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Molecular mechanisms of mammalian circadian clocks, its sensitivity to constant light and aging

Molekulární mechanismy savčích cirkadiánních hodin, jejich sensitivity na stálé světlo a stárnutí

Doctoral thesis

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Consultant: Mgr. Martin Sládek, PhD.

Prague 2019
I declare, that I compiled this thesis on my own and that I properly cited all used resources and literature. Neither this work, nor its major part was presented to gain another or the same academic degree.

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In Prague 20.8.2019

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Author’s signature
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<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AANAT</td>
<td>arylalkyl N-acetyltransferase</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP dependent kinase</td>
</tr>
<tr>
<td>AUC</td>
<td>area under curve</td>
</tr>
<tr>
<td>BMAL</td>
<td>brain and muscle ARNT-like 1</td>
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<tr>
<td>BW</td>
<td>body weight</td>
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<tr>
<td>CCG</td>
<td>clock controlled gene</td>
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<tr>
<td>CKI</td>
<td>caseine kinase</td>
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<tr>
<td>CRY</td>
<td>cryptochrome</td>
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<tr>
<td>DBP</td>
<td>D-box binding protein</td>
</tr>
<tr>
<td>DD</td>
<td>constant darkness regimen</td>
</tr>
<tr>
<td>E4BP4</td>
<td>nuclear factor, interleukin 3 regulated</td>
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<tr>
<td>FBXL</td>
<td>F-box and leucine rich repeat proteins</td>
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<tr>
<td>GIP</td>
<td>gastric inhibitory polypeptide</td>
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<tr>
<td>GIT</td>
<td>gastrointestinal tract</td>
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<tr>
<td>GLP-1</td>
<td>glucagon-like peptid</td>
</tr>
<tr>
<td>Glut2</td>
<td>glucose transporter 2</td>
</tr>
<tr>
<td>GSIS</td>
<td>glucose stimulated insulin secretion</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
</tr>
<tr>
<td>HIF1</td>
<td>hypoxia-inducible factor 1</td>
</tr>
<tr>
<td>IPGTT</td>
<td>intraperitoneal glucose tolerance test</td>
</tr>
<tr>
<td>LD 12:12</td>
<td>light:dark regimen (12h light:12h darkness)</td>
</tr>
<tr>
<td>LL</td>
<td>constant light regimen</td>
</tr>
<tr>
<td>MTNR1B</td>
<td>melatonin receptor 1B</td>
</tr>
<tr>
<td>NAMPT</td>
<td>nicotinamide phosphoribosyltransferase</td>
</tr>
<tr>
<td>NR1D1</td>
<td>nuclear receptor subfamily 1 group D member 1</td>
</tr>
<tr>
<td>PAS</td>
<td>PER-ARNT-SIM</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCLO</td>
<td>picollo</td>
</tr>
<tr>
<td>PDX1</td>
<td>pancreatic and duodenal homebox</td>
</tr>
<tr>
<td>PER</td>
<td>period</td>
</tr>
<tr>
<td>PP1</td>
<td>protein phosphatase 1</td>
</tr>
<tr>
<td>PP1R3C</td>
<td>protein phosphatase 1</td>
</tr>
<tr>
<td>PPS</td>
<td>protein phosphatase 5</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus</td>
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<tr>
<td>RPS18</td>
<td>ribosomal protein S18</td>
</tr>
<tr>
<td>RT qPCR</td>
<td>real-time quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>SCN</td>
<td>suprachiasmatic nuclei</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SGK1</td>
<td>serum and glucocorticoid-induced kinase 1</td>
</tr>
<tr>
<td>SLC2A2</td>
<td>solute carrier family 2 member</td>
</tr>
<tr>
<td>T2DM</td>
<td>type 2 diabetes mellitus</td>
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<tr>
<td>TTFL</td>
<td>transcriptional-translational feedback loops</td>
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Abstract:

Many processes in mammalian body exhibit circadian rhythms. These rhythms are driven by an intricate system composed of the central pacemaker, suprachiasmatic nuclei (SCN) in the brain, which entrains the peripheral oscillators in various organs, such as pancreas, liver, colon and lungs. Circadian clocks are autonomously driven in each cell based on molecular circuits involving so called clock genes, such as BMAL, CLOCK, PER and CRY. Age-dependent impairment of physiological functions of mammalian body, such as behaviour and metabolic functions, has been well documented. However, it has not been fully elucidated whether the impairment is linked with worsening of the circadian clock function.

The aim of our study was to find out whether i) aging affects basic properties of the circadian clock in SCN and peripheral organs, such as pancreas, colon, liver and lungs, ii) aging-induced changes in glucose homeostasis affect the properties of the circadian clock in the pancreas, and iii) the sensitivity of circadian clock in SCN and peripheral organs to disturbances in environmental lightning conditions is altered during aging.

We used groups of adult (9 months) and aged (25 months) animals which were subjected to 3 different light regimes, namely to light/dark regime (LD 12:12), constant light (LL) and constant darkness (DD) in order to study the synchronization of the circadian system during aging. We assessed properties of their peripheral circadian clocks in vivo by creating expression profiles of clock genes by RT qPCR. Metabolic wellbeing of experimental animals was assessed by IPGTT tests and by determining the levels of expression of genes linking circadian clock with metabolism. Furthermore, we studied the aging clock in vitro using organotypic explants from SCN, pancreas and lungs of genetically modified mice expressing clock gene PER2 fused with gene for luciferase enabling us to detect bioluminescence rhythms of the clock. Additionally, synchronization capacity of peripheral clocks was tested in vitro by repeated 8-h treatments.

We revealed that the exposure to LL worsened age dependent decline in behavioural rhythms. The ability to produce robust Per2\textsubscript{LUC} bioluminescence rhythms in vitro in SCN explants was not compromised under any of applied light regimes. The results also revealed tissue-specific effects of aging on the rhythmic production of Per2\textsubscript{LUC} protein. Aged pancreas showed robust oscillation of circadian clock; however, pancreas exhibited high sensitivity to in vivo lightning conditions, which did not improve after providing rhythmic signal in vitro. On the other hand, aging significantly affected the period of the clock in the lungs under all lightning conditions and the LL exposure did not have effect on the clock in the lungs.

Our aged mice developed hyperinsulinemic hypoglycaemia which was likely due to the Pclo-mediated insulin hyper-secretion together with Slc2a2-mediated glucose transport impairment in pancreas. Pp1r3c-related glycogen storage and Sgk1-related glucose transport in the liver were also impaired. Expression of clock genes in pancreas was only marginally affected by aging, it upregulated the expression of BMAL1 and downregulated the expression of CLOCK.

Altogether, our results demonstrate that since the SCN-driven locomotor activity was clearly affected by aging and the molecular circadian core clock mechanism was relatively resilient to aging, possibly the pathways responsible for this phenomenon are downstream of the core clock mechanism of SCN. We also demonstrated, that aging does not compromise pancreatic clock, but significantly affects formal properties of the in vitro circadian clock in the lungs. Our findings provide a possible explanation for previously demonstrated relationship
between disturbance of the circadian system and disordered glucose homeostasis. This includes diabetes mellitus type 2 in subjects exposed to long-term shift work.

**Key words:** circadian clock, aging, pancreatic clock, SCN, Per2\textsuperscript{Luc}, T2DM
Abstrakt:

Mnoho procesů v savčím organismu probíhá v cirkadiánních rytmech. Tyto rytmy jsou ovládány systémem složeným z hlavního pacemakeru udávajícího rytmus, suprachiasmatických jader (SCN) v mozku, který dále nastavuje periferní oscilátor v dalších orgánech, jako je slinivka, játra, střevo a plíce. Cirkadiánní hodiny fungují autonomně v každé buňce na základě molekulárních mechanismů složených z tzv. hodinových genů, například BMAL, CLOCK, PER a CRY. Narušení fyziologických funkcí savčího organismu v důsledku stárnutí, například narušení metabolicích funkcí a chování, bylo již dobře zdokumentováno. Nicméně, ještě nebylo plně objasněno, zda je toto narušení spojeno i se zhoršenou funkcí cirkadiánních hodin.

Cílem naší studie bylo zjistit zda i) stárnutí ovlivňuje základní vlastnosti cirkadiánních hodin v SCN a periferních orgánech, zejména ve slinivce, tlustém střevu, játrait a plicích, ii) změny glukózové homeostázy v důsledku stárnutí ovlivňují cirkadiánní hodiny ve slinivce a iii) zda je citlivost cirkadiánních hodin v SCN a periferních hodinách ke změnám světelného režimu v prostředí ovlivněna stárnutím.

V našich experimentech jsme použili skupinu dospělých (9 měsíců) a starých (25 měsíců) zvířat, které byly vystaveny třem různým světelným režimům, konkrétně režimu světlo/tma (LD 12:12), stálému světlu (LL) a stálé tmě (DD), abychom zkoumali synchronizaci cirkadiánního systému ve stárnutí. Vlastnosti periferních cirkadiánních hodin in vivo jsme zkoumali tak, že jsme vytvořili expresní profily hodinových genů pomocí RT qPCR. Metabolický stav experimentálních zvířat byl zjišťován pomocí IPGTT testů a také určením hodnot expresí genů spojujících cirkadiánny hodiny s metabolismem. Dále jsme studovali stárnoucí hodiny in vitro za použití organotypických explantátů z SCN, slinivky a plic geneticky modifikovaných myší exprimujících hodinový gen PER2 fúzovaný s genem pro luciferázu, který nám umožňuje detekovat biolumíniscenční rytmy hodin. Synchronizační kapacita periferních hodin byla testována in vitro opakovanými 8h ošetřeními.

Zjistili jsme, že vystavení zvířat stálemu světlu ještě více zhoršilo rytmy v chování u starých jedinců. Schopnost produkce robustní Per2LUC bioluminiscenční rytmy in vitro v explantátech z SCN ale nebyla změněna ani při jednom z použitých světelných režimů. Výsledky také ukázaly, že vliv stárnutí na rytmickou produkci Per2LUC proteinu je závislý na druhu tkáně. Slinivka ze starých jedinců vykazovala robustní oscilace cirkadiánních hodin, nicméně právě slinivka byla nejvyšším cirkadiánně vlivem podmínkou, což se nezlepšilo ani po rytmickém signálu in vitro. Na druhou stranu, stárnutí signifikantně ovlivnilo periodu hodin v plicích ve všech použitých světelných režimech, ale LL na hodiny v plicích vliv nemělo.

U našich starých myší se rozvinula hyperinsulinemická hypoglykémie, což bylo nejspíše kvůli hyper-sekreci insulínu přes Pclo, dohromady s narušeným transportem glukózy prostřednictvím Slc2a2 ve slinivce. V játrech byl také narušen mechanismus ukládání glykogenu spojený s Pp1r3c a také transport glukózy spojený s Sgk1. Exprese hodinových genů ve slinivce byla jen minimálně ovlivněna stárnutím, došlo ke zvýšení expresie BMAL1 a ke snížení expresie CLOCK.

Celkem vzato, naše výsledky ukazují, že ačkoliv pohybová aktivita ovládaná SCN byla jasně ovlivněna stárnutím, centrální molekulární mechanismus cirkadiánních hodin byl relativně odolný ke stárnutí, tedy dráhy zodpovědné za tento fenomén jsou pravděpodobně dále od centrálního molekulárního mechanismu v SCN. Dále jsme také ukázali, že stárnutí nenarušuje hodiny ve slinivce, ale signifikantně ovlivňuje vlastnosti in vitro cirkadiánních hodin v plicích. Naše zjištění poskytují možné vysvětlení dříve zjištěných souvislostí mezi narušením
cirkadiánního systému a nefunkční homeostázou glukózy v savčím organismu. Tento fenomén zahrnuje diabetes mellitus typu 2 v jedincích dlouhodobě vystavených práci na směny.

**Klíčová slova:** cirkadiánní hodiny, stárnutí, slinivka, pankreatické hodiny, SCN, Per2<sub>LUC</sub>, T2DM
1 Introduction

The rotation of planet Earth around its central axis causes a daily rhythm in environmental factors; therefore, organisms developed endogenous clock mechanism to anticipate regular changes in the environment. The proper anticipation of upcoming events provide valuable signal, e.g. about the availability of nutrients, and other vital parts of life. These inner clocks are ticking with period \( \approx 24 \) hours in order to maintain the flexibility to adapt to changing conditions during the evolution of life on Earth; thus the inner clocks are called circadian from Latin circa, meaning approximately, and diem, meaning day.

Circadian clock temporally drives physiological, metabolic and behavioural processes of the body, such as rhythms in activity, hormone secretion, temperature, sleep/wake cycles etc. Endogenous nature of the clock was revealed in constant, nonperiodic conditions, where the rhythms still persisted (Aschoff et al. 1971). The period of endogenous rhythms is corrected in natural conditions to 24h cycles to ensure synchronicity with solar day. The synchronizing cue, so called Zeitgeber, is mainly light/dark cycle, but there are also nonphotic cues, such as timing of food intake, social interactions, physical activity or chemical substances (reviewed in Green et al. 2008).

Circadian clocks are based on auto-regulatory feedback loops at the cellular level employing transcription, translation and post-translation mechanisms to produce rhythmic oscillations. Rhythms can control various processes in the cell, e.g. cell cycle, DNA damage, energetic metabolism or epigenetic regulation (reviewed in Takahashi 2015).

