UNIVERSIDADE D
COIMBRA

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RESETTING THE CLOCK ON METABOLIC DYSFUNCTION

Dissertação no âmbito do Mestrado em Química Medicinal,
orientada pela Professora Doutora Cláudia Margarida Gonçalves Cavadas
e pela Doutora Sara Matias Carmo Silva
e apresentada ao Departamento de Química da Faculdade de Ciências e Tecnologia
da Universidade de Coimbra.

Julho de 2019

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This present work was performed in the Neuroendocrinology and Aging group of the Center for Neuroscience and Cell Biology (University of Coimbra, Portugal), headed by Professor Doctor Cláudia Cavadas, and under the supervision of Doctor Sara Carmo Silva (Center for Neuroscience and Cell Biology, University of Coimbra).

This work was supported by European Regional Development Fund (FEDER), through Programa Operacional Factores de Competitividade COMPETE2020 and National funds via Fundação para a Ciência e a Tecnologia (FCT) under the project CENTRO-01-0145-FEDER-000012-Healthy Aging and Jansen Innovation Award 2018.

Acknowledgements/Agradecimentos

Primeiramente, gostaria de agradecer à Professora Doutora Cláudia Cavadas por me ter acolhido no seu grupo para a realização deste projeto. Agradecer por todo o tempo despendido, por estar sempre disposta a ajudar em qualquer problema, pela sabedoria e força que transmite.

Gostaria também de agradecer à Doutora Sara Silva por me ter acompanhado e acreditado nas minhas capacidades. Agradecer por me ter feito crescer e por me ter ensinado resiliência e que com esforço e dedicação conseguimos sempre atingir os nossos objetivos.

À Doutora Ângela Relógio e à Rukeia El-Athman do Instituto Charité pela colaboração neste projeto e pela ajuda na análise bioinformática.

À Célia, que com toda a paciência e carinho, sempre se prontificou a ajudar com aquela palavra de atenção e sabedoria que me fazia sempre acreditar que mais é possível. Marisa, o furacão do laboratório. Obrigada por me teres acompanhado, sempre pronta a ajudar, mesmo quando ias embora e deixavas o recado "alguma coisa manda logo mensagem!". Obrigada pelo carinho e por todas as palavras que batiam sempre certas. Helena, a pessoa que me ensinou que a primeira impressão nem sempre é a correta. A todas as outras meninas do laboratório, Laetitia, Ana Carvalho, Ana Rita Álvaro, Inês, e claro, aos poucos rapazes que seguram as pontas Alex e Flávio, obrigada pela ajuda e apoio.

Quero agradecer aos meus colegas da "sala de mestrados" pois, sem eles, este percurso não teria sido o mesmo. Jéssica, a minha loira de eleição sempre com aquele beijo de "Bom dia, hoje vai correr bem", Patrick, Carolina e Vanessa, obrigada pelo apoio. Ao Ricardo, ao João Brás, à Andreia e à Bárbara, os meus amores que vieram, marcaram posição e mostraram que do meu lado não iriam sair. Pelos abraços de consolo, pelas gargalhadas, pelas conversas mais sérias e claro, pelas boleias. Um obrigada especial ao Brás por, nas longas noites de trabalho, não se ir embora para casa sem se esquecer de mim como se esquecia da Mariana para o almoço. À Carina, tu que com esses olhos me ensinaste como alterar imagens e com esse abraço me fazias sempre sentir em casa cada vez que voltava. Obrigada por me ensinares a fazer timelines e por me ensinares a ser dura e forte, obrigada pelo amor e carinho que nunca me deixaram cair. Mariana, o meu amuleto da sorte, obrigada por me ensinares que me deixar ficar pelo que os outros dizem não é opção, que consigo sempre mais do que imagino. Obrigada por me

perdoares cada vez que ia primeiro à Carina mostrar as pequenas vitórias, continuas a ser a mãe de eleição! Ao Daniel, ao *nerd* do Bairro, obrigada por ficares noites e noites no CNC a ouvir o meu trabalho que tentavas sempre perceber, pelas opiniões e críticas honestas. Obrigada por me desencaminhares sempre da minha dieta e por em compensação me obrigares a subir os arcos. Ana Catarina Franco, uma verdadeira tia merece mais do que o primeiro nome! Tu, o meu braço direito em tudo, obrigada pelo apoio incondicional, pelas boleias, pelas noites intermináveis, por chorares e sorrires comigo, por esse amor que só tu sabes dar desse teu jeito. Prometo andar sempre com um copo para que não tenhas de beber da garrafa, pois ambas sabemos que não ficavas bem a trabalhar nas obras. Obrigada, amiga.

Não posso deixar de agradecer ao Pedro Paulino, tu que apareceste, mexeste com o meu pequeno mundo e me ensinaste que uma boa amizade não se mede nem pela distância nem pela altura. Obrigada também ao Diogo, Malva e Inês Fonseca que sempre se mostraram disponíveis a ajudar e a me receber com um abraço.

Às minhas amigas do coração, Mariana, Telma, Sofia, Susana, Catarina (Limão) e Linda Inês, que me acompanharam desde sempre, a minha Tribo que muitas vezes troquei pelo trabalho. Obrigada por esse amor que me perdoava sempre, por esse lado doce e meigo que me animava em noites de desespero. Prometo voltar aos cafés e às nossas longas noites de conversas e gargalhadas. A vocês, um obrigada profundo!

À minha tia do coração, à tia Rosário e ao Nuno, que me adotaram como família. Tia, obrigada por esse coração doce que não tem preço, pelo amor sem fim, os telefonemas de preocupação quando eu ficava muito tempo sem dar notícias, pelas palavras de ternura, obrigada!

Um agradecimento especial ao Mário, ao Marco e à Maria João que, mesmo longe, me guiaram e acompanharam em todas as etapas. Obrigada pelas palavras sábias que me mostravam sempre qual o caminho certo a seguir, que não me deixavam cair e me diziam sempre "És mais forte do que pensas, tens de passar por isso para crescer". Obrigada por nunca me terem abandonado.

Ao André, a pessoa com mais paciência que já alguma vez vi! Obrigada por esse amor incondicional e por me relembrares todos os dias que, no fim de tudo e por mais luta que a vida dê, tu estás lá para me receber com o abraço mais reconfortante. Obrigada pelos gestos de carinho e de apoio, pela calma e tranquilidade que me transmites e por não desistires de mim e desta luta que é a distância.

Por fim, quero agradecer aos meus maiores companheiros de vida, à minha família! Começo por ti mano, tu que do teu jeito me ensinaste que não é preciso ser lamechas para se mostrar que se ama. Obrigada pela tua força que me inspira todos os dias. Por alinhares em tudo comigo, mesmo nas maiores parvoíces e acredita, vás tu para onde fores, eu vou sempre contigo. Deste-me sempre tudo, mas principalmente deste-me a irmã que sempre pedi, a Ana Cristina e a pequena pessoa que mais mexeu comigo, a Matilde. Ana, obrigada por olhares sempre por mim e por todo o amor e carinho. Matilde, és a afilhada que nunca imaginei ter, obrigada por confiares o teu pequeno mundo em mim e me permitires fazer parte de ti. Mãe e pai, uma coisa eu sei, quando for grande quero ser metade de vocês e ter metade do amor e compaixão que vocês têm. Mãe, a pessoa mais lutadora que conheço, que depois de tudo que passou anda sempre com o sorriso mais contagiante e que tem o melhor abraço, obrigada por me mostrares o quão bonita a vida pode ser. Obrigada por me ensinares que sem luta não chego onde quero, obrigada pelas palavras certas nos momentos difíceis, por caíres e te levantares comigo e por tomares as minhas dores como tuas. Pai, o melhor colo do mundo! Obrigada pelo carinho, por teres aceite andar num carrossel para crianças só para me fazer feliz, pelos abraços de consolo quando as palavras faltavam, por me acompanhares e me protegeres do Mundo. Aos dois, obrigada pelos sacrifícios e pelo amor incondicional, sem vocês não estaria onde estou e não seria a pessoa que hoje sou. Obrigada!

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Abbreviations

AgRP – Agouti-related peptide

ALS – amyotrophic lateral sclerosis

ARC – Arcuate Nucleus

ARNT-Like1 – Aryl hydrocarbon receptor nuclear translocator-like protein 1

ATXN2, Atx2 – Ataxin-2

BBB – Blood brain barrier

BCA – Bicinchoninic Acid

Bmal1 – Brain and muscle Arnt-like protein-1

BSA – Bovine Serum Albumine

CART – Cocaine-and Amphetamine-Regulated Transcript

CCGs – Clock Controlled genes

CLOCK – Circadian Locomotor Output Cycles Kaput

Cry – Cryptochrome

DMEM – Dulbecco's Modified Eagle Medium

DTT – Dithiothreitol

E-box – Enhancer box

ECF – Enhanced Chemiofluorescence

FBS – Fetal Bovine Serum

FC – Fold Change

fcHFHS – free choice High-Fat High-Sugar

HFD – High-Fat Diet

Hprt – Hypoxanthine phosphoribosyltransferase

KDa – Kilo Dalton

KO – Knock Out

LHA – Lateral Hypothalamic Area

Lsm – Like-Sm

MEFs – Mouse Embryonal Fibroblasts

mHypoE-N42 – embryonic mouse hypothalamic cell line N42

mTOR – mammalian/mechanistic target of rapamycin

NES – Night Eating Syndrome

NPY – Neuropeptide Y

NTS – Nucleus Tractus Solitarius

OD – Optical Density

PAPB – PolyA-binding protein

PAM2 – PolyA-binding protein-interacting motif

PBN – Parabrachial Nucleus

PBS – Phosphate-buffered saline

Per – Period

PMSF – Phenyl-Methylsulphonylfluoride

POMC - Pro-opiomelanocortin

PVN – Paraventricular Nucleus

PVT – Paraventricular Thalamus

qRT-PCR – quantitative Real Time Polymerase Chain Reaction

RAIN – Rhythmicity Analysis Incorporating Nonparametric Methods

REV-ERBα – Reverse erythroblastosis virus alpha

RHT – Retinohypothalamic Tract

RIPA – Radio-Immunoprecipitation Assay

RISC – RNA-induced silencing complex

RNA – Ribonucleic Acid

ROR – Retionic acid receptor-related orphan receptors

Rpm – rotations per minute

Rps6K – Ribosomal protein S6 Kinase

SCA2 – Spinocerebellar ataxia type 2

SCN – Suprachiasmatic Nucleus

SDS – Sodium Dodecyl Sulphate

SEM – Standard Error of the Mean

SRED – Sleep-Related Eating Disorder

SV40TAG – Simian Vacuolating Virus 40 T-Antigen

TORC1 – mTOR complex 1

TORC2 – mTOR complex 2

TRI Reagent – Trizol Reagent

TYF – TWENTY-FOUR protein

VMH – Ventromedial Hypothalamus

VTA – Ventral Tegmental Area

VMH – Ventromedial Hypothalamus

WAT – White Adipose Tissue

WT – Wild-Type

ZBRK1 – KRAB-containing Zinc-finger Transcriptional Regulator

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Abstract

Circadian rhythm is the intrinsic clock within cells, responsible for the maintenance of body homeostasis and energy metabolism. This internal clock is synchronized by a master clock located in the suprachiasmatic nucleus, responsible for the regulation of the peripheral clocks, present in various tissues and cells. When disturbances to this circadian clock occur, they can lead to metabolic disorders, such as obesity. In turn, obesity *per se*, can also promote circadian deregulation. Finding new intermediary proteins that can act both on circadian rhythm and metabolism, might be a good strategy for better therapeutic approaches for obesity.

The aim of this study was to understand the role that saturated fatty acids have on the molecular circadian system *in vitro*. We used the hypothalamic mHypoE-N42 cell line and evaluated for circadian rhythmicity of core clock genes.

Results obtained demonstrate that a saturated fatty acid alters the circadian rhythmicity of clock genes. And an endogenous molecule X displays circadian rhythmicity in protein levels, that upon metabolic dysfunction are lost, promoting arrhythmicity. The overexpression of endogenous molecule X in mHypoE-N42 cells prevents the effects induced by saturated fatty acid in clock genes. These results may suggest a possible role for this protein as a new modulator of circadian rhythm.

Key words: Circadian rhythm, Hypothalamus, Clock genes

Resumo

O ritmo circadiano é o relógio intrínseco presente nas células, responsável pela manutenção da homeostase do corpo e do metabolismo energético. Este relógio interno é sincronizado por um relógio mestre, localizado no núcleo supraquiasmático e responsável pela regulação dos relógios periféricos, presentes em várias células e tecidos. Quando ocorrem perturbações no ritmo circadiano, estas podem levar a distúrbios metabólicos como a obesidade. Por sua vez, a obesidade, por si só, pode promover a desregulação no ritmo circadiano.

O objetivo deste trabalho foi perceber o papel que os ácidos gordos saturados desempenham no sistema circadiano molecular *in vitro*. Para isso utilizamos a linha celular hipotalâmica mHypoE-N42 e avaliamos a ritmicidade circadiana dos genes relógio.

Os resultados obtidos demonstram que um ácido gordo saturado altera a ritmicidade circadiana dos genes relógio e que, uma molécula endógena X, exibe ritmicidade circadiana nos níveis proteicos. Sob disfunção metabólica, estes ritmos perdem-se tornando-se esta proteína arrítmica. A sobre expressão da molécula endógena X nas células mHypoE-N42, previne os efeitos induzidos pelo ácido gordo saturado nos genes relógio. Estes resultados parecem apontar para um possível papel como modulador do ritmo circadiano, para a molécula X.

Palavras-chave: Ritmo circadiano, Hipotálamo, Genes relógio

Chapter I – Introduction

1 - Circadian rhythm

The rotation of the earth on its own axis causes light and dark cycles that impact the life of human beings. These changes allowed organisms in our planet to develop an anticipatory mechanism, responsible for the maintenance of the body homeostasis in order to respond to environmental changes (Panda, Hogenesch et al. 2002). Variations throughout the day in behavioral and physiological processes are dictated by this intrinsic mechanism, named circadian rhythm (Reinke and Asher 2019). Circadian derives from Latin where *circa* means around and *dies* means a day, referring to the periodicity of approximately 24 h of biological cycles (Eckel-Mahan and Sassone-Corsi 2013).

The circadian rhythm is present in a large range of species including plants, animals, humans, and bacteria allowing organisms to anticipate day-to-day changes in temperature and light. This rhythm works as the intrinsic clock within cells that maintains body homeostasis, in which the activities of the biological cycle are completed. In other words, this system is answerable for the determination of biological and behavioural activities by the control of our peaks of physiological activities (Figure 1). This internal clock justifies some occurrences in the life of human beings: for instance, most car accidents occur at 2 am, when our body enters the supposedly deepest phase of sleep; the highest risk of stroke and most births happens at dawn, concordant with an intrinsic peak of blood pressure (Froy 2011). Circadian system is also responsible for the oscillation of many physiologic processes such as active-rest period, body temperature, insulin secretion, glucose metabolism, blood pressure, etc. (Damiola, Le Minh et al. 2000).

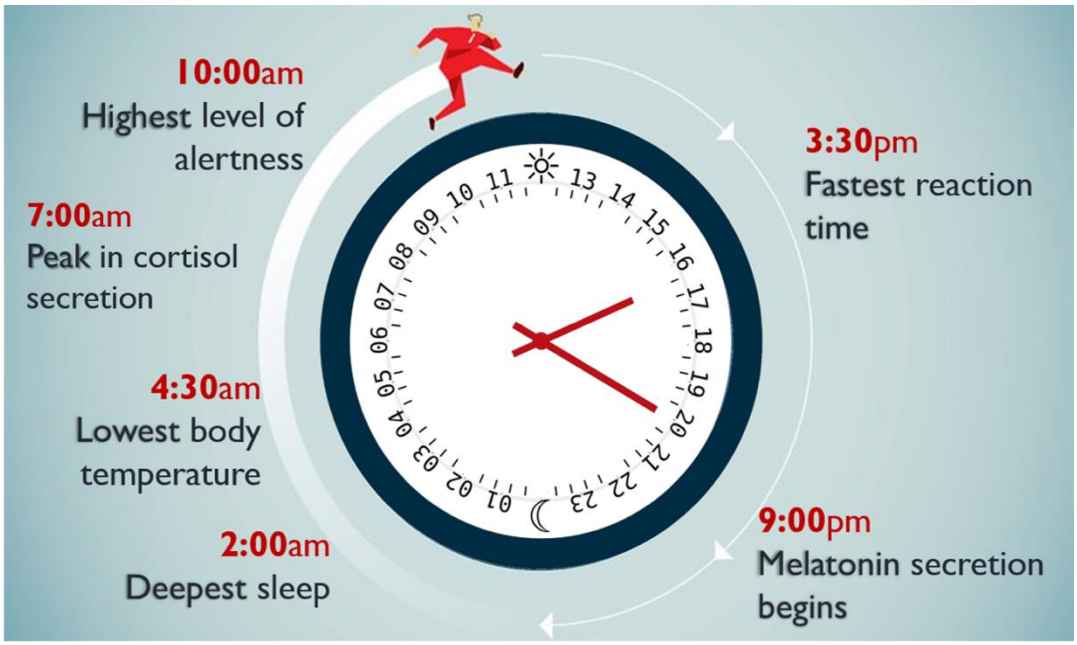

Figure 1. Circadian rhythm controls and dictates biological and physiological activities in the life of a human being.

Circadian rhythm is the intrinsic clock within cells, responsible for the maintenance of body homeostasis. (Adapted from (Farhud and Aryan 2018)).

1.1 – Organization of the molecular circadian machinery

At the molecular level, circadian rhythm is comprised by a set of genes denominated clock genes that are present in most cells of the body, tissues, and organs. As for the regulation of this cycle, the core mechanism of the circadian clock leans on intracellular mechanisms, namely a negative transcriptional-translational feedback loop (Figure 2) that involves various key players (Schibler, Ripperger et al. 2003).

In mammals, the circadian oscillator consists of an interplay between the positive and the negative transcriptional regulators. *CLOCK* (circadian locomotor output cycles kaput) and *ARNT-Like1* (aryl hydrocarbon receptor nuclear translocator-like protein 1or *Bmal1*; herein referred to as *Bmal1*) comprise the positive flank of the cycle, acting as transcription activators. As for the negative course of this rhythm, it is composed by period (*Per1*, *Per2*, *Per3*) and cryptochrome (*Cry1* and *Cry2*) (Lowrey and Takahashi 2000), that in turn, act as transcription inhibitors.

Positive regulators, *CLOCK* and *Bmal1*, form a heterodimer and promote the expression of clock controlled genes (CCGs), by binding to their promoters at the E-box. Some

CCGs are transcription factors like RAR-related Orphan Receptor alpha (*RORα*) and Nuclear Receptor Subfamily 1 Group D (more frequently known as *REV-ERBα*), which form a secondary feedback loop, further ensuring the regulation of clock factors. For example, *REV-ERBα* negatively regulates the expression of *Bmal1* while *RORα* regulates it positively (Takahashi 2017). Therefore, these transcription factors work like clock mediators, providing control feedback factors in order to ensure the maintenance of the rhythm (Preitner, Damiola et al. 2002, Sato, Panda et al. 2004).

CLOCK-BMAL1 complex activate the expression of the negative regulators *Per* and *Cry*. When these reach a concentration threshold, *Per* and *Cry* form a heterodimer and translocate into the nucleus to inhibit the activity of CLOCK-BMAL1 (Sahar and Sassone-Corsi 2012). Once *CLOCK* and *Bmal1* activity is suppressed, without activators, PER-CRY expression starts to decay. Consequently, without the transcription repressors, *CLOCK* and *Bmal1* can start being transcribed once again and a new cycle begins (Bass 2012).

In sum, circadian rhythm works as a self-sustainable cycle where clock genes are the principal regulators maintaining this oscillator working for approximately 24 h every day, thus preserving the whole-body physiology on mammals and many other organisms.

