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Interakce mezi cirkadiánními hodinami a makrofágy v tukové tkáni

Interaction between circadian clock and macrophages in the adipose tissue

Diplomová práce

Vedoucí diplomové práce: PharmDr. Alena Sumová, DSc.

Praha, 2017

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 25.4.2017

Podpis

Poděkování:

Ráda bych poděkovala své školitelce PharmDr. Aleně Sumové, DSc. za pomoc při vypracování této práce, za její čas a trpělivost. Dále bych ráda poděkovala celému Oddělení Neurohumorální regulace a Mgr. Martině Rhombaldové a RNDr. Ondřeji Kudovi, Ph.D. z Oddělení Biologie tukové tkáně Fyziologického ústavu AV ČR za ochotu, pomoc a cenné rady. V neposlední řadě bych pak ráda poděkovala i rodině a přátelům za zázemí a podporu.

Abstrakt

Dobře fungující cirkadiánní systém je důležitou součástí zdravého organismu a jeho narušení může vést k ovlivnění metabolických funkcí a následným rozvojem obezity a diabetu mellitu 2.typu. Obezita je obecně doprovázena zvýšenou migrací pro-zánětlivě polarizovaných makrofágů (M1) do tukové tkáně. V této práci jsme ukázali, že interakce makrofágů tohoto typu s tukovou tkání má významný vliv na rytmickou expresi hodinových genů v adipocytech. Dále jsme zkoumali efekt vysokotuké diety a diety obohacené o omega-3 mastné kyseliny na cirkadiánní oscilace v bílé tukové tkáni a různě polarizovaných makrofázích. Tato dieta ovlivnila chod hodin v tukové tkáni a v M0 a M2 polarizovaných makrofázích. Tyto výsledky podporují předchozí objevy týkající se efektu omega-3 mastných kyselin na metabolismus a naznačují jejich efekt na cirkadiánní systém.

Klíčová slova: cirkadiánní rytmy, tuková tkáň, makrofágy, omega-3 mastné kyseliny, high fat diet

Abstract

Well functioning circadian system is crucial component of healthy organism and its disruption can result in impairment of metabolic functions with consequential development of obesity and type 2 diabetes mellitus. Obesity is in general caused by enhanced migration of pro-inflammatory polarized macrophages (M1) into adipose tissue. We have shown, that interaction of this type of macrophages with adipose tissue had significant effect on rhythmic expression of clock genes in adipocytes. We further investigated effect of high fat diet and diet enriched by omega-3 fatty acids on circadian oscillations in WAT and differently polarized macrophages. This diet affected oscillations in adipose tissue and in M0 and M2 polarized macrophages. These results support previous findings of effect of omega-3 fatty acids on metabolism and suggest their effect on circadian system as well.

Key words: circadian rhythms, adipose tissue, macrophages, omega-3 fatty acids, high fat diet

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List of abbreviations

AANAT	Serotonin N–acetyl Transferase
ACTH	Adrenocorticotropic Hormone
AgRP	Agouti Related Protein
AMP	Adenosin Monophosphate
AMPK	AMP–activated Protein Kinase
APC	Antigen Presenting Cell
ApoB	Apolipoprotein B
ARG1	Arginase 1
ATP	Adenosine Triphosphate
AVP	Arginine Vasopressin
bHLH–PAS	Basic Helix–Loop–Helix/Per–Arnt–Sim
BAT	Brown Adipose Tissue
BMAL1	Brain and Muscle ARNT–like protein
BMDM	Bone Marrow Divided Macrophages
CAMS	Circadian Activity Monitoring System
CCL2	C–C Chemokine Ligand 2
CCR2	C–C Chemokine Receptor type 2
C/EBPα	CCAAT/enhancer–binding Protein alpha
cHF	High Fat concentrated food with casein
cHF–F	High Fat concentrated food with casein and omega 3 concentrate
CLOCK	Circadian Locomotor Output Cycles Kaput
CRE	cAMP–reactive Element

CREB	cAMP–response–element–binding Protein
CRF	Corticotropin Releasing Factor
CRP	C–reactive Protein
DAG	Diacylglyceride
DD regime	Dark–Dark regime (constant darkness)
DHA	Docosahexaenoic Acid
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
E–box	Enhancer box
EPA	Eicosapentaenoic Acid
FAS	Fatty Acid Synthase
FBS	Fetal Bovine Serum
FN–1	Fibronectin–1
GABA	Gamma–Aminobutyric Acid
GC	Glucocorticoid
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Element
GRP	Gastrin–releasing Peptide
HFD	High Fat Diet
HIF–1α	Hypoxia induced factor–1 alpha
HNF4α	Hepatocyte Nuclear Factor 4 alpha
HPA	Hypothalamic–pituitary–adrenal axis
IFN-γ	Interferon gamma
IGF–1	Insulin–like Growth Factor 1

IL	Interleukin
IML	Intermediolateral nucleus
iNOS	Inducible Nitric Oxide Synthase
LD regime	Light–Dark regime
LPS	Lipopolysaccharide
LRH–1	Liver Receptor Homolog–1
M0	Unpolarized macrophages
M1	Classically activated macrophages
M2	Alternatively activated macrophages
MAPK	Mitogen–activated Protein Kinase
MCP–1	Monocyte Chemoattractant Protein–1
MCP–1–R	Monocyte Chemoattractant Protein–1 Receptor
MHC	Major Histocompatibility Complex
MTTP	Microsomal Triglyceride Transfer Protein
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NO	Nitric Oxide
PACAP	Pituitary Adenylate Cyclase–activating Polypeptide
PBS	Phosphate Buffer Saline
PGC–1α	Peroxisome Proliferator–activated Receptor Gamma Coactivator 1–alpha
POMC	Proopiomelanocortin
PPAR	Peroxisome Proliferator–activated Receptor
PPRE	Peroxisome Proliferator hormone Response Elements

PUFA	Polyunsaturated Fatty Acids
PVN	Paraventricular Nucleus
pRGC	Photosensitive Retinal Ganglion Cells
RHT	Retinohypothalamic Tract
ROR	Retinoic acid Receptor–related Orphan Receptor
RORE	Retinoic acid–related Orphan Receptor Response Element
RPMI	Roswell Park Memorial Institute
RXR	Retinoid X Receptor
SCF	Skp–Cullin–F–box
SHP	Small Heterodimer Partner
SRE–1	Sterol Response Element 1
SREBP	Sterol Regulatory Element–binding Protein
StAR	Steroidogenic Acute Regulatory Protein
SVF	Stromal Vascular Fraction
TAG	Triacylglyceride
TIEG1	Transforming growth factor–beta Inducible Early Gene 1
TNFα	Tumor Necrosis Factor
UCP1	Uncoupling Protein 1
VIP	Vasoactive Intestinal Polypeptide
WAT	White Adipose Tissue
WT	Wild Type

1. Introduction

Circadian rhythm is an important biological process that takes place in almost every living organism on our planet, from single–cell prokaryotes [1] to plants [4] and mammals. Maintenance of circadian rhythms in behaviour and in intracellular and extracellular metabolic processes is an endogenous trait that has been developed in a form, which nowadays is in its principle common to all eukaryotic organisms. The mechanism is maintained on cellular level and is driven by molecular clock mechanism.

As it appears from its name "circadian" this system enables organisms to adapt their behaviour and internal physiological processes according to external environment that changes over day and night. The circadian rhythms are entrained with daytime mainly by external light/dark cycles.

Mammalian circadian system is hierarchically organized and it is divided in central and peripheral oscillators. The central clock is located in the hypothalamus, while peripheral subordinated oscillators can be found in organs and peripheral tissue such as liver, pancreas, adipose tissue or components of immune system etc [10].

Feeding and behaviour associated with food intake are the most powerful stimuli that sets the internal clock in numeral peripheral tissues. Changes in feeding regime have specific effect on rhythmicity of overall metabolic system, leaving the central circadian clock unaffected. As a result, disruption of the internal synchrony among circadian clocks can have deteriorating effects on our health.

The same goes for distortion in our eating habits. In modern society, obesity, in both adults and children, is steadily becoming more and more grievous problem. It is a gateway for outbreak of various diseases including cardiovascular diseases, cancer or insulin resistance [3]. Recently it turned out, that dysfunction of circadian clock system could also take part in onset of type 2 diabetes mellitus [2], which is the outcome of insulin resistance.

Aim of this thesis was to explore the properties of circadian oscillators in white adipose tissue and in components of immune system, namely macrophages – two players that among others participate in onset of type 2 diabetes. Additionally, our aim was to describe the impact of their mutual interactions. The results may contribute to considerations on dysfunction of the circadian clock in adipose tissue as a target of new approaches to treat type 2.

2. Literature summary

2.1 Molecular feedback loop

As it emerges from the name circadian itself, which comes from latin *circa diem*, meaning about a day, circadian clock govern processes that appear with daily regularity (i.e. with period about 24 hours).

These circadian rhythms can be the most easily spotted as rhythms in animal's behaviour, but they are also observable as changes of body temperature, heartbeat, blood pressure, concentrations of hormones, or blood glucose levels or even as fluctuations on molecular level [5].

All those processes are controlled by molecular mechanism that is called the "circadian clock". Genes and molecules involved in this process may vary depending on the organism, but its principle has been preserved throughout various eucaryotic species. Involved genes, also called the clock genes, are being periodically expressed and their products, proteins, form so–called transcription–translation feedback loops where proteins either positively or negatively affect expression of one another (Fig.1).

Two feedback loops are the core of the mammalian circadian clock. The key participants involved in both of these feedback loops, are proteins CLOCK (Circadian Locomotor Output Cycles Kaput) and BMAL1 (Brain and Muscle ARNT–Like Protein). Both of their secondary structure is characterised by basic helix–loop–helix motive and thus belong to the family of bHLH–PAS transcription factors (basic Helix–Loop–Helix/Per–Arnt–Sim) [7].

Upon their translation in cytoplasm these two proteins aggregate and form a heterodimeric complex, which is essential step for their translocation to the nucleus and function as transcription factors. After the transport to the cellular nucleus, they bind to deoxyribonucleic acid (DNA), namely consensus sequence known as E–box (Enhancer Box) in promoter area of target genes [6]. There they act as positive transcription factors and enhance gene expression.

Apart from other genes the CLOCK/BMAL1 heterodimer promotes expression of *Period* (homologues *Per1*, *Per2* and *Per3*) and *Cryptochrome* (*Cry1*, *Cry2*) genes, which form the second half of the first circadian feedback loop. Translated proteins PER a CRY (one homologue protein of each) then bind together and form a PER/CRY heterodimer. Their

intracellular concentration is moreover regulated by phosphorylation mediated by CKδ/ε (Casein Kinase δ or ε), which also play significant role in PER/CRY nuclear translocation [8].

In the nucleus PER/CRY heterodimer binds to CLOCK/BMAL1 heterodimer, inhibits its activity and thus acts as a negative transcription factor. The inhibitory mechanism remains to be thoroughly investigated. Recent studies point out, that CRY protein acts as the main inhibitory force and PER supports it by protecting CRY from ubiquitination and proteolysis, enabling heterodimer the nuclear translocation and promoting CLOCK/BMAL1 dissociation from chromatin [9].

The inhibition of CLOCK/BMAL1 positive transcriptional activity results in reduced gene expression, thus also in decline in expression of *Per* and *Cry* genes themselves. This leads to overall decrease in their mRNA levels and consequently to decrease in protein concentrations due to proteolysis over the time. With decreasing concentration, their inhibitive effect diminishes too, which leads to restoration of CLOCK/BMAL1 complex's positive transcriptional effect on gene expression.

CLOCK/BMAL1 heterodimers are also involved in second transcription–translation feedback loop, which affects their own expression. As mentioned above, CLOCK/BMAL1 complex binds to chromatin, namely to a sequence in genes' promoter area called the E–box. This sequence can also be found in promoting areas of genes *Rev–Erb α* and *Rev–Erb β*.

REV–ERB α and REV–ERB β belong to the Orphan nuclear receptor family. Along with them, another nuclear receptor plays role in orchestrating the molecular clock, namely ROR homologues (ROR α, β and γ). They are members of retinoic acid receptor–related orphan receptors and both REV–ERBs and RORs affect various physiological processes, including metabolism or immunity, through modulation of gene expression [11].

Both of these receptors bind to specific nucleotide sequence in gene promoters' areas called Retinoic Acid–Related Orphan Receptor Response Element (RORE), but each affects the gene transcription in a different way – while REV–ERB receptors repress it (and act thus as inhibitory transcription factors), ROR receptors enhance it (positive transcription factors). Because they both bind to the same promoter element, they compete over this binding site, which can be found in promoter area of genes *Clock* and *Bmal1*.

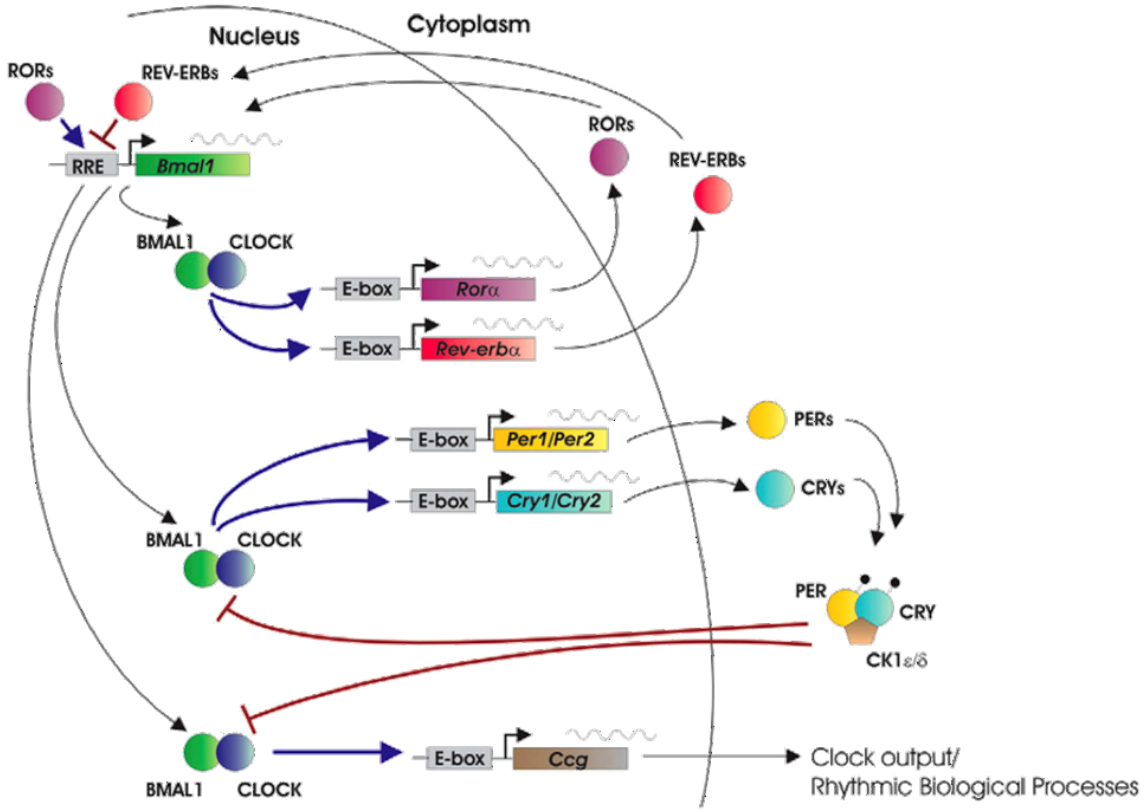


Figure 1: Simplified scheme of molecular circadian clock [6], which consists of two transcription–translation feedback loops. CLOCK/BMAL1 complex enhances translation of *Per*, *Cry*, *Rev–Erb* and *Ror* genes. In cytoplasm PER and CRY proteins form a heterodimer and after phosphorylation by Casein kinase, they are translocated to nucleus. There they inhibit CLOCK/BMAL1's positive transcriptional activity, which results in decrease in their own transcription. REV–ERB nuclear receptors on the other hand inhibit expression of *Bmal1*, which also results in decrease in REV–ERB levels. This decrease enables ROR to bind to RORE binding site and to enhance *Bmal1* expression.

They form a secondary feedback loop in which CLOCK/BMAL1 complex promotes REV–ERB α/β expression, amount of the nuclear receptors increases and represses production of CLOCK and BMAL1 proteins due to binding to RORE nucleotide sequences on promoters of their genes, resulting in gradual decrease of CLOCK/BMAL1 complexes. That consequently weakens CLOCK/BMAL1–induced transcriptional activation on *Rev–Erb* genes. RORs compete for the RORE with REV–ERB α/β and enhance expression of *Clock* and *Bmal1* genes and elevate their levels [10,12].

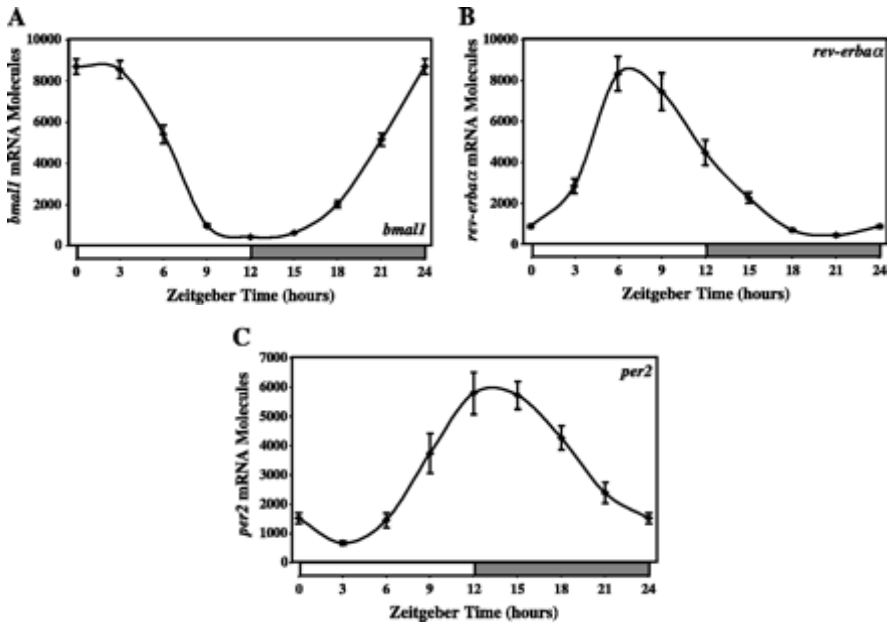


Figure 2: Daily fluctuations of selected clock genes' mRNA [13], namely *Bmal1*, *Rev–Erb α* and *Per 2*. CLOCK/BMAL1 complexes promote expression of various genes including *Per 2* and *Rev–Erb α*. REV-ERB α acts as inhibitory transcription factor and represses transcription of *Bmal1*. After gradual decrease in levels of REV–ERB α, ROR can bind to RORE binding site and again enhance transcription of *Bmal1*.