Disruptions of internal circadian clock mechanism can lead to severe defects of many functions in mammalian body. Such disruptions are one of the consequences of modern lifestyle including irregular sleeping schedule, aberrant timing of food intake, shift-work and light at night (reviewed in Roenneberg and Merrow 2016). Growing body of evidence from epidemiology studies link the increasing incidence of cardiovascular, metabolic and mental disorders with disrupted circadian system by shift work (Scheer et al. 2009; Vogel et al. 2012; Bescos et al. 2018). Several types of cancer were also linked with frequent shift work (Schernhammer and Laden 2001; Viswanathan et al. 2007). On the other hand, administration of drugs at specific times of the day, called chronotherapy, could maximize the drug effect and minimize potential harmful toxic effects (Filipski et al. 1999; Mormont et al. 2000; Granda et al. 2001, 2002; Lévi 2006; Durrington et al. 2014).

Taken together, the importance of functional circadian system for in mammals is well documented. This thesis aims to elucidate the impact of aging on molecular mechanisms of peripheral clocks. It focuses on circadian rhythms in mammals, namely in mice and rats, with emphasis on rhythmicity in gastrointestinal tract (GIT) and pancreas. Further knowledge of internal mechanisms of GIT circadian rhythm synchronization may lead to more effective treatments of GIT disorders, e.g. diabetes mellitus type 2.
2 Overview of the literature

2.1 The organization of circadian system in mammals

Circadian system in mammals consist of central clock located in suprachiasmatic nuclei (SCN), at the base of the hypothalamus above the optic chiasm in the brain, and peripheral oscillators located in various tissues, e.g. lungs, heart, liver, GIT, pancreas and kidney (Ralph et al. 1990; Dibner et al. 2010).

The SCN is the main driver of behavioural rhythms in mammals and when absent, animals lose ability to endogenously generate rhythms in locomotor activity (Stephan and Zucker 1972). Each rodent suprachiasmatic nuclei is composed of approx. 10 000 neurons and glia able to generate endogenous rhythms and ensure its proper synchrony (Welsh et al. 1995; Brancaccio et al. 2014). The dominant cue, regularly providing information about 24h day/night cycle, is light. Light is able to synchronize the SCN through retino-hypothalamic tract channelling signal from specialized retinal ganglion cells to the SCN (Moore et al. 1995; Schmidt et al. 2011). The SCN then send the signal further to other brain areas, e.g. striatum, and other parts of the body (reviewed in Mendoza and Challet 2009). The synchronization signal from the brain then propagates through neuronal pathways, employing autonomous nervous system sympathetic as well as parasympathetic (Bartness et al. 2001; Kalsbeek et al. 2008), and also through humoral pathways by rhythmic production of hormones, e.g. adrenocorticotropin, prolactin, oxytocin and antidiuretic hormone (Salvador et al. 1988; Forsling et al. 1998; Balsalobre et al. 2000; Egli et al. 2004; Oster et al. 2006). The exact mechanism of peripheral clock synchronization by the SCN is not yet fully elucidated, even though there are many studies addressing this topic. Peripheral oscillators located in various tissues, namely in lungs, liver, kidney, GIT, pancreas, heart and muscles (Sakamoto et al. 1998; Balsalobre et al. 2000; Nagoshi et al. 2004; Yamamoto et al. 2004; Yoo et al. 2004; Wu et al. 2010), convey the actual synchronization by production of hormones in paracrine or endocrine fashion regulating gene expression and cell functions (reviewed in Dibner et al. 2010). Some of rhythmically produced hormones, e.g. corticosterone, feed back to the brain and influence their own production (reviewed in Oster et al. 2017). Central clock in the SCN sends direct synchronization signals to peripheral oscillators, as mentioned above, and indirectly by regulating behaviour and body temperature. Behavioural rhythms then indicate the rhythms in feeding and fasting, which are a very important synchronization cues for peripheral oscillators in metabolically active organs (Damiola et al. 2000; Mauvoisin et al. 2017; Sinturel et al. 2017). Body temperature is also one of the important synchronization cues for peripheral clocks (Brown et al. 2002). Schematic depiction of target areas of the SCN synchronisation is in figure 1.
As described above, the SCN drives daily feeding/fasting conditions of the organism in sync with other signals. For clocks in peripheral organs in the GIT, the regularity of feeding cycles serve as the most relevant entraining signal (for extensive review see Hatori and Panda 2015). Central clock in the SCN is not affected by food intake, e.g. at improper time of day during the inactivity period. This causes desynchrony between central and peripheral clocks (Damiola et al. 2000; Stokkan et al. 2001). This desynchrony has also systemic metabolic implications (described in detail in chapter 2.3), including elevated triglyceride levels and increase in overall adiposity (Yasumoto et al. 2016). Therefore, the internal synchrony of central and peripheral clocks seem vital for the metabolic homeostasis.

2.1.1 Circadian clock in the SCN

Behavioural processes of mammals are controlled on a daily basis by central clock based in the suprachiasmatic nuclei (SCN) based in hypothalamus (Ralph et al. 1990). The SCN sense daylight via connection to retinal ganglion cells through retinohypothalamic tract (Moore et al. 1995). SCN neurons are then entrained according to obtained signal (Meijer and Schwartz 2003). The signal from retina serve solely for synchronization with environmental conditions, the SCN are able to generate 24h rhythms intrinsically (see molecular mechanisms of the clock in chapter 2.2). Each SCN is composed of heterogeneous cell population and the synchrony is achieved via precise cell-cell communication signals of pacemaker neurons (Doi et al. 2011) (for review see Welsh et al. 2010). Individual cells exhibit different levels of autonomous rhythmicity and responsiveness to external stimuli. Pacemaker neurons, i.e., neurons setting the rhythms, express various neurotransmitters. When the communication among the individual oscillators in cells is disrupted, the oscillators uncouple and the output circadian rhythm is lost (Harmar et al. 2002; Yamaguchi et al. 2003; Aton et al. 2005; Maywood et al. 2013). Not only the
communication among individual cells is important, but also the signalling integrity among specific cellular subpopulations. The communication is multidirectional between regions in the SCN, especially between the core and the shell of each nuclei. The adaptation to naturally occurring changes in lightning condition during the year seem to employ this communication to respond to changes in environment (Sumova et al. 1995; Sumová et al. 2003; Hazlerigg et al. 2005; Sosniyenko et al. 2009; Coomans et al. 2015). Upon exposure to constant light during whole 24h of the day, the rhythmicity of neuronal activity marker cFos is abolished in the entire SCN apparently due to the desynchrony among the SCN core and the shell regions (Sumová and Illnerová 2005). The drastic change of lightning regime by exposing an organism to constant light profoundly affects the amplitude of behavioural clock output, namely in locomotor activity. The period of locomotor activity in rats lengthens and after approximately 4 weeks, the animals exposed to constant light become arrhythmic (Nováková et al. 2010; Polidarová et al. 2011; Houdek et al. 2015).

The rhythmic signal from the SCN is consequently conveyed to the peripheral clocks via multiple pathways. Hereby synchronised peripheral clocks then drive tissue-specific circadian rhythms (Peirson et al. 2006). Desynchrony leading to lack of the signal from central oscillator in the SCN not only impairs the behavioural output rhythms, but also the peripheral clocks in various tissues can be disrupted. This includes disrupted circadian rhythms in heart rate, body temperature and the liver functions, increase of visceral adiposity, melatonin levels and food intake rhythms (Warren et al. 1994; Cailotto et al. 2005; Wideman and Murphy 2009; Malloy et al. 2012).

2.1.2 Circadian clock in pancreas

The pancreas harbours the peripheral circadian clocks (Mühlbauer et al. 2004) in its exocrine part (Uchiyama and Saito 1982; Damiola et al. 2000) as well as in its endocrine (Marcheva et al. 2010) part, i.e. Islets of Langerhans (further referred to solely as Islets). Circadian clock in the Islets has been extensively studied mainly due to its role in the regulation of insulin and glucagon release from ß and α cells respectively (Kalsbeek and Strubbe 1998; Sadacca et al. 2011; Malmgren and Ahrén 2016; Petrenko and Dibner 2017). Functional circadian clocks in pancreas are crucial for its proper function in mammalian body. The reduction in the percentage of large Islets in the pancreas was found in mice with deletion of the crucial clock genes BMAL1 (BMAL1\(^{-}\)) or CLOCK (CLOCK\(^{\Delta 19/\Delta 19}\)) and this alters the ability of endocrine pancreas to produce appropriate amount of hormones regulating blood glucose (Lamia et al. 2008; Marcheva et al. 2010). The disruption of BMAL1 selectively in pancreatic β-cells caused alterations in glucose tolerance in mice in vivo and in vitro. The defect in CLOCK gene also influences a wide spectrum of genes included in formation of insulin vesicles in pancreas (Marcheva et al. 2010).

Intrinsic clocks are crucial for the regulation of insulin release from β-cells in rodents and humans (Pulimeno et al. 2013; Perelis et al. 2015; Saini et al. 2015). Proper function of Islets is crucial for the maintenance of glucose homeostasis in mammalian body. Humoral signalling pathways, also play an important role in influencing the functions of pancreas, e.g. glucocorticoids influence growth and development of acinar cells in exocrine part of the pancreas (Guthrie et al. 1991).
2.2 Molecular mechanism of the circadian clock

The endogenous circadian signal is generated at the cellular level and the rhythmicity is autonomous (Yoo et al. 2004). Circadian clock based on molecular transcriptional-translational feedback loops (TTFL) tick in all nucleated cells in mammalian body (reviewed in Partch et al. 2014; Takahashi 2016). The core of cell-autonomous molecular clock in mammals is formed by two interlocked TTFLs producing a robust ~24hour rhythm of expression. One of these TTFLs consists of two activators (Clock/Npas2 and Bmal1/2) and their repressors (Per1/2 and Cry1/2). Additional mechanism employed to sustain the rhythmicity is phosphorylation, which regulates the posttranslational localisation and stability of core proteins; therefore the important role in core TTFL is played by kinases and phosphatases (kinases: CkIα, CkIδ and CkIε; phosphatases: Pp1, Pp5) (Partch et al. 2006; Meng et al. 2008; Lee et al. 2011; Kennaway et al. 2014), for review see Reischl and Kramer 2011). Activator Clock and Bmal1 proteins together form heterodimeric basic helix-loop-helix-PAS (PER-ARNT-SIM) transcription complex Clock:Bmal, which then activates transcription of the repressor genes PER and CRY together with many other so called clock controlled genes (CCGs) which comprises of many genes included in metabolism (Bozek et al. 2009). Proteins Per and Cry then form a heterodimer, when in cytoplasm, in order to translocate to nucleus and inhibit further transcriptional activation by inhibiting Clock:Bmal (Huang et al. 2012; Ye et al. 2014). Per and Cry are gradually degraded through ubiquitin dependent pathways employing important ubiquitin ligases of Fbxl family (Buhr and Takahashi 2013; Yoo et al. 2013; Reischl and Kramer 2015). After the degradation of negative elements Per and Cry, the repression of the production of positive elements Clock and Bmal is relieved and the ~24 hour cycle can begin again (depicted in figure 2).

Intrinsic period of circadian clock is controlled by regulating the rate of the action of negative elements. Casein kinases CkIδ and CkIε control the rate of Per:Cry degradation and their entrance to nucleus. Their activity can be opposed by phosphatase Pp1 or controlled by phosphatases Pp5 (Partch et al. 2006; Meng et al. 2008; Lee et al. 2011).

Additional regulation is provided by a second TTFL through activation by retinoid-related orphan receptors (Rora, β) and repression by Rev-erbα and β. This second TTFL drives rhythmic changes in transcription of BMAL1 and also causes a delay in CRY1 mRNA expression. The delay in Cry1 production is crucial for proper timing of molecular circadian clock (Yin et al. 2010; Ukai-Tadenuma et al. 2011; Takeda et al. 2012).

The core TTFL is much more complex, than described above. Other known feedback loops are based on posttranslational modifications, which help to give more flexibility, yet robustness, to the molecular circadian clock to generate accurate timing for local physiology. In recent years, researchers found additional feedback loop within core clock TTFL which employs Cry1 driven phosphorylation of Bmal1 by Ck2 (Tamaru et al. 2015). This complexity might have raised through evolution by linking more feedback loops with limited time-telling capacity together forming a robust system of more intertwined feedback loops (Brown et al. 2012).

Important role in circadian clock molecular wiring is played by epigenetic factors. One of core genes in main clock TTFL is CLOCK encoding basically histone acetyltransferase targeting many sites of the genome (~ 1 629 genes in the liver) on so called E-boxes (Doi et al. 2006; Yoshitane et al. 2014). E-boxes consist of an upstream sequence shared by many genes including those encoding major players of metabolic pathways in the cell (Panda et al. 2002).
Figure 2: Central TTFL of molecular circadian clock in mammalian cells. Core mechanism employs two activators (Clock and Bmal1) and two repressors (Per and Cry). This feedback loop is closely linked with additional TTFL composed of Rev-erbα and Rorα influencing the transcription of BMAL1 and CRY1. Adapted from (Partch et al. 2014).