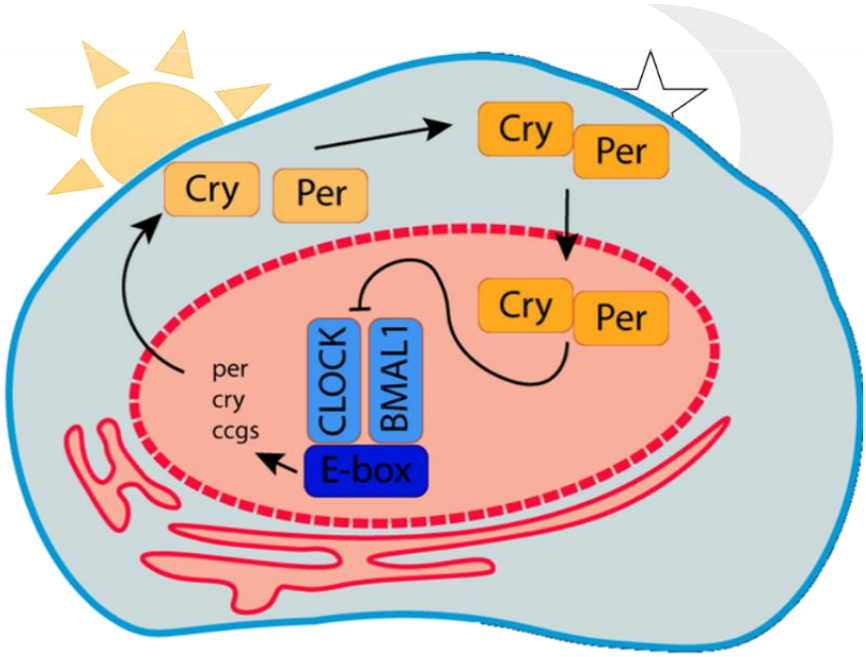

Figure 2. Negative transcription- translation feedback loop that forms mammalian molecular circadian machinery.

CLOCK and *Bmal1* are transcription factors that compose the positive side of the cycle that regulate the expression of *CRY* and *PER*, the negative elements. *CLOCK* and *Bmal1* induce the expression of clock controlled genes (CCGs) by binding to their promoters in the E-BOX. *Per* and *Cry*, in turn, form a heterodimer and translocate into the nucleus inhibiting the expression of the CLOCK-BMAL1 complex. Once *Per* and *Cry* decay, the cycle resets. This cycle takes approximately 24 hours (Adapted from (Paschos and FitzGerald 2017).

1.2 – Anatomy of the central clock

Clock genes are expressed in all tissues and cells of the body. These tissues have the competence to express and maintain autonomous circadian oscillations/rhythms (Yoo, Yamazaki et al. 2004). In order to maintain body homeostasis as well as to keep all of the peripheral oscillators synchronized, there is a master synchronizer, a master clock. This central pacemaker is located in the anterior hypothalamus, more precisely in the hypothalamic suprachiasmatic nucleus (SCN) (Froy 2012). Studies in rodents without SCN showed that circadian rhythmicity is maintained in most tissues, but becomes dampened after some time (Yoo, Yamazaki et al. 2004)

Oscillations in circadian rhythm are entrained by *Zeitgebers* (German term for "time givers"), external stimuli that can be defined as synchronizers. *Zeitgebers* allow the synchronization between the body and the environment. Light, food, drugs, and temperature, are examples of these external stimuli (Albrecht 2012). Light performs as the main *Zeitgeber* since it works directly in the central pacemaker. The SCN synchronization depends on the reception of photic information, recognized by the retina and transmitted through the retinohypothalamic tract (RHT) (Blancas-Velazquez, Mendoza et al. 2017).

1.2.1. The Hypothalamus

In regard to the hypothalamus, this brain region is not only responsible for the regulation of circadian rhythm, but it is also responsible for the maintenance of body homeostasis. This key role in body homeostasis derives from the hypothalamus ability to regulate a variety of processes such as sleep, fatigue, stress response, body temperature, fertility, aging, metabolic regulation, and circadian rhythm (Machluf, Gutnick et al. 2011). Hypothalamus is constituted by three predominant regions: periventricular, medial and lateral, where each one of these is comprised of an assorted of nuclei with distinct physiological activities. Every nuclei are comprised of different neuronal cells that communicate within the nucleus in order to form neuronal networks and maintain physiological functions (Carmo-Silva and Cavadas 2017).

One of the essential physiological functions of the organism is feeding. Feeding patterns are directly responsible for the body homeostasis, where homeostasis reflects the energy status. In other words, a condition of energy homeostasis is based on an equilibrium between energy intake and energy expenditure. This balance is controlled by environmental cues, behavioural conditions, and peripheral signs, where the hypothalamus acts as a sensor that integrates all of these factors (Ueno and Nakazato 2016). This brain region is going to receive peripheral informations from organs such as the white adipose tissue (WAT), stomach, pancreas, and gut and orchestrate these signals in order to respond organism's needs (Blouet and Schwartz 2010).

In regard to the regulation of food intake by the hypothalamus, there are several nuclei involved in this process such as the hypothalamic arcuate nucleus (ARC), ventromedial hypothalamus (VMH), paraventricular nucleus (PVN) and lateral hypothalamic area (LHA) (Schwartz, Woods et al. 2000). The hypothalamic arcuate nucleus is recognized as an energy balance sensor due to its priveleged position within the hypothalamus. The ARC is in close contact with the blood brain barrier (BBB) and besides that, this nucleus also receives peripheral signals which are integrated by two predominant nuclei: the orexigenic and anorexigenic neurons (Schwartz, Woods et al. 2000, Langlet 2014, Carmo-Silva and Cavadas 2017).

Orexigenic molecules present in the ARC such as neuropeptide Y (NPY) and agouti-related peptide (AgRP) are the ones responsible for the ingestion of food. When our stomach is empty and consequently we feel hunger, these are the neurons that send the

signals in order to promote food intake. On the other side, when we feel satisfied in regard to feeding/food, the other population of neurons present in the ARC are going to act. These are anorexigenic molecules such as proopiomelanocortins (POMC) and cocaine-and amphetamine-regulated transcript (CART) that decrease the ingestion of food and, as a result, promote energy expenditure and provides indications of saciety (Schwartz, Woods et al. 2000, Langlet 2014).

There are other brain areas that take part in the process of food intake and energy expenditure. Some of them are ventral tegmental area (VTA), paraventricular thalamus (PVT), parabrachial nucleus (PBN) and the nucleus tractus solitarius (NTS) (Waterson and Horvath 2015). Despite all of these nuclei present in these brain areas, it is in the hypothalamus that the main energy center is placed included ARC, PVN, VMH and LHA nuclei that interact to form responses according to organism's needs (Williams and Elmquist 2012).

However, as most of the processes in our organism, feeding is also regulated by circadian rhythm. This is of special importance if we think that the central clock and the central energetic regulator are comprised within the same brain region. Furthermore, when feeding patterns are altered, for example in the consumption of a high fat diet (HFD), dysfunction and inflammation occurs in the hypothalamus. The hypothalamic dysfunction will ultimately compromise all functions of the hypothalamus, namely circadian rhythm and energy balance.

1.3 – Circadian regulation and metabolism

The circadian cycle has a direct impact on energy homeostasis and metabolism regulation. The expression of most metabolites, enzymes and key players in metabolic pathways, display circadian rhythmicity (Ramsey, Marcheva et al. 2007, Froy 2010). These metabolites, such as lipids, amino acids, and glucose oscillate in a circadian manner in the blood, with peaks occurring during the active period and wakefulness (Reinke and Asher 2019). Likewise, feeding patterns have the ability to regulate the circadian clock. The major amount of food should be consumed in the active period, not just because of the organization of our circadian cycle but also in order to our body restore energies and

nutrients during the rest phase (Reinke and Asher 2019). When food is consumed outside the active period, metabolic alterations occur (Froy 2010).

Glucose is the energy source of plenty cells, is mainly driven by the SCN and is one of the responsible for scheduling food intake to the active period (Dyar, Ciciliot et al. 2014). This monosaccharide shows circadian rhythmicity in peripheral tissues and brain, with a peak occurring at the beginning of the active phase, more precisely in the course of transition from inactive phase to active period (Cailotto, La Fleur et al. 2005). A study in rats with lesions in the master clock (SCN) showed alterations in blood glucose homeostasis, with modifications in liver glucose uptake and production (Kalsbeek, Ruiter et al. 2006).

Insulin also presents circadian rhythmicity, not only in its secretion from the pancreas but also at the systemic level, in its action (impacting on insulin sensitivity) (Ding, Gong et al. 2018). Pancreatic ß cells, responsible for insulin secretion also oscillate in a circadian manner (Perelis, Marcheva et al. 2015). Studies on pancreatic islet cells demonstrated that deletion of *CLOCK* or *Bmal1* leads to defective insulin secretion, lower glucose tolerance, and modifications in the proliferation of these cells (Marcheva, Ramsey et al. 2010, Eckel-Mahan and Sassone-Corsi 2013).

Metabolic mediators secreted by adipocytes, such as leptin, adiponectin, and resistin also oscillate in a circadian manner. Leptin acts on the hypothalamus, promoting appetite suppression (Froy 2012). As levels of leptin in plasm are circadian, the peak in leptin is set to occur early in the inactive phase (to ensure satiety during the inactive period) (Sukumaran, Almon et al. 2010). Upon SCN lesion, leptin loses its circadian rhythmicity (Kalsbeek, Fliers et al. 2001).

Glucocorticoids also exhibit circadian rhythmicity, with a peak occurring at the beginning of the active period, both in humans and rodents (Cheifetz 1971). Experiments show that rhythmicity in glucocorticoid levels drop in *CLOCK* mutant mice. On the other hand, glucocorticoids can also reset the phase of circadian rhythm (Balsalobre, Brown et al. 2000).

The mammalian/mechanistic target of rapamycin (mTOR) acts as a signaling pathway in the modulation of metabolic processes, whereas its activity is regulated by nutrients (Ramanathan, Kathale et al. 2018). Besides metabolic regulation and along with ribosomal protein S6 kinase (RPS6K) (downstream target), mTOR is also implicated in cellular processes such as cell growth, proliferation or death, autophagy and protein synthesis

(Hu, Xu et al. 2016). Deregulation of this signaling pathway can promote diseases associated with metabolic dysfunction, such as type 11 diabetes and obesity, but also neurodegenerative diseases and cancer (Haissaguerre, Saucisse et al. 2014). In mice and *Drosophila*, genetic modifications in the mTOR pathway promote alterations in the circadian cycle (Liu, Stowie et al. 2018). Curiously, mTOR plays many roles in several tissues and cells external to the master clock, such as adipocytes, skeletal and cardiac muscles and renal carcinoma cells, where it exhibits circadian rhythmicity (Chang, Yoshihara et al. 2017). Considering that the mTOR pathway is connected with energy rank in cells and controlled by nutrients, these results suggest that through the mTOR pathway, cellular metabolism can also hint the circadian cycle (Ramanathan, Kathale et al. 2018).

1.4 – Dysfunction of circadian rhythm conducts to metabolic disturbances

In the modernized society that we live in, it is easy to somehow disrupt the circadian cycle. For example, sleeping behaviors are frequently disturbed, being these the main disrupters of our internal clock (Peplonska, Bukowska et al. 2015). When this and other perturbations occur, the major impact will show off in body homeostasis leading to serious illness such as neurodegenerative diseases, psychiatric and metabolic disorders (Navara and Nelson 2007).

Disorders connected to metabolism, for instance, obesity, hyperlipidemia, and hyperinsulinemia are a result of an impairment in the balance between energy intake and energy expenditure (Carmo-Silva and Cavadas 2017). Nowadays, feeding became more reckless where access to caloric food is now more frequent and lifestyle evolved into a sedentary pattern. This imbalance increases the number of obese people becoming this one of the diseases of the century (Shimizu, Yoshida et al. 2016). We know more about obesity causes, namely that meal timing is as important as the constitution of the meal *per se* (Wang, Patterson et al. 2014). Meal timing is tightly correlated with the circadian clock and it is well established that overall metabolism is under circadian regulation (Kornmann, Schaad et al. 2007).

Meal timing ensures that we ingest calories during our active period, in opposition to feeding in the inactive period. The latter option increases the predisposition for obesity (Wang, Patterson et al. 2014). There are many circadian disruptors that can also lead to metabolic dysfunction, for instance, exposure to light in the dark period, which happens when we are exposed to artificial lights (telephones and laptops), or jet lag (Summa and Turek 2014, Hatori, Gronfier et al. 2017).

Rotating shifts and night shifts have been associated with the increased predisposition for obesity, but also cancer, type 11 diabetes, metabolic syndrome, and cardiovascular disease. Such alterations in the normal schedule impact on the molecular and physiological rhythms within the body (Eckel-Mahan and Sassone-Corsi 2013, Wyse, Celis Morales et al. 2017). Experimental models support this link between circadian rhythm and metabolism. When mice are put in dim light instead of darkness, they suffer a pronounced increase in body mass (Fonken, Finy et al. 2009). In a groundbreaking study, Hatori, et al., observed that mice fed a high fat diet *ad libitum* became morbidly obese, however, when this access was restricted to the active period, the weight gain and metabolic dysfunction was not observed (Hatori, Vollmers et al. 2012). These results indicate for weight and metabolism, is not only what we eat that matters, but also when we eat it. The restriction of food to the activity period is enough to ensure both the functioning of circadian clock and metabolic homeostasis (Hatori, Vollmers et al. 2012). Nocturnal mice fed solely during their supposedly inactive period, had an inversion in the normal expression of clock genes in heart, liver, lung, kidneys, and skeletal muscle (Laermans and Depoortere 2016). Taking all these studies into consideration, inversion of the normal day-night patterns and shifts in meal timing, lead to the deregulation of the circadian cycle and increase the predisposition for metabolic disorders (Eckel-Mahan and Sassone-Corsi 2013, Guerrero-Vargas, Espitia-Bautista et al. 2018).

1.5 – Obesity alters circadian rhythmicity

The feeding cycle in mammals is under homeostatic control being coordinated by the limbic system and hypothalamus, which in turn regulates the impulse to eat or not eat (Elmquist, Coppari et al. 2005). The hypothalamus, due to its role in the regulation of

circadian rhythm and in energy balance, is responsible for managing and timing of behavioural rhythms such as feeding (Schwartz, Woods et al. 2000). However, feeding is not as simple as what we eat and when we eat it, or even when we are hungry or satiated. The limbic system is responsible for the process of reward, which connects high-palatable food with the feeling of pleasure (Avena, Bocarsly et al. 2012). The process of reward may be one of the most important processes in the development of diet-induced obesity. For example, a meal enriched in high fat or high sugar, stimulates the limbic system, triggering our senses and the feeling of satiety is attenuated. In the end, this process leads to a compulsion of high palatable food and to a development of obesity and other metabolic disorders (Zheng, Lenard et al. 2009, Avena, Bocarsly et al. 2012).

In this context, there two eating disorders, night-eating syndrome (NES) and sleep-related eating disorder (SRED), which support the interesting link between obesity and circadian rhythm. These two eating syndromes are both related to feeding at the wrong period of the day, the night, and are characterized by continual awakenings during the sleep and consequent desire to eat mostly high fat and/or high sugar foods. They are also typified by evening hyperphagia and morning anorexia (O'Reardon, Peshek et al. 2005, Kucukgoncu, Midura et al. 2015). People who suffer from NES display an advance in the phase of ghrelin rhythms and a delay in melatonin rhythm. This and other consequences, such as insomnia, incoherent sleep ,and a delay in the onset of sleep, leads patients to the development of depressive symptoms and mood swings (de Zwaan, Roerig et al. 2006, Eckel-Mahan and Sassone-Corsi 2013). Moreover, evidences show that more than 40% of NES individuals become obese (Gallant, Lundgren et al. 2012, Eckel-Mahan and Sassone-Corsi 2013).

Nevertheless, there are various evidence linking circadian rhythm and metabolic disorders, like obesity (Eckel-Mahan and Sassone-Corsi 2013). Taking this into consideration, researchers developed animal models to study the factors involved in this relation. Rodents with a free choice between high-fat high-sugar (fcHFHS) or standard chow food in the day-to-day diet, develop a "snacking behaviour", and consequently altered circadian patterns of feeding (la Fleur, Luijendijk et al. 2014). This snacking behaviour coincides with the consumption of food in their inactive period, which promotes and exacerbates obesity and metabolic dysfunction (la Fleur, Luijendijk et al. 2014). Obese individuals also display this type of behaviour, cravings for high-fat or high-sugar foods at night (Berteus Forslund, Torgerson et al. 2005). In mice fed a high fat diet

in the supposedly inactive period, *BMAL1* and *Per2* were decreased in the liver, adipose tissue, and hypothalamus (Kohsaka, Laposky et al. 2007). As a matter of fact, clock gene alterations can be observed just after 3 days of exposure to high-fat diet (Eckel-Mahan, Patel et al. 2013).

A diet rich in fat consists in an excessive consume of calories resulting in an increase in the levels of toxic lipids in the blood circulation. As a result, chronic inflammation occurs leading to pathogenic risk factors like cardiovascular diseases, obesity and diabetes (Kwon and Querfurth 2015). The most common saturated fatty acid present in a high fat diet is palmitate (Tse, Salehi et al. 2018). Circulating palmitate can promote insulin resistance, metabolic pathways disruption (mTOR), inflammation and endoplasmic reticulum stress, leading to dysfunction in metabolic tissues (Mayer and Belsham 2010, Dalvi, Chalmers et al. 2017). Gao et al. performed a study in hepatocytes that, when treated with palmitate, developed insulin resistance (Gao, Nong et al. 2010). Palmitate also impacts the hypothalamus, the main region that integrates and regulates many physiological processes such as feeding and circadian rhythm (Tse, Salehi et al. 2018). A study performed in mHypoE-37 neurons demonstrated that, upon palmitate treatment, these neurons exhibited disrupted circadian behavior at the molecular level (Greco, Oosterman et al. 2014).

These results support the link between circadian rhythm and metabolic alterations, indicating that upon obesity there is the disruption of the normal circadian rhythm. And that on the other hand, as discussed, the disruption of the normal circadian rhythm can predispose for obesity. Thus, the restoration of circadian rhythm might be a useful strategy to prevent or treat obesity.

1.6 – Understanding the circadian clock: benefits for obesity therapy?

As previously established, the mammalian circadian system is composed by a master clock located in the SCN which is responsible for the maintenance of the whole body homeostasis and the regulation of peripheral clocks present in most tissues and cells (Shimizu, Yoshida et al. 2016, Mendoza 2019).

Given the inter-connection between circadian rhythms and metabolism, it is necessary to take a closer look and further investigate circadian machinery at the physiological level to better understand how clock genes work in a case of metabolic dysfunction. *In vitro* studies are the first step to understand this bridge but are still not clear in a way that do not provide enough information. Nevertheless, researchers developed into animal models with genetic alterations lacking specific clock genes (Tsang, Astiz et al. 2017). These mouse models are of special interest for the study of the impact of circadian rhythm alterations on metabolism.

1.6.1 – Genetic mouse models to study circadian rhythm disruption

Bmal1 is one of the clock genes that composes the positive flank of the circadian rhythm and it was identified as an essential element in the regulation of this machinery. *Bmal1* knockout (KO) mice in conditions of constant dark displayed arrhythmicity with slight differences between the active (dark) and inactive (light) phase (Bunger, Wilsbacher et al. 2000). These mice, when exposed to a condition of 6 hours of constant light during the dark period, did not show differences in behavior, while wild-type (WT) mice suffered a decrease in activity (Bunger, Wilsbacher et al. 2000). *Bmal1* KO mice did not exhibit differences in food intake, nevertheless, present a decrease in serum insulin, an increase in visceral fat and glucose intolerance (Lamia, Storch et al. 2008). Further studies connected this circadian activator with lipid metabolism in mature adipocytes and adipogenesis (Guo, Chatterjee et al. 2012). For instance, in *Bmal1* KO mice embryonic fibroblasts did not differentiate into adipocytes leading to a consequent decrease in adipogenesis. However, the overexpression of *Bmal1* in adipocytes resulted in an increase in lipid synthesis activity (Froy 2012). These and other findings have shown that *Bmal1* might be involved in the process of adipocyte differentiation and glucose metabolism acting also as an important factor in insulin production (Yang, Chen et al. 2016).

CLOCK is also part of the positive heterodimer from circadian regulation. Studies in *Clock* mutant mice in conditions of constant darkness show an increase in the activity during the rest period, however, these mice become arrhythmic over time (Turek, Joshu et al. 2005). These changes in activity lead to alterations in feeding patterns. *Clock* mutant mice

both on chow and high fat diet are obese, with hyperphagia and decreased energy expenditure (Turek, Joshu et al. 2005).