Both feedback loops are interlocked one to each other and the whole system results in ongoing fluctuations in proteins' concentration (Fig. 2). Completion of one full cycle of these oscillations takes in constant conditions circa 24 hours and is what we call a circadian clock. This molecular mechanism is what can be found in almost every living cell of our body.

2.2 Organisation and synchronization of circadian clock

The biological clock is not as precise as for example atomic clock. It is a flexible system and clock's free–running period (i.e. time it takes to complete one full cycle without being adjusted by external cues, also called τ) is not exactly 24 hours and may vary not only among various species but also among different tissues within single organism. So how is it possible that clocks in individual cells can synchronize with each other?

As mentioned above, mammalian biological clock system is hierarchically organized – it consists of master pacemaker clock, that synchronises its activity to the environment and sends signals of various nature throughout the body to the peripheral subordinate oscillators (described further in chapter 2.2.3 Synchronization of peripheral oscillators).

2.2.1 SCN

Master pacemaker clock is situated in the anterior hypothalamus, concretely in two areas that are collectively called the suprachiasmatic nuclei (SCN). They are located superior to the crossing of the optic nerves, known also as optic chiasm, bilaterally on both sides of the third ventricle.

The significance of the SCN as the master pacemaker, i.e. the central circadian clock was finally confirmed in 1990. At that time, Ralph, et al. [14] described that not only the animal's circadian rhythms were abolished after the excision of their SCN, but with series of SCN transplantation experiments, they showed, that the length of animal's free–running period was dependent on the genetic background of the donor SCN neurons.

Since then the research on its functions and anatomy has become field of interest and progressed greatly. Each unit of human SCN consists of ~45,000 neurons (rodent's SCN consists of ~10,000 neurons) [15]. These neurons form two distinguishable clusters composed of neurons of different phenotype.

SCN is commonly divided on dorsomedial and ventrolateral parts and they mainly differ in morphology, polypeptide expression, nature of their neural connections and each cluster plays its own specific role in clock synchronization to the environment [15].

Neurons in ventrolateral SCN, also called the "core" neurons, are most distinctly characterized by expression of vasoactive intestinal polypeptide (VIP) and gastrin–releasing peptide (GRP) alongside with neurotransmitter γ–Aminobutyric acid (GABA) and neuromodulator called substance P. These neurons lie in close proximity to the optic chiasm and they are first to process the information about light cues from the environment.

Neurons in dorsomedial SCN, also called the "shell" neurons on the other hand express mainly arginine vasopressin (AVP) polypeptide. These cells do not receive direct input from retinal neurons, but from those neurons in SCN core. [15]

2.2.2 Clock synchronization to photic stimuli

As mentioned above, the most significant cue for setting of the circadian clock (also called a *Zeitgeber* throughout the literature) is the light, either from natural or artificial source.

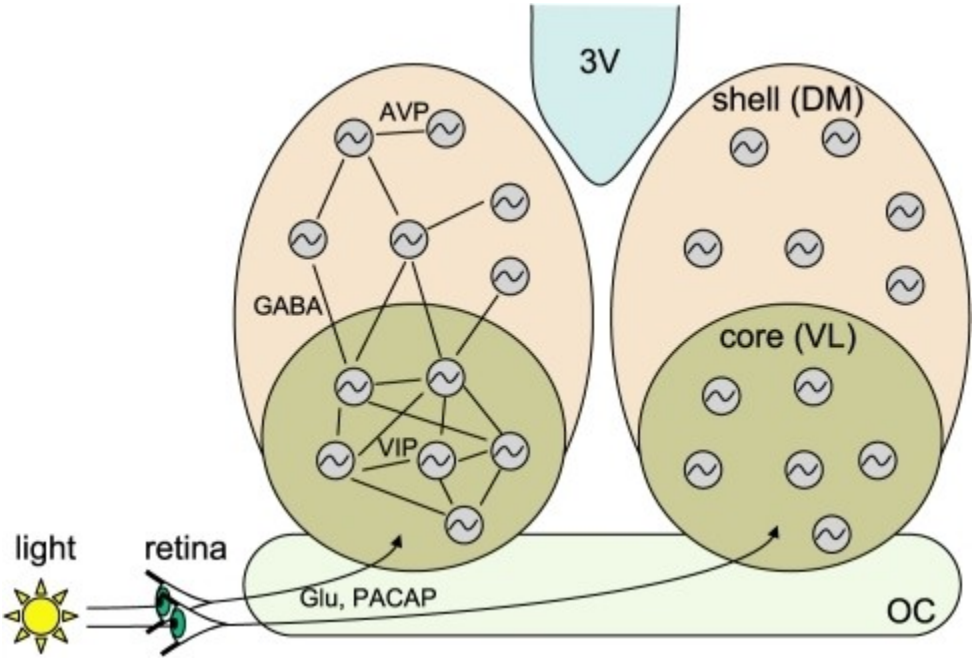


Figure 3: Organization of the suprachiasmatic nucleus (SCN) and representation of its neuronal pathways [16]. SCN neurons are being morphologically divided into two clusters – ventrolateral "core" neurons and dorsomedial " shell" neurons. Core neurons are directly innervated by pRGC, which upon the exposure to light stimulus transmit the signal via RHT to SCN. Signal, that activates molecular signal cascade and that then affects the molecular clock feedback loop, is gradually transmitted throughout the SCN using various neurotransmitters depending on the origin of neuron (VIP expression prevails in core neurons, AVP expression in shell neurons).

Light is perceived in photoreceptive cells. In mammals, those cells can only be found in the retina, namely in rods, cones and a subset of retinal photosensitive ganglion cells (pRGC). Rods and cones perceive visual stimuli, and forward information about it to the visual cortex. Retinal ganglion cells are those contributing to the clock synchronization [29].

Transformation of the presence of the light stimulus into neural information in pRGC is mediated via photopigment melanopsin [27, 28]. This information is then forwarded through the retinohypothalamic tract (RHT). These tracts originating from each eye lead to the area of the optic chiasm, where the information is passed to SCN core neurons. This transmission is mediated by glutamate and pituitary adenylate cyclase–activating polypeptide (PACAP) neurotransmitters [30].

After the light exposure, in the SCN neurons a signal cascade is activated – activation of NMDA glutamate receptors initiates influx of Ca^{2+} inside the cells, this leads to gradual activation of various enzymes including calmodulin, adenylyl cyclase, protein kinase A, which phosphorylates, and thus activates cAMP–response–element–binding protein (CREB).

CREB is, similarly to CLOCK/BMAL1 complex, a transcription factor, that binds to cAMP–reactive element (CRE) and enhances expression of *Per1* and *Per2* genes [17].

From this point the signal is gradually spread among the SCN neurons, both those in the core and shell. Due to their mutual connection, the SCN neurons are capable of generation of rhythmic signals that are broadcasted not only throughout the other parts of brain, but also to the rest of the body (Fig.3) [18, 19].

2.2.3 Synchronization of peripheral oscillators

Once the master clock oscillator (i.e. the SCN) is synchronized to the light stimulus from the environment, its role is to deliver the information about the day time to the peripheral oscillators, thus to the remaining body tissues, whose cells also possess the molecular clock, but are not able to entrain their activity to the external conditions.

The brain centre that functions as the middle–station between the SCN and the periphery is the paraventricular nucleus (PVN). These two neuroanatomical nuclei are not only situated in quite close proximity (PVN is situated superior to the SCN, adjacent to the third ventricle), but they are also strongly connected through neural wiring. The neural transmission between SCN and PVN is mediated predominantly by neurotransmitters GABA, glutamate and AVP [23, 24, 25].

PVN consists of 3 main cell types – magnocellular neurosecretory neurons, parvocellular neurosecretory neurons and so–called preautonomic interneurons. The magnocellular neurons characteristically secrete the peptide hormones AVP and oxytocin. These hormones are being released from cells' axons inside the neurohypophysis, where they stimulate further expression of these endocrine hormones [25, 26].

Parvocellular neurons are involved mainly in the endocrinal pathway of entrainment of peripheral circadian oscillators, namely by modulation of the hypothalamic–pituitary–adrenal axis (HPA). These neurons express corticotropin releasing factor (CRF), a neuropeptide, stimulates endocrine cells in the anterior pituitary. These cells start expressing the adrenocorticotropic hormone (ACTH), that activates expression of glucocorticoids (GC) in the cortex of the adrenal gland [31]. Newly produced GCs are secreted into bloodstream and distributed to the peripheral tissues. There GCs diffuse into the cellular cytoplasm and bind to the glucocorticoid receptors (GR), which then affect the cellular transcription and they also create a negative feedback that causes decrease in CRF and ACTH production [31].

GCs are steroid hormones, which are derived from cholesterol, and they are body's main regulators of stress response, which is primarily governed by mentioned HPA axis. The release of cortisol and corticosterone, which are prevailing GCs in humans and rodents respectively [31], as a reaction to stress affects many physiological processes including metabolism and immune system [47] and many more and also play important role in peripheral clock entrainment.

The link between GC's production and circadian clock is apparent in levels of plasma GCs, which show robust circadian rhythm even in non–stressful environment, as well as do its expression mediators – ACTH and CRF [33, 34]. As mentioned before, GC bind in cytoplasm to GR, which is in absence of ligands usually associated with chaperons of heat shock protein family [35], that dissociate upon forming a complex with the ligand. The GC/GR complex is then transported to the nucleus, where in form of homodimer it binds to a DNA promoter sequence called the glucocorticoid response element (GRE) [36]. This sequence can be found in promoter area of core clock genes, namely *Per1* and *Per2*. The GC/GR complex bound to the GRE acts as a positive transcription factor and elevates levels of both proteins PER1 and PER2 and thus causes a phase shift in clock oscillations [32, 37].

However, the circadian rhythm in production of GCs can also be observed even after hypophysectomy, i.e. surgical removal of the pituitary gland [38]. This shows that production of GCs is regulated by circadian clock system through different pathways, than just by activation of the HPA axis, which brings us to the last way of clock entrainment mediated through the last type of PVN neurons called preautonomic interneurons [33].

Preautonomic interneurons project to the intermediolateral (IML) nucleus, which is located in the spinal cord, and they transfer input received from hypothalamus to both sympathetic and parasympathetic autonomous nervous system [31, 39]. These so–called splanchnic nerves then innervate various peripheral organs and affect their function and number of related physiological processes.

The GC–producing cells in cortex of adrenal gland are innervated by autonomic sympathetic neurons. These neurons transmit the information by release of neurotransmitter adrenaline. Its binding to the adrenergic receptor activates mitogen–activated protein kinase (MAPK) cascade [41]. Neural sympathetic stimulation promotes expression of PER1 protein and thus adjusts molecular clock oscillations in adrenal gland. Clock mechanism then governs transcription of proteins and enzymes responsible for GC production, for example

steroidogenic acute regulatory protein (StAR), whose expression also shows circadian rhythm and which is responsible for transport of GC precursor cholesterol into cell's mitochondria [40].

Another important organ that is innervated by sympathetic neurons projecting from the IML nucleus through the superior cervical ganglion is the pineal gland. Increased levels of cAMP caused by stimulation of adrenergic receptors and onset of related signaling cascade leads to increased expression of serotonin N–acetyl transferase (AANAT) which converts serotonin into hormone melatonin [42].

Melatonin is probably the most associated hormone with biological rhythms. Its secretion is linked strictly to the dark part of the day. Length of the interval of melatonin production changes over the year and acts as an indicator for season–related physiological processes, for example in hibernating animals [42].

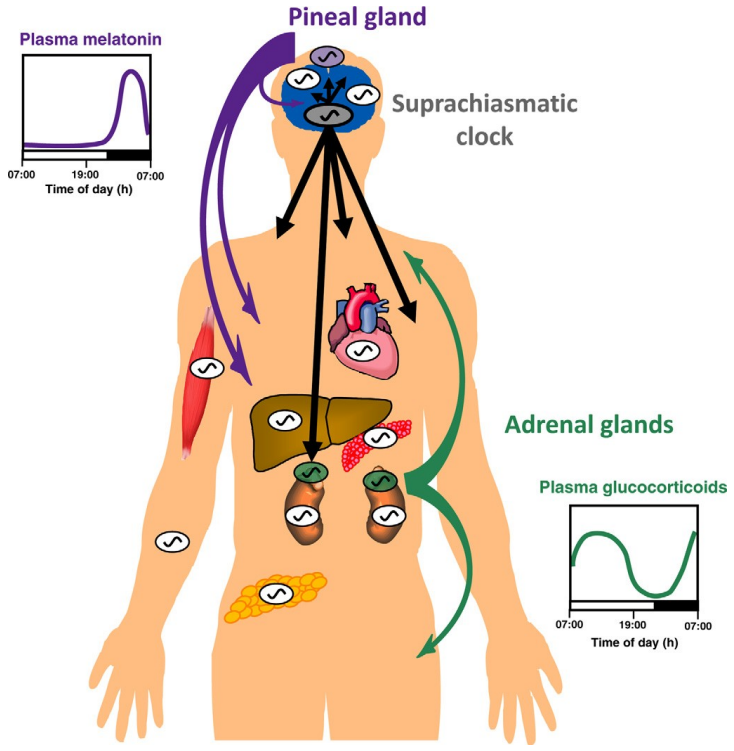


Figure 4: Hormonal entrainment of peripheral oscillators [43] SCN, i.e. the master pacemaker, uses hormonal signalization pathways to entrain peripheral oscillators. Hormones responsible for this signalization are glucocorticoids (GCs; cortisol in humans, corticosterone in rodents) and melatonin. Production of GCs in adrenal gland is stimulated by SCN's activation of so–called hypothalamic–pituitary–adrenal axis and its levels reach peak during onset of animal's active period. Melatonin is secreted from pineal gland exclusively during the dark period of the day. Both of these hormones contribute to synchronization of peripheral oscillators by activation of specific signalling cascades that affect level of transcription of target clock genes.

Melatonin receptors MT1 and MT2 can be found in various types of tissue including brain structures such as SCN, to which it provides a feedback signal, and other peripheral organs. Binding of a ligand activates associated G–proteins and activates signalling cascades that may result in increased transcription of *Rev–Erbα* gene [43].

SCN can project its entrainment signals not only via the hormonal signalling by GCs or melatonin as described above (Fig.4), but also by direct innervation of target organs. One of the first peripheral structures proven to have neural connection to the SCN was an adipose tissue [22], but many other are innervated as well.

2.3 Clock and metabolism

Function of the organism's metabolic system is a very complex process that involves numerous molecules, proteins and enzymes of different nature. Metabolism's overall effectiveness is heavily dependent on their availability at the time, when they are most needed. It is no wonder that metabolic related actions are influenced and synchronized by the ticking of the circadian clock.

2.3.1 Feeding dependent clock entrainment

Despite being entrained by both hormonal and neural signals from the master pacemaker, compartments of the gastrointestinal tract and related organs (such as adipose tissue) have unique way of clock synchronization, which is not mediated through the SCN. Although SCN mediated signals are strong clock synchronizing cues, the one that is able to surpass their effects is food intake.

In 2000, Damiola and colleagues [44] in their experiment subjected mice to forced change in feeding habits, also called "restricted feeding". During this protocol, food is available to the animals only for a restricted time period, in this case during the day, since mice are nocturnal animals and thus feed preferably during the night. This resulted in inversion of phases of rhythms of core clock proteins as well as of rhythms of clock controlled genes in peripheral organs but not in the SCN. The circadian rhythms are also preserved in animals after SCN lesions [52]. The feeding behaviour is thus capable of uncoupling of the peripheral oscillators from the central clock and even of generation of sufficient entraining signal in its absence.

This phenomenon has been observed also on a cellular level, when it has been shown that income of nutrients in form of serum shock is able to restore otherwise dampened molecular clock oscillations in tissue cell cultures [45].