The complex mechanism of intertwined feedback loops in molecular clock in the cell is increasingly attracting the attention of groups focused on theoretical approaches. They employ modelling to raise new questions for biologists to answer, e.g. how to designate single cell oscillators self-sustainable (Westermark et al. 2009). Interdisciplinary approach employing also in silico methods, like bioinformatics and mathematical modelling, could point toward new directions of in vivo and in vitro research in chronobiology (Relógio et al. 2011; Pett et al. 2016).
2.3 Circadian clock in mammalian metabolism

Core molecular mechanism of circadian clock, comprised of intertwined TTFLs, is both driving the rhythms in metabolism of the cell, but also receiving various signals about energy homeostasis. Core clocks are able to modify its ticking according to specific needs of each cell in the body. Molecular mechanism, comprising of clock genes PER, CRY, CLOCK and BMAL, also influences the expression patterns of CCGs. These clock controlled genes serve as downstream transcription factors rhythmically switching on or off a great array of tissue specific CCGs relevant to distinct metabolic functions of affected organs (Reinke and Asher 2019). The ticking does not only influence processes on cellular level, but also acts in synchrony in whole organs. For example, in order to ensure proper functioning of digestive processes, the phase of the clock controlled expression of kinase Wee1 is slightly shifted along cranio-caudal axis of intestine according to anticipated food, suggesting important role of clock in cell cycle (Polidarova et al. 2009).

Molecular clock mechanism is robust, but the clocks are still able to respond to extrinsic cues. Energetic status of the cell, and energy intake by feeding, can influence the core clock feedback loops mechanism (reviewed in Asher and Schibler 2011). Several key points are considered as main inputs into the clock. AMP dependent kinase (AMPK), which directly influences the phosphorylation of the core-clock component Cry, is believed to be one of the key molecules mediating the interconnection of metabolism and circadian clock (Lamia et al. 2009).

One of the signals providing information channel between the central and peripheral clocks are hormones. Many hormones are produced with distinct circadian rhythmicity, such as glucocorticoids, melatonin, ghrelin, leptin, incretins, insulin and glucagon (reviewed in Challet 2015) (depicted in figure 3).

Glucocorticoids (GCs) are recognised as one of the most significant entraining cues for various peripheral clocks, including liver, kidney and heart (Balsalobre et al. 2000; Pezük et al. 2012). The most studied GC is the main glucocorticoid in rodents, corticosterone. Corticosterone shows elevated levels during active part of the day and the loss of the SCN results in loss of corticosterone rhythm (Abe et al. 1979). Glucocorticoid receptors are present almost in every cell in the body (one of known exceptions include adult SCN; ROSENFELD et al. 1988) which allows them to affect expression on many sites throughout the body.
Figure 3: Schematic depiction of the circadian timing system of nocturnal rodent. Figure emphasizes the hormonal outputs from peripheral oscillators regulated by signals from central clock in the SCN via autonomous nervous system. Synchronization inputs by brain-controlled feeding/fasting, sleep/wake cycles are depicted on the right. Peripheral clocks and secondary clocks in brain are phase controlled by central clock and rhythmically release hormones. Adapted from (Challet 2015).

The disruptions of clock mechanism can cause severe metabolic disorders (reviewed in Maury et al. 2010). These disorders include pre-diabetes state in rodents as well as in human (see in more detail in chapter 2.3.1) (Rudic et al. 2004; Scheer et al. 2009; Marcheva et al. 2010). In the modern society many common behaviours are leading to disruptions of circadian rhythmicity, including irregular high-energy meals together with impaired sleep-wake cycles. Till Roenneberg and his team introduced the term “social jetlag” implying common misalignment of inner time and the timing of our lifestyle. Therefore, our modern lifestyle forces us to be constantly in other time-zone, then our bodily needs (Wittmann et al. 2006).

2.3.1 Metabolism of glucose and circadian rhythms

Levels of glucose and associated hormones, e.g. insulin, glucagon and incretins (e.g. GLP-1 and GIP), are under tight control in order to maintain functional homeostasis in the body. Even small disharmony in the system, especially long-term, can have detrimental effects for peripheral organs as well as for brain (reviewed in Epstein 1967; Cryer et al. 2003). The incidence of T2DM in humans is rising worldwide reaching the levels of epidemics, which is often linked with irregular sleep, unhealthy lifestyle and obesity (Martins et al. 2008;
Schroeder and Colwell 2015; Grandner et al. 2016; Forouhi and Wareham 2019). Social jetlag, i.e. the misalignment of internal and external time, was found to add to the development of obesity and adjacent metabolic disorders (Roenneberg et al. 2012). Frequent disruptions of circadian system, for example due to working night shifts, has been also linked to the development of T2DM in humans (Kalsbeek et al. 2014). Changes to the schedule of food intake may also contribute to the T2DM development (Halberg et al. 2005). The shift and restriction of feeding time to different time of the day, e.g. from the day to the night, causes complete phase shift of the expression rhythms of clock genes in the liver and many other organs of the GIT including pancreas (Damiola et al. 2000; Polidarová et al. 2013).

Molecular link between circadian clock and glucose maintenance can be mediated through AMPK. Functional AMPK is vital for glucose responsiveness in pancreatic β-cells, since it reacts to changes of energy homeostasis in the cell, which can be changed by rising intake of glucose from blood (Fu et al. 2013; Grahame Hardie 2014). AMPK directly influences several key players in TTFL by phosphorylation, therefore tightly links the glucose regulation and circadian clock together (Lamia et al. 2009; Jordan and Lamia 2013).

The production of insulin, which is produced mainly upon the rise of glucose in blood, follows a low-amplitude circadian rhythm (Boden et al. 1996). Insulin production from perfused Islets of Langerhans in vitro follow circadian rhythm, even though the rhythm is very shallow (Peschke and Peschke 1998). Insulin sensitivity, i.e. the ability of cells to react properly upon insulin in blood, varies with the time of the day as well (Coomans et al. 2013). Patients suffering from T2DM and even their relatives without developed T2DM show altered rhythms of glucose tolerance (Polonsky et al. 1988; Boden et al. 1999).

Insulin is also able to feedback to the brain and influence the neuronal firing rate (Shibata et al. 1986). Recent study show the direct effects of insulin and insulin-like growth factor 1 (IGF 1) on production of Per protein, thus affecting the molecular core of the clock. Insulin and IGF1 production depends on food timing, therefore, disrupted food timing can disrupt clock also through the production of these two hormones (Crosby et al. 2019).

Glucocorticoids affect vital pancreatic functions. Their administration to cultured Islets in higher concentrations for longer period of time, mimicking chronic stress, down-regulates insulin synthesis and repeated administration of glucocorticoids can impair insulin sensitivity (Beaudry and Riddell 2012). Dexamethasone, i.e. glucocorticosteroid drug, was found to supress the glucose-dependent insulin secretion through the action of serum and glucocorticoid-inducible kinase 1 (Sgk1) (Ullrich et al. 2005). Other line of glucocorticoid effect on pancreas goes through the regulation of clock genes influencing leptin production pattern (Sinha et al. 1996; So et al. 2009).

Hormones called incretins are produced by intestinal L-cells and actively participate in regulation of blood glucose levels. Incretin glucagon-like peptide-1 (GLP-1) improves blood glucose levels by stimulating insulin secretion and is produced simultaneously with the rise of glucose in blood (Reimann et al. 2008). The secretion of GLP-1 exhibits daily rhythms with increased levels preceding feeding period (Gil-Lozano et al. 2014). Incretin oxyntomodulin plays important role in glucose homeostasis maintenance by resetting hepatic circadian clock according to daily changes in the intake of food (Landgraf et al. 2015). Other hormones, namely melatonin, can modulate the effect of incretins, e.g. by sensitizing the cAMP signalling pathways to mediate the response to GLP-1 (Kemp et al. 2002).
2.3.2 Melatonin in relation to metabolism

Melatonin is so called “night hormone” implying its production pattern. Its production by pineal gland is controlled by the SCN and occurs rhythmically with low levels during the day and high levels during the night (Illnerova and Sumova 1997). This hormone also provides a signal of the seasonal time according to the length of the photoperiod (Illnerova and Vaněček 1980). Melatonin is synthesised from L-tryptophan originating from serotonin. The main regulation point of melatonin production is aralkylamin N-acetyltransferase (AANAT) which activity is regulated by signals from the SCN. Therefore, the light provides direct inhibition cue to the production of melatonin (Illnerova and Sumova 1997; Zawilska et al. 2009). Insufficient levels of melatonin in night, for example due to light at night mostly in cities, might be linked with elevated risk of developing several diseases, e.g. supporting the tumour growth (Blask et al. 2014). Melatonin is often denoted as a “universal cure” for many diseases of affluence, including cancer or T2DM. This is mainly due to its role as a scavenger of free radicals and its role in influencing the signalling pathways in many different cell types (Hardeland et al. 2011; Reiter et al. 2014). In the exocrine pancreas, melatonin affects excretion of pancreatic enzymes and might possibly protect exocrine pancreas against pancreatitis (Nawrot-Porąbka et al. 2013; Jaworek and Leja-Szpak 2014).

Melatonin signal is likely involved in entrainment of the clock in GIT acting mainly through two membrane receptors (Reppert et al. 1996). In the intestine, melatonin is produced by enterochromaffin cells of the mucosa influencing the motility of the intestine (Thor et al. 2007). The role of melatonin in pancreatic functions is still not fully clear, several studies addressed this topic; however, with conflicting results. Some studies refer to melatonin as the suppressor of insulin production (Rasmussen et al. 1999; Wolden-Hanson et al. 2000), other studies contradict the role of melatonin in insulin production (Bizot-Espiard et al. 1998). As noted above in this chapter, some studies credit melatonin to play a protective role against pancreatitis (Carrasco et al. 2014). According to study from Conti and Maestroni, melatonin could even suppress the diabetes mellitus type 1 (Conti and Maestroni 1998).

Pancreas harbours both melatonin receptors and the loss of melatonin signalling to pancreas affected also insulin secretion (Peschke et al. 2007; Mühlbauer et al. 2009; Stebelová et al. 2010). Furthermore, melatonin receptors exhibit altered expression patterns in pancreas of patients suffering from T2DM (Peschke et al. 2007). Common variant in melatonin receptor gene MTNR1B is considered a risk factor for T2DM, carriers show impaired insulin secretion (Lyssenko et al. 2009). This MTNR1B variant causes higher expression of melatonin receptor in pancreas and is linked with higher possibility of metabolic disorders (Mulder et al. 2009; Dupuis et al. 2010; Zhang et al. 2014; Mulder 2017). However; even in vivo studies with perfused pancreatic islets are not consistent in the role of melatonin on insulin secretion. One of the studies by Peschke indicates the phase advance of insulin secretion in isolated rat β-cells upon the melatonin administration (Peschke and Peschke 1998), on the other hand different study contradicts this effect (Frankel and Strandberg 1991). The possible effect of melatonin, studied in rat pancreatic cell lines INS-1, is through melatonin receptors influencing the levels of cAMP regulating the signalling pathways in β-cells (Peschke et al. 2002; Bazwinsky-Wutschke et al. 2012). Lowering of cAMP levels may lead to the decrease of insulin production (Kemp et al. 2002). Most of the results, crediting melatonin with effects on insulin production, are from studies of one laboratory, so the functioning of these pathways remains to be confirmed.
2.4 Aging of the circadian clock

Aging of mammals is often accompanied by aggravation of some of its functions. Elderly subjects also often suffer from various diseases, such as diabetes mellitus type 2. Since the circadian clocks are vital for proper functions of human body, the aging of circadian clock has to be taken into account. While studying the cohorts of aged subjects, in order to get unbiased results, it is important to closely inspect various variables, e.g., lifestyle, amount of exercise and amount of visceral fat, between young and elderly and take them into account (Hood and Amir 2017). Chronotype, i.e., the individual preference of sleep and wake time, changes with age, tending to point to earlier awakening time and shorter activity period in older individuals (Foster and Roenneberg 2008). Although, when being a lark as a young, i.e., preferring early waking hours, it only gets more robust in older age (Broms et al. 2014). Rhythms in body temperature was found to be equally robust in young and in elderly showing lower amplitudes with increasing age (Czeisler et al. 1999). Melatonin production is tightly linked with body temperature regulations since it directly influences it (reviewed in Foster and Roenneberg 2008). Total melatonin levels are lower in older individuals and also the peak of its production tends to be shifted towards earlier time of the day (Touitou et al. 1981; Kennaway et al. 1999; Zhdanova et al. 2011). Example outputs of circadian rhythms in young and elderly are summarised in figure 4. However, the mechanisms of aging clock have not been fully resolved yet.

![Figure 4: Scheme of example output rhythms of circadian clock in younger and older adults depicted in blue and red respectively. Circadian rhythms, including waking activity, temperature control, SCN neuron firing rate, cortisol, melatonin and glucose levels, exhibit lower amplitudes in older individuals compared with younger. Adapted from (Hood and Amir 2017).](image)

The communication between individual cellular oscillators in the SCN is vital for its proper function in driving the output rhythms. The disruption of such communication can lead to the loss of the synchrony at the cell population level (Harmar et al. 2002; Yamaguchi et al. 2003; Aton et al. 2005). Aged SCN loses the ability to drive the output rhythms at systemic behavioural level (Valentinuzzi et al. 1997) and also at the level of neuronal activity (Farajnia et
Phase-synchrony of the SCN cells at tissue level is disturbed in aged rodent brain (Farajnia et al. 2012). Neural activity rhythms at the main SCN output site, leading to PVN, are degraded, possibly causing the disruptions in behaviour, and this occurs before any disruptions in key components of molecular clock (Nakamura et al. 2011). The period of free running rhythms in behaviour of aging hamsters and deer mouse is shorter and declines systematically alongside the age (Pittendrigh and Daan 1974). On the other hand, the period of circadian rhythms in common mouse tend to be longer in aged animals (Valentinuzzi et al. 1997). The re-entrainment to LD cycle after constant darkness was found to be slower in aged animals compared with young. The adjustment to seasonal changes in photoperiod tend to be different and slower in aged animals too (Scarbrough et al. 2017). Adjustment of circadian rhythms after light pulses is also impaired in aged animals (Turek et al. 1995). Aged animals show the fragmentation of output rhythms (Farajnia et al. 2012). The rhythms of clock ex vivo in the aged SCN exhibit lower amplitudes compared with young ones (Nakamura et al. 2015). Recent literature also hinted the role of PER1 expression regulation by epigenetic modifications in age-related changes of hippocampal memory formation (Rhee et al. 2018).

