels are also affected in humans as well as in murine models of circadian misalignment (Birketvedt et al. 1999, Salgado-Delgado et al. 2010).

Seldom will it be possible to indicate a single cause for NAFLD; however, inasmuch as the ANS regulates several of the above mentioned processes, it can be expected that many metabolic derangements found under disrupted circadian conditions are associated with the imbalanced action between the autonomic branches at specific times of day. Indeed, studies in humans have shown that circadian misalignment induces an imbalance between the sympathetic and parasympathetic activities (Chung et al. 2009, Wehrens et al. 2012).

Autonomic dysfunction is known to be associated with or even precede diseases such as obesity, diabetes and hypertension (Weyer et al. 2001, Dauphinot et al. 2010). Actually, abnormal circadian patterns are found in certain metabolic variables in NAFLD patients (Jin et al. 2012), and several parameters associated with autonomic dysfunction are prevalent in people with the disease (Oliver et al. 1997, Newton et al. 2009, Sun et al. 2015). Therefore, we propose that circadian misalignment and autonomic imbalance may have a *causal* role in NAFLD. In this regard, a key question is whether the strengthening of the circadian signal by simple changes in the lifestyle, such as a stringent alignment of the feeding schedule to the activity period, could reinstate the correct balance in the ANS, preventing the progression of NAFLD. Indeed, some associated therapies that may attenuate the autonomic dysfunction, such as the administration of melatonin (Gonciarz et al. 2012, Hatzis et al. 2013), and resistance exercise (Jakovljevic et al. 2013), have already been proposed.

CHAPTER 5.

GENERAL CONCLUSIONS

In this thesis we aimed to contribute to the understanding of how the temporal control of metabolism is achieved (Chapter 2) and why it is important for metabolic health (Chapter 4).

In support of our initial hypothesis, we demonstrated that oscillations in the expression of liver clock genes (which are thought to be in charge of driving the tissue-specific circadian metabolism) and those from the accessory loop formed by *Rev-erbα* and *PPARα* are induced by a very similar to normal feeding schedule in SCNxx animals. Once established these oscillations can be sustained by themselves at least for a cycle. However, in contrast to what we expected, we also found that the normal diurnal expression of *Nampt* and *Sirt1* (that are considered as direct clock-controlled genes) as well as their changes in response to the feeding/fasting state normally seen in intact animals cannot be recovered in SCNxx rats, despite having an apparently functional local liver clock. Additionally, we found that the rhythm in Id2 expression in the liver cannot be reinstated by the feeding schedule in animals devoid of SCN. Altogether these results suggest that not all the interactions between clock and metabolic genes are so tightly interconnected as has been proposed –at least at the mRNA level, and shows that the SCN uses different pathways to regulate the diurnal pattern of gene expression in the periphery.

Thus, our results allow us to conclude that in rat liver there are at least three types of genes according to the mechanisms that control their differential expression throughout the day (Fig. 18):

1) Clock genes. Those that are part of a robust oscillatory network and can be induced by the feeding schedule independent of the SCN; they comprise the core clock genes and the *PPARα* and *Rev-erbα* accessory loops.

2) Metabolic genes. Those whose diurnal oscillations are responsive to feeding-fasting associated cues rather than belonging to an intrinsic local clock mechanism, and **in addition** require the SCN to fully reflect the nutritional status. To this group belong *Nampt* and *Sirt1*.

3) SCN-dependent genes prototyped by *Id2*. Those whose time-of-day differential expression do not depend on the local clock, nor in the feeding schedule, but rather on other yet to be identified SCN-associated pathway(s).