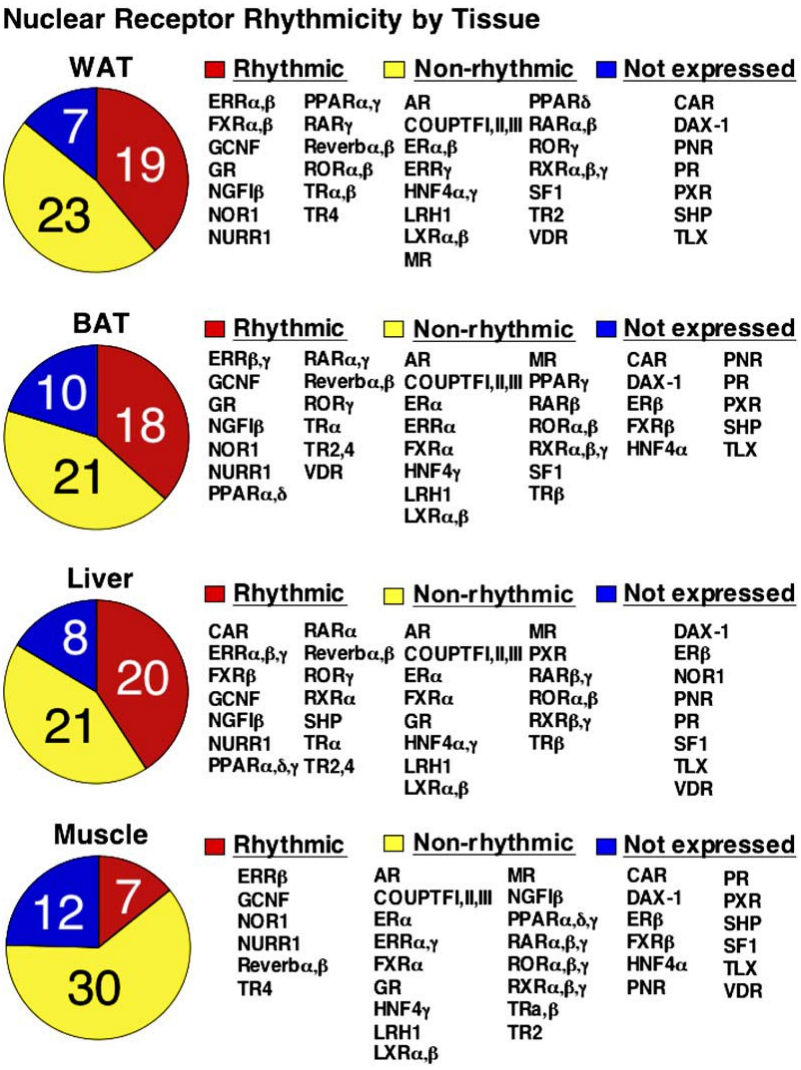


Figure 5: Rhytmicity in expression of nuclear receptors [58] Yang et. al. conducted analysis of diurnal variations in expression of nuclear receptor genes. They analysed transcriptome in four different kinds of peripheral tissue – white adipose tissue (WAT), brown adipose tissue (BAT), liver and skeletal muscle and assessed rhythmicity of nuclear receptor's expression. Included pie charts depict percentual representation of rhythmic and non–rhythmic members of nuclear receptor superfamily.

Food intake causes changes in redox and energetic state of cells, concentration of various hormones and glucose. These changes are perceived mainly through nuclear receptors, which then directly modify gene expression. Nuclear receptors RORs and REV–ERBs are outright components of the circadian transcription–translation feedback loop, but many other interact with the clock mechanism. Almost a fifth of members of the nuclear receptors superfamily is being expressed in diurnally rhythmic fashion (Fig.5) [58]

Reciprocally some of nuclear receptors can affect the run of the molecular clock and thus form a link between their "timekeeping" and metabolism. Peroxisome proliferator–activated receptors (PPARs) belong to the most prominent ones, especially homologues PPARα and PPARγ. These nuclear receptors are sensitive to changes in concentrations of fatty acids and eicosanoids, which are their natural ligands [60]. All homologues of PPARs are expressed in circadian fashion. Their expression is modulated by CLOCK/BMAL1complex which binds to E–box in their promoter. Mature PPAR incorporated in nuclear membrane heterodimerizes

with retinoid X receptor (RXR) and newly formed complex is then able to bind to DNA sequence known as peroxisome proliferator hormone response elements (PPRE), which can be found in promoter areas of various metabolism–linked genes. This response element is also situated in promoter area of *Bmal1* and Rev–Erbα genes and their expression is thus regulated by PPARs [59]. PPARs regulatory effect on *Bmal1* transcription seems to be modulated by interaction with PER2 [61].

Peroxisome proliferator–activated receptor gamma coactivator 1–α (PGC–1α) is a transcriptional coactivator that binds with various nuclear receptors including PPAR–γ and RORα. This co–activator enables PPAR–γ to interact with various transcription factors, and enlarges its field of transcriptional activity. Disruption of rhythmic production of various metabolically important proteins has been observed in PGC–1α deficient mice and their ability to entrain to restricted feeding deteriorated. Through coactivation of RORα, PGC–1α promotes expression of BMAL1 and plays role in proper expression of other core clock proteins as well [62, 63].

PGC–1α coactivator also associates with so–called Sirtuins [63]. Histone deacetylases of Sirtuin protein class (short for silent information regulator), namely SIRT1 and SIRT6, are NAD+ dependent and linked to molecular mechanism of circadian clock. With food intake, cellular ratio of oxidized to reduced forms of NADH (Nicotinamide adenine dinucleotide) and NADPH (Nicotinamide adenine dinucleotide phosphate) coenzymes shifts. Both levels of NAD+ and SIRT1 fluctuate throughout the day. Sirtuins are activated by elevated level of NAD+ coenzyme, which is more present during periods of fasting. They ensure rhythmical deacetylation of histone H3 and this way they negatively regulate transcription of either various metabolism–linked clock controlled genes and regulate for example gluconeogenesis, glucose homeostasis, cholesterol and fatty acids metabolism etc., or even core clock genes *Bmal1* and *Per2* [46, 64].

Change of redox ratio can also influence strength, with which the essential CLOCK/BMAL1 heterodimer binds to the E–box binding site. Increased reduced levels of NADH and NADPH cofactors enhance CLOCK/BMAL1's binding ability, whereas levels of their oxidized forms (i.e. NAD+ and NADP+) reduce it [65].

Another change in the energetic state of the cell, that affects molecular clock is the shift in AMP to ATP ratio. High cellular energetic demand, which is caused by metabolic processes such as glycolysis or due to prolonged fasting, leads to increased consumption of the

macroergic molecule adenosine triphosphate (ATP), leaving energetically much less rich adenosin monophosphate (AMP). AMP–activated protein kinase (AMPK) responds to this change in AMP to ATP ratio and upon activation it regulates cellular metabolism through phosphorylation. AMPK also has a role in regulation of the clock system. Phosphorylation of CRY1 and CRY2 promotes their mediated degradation by Skp–Cullin–F–box (SCF) ubiquitin ligases and phosphorylation of CKε increases its enzymatic activity, promotes this way phosphorylation of PER2, which leads to its degradation. This way AMPK reduces negative feedback on CLOCK/BMAL1 complex [66].

The most elemental nutrient present in food is glucose, whose blood serum levels arises immediately after feeding, before glucose is distributed to cells by insulin mechanism. Glucose itself seem to have effect on clock entrainment, Hirota and his colleagues [67,68] showed that glucose administration down–regulated levels of *Per1*, *Per2* and *Bmal1* mRNA in rat fibroblasts. They concluded, that decrease in *Bmal1* mRNA was mediated by protein TIEG1 (transforming growth factor–beta inducible early gene 1), which binds to GC–box in *Bmal1* promoter. However exact mechanism by which glucose regulates levels of *Per1* and *Per2* levels and affects the molecular clock still remains unclear.

2.3.2 Liver synchronization

Liver and other peripheral tissues that are parts of metabolic system including pancreas or adipose tissue can process inputs from all above mentioned mechanisms and effectively entrain its clock gene's oscillations to feeding and regulate metabolic processes, such as glucose or fatty acids homeostasis accordingly.

Liver is an organ that produces majority of feeding–related enzymes. It has been shown that over 3000 genes in liver transcriptome is expressed in circadian manner, which is more than in other examined organs including aorta, adrenal gland, brain, adipose tissue, kidney, lung or skeletal muscle [56]. That includes genes whose proteins are not only responsible for glucose homeostasis, but also for lipid metabolism, bile acids production or regulation of toxins elimination [44].

Despite being connected with SCN through neuronal pathway, liver's synchronization of its molecular clock is more affected by entraining signals set by feeding behaviour or glucocorticoid release [52].

Neural input, concretely administration of adrenaline, elevates levels of PER1 [55]. As mentioned before, liver is place where glucose is stored in form of glycogen. Hepatic

circadian clock is then responsible for regulation of expression of glycogen synthase, an enzyme that is one of key participants of glycogenesis, and modulates activity of glycogen phosphorylase, a rate–limiting enzyme in glycolysis [57].

2.3.3 Clock regulation of daily glucose rhythm and lipid homeostasis

Blood glucose concentration varies throughout the day and exhibits a daily pattern, as well as the liver glycogen [21,48,51]. This is caused by rhythmic secretion of hormones responsible for their transport and metabolism, which is orchestrated by circadian clock (Fig.6). We are talking in particular about insulin and glucagon, although exact influence of the latter on daily oscillating glucose concentration in blood plasma is still being speculated [48].

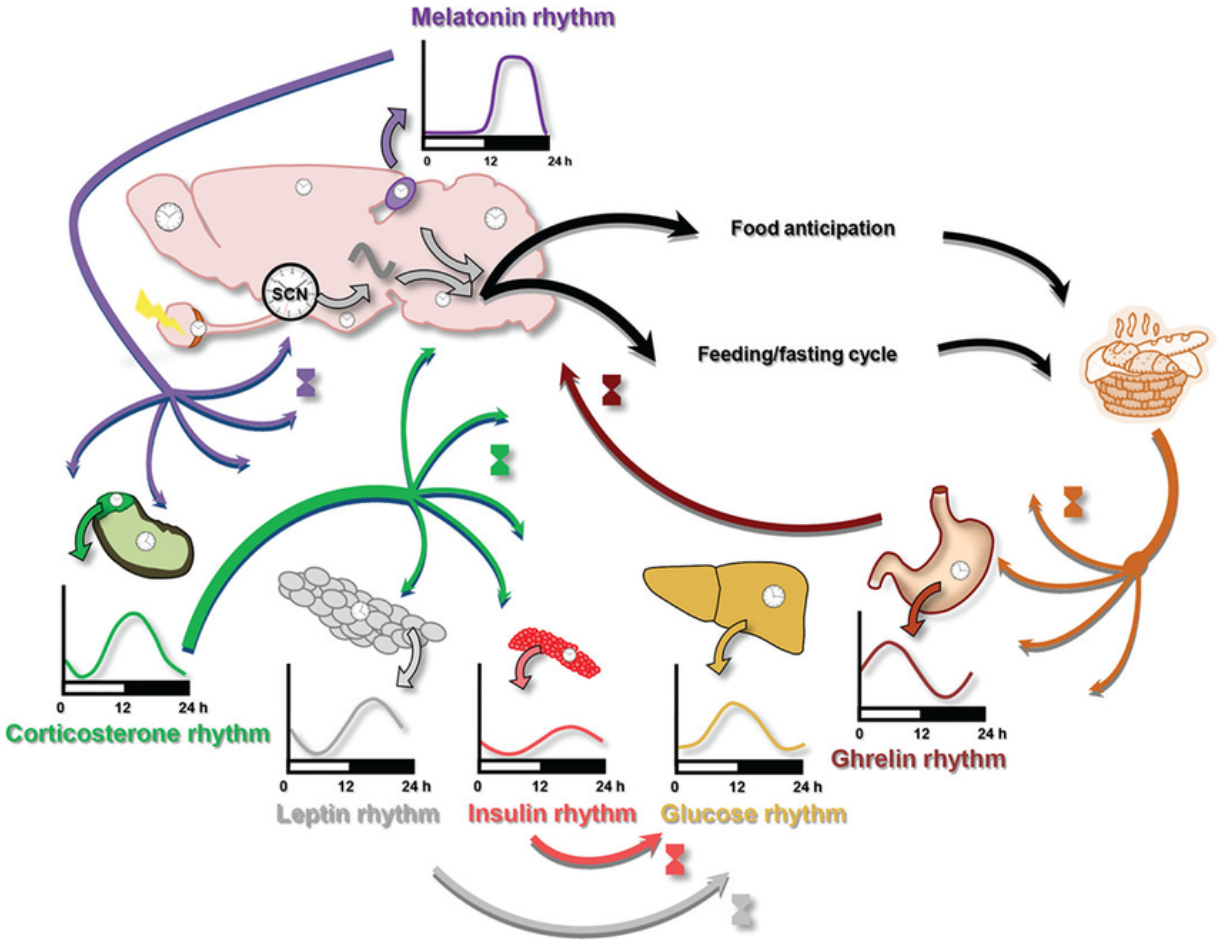


Figure 6: Hormonal synchronization within circadian clock system [84] Once synchronized to photic cues, the SCN regulates circadian rhythm in expression of melatonin and GCs. GCs play essential role in regulation of peripheral oscillators. Entrained peripheral clocks regulate rhythmic expression of metabolic hormones such as leptin, insulin or ghrelin. Together they regulate levels of glucose and fatty acids in blood plasma. Metabolic hormones feedback back to hypothalamus and participate on regulation of feeding related behaviour. Feeding itself then entrains the peripheral clocks and affects hormonal expression.

Both of these peptide hormones are secreted by pancreas. Glucagon and insulin are secreted by specialized endocrine cells named alpha cells and beta cells, respectively. Both are responsible for regulation of plasmatic glucose levels, although they act in an opposing manner. Insulin binds to insulin receptor and by activation of signalling cascade it regulates amount of insulin–mediated glucose transporters in the plasmatic membrane and promotes glucose cellular uptake. Insulin–regulated glucose transporters GLUT4 are for example abundantly present in the liver, where imported glucose is stored in form of glycogen [49]. Glucagonbinds to its receptor and by activation of PKA and CREB it enhances expression of enzymes that promote glycolysis and gluconeogenesis. This leads to elevated levels of plasma glucose [50].

The way in which daily fluctuations in concentrations of these hormones affect the overall metabolic process is possibly best observable in different effectiveness of glucose uptake during glucose or insulin tolerance tests depending on time of the light/dark cycle during experiments [48].

The daily secretion of glucagon and insulin is promoted by neuronal signals transmitted from the SCN via autonomic nervous system. It is worth noting that molecular clock in both alpha and beta cells, despite being organized close together in structures called the Langerhans islets, seem to react differently to entraining signals of sympathetic neurotransmitter adrenalin, probably due to different composition of membrane receptors [51]. In case of beta cells Saini, C. and colleagues [53] demonstrated that clock disruption caused reduced expression of proteins responsible for insulin secretion and formation of insulin granules which resulted in decrease of secreted insulin. The process of clock–dependent glucagon release regulation in alpha cells is based on a similar principle – by regulation of exocytotic process [54].

Lipid plasma concentrations, namely of triacylglycerides (TAG) and diacylglycerides (DAG) carrying polyunsaturated fatty acids (PUFA) also show strong circadian rhythm, peaking near onset of active period [69].

Circulating lipid levels depend on balance of lipid release and uptake. Ingested lipids are being emulsified by bile salts and processed by lipases producing free fatty acids and monoacylglyceroles. These are then transported inside the intestine enterocytes. The monoacylglycerols and free fatty acids are esterified to the forms of TAGs and DAGs by

enzymatic activity of monoacylglycerol acyltransferases and diacylglycerol acyltransferases respectively. TAGs are then packed into structures called chylomicrons [71].

Rhythm in lipid release and storage is primarily caused by oscillating concentrations of apolipoprotein B (ApoB) microsomal triglyceride transfer protein (MTTP), which is responsible for lipoprotein assembly and formation of chylomicrons. MTTP production is regulated by clock controlled gene, which is coding small heterodimer partner (SHP) protein. CLOCK/BMAL1 heterodimer binds to *Shp*'s E–box and SHP protein binds to the *Mttp* promoter with hepatocyte nuclear factor 4 alpha (HNF4α) and liver receptor homolog–1 (LRH–1) nuclear receptors and together they suppress its expression. MTTP is responsible for transportation of TAGs through plasmatic membrane of liver cells. Its downregulation is linked with diminished blood plasma TAG levels [70].

Another protein that emerged to be both responsible for lipid metabolism and intertwined with molecular clock is sterol regulatory element–binding protein (SREBP). This transcription factor is essential player in activation of various genes responsible for cholesterol (SREBP–2) and fatty acid metabolism and lipogenesis (SREBP–1c), such as fatty acid synthase (FAS). Its unique amino acid sequence enables it recognise not only sterol response element 1 (SRE–1) element in promoter areas, but also E–box, which is key regulatory compartment in clock mechanism. Through these response elements SREBP regulates expression of numerous genes, of which almost two thirds are being expressed in circadian fashion [76]. Transcriptome analysis by Le Martelot et. al [77] showed, that *Rev–Erbα* knock out in mice impaired otherwise circadian expression and nuclear accumulation of SREBP.

2.4 Clock in adipose tissue

The main storage depot of TAGs is adipose tissue. Chylomicrones are absorbed by adipocytes where lipids are stored in forms of lipid droplets. TAGs are there further processed by lipases and eventually released back into bloodstream.

2.4.1 Types of adipose tissue

Adipose tissue is according to its location being divided to visceral fat placed in abdominal cavity (including epididymal fat in males or parametrial fat in females, retroperitoneal fat, perirenal fat, mesenteric fat and omental fat), or subcutaneous fat situated in hypodermis (most experimentally significant is its inguinal depot) (Fig.7) [73].

There are two distinguished phenotypes of adipose tissue – white adipose tissue (WAT) and brown adipose tissue (BAT). Recently, transitional form of adipose tissue converting between

white and brown forms, also called "beige" adipose tissue, has become a subject of interest. BAT is characterized by its nonshivering thermogenic activity. BAT compared to WAT contains abundant number of mitochondria with characteristic uncoupling protein 1 (UCP1) that uncouples respiratory chain and enables flow of protons in the mitochondrial matrix and generates heat. Growth of BAT is triggered by exposition to cold environment which causes enhanced adrenergic stimulation. In humans, BAT is present primarily during early ontogenesis whereas smaller mammals such as rodents posses BAT depots throughout their whole life. BAT is characteristically stored in depot located in scapulae and thoracic areas [74].

WAT can be transformed into BAT by adaptive process called "browning". Both beige and brown adipocytes express UCP among respective specific proteins. This change in adipocyte phenotype is orchestrated by two key players – CCAAT/enhancer–binding protein α (C/EBPα) and PPARγ, which promotes expression of *Ucp1* gene via PGC–1α mediated activation [75].