Effect of aging on internal synchrony of circadian clocks in peripheral organs has not yet been fully elucidated. However, there is some evidence hinting at the clock decline, e.g. the sympathetic tonus is attenuated in aged animals (Tahara et al. 2017). Tissue-specific effects of aging were reported in vitro, using transgenic PER1LUC rats (Yamazaki et al. 2002). Aging was found to be linked with altered synchronisation of peripheral clocks in tissue-dependent manner, which might be due to the disrupted SCN output systems (Sellix et al. 2012). Liver transcriptome was recently shown to be acetylated in a cyclic fashion and reprogrammed in aging (Sato et al. 2017).

Organs of GIT are vital for proper utilization of nutrients from food. Physiological properties of GIT organs change with age. Exocrine pancreas weight decreases with age and it was described to be prone to defects, e.g. acinar cell degranulation, in aging; however there is lacking evidence of decreased exocrine functions (Majumdar et al. 1997; Chantarojanasiri et al. 2015). Endocrine part of pancreas also undergo morphological changes during aging. Different distribution of Islet size was found comparing young and aged subjects. Aged rat pancreas harbours increased number of large islets compared to young rats, in addition, small Islets tend to loose ability to rapidly respond to glucose even in humans (Hellman 1959; Chen et al. 1985). Larger Islets exhibit higher insulin secretion rate, which may represent compensatory mechanism to maintain functional secretion capacity of insulin throughout aging (Kitahara and Adelman 1979).

The literature referring to overall insulin levels in blood during aging is not following a uniform view on whether it changes during lifespan or not. As Adelman noted in his review from 1989, this can be caused by numerous factors complicating the interpretation of data including the heterogeneity of the distribution of Islets in pancreas. He also questions the extent to which it is possible to evaluate the secretion of insulin by measurements of hormone levels in peripheral blood (Adelman 1989). The results concerning insulin levels and glucose tolerance heavily depend on the type of glucose administration, such as the oral administration of glucose gives low specificity and sensitivity of resulting insulin levels (Chang and Halter 2003). Also, when increasing insulin levels in aged subjects are correlated to visceral fat or other factors such as diet, there appears to be no significant changes during aging (DeFronzo 1981; Reaven
and Reaven 1985; Kohrt et al. 1993; Shimizu et al. 1996; Imbeault et al. 2003; Basu et al. 2003). Different insulin secretion in aging might also be caused by enhanced somatostatin actions or higher glucagon levels found in aged subjects (Klug et al. 1979; Casad and Adelman 1992). Aging in all studied models (humans, rats and mice) is associated with impaired glucose tolerance, insulin resistance and altered pancreatic secretory capacity. Whether and/or how these changes might be caused by aging per se, rather than other factors like body composition, diet or level of physical activity, is not clear (Evans and Farrell 2011).

2.5 Rationale of this thesis

Based on thorough literature research we found the gaps in the understanding of aging mechanisms of central and peripheral circadian clock. It was still poorly understood, if the defects in output rhythms are caused by malfunctioning molecular clock in individual cells of the SCN, by non-functional communication between cell subpopulations or by the defects in the signal output pathways. Therefore, we designed a set of experiments to study the signal integrity within the SCN. We used genetically modified Per2LUC mice to study the properties of aged SCNs in vitro. We wished to answer the question whether the ability to generate rhythmic signal at the level of the entire nucleus is affected by age.

None of the previous studies addressed aging specifically in the pancreas, therefore, we designed experiments to advance our understanding of it. We used Per2LUC mice to reveal the effects of aging and we also tested glucose levels maintenance in those animals. In order to study functional properties of pancreatic clock in vitro, we aimed to develop a sustainable model of organotypic explant cultivation also to study the responsiveness of aged clock. Because the pancreatic clock is under control of the central clock, we also tested the effect of the disruption of central clock. These experiments were aimed at answering the question whether the rhythmic environment contributed to the functioning of pancreatic clock in aged individuals and whether pancreatic clock itself changed with age.
3 Aims of the thesis

- To set up the long-term cultivation of pancreatic explants in vitro
- To characterize ability to generate circadian signal in central SCN clock of aged animals
- To characterize peripheral clocks in peripheral tissues of aged animals
- To test the hypothesis that the exposure to constant light affects clocks in aged animals
4 Methods

4.1 Experimental animals

Our laboratory maintains the breed of genetically modified Per2\textsubscript{LUC} mice (strain B6.129S6-Per2\textsuperscript{tm1Jt/J}, JAX, USA) (Yoo et al. 2004) which were used for in vitro experiments. Briefly, these mice produce Per2 protein with linked luciferase; therefore, every dissected part of the mouse can show the production of Per2\textsubscript{LUC}, when luciferin is administered.

Male and female Per2\textsubscript{LUC} mice were maintained in a light/dark cycle consisting of 12h of light and 12h of darkness (LD 12:12), with lights on at 6:00 and off at 18:00. Light was provided by overhead 40W fluorescent tubes and the illumination was approximately 500 lx depending on the exact cage position in the animal room. Mice were housed separately. The animal facility was temperature controlled with 21 ± 2°C temperature range. Mice had free access to food and water throughout the experiment. For consistency, the time in all experiments is expressed as relative circadian time, i.e. circadian time 0 (CT0) corresponds to lights on and circadian time 12 (CT12) corresponds to lights off during the LD regimen.

To test the effect of different light regimes on adult and aged animals, the light regimen was changed. At various ages, experimental mice were subjected to constant light (LL) or constant darkness (DD) regimen, i.e. lights were either left on or off respectively. When experimental mice reached desired age, they were exposed to LL regimen for up to 9 months. Mice up to 8-13 months of age were designated as “adult” and mice 24-26 months old were designated as “aged” group.

To study the gene expression \textit{in vivo}, Wistar rats (Bio Test Konarovice, Czech Republic and Institute of Physiology, Czech Academy of Sciences) were used and designated into two groups according to their age: “adult” (6 months old) and “aged” (22-26 months old).

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

4.2 Locomotor activity monitoring

To test the behaviour of experimental animals, hereby the function of circadian aspects of locomotor activity. The locomotor activity of experimental animals was monitored as described previously in our study (Polidarová et al. 2011). Briefly, animals were maintained individually in cages equipped with infrared movement detectors. These detectors were attached above the centre of the top of the cage. Signals were gathered each minute through circadian activity monitoring system (Dr. H.M. Cooper, INSERM, France). The data were analysed using the ClockLab toolbox (Actimetrics, Wilmette, IL, USA) to generate double-plotted actograms and chi-squared periodograms to evaluate the activity and calculate its period and amplitude (power of the period estimation).
4.3 Glucose tolerance test and insulin ELISA

To assess the functional metabolic state of aged animals we performed intraperitoneal glucose tolerance test (IPGTT) on adult and aged Per2Luc mice (Ayala et al. 2010). Mice were fasted for 3 h before the IPGTT (Andrikopoulos et al. 2008). Mice were injected with glucose solution (2g/kg b.w., i.p.) and blood glucose was measured by glucose meter (Glucolab, Infopia Co.Ltd, Korea) before the injection (basal) and after 15, 30, 60, 120 and 180 minutes after the injection to see the glucose clearance rate. To describe overall glucose clearance rate, incremental area under curve (AUC) was calculated from glucose levels over time (Floch et al. 1990). To assess basal insulin levels, blood was drawn before the glucose injection after 3 h fasting. Insulin levels in plasma were assessed by ELISA (BioVendor, Brno, Czech Republic). All IPGTT tests were performed at noon.

4.4 Collection of tissue samples from in vivo experiments

Animals were released into constant darkness (DD) on the day of sampling and were sacrificed every 4 hours during 24h cycle starting at circadian time 0 (CT0). CT0 corresponded to the time of lights on in previous days. Rats were euthanized under deep anaesthesia, which occurred within < 1-2 minutes after the thiopental injection (50mg/kg b.w., i.p.). At each time point (consisting of 7 time points per day) 4-5 rats were sampled and liver, lung, pancreas and colon were collected.

The samples of liver and lungs were immersed immediately into RNAlater stabilisation reagent (Sigma-Aldrich, St. Luis, MO, USA). The procedure of colon epithelial cells sampling was confirmed previously (Sládek et al. 2007b). Briefly, distal colon sample was rinsed with phosphate-buffered saline and cut longitudinally to scrape the mucosa layer rich in epithelial cells, which were immediately immersed in RNAlater stabilisation reagent. The samples were stored in RNAlater in the freezer at -20°C until RNA isolation. The pancreatic tissue was homogenised, immediately after the sampling, using ceramic beads (1.4mm, MoBio Laboratories; Magna Lyser, Roche) in isolation buffer with β-mercaptoethanol according to manufacturer’s instructions (GenElute™ Mammalian Total RNA Miniprep Kit; Sigma-Aldrich) to ensure immediate inactivation of digestive enzymes.

4.5 Real-time RT-qPCR

Samples of liver, lung and colon were homogenized by ultrasound sonication or by mechanical disruption in isolation buffer (Sigma-Aldrich). Total RNA was purified from all homogenates using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) according to the manufacturer’s instructions. Concentrations of isolated RNA were determined by Nanodrop spectrophotometry (ThermoFisher, Waltham, MA, USA) at 260 nm. The quality of RNA was assessed by electrophoresis on a 1.5% agarose gel; moreover, randomly selected samples of isolated total RNA were tested for integrity using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

The real-time RT qPCR method was done as described previously (Sládek et al. 2007a). Briefly, 1µg of isolated total RNA was reverse transcribed using the High Capacity cDNA RT kit (ThermoFisher) according to manufacturer’s instructions. Diluted cDNA (2µl) was then
amplified on the LightCycler480 in 14µl reaction containing SYBR Select qPCR Master Mix (ThermoFisher) and corresponding primers. Primers were designed as intron-spanning in our laboratory using LaserGene PrimerSelect program (DNAStar, Madison WI, USA), designed using online NCBI’s tools for primer design (NCBI, Bethesda MD, USA) or ordered from a commercial vendor (Sigma KicqStart primers). Primer sequences can be found in chapter 4.5.1. Relative quantification of cDNA concentration was calculated using ΔΔCt method (Pfaffl 2001). The measured gene expression of selected genes was normalised to the mean relative expression of two house-keeping genes, 62-microglobulin (B2M) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for colon, lungs and liver and TATA binding protein (TBP) together with ribosomal protein S18 (RPS18) for pancreas. These reference genes were proven to be stable throughout the 24h and were used in our previous study (Polidarová et al. 2017a). Samples of compared groups (adult and aged animals) were assayed in the same real-time RT qPCR run.

4.5.1 Primers

<table>
<thead>
<tr>
<th>Primers for house-keeping genes</th>
<th>sequence</th>
</tr>
</thead>
</table>
| GAPDH - NM_017008                | Forward: 5’ – TGATTCTACCACGGCAAG – 3’  
Reverse: 5’ – TGATGCGTTCCTCATTGAT – 3’ |
| RPS18 - NM_213557.1             | Forward: 5’ – ACTGCATTAAGGGTGGTGC – 3’  
Reverse: 5’ – GTCAAGAGATTTGTATTGTC – 3’ |
| B2M - NM_012512                 | Forward: 5’ – CGCTCGGGTGAC-CGTGATTTTCTG – 3’  
Reverse: 5’ – CTGAGGTGGGGAACCTGACAGC – 3’ |

*Table 1:* sequences and GenBank accession numbers of primers used in RT qPCR in this thesis for normalisation of clock gene expression profiles during the day.