Period gene family belongs to the negative side of the feedback loop responsible for the regulation of the circadian cycle. *Per2* mutant mice in constant darkness, display, during the supposedly active period, a decrease in activity and attenuated circadian rhythmicity (Yang, Liu et al. 2009). Besides that, these mice demonstrate impaired stress response, due to the loss of diurnal corticosterone oscillations (Yang, Liu et al. 2009). These mice show increased adipose tissue and hyperphagia when exposed to a high-fat-diet (Yang, Liu et al. 2009).

With respect to the secondary loop, ROR and REV-ERBs, also play a role in lipid metabolism and adipocyte differentiation (Froy 2012). REV-ERBs act as a negative regulator of clock gene *Bmal1* and RORα as a positive regulator (Preitner, Damiola et al. 2002, Sato, Panda et al. 2004). Mice with diet-induced obesity, when treated with a REV-ERB agonist, display a decrease in fat mass and an improvement in hyperglycemia and dyslipidemia. This evidence suggest that inhibition of Bmal1 levels might be benefic in obesity (Froy 2012).

This evidence show that circadian clock genes not only act as circadian regulators but also play a role in metabolism. However, more studies are necessary in order to better understand how we can target circadian cycle to prevent metabolic disturbances. With this aim in consideration, it is necessary to find secondary targets that can provide a link between these two mechanisms.

1.7 – Ataxin-2 protein

Ataxin-2 is an mRNA binding protein encoded by the ATXN2 gene with various functions at the cellular level with an estimated molecular mass of 140 kDa and 1312 amino acid residues (Sahba, Nechiporuk et al. 1998, Carmo-Silva, Nobrega et al. 2017). Mutations in this gene lead to neurodegenerative diseases such as spinocerebellar ataxia type 2 (SCA2) and to an increased risk in the development of amyotrophic lateral sclerosis (ALS) (Carmo-Silva, Nobrega et al. 2017, Pfeffer, Gispert et al. 2017). Ataxin-2 is expressed in almost every tissue such as the liver, gut, lung, muscle, brain, and heart. Ataxin-2 major expression occurs in the Purkinje cells present in the cerebellum, but is

also present in the hippocampus, thalamus and hypothalamus (Nechiporuk, Huynh et al. 1998, Huynh, Del Bigio et al. 1999). The main location of this polyglutamine protein is in the cytoplasm, however, there is evidence proving an association between ataxin-2 and polysomes in the endoplasmatic reticulum and its presence in nuclear and plasma membranes (Hallen, Klein et al. 2011).

Ataxin-2 plays several roles in the cell, namely RNA metabolism through mRNA splicing, mRNA stabilization and mRNA decay (Albrecht, Golatta et al. 2004). Ataxin-2 contains an N-terminal like-Sm (Lsm) domain and a C-terminal polyA-binding protein-interacting motif (PAM2), motif for regulation of RNA metabolism (Albrecht, Golatta et al. 2004). This protein also acts as a transcription modulator and has the ability to modulate its own expression, demonstrating that can modulate the process of transcription itself (Hallen, Klein et al. 2011). Another function is related to cytoskeleton reorganization where studies in *C. elegans* provide information about the cell cycle, where ataxin-2 displayed a role in microtubule dynamics (Stubenvoll, Medley et al. 2016). Moreover, ataxin-2 also plays a role in endocytosis, interacting with endophilins, proteins known to be implicated in the endocytosis process (Wiley and Burke 2001). Mutation in the ATXN2 gene leads to an impairment in calcium homeostasis, proposing a role in this function (Halbach, Gispert et al. 2017)

1.7.1 – Ataxin-2 in metabolism

The idea of ataxin-2 as a metabolic regulator arisen almost by chance, by the observation that ATXN2 KO mice develop insulin resistance, alterations in lipid metabolism and dyslipidemia, and abdominal obesity (Kiehl, Nechiporuk et al. 2006, Meierhofer, Halbach et al. 2016). However, when this protein is mutated, it results in a decrease in body weight (Damrath, Heck et al. 2012).

Studies in *Caenorhabditis elegans* suggest that the ataxin-2 homologue ATX-2, is necessary for the weight-loss promoted by dietary restriction (Bar, Charar et al. 2016). In this case, if ATX-2 is suppressed even upon dietary restriction, animals display an increase in fat content and body mass. Bar, Charar et. al. also proposed that the impact of ataxin-2 on body size might occur through the mTOR pathway (Bar, Charar et al. 2016). Regarding

this evidence and considering ataxin-2 functions, this protein has been suggested as a new metabolic player (Carmo-Silva, Nobrega et al. 2017).

1.7.2 – Ataxin-2 and mTOR pathway

As mentioned, mTOR (mammalian target of rapamycin) is a nutrient sensing protein involved in the maintenance and regulation of metabolism and energy homeostasis, involved in obesity and insulin resistance (Hu, Xu et al. 2016).

There are a few evidence connecting this serine/threonine protein kinase with ataxin-2, in the context of stress response (Takahara and Maeda 2012). For instance, some of this evidence propose that ataxin-2 can inhibit mTOR pathway, either indirectly through stress granules, or directly through a reduced phosphorylation of the ribosomal protein S6 (RPS6), which in turn is responsible for the regulation of cell proliferation and protein synthesis, as a consequence of a block in mTOR pathway by ataxin-2 (Swisher and Parker 2010, Takahara and Maeda 2012, DeMille, Badal et al. 2015, Lastres-Becker, Nonis et al. 2016, Carmo-Silva, Nobrega et al. 2017).

Stress granules are responsible for cell survival being formed in a situation of cellular stress by mRNA protein complexes (Sfakianos, Mellor et al. 2018). Ataxin-2 protein is determinant for the formation of stress granules, being a part of its components. When nutritional stress occurs, ataxin-2 sequester TORC1, a subunit of mTOR, into stress granules, thus inhibiting this pathway (Swisher and Parker 2010, Takahara and Maeda 2012, Carmo-Silva, Nobrega et al. 2017).

Nevertheless, studies in mouse embryonal fibroblasts (MEFs) from ATXN2 KO mice suggest a possible role for ataxin-2 in the regulation of S6 kinase. Absence of ataxin-2 leads to the increase in RPS6K phosphorylation, as a result of an activation of the PI3K/mTOR pathway (Lastres-Becker, Nonis et al. 2016). Thereby, through this modulation of RPS6K activity by ataxin-2, this polyglutamine protein also impacts on mTOR signaling. It is well established that the mTOR pathway is a crucial component for metabolic regulation, the role of ataxin-2 in metabolic homeostasis is further supported by the fact that this protein can impact this metabolic pathway (Carmo-Silva, Nobrega et al. 2017).

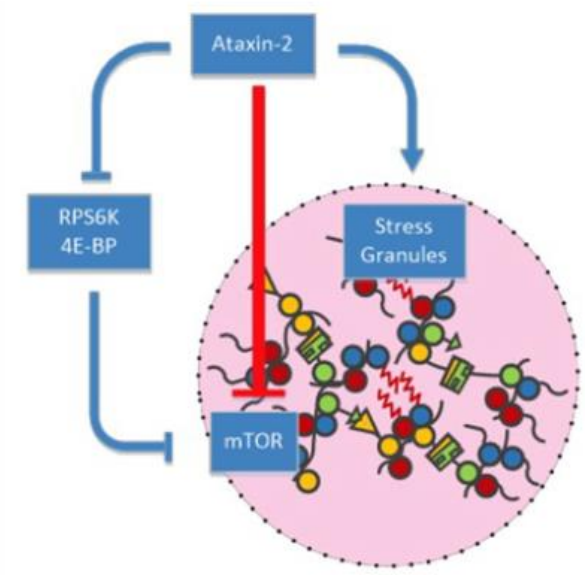

Figure 3. Ataxin-2 has a regulator of the metabolic pathways such as mTOR.

A lack of ataxin-2 results in metabolic dysfunction in mice and worms. This suggests a possible role for ataxin-2 in metabolic pathways whereas the metabolic actions of ataxin-2 might be modulated through the mTOR pathway. Ataxin-2 has the capability to block mTOR with a consequent decrease in the phosphorylation of RPS6K and 4E-BP. It is also known that ataxin-2 acts in the formation of stress granules. Likewise, mTOR might be sequestered by stress granules blocking its action where ataxin-2 expression might be a part of this sequestration. (Image from Carmo-Silva et al., 2017. Usage approved by the author).

1.7.3 – Ataxin-2 in circadian rhythm

Circadian rhythms in mammals are more complex than in invertebrates, where clock genes play distinct roles. For instance, flies only have one PERIOD gene where it acts as the major negative regulator (Rothenfluh, Young et al. 2000).

Ataxin-2 in *Drosophila melanogaster* has a single homologue, dATX-2, which has been related to the maintenance of circadian rhythms (Lim and Allada 2013, Zhang, Ling et al. 2013). For instance, dATX-2 silencing promoted a depletion in PER expression, destabilizing the circadian rhythm. Consequently, flies behaved as if they were in their inactive period (Lim and Allada 2013, Zhang, Ling et al. 2013). Other studies further support the role of dATX-2 in the management of the circadian cycle, proving its dual role in translation (Lee, Yoo et al. 2017). Others saw that ataxin-2 is a translation-

activator of clock gene *Per* through the interaction with TWENTY-FOUR (TYF) protein, a *Per* translation promotor (Zhang, Ling et al. 2013). On the other hand, *CLOCK* gene can be silenced through the binding of dATX2 with RNA-induced silencing complex (RISC) (Figure 4) (Lee, Yoo et al. 2017). This evidence propose a role for ataxin-2 in the regulation of circadian rhythm, a connection that could be a result of the dual role of this protein in translation regulation.

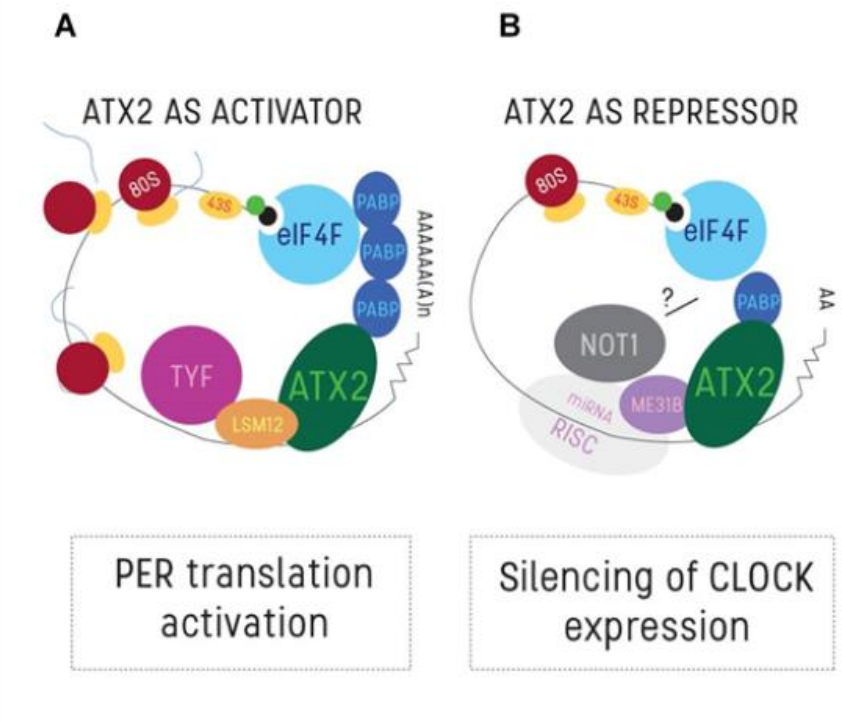

Figure 4. Role of dATX2 in the regulation of the circadian cycle.

In order to manage the expression of the two clock genes PER and CLOCK, dATX2 in Drosophila formed two complexes. The first one (A) to promote PER translation through the connection between TYF (TWENTY-FOR protein) and PABP (PoyA-binding protein). The second complex (B) acts as a repressor for the expression of CLOCK through the binding to RISC (RNA-induced silencing complex) components (adapted from Lee et al., 2017).

Pfeffer et al. studied circadian cycle in ATXN2 KO mice, where they observed no alterations in the expression of clock gene *Per*, although circadian behaviour seemed to be modified in these rodents. Under light-dark conditions, ATX2 KO mice displayed higher daytime activity compared to wild-type (WT) mice. (Pfeffer, Gispert et al. 2017).

They also observed a prolonged period length in KO, an observation already shown by Lim et al, (Lim and Allada 2013) in *Drosophila melanogaster*. Another observation by Pfeffer was that, when both KO and WT mice suffered a 6 h delay in the light-dark cycle, KO entrained slower than WT in the new photoperiod (Pfeffer, Gispert et al. 2017), Unpublished data from our lab, further support these findings, where we found that Ataxin-2 KO mice exhibit diurnal hyperactivity when compared to WT littermates. This diurnal hyperactivity, with concomitant changes on clock gene expression in the hypothalamus, might explain the metabolic dysfunctions displayed by this mouse model (Carmo-Silva et al., 2019, in revision in International Journal of Obesity). Other studies in our lab, observed that ataxin-2 re-establishment in the hypothalamus of ATXN2 KO mice, increased the activity of two clock genes: *Bmal1* and *Per2* (Carmo-Silva et al., 2019, in revision in International Journal of Obesity).

Taking all this information into consideration, these findings suggest that ataxin-2 might act both in circadian rhythm and metabolism homeostasis. Furthermore, it is well established that both of these mechanisms are regulated and controlled by the hypothalamus, a brain region where ataxin-2 is also present. Due to the role of ataxin-2 in the transcription/translation of clock genes, we hypothesized that this hypothalamic protein might be acting as a regulator of circadian rhythm.

2. Objectives

Circadian rhythm is one of the main responsible for several physiological processes and for the maintenance of body homeostasis. Alterations in the circadian cycle can lead to several conditions, namely to metabolic dysfunction and obesity. The circadian cycle and metabolism are intrinsically connected in a way that, the simple act of eating in the wrong period of the day can lead to disruptions in the normal function of this circadian mechanism.

With these observations in account, molecules that can act both on circadian rhythm and metabolism could be a promising therapeutic approaches to re-establish body homeostasis. Ataxin-2 is a protein encoded by the ATXN2 gene, involved in the regulation of various cellular processes. This protein has been described as a player in metabolism and also in circadian rhythm.

Taking all the information into consideration, the aims of this project are:

- Study the effect of saturated fatty on the molecular circadian system in a hypothalamic cell line;
- Explore the circadian alterations of mTOR pathway upon nutritional stress with saturated fatty acids, in a hypothalamic cell line;
- Understand if ataxin-2 can regulate circadian rhythm, and rescue the effects of palmitate on the circadian machinery and the mTOR pathway.

Chapter II – Methods

2-Cellular models

2.1-Embryonic Mouse Hypothalamic Cell Line N42 (mHypoE-N42)

The hypothalamic cell line used in this project is denominated as mHypoE-N42 (CELLutions Biosystems Inc./Cederlane). This adherent cell line was obtained through retroviral transfer of Simian Vacuolating Virus 40 T-Antigen (SV40 Tag) of hypothalamic primary cultures from immortalized mouse embryonic day 15-18 (E15-18). Cells take approximately 24h to double themselves. Cells were preserved in a high glucose Dulbecco's Modified Eagle Medium (DMEM; 4.5 g/L/D-glucose, Sigma-Aldrich), which was supplemented with 10 % of heat inactivated fetal bovine serum (FBS; Gibco, Life technologies), 100 µg/mL of streptomycin and 100 U/mL of penicillin (all from Invitrogen, Carlsbad, CA, USA). Then, these cells were kept in 75 cm^2 tissue culture flasks preserved in a humidified atmosphere with 5 % CO_2 and 95 % air with an ambient temperature of 37 °C. When cells reached a confluence of approximately 90 % they were washed twice with pre-warmed PBS and through trypsinization they were detached (incubation of 3-5 min at 37 °C). Growth medium was added in order to inhibit trypsin (Life Technologies, from Invitrogen) and in order to sediment cells, a centrifugation was performed at 1000 rotations per minute (rpm) for 5 minutes. Supernatant was then discarded and pre-warmed fresh growth medium added in order to resuspend the cells. Cell density was determined with a haemocytometer, by direct counting. Cells were plated in uncoated 60 mm^2 plates or 6-well multiwells. Cells were maintained in a new culture flask, for subsequent experiments.

2.2- mHypoE-N42 cells synchronization

Cells were kept in an incubator at 37 °C with 5 % CO_2 air after plating (300 000 cells/plate). After 24 h of incubation, cells were submitted to a 12 h serum starvation and then synchronized so they are all in the same phase of the cellular cycle. As for the synchronization, it consisted in an incubation of cells with 50 % of horse serum (Gibco,

Life Technologies) for 2h, which was diluted in cell culture medium without serum. After this process, cells were placed back in normal medium. In the end of this protocol, cells were treated (more detailed in the next topic) and then collected every 4 h in a period of 48 h after synchronization assay. This protocol was adapted from published data (Balsalobre, Damiola et al. 1998, Guo, Chatterjee et al. 2012).

2.3- Cellular treatments

After synchronization assay, cells were treated either with palmitate or with vehicle, BSA (bovine serum albumin). Palmitate solution was obtained through a solubilization of 167 µM of free-fatty-acid BSA (incubation at 37 °C for 2 h; Sigma) and 500 µM of acid palmitic (Sigma). Control condition resulted from the free-fatty-acid BSA, used as vehicle.

Cell were incubated either with vehicle or palmitate, a treatment that consists in two different conditions distinguished by concentrations. Cells were treated either with 500 µM (BSA and palmitate) or with 50 µM (BSA and palmitate), right after synchronization. For 500 µM incubation, cells were collected right after synchronization every 4 h in a period of 48 h. For 50 µM, cells were incubated for 24 h and then collected every 4 h in a period of 48 This protocol was adapted from Greco et al., 2014 (Greco, Oosterman et al. 2014).

2.4- mHypoE-N42 cells transduction with lentiviral vectors

Twenty four hours after plating, mHypoE-N42 cell line was transduced with lentiviral vectors encoding for an Ataxin-2 construct (200 000 ng/ 100 000 cells). After transduction and before palmitate and vehicle treatment, cells were again incubated at 37 °C for 24 h in a 5 % CO_2 air incubator. After this period, cells were then treated either with palmitate or vehicle solution in the same conditions described above (1.3).

2.5-Gene expression analysis

2.5.1-Purification and quantification of total RNA: TRI Reagent protocol

TRI Reagent (Sigma-Aldrich) was used in order to disrupt cells and dissolve cell components during sample homogenization which will be placed into an Eppendorf. In order to distinguish the deposition of the insoluble material, samples were centrifuged at 12 000 g for 10 minutes with a temperature of 4 °C. Supernatant was collected to a new Eppendorf followed by the addition of 200 µL of chloroform. Samples were vortexed vigorously and left at room temperature for 3 minutes. Then, samples were centrifuged for 15 minutes at 12 000 g with a temperature of 4 °C. This resulted in a mixture with 2 separated phases: an organic phase with protein content and an aqueous phase, the one that contains the total ribonucleic acid (RNA). The first phase was transferred into a new Eppendorf for further protein quantification (presented in detail in 1.5) and then the second to another tube where ethanol 75 % was added with the same volume as the content in the tube. Mixture was then transferred into columns with silica matrix where purification of RNA occurred in accordance with the manufacture's specifications. In resume, after silica matrix adsorbed all the RNA content and the recommended washes, RNA was eluted with 60 µL of water RNAse-free with a final centrifugation of 11 000 g for 2 minutes. Through an optical density (OD) measurements, total RNA was quantified using a ND-1000 Nanodrop Spectrophotometer (Termo Sientific). In the end of this protocol, cells were kept at -80 °C until further use.

2.5.2-Purification and quantification of total RNA from cultured cells

For purposes of obtaining higher amounts of RNA, another RNA extraction method was used. RNA was isolated using the NucleoSpin RNA protocols according to the manufacturer's instructions. In brief, cells were collected, medium was discarded through aspiration and then washed twice with ice cold PBs. Then, disrupted with 350

µL buffer RA1 and 3.5 µL β-Mercaptoethanol (Sigma) and placed into an Eppendorf where the content was homogenized by pippeting up and down. Lysate was placed into a collection tube with a NucleoSpin Filter (violet ring) and centrifuged for 1 min at 11,000 x g. After, 70 % of ethanol was added to the homogenized lysate, mixed through up and down and in turn placed into a NucleoSpin RNA Column (light blue ring) placed in a Collection tube. Mixture was centrifuged at 11,000 x g for 30 s being the total RNA adsorbed to a silica matrix. In the end, total RNA was washed with the recommended buffers followed by an elution with 60 µL of RNase-free water by centrifugation for 1 min at 11,000 x g. RNA quantification was determined through ND-1000 Nanodrop Spectrophotometer (Thermo Fisher Scientific) and samples were stored at -80 °C until use.