WAT is the main storage unit of TAG. Their cellular volume varies depending on the body

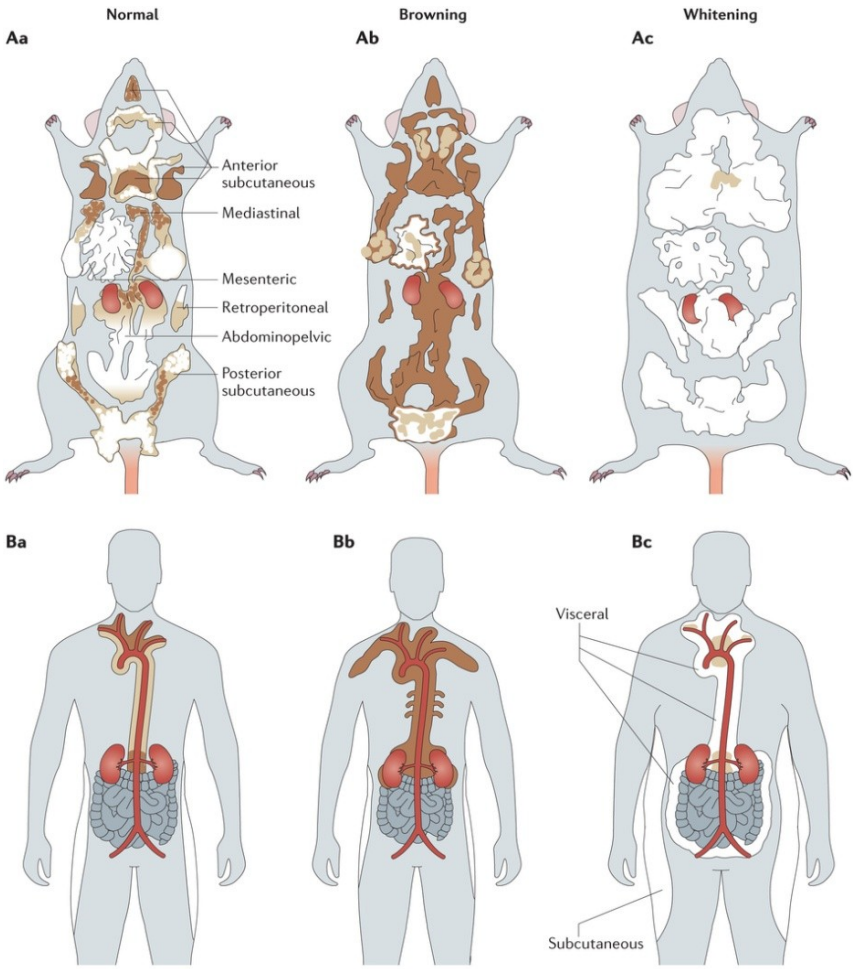


Figure 7: Distribution of adipose tissue throughout the mouse and human body [72] Schematic image of adipose tissue distribution in adult individuals of normal phenotype (Aa, Ba), enhanced amount of brown adipose tissue in mouse kept in 6°C for 10 days (Ab) and in human after adrenergic stimulation (Bb) and enhanced amount of white adipose tissue in leptin deficient mouse (Ac) or obese individual (Bc)

weight, with increasing body mass the adipocyte volume increases as well. However their number is set during adolescence and remains static even after radical weight loss [78].

2.4.2 Regulation of lipid metabolism

As mentioned before, adipose tissue of both types is richly innervated, including neural inputs from SCN, however it appears to be innervated only by sympathetic nervous system. This type of innervation is essential in season-dependent lipid mobilization, it reduces fasting induced lipolysis and plays a role in transformation of WAT into heat–producing BAT [72, 79]. The metabolic pathway, which is activated through release of neurotransmitters adrenalin and noradrenalin, is heavily dependent on composition of adrenergic receptors on plasmatic membrane. Activation of β3–adrenergic receptors onsets signalling cascade including adenylyl cyclase and PKA, which activate various lipases and promote lipolysis, whereas higher α_2/β–adrenergic receptor ratio on plasmatic membrane contributes to lipid enhanced storage [80].

Endocrine signalization has great impact on lipid metabolism in adipose tissue. Impairments of daily GC fluctuations, which can be observed during pathological conditions affecting feedback loop of HPA axis such as Cushing's syndrome or hypercortisolemia, have indisputable effect on lipid storage. GC overexpression, which is observable during both of these conditions, increases activity of lipoprotein lipases and promotes fatty acids uptake [83].

GCs also enhance production of leptin. Leptin is one of several hormones secreted exclusively by adipocytes, also referred to as adipokines. Leptin plasma levels oscillate throughout the day and participates in maintaining the right functioning of metabolism and provides so–called post–feeding time cue. Leptin is largely secreted after the meal time. Its deficiency however doesn't have effect on oscillations in plasma fatty acids level, but seems to have attenuating effect on food–anticipatory activity (i.e. enhanced animals' behavioural activity prior feeding times during restricted feeding protocols) [85].

Leptin's main function is signalisation to a hypothalamic structure called the Arcuate nucleus. Here leptin inhibits AgRP (Agouti related protein) expressing population of neurons and activates proopiomelanocortin (POMC) expressing neurons through leptin–sensitive receptors. This results in reduction of food seeking behaviour and food intake. Orexigenic hormones such as insulin or ghrelin have an exact opposite effect on both AgRP and POMC neuron populations in Arcuate nucleus and promotes food related behaviour and hyperphagy [86].

Leptin thus contributes to regulation of food income on behavioural level and this way it contributes to feeding–mediated peripheral clock synchronization. It is still hypothesized, whether oscillations of leptin blood levels play role in modulation of core circadian clock in the SCN [87].

Neural, endocrine and behavioural signals set peripheral clock inside adipocytes and modulate lipid metabolism. It has been shown, that disruption of clock mechanism in adipose tissue has different effect on metabolism depending on the clock gene that has been affected. *Bmal1* knock–out causes reduced ability of lipid storage, which results in increased levels of circulating TAGs [88]. Similar symptoms are observable in mice with Clock δ^{19} mutation, that causes obesity and hypertriglyceridemia [81].

On the other hand *Per2*$^{-/-}$ mutation in mice results in decrease of both adipose tissue mass and plasma TAG, which is caused by increased PPARγ transcriptional activity, which would be otherwise down–regulated by functional PER2 [82]. RORα knock–out has analogical effect on size of adipose tissue pads and levels of circulating TAGs. RORα promotes expression of *Srebp–1c,* but its deficiency results in decrease of its mRNA levels and consequently to impaired downstream SREBP–1c regulation of metabolic genes [89].

2.5 Obesity, type 2 Diabetes Mellitus and its effect on circadian clock

From all the previously listed ways of interaction between metabolism and circadian clock system it emerges how closely they are intertwined and that disturbance in one leads to disturbance in the other – chronic desynchronization of peripheral oscillators leads to metabolic disorders, and *vice versa*.

In modern society obesity and related insulin resistance, which gradually results in type 2 diabetes mellitus, becomes more and more serious problem. Nowadays there are numerous studies which confirm, that clock disturbance and desynchronization is one of the key non–genetic factors that stand by onset of metabolic–related dysfunctions.

Poor clock entrainment is usually a consequence of improper lifestyle organisation such as light exposure during night (blue light especially has been proven to significantly reduce night time melatonin levels [90]), irregular timing of food intake and sleep schedules or travelling between different time (which is also main cause of clock dysrhythmia called jet lag).

Three main types of diabetes are being recognized – type 1, type 2 and gestational diabetes. Type 1 diabetes is a genetically caused autoimmune disease during which pancreatic β–cells

are being targeted by body's immune system and their destruction leads to insufficient insulin production [91]. Gestational diabetes is a health condition that affects women during pregnancy. Afflicted women, which are otherwise not diagnosed with other type of diabetes, show high levels of blood glucose and insulin insufficiency. This medical condition may furthermore cause other related complications [92].

According to International Diabetes Federation, at least 90% of all causes of diabetes are those of the last type, i.e. type 2 diabetes [93]. Type 2 diabetes usually occurs as a result of obesity in combination with genetic predispositions and may develop at any age. Impaired circadian factors play major role in onset of this type of diabetes.

Circadian clock disruption is serious problem affecting groups of people, who are frequently exposed to night shift working schedules, such as for example nurses. Published data have shown, that shift workers are more prone to development of several health problems including cancer, cardiovascular diseases, obesity, metabolic syndrome and diabetes [97].

Obesity and diabetes in human patients is linked to impaired sleeping patterns and insomnia [94]. Similar behavioural change is also observable on animal models of obesity. Mice, which are naturally nocturnal animals, kept on diet with high content of lipid (i.e. high fat diet, HFD) tend to be more active during the light phase and consume more food during this phase than lean controls [95].

This atypical food consummation contributes to disorganisation of metabolic processes and disruption of circadian clock in peripheral organs. Experiments have shown that, although being kept on high fat diet, when food was available only during the dark phase of the day, mice gained less weight than those fed during light phase or *ad libitum*. Restricted feeding also restored circadian rhythms of clock genes in liver, which would otherwise be disrupted by HFD [96].

Impaired circadian rhythmicity in expression of various clock controlled genes related to metabolism has been observed in animal models of obesity as well. Circadian profiles of mRNA levels from mice kept on HFD show attenuated rhythms of PPARγ or SREBP–1c in adipose tissue or acetyl–CoA carboxylase (ACC), FAS or fatty–acid binding protein (FABP) in liver. Plasma levels of glucose, insulin, leptin and corticosterone dampen too [95].

Ongoing desynchrony between circadian core clock in SCN and clock in the peripheries creates somewhat a *circulus vitiosus*. Due to impaired feeding behaviour peripheral organs

lack important synchronizing cues set by rhythmic secretion of hormones, lipid and glucose homeostasis or gene expression, which leads to increasing clock desynchrony accompanied by worsened diabetic symptoms, if not treated properly.

2.6 Immune system

Type 2 diabetes occurs as an escalation of an insulin resistance and pancreatic β–cells dysfunction, which causes impaired insulin secretion. One of crucial factors responsible for development of these medical conditions is ongoing chronic inflammation, one of symptoms of severe obesity.

2.6.1 Characterization of immune system

Immune system is compartment of the organism, which is responsible for body's defence against alien pathogens. It is composed of two main anatomical types of structures – primary and secondary lymphatic organs. Primary lymphatic organs, i.e. bone marrow and thymus, are where immature lymphatic cells develop from proliferating pluripotent stem cells. Secondary lymphatic organs, i.e. spleen, lymph nodes and mucosa–associated lymphoid tissue found for example in appendix, are places, where lymphocytes mature and where initiation phases of immune reactions take place.

Two main developmental leukocyte lines, that differentiate from stem cell, are distinguished – myeloid line and lymphoid line, each giving origin to a different immune cell types.

Precursor of the lymphoid line further differentiates either into B–cells, T–cells or so–called "natural killer" cells. These cell types mediate antigen specific immune reaction. This type of reaction is based on interaction between T and B–cells. T–cells recognize peptide fragments of produced proteins presented by cells or specialized APC (antigen presenting cells) through

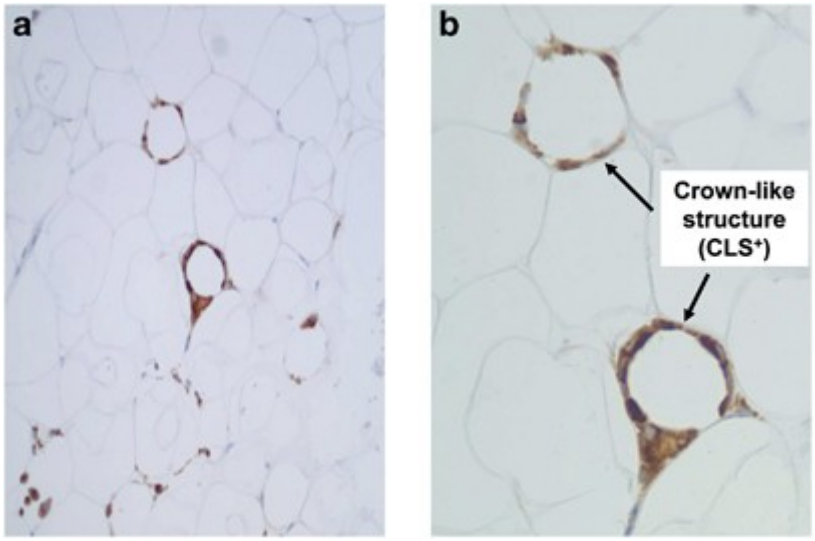


Figure 8: Crown–like structures [99] Macrophages (brown colour) acumulate around adipocytes and form so–called crown–like structures, which can be observable on imunohistological sections (stained by CD68 antibodies) displayed in (a) 20x and (b) 40x magnification

transmembrane MHC (major histocompatibility complex) glycoproteins of class 1 and class 2 respectively. When T–cells recognise presented fragment as pathogen, they trigger either cytotoxic reaction, or they interact with B–cells and stimulate production of antibodies.

Myeloid precursor gives origin to monocytes which differentiate into macrophages, dendritic cells and to three types of granulocytes – neutrophils, eosinophils and basophils. These cells are responsible for non–specific immune reaction.

Both granulocytes and macrophages are capable of phagocytosis, but whereas granulocytes can be found circulating in bloodstream and migrate to afflicted tissue, macrophages, tissue form of monocytes, reside inside tissue and are mainly responsible for removal of apoptotic cells or alien organisms such as bacteria [98].

2.6.2 Macrophages and their polarizations

As mentioned above, macrophages reside inside the tissue of metabolic organs, where they act as first defensive force against pathogens. On histological sections of adipose tissue they can be observable as cells accumulated around large round adipocytes. These accumulations are due to their resemblance to a wreath or a crown called crown–like structures (Fig.8). Cells accumulating adipocytes, which are also collectively being called stromal vascular fraction (SVF), compose beside macrophages mainly of mesenchymal stem cells, pre–adipocytes and vascular endothelial cells [81].

Newly formed unpolarized macrophages (M0) can exhibit a different functional phenotype depending on the environmental signals, namely cytokines and growth factors, to which they have been exposed. It has been described, that macrophages polarize into two main distinguishable states (with range of intermediate states) – classically activated macrophages (M1) and alternatively activated macrophages (M2). The main difference between these two states is whether or not they mediate inflammatory response [100].

M1–polarized macrophages initiate onset of inflammatory as a response to pro–inflammatory signals such as lipopolysaccharides (LPS), endotoxine characteristic for bacterial infection, IFNγ (interferon) or TNFα (tumor necrosis factor), cytokines secreted as a part of Th1/Th17 mediated immune reaction. They are characterized by high production of inflammation–promoting cytokines such as interleukine (IL)–6, IL–12, IL–23, IL–1α and β or TNFα [100, 101, 102]. They also express C–C chemokine receptor type 2 (CCR2, in some literature also often alternatively named as MCP–1–R, monocyte chemoattractant protein–1

receptor), which enables macrophages to infiltrate afflicted tissue and migrate in place of inflammation [102], which is marked by high production of CCR2's ligand CCL2 (i.e. MCP–1). CCL2 is being produced by various types of cells, including macrophages themselves, as a result of exposure to oxidative stress, cytokines, or growth factors [103].

Increased oxidative stress is one of consequences of increased M1–polarization. The phenotypic switch, that is the change in macrophage polarization, is accompanied by change in cell's energy metabolism and anaerobic glycolysis becomes its main source of ATP. Shift in favour of anaerobic glycolysis causes increased production of ROS (reactive oxygen species). It also causes increased accumulation of succinate, which induces transcription of HIF–1α (hypoxia induced factor). HIF–1α transcription factor promotes expression of inducible nitric oxide synthase (iNOS), leading to production of cytotoxic nitric oxide (NO) from L–arginine amino acid [104].

Alternatively activated M2 macrophages on the other hand act in an anti–inflammatory manner. Their role is to phagocyte cellular debris, they promote healing of wounded tissue and suppress negative effects of inflammation [100]. Phenotypic switch leading to this polarization is promoted by their exposition to anti–inflammatory cytokines, mainly IL–4, IL–10 or IL–13. Depending on the cytokines that activate this switch, we can further divide M2 macrophages into 3 subtypes with different function. M2a (IL–4, IL–13) and M2b macrophages (IL–1, LPS, immune complexes) are involved in Th2–mediated immune reaction and M2c (IL–10) are involved in tissue repair and remodelling. However their functional division isn't strict. M2a macrophages for example produce fibronectin–1 (FN–1), coagulation factor XIII or insulin like growth factor 1 (IGF–1), which take part in tissue repair [101, 105].

M2 macrophages are generally characterized by enhanced production of IL–10 and lowered secretion of IL–1, IL–12 and IL–6 pro–inflammatory cytokines [101]. Their expression of arginase 1 (ARG1) is increased. ARG1 enzyme competes with iNOS for L–arginine as substrate, which leads to a decrease in NO production. Exposition to anti–inflammatory cytokines also promotes oxidative phosphorylation and thus lowered production of ROS [104]. M1 and M2 polarizations also appear to differ in their migratory abilities, since macrophages, that don't produce CCR2 exhibit anti–inflammatory properties [102].

Anti–inflammatory effect of M2 polarized macrophages seem to be dependent on PPARγ and PGC–1β, whose expression increases in alternatively activated macrophages. It has been

shown that increased PPARγ levels correlate with increased levels of anti–inflammatory markers [106] and attenuate production of pro–inflammatory cytokines [107].

2.6.3 Chronic inflammation in obesity

Chronic inflammation is one of typical symptoms accompanying obesity and increased adiposity. Low–grade inflammation causes altered plasma levels of various factors such as TNF–α, IL–6, C–reactive protein (CRP) or other markers of inflammation as well as adipokines leptin and adiponectin [108, 110].

Increased levels of circulating pro–inflammatory cytokines promote M1 macrophage polarization. Phenotypic switch in favour of this polarization is also accompanied by their increased expression of MCP–1/CCL2, chemoattractant, which promotes macrophage migration and accumulation in place of inflammation. Their activation is accompanied by increased activity of iNOS and production of NO [109].

Ongoing chronic inflammation of adipose tissue results in adipocyte lipolysis, necrosis and insulin resistance in cells of various peripheral tissue. Systemic insulin resistance is characterized as cells inability to react accordingly to hormone insulin. This leads to impaired glucose metabolism. Insulin resistance is one of symptoms of so–called metabolic syndrome, which is apart insulin resistance characterized also by glucose intolerance, obesity, hypertriglyceridemia, low HDL cholesterol, hypertension, and atherosclerosis [110].