<table>
<thead>
<tr>
<th>Primers for metabolic or clock-controlled genes (CCGs)</th>
<th>sequence</th>
</tr>
</thead>
</table>
| PCLO - NM_020098.1                                     | Forward: 5’ – GATCGATTCAGAAGAAAGATG – 3’  
Reverse: 5’ – CCCAGATCGACCTCAGCTAAGC – 3’ |
| PDX1 - NM_022852.3                                    | Forward: 5’ – TCATCTCCCTTTCCGGTGGAT – 3’  
Reverse: 5’ – TATTCTCTCTGCTGTGCTG – 3’ |
| DBP - NM_012543                                       | Forward: 5’ – GCTAATGACCTTTGAACC – 3’  
Reverse: 5’ – AGTACTTCTCATCTCCTGTT – 3’ |
| NAMPT - NM_177928                                     | Forward: 5’ – CTTTGGTTCATGCGCTGTAC – 3’  
Reverse: 5’ – GCCGCCCTTTTGCACTGT GG – 3’ |
| HIF1A - NM_024359                                     | Forward: 5’ – AGCGGCTGGGGGAGACATG – 3’  
Reverse: 5’ – TGCCGGCTTTTGCACTGTGG – 3’ |
| SGK1 - NM_001193569.1                                 | Forward: 5’ – AAGAAGATACGCCCCCATTTA – 3’  
Reverse: 5’ – AGGGCTCCGGGTCATGCAAA – 3’ |
| PP1R3C - NW_047565                                    | Forward: 5’ – CCGCTAAATGCGCTGCGAC - 3’  
Reverse: 5’ – GGGGTGATTGGATGTGTCAC – 3’ |
| E4BP4 - NM_053727                                     | Forward: 5’ – ACCGGTGTAAGGGGCGTGAGT – 3’  
Reverse: 5’ – GTGGCGGTGGGGTGTGCTC – 3’ |

*Table 2:* sequences and GenBank accession numbers of primers used in RT qPCR in this thesis to assess metabolic gene and CCG expression profiles during the day.
### Primer for clock genes

<table>
<thead>
<tr>
<th>Primer for clock genes</th>
<th>sequence</th>
</tr>
</thead>
</table>
| **PER1** - AB002108    | Forward: 5’ – CGCACTCAGGGAAGCTCAAACCTC – 3’  
Reverse: 5’ – GTCCATGCTACAGGGCTCAC  – 3’ |
| **PER2**- NM_031678    | Forward: 5’ – CACGCAACGGGAGTGACATCACAC – 3’  
Reverse: 5’ – CAAGGGAGGCTGCGAACEACAT – 3’ |
| **REV-ERB α** - NM_145775 | Forward: 5’ – GCTGTGCGGGAGGTACATCACAC – 3’  
Reverse: 5’ – TGTAGGGTGTGGCTGAGGAA – 3’ |
| **BMAL1** - AB012600   | Forward: 5’ – CAATGCGATGTCCCGGAAGTTAGA – 3’  
Reverse: 5’ – TCTTCTCGGTCTGACATCTTCTC – 3’ |
| **CLOCK** - NM_021856  | Forward: 5’ – TTCGATCACAGCCCAACT  
Reverse: 5’ – ACCTCCGCTGTGCTCAGGAA – 3’ |
| **CRY1** - NM_198750   | Forward: 5’ – GTGGTGGGAGGGCTCAGT – 3’  
Reverse: 5’ – ACTCTGTTGCGTCTTCTGTA – 3’ |

Table 3: sequences and GenBank accession numbers of primers used in RT qPCR in this thesis assess circadian gene expression profiles during the day.

#### 4.6 Organotypic explants preparation and real-time bioluminescence monitoring

Adult and aged Per2LUC mice were sacrificed between 12:00 and 13:00, i.e. CT6 – CT7, via rapid cervical dislocation under isoflurane anaesthesia. Their lung and pancreas were removed, rinsed thoroughly in cool HBSS medium (Sigma-Aldrich, St. Luis, MO, USA) and cut into approximately 1x1 fragments. Individual explants, approximately 6 per animal, were then placed onto Millicell Culture Inserts (Merck, Darmstadt, Germany) and inside Ø35mm Petri dishes each with 1ml of air-buffered recording medium (Yamazaki and Takahashi 2005) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 10 µg/ml gentamicin, 1x GlutaMAX (ThermoFisher), 15% foetal calf serum (Sigma) and 0.1 mM Luciferin-EF (Promega, Madison, WI, USA). Organotypic explants of pancreas were pre-incubated for 1.5h in 1ml of air-buffered recording medium to ensure throughout rinse of digestive enzyme, which could possibly prevent long-term cultivation. Pancreatic explants were then placed onto fresh recording medium (on the same Millicell Culture Insert).

The brains of experimental mice were removed and 250-µm-thick slices of the SCN were sectioned in ice-cold HBSS medium (Sigma-Aldrich) using a vibratome (Leica, Wetzlar, Germany). Two explants containing the SCN from each brain were prepared and then individual explants were placed onto Millicell Culture Insert (Merck).

The dishes containing organotypic explant on Millicell Culture Insert (Merck) were sealed with vacuum grease and glass coverslips and placed inside a LumiCycle apparatus (Actimetrics, Wilmette, IL, USA) for bioluminescence recording. In all experiments presented in this thesis, the recording of bioluminescence started immediately after the culturing of the explants.
4.6.1 The treatment of in vitro organotypic explants

In a separate experiment designed to test the entrainment of the circadian clock in vitro to external cues, the explants were exposed to in vitro treatment after 3 days of bioluminescence recording. The treatment procedure of pancreatic and lung explants involved the addition of 1µl of solution [1 % ethanol/saline phosphate buffer with or without 1 mM melatonin (Sigma-Aldrich, St. Luis, MO, USA); final melatonin concentration in the recording medium was 1 µM] at 8:00 and the replacement with fresh medium 8 h later. This treatment procedure was performed in 5 subsequent days and then the explants were left in the recording medium for another 5 days to ensure robust data for analysis.

The organotypic explants of the SCN were subjected to treatment after 3 days of recording. The phosphate-buffered saline was added at 8:00 and medium was replaced with fresh medium 8h later at 16:00. The treatment was repeated for 5 subsequent days. After the last day of treatment the explants were left in the recording medium for another 5 days. SCN explants were also subjected to treatment with melatonin containing solution; however since the experiments did not show us any significant effect of such treatment, these results were omitted from this thesis.

For each of the treatments, the experiment was repeated and the data were pooled. Only the explants surviving the treatment with measurable oscillations after the treatment procedures were considered for subsequent statistical analyses (cca. 90%). The raw luminescence traces were assessed and analysed using LumiCycle Analysis software (Actimetrics).

In a separate experiment, designed to assess the dampening rate of bioluminescence rhythms, pancreatic and lung explants from adult and aged mPer2LUC mice maintained in various light regimes were dissected and left untreated in the LumiCycle apparatus for at least 5 days. The calculations of amplitudes were performed using the LumiCycle Analysis software. The amplitude of the first peak of mPer2LUC rhythm measured from each explant was set to 1 and the amplitudes of subsequent peaks were normalised to this value.

4.6.2 Measurement of insulin production from pancreatic explants

The aim was to test the viability of Islets of Langerhans inside of the organotypic explant of pancreas from Per2LUC mice during prolonged incubation. In order to test this, we used modified glucose stimulated insulin secretion (GSIS) protocol and measured the ability of Islets to respond to glucose stimuli. Organotypic explants were cultivated in air-buffered recording medium (Air-medium, for details see chapter 4.6) with 11mM glucose. After five days of cultivation the explants were transferred into Air-medium with 5mM glucose. Then, after another 24 hours, the explants were transferred into Air-medium with 20mM glucose to stimulate insulin secretion. Medium was then collected after another 24 hour period and insulin concentrations in media were assessed by ELISA (BioVendor, Brno, Czech Republic).
4.7 Immunohistochemistry of pancreas

In order to compare the spatial distribution and overall levels of Bmal1 in the pancreatic tissue slices of adult and aged rats, we performed immunohistochemistry. 12 µm thick sections of pancreas were cut in Cryostat microtome (Leica), mounted on slides, fixed in 4% paraformaldehyde in phosphate-buffered saline solution (PBS) and processed for immunohistochemistry using the standard avidin-biotin method with diaminobenzidine as chromogen (Vector Laboratories, Eterborough, UK). Used antibodies (Bmal1) were raised against the C-terminal 15 residues of mBmal1 (GLGGPVDFSDLPWPL) using the Sigma-Aldrich custom peptide antibody service, as specified previously (Reddy et al. 2006). Parallel sections were treated simultaneously through immunohistochemical procedure without the incubation with the specific 1°Ab, serving as controls. To simplify the orientation in the tissue by visualising the nuclei and overall structure of the tissue, we performed hematoxylin and eosin staining according to manufacturer’s protocols (Sigma-Aldrich, USA).

4.8 Statistical analyses and used software

Several types of data obtained from experiments are presented in this thesis. All statistical analyses were implemented in commercial Prism 6 software (GraphPad, Inc., La Jolla, CA, USA).

Analysis of in vitro profiles of Per2\textsuperscript{LUC} production in organotypic explants from Per2\textsuperscript{LUC} mice was done using LumiCycle Analysis software (Actimetrics). The data were baseline-subtracted by using the 24h running average and smoothed prior to fitting. Then the data were fitted with damped sine wave to calculate the rhythm parameters before and after the treatments, i.e. the period and amplitude of the rhythm. A dampening, i.e. the lowering of rhythm amplitude in time, was assessed according to previously described method (Nakamura et al. 2015). The dampening analysis was performed whenever at least 5 full circadian cycles were available for inspection. The data were evaluated by repeated measures and compared using two-way ANOVA with Šídák’s multiple comparison test.

Periods calculated in LumiCycle Analysis software are depicted as single values in this thesis. Group means of periods were then compared by Student’s t-test with Welch’s correction. The Mann-Whitney test was used for mean comparison when at least one of the compared groups did not pass D’Agostino and Pearson omnibus normality test (only group not passing this test was due to limited number of samples from animals held under constant light). To test the difference in variances of period values before and after the treatment we used F test. The value of p < 0.05 was required for significance; however it was adjusted for multiple comparison when relevant.

The same statistical tests, to assess the differences of group means, were applied for comparison of glucose and insulin levels in blood in vivo and also in comparison of metabolic-relevant gene expression in vivo in the liver (fig 8 and 11).

Daily profiles of clock gene expression from in vivo experiments are usually depicted as means of four to five animals ± SD per every time point, if not indicated otherwise. The circadian rhythmicity of such profiles was assessed by cosine analysis (for details see Polidarová et al.
Briefly, the data were fitted with two alternative regression models, straight line representing null hypothesis or a single cosine curve with a constant wavelength of 24h to distinguish rhythmic and non-rhythmic gene expression. The differences between groups were analysed by regular two-way ANOVA followed by the Holm-Šidák post hoc multiple-comparison test to assess individual difference between groups in each time point. The amplitudes, i.e. the difference between the trough or peak and the mean value of fitted cosine curve, in expression profiles were compared by multiple t-tests with Holm-Šidák correction. The value of $p < 0.05$ was required for significance. In figures 10B and 11B the values of amplitudes were normalized by setting amplitude from adult group as reference value of 1.

The analysis of locomotor activity was done using ClockLab plugin in MATLAB Software (The MathWorks, Inc., USA). Measured locomotor activity parameters were compared using unpaired t-tests with Welch’s correction. These tests were used in case of comparisons of 2 groups, e.g. between free-running periods, while one-way ANOVA with Šidák’s multiple comparison test was used to compare 3 or more groups.

Resulting $P$-values are depicted in figures included in this thesis by asterisks in graphs as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. In case of the results of F test, a symbol of hash mark (#) instead of asterisk was depicted following the same logic.
5 Results

5.1 Setting of long-term cultivation of organotypic explants in vitro and identification of the circadian clocks in pancreas

Long term cultivation of organotypic explants of brain and peripheral tissues for purposes of circadian biology studies has been established in many laboratories around the world including the laboratory of Dr. Sumová (Jin et al. 2003; Kováčiková et al. 2005; Asher and Schibler 2011). However, the long term cultivation of pancreatic explants for circadian studies has not been reported previously. Mice expressing circadian protein Per2 together with luciferase allowed us to observe the ticking of the circadian clock in vitro (Yoo et al. 2004).

Pancreas is a tricky organ to explant from mammalian body due to extensive amount of digestive enzymes that actively digest the tissue from the very moment of its dissection. We tested several approaches before were able to record the bioluminescence of pancreatic tissue in vitro for more than 20 days (Fig 5).

![Figure 5: Organotypic explants of pancreas from PER2\textsuperscript{LUC} mice show robust circadian oscillations sustainable for more than 20 days. (A) Representative raw bioluminescence trace of pancreatic explant from LumiCycle.](image)

One of the main features of pancreas in mammalian body is the secretion of hormones from its endocrine part. In order to test the ability of Islets of Langerhans to produce insulin in reaction to glucose in our in vitro system, we subjected the explants to modified GSIS. Organotypic explants were able to produce more insulin in response to elevated glucose concentration in cultivation media even after several days of cultivation in vitro (Fig 14B). Such system of cultivation of pancreatic organotypic explants was a useful tool in our further in vitro studies of circadian clock.
5.2 Characterization of circadian clock in adult and aged rodents

5.2.1 Effect of aging on locomotor activity and circadian clock in SCN

Our experimental Per2\textsuperscript{LUC} mice were subjected to long-term monitoring of spontaneous locomotor activity. Activity was recorded in adult (8 – 9 months old) and aged (25 – 26 months old) mice housed under LD 12:12 regimen. To assess the internal free-running period of circadian clock, mice were subjected to DD regimen for 35 days. Representative behaviour of mice in LD 12:12 and DD is depicted as actograms and periodograms together with the analysis of the records and shown in figure 6A,B.

The levels of total activity did not differ between groups of mice in LD 12:12 regimen; however, total activity levels in mice under DD were significantly lower in aged mice compared with adult (figure 6C). The mean period of free-running activity (in DD) was in adult mice 23.98 ± 0.13 h and in aged mice 23.9 ± 0.07 h, so they did not differ; however, the activity/rest ratio was significantly lower in aged mice in comparison with adult mice. Our results clearly show the impairment of behavioural rhythms in aged mice compared with young mice.