2.5.3-Reverse transcriptase

Reverse transcription into cDNA was achieved through the iScript cDNA Synthesis Kit (Bio-Rad) according with the manufacturer's instructions. In brief, samples of total RNA were reverse transcribed into cDNA in 20 µL of volume 1x iScript reaction buffer and 1 µL of iScript reverse transcriptase. This procedure was performed by a thermocycler at 25 °C for 5 minutes, 46 ° C for 20 minutes, 95 °C for 5 minutes and in the end 4 °C in order to stabilize until store at -20 °C. Negative control was also performed (NoRT which is composed by the same components of the reaction, except for reverse transcriptase).

2.5.4-Quantitive real-time polymerase chain reaction

Quantitative Real Time PCR (qRT-PCR) was conducted in an iQ5 thermocycler (Bio-Rad) using IQ SYBR Green Supermix (BioRad) and predesigned primers, used for target genes validated by QIAGEN and Sigma (Table 1). Each primer set had a master mix previously prepared containing the adequate volume of 2x QuantiTect SYBR Green PCR Master Mix and 10x the respective primer. All reactions were executed according to

the manufacturer's instructions. Melting curve protocol started right after amplification. The iQ5 Optical System Software (Bio-Rad) automatically determines the threshold values for threshold cycle determination (Ct) and also the amplification efficiency for each gene. Relative mRNA quantification was carried out through the ΔΔCt method, using Hypoxanthine Phosphoribosyltransferase (Hprt) and ß-Actin combination, for endogenous housekeeping genes.

Table 1. Primers used for gene expression analysis

Primer	Temperature	Concentration	Sequence	Function	Source
Ataxin-2	55 ºC	10 µM	Not provided	Interest gene	Qiagen
Hprt	55 ºC	10 µM	Not provided	Reference gene	Qiagen
Bmal1	60 ºC	3 µM	F: AAATCCACAGGATAAGAGGG R: ATAGTCCAGTGGAAGGAATG	Clock gene	Sigma
Clock	58 ºC	3 µM	F: AAGTGACTCATTAACCCCT R: CTATGTGTGCGTTGTATAGTTC	Clock gene	Sigma
Cry	59 ºC	3 µM	F: AGAAGGGATGAAGGTCTTTG R: CTCTTAGGACAGGTAAATAACG	Clock gene	Sigma
Per1	58 ºC	3 µM	F: GTTCTCATAGTTCCTCTTCTG R: GTGAGTTTGTACTCTTGCTG	Clock gene	Sigma
Per2	55 ºC	5 µM	F: CTTTCACTGTAAGAAGGACG R: GTGAGTTTGTACTCTTGCTG	Clock gene	Sigma

2.6-Protein expression analysis

2.6.1- Cell lysates

2.6.1.1- Protein lysates of culture cells – RIPA method

After cell treatments, cells were collected every 4 h during a period of 48 h. When collected, cells were placed on ice, culture medium was discarded through aspiration and cells were washed twice with ice-cold PBS. As for the protein extraction protocol,

cells were lysed in 180 µL of radio-immunoprecipitation assay-buffer (RIPA) solution (50 mM Tris-HCL, pH 7.4, 150 nM NaCl (Thermo Fisher Scientific), 1 % Triton X-100; 0.5 % deoxycholate (Sigma-Aldrich); 0.1 % sodium dodecyl sulphate (SDS, Sigma-Aldrich); 200 µM phenyl – methylsulphonylfluoride (PMSF, Sigma – Aldrich) also supplemented with proteases inhibitors (Roche). Content was placed into 1,5 mL tubes and then submitted to a 4 s ultra-sound pulse (1 pulse/s). In the end of this process, cells were incubated for 15 min at 4 °C and stored at -20 °C until use.

2.6.1.1.2 – Protein quantification - Bicinchoninic Acid (BCA) protein assay

From each sample, protein concentration was determined using the Bicinchoninic Acid (BCA) (Thermo Fisher) according with the manufacturer's instructions. Standard curve is performed using bovine serum albumin (BSA) (2 mg/mL) which in turn was submitted to a series of dilutions. Therefore, samples were denaturized through the addition of 6 times concentrated sample buffer (0.5 M Tris, 30 % glycerol, 10 % SDS, 0.6 M DTT, 0.012 % bromophenol blue) and heated for 5 min with a temperature of 95 °C. Samples were then stored at – 20 °C until further use.

2.6.1.2-TRI reagent protocol

After cell treatments, cells were collected every 4 h during a period of 48 h. When collected, cells were placed on ice, culture medium was discarded through aspiration and cells were washed twice with ice-cold PBS. Through mechanical disruption of the cells, these were scrapped TRI reagent (Sigma-Aldrich) in order to promote lysis and then collected into a microcentrifuge 1.5 mL tube (Eppendorf). To deposit insoluble material, the content was centrifuged for 10 min at 12,000 x g with a temperature of 4 °C. Supernatant was collected into a new tube and 200 µL of chloroform was added. A vigorous vortex was performed and samples were left at room temperature for 3 min in order to perform a new centrifugation. After this, the content was divided in 2 distinguished phases: an organic phase which contains protein, and an aqueous phase.

Organic phase (containing protein) was transferred into a new tube and 300 µL of ethanol 100 % was added and the mixture was left at room temperature for 3 minutes. Samples were centrifuged at 2,000 x g for 10 min at 4 °C. Protein was precipitated: 1.5 mL of isopropanol were added followed by vortex and left for 10 min at room temperature. After this time, content was again centrifuged at 12,000 x g for 10 min at 4 °C. Pellet was resuspended in 2 mL of guanidine solution followed by incubation for 20 min and then again centrifuged at 7,500 x g for 5 min at 4 °C, where supernatant was discarded. This process was repeated 2 times more. To this protein phase, was added 1.6 mL of ethanol 100 % and mixed vigorously. After this, samples were incubated for 20 min at room temperature and then centrifuged at 7,500 x g for 5 min at 4 °C. Afterwards, supernatant was discarded and the pellet left to dry for 5-10 min at room temperature. The pellet was then resuspended in 200 µL of DTT solution and urea followed by vigorous vortex. In the end, solution was left for 1 h, and occasionally vortexed and then incubated at 50 °C for 3 min in the dry bath.

2.6.1.2.2 - Protein quantification - Bradford protein assay

Concentration of the protein resulting from TRI reagent protocol was determined by the Bradford protein assay (Bio-Rad) with accordance to the manufacturer's instructions. Standard curve is based in bovine serum albumin (BSA) which was submitted into a series of dilution. After quantification, cells were denaturized by adding 6 times concentrated sample buffer (0.5 M Tris, 30 % glycerol, 10 % SDS, 0.6 M DTT, 0.012 % bromophenol blue) and heated for 5 min with a temperature of 95 °C. Samples were then stored at – 20 °C until further use.

2.6.2- Western blotting analysis

Western Blotting protocol is used in order to immunodetect the expression of proteins in cells and tissues extracts. Total protein was loaded per lane with the same amount (50 µg), separated by electrophoresis in sodium dodecyl sulphate-polyacrylamide (SDS-

PAGE) gels (4 % stacking, 10 % running) and transferred in CAPS buffer (0.1 M CAPS, pH 11.0; 10 % methanol) into polyvinylidene (PVDF) membranes (Merck Millipore). After blot procedure, membranes were blocked in 5 % non-fat milk or 5 % bovine serum albumin (BSA), depending on the antibody, together with Tris-buffered saline (137 mM NaCl, 20 mM Tris-HCL, pH 7.6) containing 0.1 % Tween 20 (Acros Organics) (TBS-T) for one hour at room temperature. Overnight and with a temperature of 4 °C, immunoblotting was performed where membranes were incubated with the primary monoclonal antibodies (see table below). After the overnight incubation, membranes were three times washed with TBS-T followed by incubation with an alkaline phosphatase-linked secondary antibody, specific mouse IgG or rabbit IgG in a 1:1 000 dilution (Invitrogen). Bands represent the protein immunoreactive and can be visualized by chemifluorescence with the help of ECF (enhanced chemiofluorescence; GE Healthcare) substrate (GE Healthcare) in a VersaDoc Imaging System (Bio-Rad) where the optical density of the bands was quantified with the Quantity One Software (Bio-Rad). In the end, results were normalized to β-tubulin and expressed as the relative amount compared with control.

Table 2. List of antibodies used in Western Blot procedure

Antibody	Reference	Brand	Dilution
Ataxin-2 (clone 22)	611378	BD Biosciences	1:500
Bmal1	Sc-365645	Santa Cruz	1:500
mTOR (Ser2448)	2972	Cell Signalling	1:1000
Phospho-mTOR	2971	Cell Signalling	1:1000
RPS6K	9202	Cell Signalling	1:1000
Phospho-RPS6K	9204	Cell Signalling	1:1000
Tubulin (clone SAP.4G5)	T8328	Sigma	1:10 000

2.7-Statistical and mathematical data analysis

Mathematical and statistical analysis was performed in collaboration with Professora Angela Relógio's Group, from Charité Institute, accordingly with already published methods for this type of analysis (El-Athman, Fuhr et al. 2018). Circadian rhythmicity was calculated by fitting a sine-cosine function to our gene expression data, obtained from the calculation of ΔΔCt of qRT-PCR and optic densitometry data from Western Blotting analysis. Circadian Rhythmicity was determined in RAIN -Rhythmicity Analysis Incorporating Nonparametric Methods- software. *p* values under 0.05 indicated significant circadian rhythmicity.

Other circadian rhythm parameters such as acrophase and amplitude, where also analysed in RAIN software. Acrophase was determined through fitting a robust harmonic regression ($p < 0.05$) to the time-course or determined by RAIN. Amplitudes were also determined through fitting a robust harmonic regression ($p < 0.05$) and calculating the fold change (FC) amplitude from the resulting relative amplitude (AmpFC = (1 + Amprel)/(1 – Amprel)) or calculated as the peak-to-trough ratio of the maximum and the minimum expression value.

Chapter III – Results

3. Results

3.1. Validation of mHypoE-N42 cell line synchronization and treatment

To evaluate the expression of clock genes and their circadian oscillations, we used an *in vitro* approach, in the embryonic hypothalamic mouse cell line, mHypoE-N42. This cell line was submitted to a synchronization assay (Figure 5A) as described in Methods section 2.1. Synchronization allows us to work with cells in the same stage of the cellular cycle (Balsalobre, Damiola et al. 1998) and therefore obtain more accurate results in respect of circadian oscillations. Since we wanted to understand how metabolic stress could affect the circadian machinery, we used palmitate treatment as described in Methods section 2.3. As already mentioned, palmitate is the most common saturated fatty acid present in a high fat diet. This fatty acid, besides promoting metabolic dysfunction and insulin resistance, it also impacts the metabolic pathway mTOR (Mayer and Belsham 2010). We used two approaches to induce metabolic dysfunction: i) a higher concentration of Palmitate (500 μM) to mimic an acute exposure to high fat diet (comparable to one sporadic fat enriched meal); and ii) a lower concentration of Palmitate (50 μM) with an increased exposure time, to mimic a continued exposure to fat enriched foods (comparable to fat enriched snacks throughout the days) (Greco, Oosterman et al. 2014).

To validate the synchronization assay, we evaluated the expression of *Bmal1* and *Per2* (Figure 5B). Since *Bmal1* belongs to the positive side of the cycle and *Per2* belongs to the negative one, we expected that their circadian expression should oppose one another. Considering this, we used the tracing of their expression to validate the synchronization of each set of experiments (Figure 5B). If after synchronization, *Bmal1* and *Per2* did not show the expected pattern of expression (as shown in Figure 5B), the assay was excluded from the results.

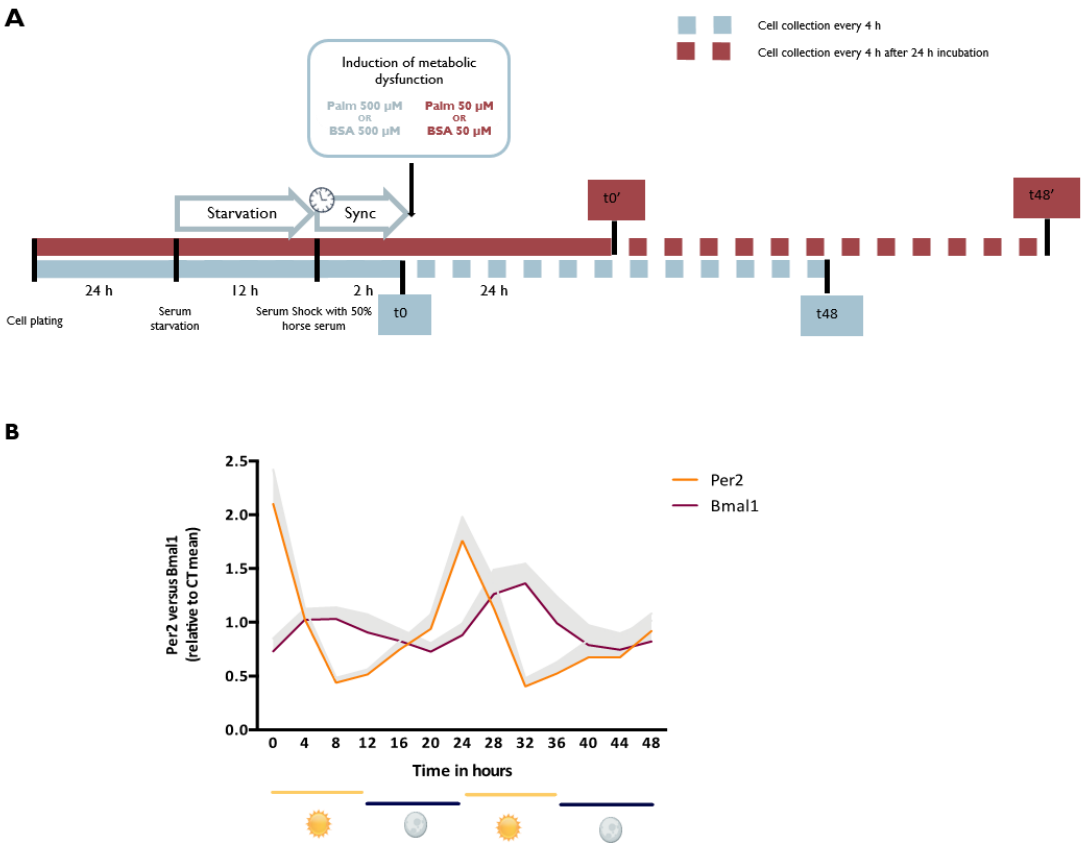

Figure 5. Synchronization protocol and consequent mRNA fluctuations of clock genes.

Synchronization protocol (A), mHypoE-N42 cell line was submitted to a process of synchronization including starvation followed by a serum shock provoked by 50 % of horse serum. This process is done in order to place cells in the same phase of the cycle. After this process, cells were treated with palmitate and collected every 4 h in a period of 48 h. mRNA and protein analysis were performed. (B) Synchronization validation through *Bmal1* and *PER2* mRNA expression. Experimental sets with the represented tracing were used for further analysis, those that had different patterns of expression were excluded. Colored tracing obtained from plot of gene relative expression at each *Zeitgeber*, calculated through ΔΔCT analysis. Grey shadows refer to error (SEM for each *Zeitgeber*). n= 7

3.2. Palmitate alters circadian oscillations of core clock genes

Disorders connected to metabolism, for instance obesity, result mainly from an impairment in the balance between energy intake and energy expenditure. However, current evidence show us that obesity is not only about the quantity of calories that we ingest, but also about the timing of that feeding. Meal timing is related with circadian clock, and it is well established that metabolism is under circadian regulation (Eckel-Mahan and Sassone-Corsi 2013).

Every tissue and every cell possess circadian rhythmicity, and transcript levels of the core circadian clock machinery oscillate in a period of 24 h. In order to evaluate these oscillations upon metabolic distress *in vitro*, the hypothalamic mouse mHypoE-N42 cell line was treated with palmitate in two conditions (acute versus prolonged exposure, as described above in cell line and synchronization section).

mRNA fluctuations of clock genes were evaluated within 48 h comparing control and palmitate-treated (Figure 6). The expression of mRNA was fitted into a statistic program, RAIN, and analyzed in accordance with published protocols. The traced curves are predictive tracings resulting from the harmonic regression analysis integrating the relative gene expression of the various *Zeitgebers* (*Zeitgebers* referring to each timepoint after synchronization) (El-Athman, Fuhr et al. 2018). The *p-value* represented in the graphs stands for the strength of circadian rhythmicity. A *p-value* under 0.05 represents significate circadian oscillations.

Bmal1 and *Cry1* (Figure 6) in control condition display circadian rhythmicity (p=1.6e-0.2 for *Bmal1* and p=3.0e-0.5 for *Cry1*), that is lost upon treatment with palmitate (both concentrations). The loss of rhythmicity happens with both treatments for both genes, however, its effects seem to vary differently for each one. As for *Bmal1*, *p value* goes from 0.56, in a prolonged exposure, to 0.10 in an acute exposure to palmitate. In regard to *Cry1*, *p value* goes from 0.57, in a prolonged exposure to palmitate, to 0.95 in an acute exposure. On *Bmal1*, the loss of rhythmicity is more pronounced upon the prolonged palmitate exposure of 50 µM, while on *Cry*, both exposures have similar effects. *Clock* does not show circadian rhythmicity both in control (p=0.19) and palmitate-treated groups (p=0.86 in palmitate 50 µM and p=0.95 in palmitate 500 µM). The absence of rhythmicity of this gene had already been described for an hypothalamic cell line, mHypoE-37 (Greco, Oosterman et al. 2014).

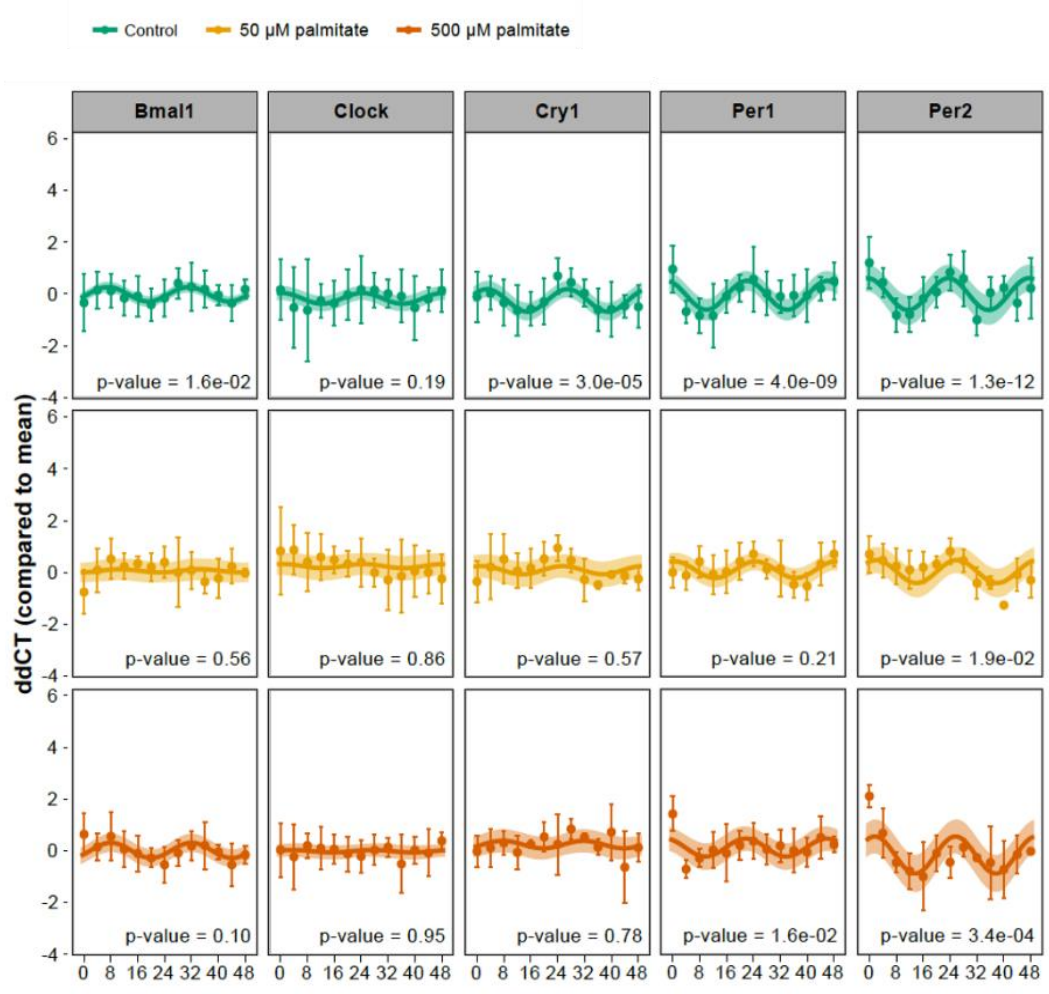

Figure 6. Palmitate changes circadian rhythmicity of core clock genes.