2.6.4 Circadian modulation of immune system

Immune system is not an exception and just as digestive system its activity is modulated by rhythmic oscillations of circadian clock. Cells of the immune system, derived from both myeloid and lymphoid line, posses the functional molecular clock.

Macrophage's molecular clock governs various functions including their phagocytosis activity which oscillates throughout the day. This is caused by synchronized rhythmic expression of pro–inflammatory markers, such as IL–1β, IL–6, TNFα, CCR2 and CCL2 [111]. The production of the latter two is crucial for macrophage recruitment. *In vitro* studies have shown that REV–ERBα and PER1 upregulation represses *Ccl2* expression, whereas BMAL1 and RORα positive transcription factors enhance it [112, 114]. PER1 also downregulates expression of CCR2 through interaction with PPARγ [114].

Cytokine expression is also regulated by circulating levels of GCs, whose rhythmic production is set by core clock in the SCN and that act as an important time–setting cue for

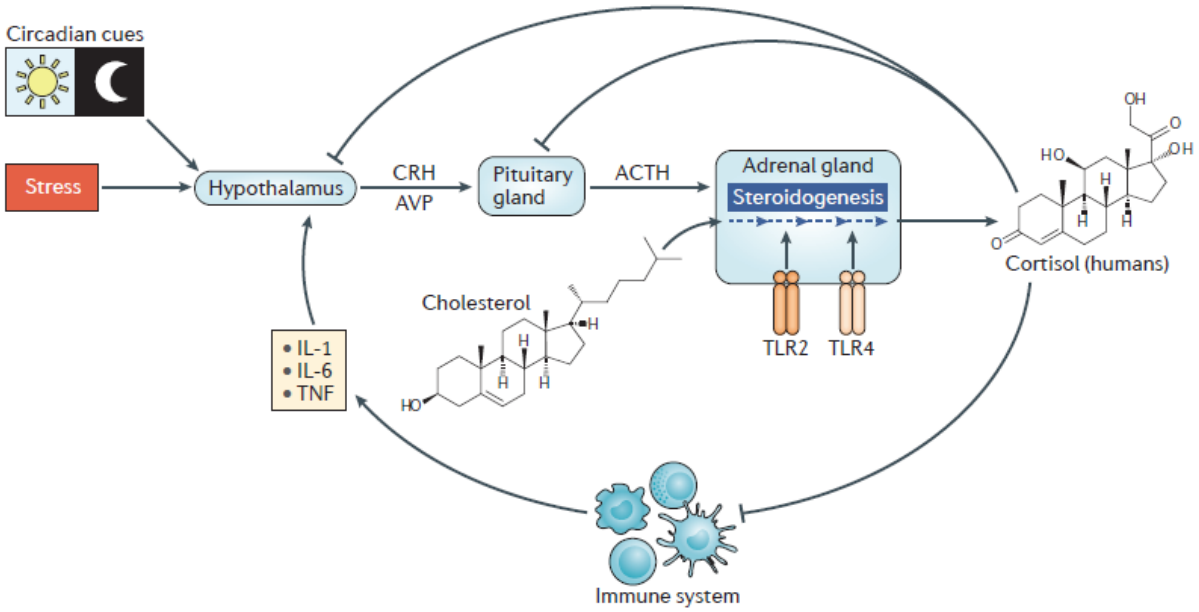


Figure 9: Regulation of immune system by HPA axis [47] Circadian rhythms in SCN and exposition to external stressors activates HPA axis by stimulation of CRH and ACTH production in hypothalamus and pituitary gland respectively. This leads to promotion of GC expression in adrenal gland. Elevated levels of GCs create a negative feedback to hypothalamus, where they suppress their own expression. They also play important role in suppression of immune system, which otherwise stimulates HPA axis through IL–1, IL–6 and TNF cytokines.

synchronization of peripheral oscillators (as described earlier in chapter 2.2.3 Synchronization of peripheral oscillators).

HPA axis is a main mediator of stress reaction. Its main role is to temporarily suppress non–vital high–energy consuming processes, so that organism is can deal with external stressors. These suppressed processes include immune system, which is why are GCs often used in anti–inflammation therapeutics [113]. Activation of HPA axis is regulated by feedback loops. GCs provide negative feedback to hypothalamus and pituitary gland and through downregulation of CRH and ACTH they suppress their own expression. Immune system on the other hand positively stimulates hypothalamus by IL–1, TNF and IL–6 cytokines. Attenuation of their secretion diminishes stimulation of hypothalamus, which also has an effect on GC production (Fig.9) [47].

Cortisol and corticosterone in humans and rodents respectively act upon the immune system in various ways. Studies have shown that GCs reduce blood flow to inflamed tissue, inhibit expression of pro–inflammatory cytokines and chemokines, such as CCL2, and promote secretion of anti–inflammatory cytokines, such as IL–10, they promote M2c macrophage

polarization, and thus contribute to tissue repair, inhibit maturation of dendritic cells, suppress immune reaction mediated by Th1 and Th17 cells, but on the other hand they also seem to promote Th2–mediated immune response, and many others. The way of their modulation of immune response also seem to depend on whether their administration is before or after exposition to pathogen [see review 47].

2.7 Alternative diets and Omega 3 fatty acids

In recent studies it has been suggested, that lipid composition in food has regulatory effect on lipid metabolism, inflammation and oscillations of circadian clock.

The main molecules of interest are polyunsaturated fatty acids (PUFA), concretely eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, which belong to the omega–3 fatty acids group, which can often be found in fish oil, which are generally believed to have beneficial effects.

Studies, that confirm that increased PUFA concentration in HFD has positive effect on hepatic inflammation and steatosis, suggest potential utilization of omega–3 fatty acids as dietary therapeutic tool against obesity [116]. However it has also been shown, that EPA's and DHA's effect on metabolism and insulin resistance is dependent on the overall lipid background of the diet [118].

Kuda and colleagues [117] demonstrated, that PUFA–enriched diet had anti–inflammatory effect on WAT in both mice and diabetic patients, and that DHA promoted macrophage anti–inflammatory polarization and reduced NO and ROS production.

Effect of alternations in dietary lipid composition on circadian clock hasn't yet been thoroughly investigated. It has been demonstrated, that treatment by pro–inflammatory saturated acids as palmitate in comparison to DHA caused cell–specific phase shifts in clock gene expression [115] or that it alleviates effects of palmitate on Bmal1 oscillations in immortalized hypothalamic neurons [119]. Exact nature of interaction between PUFAs and circadian clock however still remains to be explored.

3. Dissertation objectives

The general goal of the study is to reveal a mutual interaction between the circadian clocks in macrophages of different polarization and in the WAT of chow or high fat diet fed animals. The specific aims are as follows:

I. Using a real–time bioluminiscence recording of tissue samples from transgenic *Per2*^{Luc} mice
   - to characterize circadian clock in WAT
   - to characterize circadian clock in macrophages and describe differences in their M0, M1 and M2 polarization states

II. To ascertain diet–induced changes in oscillations of clock gene expression in WAT and polarized macrophages in samples from
   - *Per2*^{Luc} mice fed high fat diet (cHF)
   - *Per2*^{Luc} mice fed high fat diet enriched by omega–3 fatty acids (cHF–F)
   - compare findings to oscillations observed in control *Per2*^{Luc} mice fed regular chow diet (CTRL)

III. To describe the effect of interaction between WAT and macrophages of different polarizations on circadian oscillations of clock genes in WAT of different dietary backgrounds in "co–culture" experiments.

4. Material and methods

4.1 Experimental animals and procedures

In experiments were used male *Per2*^{Luc} mice (strain B6.129S6–*Per2*^{tm1Jt}/J, JAX, USA) and wild type (WT) male C57BL/6J mice. All were kept in environment with light regime, which consisted of 12 hours of light and 12 hours of darkness (LD 12:12).

At the age of four to six months, *Per2*^{Luc} mice were kept on a special diet for 9 following weeks. All mice were fed *ad libitum* and they were continuously weighted during the period of the experiment.

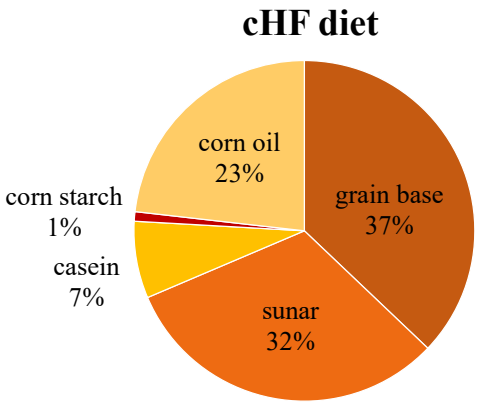
At the beginning of high fat diet (HFD) experiments *Per2*^{Luc} mice were randomly divided into groups, which were later fed different diets.

The Animal Care and Use Committee of the Institute of Physiology, in agreement with the Animal Protection Law of the Czech Republic as well as European Community Council directives 86/609/EEC, approved all experiments. All efforts were made to alleviate the suffering of the animals.

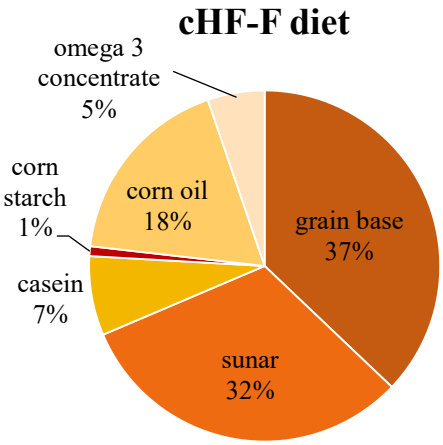
4.2 Diets

Transgenic *mPer2*^{Luc} mice from cHF group were fed with food containing high portion of fat and added casein (i.e. cHF diet). *mPer2*^{Luc} mice from cHF–F group were fed cHF diet for the first two weeks, which for remaining 6 weeks of study was switched to diet with high portion of fat, added casein and enriched by omega 3 amino acid concentrate containing concretely eicosapentaenoic acid and docosahexaenoic acid (i.e. cHF–F diet). Control mice were kept on regular maintenance "chow" diet (for composition of all three diets, see Fig.10).

cHF diet		
Additive	**Amount [g/kg]**	**Details, origin**
grain base	371	ssniff–Spezialdiäten GmbH
sunar	315	Complex 3, HERO CZECH s.r.o.
casein	73	ssniff–Spezialdiäten GmbH
cornstarch	9	Dr. Oetker, spol. s r.o.
corn oil	232	Kačaby
tocopherol	0.194	Sigma–Aldrich Co.



cHF–F diet		
Additive	**Amount [g/kg]**	**Deatils, origin**
grain base	371	ssniff–Spezialdiäten GmbH
sunar	315	Complex 3, HERO CZECH s.r.o.
casein	73	ssniff–Spezialdiäten GmbH
cornstarch	9	Dr. Oetker, spol. s r.o.
corn oil	179.5	Kačaby
omega 3 concentrate	52.5	Epax 1050 TG, Epax



Chow diet Altromin Spezialfutter GmbH & Co. KG	
Additive	**Amount [g/kg]**
Protein	192.0
Fat	40.8
Fibre	60.5
Ash	69.4
Moisture	112.9
Disacharids	49.5
Polysacharids	358.9

Figure 10: Composition of cHF, cHF–F and chow diets. Used additives for concrete diets, their amount per kg and place of their origin are shown in provided tables. Percentual representation of additives in cHF and cHF–F diets is depicted in attached pie–charts. cHF diet consisted of grain base, sunar – dried milk substance, casein (i.e. milk protein), cornstarch, corn oil and tocophelrol. cHF–F diet had similar composition, but portion of corn–oil was substituted by omega–3 concentrate containing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Control mice were fed regular chow diet.

4.3 Weight monitoring

Mice were weighted three times a week for 6 weeks starting from the beginning of the experiment. For next 3 weeks mice weren't disturbed or weighted in order to monitor their spontaneous locomotor activity (see 4.4 Locomotor activity monitoring) during this time. They were weighted again at the end of the 9th week of the experiment, i.e. before sampling.

Weight gain was expressed as percentual increase in body weight related to initial body weight. Weight gain curves exhibit mean values which were calculated for the animal group at each measure point.

4.4 Locomotor activity monitoring

mPer2^{Luc} mice from each dietary group (n_{CTRL}=10, n_{cHF}=5, $n_{cHF\text{-}F}$=6) were monitored for one week in LD 12:12 regime which was then switched to constant darkness (DD regime) for two weeks.

Mice were kept individually in cages, which were equipped with infrared movement detectors. Monitoring system CAMS (Circadian Activity Monitoring System, by Dr. H. M. Cooper, Inserm, France) was used for measurement of circadian activity; activity of tested subjects was measured each minute. Actograms were evaluated in MATLAB using ClockLab analysis, to determine freeruning period (subtracted from Chi–squared periodogram, which was made for each animal) and light/dark activity ratio (calculated ratio between measured counts during light phase and during dark phase) in tested subjects.

4.5 WAT explants isolation and *Per2*^{Luc} luminescence monitoring

Samples of epididymal adipose tissue were used in following experiments. After excision, the adipose tissue was temporarily placed in preheated PBS (Phosphate buffer saline, Sigma–Aldrich) (37°C). Tissue was then divided to smaller explants (~ 20 mg, 3–5 samples were taken from each animal) and then placed in 35 mm Petri dishes with 1 ml of preheated (37°C) Air–buffered recording medium (for composition see Fig.11). The dishes were then sealed with vacuum grease and glass coverslips. Covered dishes were placed inside LumiCycle apparatus (Actimetrics) for bioluminescence recording.

Air–buffered medium		
Additive	**Amount/l**	**Origin**
Dry DMEM	8.3 g	Sigma–Aldrich
$NaHCO_3$ 7,5%	4.7 ml	Sigma–Aldrich
Hepes 1M	10 ml	Sigma–Aldrich
Glucose	4.5 g	Sigma–Aldrich
Penicillin/streptomycin	10 ml	Sigma–Aldrich
Gentamycin	1.1 ml	Sigma–Aldrich
FBS	100 ml	Sigma–Aldrich
GlutaMax	10 ml	Gibco
Luciferin–EF	1 ml	Promega
dH_2O	860 ml	

Figure 11: Composition of Air–buffered recording medium used for luminiscence monitoring.

BMDM medium		
Additive	**ml/l**	**Origin**
L929 supernatant	100	Rhombaldová, M., Adipose tissue biology department, Institute of physiology CAS
FBS	90	Sigma–Aldrich
RPMI 1640	810	Sigma–Aldrich
penicillin/streptomycin	1	Sigma–Aldrich

Figure 12: Composition of BMDM medium, which selectively promotes growth of macrophages. For preparation of this medium was used supernatant from L929 cell line, which contains factors supporting growth of macrophages as described earlier [120] and which was kindly provided to us by Mgr. Rhombaldova, M., Adipose tissue biology department, Institute of physiology CAS ; RPMI 1640 medium, containing l–glutamine and 20 mM HEPES; fetal bovine serum (FBS) and penicillin and streptomycin.

4.6 Isolation of murine bone marrow and cultivation of bone–marrow–divided macrophages (BMDM)

Murine bone marrow was isolated from their femurs and tibias. Bones were taken and carefully cleansed of muscle tissue. Bones were cut at metaphysis and bone marrow was washed out from medullary cavity by medium, which selectively promotes growth of macrophages (i.e. BMDM medium, for composition see Fig.12).

Bone marrow cells from single bone were incubated in 100 mm Petri dish in 15 ml of BMDM medium in 37°C in 5% CO_2 atmosphere for a week. The third and the fifth day of their incubation, their medium was changed.

At the end of the incubation time the cells were harvested for further experiments.

Any leftover BMDM medium was removed and adherent cells were washed over by 6 ml PBS. 3ml of Accutase solution (Sigma–Aldrich) was added and cells were incubated at the room temperature for 5–10 min. Suspended cells were harvested and centrifuged for 6 min, 20°C, 500G. Supernatant was then removed and pelleted cells were resuspended in BMDM medium.

4.7 Macrophage polarization

Mature BMDM were counted and divided in 35 mm Petri dishes (~ 500 000 cells/dish) in 1 ml of BMDM medium. 3–5 samples of were made from macrophages from each animal. Cells were incubated overnight (37°C, 5% CO_2), to let the cells adhere. Next day medium was removed and replaced by either Air–buffered medium alone for maintenance of unpolarised M0 macrophages, or Air–buffered medium with IFN-γ (20 ng/ml, Protech) and LPS (100 μM, Protech) for induction of M1 proinflammatory polarization or IL-4 (20 ng/ml, Protech) for induction of M2 anti–inflammatory polarization. The dishes were sealed with vacuum grease and glass coverslips and cells were incubated overnight (37°C, 5% CO_2). Covered dishes were placed inside LumiCycle apparatus for bioluminescence recording.

4.8 Isolation of murine peritoneal cells

Mice were sedated by 5% isofluran (AbbVie). After the confirmation, that mouse is no longer responsive to painful stimuli, 5ml of ice cold PBS was injected in its peritoneal cavity. Peritoneal fluid was then retracted back and kept on ice. Mice were decapitated and used for other sampling. Fluid was centrifuged for 8 min, 1500 RPM, room temperature. Supernatant was removed and pelleted cells were resuspended in Air–buffered recording medium. Harvested cells were equally divided into two 35 mm Petri dishes with 1 ml of Air–buffered recording medium each. Dishes covered with grease and glass coverslips, and they were placed inside LumiCycle apparatus for bioluminescence recording.

4.9 WAT and BMDM co–cultivation

BMDM were cultured, harvested, divided and polarized as described above (see sections 4.5 Isolation of murine bone marrow and cultivation of bone–marrow–divided macrophages

and 4.6 Macrophage polarization). After overnight polarization of macrophages WAT explant was inserted in the dish into unchanged medium containing polarizing cytokines. Dishes were then covered again with glass coverslip and inserted into Lumicycle apparatus for bioluminescence recording.