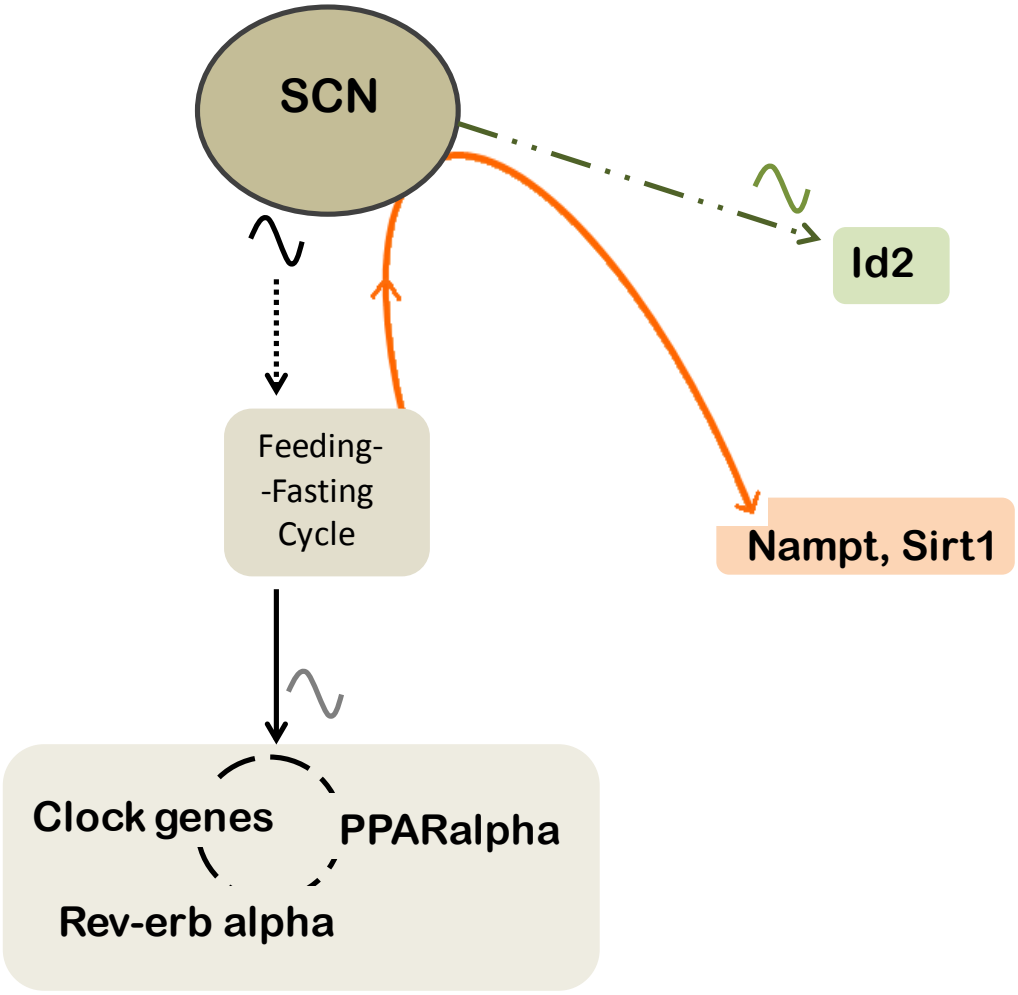

FIGURE 18: We identified three types of control over the temporal profile of liver genes involved in metabolism. One includes the self-sustained oscillations of the clock genes and the accessory loop formed by *PPARα* and *Rev-erbα* which can be induced by a physiological feeding schedule even independently of the SCN. The time-of-day dependent expression of other sets of genes, where *Nampt* and *Sirt1* are included, depend on the feeding-fasting conditions but require the SCN to display expression levels according to these states. Finally, there is another group, represented by *Id2*, whose oscillatory expression in the liver depends on yet to be determined SCN-derived signals that are neither related with the feeding-fasting cycles nor with the local clock.

The general significance of our studies is that this differential dependence on SCN or feeding/fasting related signals could be a factor involved in the development of metabolic disorders associated to circadian misalignment, as suggested by the case of *Nampt* presented at the end of Chapter 3, and as can be noticed with the following hypothetical example:

> If a transcription factor involved in fatty acid oxidation is driven by the fasting-feeding cycles and its coactivator proteins rely most on other SCN-derived cues, then situations of circadian desynchronization such as eating at the wrong phase would result in a lack of temporal coincidence between these factors, probably decreasing beta-oxidation efficiency. If such situation occurs frequently then there will be a time when the consequences of these misalignments will become manifested as a metabolic disorder, for example, decreased use of fatty acids and consequent accumulation either in adipose tissue or ectopic deposition.

Current studies in our laboratory are aimed to measure metabolic parameters such as liver lipid accumulation, blood levels of hormones and metabolites, etc. in rats under the conditions presented here in order to understand their physiological repercussions.

As many features of the metabolic syndrome are developed not only in animal models but also by humans under conditions of circadian desynchronization (Lowden et al. 2010, Salgado-Delgado et al. 2013), it is generally assumed that similar processes may take place in humans (Lowden et al. 2010, Salgado-Delgado et al. 2013).

Our work is part of a bigger picture trying to solve how circadian functions are established in the body and why they are important for health; therefore in chapter 4 we have focused on the possible mechanisms whereby the SCN controls many aspects involved in lipid metabolism through the organization and coordination of the daily cycles of feeding-fasting, hormonal secretion and the activity of the sympathetic-parasympathetic branches. We hope these considerations will lead to applied research, to tackle how the circadian imbalance of these pathways within and between organs may lead to diseases such as non-alcoholic fatty liver.

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