Circadian flutuactions of clock genes *Bmal1, Clock, Cry1, Per1* and *Per2* under control (BSA) and palmitate conditions. mHypoE-N42 cell line treated either with 500 µM or 50 µM of palmitate, RNA was harvested every 4 h for 48 h and used for qRT-PCR. This analysis was performed in the program RAIN - Rhythmicity Analysis Incorporating Nonparametric methods- using a fitted sine-cosine function to our qRT-PCR data. Harmonic regression analysis with a 24h period analysis. Tracing obtained by fitting a sine-cosine function to gene relative expression, calculated from ΔΔCT analysis. The points represent our gene expression data and tracing refers to the prediction of harmonic oscillations calculated by RAIN software . n=7

Per1 and *Per2*, clock genes belonging to the negative side of the circadian cycle, display different behaviours. Both present circadian oscillations in a non-treated condition (p=4.0e-0.9 for *Per1* and p=1.3e-12 for *Per2*). However, when subjected to a prolonged palmitate incubation, *Per1* loses its rhythmicity (p=0.21) while *Per2* continues oscillating

in a circadian fashion (*p*=1.9-0.2). Although *Per2* maintains its rhythmicity with 50 μM of palmitate, we can observe a huge difference in the *p value* where it suffers a decrease from 1.3e-12 to 1.9e-0.2. However, for both genes, with an acute palmitate exposure (500 μM) the circadian rhythmicity was maintained. These results might indicate that PER isoforms are resistant to transient changes on the nutritional uptake.

Looking at *Bmal1*, despite both palmitate conditions provoked a phase delay, that is, a delay in the time of peak expression, this effect is more pronounced in palmitate 50 μM, inducing a phase delay of 3.8 h (Table 3). These accentuated alterations can also be observed in amplitude where the bigger difference is observed upon a higher permanence of this fatty acid. Palmitate effects in *Bmal1* were already observed in Figure 6 where this clock gene become arrhythmic upon metabolic dysfunction. Through the evaluation of these other parameters, we can better understand how palmitate is changing the circadian oscillations, and which treatment is having a more pronounced effect.

As for *Clock*, as shown in Figure 6, this gene maintains arrhythmicity in control and treatment conditions. However, acrophase indicates that this clock gene suffers a phase advance (advance on acrophase peak), when treated with palmitate. This difference is more accentuated in palmitate 50 μM where this phase advance is up to 22 h (Table 3). These results indicate that, although its arrhythmicity, palmitate can still interfere with *Clock* expression.

In regard to the negative side of the circadian cycle, *Cry1* suffered a pronounced phase delay of 5.3 h when treated with palmitate 500 μM, having also the lowest amplitude between control and palmitate conditions (Table 3). As for *Per1*, we observed alterations in rhythmicity only in the prolonged exposure to this saturated fatty acid (Figure 6). Acrophase results corroborate these observations, presenting a phase delay of 21.8 h. On *Per2*, we saw that despite this clock gene not becoming arrhythmic, it had a decrease in the significance (*p value*) of rhythmicity for both palmitate concentrations. Acrophase analysis indicate a similar phase delay in both palmitate conditions, of approximately 22 h.

Table 3. Evaluation of circadian parameters of mRNA expression upon palmitate

Gene	Treatment	p_value	Acrophase in hours	Relative Amplitude	Period
Bmal1	Control	0.0162115439	7.3915740732	0.269892482	24
Bmal1	Palmitate 500uM	0.1037360208	7.7992888292	0.2925101666	24
Bmal1	Palmitate 50uM	0.5593085359	11.2060005371	0.1489568623	24
Clock	Control	0.1868895964	22.8918247172	0.241731439	24
Clock	Palmitate 500uM	0.9463289378	8.3850311034	0.0460267919	24
Clock	Palmitate 50uM	0.8562793155	0.8485828888	0.0983508272	24
Cry1	Control	3.02734919158211E-005	2.2896676033	0.4484772732	24
Cry1	Palmitate 500uM	0.7838352999	7.5446192848	0.1024968596	24
Cry1	Palmitate 50uM	0.5673866007	1.3124639334	0.1589992964	24
Per1	Control	0.000000004	21.9214697083	0.6762321125	24
Per1	Palmitate 500uM	0.0155084807	21.5220586419	0.433150016	24
Per1	Palmitate 50uM	0.2082279527	0.1480319545	0.2280572841	24
Per2	Control	1.29993742867722E-012	23.4876538514	0.7794576063	24
Per2	Palmitate 500uM	0.0003425075	1.5275451162	0.7794913287	24
Per2	Palmitate 50uM	0.0186261116	1.6094811668	0.3727870521	24

Taken together, the results in Figure 6 and Table 3 suggest that palmitate can differentially affect the circadian oscillations of core clock genes. These alterations are concentration dependent and differ between genes. The lower concentration with prolonged exposure seems to have a higher impact on the molecular circadian cycle.

3.3. Palmitate affects the metabolic pathway mTOR in a hypothalamic cell line

The consumption of high-fat or high-sugar diets is increasing, leading to an increase in the circulating fatty acids in human's organism. One of these fatty acids is palmitate, impacting in metabolism leading to metabolic dysfunction (Tse, Salehi et al. 2018). Hypothalamic mTOR functions as a homeostatic sensor (Cao, Robinson et al. 2013) being responsible for the regulation of metabolism, cell growth and protein synthesis. Upon palmitate stimuli, this metabolic pathway is over-activated (Khapre, Patel et al.

2014). Some studies suggest a possible role of this protein in the regulation of circadian rhythm (Ramanathan, Kathale et al. 2018).

Using the same approach as before, we intended to observe the effects of palmitate on the circadian expression of mTOR and its substrate, the ribosomal protein S6 kinase (Rps6K). Protein fluctuations were evaluated over 48 h, for control and palmitate-treated (Figure 7). The same analysis (Figure 6, Table 3) was applied to protein circadian oscillations.

Bmal1 has significant protein circadian rhythmicity (p=1.9e-0.3), lost upon palmitate treatment (both conditions) (p=0.32 in palmitate 50 µM and p=0.15 upon 500 µM of palmitate) (Figure 7A and B). These results are in accordance to those observed in mRNA expression. Observing the acrophase values we see that from control to palmitate 500 µM, Bmal1 suffered a phase advance up to 1.3 h, while with a prolonged exposure, this protein suffered a phase delay of 0.4 h (Table 4). As for amplitude, *Bmal1* suffered a bigger decrease upon palmitate 500 µM. We conclude that palmitate can alter the circadian expression of Bmal1 in both prolonged and acute exposures. Besides its role on circadian rhythm, Bmal1 has also been related with protein synthesis, especially by functioning as a substrate for RPS6K (Lipton, Yuan et al. 2015). This can explain why Bmal1 is deeply affected by palmitate treatment, considering its known effect on this metabolic pathway.

In fact, we evaluated protein levels to major players of the mTOR pathway and we observed that mTOR and its substrate suffered alterations in the circadian patterns upon palmitate treatment (Figure 7A and B). Both had similar rhythmicity in control group (p=3.3e-0.2 for mTOR and p=2.2e-0.2 for Rps6K) but when treated with palmitate both mTOR (p=0.80, prolonged exposure and p=0.17, acute exposure to palmitate) and RPS6K (p=0.51, prolonged exposure p=0.24, acute exposure) become arrhythmic. This similarity in circadian patterns on control conditions were expected due to their interaction as downstream target. Furthermore, as observed for mRNA, the prolonged exposure to palmitate (even in lower concentration), had the higher impact on rhythmicity.

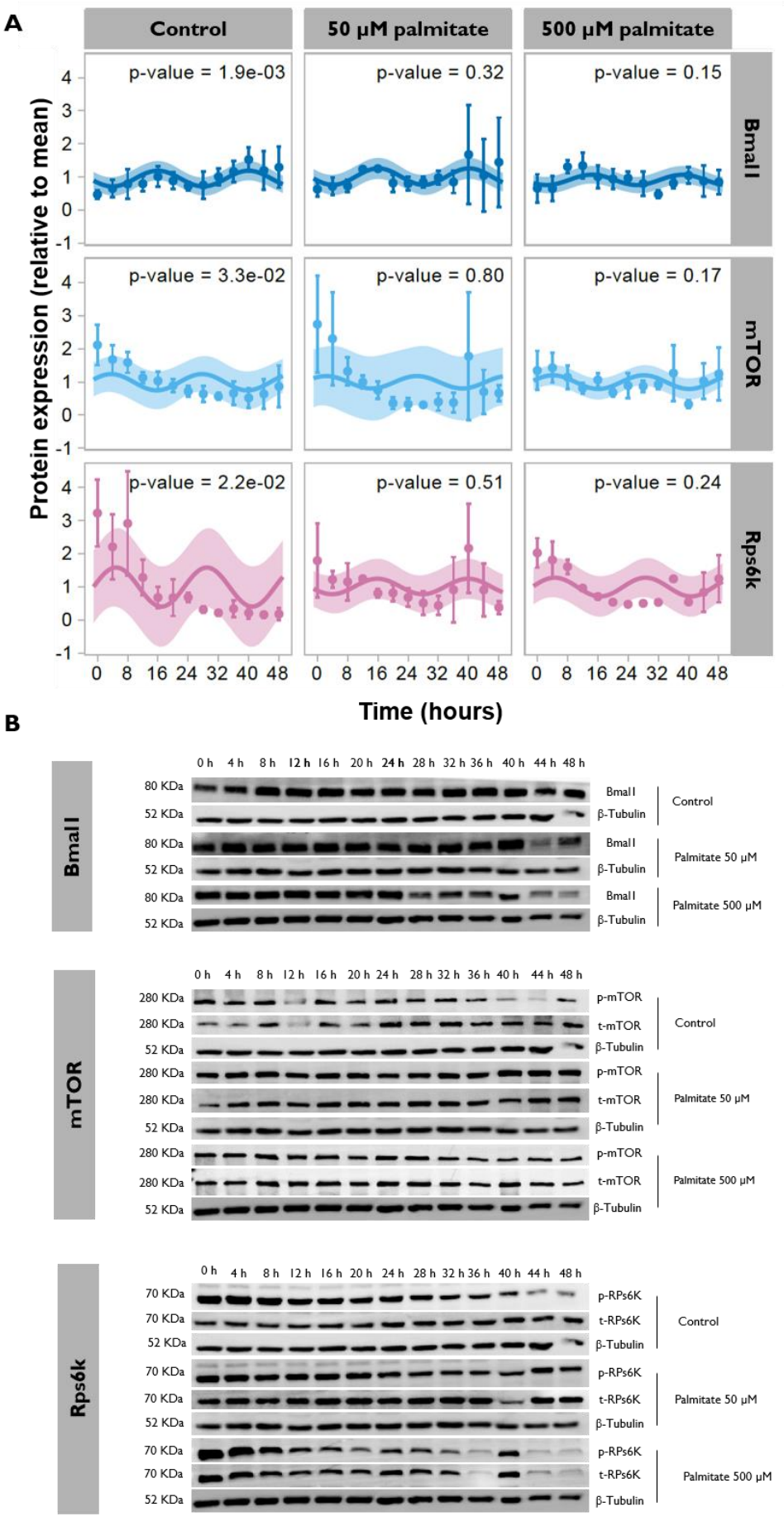
A
Control
50 µM palmitate
500 µM palmitate
p-value = 1.9e-03
p-value = 0.32
p-value = 0.15
Bmal1
p-value = 3.3e-02
p-value = 0.80
p-value = 0.17
mTOR
p-value = 2.2e-02
p-value = 0.51
p-value = 0.24
Rps6k
Protein expression (relative to mean)
Time (hours)
B
Bmal1
0 h 4 h 8 h 12 h 16 h 20 h 24 h 28 h 32 h 36 h 40 h 44 h 48 h
80 KDa
52 KDa
Bmal1
β-Tubulin
Control
Palmitate 50 µM
Palmitate 500 µM
mTOR
280 KDa
p-mTOR
t-mTOR
Rps6k
70 KDa
p-RPs6K
t-RPs6K

Figure 7. Palmitate disrupts protein circadian fluctuations of Bmal1, mTOR and Rps6k.

Circadian flutuactions Bmal1 (A and B), mTOR (A and C), and Rps6k (A and D) under control and palmitate conditions. mHypoE-N42 cell line was treated either with 500 µM or 50 µM of palmitate. Protein was harvested every 4 h for 48 h and used for western blot. (B-D) Whole-cell extracts were assayed for Bmal1, phospho-mTOR (p-mTOR), total mTOR (mTOR), phospho-RPS6K (p-RPS6K), RPS6K and tubulin immunoreactivity by Western blotting. (A) Rhythmicity analysis was performed in the program RAIN - Rhythmicity Analysis Incorporating Nonparametric methods- using a fitted sine-cosine function to our western blot data. Harmonic regression analysis with a 24h period analysis. Tracing obtained by fitting a sine-cosine function to relative protein levels. The points represent our gene expression data and tracing refers to the prediction of harmonic oscillations calculated by RAIN software. n=3

Looking at acrophase results for mTOR, interestingly, we can observe an opposite pattern relative to Bmal1 (Table 4), mTOR suffered a phase delay of 0.5 h from control to palmitate 500 µM but a phase advance of 1.3 h from control to palmitate 50 µM conditions. RPS6K on the other hand, suffered an 11 h delay with a prolonged exposure to palmitate (Table4). Furthermore, amplitudes are lower with a prolonged exposure complementig the rhythmicity results.

Table 4. Evaluation of circadian parameters of protein levels upon palmitate treatment

Protein	Treatment	p_value	Acrophase in hours	Relative Amplitude	Period
Bmal1	no_treatment	0.001856381274737	15.7517514653827	0.246716267758664	24
Bmal1	palmitate_500	0.1456268196919	14.4963295506886	0.156904552741858	24
Bmal1	palmitate_50	0.315744024453041	16.1181042549688	0.249007908050337	24
Mtor	no_treatment	0.033133396505925	3.6601193001119	0.245805780000149	24
Mtor	palmitate_500	0.167810110342119	4.15451876482356	0.207002683825865	24
Mtor	palmitate_50	0.80072758454713	2.32606595678862	0.187986562101827	24
Rps6k	no_treatment	0.021901701690546	4.84014102465315	0.594744040560543	24
Rps6k	palmitate_500	0.240385655625573	4.62138607683972	0.283098862677657	24
Rps6k	palmitate_50	0.506803369222207	15.8580316799144	0.234356295961491	24

Taken together these observations, we can say that palmitate also impacts on mTOR metabolic pathway circadian expression, whith a more pronounced effect when in a longer time of exposure, even if in a lower concentration.

3.4. Ataxin-2 mRNA and protein fluctuations upon metabolic dysfunction

Ataxin-2 is involved in the regulation of various cellular processes such as translation regulation, RNA metabolism, calcium homeostasis and cytoskeleton reorganization. (Carmo-Silva, Nobrega et al. 2017). Ataxin-2 can regulate circadian rhythm in *Drosophila* (Lee, Yoo et al. 2017) and act as an mTOR repressor (Bar, Charar et al. 2016). Unpublished data from our lab suggest that ataxin-2 acts as a metabolic sensor, able to prevent metabolic dysfunction when overexpressed in the hypothalamus of mice (Carmo-Silva et al., 2019 *in review*). Taking these observations in consideration, we intended to understand if ataxin-2 was regulated in a circadian fashion and how its expression was affected under metabolic dysfunction induced by palmitate.

Using the same approach as before, we observed no significant circadian rhythmicity on *Ataxin-2* mRNA expression (Figure 8A). However, we can observe circadian fluctuations on protein levels of ataxin-2 under control conditions (p=9.3e-0.2) (Figure 8B). These circadian oscillations are lost upon palmitate treatment (p=0.85 in palmitate 50 μM and p=0.40 in palmitate 500 μM conditions) (Figure 8B and C), similarly to what happened to Bmal1, mTOR and Rps6K (Figure 7). Once again, palmitate prolonged exposure promoted more profound alterations on the circadian oscillations.

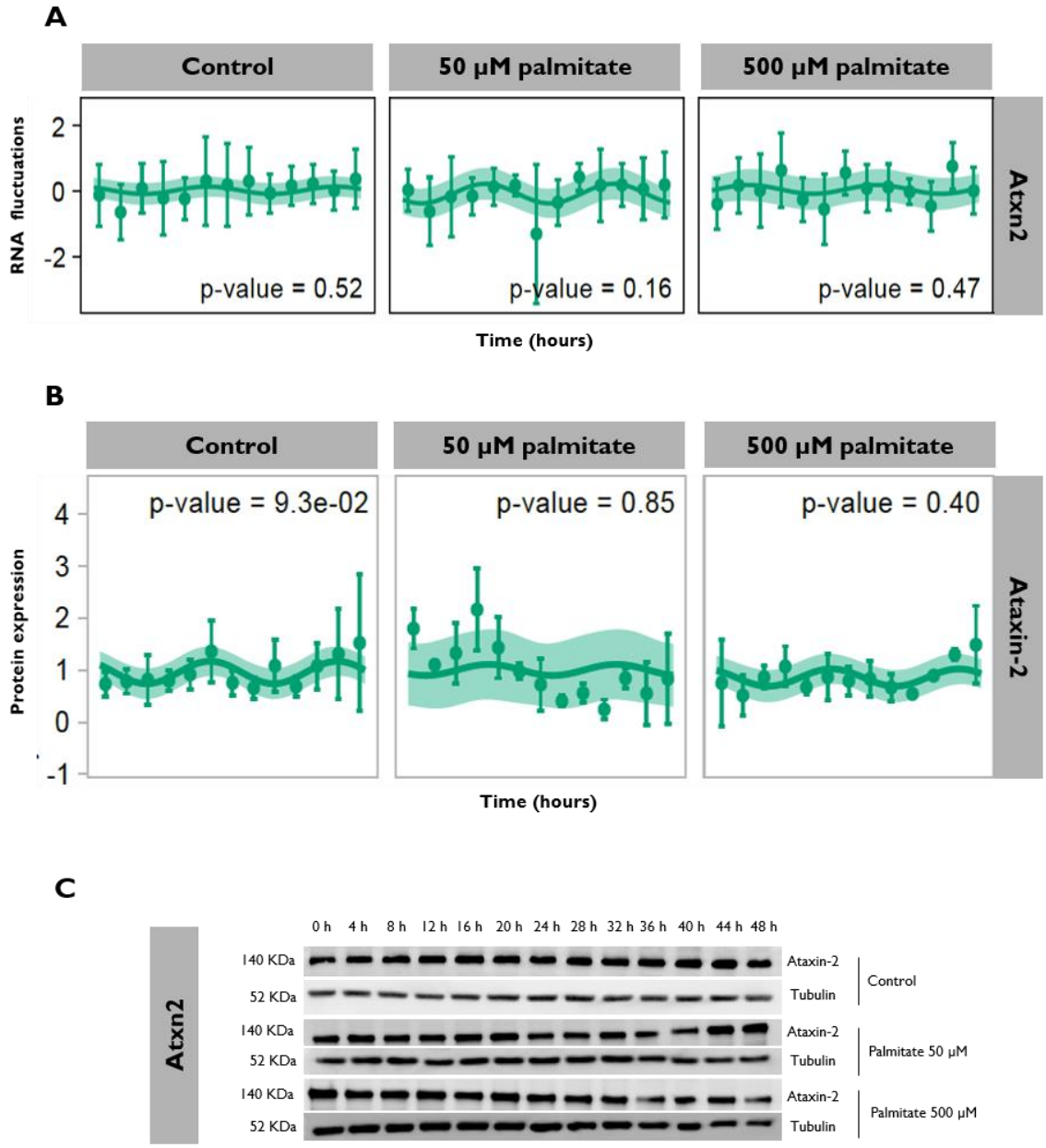

Figure 8. Ataxin-2 mRNA and protein display different rhythmicity and are affected by palmitate treatment.

mHypoE-N42 cell line was treated either with 500 µM or 50 µM of palmitate in order to understand the effects that this saturated fatty acid has on the circadian expression of ataxin-2. RNA (A) and protein (B and C) were harvested every 4 h for 48 h and used for qRT-PCR (A) and western blot (B and C). This analysis was performed in the program RAIN - Rhythmicity Analysis Incorporating Nonparametric methods- using a fitted sine-cosine function. Harmonic regression analysis with a 24h period analysis. Tracing obtained by fitting a sine-cosine function to gene relative expression, calculated from ΔΔCT analysis. Tracing refers to the prediction of harmonic oscillations calculated by RAIN software. n=7 for mRNA and n=3 for protein.