4.10 Bioluminiscence statistical analysis

Recorded data from LumiCycle apparatus (Actimetrics) were evaluated using Lumicycle Analysis programme (Actimetrics). Period values were subtracted by Lumicycle analysis. Period values were used for statistical evaluation when goodness of fit of subtracted dampened sin curve was ≥75%.

In rhythmicity analysis, PER2 expression was considered rhythmical when at least one full oscillation cycle was visible.

Gaussian distribution of measured data was tested using D'Agostino–Pearson normality test. One–way ANOVA was used to determine statistically significant differences in variances of recorded values, Bartlett's and Brown–Forsythe's tests of variance were used. Differences between mean values of recorded data were evaluated using multiple comparisons test with Holm–Sidak corrections. Tests were evaluated as statistically significant when $p \leq 0.05$.

5.Results

5.1 Diet–induced changes in body mass and activity rhythms

5.1.1 Weight gain

Multiple comparisons test showed a significant effect of diet composition on percentual weight gain which was higher in cHF than in cHF–F group of *Per2*^{Luc} mice (p=0.0368). One–way ANOVA multiple comparisons test showed significant difference between cHF and CTRL group (p=0.0235), but no significant difference between cHF–F and CTRL group (Fig.13). Weight gain was monitored for 6 weeks by weighting the mice of all groups three times a week (Fig.14).

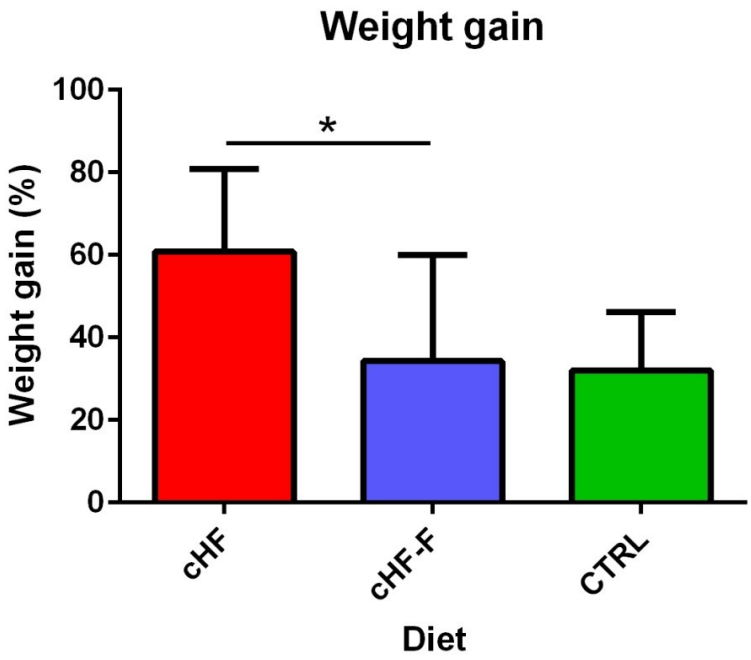


Figure 13: Weight gain (percentage). Values were calculated as group mean of weight increase (percentage) values, which were calculated individually for each animal. Group of mice fed cHF diet (n=10) are represented by red column, mice fed cHF–F diet (n=6) are represented by blue column, control CTRL animals (n=5) are represented by green column. Each column is showed with SD± for each group. Significant difference between values of cHF and cHF–F group, which was shown by Student's t–test (p=0.0368) is indicated by a star–sign.

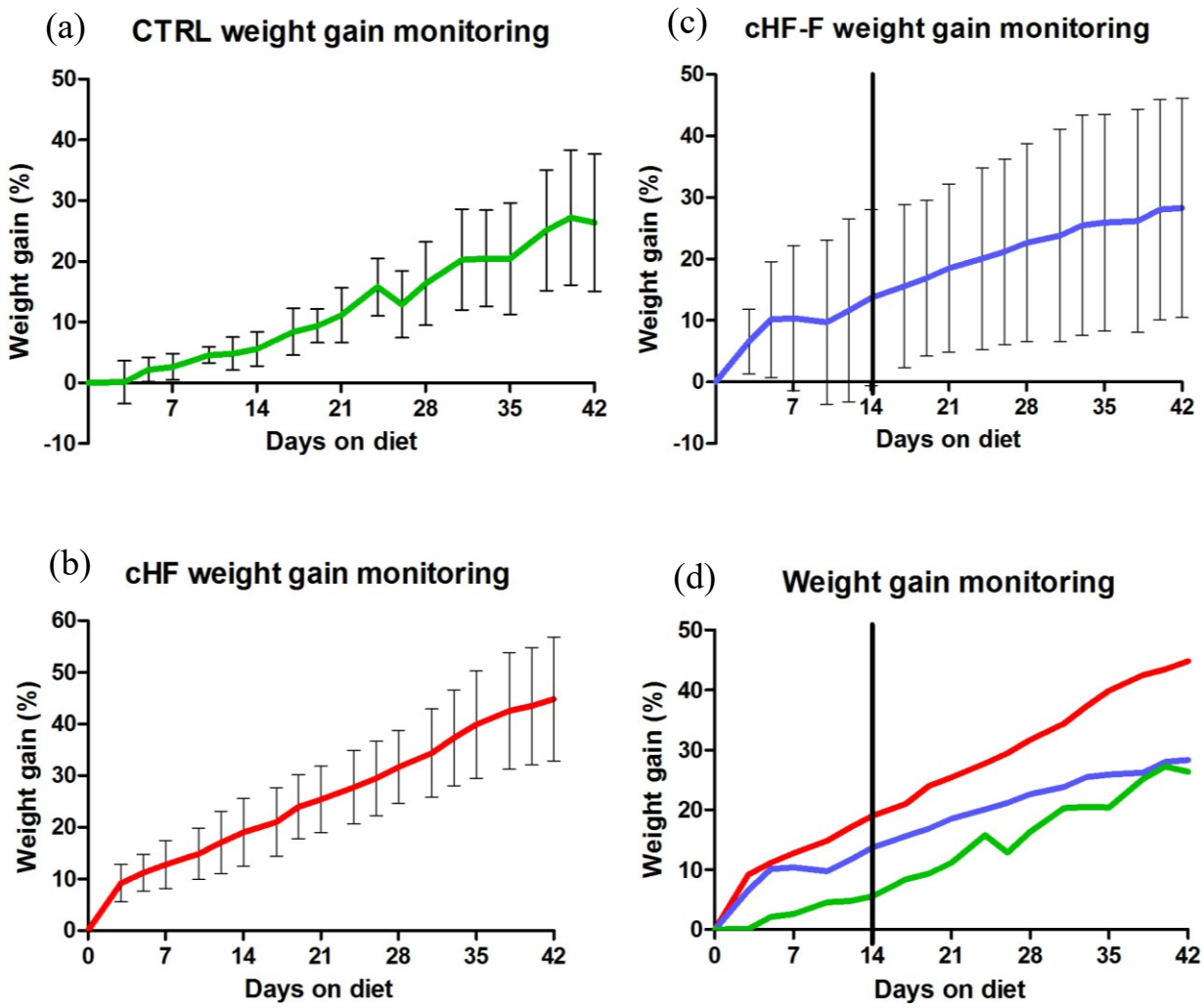
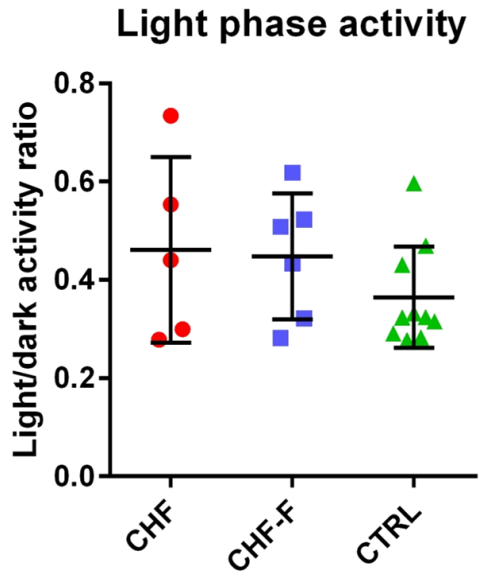


Figure 14: Weight gain curves Weight gain (percentual increase in body weight related to initial body weight) was monitored for 6 week, when mice from all groups were weighted three times a week. Measured values were used for compilation of weight gain curves for each diet group, green for CTRL group (a), red for cHF group (b) and blue for cHF-F group (c). Curves were constructed by connection of means, which were calculated for each measure point. Each measure point is showed with SD±. All curves were for comparison compiled into one graph (d). *mPer2*^{Luc} mice from cHF–F group were fed for first two weeks cHF diet, which was then for remaining 6 weeks switched to diet with high portion of fat enriched by omega 3 fatty acid concentrate. Vertical black line (c, d) indicates time of this diet transition.

5.1.2 Activity monitoring

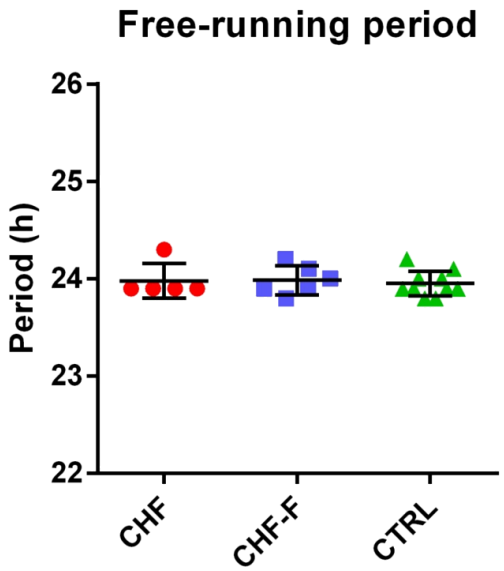
Due to non–Gaussian distribution of collected values for light dark activity ratio, non–parametric Kruskal–Wallis test was used during ANOVA analysis. No significant difference of variances or between mean values was detected (Fig.15).

Parametric one–way ANOVA was used for evaluation of measured lengths of free–running period of tested animals in CTRL (n=10), cHF (n=5) and cHF–F (n=6) groups. No significant differences between mean values or in variances of measured data were detected (Fig.16).



Light/dark activity ratio		
Diet	**Mean**	**±SD**
CTRL	0.36	0.097
cHF	0.46	0.11
cHF–F	0.44	0.17

Figure 15: Light/dark activity ratio. Values were calculated as ratio between measured counts during light phase and during dark phase for each monitored animal in CTRL (n=10), cHF (n=5) and cHF–F (n=6) group. Calculated mean values of light/dark activity ratio are presented in table with ±SD.



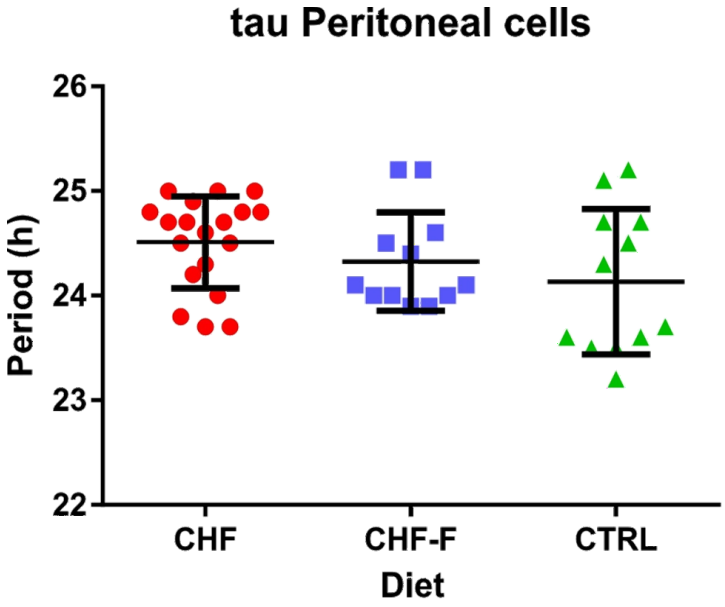
Free–running period		
Diet	**Mean**	**±SD**
CTRL	23.98	0.12
cHF	23.98	0.16
cHF–F	23.95	0.13

Figure 16: Free–running period. Values were subtracted from Chi–squared periodograms using MATLAB Clock lab program for each monitored animal in CTRL (n=10), cHF (n=5) and cHF–F (n=6) group. Calculated mean values of free–running period are presented in table with ±SD.

5.2 Bioluminiscence monitoring

5.2.1 Peritoneal cells

One–way ANOVA didn't reveal any significant differences between the mean values or differences in variances of period lengths (tau) of PER2 oscillations in the cells isolated from peritoneal cavity (Fig.17).

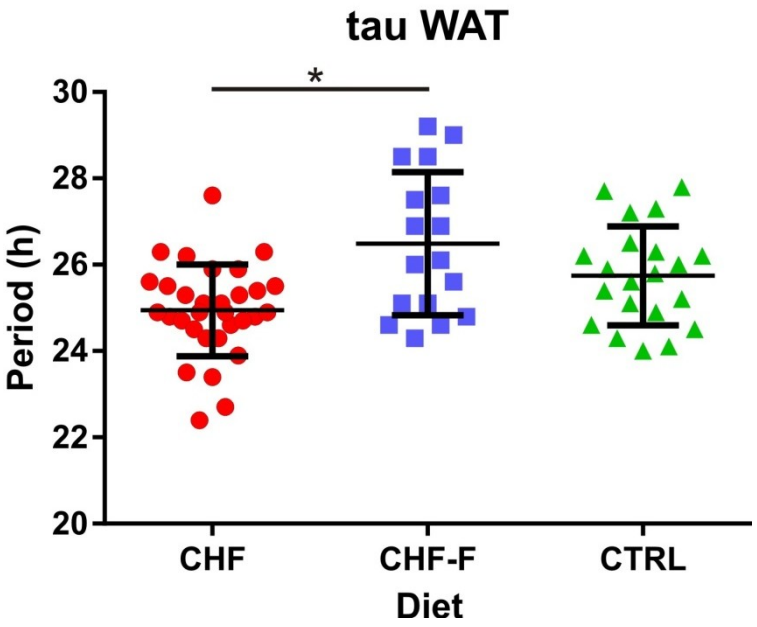


tau Peritoneal cells		
Diet	**Mean**	**±SD**
CTRL	24.13	0.66
cHF	24.51	0.43
cHF–F	24.33	0.45

Figure 17: Period of PER2 oscillations in cells isolated from peritoneal cavity. PBS was injected into mouse's peritoneal cavity and collected back. Cells isolated from retracted fluid from single animal were equally divided into two samples. Period of PER2 oscillation was measured in for each sample from animals from CTRL (n=6), cHF (n=10) and cHF–F (n=6) groups. Calculated values of tau of PER2 oscillations from cells isolated from peritoneal cavity are presented in table with ±SD.

5.2.2 WAT

One–way ANOVA showed significant difference in variances of measured values (p=0.0005) and so did Brown–Forsythe test of variances (p=0.0195). Multiple comparisons test showed significant difference between the mean values of period lengths (tau) of PER2 oscillations in WAT explants from mice of cHF and cHF–F groups (p=0.0005) (Fig.18).

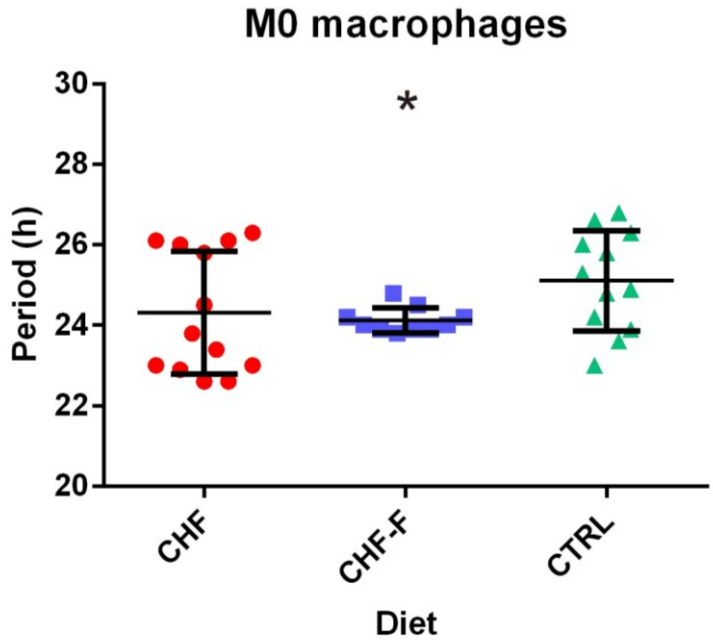


tau WAT		
Diet	**Mean**	**±SD**
CTRL	25.74	1.12
cHF	24.94	1.05
cHF–F	26.49	1.61

Figure 18: Period of PER2 oscillation from WAT explants. 3–5 samples were taken from WAT isolated from animals from CTRL (n=7), cHF (n=10) and cHF–F (n=6) groups. Period values of PER2 oscillation were used for statistical evaluation when goodness of fit of subtracted dampened sin curve was ≥75%. Significant difference between mean values showed by multiple comparisons test (p=0.0005) is indicated by a star–sign. Calculated values of tau of PER2 oscillations from WAT explants are presented in table with ±SD.

5.2.3 Macrophages, M0 polarization

Brown–Forsythe's test of variances (p=0.008) and Bartlett's test (p=0.0002) showed that the period lengths of PER2 oscillations of unpolarized M0 macrophages from the cHF–F group were significantly less variable than those from CTRL and cHF groups (Fig.19).