\textit{Figure 6:} Recording of spontaneous locomotor activity of Per2\textsuperscript{LUC} mice in constant darkness (DD). Double plotted representative actograms (A) and periodograms (B) for each group of mice. (C) The analyses of the locomotor activity records comparing young and adult mice. Group of aged animals is depicted in green and group of adult animals in black. Data are depicted as mean ± SD. P values of Šidák’s multiple comparison test are specified in each graph. Adapted from (Polidarová \textit{et al.} 2017b).
From each of the observed animals, the organotypic slices of SCN and also organotypic explants of peripheral organs were cultivated to study the rhythmicity in vitro. The SCN slices (2 explants per mouse) were taken after maintaining the mice in LD 12:12 for at least 14 days (adult: n = 18; aged: n = 11) or in DD for 35 days (adult: n = 5; aged: n = 5). Representative bioluminescence traces are shown in figure 7A. The analysis of Per2\textsuperscript{LUC} rhythms measured during first three cycles after culturing in vitro did not show any significant difference either in period (figure 7B) or in the amplitude of the rhythm (figure 7C) between adult and aged animals maintained in LD 12:12 or DD. Light regime in vivo did not affect the period either in adult (Šidák’s, p = 0.6843) or in aged (Šidák’s, p = 0.7089) explants in vitro. A dampening, i.e. the rate of amplitude decline in rhythms, was measured during first 5 cycles after explanting (figure 7D). There was no significant effect of aging on the dampening in LD (two-way ANOVA, p = 0.6103; F\textsubscript{1,12} = 0.27). In DD the dampening didn’t show overall difference (two-way ANOVA, p = 0.1150; F\textsubscript{1,18} = 2.74) only a slightly lower amplitude was detected in the second cycle (Šidák’s, p = 0.0232).

![Figure 7](image)

**Figure 7:** (A) the representative Per2\textsuperscript{LUC} bioluminescence traces of SCN organotypic explants from adult and aged mice. Mice were maintained in LD 12:12 and DD. Period (B) and amplitude (C) of bioluminescence rhythms. (D) Dampening of bioluminescence rhythms over 5 consequent days after explanting. Amplitudes were analysed in first 5 cycles and were normalised to the amplitude of the first cycle. Group of aged animals is depicted in green and group of adult animals in black. Data are depicted as mean ± SD. P values of t-test are specified in each graph. Adapted from (Polidarová et al. 2017b).

Taken together, the endogenous rhythms of organotypic SCN slices were not affected by the light regimen prior to explanting either in adult animals or in aged. The decrease in behavioural amplitude was not detectable in explanted SCN in vitro.
5.2.2 Aging affects pancreatic function and glucose homeostasis mechanisms in vivo

To assess the effects of aging on physiological endocrine functions of pancreas, we performed IPGTT tests (for details see chapter 4.3) in adult and aged Per2\textsuperscript{LUC} mice. Basal plasma glucose levels were lower in aged animals compared to young (p = 0.0117). On the other hand, the insulin levels were higher in aged (p = 0.0033) compared to young (figure 8A). Additionally, the IPGTT revealed slightly impaired glucose handling in aged animals (figure 8B). Glucose levels 30 minutes after glucose administration were higher in aged animals (p < 0.0001). Incremental area under the curve (AUC) was significantly higher in aged animals as well (p = 0.0045). Taken together, aging did change the response to glucose in our experimental animals.

![Figure 8](image.png)

Figure 8: The measurement of glucose handling in adult and aged Per2\textsuperscript{LUC} mice. (A) The basal levels of glucose and insulin. The basal insulin levels are expressed as relative to body weight (BW). (B) The analysis of glucose clearance rate by intraperitoneal glucose tolerance test (IPGTT). The levels of glucose in blood were measured before the glucose bolus and then after 15, 30, 60, 120 and 180 minutes. Values are normalised to basal glucose for each individual. Group of aged animals is depicted in green and group of adult animals in black. Data are depicted as mean ± SD. Adapted from (Novosadová et al. 2018).

We also analysed the expression throughout 24 h cycle of several genes regulating glucose and insulin in pancreas. The expression profiles are depicted in figure 9A. The expression of piccolo (PCLO), i.e. the gene crucial for the release of insulin containing vesicles, was upregulated in aged animals throughout the 24h cycle. Expression of pancreatic and duodenal homeobox (PDX1) gene was significantly (p = 0.0022) upregulated in aged animals, although post hoc analysis did not find significant differences at individual time points. PDX is crucial for development of pancreatic β cells and is also known as insulin promoter factor 1. Interestingly, the expression of solute carrier family 2 member (SLC2A2) gene coding glucose transporter Glut2, was downregulated by aging, although again the post hoc analysis did not find significant differences at individual time points.

Apart of genes regulating the glucose and insulin, we also assessed the expression patterns of genes linking circadian clock with metabolism of the cell (figure 9B). Maximal expression level of nuclear factor, interleukin 3 regulated (E4BP4) was not affected by aging. Similarly, the maximal expression level of D-box binding protein (DBP) was not significantly different between adult and aged animals. Thus, aging did influence the expression of genes involved in glucose metabolism, but did not affect selected genes linking circadian clock to metabolism.
5.2.3 Aging affects clock gene expression in a gene-specific and peripheral tissue-specific manners in vivo

In order to ascertain whether the impairment of glucose homeostasis in mice is linked with changes in pancreatic clock, we compared the daily expression profile of key molecular components of circadian clock PER1, PER2, BMAL1, NR1D1 and CLOCK in pancreas, liver, colon and lungs between tissues from young and aged Wistar rats (figures 10, 11). We included lungs as an example of non-metabolic tissue.

In the pancreatic tissue, aging had a significant effect on the amplitude of BMAL1 expression profile, whereas it did not show the same effect in lungs (figure 10B). Moreover, the amplitude of the expression profile of BMAL1 was also significantly elevated in other tissue linked with glucose metabolism, i.e. in the liver, of aged animals (figure 10A). However, the expression of BMAL1 in the colon did not show similar pattern. Interestingly, the expression of CLOCK was significantly downregulated in both pancreas and lungs of aged animals, even not expressing any robust rhythms in its expression (figure 10A).
Figure 10: Daily expression pattern of circadian genes in pancreas and lungs. (A) Daily profiles in relative expression detected by RT qPCR of circadian genes PER1, PER2, BMAL1, NR1D1 and CLOCK. 4 - 5 animals were sacrificed per each point. The results of the post hoc analysis and a result of 2-way ANOVA comparison between expression levels in adult and aged animals are depicted in each graph. (B) The analysis of gene expression rhythms amplitudes in pancreas and lungs. The results of a multiple comparison t-test with Holm-Šidák correction are depicted as asterisks. Circadian time 0 corresponds to lights on of the LD12:12 regimen prior to the animal sacrifice. Group of aged animals is depicted in green and group of adult animals in black. Data are depicted as mean ± SD. Asterisks indicate the results of statistical analyses *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Adapted from (Novosadová et al. 2018).

We also investigated the effects of aging on other GIT tissues, namely in the colon and in the liver (figure 11). Apart from the effect of aging on the expression of BMAL1 (as mentioned above), we didn’t find dramatic changes of clock gene expression in aged animals (figure 11A). However, the amplitude of clock genes in the liver was significantly different between adult and aged animals, although it was not following a uniform pattern (figure 11B). The amplitude of PER1 expression rhythm was significantly higher in aged animals, whereas this property of PER2 expression rhythm was lower in aged animals. Aging significantly suppressed (p = 0.008) the expression of hypoxia-inducible factor 1 (HIF1A) which is involved in clock-metabolism interactions (figure 11C). Aging also significantly elevated (p = 0.0007) the expression of protein phosphatase 1 (PP1R3C), also known as protein targeting to glycogen (PTG), which is playing an important role in metabolism. Aged animals also exhibited significantly elevated (p = 0.0312) expression of nicotinamide phosphoribosyltransferase (NAMPT) which encodes rate-limiting enzyme of NAD+, being an important part of energy homeostasis processes of each cell. Aging completely suppressed (p = 0.0007) the expression of serine/threonine protein kinase 1 (SGK1), i.e. gene encoding kinase activated by glucocorticoids and also by insulin, providing valuable input into the cellular metabolism. From studied genes linking the circadian clocks to metabolism, only the levels of E4BP4 were significantly upregulated in aged animals (p = 0.0007), whereas aging did not significantly affect (p = 0.2857) the expression of DBP.
Figure 11: Daily expression pattern of circadian genes in liver and colon. (A) Daily profiles in relative expression detected by RT qPCR of circadian genes PER1, PER2, BMAL1, NR1D1 and CLOCK. 4 - 5 animals were sacrificed per each point. The results of the post hoc analysis and a result of 2-way ANOVA comparison between expression levels in adult and aged animals are depicted in each graph. (B) The analysis of gene expression rhythms amplitudes in liver and colon. The results of a multiple comparison t-test with Holm-Šidák correction are depicted as asterisks. (C) Analysis of gene expression of metabolically relevant genes E4BP4, SGK1, HIF1, NAMPT, DBP and PP1R3C in the liver. The expression was measured in one time point – circadian time 0 for E4BP4, SGK1, HIF1, NAMPT and PP1R3C; and in circadian time 12 for DBP. The differences between groups were assessed by t-test. Circadian time 0 corresponds to lights on of the LD12:12 regimen prior to the animal sacrifice. Group of aged animals is depicted in green and group of adult animals in black. Data are depicted as mean ± SD. Asterisks indicate the results of statistical analyses *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Adapted from (Novosadová et al. 2018).

In order to study the effects of aging on circadian clock in pancreatic islets and also in exocrine part of pancreas, we performed an immunohistology on cryosections of pancreatic tissue. Representative sections, stained by haematoxylin and eosin stain and by immunohistochemistry, of pancreatic tissue are depicted in figure 12A and 12B. The analysis of Bmal1-immunopositive cells in pancreatic islets did not show any significant difference between adult and aged animals (figure 12C). Moreover, the analysis of Bmal1-immunopositive cells in exocrine pancreas did not show any significant difference between adult and aged animals either.
Figure 12: Immunohistochemistry of Per2\textsuperscript{LUC} mice pancreas. (A) Representative sections of snap frozen pancreatic tissue of adult and aged Per2\textsuperscript{LUC} mice (n = 3) stained by Haematoxylin and Eosin (HISTO) and processed by immunohistochemistry with anti-Bmal1 Ab (BMAL1). Islets of Langerhans are demarcated by black line in each picture. White bar corresponds to 100µm. (B) Immunopositive nuclei were quantified as a percentage of total nuclei number in each pancreas section. Group of aged animals is depicted in green and group of adult animals in black. Data are depicted as mean ± SD. Adapted from (Novosadová et al. 2018).

We studied the effects of aging on circadian clock in peripheral organs both in vivo and in vitro using Wistar rats for in vivo gene expression profiles and Per2\textsuperscript{LUC} mice for in vitro experiments with organotypic explants. In order to ascertain the similarity of the clock
resilience in both used animal models, we performed also in vivo analysis of the clock in peripheral organs of Per2^{LUC} mice and compared it with expression profiles of Wistar rats. The representative comparison in depicted in figure 13. We did not find any significant difference between the expression profiles of studied animal models.

![BMAL1 expression in mouse and rat pancreatic tissue](image.png)

**Figure 13:** Daily expression pattern of circadian gene BMAL1 in mouse and rat pancreatic tissue. Daily profiles in relative expression detected by RT qPCR include 4 - 5 animals sacrificed per each point. The results of 2-way ANOVA comparison is depicted in the graph. Circadian time 0 corresponds to lights on of the LD12:12 regimen prior to the animal sacrifice. Data are normalised and are depicted as mean ± SD. Adapted from (Novosadová et al. 2018).

5.2.4 Circadian clocks in pancreas and lungs in vitro show differences in their resilience to aging

Organotypic explants of pancreas and lungs were prepared from adult and aged Per2^{LUC} mice to study the properties of circadian clock in aging in vitro. Representative traces of Per2^{LUC} bioluminescence from adult and aged mice maintained in LD 12:12 regimen are depicted in figure 14A. First crucial point of our investigation was to determine, that organotypic explants of pancreas retained their ability to respond to glucose application with a significant increase in insulin in the media. Application of modified protocol of GSIS (for details see methods section) revealed a significant increase of insulin in media after administration of elevated glucose concentration into media (figure 14B). Thorough analyses of bioluminescence rhythms are depicted in figure 14C – E. The effect of aging was highly tissue – specific. Aging did not significantly affect either the period or the amplitude of the rhythms in pancreas. The rate of amplitude dampening was not significantly affected by aging either (p = 0.4502). On the other hand, aging significantly lengthened the period of the rhythms in lungs. Although the overall amplitude was not affected by aging, the amplitude dampened more rapidly (p = 0.0088). Taken together, the aging in animals maintained in LD 12:12 regimen did not affect the properties of circadian clock in pancreas in vitro whereas it negatively affected clocks in lungs.
5.3 The effect of exposure to constant light on circadian clock in aged animals

5.3.1 Constant light exposure affects circadian clock at the behavioural level

Adult and aged Per2\textsuperscript{LUC} mice were exposed to constant light (LL) to assess the effect of age on the sensitivity of circadian clock. Mice were subjected to LL for two different intervals, i.e. a short interval of 40 days and a long interval of 7-9 months. Most of the animals were subjected to the monitoring of behaviour. Explants of SCN and various peripheral organs were taken from these animals and inner properties of circadian clocks were assessed (see chapters 5.2.1 and 5.2.4).