Looking at acrophase values for ataxin-2, we can observe that despite mRNA levels do not express circadian rhythmicity (Figure 8A), ataxin-2 suffered a phase advance (Table 5) when treated with palmitate. This phase shift was more pronounced with a prolonged exposure to this fatty acid (50 μM). However, looking at acrophase results in regard to protein content, ataxin-2 suffered a phase delay of 0.9 h upon palmitate treatment of 500 μM and a phase advance of 5.5 h with the lower concentration of this fatty acid (50 μM). As for amplitude levels in protein expression, it was upon a prolonged exposure to palmitate that amplitude suffered a higher decrease.

Table 5. Evaluation of ataxin-2 of mRNA expression (ATXN2) and protein levels upon palmitate treatment

	Treatment	p_value	Acrophase in hours	Relative Amplitude	Period
ATXN2	no_treatment	0.5181172632	19.963539364	0.1192022451	24
ATXN2	palmitate_500	0.1582599329	14.3370939022	0.3466161171	24
ATXN2	palmitate_50	0.4695352041	7.5299620286	0.1817573579	24
Ataxin-2	no_treatment	0.092533736327043	19.7436339441007	0.209691755122849	24
Ataxin-2	palmitate_500	0.397960569872115	20.6604636400829	0.1435073068129	24
Ataxin-2	palmitate_50	0.846134347035067	14.2491162851094	0.106555207970699	24

In sum, ataxin-2 protein levels present circadian oscillations, but not mRNA. However, this circadian rhythmicity is lost upon metabolic dysfunction induced by palmitate. Such as clock genes and the mTOR pathway, this protein is deeply affected upon a prolonged exposure to this saturated fatty acid. Nevertheless, despite the non-oscillatory patterns in mRNA levels of ataxin-2, palmitate treatment promoted a shift advance on its peak, in both palmitate conditions (500 μM and 50 μM).

3.5. mHypoE-N42 cell line transduced with lentiviral vectors encoding for Ataxin-2

Considering the potential role of ataxin-2 as a metabolic sensor and a regulator of circadian rhythm, we wanted to investigate if ataxin-2 could prevent the palmitate-induced circadian dysfunctions above described.

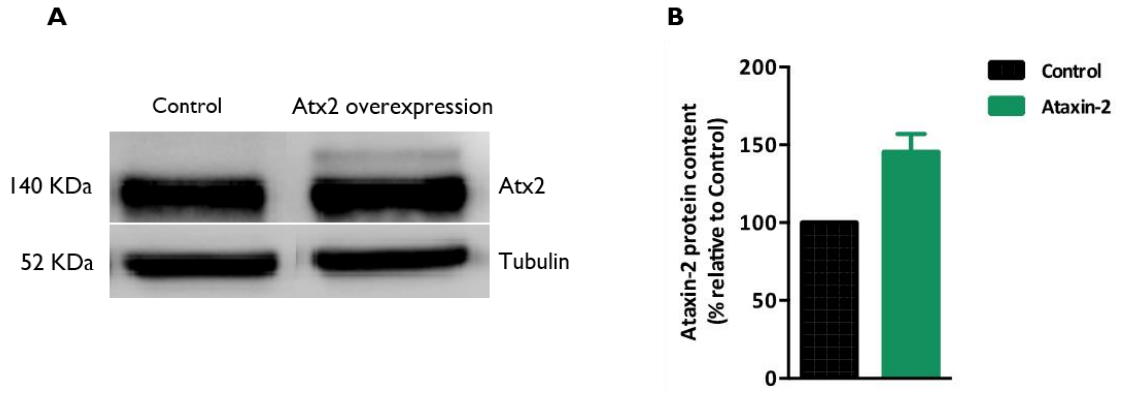

Figure 9. Western blot analysis for the mHypoE-N42 cell line transduced with lentiviral vectors encoding for an Ataxin-2 construct.

Protein analysis of ataxin-2 in control (cells that were not transduced) and in Ataxin-2 overexpression (cells transduced by the lentiviral vector encoding for Ataxin-2). (A and B) Whole-cell extracts were assayed for ataxin-2 and tubulin immunoreactivity by Western blotting. n= 2 Data is expressed as the mean ± SD.

With this purpose, mHypoE-N42 cell line was transduced using lentiviral vectors encoding for ataxin-2 overexpression. Seven days after transduction, protein was collected and western blotting analysis performed. With this approach we effectively transduced this cell line, now overexpressing ataxin-2 (Figure 9).

With the mHypoE-N42 cell line overexpressing for ataxin-2, we wanted to evaluate the circadian pattern of ataxin-2 mRNA and protein levels, in both control and palmitate

conditions. This evaluation was done in order to understand if the transduction could affect the circadian behaviour of ataxin-2, after its overexpression.

Regarding ataxin-2, we had previously seen in our lab (data not shown), that palmitate decreases ataxin-2 mRNA expression, and in this study, we observed a shift on circadian rhythmicity on protein levels of ataxin-2 upon palmitate treatment (Figure 8). Here, we observe that palmitate does not have much impact on the circadian patterns on cells overexpressing ataxin-2, where control and treatment groups display similar patterns both in mRNA and protein content (Figure 10A and B). However, when treated with palmitate 500 µM, a delay in the increase of ataxin-2 protein levels that at *Zeitgeber* 32 appears to reach the same levels as other conditions. Despite the lower set of experiments concerning protein circadian oscillations, and looking at both mRNA and protein levels, we can speculate that ataxin-2 overexpression can prevent palmitate induced changes on its own expression.

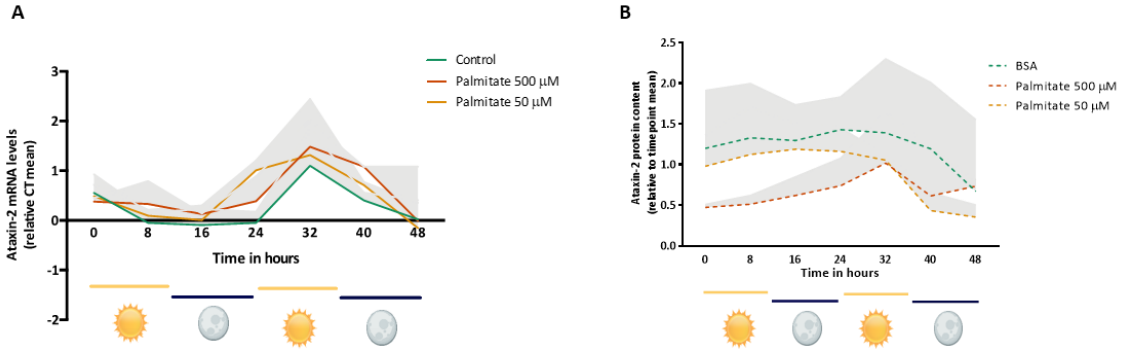

Figure 10. Circadian patterns of the mHypoE-N42 cell line overexpressing ataxin-2. Circadian fluctuations of ataxin-2 under control and palmitate conditions were assessed where the mHypoE-N42 cell line transduced with the lentiviral vector of ataxin-2. This cell line was treated either with 500 µM or 50 µM of palmitate in order to understand if ataxin-2 might mediate the effects induced by palmitate on the molecular circadian clock. mRNA analysis was done by qRT-PCR. (A) Coloured tracing obtained from plot of gene relative expression at each zeitgeber, calculated through ΔΔCT analysis. Grey shadows refer to error (SEM for each timepoint), n=3. (B) Dashed tracing obtained whole-cell extracts Western Blotting, assayed for Ataxin-2 and tubulin immunoreactivity. Grey shadows refer to error (SD for each timepoint), n= 2.

3.6. Ataxin-2 overexpression rescued the effects induced by palmitate

The independent studies relating ataxin-2 with circadian rhythm and metabolism (Carmo-Silva et al., 2017), point to a possible link between this two separate actions of ataxin-2. Meaning that ataxin-2 might have a beneficial metabolic effect through the regulation of circadian rhythm, or vice versa.

Using ataxin-2-overexpressing cells, we used the same synchronization and treatment conditions as before.

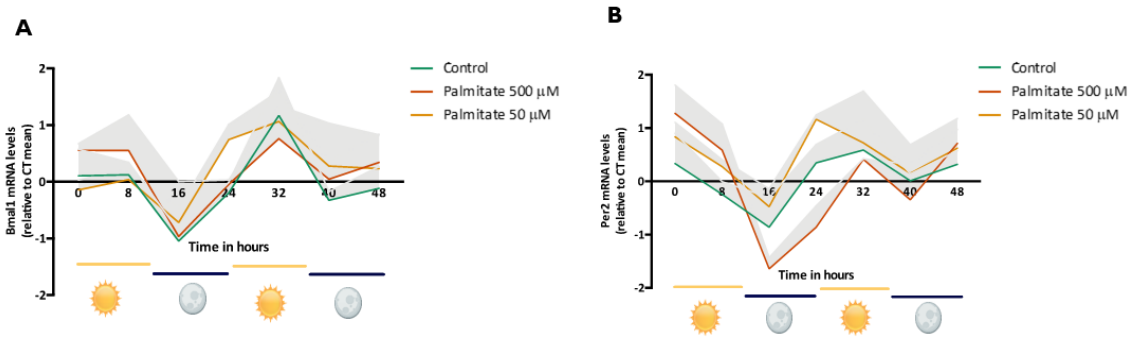

Figure 11. Ataxin-2 overexpression can prevent palmitate-induced circadian alterations in mRNA fluctuations.

Circadian fluctuations of clock genes *Bmal1*(A) and *Per2*(B) under control and palmitate conditions were assessed where the mHypoE-N42 cell line transduced with the lentiviral vector of ataxin-2. This cell line was treated either with 500 µM or 50 µM of palmitate in order to understand if ataxin-2 might mediate the effects induced by palmitate on the molecular circadian clock. mRNA analysis was done by qRT-PCR. Coloured tracing obtained from plot of gene relative expression at each Zeitgeber, calculated through ΔΔCT analysis. Grey shadows refer to error (SEM for each timepoint). n= 3

Considering the major effects on *Bmal1* and *Per2*, we looked at the expression of these clock genes on cells transduced with ataxin-2 and treated with palmitate (Figure 11A and B). Since these genes are displayed in different phases of the circadian clock, they

can be seen as representative. We can observe that both *Bmal1* (Figure 11A) and *Per2* (Figure 11B) display a similar pattern of expression on control and palmitate conditions. We can observe that the major peaks in expression are similar to all conditions (control, palmitate 500 μM and palmitate 50 μM). These observations are opposite to what observed before (Figure 6), where palmitate changed the circadian patterns of these clock genes. These results might suggest that the overexpression of ataxin-2 is preventing the palmitate induced alterations in the circadian oscillations of the clock genes.

Considering the potential effect of ataxin-2 on mTOR signaling, where this protein might inhibit this serine/threonine protein kinase through stress granules or trough regulation of RPS6K activity (Carmo-Silva, Nobrega et al. 2017), we further analyzed Bmal1, mTOR and RPS6K protein levels in the ataxin-2 overexpressing cells. Using the same synchronization and treatment protocol, our aim was that these results would complement the observations obtained earlier (Figure 7). Bmal1 protein circadian expression displays some differences in terms of levels, but not in terms of pattern of expression. Both control and palmitate conditions, peak almost at the same *Zeitgeber* and have similar fluctuations, indicating that ataxin-2 might be preventing palmitate-induced alterations (Figure 12A and B). This preventive effect is more pronounced in the mTOR protein levels where both control and test groups display similar patterns almost oscillating in the same way (Figure 12C and D). As for RPS6K, the mTOR's downstream target, we can also observe that the circadian patterns of this protein, both in control and palmitate conditions, seem to oscillate in the same manner, despite in a prolonged exposure there seems to be a delay in gaining rhythms (Figure 12E and F).

Although we did not perform the same analysis as on the other sets of experiments, ataxin-2 overexpression seems to protect cells against the palmitate induced alterations. Ataxin-2 overexpression can on one hand, prevent changes on the mRNA expression of clock genes *Bmal1* and *Per2,* and on the other, prevent changes on the mTOR pathway activation. These results further support the hypothesis of ataxin-2 as a mediator of both circadian rhythm and metabolism.

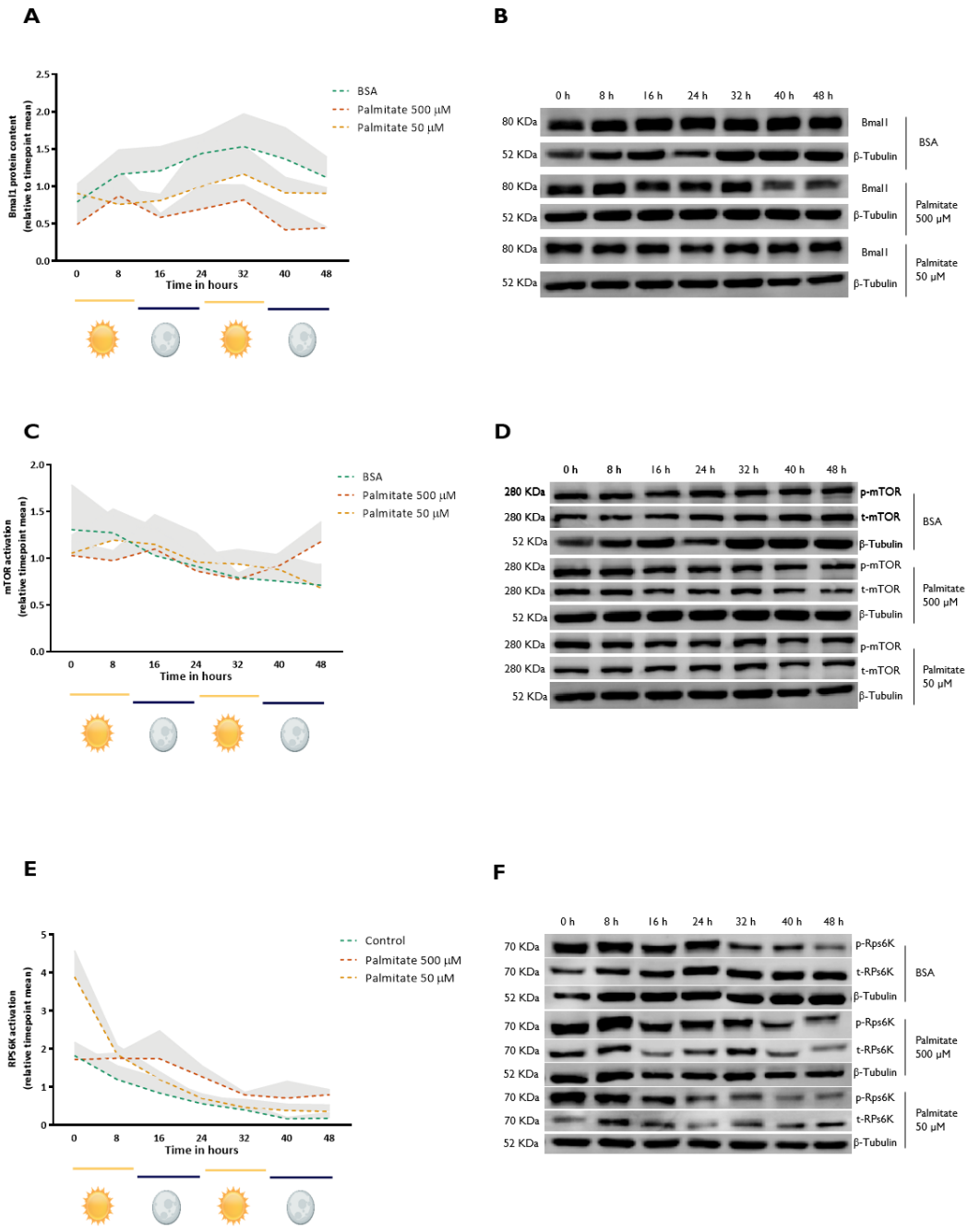

Figure 12. Ataxin-2 overexpression can prevent palmitate-induced protein circadian alterations.

Circadian fluctuations of clock gene Bmal1 (A and B), mTOR (C and D) and Rps6K (E and F) under control and palmitate conditions were assessed where the mHypoE-N42 cell line transduced with ataxin-2. This cell line was treated either with 500 µM or 50 µM of palmitate. (B, D, F) Whole-cell extracts were assayed for Bmal1, phospho-mTOR (p-mTOR), total mTOR (mTOR), phospho-RPS6K (p-RPS6K), RPS6K and tubulin immunoreactivity by Western blotting. (A) Dashed colored tracing obtained from plot of protein levels at each zeitgeber. Grey shadows refer to error (SD for each timepoint). n= 2

Chapter IV – Discussion

4. Discussion

Metabolic disorders such obesity, are reaching nearly pandemic values. So far, therapeutic strategies that can promote sustained weight loss and metabolic improvement are still scarce. Research in obesity is now focused on understanding the underlying mechanisms of the pathophysiology of the disease, in order to find new targets for better therapeutic approaches.

One of the main reasons for this difficulty has to do with the complex etiology of obesity. In this work, we focused on one of the aspects that has been tightly correlated with this disease: circadian rhythm alterations. We now know that obesity is not only about the calories that we ingest, but also the time that we eat (Eckel-Mahan and Sassone-Corsi 2013). On one hand, meal timing is related with circadian rhythm and on the other, metabolism is under circadian regulation (Sahar and Sassone-Corsi 2012, Albrecht 2017). So, when metabolic dysfunction occurs, it might lead to circadian disruption and vice-versa (Froy 2012, Eckel-Mahan and Sassone-Corsi 2013). With the purpose to understand the role of circadian rhythm in the development of metabolic disorders, one strategy might be to find new targets that are involved in the regulation of both metabolism and circadian rhythm. In this context, we suggest that ataxin-2 might be a promising target, regulating the physiology of the hypothalamus, acting both on metabolic homeostasis (Kiehl, Nechiporuk et al. 2006, Lastres-Becker, Brodesser et al. 2008, Carmo-Silva, Nobrega et al. 2017) and circadian rhythm (Lim and Allada 2013, Lee, Yoo et al. 2017, Pfeffer, Gispert et al. 2017). Data obtained suggests that, ataxin-2 through the regulation of clock genes, is a promising target in the modulation of metabolic pathways.

One of the aspects known to alter the hypothalamic circadian cycle is nutrient status (Eckel-Mahan and Sassone-Corsi 2013). For instance, a high fat diet alters circadian behaviour and clock genes (Barnea, Madar et al. 2009, Kwon, Lee et al. 2014). Kohsaka et. al showed that, mice under a high-fat diet exhibited alterations in circadian rhythm behaviour, thus illustrating that a HFD can provoke alterations in the circadian cycle (Kohsaka, Laposky et al. 2007). Fat enriched diets are based on saturated fatty acids, like palmitate. Studies demonstrate that palmitate can cross the blood-brain barrier, reaching the hypothalamus and promoting dysfunction (Kasser, Deutch et al. 1986, Spector 2001).

Therefore, inducing metabolic dysfunction through palmitate is a valid model to test the effects that a high fat diet might have on the molecular circadian system.

Similar to those observations, mHypoE-N42 hypothalamic cell line displayed alterations in the circadian patterns of clock genes upon metabolic dysfunction, induced by palmitate. The exception to that was clock gene *CLOCK* that did not oscillate in a circadian fashion. The nonrhythmic expression of this gene, had previously been demonstrated by others in different cell types, such as GT1-7 neurons, primary SCN tissues and primary retinal tissue (Gekakis, Staknis et al. 1998, Kamphuis, Cailotto et al. 2005). The fact that *Per* rhythmicity was not loss upon palmitate treatment, was also another result already reported by Greco et. al (Greco, Oosterman et al. 2014). As for *Bmal1*, clock gene belonging to the positive limb of the circadian cycle, suffered alterations in rhythmicity induced by palmitate, with a significant phase delay. This observation corroborates what was previously described for other hypothalamic cell line (mHypoE-37) (Greco, Oosterman et al. 2014). This clock gene is of particular importance since has been pointed as a translation factor, responsible for the regulation of the rhythms of protein synthesis, in response to phosphorylation by S6K1 (Lipton, Yuan et al. 2015).