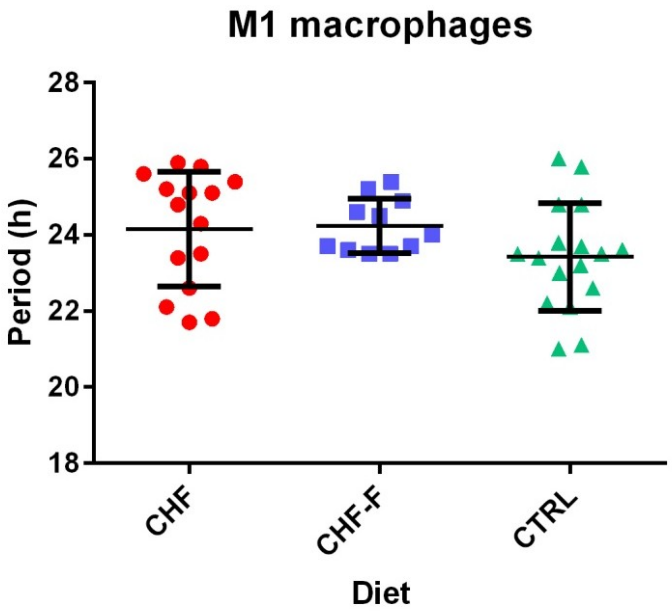


tau M0 macrophages		
Diet	**Mean**	**±SD**
CTRL	25.10	1.19
cHF	24.32	1.46
cHF–F	24.12	0.30

Figure 19: Period of PER2 oscillations in M0 unpolarized macrophages. Macrophages were cultivated from bone marrow, which was isolated from *Per2*^{Luc} mice. 3–5 samples (~500 000 cells/sample) of macrophages were made from each animal from CTRL (n=7), cHF (n=10) and cHF–F (n=6) groups. Period values of PER2 oscillation were used for statistical evaluation when goodness of fit of subtracted dampened sin curve was ≥75%. Significant difference in variance of values showed by Brown–Forsythe's test (p=0.008) and Bartlett's test of variance (p=0.0002) is indicated by a star–sign. Calculated values of tau of PER2 oscillations in M0 unpolarized macrophages are presented in table with ±SD.

5.2.4 Macrophages, M1 polarization

One–way ANOVA didn't show any significant differences between the mean values of measured data or difference in variances of period lengths (tau) of PER2 oscillations in M1 polarized macrophages isolated from CTRL, cHF and cHF–F groups (Fig.20).

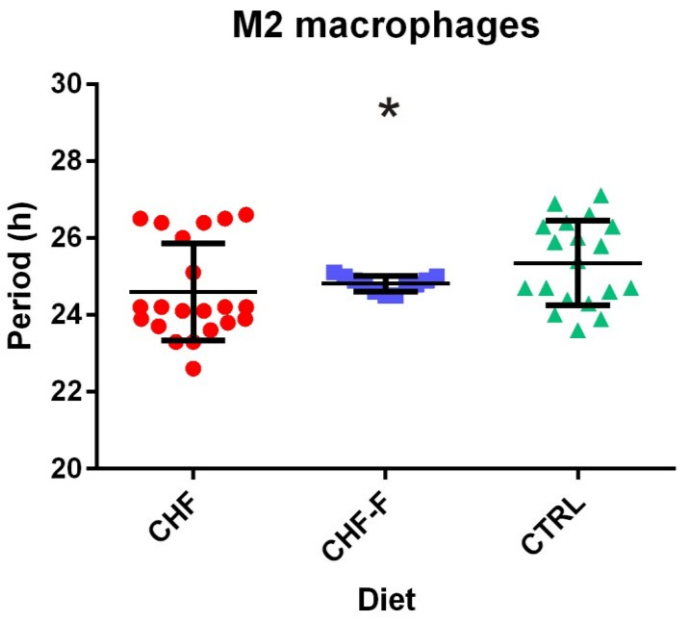


tau M1 macrophages		
Diet	**Mean**	**±SD**
CTRL	23.42	1.37
cHF	24.15	1.46
cHF–F	24.24	0.68

Figure 20: Period of PER2 oscillations in M1 polarized macrophages. Macrophages were cultivated from bone marrow, which was isolated from *Per2*^{Luc} mice. Macrophages were exposed to LPS and IFN–γ to induce their M1 polarization. 3–5 samples (~500 000 cells/sample) of macrophages were made from each animal from CTRL (n=7), cHF (n=10) and cHF–F (n=6) groups. Period values of PER2 oscillation were used for statistical evaluation when goodness of fit of subtracted dampened sin curve was ≥75%. Calculated values of tau of PER2 oscillations in M1 polarized macrophages are presented in table with ±SD.

5.2.5 Macrophages, M2 polarization

Brown–Forsythe's test of variances (p=0.0033) and Bartlett's test (p=0.0001) showed significant difference in variances of measured period lengths of PER2 oscillations in samples of M2 polarized macrophages isolated from mice from CTRL, cHF and cHF–F groups (Fig.21). The variation was significantly lower for the cHF-F group compared to CTRL and cHF groups.



tau M2 macrophages		
Diet	**Mean**	**±SD**
CTRL	25.35	1.07
cHF	24.60	1.23
cHF–F	24.81	0.19

Figure 21: Period of PER2 oscillations in M2 polarized macrophages. Macrophages were cultivated from bone marrow, which was isolated from *Per2*^{Luc} mice. Macrophages were exposed to IL–4 to induce their M2 polarization. 3–5 samples (~500 000 cells/sample) of macrophages were made from each animal from CTRL (n=7), cHF (n=10) and cHF–F (n=6) groups. Period values of PER2 oscillation were used for statistical evaluation when goodness of fit of subtracted dampened sin curve was ≥75%. Significant difference in variance of values showed by Brown–Forsythe's test (p=0.0033) and Bartlett's test of variances (p=0.0001) is indicated by a star–sign. Calculated values of tau of PER2 oscillations in M2 polarized macrophages are presented in table with ±SD.

5.3 Co–culture experiments

5.3.1 Effect of cytokines in BMDM polarization medium on circadian *Per2* oscillations in WAT explants

WAT samples were inserted in dishes with Air–buffered medium (n=4), M1 polarization medium with added LPS and IFN–γ (n=4) or in M2 polarization medium with added IL–4 (n=4). PER2 expression was considered rhythmical, when at least one full oscillation cycle was visible. In all monitored samples rhythmical expression of PER2 was preserved. One–way ANOVA and multiple comparisons test showed no significant differences in measured period values of PER2 expression in WAT samples (Fig.22).

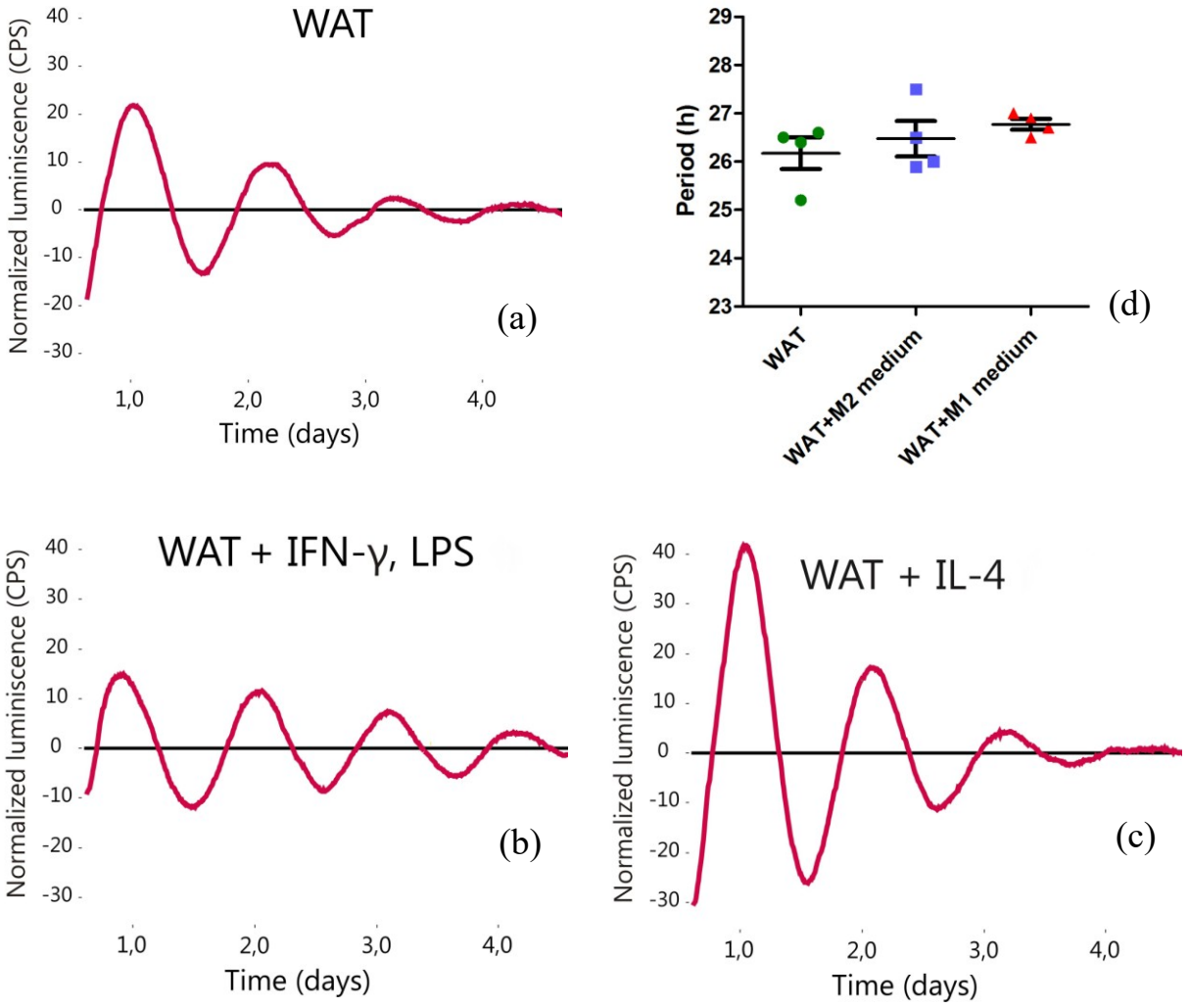


Figure 22: Real–time bioluminiscence monitoring of PER2 expression in WAT explants. WAT samples were inserted in dishes with Air–buffered medium (a), M1 polarization medium with added LPS and IFN–γ (b) or in M2 polarization medium with added IL–4 (c). Mean representative curves were picked for presentation. One–way ANOVA and multiple comparisons test showed no significant difference in measured period values of PER2 expression in WAT samples cultured in different medias (d).

5.3.2 WAT co–culture with M1 polarized macrophages

Rhythms of PER2 expression vanished in majority of samples of WAT explants isolated from *Per2*^{Luc} mice, when they were co–cultivated with M1 polarized macrophages. Only 22.7% of samples from CTRL, 27.0% from cHF and 31.3% from cHF–F groups showed at least one full visible oscillation cycle in PER2 expression. Period length analysis was not performed due to insufficient number of samples, that would suit selected criteria (oscillations being rhythmical, goodness of fit of subtracted dampened sin curve ≥75%) (Fig.23).

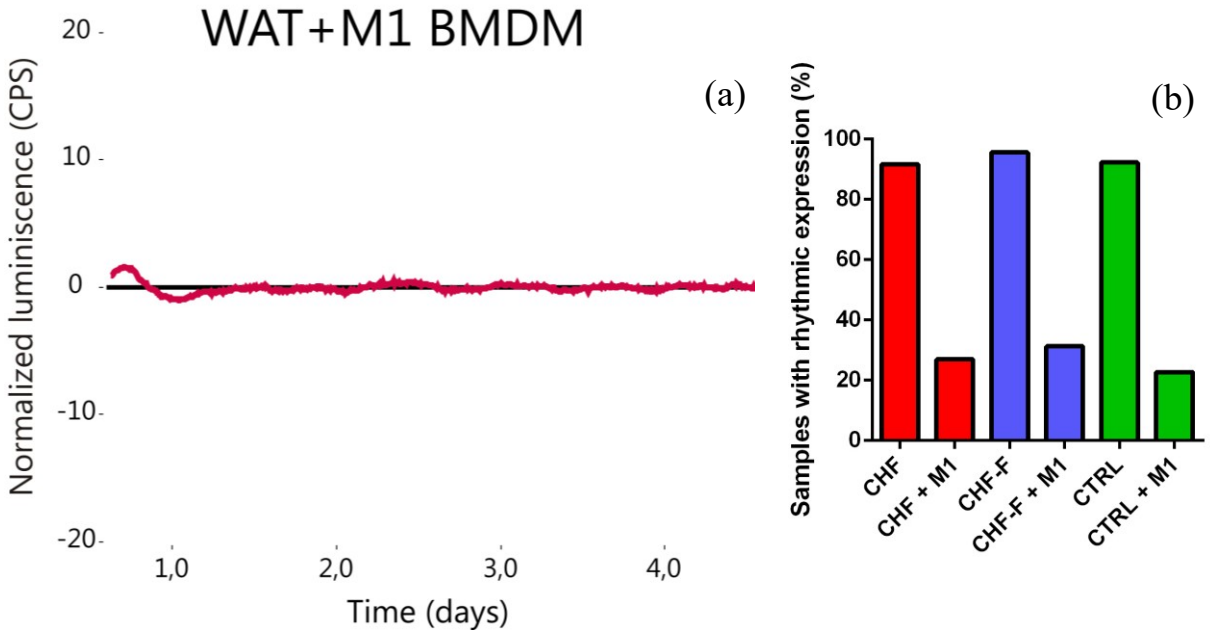
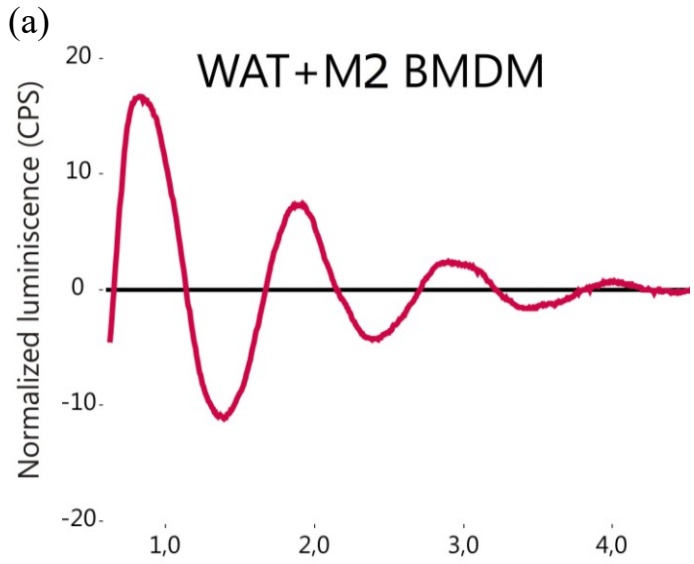


Figure 23: Rhythmic expression vanished in most samples of WAT explants co–cultured with M1 macrophages. In majority of WAT samples rhythmic expression of PER2 vanished. Mean representative curve was picked for presentation (a). Column graph shows different in percentage of rhythmic samples between samples of WAT and WAT co-cultured with M1 macrophages. Percentage of rhythmic samples from individual groups, which showed at least one full visible oscillation cycle in PER2 expression, were following: CTRL 92.3%, CTRL+M1 22.7%, cHF 91.7%, cHF+M1 27.0%, cHF-F 95.7% and cHF–F+M1 31.3% (b).

5.3.3 WAT co–culture with M2 polarized macrophages

One–way ANOVA, Brown–Forsythe's test (p=0.023) and Bartlett's test (p=0.0117) showed significant difference in variance of period values of PER2 expression in WAT samples from CTRL, cHF and cHF–F groups co–cultivated with M2 polarized macrophages (Fig.24).



tau WAT + M2 co–culture		
Diet	**Mean**	**±SD**
CTRL	26.19	1.23
cHF	25.56	0.91
cHF–F	25.41	2.15

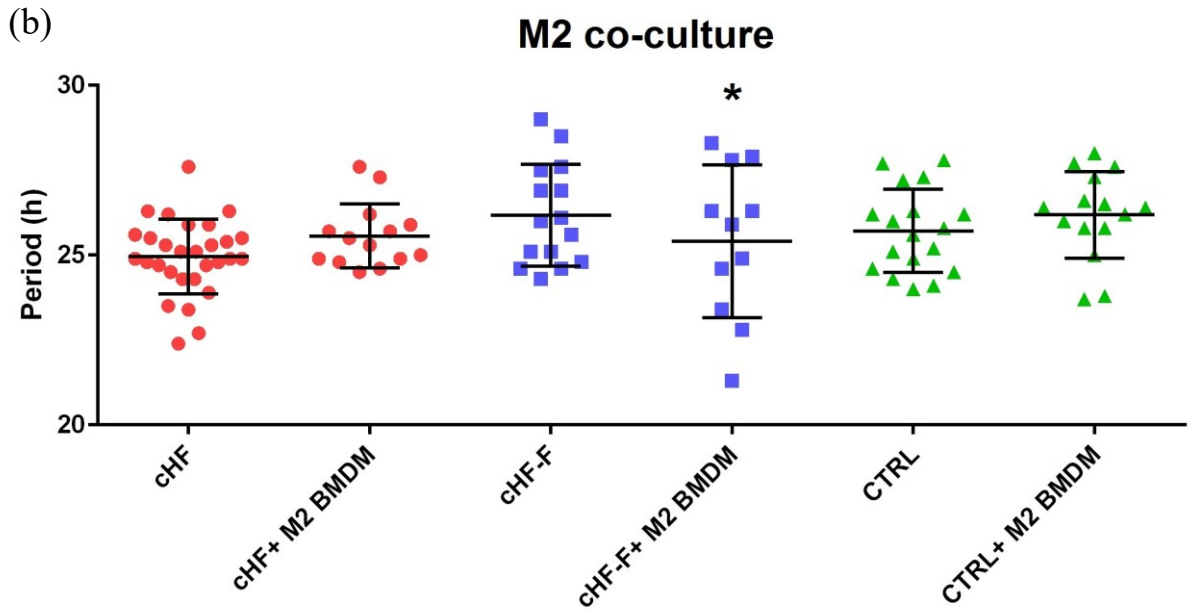


Figure 24: Real–time bioluminescence monitoring of PER2 expression in WAT explants co–cultured with M2 macrophages. Mean representative curve was picked for presentation (a). Comparison of measured tau values from WAT explants from CTRL, cHF or cHF–F groups vs. WAT explants from CTRL, cHF or cHF–F groups co–cultivated with WT M2 macrophages (b). Significant difference in variance of measured period values of PER2 expression in WAT explants showed by One–way ANOVA, Brown–Forsythe's test (p=0.023) and Bartlett's test (p=0.0117) is indicated by a star–sign. Calculated values of tau of PER2 oscillations in WAT explants co–cultivated with M2 polarized macrophages are presented in table with ±SD.