Effects of short term LL exposure on animal behaviour, the locomotor activity was measured in adult (n = 5; 8-9 months old) and aged (n = 5; 25-26 months old). Mice were entrained to LD 1:12 regimen at first and then maintained in LL for 40 days. Representative actograms and periodograms are depicted in figure 15A. Analysis of the behaviour recording revealed a persistent rhythm in adult animals with a mean period 23.48 ± 0.13h. On the other hand, of all analysed aged mice, only 2 exhibited faint rhythms and 3 of them were completely arrhythmic. Short exposure to constant light affected adult and aged animals differently, the rhythms of activity became more disrupted in aged mice compared to the adults.
Figure 15: The locomotor activity monitoring in mice subjected to LL regimen. (A) Representative actograms and periodograms from mice subjected to LL for 40 days. (B) Representative actograms and periodograms from mice subjected to LL for 7-9 months. (C) Analyses of behavioural rhythms in mice subjected to LL for 7-9 months. Group of aged animals is depicted in green and group of adult animals in black. Data are depicted as mean ± SD, where applicable. P values of t-test are specified in each graph. Adapted from (Polidarová et al. 2017b)

Effect of long term LL exposure was studied in adult mice exposed for 7-9 months to LL (n = 9; 8-13 months old at the time of tissue explantation) and in aged mice exposed to LL for 8 months prior to sampling (n = 5; 24 months old). Activity was monitored in the last 22 days before the explantation of various tissues. The representative actograms and periodograms are presented in figure 15B. The analysis of behavioural rhythms (figure 15C) revealed surprising similarity of period length between the groups; however, the power of the period estimation was significantly lower in aged mice, suggesting impaired circadian regulation of activity during aging. Total activity levels did not differ between adult and aged animals; although, the activity/rest ratios were lower in aged animals.

Taken together, these results strongly suggest the exposure to LL disrupts behavioural rhythms more in aged animals than in adults, whether exposed to LL for shorter period or longer period of time. Surprisingly, aged animals showed higher sensitivity to short-term LL exposure than to the long-term exposure to LL.
### 5.3.2 Constant light exposure effect on the endogenous rhythms in SCN in vitro

SCN slices were taken right after the monitoring of behavioural activity. The Per2\textsuperscript{LUC} bioluminescence in SCN slices from mice subjected to short term LL exposure (40 days) exhibited low amplitude rhythms. The period of these rhythms was in slices from adult animals 26.13 ± 1.7 h and in slices from aged animals 27.43 ± 4.26 h; therefore, there was no significant difference between the groups (figure 16A). Amplitude analysis of bioluminescence in vitro rhythms did not show significant difference between adults and aged animals. The analysis of dampening did not show any differences between the groups either (two-way ANOVA, \( p = 0.3045, F_{1,18} = 1.12 \)).

The analysis of in vitro rhythmicity, namely the period and amplitude of Per2\textsuperscript{LUC} rhythms, did not show the impairment of rhythms in SCN explants from aged animals as could be expected due to behavioural disruptions in aged animals after the exposure to LL.

SCN explants prepared from adult (\( n = 15 \)) and aged (\( n = 15 \)) subjected to long term LL exposure exhibited robust circadian rhythms in Per2\textsuperscript{LUC} oscillations in vitro. Some of these animals were monitored for locomotor activity (see chapter 5.3.1). The analysis of the in vitro rhythms did not reveal any significant difference in mean period of the explants from adult (25.61 ± 5.85 h) and aged (29.22 ± 9.92 h) animals (figure 16B). Mean amplitude of these rhythms did not show any significant difference between groups either.

![Figure 16](image_url)

**Figure 16:** The analysis of Per2\textsuperscript{LUC} bioluminescence traces of SCN organotypic explants from adult and aged mice maintained in LL for 40 days (A) and for 7-9 months (B). Group of aged animals is depicted in green and group of adult animals in black. Data are depicted as mean ± SD. \( P \) values of Mann-Whitney tests are specified in each graph, when applicable. Adapted from (Polidarová et al. 2017b).

Some of the SCN explants exhibited rhythmicity with extremely long period over 30 h or were completely arrhythmic, i.e. during the first 50 h after explanting, no significant periodicity was detected. This was applicable for 4 out of 30 in explants from adult animals and in 7 out of 30 in explants from aged animals. However, as detailed in methods, we prepared two SCN explants from each mouse to maximize the number of samples available. The other explant from the same SCN always showed a period within circadian range. This pattern was found in 27% of explants from adult mice and in 47% of explants from aged mice (figure 17). Interestingly, the periods of all explants from mice kept in LD 12:12 and DD (figure 17A) did not
deviate significantly in any of the adult or aged animals. This was not the case for explants from animals kept in LL for 7-9 months, there were big differences between the pairs of explants from single SCN (figure 17B). Closer microscopic inspection revealed the anatomical difference among the explants in each pair (figure 17D). These pairs differed in their rostro-caudal position and the size of the SCN area in the explant correlated to the level of emitted bioluminescence. Explants containing the middle region of the SCN, i.e. the largest area possible, emitted higher levels of bioluminescence (200-1200 counts/s), whereas other explants, not containing the middle region, emitted lower levels (50-200 counts/s). Careful matching of explants with their rostro-caudal position and the period of bioluminescence rhythms revealed that all arrhythmic explants contained middle SCN regions; therefore, the rhythm disruption was not due to the size of the SCN area in each explant (figure 17E).

**Figure 17:** The comparison of the period of in vitro Per2\textsuperscript{LUC} bioluminescence of two explants prepared from each individual SCN. From each SCN, two explants were prepared from coronal sections; matched explants are represented by adjacent columns. (A) Periods of bioluminescence rhythms in SCN explants from mice kept in LD 12:12. (B) Periods of SCN explants from mice kept in LL for 7-9 months. (C) Periods of bioluminescence rhythms in SCN explants from mice kept in DD for 35 days. Horizontal dashed line represents 24 hour period for comparison. (D) Representative microphotograph from bright-field microscopy of explants containing the rostral, middle and caudal SCN used for bioluminescence recording. (E) Representative bioluminescence records of the SCN explant pairs of adult and aged mice over 4 days of culturing. Shown explants contained middle and rostral/caudal sections of the SCN (marked in full and dashed line respectively). Similar records were obtained from the pairs containing caudal instead of rostral part of SCN. Adapted from (Polidarová et al. 2017b).
5.3.3 The effect of LL exposure on the SCN is reversible in vitro

The absence of rhythmicity of middle SCN explant from animals exposed to prolonged LL regime might have been due to low tissue viability; therefore we exposed the slices to repeated treatment procedure to verify its ability to respond to entraining stimuli. Explants were first left undisturbed after explanting to determine the period before the treatment measured in 3 cycles. Then, explants were subjected to repeated treatment by melatonin and vehicle (for details see materials and methods) for 5 subsequent days. Analysis of periods during 3 cycles after the treatment, revealed that the procedure synchronised all the explants from adult (25.02 ± 0.82) and from aged (24.86 ± 0.70) animals, regardless of its period before the treatment. The amplitudes of resulting rhythms after the treatment were 7.5 ± 9.8 for explants from adult animals and 5.2 ± 4.9 for aged mice. Representative traces of arrhythmic explants, which became rhythmic after the treatment are depicted in figure 18. The finding of restored rhythmicity in explants subjected to this treatment ruled out the possibility that the absence of the rhythmicity in explants from animals after LL was due to a technical problem influencing the viability of explants.

![Figure 18](image)

Figure 18: Representative traces of Per2\textsuperscript{LUC} bioluminescence from SCN slices. Slices were subjected to repeated treatment for 5 subsequent days (marked by arrows). The treatment consisted of repeated administration of 1µl of the phosphate-buffered saline (marked by full arrow) and media exchange 8 h later (marked by open arrow). Adapted from (Polidarová et al. 2017b).

5.3.4 Pancreatic clocks are affected by LL independently of age

Previous analyses of pancreatic clock properties in vitro revealed their resilience to aging (for details see chapter 5.2.4). We also tested, whether the exposure of animals to LL affects the properties of circadian clock in vitro and whether that effect is influenced by aging. Since explants of SCN show robust rhythms even in explants from aged animals (for details see chapter 5.2.1), we detected circadian rhythms also in organotypic explants of pancreas and lungs from adult and aged animals maintained in LL prior to explanting. Representative bioluminescence traces are depicted in figure 19A.

Exposure to LL prior to explanting shortened the period of rhythms in pancreas compared to the period in animals maintained in LD 12:12 both in adult (24.8 ± 0.4 h in LL compared to 25.5 ± 0.9 h in LD 12:12; P = 0.0108) and in aged (25.0 ± 0.5 h compared to 25.6 ± 1.2 h respectively; P = 0.0359) mice. Aging had no additional effect to LL exposure on the length
of period of bioluminescence rhythms in pancreas, although it significantly decreased the amplitude of the rhythms (figure 19B-C). Surprisingly, the amplitude dampening was quicker in adult than in aged pancreas (two-way ANOVA, p = 0.0048) as depicted in figure 19D. In fact, the exposure to LL prior to explanting had much larger effects on the rhythms than aging itself. The bioluminescence rhythms dampened significantly faster in the pancreas of the adult and aged mice (two-way ANOVA, p < 0.0001 for both groups) maintained in LL compared to mice maintained in LD 12:12 regimen (figure 19E).

Exposure to LL prior to explanting significantly lengthened the periods of bioluminescence rhythms in lungs compared to LD 12:12. This effect was notable in adult 27.0 ± 0.7 h in LL compared to 26.1 ± 0.6 h in LD 12:12; P = 0.0004) and in aged (28.3 ± 1.3 h compared to 27.0 ± 0.7 h respectively; P < 0.0001) mice. Thus, aging had an additional effect on period of lungs affected by LL further lengthening the period of bioluminescence rhythms (figure 19B). Aging also affected rhythms by supressing the amplitude of bioluminescence rhythms in lungs (figure 19C). The rate of the rhythm amplitude dampening in explants of lungs was affected neither by exposure to LL (p = 0.2995) nor by age (p = 0.2985) of experimental animals (figure 19D,E).

Figure 19: The analysis of Per2LUC bioluminescence traces of organotypic explants of pancreas and lungs from adult and aged mice maintained in LL. (A) Representative bioluminescence traces of Per2LUC recorded for 7 consequent days without media change or any other disturbances. The analysis of period (B) and amplitude (C) of in vitro bioluminescence rhythms. (D) Comparison of amplitude dampening rate of bioluminescence rhythms in vitro between adult and aged animals. (E) Comparison of the dampening of bioluminescence amplitude between rhythms of explants from animals kept in LL and in LD 12:12. Amplitudes were analysed in first 5 cycles and were normalised to the amplitude of the first cycle. Group of aged animals is depicted in green and group of adult animals in black. Data are depicted as mean ± SD. P values of Mann-Whitney tests are specified in each graph, when applicable. Adapted from (Novosadová et al. 2018).
5.3.5 The effect of LL on circadian clock in pancreas and lungs is reversible in vitro

The exposure of animals to LL impaired the coherence of the rhythms in organotypic explants from pancreas and lungs (for details see previous chapter). Thereby, the next question was whether LL also affected the ability of these explants to be synchronised in vitro by rhythmic cues. A rhythmic signal, consisting of repeated administration of 1 µl of the PBS and media exchange 8 h later, was imposed on the organotypic explants in vitro. This treatment was repeated for 5 subsequent days. Representative raw bioluminescence traces of the treatment procedure are depicted in figure 18. The treatment had entraining effect and it caused significant change in the period lengths and/or it decreased the inter-individual variability between the explants (figure 20). The treatment significantly decreased the period in most cases and the individual variability in pancreatic explants from adult (period: \( p = 0.0006 \); variability: F test, \( p < 0.0001 \)) and also in aged (period: \( p = 0.4071 \); variability: F test, \( p = 0.4071 \)) animals (figure 20A). In pancreatic explants from adult mice, the period before the treatment was 29.4 ± 6.3 h and after the treatment 24.9 ± 0.7 h. Explants from aged mice kept in LD 12:12 exhibited period 25.3 ± 1.5 h before the treatment and 25.6 ± 0.6 h after the treatment.

Important, after the exposure of mice to LL, the explants of pancreas were not exhibiting the same response to the treatment (figure 20A). The treatment was not potent enough to entrain the clock in the pancreas from those animals. The mean period length changed after the treatment in adult (\( p = 0.0002 \)) as well as in aged (\( p = 0.039 \)) mice; however, the variability among explants did not change in adults (F test, \( p = 0.5825 \)) and was even larger in explants from aged animals (F test, \( p < 0.0001 \)). The periods of bioluminescence rhythms of pancreatic explants from adult mice were 24.5 ± 0.8 h before the treatment and 25.8 ± 0.9 h after it. In case of explants from aged mice, the periods before were 24.8 ± 0.6 h before and after the treatment the periods were 27.5 ± 3.3 h.

In organotypic explants of lungs from animals maintained in LD 12:12, the period did not significantly change after the treatment in adults (\( p = 0.4297 \); variability: F test, \( p = 0.7631 \)); however, the period significantly shortened in aged animals (\( p = 0.0003 \); variability: F test, \( p = 0.1212 \)) as depicted in figure 20B. The periods of bioluminescence rhythms of lung explants from adult mice before the treatment were 25.9 ± 0.8 h and 26.1 ± 0.9 h after the treatment. In aged group, the periods before the treatment were 27.7 ± 0.5 h and after the treatment shortened to 26.3 ± 0.3 h.

The treatment had a significant effect on lung organotypic explants from adult mice kept in LL (\( p = 0.0001 \), variability: F test, \( p = 0.0036 \)) (figure 20B). The period of rhythms of adult lung explants was 27.1 ± 0.9 h before the treatment and 25.9 ± 0.4 h after. The treatment had less robust effect on explants from aged animals (\( p = 0.0013 \); variability: F test, \( p = 0.4919 \)). Bioluminescence rhythms of lung explants from aged animals exhibited period before the treatment 27.8 ± 1.0 h and 25.8 ± 1.3 h after.