The Ribosomal protein S6 Kinase (RPS6K), is one of the main substrates of mTOR. mTOR is an intracellular sensor responsible for the regulation of metabolism and energy homeostasis (Hu, Xu et al. 2016). This pathway is implicated in protein synthesis, lipid metabolism, autophagy and cell growth (Wullschleger, Loewith et al. 2006). There are a few evidence on mTOR displaying circadian rhythmicity in various cells and tissues, as well as in the SCN (Chang, Yoshihara et al. 2017, Ramanathan, Kathale et al. 2018). Furthermore, *in vitro* approaches demonstrate that its inhibition lengthens the period and dampens amplitude, whereas its activation shortens period and increases amplitude. mTOR constitutive activation can also increase expression of clock proteins, such as Bmal1, Cry and Clock. (Ramanathan, Kathale et al. 2018). In our hypothalamic cell line, both mTOR and its substrate present circadian rhythmicity that is loss upon metabolic stress. mTOR can be activated by palmitate (Kwon and Querfurth 2015), so we can suppose an over-activation of this pathway upon treatment. However, different concentrations of palmitate promoted opposite outcomes in both mTOR and its substrate, with a phase delay in an acute exposure (500 μM) and a phase advance in the prolonged exposure (50 μM). This circadian behaviour is similar to the one observed in

the protein levels of Bmal1, that also displays a phase advance upon an acute exposure and a phase delay upon a prolonged exposure. Besides that, Bmal1 and RPS6K peak in the same phase of the cycle upon when treated with palmitate 50 μM. This might be due to the fact that Bmal1 and mTOR downstream target are connected. Studies in mouse liver demonstrate that both Bmal1 and Phospho-S6 peak in the same phase, the active period (Cornu, Oppliger et al. 2014, Lipton, Yuan et al. 2015). Bmal1 can interfere directly with RPS6K and the mTOR pathway (Lipton et al., 2015), an interesting fact in light of our results, observing these proteins behave in a similar pattern. This interaction further strengthens the idea that the circadian machinery and the molecular clock are tightly interconnected. This might also explain the slight differences between mTOR and RPS6K behaviour upon palmitate treatment (peaking at different times); meaning that Bmal1 interference with RPS6K might be as determinant for RPS6K fluctuations, as mTOR activation. Furthermore, different palmitate-induced responses between mTOR and its substrate, might also be related to the cellular responses to "fuel" overload. With palmitate, we have an abundance of nutrients and an over-activation of RPS6K phosphorylation, thus, a mediator might regulate mTOR activation to prevent overgrowth, without directly interfering with its substrate.

A possible modulator of the mTOR pathway is ataxin-2 (Carmo-Silva et al., 2017). This protein is of special interest in the context of this study for numerous reasons: i) in *Drosophila,* the depletion of dATX2 leads to alterations in circadian behaviour with consequent arrhythmicity (Lim and Allada 2013); ii) Ataxin-2 KO mice are insulin resistant and obese (Kiehl, Nechiporuk et al. 2006, Lastres-Becker, Brodesser et al. 2008) and iii) in periods of cell stress, PBP1 (the yeast ortholog of ATX2) is able to sequester the nutrient sensor TORC1 and inhibit its activity (Ostrowski, Hall et al. 2017). Furthermore, unpublished data from our lab showed that the metabolic dysfunction presented by the ATXN2 KO mice, is secondary to dysfunction in the circadian clock (Carmo-Silva et al., 2019, under review at International Journal of Obesity).

There was a lack of description in the literature regarding ataxin-2 circadian oscillations, interestingly, we observed rhythmicity in protein levels but not mRNA. mRNA decay, post-transcriptional modifications and translation rates might account for differential expressions between mRNA and protein (Denti, Viero et al. 2013). Furthermore, ataxin-2 can regulate its own transcription: ataxin-2 (protein) associates with the transcription

regulator ZBRK1 (KRAB-containing zinc-finger transcriptional regulator), to promote its own transcription (ATXN2 gene) (Hallen, Klein et al. 2011). This fact might also account for differential oscillations between ataxin-2 mRNA and protein levels. Nevertheless, protein levels of ataxin-2 lost rhythmicity upon metabolic stress induced by palmitate. These results align with the idea that ataxin-2 can function as a metabolic sensor, being decreased in the hypothalamus of obese mice (Carmo-Silva et al., 2019, under review at International Journal of Obesity). The loss of circadian rhythmicity on ataxin-2 might also be partly responsible for the loss of rhythmicity of mTOR, since ataxin-2 can modulate the activation of this protein (Bar, Charar et al. 2016). If ataxin-2 oscillations are dampened upon metabolic stress, then its functions might also be compromised.

Data from our lab showed that ataxin-2 hypothalamic overexpression can rescue changes on *Bmal1* and *Per2* that occur in the ATXN2 KO mice (Carmo-Silva et al., 2019, under review at International Journal of Obesity). Considering this fact and its metabolic regulator potential, we investigated if ataxin-2 overexpression could prevent palmitate-induced alterations (on circadian rhythm and the mTOR pathway). Our data shows that on ataxin-2 overexpressing mHypoN42 cells, *Bmal1* and *Per2* circadian oscillations are not disrupted by palmitate. In these cells, palmitate treatment has no major effect on the circadian fluctuations of these two genes when compared to control. However, we also observed that, upon ataxin-2 overexpression, *Bmal1* and *Per2* displayed similar circadian patterns. For instance, they present peaks at *Zeitgeber* 16 h and 32 h. This result is of particular interest since *Bmal1* and *Per2* are supposedly expressed in opposite times of the circadian cycle (Sulli, Manoogian et al. 2018). This might indicate that ataxin-2 is regulating the transcription of these clock genes. Researchers described this role for ataxin-2 in *Drosophila,* where it was implicated in the maintenance of the circadian cycle acting as an activator of dPER translation (Lim and Allada 2013, Lee, Yoo et al. 2017, Pfeffer, Gispert et al. 2017). On the opposite, studies performed in mice show that ataxin-2 was not directly implicated in the transcriptional activation of *PER1* and *PER2* (Pfeffer, Gispert et al. 2017). Although we need more studies to understand the implications of this regulation in clock genes transcription, ataxin-2 prevented palmitate-induced alterations, further supporting the idea that ataxin-2 might function as a regulator of circadian rhythm. This is further demonstrated on protein results, where in cells expressing ataxin-2, palmitate does not have much impact on the circadian oscillations of protein content of Bmal1, mTOR and RPS6K. This is in line with a study

where Bar et. al demonstrated that when ataxin-2 is upregulated, the mTOR pathway is consequently inhibited (Bar, Charar et al. 2016). So, the fact that mTOR circadian pattern is not affected by palmitate might come from the ability of ataxin-2 to inhibit the overactivation of this pathway, but also from its possible regulation of clock genes, namely Bmal1. Bmal1 protein levels are also not deeply affected by palmitate incubation in cells overexpressing ataxin-2. This, allied with the mRNA results, tell us that ataxin-2 might in fact regulate the transcription-translation feedback loop of Bmal1. A clock gene that also has direct implications in the mTOR pathway, hence in metabolism.

In brief, this project supports that there is a connection between circadian rhythm and metabolism, as previously demonstrated by other researchers. Alterations induced by metabolic dysfunction, palmitate in this case, can alter the normal circadian patterns of the circadian machinery and the metabolic pathway, causing mTOR deregulation. Nevertheless, this project additionally supports the hypothesis that ataxin-2 can act as a potential metabolic mediator by preventing the effects induced by palmitate on the circadian cycle.

Chapter V – Conclusions

5. Conclusions

In this project, we aimed to understand the role that saturated fatty acids, in this particular case, palmitate, have on the molecular circadian system *in vitro* and also in metabolic pathway mTOR. Besides that, we also intended to evaluate the effect that palmitate had on the circadian expression of ataxin-2, and if this protein overexpression could prevent palmitate-induced effects. This was done in order to investigate the possible role of ataxin-2 in metabolism regulation and maintenance of the circadian cycle. This is related with studies that connect ataxin-2 with metabolism, being involved in metabolic processes such as lipid metabolism, insulin signaling and protein synthesis (Carmo-Silva, Nobrega et al. 2017). In regard to the possible role of ataxin-2 in circadian rhythm, several studies show that ataxin-2 orthologs act as players in circadian rhythm regulation (Lim and Allada 2013, Zhang, Ling et al. 2013, Lee, Yoo et al. 2017, Pfeffer, Gispert et al. 2017).

Here, we demonstrate that palmitate can disrupt circadian machinery. Palmitate compromises the circadian fluctuations of clock genes, such as *Bmal1*, *Cry* and *Per*, but also the circadian fluctuations of the mTOR pathway (mTOR and its substrate, Rps6K). We show that ataxin-2 presents circadian rhythmicity, but only for protein levels and not for mRNA. We also demonstrate that ataxin-2 overexpression can prevent the circadian misalignments caused by palmitate, preserving the circadian fluctuations of main clock genes *Bmal1* and *Per2*. Furthermore, we found evidence for a possible role of ataxin-2 in the regulation of the transcription of these genes.

Strengthening the role of ataxin-2 as a modulator of the mTOR pathway (Bar, Charar et al. 2016), we found that ataxin-2 overexpression can also prevent the effects of palmitate on the activation of this pathway, thus preserving its circadian oscillations. Furthermore, Bmal1 protein content is also regulated by ataxin-2, proving its potential in the regulation of clock genes expression and levels.

This interconnection between ataxin-2, clock genes and the metabolic signaling pathway of mTOR, is of special interest since it relates this protein as an intermediary between circadian rhythm and metabolism. The results obtained propose a possible role of ataxin-2 as a new potential therapeutic target for diseases such as obesity, that presents in its pathophysiology, both metabolic dysfunction and circadian rhythm disruption.

The next step of this project is to better understand the underlying mechanisms connecting ataxin-2 with the core clock genes, and how ataxin-2 can regulate them. This will allow us to better understand the protective effects of ataxin-2 on circadian rhythm under metabolic stress.

In general, the main conclusions from this project are:

- Palmitate disrupts the circadian oscillatory profiles of clock genes and mTOR pathway.
- Different types of exposure to palmitate lead to distinct responses from circadian clock genes.
- Ataxin-2 protein levels present circadian rhythmicity, but mRNA levels do not oscillate in a time-dependent manner.
- Ataxin-2 overexpression may rescue the effects induced by palmitate on *Bmal1* and *Per2*, but also on the mTOR pathway.
- The results observed give a hint for ataxin-2 as a modulator of circadian rhythm.
- Ataxin-2 beneficial metabolic effects might derive from its ability to rescue circadian oscillations upon metabolic dysfunction.

Chapter VI – Reference

Albrecht, M., M. Golatta, U. Wullner and T. Lengauer (2004). "Structural and functional analysis of ataxin-2 and ataxin-3." Eur J Biochem **271**(15): 3155-3170.
Albrecht, U. (2012). "Timing to perfection: the biology of central and peripheral circadian clocks." Neuron **74**(2): 246-260.
Albrecht, U. (2017). "The circadian clock, metabolism and obesity." Obes Rev **18 Suppl 1**: 25-33.
Avena, N. M., M. E. Bocarsly and B. G. Hoebel (2012). "Animal models of sugar and fat bingeing: relationship to food addiction and increased body weight." Methods Mol Biol **829**: 351-365.
Balsalobre, A., S. A. Brown, L. Marcacci, F. Tronche, C. Kellendonk, H. M. Reichardt, G. Schutz and U. Schibler (2000). "Resetting of circadian time in peripheral tissues by glucocorticoid signaling." Science **289**(5488): 2344-2347.
Balsalobre, A., F. Damiola and U. Schibler (1998). "A serum shock induces circadian gene expression in mammalian tissue culture cells." Cell **93**(6): 929-937.
Bar, D. Z., C. Charar, J. Dorfman, T. Yadid, L. Tafforeau, D. L. Lafontaine and Y. Gruenbaum (2016). "Cell size and fat content of dietary-restricted Caenorhabditis elegans are regulated by ATX-2, an mTOR repressor." Proc Natl Acad Sci U S A **113**(32): E4620-4629.
Barnea, M., Z. Madar and O. Froy (2009). "High-fat diet delays and fasting advances the circadian expression of adiponectin signaling components in mouse liver." Endocrinology **150**(1): 161-168.
Bass, J. (2012). "Circadian topology of metabolism." Nature **491**(7424): 348-356.
Berteus Forslund, H., J. S. Torgerson, L. Sjostrom and A. K. Lindroos (2005). "Snacking frequency in relation to energy intake and food choices in obese men and women compared to a reference population." Int J Obes (Lond) **29**(6): 711-719.
Blancas-Velazquez, A., J. Mendoza, A. N. Garcia and S. E. la Fleur (2017). "Diet-Induced Obesity and Circadian Disruption of Feeding Behavior." Front Neurosci **11**: 23.
Blouet, C. and G. J. Schwartz (2010). "Hypothalamic nutrient sensing in the control of energy homeostasis." Behav Brain Res **209**(1): 1-12.
Bunger, M. K., L. D. Wilsbacher, S. M. Moran, C. Clendenin, L. A. Radcliffe, J. B. Hogenesch, M. C. Simon, J. S. Takahashi and C. A. Bradfield (2000). "Mop3 is an essential component of the master circadian pacemaker in mammals." Cell **103**(7): 1009-1017.
Cailotto, C., S. E. La Fleur, C. Van Heijningen, J. Wortel, A. Kalsbeek, M. Feenstra, P. Pevet and R. M. Buijs (2005). "The suprachiasmatic nucleus controls the daily variation of plasma glucose via the autonomic output to the liver: are the clock genes involved?" Eur J Neurosci **22**(10): 2531-2540.
Cao, R., B. Robinson, H. Xu, C. Gkogkas, A. Khoutorsky, T. Alain, A. Yanagiya, T. Nevarko, A. C. Liu, S. Amir and N. Sonenberg (2013). "Translational control of entrainment and synchrony of the suprachiasmatic circadian clock by mTOR/4E-BP1 signaling." Neuron **79**(4): 712-724.
Carmo-Silva, S. and C. Cavadas (2017). "Hypothalamic Dysfunction in Obesity and Metabolic Disorders." Adv Neurobiol **19**: 73-116.
Carmo-Silva, S., C. Nobrega, L. Pereira de Almeida and C. Cavadas (2017). "Unraveling the Role of Ataxin-2 in Metabolism." Trends Endocrinol Metab **28**(4): 309-318.
Chang, S. W., T. Yoshihara, S. Machida and H. Naito (2017). "Circadian rhythm of intracellular protein synthesis signaling in rat cardiac and skeletal muscles." Biochem Biophys Rep **9**: 153-158.
Cheifetz, P. N. (1971). "The daily rhythm of the secretion of corticotrophin and corticosterone in rats and mice." J Endocrinol **49**(3): xi-xii.
Cornu, M., W. Oppliger, V. Albert, A. M. Robitaille, F. Trapani, L. Quagliata, T. Fuhrer, U. Sauer, L. Terracciano and M. N. Hall (2014). "Hepatic mTORC1 controls locomotor activity, body temperature, and lipid metabolism through FGF21." Proc Natl Acad Sci U S A **111**(32): 11592-11599.
Dalvi, P. S., J. A. Chalmers, V. Luo, D. Y. Han, L. Wellhauser, Y. Liu, D. Q. Tran, J. Castel, S. Luquet, M. B. Wheeler and D. D. Belsham (2017). "High fat induces acute and chronic inflammation in the hypothalamus: effect of high-fat diet, palmitate and TNF-alpha on appetite-regulating NPY neurons." Int J Obes (Lond) **41**(1): 149-158.

Damiola, F., N. Le Minh, N. Preitner, B. Kornmann, F. Fleury-Olela and U. Schibler (2000). "Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus." Genes Dev **14**(23): 2950-2961.
Damrath, E., M. V. Heck, S. Gispert, M. Azizov, J. Nowock, C. Seifried, U. Rub, M. Walter and G. Auburger (2012). "ATXN2-CAG42 sequesters PABPC1 into insolubility and induces FBXW8 in cerebellum of old ataxic knock-in mice." PLoS Genet **8**(8): e1002920.
de Zwaan, M., D. B. Roerig, R. D. Crosby, S. Karaz and J. E. Mitchell (2006). "Nighttime eating: a descriptive study." Int J Eat Disord **39**(3): 224-232.
DeMille, D., B. D. Badal, J. B. Evans, A. D. Mathis, J. F. Anderson and J. H. Grose (2015). "PAS kinase is activated by direct SNF1-dependent phosphorylation and mediates inhibition of TORC1 through the phosphorylation and activation of Pbp1." Mol Biol Cell **26**(3): 569-582.
Denti, M. A., G. Viero, A. Provenzani, A. Quattrone and P. Macchi (2013). "mRNA fate: Life and death of the mRNA in the cytoplasm." RNA Biol **10**(3): 360-366.
Ding, G., Y. Gong, K. L. Eckel-Mahan and Z. Sun (2018). "Central Circadian Clock Regulates Energy Metabolism." Adv Exp Med Biol **1090**: 79-103.
Dyar, K. A., S. Ciciliot, L. E. Wright, R. S. Bienso, G. M. Tagliazucchi, V. R. Patel, M. Forcato, M. I. Paz, A. Gudiksen, F. Solagna, M. Albiero, I. Moretti, K. L. Eckel-Mahan, P. Baldi, P. Sassone-Corsi, R. Rizzuto, S. Bicciato, H. Pilegaard, B. Blaauw and S. Schiaffino (2014). "Muscle insulin sensitivity and glucose metabolism are controlled by the intrinsic muscle clock." Mol Metab **3**(1): 29-41.
Eckel-Mahan, K. and P. Sassone-Corsi (2013). "Metabolism and the circadian clock converge." Physiol Rev **93**(1): 107-135.
Eckel-Mahan, K. L., V. R. Patel, S. de Mateo, R. Orozco-Solis, N. J. Ceglia, S. Sahar, S. A. Dilag-Penilla, K. A. Dyar, P. Baldi and P. Sassone-Corsi (2013). "Reprogramming of the circadian clock by nutritional challenge." Cell **155**(7): 1464-1478.
El-Athman, R., L. Fuhr and A. Relogio (2018). "A Systems-Level Analysis Reveals Circadian Regulation of Splicing in Colorectal Cancer." EBioMedicine **33**: 68-81.
Elmquist, J. K., R. Coppari, N. Balthasar, M. Ichinose and B. B. Lowell (2005). "Identifying hypothalamic pathways controlling food intake, body weight, and glucose homeostasis." J Comp Neurol **493**(1): 63-71.
Farhud, D. and Z. Aryan (2018). "Circadian Rhythm, Lifestyle and Health: A Narrative Review." Iran J Public Health **47**(8): 1068-1076.
Fonken, L. K., M. S. Finy, J. C. Walton, Z. M. Weil, J. L. Workman, J. Ross and R. J. Nelson (2009). "Influence of light at night on murine anxiety- and depressive-like responses." Behav Brain Res **205**(2): 349-354.
Froy, O. (2010). "Metabolism and circadian rhythms--implications for obesity." Endocr Rev **31**(1): 1-24.
Froy, O. (2011). "Circadian rhythms, aging, and life span in mammals." Physiology (Bethesda) **26**(4): 225-235.
Froy, O. (2012). "Circadian rhythms and obesity in mammals." ISRN Obes **2012**: 437198.
Gallant, A. R., J. Lundgren and V. Drapeau (2012). "The night-eating syndrome and obesity." Obes Rev **13**(6): 528-536.
Gao, D., S. Nong, X. Huang, Y. Lu, H. Zhao, Y. Lin, Y. Man, S. Wang, J. Yang and J. Li (2010). "The effects of palmitate on hepatic insulin resistance are mediated by NADPH Oxidase 3-derived reactive oxygen species through JNK and p38MAPK pathways." J Biol Chem **285**(39): 29965-29973.
Gekakis, N., D. Staknis, H. B. Nguyen, F. C. Davis, L. D. Wilsbacher, D. P. King, J. S. Takahashi and C. J. Weitz (1998). "Role of the CLOCK protein in the mammalian circadian mechanism." Science **280**(5369): 1564-1569.
Greco, J. A., J. E. Oosterman and D. D. Belsham (2014). "Differential effects of omega-3 fatty acid docosahexaenoic acid and palmitate on the circadian transcriptional profile of clock genes in immortalized hypothalamic neurons." Am J Physiol Regul Integr Comp Physiol **307**(8): R1049-1060.