6.Discussion

6.1 Effect of diet composition on body mass and activity rhythm

In our experiments, mice were kept on either high fat diet (i.e. cHF diet), high fat diet enriched by omega–3 fatty acids (i.e. cHF–F diet) or on regular chow diet (i.e. CTRL group) for 9 weeks.

Our results demonstrated, that addition of omega–3 fatty acids in high fat diet significantly reduced weight gain increase due to high fat diet. This result was in accordance with previous experiments conducted on rodents [121]. There are also studies that show similar effect of increased PUFA intake on body weight and adiposity in human participants [122]. These studies altogether propose applicability of omega–3 fatty acids supplementation in diet to reduce weight as promising therapeutic treatment.

Previous studies have demonstrated, that HFD diet has a significant effect on the locomotor activity rhythms. The obese mice, which were fed with HFD, tended to be more active during their resting period (i.e. during the light phase of the day) than lean mice which were fed with standard chow diet [95]. Analysis of our data didn't reveal any significant difference in activity/rest ratios between groups of our tested animals. However, we cannot exclude a possibility that this was due to a limited number of monitored animals in our experiment.

Daily regime in food intake has indisputable effect on peripheral circadian oscillators [52]. However Kohsaka and colleagues [95] demonstrated that diet composition has an effect on free-running period of locomotor activity rhythm which is driven by the central clock in the SCN. To verify these findings, we monitored activity rhythms of tested animals for two weeks in constantl darkness (i.e. DD regime). Our data did not confirm the previous finding because we did not found any significant difference in the free-running period lengths of activity rhythms between the animals from cHF, cHF–F and CTRL groups. Again, it might be caused by small number of monitored animals.

6.2 PER2 oscillations in adipose tissue

Since the discovery that impairment in the circadian system is linked to obesity and consequential onset of various metabolic disorders [132], observation of molecular clock mechanism residing in the adipose tissue has become a topic of interest.

Effect of HFD on oscillations of clock genes expression in the adipose tissue has already been described in various studies. Yanagihara and colleagues [125] have shown that mice, which were kept for 8 weeks on lipid-rich diet, had only slightly or not at all affected circadian rhythms in levels of clock gene's mRNA. Our own results are in accordance with these findings. Period lengths of PER2 expression *in vitro* didn't show significant differences between control (CTRL) group and group of mice kept on HFD (cHF group).

There are only few studies that focused on the effect of omega-3 fatty acids in HFD on the adipose tissue but those that examined the effect on circadian clock are only sparse.

Multiple comparisons analysis of our data have shown, that the mean value of periods of PER2 expression rhythms in the adipose tissue isolated from mice which were kept on diet enriched by omega-3 fatty acids (cHF-F group) wasn't significantly different compared to control (CTRL) group. These results were similar to those we observed in cHF group which did not differ significantly to CTRL group as well. However, our data revealed significantly longer period for cHF-F compared to cHF diet.

This might indicate, that although HFD has only mild effect on circadian oscillations in adipose tissue, the nature of this effect is dependent on composition of lipids in diet. Based on our results it seems important to investigate in future whether prolongation of the feeding time would lead to more significant effect.

Additionally, in the cHF-F group we found significantly increased variance in the measured data compared to other groups. It has been shown that increased amount of omega-3 fatty acids in diet reduces adipocyte size by about 10% in epididymal adipose tissue after 18 weeks on special diet [126]. Thus it is likely that although the isolated tissue samples were approximately of the same size, there was a greater number of adipocytes in samples from cHF-F group. Another factor that we could only speculate about is whether deposition of omega-3 fatty acids was homogenous within individual adipocytes or within epididymal adipose tissue. Increased number of circadian oscillators (which reside in each individual adipocyte) and possible different amount of stored omega-3 fatty acids could explain the increased variability between examined samples.

The effect of omega-3 fatty acids on circadian oscillations of clock genes is most probably mediated through PPARγ receptor. PPARs are nuclear receptors, that are sensitive to changes in concentrations of fatty acids and have proven to be able to affect expression of specific

clock genes (as described in chapter 2.3.1 Feeding dependent clock entrainment). It has been shown in previous studies, that PPARγ prefers PUFA for binding and that eicosanoids (group of derived fatty acids, among others it includes fatty acids derived from omega-3 fatty acids) increase PPARγ-mediated gene activation [131].

6.3 PER2 oscillations in macrophages

Macrophages, just like majority of body cells posses functional circadian clock mechanism. Unlike adipose tissue, they don't cluster in form of intact organs, but they either circulate through bloodstream or reside within various types of peripheral tissue [98]. For our experiments we isolated cells from bone marrow, place of macrophage differentiation, and cells from peritoneal cavity.

6.3.1 Characterization of circadian oscillations in peritoneal cells

Immune cells reside in abundant numbers in peritoneal cavity. Phenotypic characterisation and flow–through cytometric analysis of cells, which were isolated from peritoneal cavity, have shown, that population of peritoneal cells consists predominantly of macrophages, B–cells and T–cells [123].

Our bioluminescence monitoring from the peritoneal cavity cells didn't show significant differences in period lengths between dietary groups. This might have been caused by above mentioned phenotypic variance of present immune cells because differences in circadian expression of clock genes, including *Per2*, in various types of immune cells have been observed before [124].

In another part of our experiment, we monitored PER2 oscillations in macrophages, which were derived from the murine bone marrow and which matured in controlled conditions. Unlike these macrophages, peritoneal cells might have been also affected by exposition to various systemic time-setting cues while still residing within the body, which might explain differences in data between these two sets of immune cells of different origin.

6.3.2 Circadian oscillations in macrophages derived from bone marrow

We monitored circadian rhythms in bioluminescence in macrophages derived from bone marrow. Preferred differentiation of isolated myeloid precursor cells into macrophages was induced by supplementation of L929 line supernatant in culture medium as it has been described earlier [120]. These cells were cultured in absence of externally supplied signalling

cues such as hormones including glucocorticoids, and cytokines, which are usually present in vivo conditions during macrophage maturation time.

Macrophages of different polarizations were monitored in i) non–polarized M0 state, which were macrophages that weren't exposed to any externally administered cytokines, ii) pro-inflammatory M1 state, which was induced by exposition to LPS and IFN-γ, and iii) anti-inflammatory polarized M2 macrophages induced by IL-4.

Our data have shown, that lipid composition in diet had an effect on period of PER2 rhythmic expression. Enrichment of high fat diet by omega-3 fatty acids resulted in significant decrease in variance of measured period values within examined samples. In particular this effect was visible in M0 and M2 polarized macrophages in particular.

Kuda and colleagues [117] showed that addition of omega-3 polyunsaturated fatty acids into media used for cultivation of BMDM promoted decline in levels of pro-inflammatory IL-6 cytokine released macrophages in media. DHA administration affected metabolic processes related to NO and ROS production and promoted anti-inflammatory metabolic state, referred to as M2.

As mentioned before (in chapter 2.6.2 Macrophages and their polarizations), macrophages of M1 and M2 polarization states differ in preferred ways of acquiring ATP, either via anaerobic glycolysis in M1 state or oxidative phosphorylation in M2 state [104].

It has also been demonstrated that changes in cell's energetic system affect clockwork of the circadian system through various pathways (described more thoroughly in chapter 2.3.1 Feeding dependent clock entrainment). Changes in metabolism caused by omega-3 fatty acids as metabolic substrate could be the link to altered circadian oscillations, that we observed in our experiments. This hypothesis is furthermore supported by the fact, that statistically significant results were observed for data measured from M0 and M2 polarizations.

The rhythms measured in the group of mice that was fed non-enriched high fat diet (i.e. cHF) didn't show any significant difference compared to the control group (i.e. CTRL group). This might indicate that not the amount of lipids in diet but rather composition of fat component of diet plays a role in regulation of circadian clock system in macrophages.

6.4 Co-cultivation experiments

The goal of our final experiment was to examine whether interaction between epididymal adipose tissue and differently polarized macrophages could affect oscillations of circadian gene's expression inside adipocytes.

We observed this interaction in so-called "co-cultivation experiments". We cultivated samples of WAT, isolated from *Per2*^{Luc} mice from each dietary group, together with macrophages isolated from WT mice (Fig.25).

6.4.1 Co-cultivation with M1 polarized macrophages

As mentioned before, macrophage's polarization switch was induced by addition of cytokines for either M1 (LPS, IFN-γ) or M2 (IL-4) polarization in culture medium. Since samples of WAT were simultaneously cultivated in media with macrophages, we first conducted a test whether cytokines alone interfere with circadian molecular mechanism within adipocytes. Previously there have been conducted numerous experiments which were focused on effect of cytokines on circadian clock, however, these experiments were performed mostly *in vivo* and were focused mainly on core clock residing in the SCN. These experiments demonstrated for example, that LPS treatment suppressed expression of *Per2* in the SCN or that microinjection

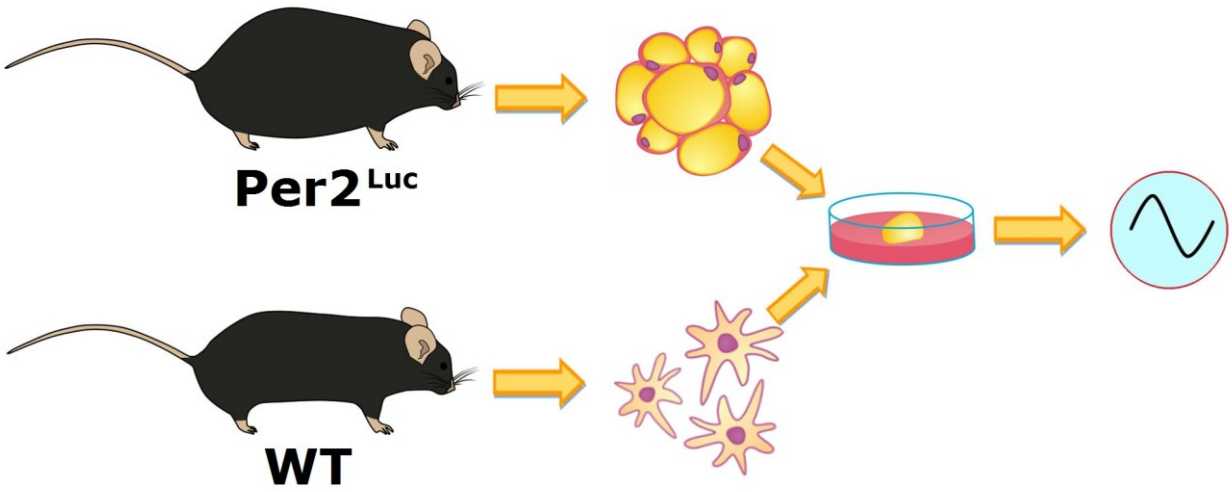


Figure 25: Scheme describing design of co–culture experiments. WAT explants (~ 20 mg) taken from *Per2*^{Luc} mice and macrophages cultured from bone marrow (~500 000 cells/dish), which was taken from wild type mice, were co–cultivated in single Petri dish. WAT explants and macrophages were cultivated in Air–buffered medium with either IFN-γ (20ng/ml, Protech) and LPS (100μM, Protech) for induction of M1 proinflammatory macrophage polarization, or IL-4 (20 ng/ml, Protech) for induction of M2 anti–inflammatory macrophage polarization. Covered dishes were placed inside LumiCycle apparatus for bioluminescence recording.

of IFN-γ in hamsters caused phase-advances in locomotor activity [127]. Adipocytes also possess receptors, which are responsive to various cytokines and contribute to induction of pro-inflammatory response and production of inflammatory cytokines upon exposition to pro-inflammatory agens such as LPS [128]. These studies however doesn't study the effect of cytokines on either molecular clock oscillations or effect on target tissue which is mediated through cytokine-activated immune cells. Our data have shown that none of the sets of cytokines added to the medium, in which we cultivated WAT explants from control animal alone, affected rhythmicity of the monitored samples (all remained rhythmic for several days). Period of their PER2 oscillations hasn't been evaluated as significantly different either.

However, co-cultivation of WAT with M1 polarized macrophages induced apparent changes. Almost three quarters of monitored samples from all dietary groups didn't show any rhythm in clock gene expression. This could have been a result of macrophage's cytotoxic activity. As mentioned before, inflammatory M1 state of macrophages is coupled with altered metabolic pathways and results in increased production of cytotoxic NO and ROS. Complete destruction of adipocytes by the molecules and phagocytosis would explain disappearance of PER2 rhythms.

Since NO and ROS are toxic to macrophages as well, their exceedingly increased production should destroy them as well. Although macrophages are able to partially protect themselves against the cytotixic effect of ROS [129], the survival rate and circadian rhythms in the LPS–activated macrophages should be impaired as well. However it apparently didn't happen in bioluminescence records of M1 macrophages, since they stayed rhythmic for several days.

Moreover, rhytmicity of some of the WAT samples was rescued after stimulation by artificial GC agonist dexamethasone, despite being originally non-rhythmical (data not shown). All this could indicate that presence of immune cells, not just of pro-inflammatory cytokines, is important for modulation of circadian clock inside adipocytes and that disappearance of circadian rhythm in their PER2 expression is caused other factors than increased production of NO and ROS by macrophages. These hypotheses would of course require further examination.

6.4.2 Co-cultivation with M2 polarized macrophages

In contrast to the effect of M1 macrophages, co-cultivation with alternatively activated M2 macrophages didn't affect rhythmicity of monitored WAT samples, and we could observe the PER2 oscillations for several days. Our original hypothesis was, that cultivation with M2

macrophages, that contribute to tissue repair and remodelling, would help to reverse effect inflicted on the circadian system in adipose tissue by HFD.

Comparision of period lengths of PER2 rhythms of WAT samples from CTRL and cHF group with and without co-cultivation with M2 macrophages didn't reveal significant difference. Therefore, to determine, whether and how M2 co-cultivation may affect circadian oscillations in WAT samples, additional are required because the effect of diet which was rich on lipids on circadian system in WAT was only faint and did not reach statistical significance (as mentioned above in chapter 6.2 PER2 oscillations in adipose tissue).

Nevertheless, we observed significantly higher variance in data measured from group of mice fed HFD enriched by omega-3 fatty acids. Increased variance of period lengths of PER2 rhythms we already observed in samples of WAT which were cultivated alone. M2 co-cultivation seemed to deepen this effect.

Above presented results have shown, that cytokines (in this case IL-4), which were present in culture media, didn't affect rhythmicity (i.e. presence of rhythms in amount of PER2) in monitored samples from lean control mice.

However, in samples from mice which were fed diet enriched by omega-3 fatty acids, the observed variance of measured lengths of PER2 periods could be explained by presence of cytokines in the medium, namely anti-inflammatory cytokine IL-4. Tsao and colleagues [130] have demonstrated that IL-4 possess ability to affect lipid metabolism by promotion of lipolysis in mature adipocytes. They have also shown, that IL-4 treatment caused changes in PPARγ expression in adipocytes. This effect of IL-4 on PPARγ, which is the most probable candidate to mediate effect of lipid composition in diet on clock in adipose tissue (as mentioned before in chapter 6.2 PER2 oscillations in adipose tissue), could explain why co-cultivation with M2 macrophages resulted in deepening of the effect which was already caused by enrichment of high fat diet by omega-3 fatty acids.

6.5 Results' overview of effect of omega-3 fatty acids on metabolism and circadian clock

The results of our study allow us to propose a hypothesis as follows: The alternative diet and different composition of fatty acids (namely enrichment with omega-3 fatty acids) affects metabolism inside adipocytes. This modulation of cellular metabolism impacts on the adipocyte circadian clock, which gradually shortens its period and it consequently affects

expression of various clock controlled metabolism-related genes. This could result in a more effective lipid metabolism and enhanced lipolysis, which would explain the observed reduced body weight gain of our mice on HFD with omega-3 fatty acids. Furthermore, a result of the alternative diet, there is a preferable shift toward M2 polarization of macrophages that contribute to tissue's repair and may help to prevent the outbreak of inflammation, and also their rhythmicity is better synchronized.

7.Conclusion

In this study we demonstrated, that composition of lipids in diet had effect on oscillations in clock gene's expression, namely in observed levels of PER2 protein, in adipose tissue and macrophages of different polarizations.

Our data confirmed that administration of HFD enriched by omega-3 fatty acids resulted in lower weight gain compared to mice that were fed regular HFD. We have shown, that increased amount of omega-3 fatty acids in food affected molecular clock in adipose tissue. Its effect on the clock rhythmicity was enhanced by simultaneous cultivation with M2 polarized macrophages. We observed this effect as gradual shortening in period of PER2 expression within WAT samples. We have demonstrated that omega-3 fatty acids-rich diet had positive effect on synchronization among clocks of M0 and M2 polarized macrophages. We have also shown that co-cultivation M1 polarized macrophages abolished PER2 rhythmic expression independently of type of diet.

Altogether, our results support previous findings related to the effect of omega-3 fatty acids on metabolism and their anti-inflammatory properties and we confirmed their effect on the circadian system in adipose tissue and macrophages. Furthermore our findings demonstrating the effect of M1 polarized macrophages on circadian system propose new field worth of investigation, i.e. the mechanism by which chronic inflammation caused by obesity may pronounce overall clock desynchrony among circadian clocks at the systemic level.

Results of this diploma thesis will be used as a background for further studies on the molecular mechanism underlying our findings. The results provide a basis for investigation of future targeting of immune cells by therapeutic procedures to alleviate symptoms caused by obesity or circadian clock dysfunction.

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