Guerrero-Vargas, N. N., E. Espitia-Bautista, R. M. Buijs and C. Escobar (2018). "Shift-work: is time of eating determining metabolic health? Evidence from animal models." Proc Nutr Soc **77**(3): 199-215.
Guo, B., S. Chatterjee, L. Li, J. M. Kim, J. Lee, V. K. Yechoor, L. J. Minze, W. Hsueh and K. Ma (2012). "The clock gene, brain and muscle Arnt-like 1, regulates adipogenesis via Wnt signaling pathway." FASEB J **26**(8): 3453-3463.
Haissaguerre, M., N. Saucisse and D. Cota (2014). "Influence of mTOR in energy and metabolic homeostasis." Mol Cell Endocrinol **397**(1-2): 67-77.
Halbach, M. V., S. Gispert, T. Stehning, E. Damrath, M. Walter and G. Auburger (2017). "Atxn2 Knockout and CAG42-Knock-in Cerebellum Shows Similarly Dysregulated Expression in Calcium Homeostasis Pathway." Cerebellum **16**(1): 68-81.
Hallen, L., H. Klein, C. Stoschek, S. Wehrmeyer, U. Nonhoff, M. Ralser, J. Wilde, C. Rohr, M. R. Schweiger, K. Zatloukal, M. Vingron, H. Lehrach, Z. Konthur and S. Krobitsch (2011). "The KRAB-containing zinc-finger transcriptional regulator ZBRK1 activates SCA2 gene transcription through direct interaction with its gene product, ataxin-2." Hum Mol Genet **20**(1): 104-114.
Hatori, M., C. Gronfier, R. N. Van Gelder, P. S. Bernstein, J. Carreras, S. Panda, F. Marks, D. Sliney, C. E. Hunt, T. Hirota, T. Furukawa and K. Tsubota (2017). "Global rise of potential health hazards caused by blue light-induced circadian disruption in modern aging societies." NPJ Aging Mech Dis **3**: 9.
Hatori, M., C. Vollmers, A. Zarrinpar, L. DiTacchio, E. A. Bushong, S. Gill, M. Leblanc, A. Chaix, M. Joens, J. A. Fitzpatrick, M. H. Ellisman and S. Panda (2012). "Time-restricted feeding without reducing caloric intake prevents metabolic diseases in mice fed a high-fat diet." Cell Metab **15**(6): 848-860.
Hu, F., Y. Xu and F. Liu (2016). "Hypothalamic roles of mTOR complex I: integration of nutrient and hormone signals to regulate energy homeostasis." Am J Physiol Endocrinol Metab **310**(11): E994-E1002.
Huynh, D. P., M. R. Del Bigio, D. H. Ho and S. M. Pulst (1999). "Expression of ataxin-2 in brains from normal individuals and patients with Alzheimer's disease and spinocerebellar ataxia 2." Ann Neurol **45**(2): 232-241.
Kalsbeek, A., E. Fliers, J. A. Romijn, S. E. La Fleur, J. Wortel, O. Bakker, E. Endert and R. M. Buijs (2001). "The suprachiasmatic nucleus generates the diurnal changes in plasma leptin levels." Endocrinology **142**(6): 2677-2685.
Kalsbeek, A., M. Ruiter, S. E. La Fleur, C. Cailotto, F. Kreier and R. M. Buijs (2006). "The hypothalamic clock and its control of glucose homeostasis." Prog Brain Res **153**: 283-307.
Kamphuis, W., C. Cailotto, F. Dijk, A. Bergen and R. M. Buijs (2005). "Circadian expression of clock genes and clock-controlled genes in the rat retina." Biochem Biophys Res Commun **330**(1): 18-26.
Kasser, T. R., A. Deutch and R. J. Martin (1986). "Uptake and utilization of metabolites in specific brain sites relative to feeding status." Physiol Behav **36**(6): 1161-1165.
Khapre, R. V., S. A. Patel, A. A. Kondratova, A. Chaudhary, N. Velingkaar, M. P. Antoch and R. V. Kondratov (2014). "Metabolic clock generates nutrient anticipation rhythms in mTOR signaling." Aging (Albany NY) **6**(8): 675-689.
Kiehl, T. R., A. Nechiporuk, K. P. Figueroa, M. T. Keating, D. P. Huynh and S. M. Pulst (2006). "Generation and characterization of Sca2 (ataxin-2) knockout mice." Biochem Biophys Res Commun **339**(1): 17-24.
Kohsaka, A., A. D. Laposky, K. M. Ramsey, C. Estrada, C. Joshu, Y. Kobayashi, F. W. Turek and J. Bass (2007). "High-fat diet disrupts behavioral and molecular circadian rhythms in mice." Cell Metab **6**(5): 414-421.
Kornmann, B., O. Schaad, H. Reinke, C. Saini and U. Schibler (2007). "Regulation of circadian gene expression in liver by systemic signals and hepatocyte oscillators." Cold Spring Harb Symp Quant Biol **72**: 319-330.

Kucukgoncu, S., M. Midura and C. Tek (2015). "Optimal management of night eating syndrome: challenges and solutions." Neuropsychiatr Dis Treat **11**: 751-760.
Kwon, B., H. K. Lee and H. W. Querfurth (2014). "Oleate prevents palmitate-induced mitochondrial dysfunction, insulin resistance and inflammatory signaling in neuronal cells." Biochim Biophys Acta **1843**(7): 1402-1413.
Kwon, B. and H. W. Querfurth (2015). "Palmitate activates mTOR/p70S6K through AMPK inhibition and hypophosphorylation of raptor in skeletal muscle cells: Reversal by oleate is similar to metformin." Biochimie **118**: 141-150.
la Fleur, S. E., M. C. Luijendijk, E. M. van der Zwaal, M. A. Brans and R. A. Adan (2014). "The snacking rat as model of human obesity: effects of a free-choice high-fat high-sugar diet on meal patterns." Int J Obes (Lond) **38**(5): 643-649.
Laermans, J. and I. Depoortere (2016). "Chronobesity: role of the circadian system in the obesity epidemic." Obes Rev **17**(2): 108-125.
Lamia, K. A., K. F. Storch and C. J. Weitz (2008). "Physiological significance of a peripheral tissue circadian clock." Proc Natl Acad Sci U S A **105**(39): 15172-15177.
Langlet, F. (2014). "Tanycytes: a gateway to the metabolic hypothalamus." J Neuroendocrinol **26**(11): 753-760.
Lastres-Becker, I., S. Brodesser, D. Lutjohann, M. Azizov, J. Buchmann, E. Hintermann, K. Sandhoff, A. Schurmann, J. Nowock and G. Auburger (2008). "Insulin receptor and lipid metabolism pathology in ataxin-2 knock-out mice." Hum Mol Genet **17**(10): 1465-1481.
Lastres-Becker, I., D. Nonis, F. Eich, M. Klinkenberg, M. Gorospe, P. Kotter, F. A. Klein, N. Kedersha and G. Auburger (2016). "Mammalian ataxin-2 modulates translation control at the pre-initiation complex via PI3K/mTOR and is induced by starvation." Biochim Biophys Acta **1862**(9): 1558-1569.
Lee, J., E. Yoo, H. Lee, K. Park, J. H. Hur and C. Lim (2017). "LSM12 and ME31B/DDX6 Define Distinct Modes of Posttranscriptional Regulation by ATAXIN-2 Protein Complex in Drosophila Circadian Pacemaker Neurons." Mol Cell **66**(1): 129-140 e127.
Lim, C. and R. Allada (2013). "ATAXIN-2 activates PERIOD translation to sustain circadian rhythms in Drosophila." Science **340**(6134): 875-879.
Lipton, J. O., E. D. Yuan, L. M. Boyle, D. Ebrahimi-Fakhari, E. Kwiatkowski, A. Nathan, T. Guttler, F. Davis, J. M. Asara and M. Sahin (2015). "The Circadian Protein BMAL1 Regulates Translation in Response to S6K1-Mediated Phosphorylation." Cell **161**(5): 1138-1151.
Liu, D., A. Stowie, N. de Zavalia, T. Leise, S. S. Pathak, L. R. Drewes, A. J. Davidson, S. Amir, N. Sonenberg and R. Cao (2018). "mTOR signaling in VIP neurons regulates circadian clock synchrony and olfaction." Proc Natl Acad Sci U S A **115**(14): E3296-E3304.
Lowrey, P. L. and J. S. Takahashi (2000). "Genetics of the mammalian circadian system: Photic entrainment, circadian pacemaker mechanisms, and posttranslational regulation." Annu Rev Genet **34**: 533-562.
Machluf, Y., A. Gutnick and G. Levkowitz (2011). "Development of the zebrafish hypothalamus." Ann N Y Acad Sci **1220**: 93-105.
Marcheva, B., K. M. Ramsey, E. D. Buhr, Y. Kobayashi, H. Su, C. H. Ko, G. Ivanova, C. Omura, S. Mo, M. H. Vitaterna, J. P. Lopez, L. H. Philipson, C. A. Bradfield, S. D. Crosby, L. JeBailey, X. Wang, J. S. Takahashi and J. Bass (2010). "Disruption of the clock components CLOCK and BMAL1 leads to hypoinsulinaemia and diabetes." Nature **466**(7306): 627-631.
Mayer, C. M. and D. D. Belsham (2010). "Palmitate attenuates insulin signaling and induces endoplasmic reticulum stress and apoptosis in hypothalamic neurons: rescue of resistance and apoptosis through adenosine 5' monophosphate-activated protein kinase activation." Endocrinology **151**(2): 576-585.
Meierhofer, D., M. Halbach, N. E. Sen, S. Gispert and G. Auburger (2016). "Ataxin-2 (Atxn2)-Knock-Out Mice Show Branched Chain Amino Acids and Fatty Acids Pathway Alterations." Mol Cell Proteomics **15**(5): 1728-1739.

Mendoza, J. (2019). "Eating Rewards the Gears of the Clock." Trends Endocrinol Metab **30**(5): 299-311.
Navara, K. J. and R. J. Nelson (2007). "The dark side of light at night: physiological, epidemiological, and ecological consequences." J Pineal Res **43**(3): 215-224.
Nechiporuk, T., D. P. Huynh, K. Figueroa, S. Sahba, A. Nechiporuk and S. M. Pulst (1998). "The mouse SCA2 gene: cDNA sequence, alternative splicing and protein expression." Hum Mol Genet **7**(8): 1301-1309.
O'Reardon, J. P., A. Peshek and K. C. Allison (2005). "Night eating syndrome : diagnosis, epidemiology and management." CNS Drugs **19**(12): 997-1008.
Ostrowski, L. A., A. C. Hall and K. Mekhail (2017). "Ataxin-2: From RNA Control to Human Health and Disease." Genes (Basel) **8**(6).
Panda, S., J. B. Hogenesch and S. A. Kay (2002). "Circadian rhythms from flies to human." Nature **417**(6886): 329-335.
Paschos, G. K. and G. A. FitzGerald (2017). "Circadian Clocks and Metabolism: Implications for Microbiome and Aging." Trends Genet **33**(10): 760-769.
Peplonska, B., A. Bukowska and W. Sobala (2015). "Association of Rotating Night Shift Work with BMI and Abdominal Obesity among Nurses and Midwives." PLoS One **10**(7): e0133761.
Perelis, M., B. Marcheva, K. M. Ramsey, M. J. Schipma, A. L. Hutchison, A. Taguchi, C. B. Peek, H. Hong, W. Huang, C. Omura, A. L. Allred, C. A. Bradfield, A. R. Dinner, G. D. Barish and J. Bass (2015). "Pancreatic beta cell enhancers regulate rhythmic transcription of genes controlling insulin secretion." Science **350**(6261): aac4250.
Pfeffer, M., S. Gispert, G. Auburger, H. Wicht and H. W. Korf (2017). "Impact of Ataxin-2 knock out on circadian locomotor behavior and PER immunoreaction in the SCN of mice." Chronobiol Int **34**(1): 129-137.
Preitner, N., F. Damiola, L. Lopez-Molina, J. Zakany, D. Duboule, U. Albrecht and U. Schibler (2002). "The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator." Cell **110**(2): 251-260.
Ramanathan, C., N. D. Kathale, D. Liu, C. Lee, D. A. Freeman, J. B. Hogenesch, R. Cao and A. C. Liu (2018). "mTOR signaling regulates central and peripheral circadian clock function." PLoS Genet **14**(5): e1007369.
Ramsey, K. M., B. Marcheva, A. Kohsaka and J. Bass (2007). "The clockwork of metabolism." Annu Rev Nutr **27**: 219-240.
Reinke, H. and G. Asher (2019). "Crosstalk between metabolism and circadian clocks." Nat Rev Mol Cell Biol **20**(4): 227-241.
Reinke, H. and G. Asher (2019). "Crosstalk between metabolism and circadian clocks." Nature Reviews Molecular Cell Biology **20**(4): 227-241.
Rothenfluh, A., M. W. Young and L. Saez (2000). "A TIMELESS-independent function for PERIOD proteins in the Drosophila clock." Neuron **26**(2): 505-514.
Sahar, S. and P. Sassone-Corsi (2012). "Regulation of metabolism: the circadian clock dictates the time." Trends Endocrinol Metab **23**(1): 1-8.
Sahba, S., A. Nechiporuk, K. P. Figueroa, T. Nechiporuk and S. M. Pulst (1998). "Genomic structure of the human gene for spinocerebellar ataxia type 2 (SCA2) on chromosome 12q24.1." Genomics **47**(3): 359-364.
Sato, T. K., S. Panda, L. J. Miraglia, T. M. Reyes, R. D. Rudic, P. McNamara, K. A. Naik, G. A. FitzGerald, S. A. Kay and J. B. Hogenesch (2004). "A functional genomics strategy reveals Rora as a component of the mammalian circadian clock." Neuron **43**(4): 527-537.
Schibler, U., J. Ripperger and S. A. Brown (2003). "Peripheral circadian oscillators in mammals: time and food." J Biol Rhythms **18**(3): 250-260.
Schwartz, M. W., S. C. Woods, D. Porte, Jr., R. J. Seeley and D. G. Baskin (2000). "Central nervous system control of food intake." Nature **404**(6778): 661-671.

Sfakianos, A. P., L. E. Mellor, Y. F. Pang, P. Kritsiligkou, H. Needs, H. Abou-Hamdan, L. Desaubry, G. B. Poulin, M. P. Ashe and A. J. Whitmarsh (2018). "The mTOR-S6 kinase pathway promotes stress granule assembly." Cell Death Differ **25**(10): 1766-1780.
Shimizu, I., Y. Yoshida and T. Minamino (2016). "A role for circadian clock in metabolic disease." Hypertens Res **39**(7): 483-491.
Spector, A. A. (2001). "Plasma free fatty acid and lipoproteins as sources of polyunsaturated fatty acid for the brain." J Mol Neurosci **16**(2-3): 159-165; discussion 215-121.
Stubenvoll, M. D., J. C. Medley, M. Irwin and M. H. Song (2016). "ATX-2, the C. elegans Ortholog of Human Ataxin-2, Regulates Centrosome Size and Microtubule Dynamics." PLoS Genet **12**(9): e1006370.
Sukumaran, S., R. R. Almon, D. C. DuBois and W. J. Jusko (2010). "Circadian rhythms in gene expression: Relationship to physiology, disease, drug disposition and drug action." Adv Drug Deliv Rev **62**(9-10): 904-917.
Sulli, G., E. N. C. Manoogian, P. R. Taub and S. Panda (2018). "Training the Circadian Clock, Clocking the Drugs, and Drugging the Clock to Prevent, Manage, and Treat Chronic Diseases." Trends Pharmacol Sci **39**(9): 812-827.
Summa, K. C. and F. W. Turek (2014). "Chronobiology and obesity: Interactions between circadian rhythms and energy regulation." Adv Nutr **5**(3): 312S-319S.
Swisher, K. D. and R. Parker (2010). "Localization to, and effects of Pbp1, Pbp4, Lsm12, Dhh1, and Pab1 on stress granules in Saccharomyces cerevisiae." PLoS One **5**(4): e10006.
Takahara, T. and T. Maeda (2012). "Transient sequestration of TORC1 into stress granules during heat stress." Mol Cell **47**(2): 242-252.
Takahashi, J. S. (2017). "Transcriptional architecture of the mammalian circadian clock." Nat Rev Genet **18**(3): 164-179.
Tsang, A. H., M. Astiz, B. Leinweber and H. Oster (2017). "Rodent Models for the Analysis of Tissue Clock Function in Metabolic Rhythms Research." Front Endocrinol (Lausanne) **8**: 27.
Tse, E. K., A. Salehi, M. N. Clemenzi and D. D. Belsham (2018). "Role of the saturated fatty acid palmitate in the interconnected hypothalamic control of energy homeostasis and biological rhythms." Am J Physiol Endocrinol Metab **315**(2): E133-E140.
Turek, F. W., C. Joshu, A. Kohsaka, E. Lin, G. Ivanova, E. McDearmon, A. Laposky, S. Losee-Olson, A. Easton, D. R. Jensen, R. H. Eckel, J. S. Takahashi and J. Bass (2005). "Obesity and metabolic syndrome in circadian Clock mutant mice." Science **308**(5724): 1043-1045.
Ueno, H. and M. Nakazato (2016). "Mechanistic relationship between the vagal afferent pathway, central nervous system and peripheral organs in appetite regulation." Journal of Diabetes Investigation **7**(6): 812-818.
Wang, J. B., R. E. Patterson, A. Ang, J. A. Emond, N. Shetty and L. Arab (2014). "Timing of energy intake during the day is associated with the risk of obesity in adults." J Hum Nutr Diet **27 Suppl 2**: 255-262.
Waterson, M. J. and T. L. Horvath (2015). "Neuronal Regulation of Energy Homeostasis: Beyond the Hypothalamus and Feeding." Cell Metab **22**(6): 962-970.
Wiley, H. S. and P. M. Burke (2001). "Regulation of receptor tyrosine kinase signaling by endocytic trafficking." Traffic **2**(1): 12-18.
Williams, K. W. and J. K. Elmquist (2012). "From neuroanatomy to behavior: central integration of peripheral signals regulating feeding behavior." Nat Neurosci **15**(10): 1350-1355.
Wullschleger, S., R. Loewith and M. N. Hall (2006). "TOR signaling in growth and metabolism." Cell **124**(3): 471-484.
Wyse, C. A., C. A. Celis Morales, N. Graham, Y. Fan, J. Ward, A. M. Curtis, D. Mackay, D. J. Smith, M. E. S. Bailey, S. Biello, J. M. R. Gill and J. P. Pell (2017). "Adverse metabolic and mental health outcomes associated with shiftwork in a population-based study of 277,168 workers in UK biobank<sup/>." Ann Med **49**(5): 411-420.

Yang, G., L. Chen, G. R. Grant, G. Paschos, W. L. Song, E. S. Musiek, V. Lee, S. C. McLoughlin, T. Grosser, G. Cotsarelis and G. A. FitzGerald (2016). "Timing of expression of the core clock gene Bmal1 influences its effects on aging and survival." Sci Transl Med **8**(324): 324ra316.
Yang, S., A. Liu, A. Weidenhammer, R. C. Cooksey, D. McClain, M. K. Kim, G. Aguilera, E. D. Abel and J. H. Chung (2009). "The role of mPer2 clock gene in glucocorticoid and feeding rhythms." Endocrinology **150**(5): 2153-2160.
Yoo, S. H., S. Yamazaki, P. L. Lowrey, K. Shimomura, C. H. Ko, E. D. Buhr, S. M. Siepka, H. K. Hong, W. J. Oh, O. J. Yoo, M. Menaker and J. S. Takahashi (2004). "PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues." Proc Natl Acad Sci U S A **101**(15): 5339-5346.
Zhang, Y., J. Ling, C. Yuan, R. Dubruille and P. Emery (2013). "A role for Drosophila ATX2 in activation of PER translation and circadian behavior." Science **340**(6134): 879-882.
Zheng, H., N. R. Lenard, A. C. Shin and H. R. Berthoud (2009). "Appetite control and energy balance regulation in the modern world: reward-driven brain overrides repletion signals." Int J Obes (Lond) **33 Suppl 2**: S8-13.