Exposure to LL significantly impaired the ability of the pancreatic explants to be entrained in vitro by repeated treatment, but it did not impair this ability for the explants of lungs.
Figure 20: Effect of in vitro treatment on entrainment of Per2LUC bioluminescence rhythms in organotypic explants of pancreas (A) and lungs (B) from animals maintained in LD 12:12 and LL. The treatment was performed during 5 consequent days consisting of application of 1 µl solution followed by medium exchange 8 h later. Open and filled circles apply to different solutions preceding the medium exchange as described in methods chapter. The periods were detected before and after the treatment. The resulting t-test P values for differences between the periods before and after the treatment are depicted by asterisks (*P < 0.05; **P < 0.01; ***P < 0.001), while the F test P values for differences between inter-individual period variances are depicted by hash marks (## P < 0.01; #### P < 0.0001). Adapted from (Novosadová et al. 2018).

Some of the organotypic explants of pancreas from mice maintained in LD 12:12 exhibited bioluminescence Per2LUC rhythms with extremely long periods, more than 30 h, outside the circadian range. We subjected these explants to treatment procedure as mentioned above to test their viability (figure 21A). Period of bioluminescence rhythms was restored after the treatment and successfully synchronised to rhythms fitting circadian range (figure 21B). This result confirmed, that extremely long periods of Per2LUC bioluminescence rhythms in pancreatic explants in vitro were not due to their low viability in culturing conditions.

Figure 21: A test of viability of organotypic explants of pancreas from Per2LUC mice. (A) Representative traces of Per2LUC bioluminescence from pancreatic explants of mice kept in LD 12:12 undergoing a treatment for 5 subsequent days. The treatment consisted of repeated administration of 1 µl of the phosphate-buffered saline and media exchange 8 h later. (B) The periods of bioluminescence rhythms before and after the treatment. Adapted from (Novosadová et al. 2018).
6 Discussion

6.1 Circadian regulation in adult and aged animals

In accordance with previous reports, our experimental animals exhibited aggravated regulation of locomotor activity with age (Pittendrigh and Daan 1974; Turek et al. 1995; Valentinuzzi et al. 1997; Nakamura et al. 2011, 2015). Some studies noted the changes in period lengths in aged animals (McAuley et al. 2002; Farajnia et al. 2012); however, our results did not show age-related effect on the periods of the rhythms. However, we detected relatively high inter-individual variability in period lengths, which might preclude the detection of a subtle age-dependent changes in period lengths. Similarly to our results, previous study on humans comparing aged and adult subjects did not show the differences in circadian rhythm period either (Czeisler et al. 1999).

On the other hand, it is important to note the difference in behaviour monitoring methods. Most of the rodent studies mentioned above used wheel running measurements, whereas we used more natural measurement of spontaneous open field locomotor activity in cages. Wheel running might be a biased method of measurement, providing experimental animals with reward and changing their physiological, energy balance and even the length of inner period (Shioiri et al. 1991; Novak et al. 2012).

Importantly, our aged mice exhibited less precisely controlled activity rhythms in DD. They had rhythms with lower amplitudes and showed lower total activity levels than adult mice. Therefore, the regulation of spontaneous locomotor activity rhythms by SCN was somewhat compromised in our aged mice maintained in constant darkness. The exposure of mice to LD 12:12 regimen ameliorated the age dependent decrease in total activity of aged mice but did not increase the drop in amplitudes of observed rhythms. Taken together, we observed clear signs of senescence in our aged animals at the behavioural level.

Some studies point out that the age-related progressive yellowing and thickening of the eye lens could reduce the light sensitivity, i.e. lower the signal from the strongest Zeitgeber for circadian rhythms (Hood and Amir 2017). Model of such desynchrony in inner organs is represented in figure 22. Our results presented in this thesis provided another evidence of persisting robust oscillations in circadian clock in elder individual; therefore, the lack of output rhythms consistency might be due to this lack of proper signalling cues from the environment.

Figure 22: Model representation of liver clock from healthy animal (A) and from SCN lesioned animal (B). Synchronization of peripheral oscillators is occurring on cellular level receiving the signal from SCN. The resulting rhythm from an organ homogenate is just a sum of all cellular oscillators. Therefore, the lack of signal from a tissue can be due to the internal desynchrony of individually oscillating cells (adapted from Stratmann and Schibler 2006).
6.1.1 Aging of circadian clock in the SCN

Our results clearly demonstrated remarkable resilience of central circadian clock in SCN to aging. In spite of the aggravated parameters of behavioural activity in aged animals, aging had no effect on the in vitro Per2\textsuperscript{LUC} bioluminescence rhythms in the SCN from the same animals maintained in LD 12:12 regimen. Similar results were reported also in several studies from different authors (Yamazaki \textit{et al.} 2002; Davidson \textit{et al.} 2008; Nakamura \textit{et al.} 2011; Sellix \textit{et al.} 2012).

In a study from 2015, Nakamura \textit{et al.} hypothesized that the lack of age effects on SCN rhythmicity might be due to the masking effects of persisting LD cycle providing synchronizing cues (Nakamura \textit{et al.} 2015). Authors reported that age does not affect Per2\textsuperscript{LUC} bioluminescence rhythms in SCN slices from animals maintained in LD 12:12; however they noted faster dampening of the in vitro rhythms aged animals than in younger ones maintained in DD for 10 days. Nakamura \textit{et al.} also noted the lower level of synchrony among individual oscillators in SCN from aged animals maintained in DD. In our study, the Per2\textsuperscript{LUC} bioluminescence rhythm parameters did not significantly differ between aged and adult animals even when maintained in DD for a longer interval (35 days) and we observed only a marginal effects of aging on dampening of the rhythms. Therefore, our results do not indicate masking effect of the previous LD cycle on the age-induced changes in the SCN rhythmicity. In the study of Nakamura \textit{et al.} (Nakamura \textit{et al.} 2015), the animals designated as “aged” were younger (13 – 15 months old) than we in our studies (25 - 26 months old) presented in this thesis. Theoretically, the reason of the discrepancy between their and our results could be that aging might affect the SCN earlier, before 9 months of age, which was the age of our “adult” animals, and therefore the effect might be missed in our study.

Nevertheless, previous studies indicated the negative effect of aging on the electrical activity rhythm in SCN neurons (Farajnia \textit{et al.} 2014). The electrical activity represents an output rhythm whereas the Per2-drive bioluminescence reflects behaviour of the molecular clock. It appears from our results that these two parameters – clock gene expression and electrical activity rhythms – do not always respond in parallel. Similarly, there are also a non-parallel responses of clock gene expression and neuronal activity previously demonstrated in rat SCN (Sumová and Illnerová 2005). Therefore, aging seems to affect the ability of SCN neurons to maintain output rhythms rather than the TTFL operation. Altogether, our behavioural and in vitro data indicate that the decrease of amplitude of the locomotor activity rhythms is rather due to the inability of SCN to drive the output rhythms, than due to impaired circadian rhythm generation within SCN.

6.1.2 Effects of aging in peripheral organs

The glucose homeostasis was significantly altered in our aged Per2\textsuperscript{LUC} mice compared to the adults. Our results showed higher basal levels of insulin in spite of lower basal glucose levels in aged animals; additionally, these animals had a lower rate of glucose clearance measured by IPGTT. Previous studies on the effects of aging on basal insulin levels have been controversial, both an increase (Gregg \textit{et al.} 2016) and a decrease (Santulli \textit{et al.} 2012) in insulin release have been reported. Age-dependent impairment of glucose clearance has also been previously
described (Gold et al. 1976; Kitahara and Adelman 1979; Santulli et al. 2012). The mechanisms leading to the elevated insulin levels in our aged Per2\textsuperscript{LUC} mice might be related to a previously reported increase in the ratio of large Islets in aged individuals (Hellman 1959; Chen et al. 1985). Larger Islets have a higher insulin secretion rate (Kitahara and Adelman 1979). This phenomenon could be also due to a possible age-dependent decline in the removal rate of insulin (Reaven et al. 1982), which might be linked to a decline in insulin-degrading enzyme (IDE) levels in the liver (Kochkina et al. 2015). The effect of aging on increasing insulin levels has not been confirmed in previous studies, after the correction of the results for visceral fat or used diet (DeFronzo 1981; Kohrt et al. 1993; Imbeault et al. 2003). Interestingly, in our study, aged mice fed the same diet as adults exhibited higher insulin levels even after the correction for their body weight.

We studied the expression of selected genes, involved in insulin secretion, in glucose homeostasis relevant tissues. We found upregulated expression of \textit{PCLO} in the pancreas of aged animals. \textit{PCLO} upregulation suggests this is the cause of the higher insulin levels because the downregulation of the gene selectively in pancreatic β-cells significantly impaired insulin secretion (Fujimoto et al. 2002). Our results also show upregulated expression of \textit{PDX1}, a transcription factor involved in glucose stimulated insulin secretion (Li et al. 2005), in pancreas of aged animals in accordance with previous reports (Avrahami et al. 2015).

We observed lower basal levels of plasma glucose in our aged animals. This was likely a consequence of increased glycogen storage in the liver, functioning as a compensatory mechanism to the higher insulin levels. Additionally, we found significantly higher expression of \textit{PP1R3C} in the liver, which is a gene coding protein phosphatase 1 promoting hepatic glycogen synthesis (Printen et al. 1997). In line with these results, the expression of \textit{SGK1} was dramatically suppressed in the liver of our aged animals. \textit{SGK1} was previously shown to influence glucose transport rate (Liu et al. 2014). Taken together, the downregulation of \textit{SGK1} expression may participate in the lower rate of glucose clearance as well as in the lower glucose uptake that we observed in aged mice by IPGTT. Our results indicate a possible decrease in pancreatic glucose sensitivity in aged animals, due to lower expression of \textit{SLC2A2}, which is a glucose transporter Glut2 of β-cells required for normal glucose stimulated insulin release. On the top of that, we detected significant changes in the expression of metabolism-related genes (\textit{NAMPT}, \textit{HIF1α} and \textit{E4BP4}) in the liver of aged animals, which was in accordance with previously published aging-induced changes in the liver transcriptome (Sato et al. 2017). Altogether, aged Per2\textsuperscript{LUC} mice in our study exhibited signs of hyperinsulinemic hypoglycaemia that indicates profound changes in their pancreatic functions and glucose homeostasis.

Interestingly, the expression of some clock genes was rather upregulated in some peripheral tissues due to aging, such as the expression of \textit{BMAL1} exhibited significantly higher amplitude both in the pancreas and liver (but not in the lungs). Previous study also noted a gene specific up- and down-regulation of gene expression in the liver due to aging (Sato et al. 2017). The apparent age related decrease in the expression of another key component of TTFL, \textit{CLOCK}, found in the pancreas did not seem to affect the overall quality of expression rhythms of other clock genes. This might be caused by the Bmal1 being the rate limiting partner in the transcription heterodimer in central molecular TTFL of the clock (Lee et al. 2001). Our results have shown a significantly elevated amplitude of the gene expression rhythm of clock gene
**BMAL1** in the pancreas of aged animals, which is in accordance with the previously published direct role of Bmal1 in the regulation of **PCLO** expression (Perelis et al. 2015). Apart from the pancreas, we found upregulated expression of **BMAL1** in the liver of aged animals; however, the effect was completely absent in the lungs, and interestingly, it was rather opposite in the colon. Therefore, the effect of aging on the expression of **BMAL1** was highly tissue specific. Bmal1 has a major role in the core TTFL of circadian timekeeping mechanism (Takahashi 2015), but also has a role beyond its circadian function that was also well documented (Yang et al. 2016). Circadian oscillations in other tissues and cell types, such as in epidermal and muscle stem cells, were shown to remain robust during aging; however, they undergo extensive reprogramming in the process (Solanas et al. 2017). The resilience of the circadian clock to aging would therefore favour an additional non-circadian role of Bmal1 in the pancreas.

In order to monitor the circadian clock in aged tissues in real time, we used organotypic explants of the pancreas from Per2<sup>LUC</sup> mice. Organotypic explants represent an integral tissue composed of all cell types present in the pancreas, i.e. the endocrine and exocrine part. We confirmed the viability of endocrine β-cells in the explant by the ability of the explants to produce insulin upon glucose stimulation. We did not consider isolated β-cells as a suitable model for this type of experiment due to their much shorter viability, which would preclude the long term recording necessary for studying the circadian clock properties.

Aging had no effect on the circadian clock properties in the pancreas of animals maintained in LD 12:12 regimen. There were no effects on the period and dampening rate of Per2<sup>LUC</sup> bioluminescence rhythms. We found remarkable contrast of pancreatic clock in vitro and clock in the lung explants, which became less persistent due to aging. The rhythms in lung explants from aged animals ran with prolonged periods and dampened faster. This was in agreement with previously published data showing the effect of aging on the circadian clock in lungs; the study reported total lack of rhythmicity in lung explants from aged **PER1-LUC** rats (Yamazaki et al. 2002).
6.2 Effects of constant light regimen on clock in adult and aged animals

We did not observe any major effects of aging on the in vitro rhythms of Per2\textsuperscript{LUC} bioluminescence in the SCN of animals maintained in LD 12:12 or in DD regimen. Therefore, we hypothesized, that aging might affect the SCN neuronal plasticity and its ability to send signals to peripheral organs might. To test this hypothesis, we exposed mice to LL for various intervals and tested the ability of SCN to respond to abnormal external lightning conditions. Constant light was previously proven to have disrupting effects on several features of mammalian body (Nováková \textit{et al.} 2010, 2011).

Adult and aged mice were exposed to LL and after attaining a steady-state locomotor activity rhythm (occurring approximately 2 weeks after the start of LL) we were able to detect circadian rhythms with periods shorter than 24 hours (independent of duration of LL exposure). This result was in contrast with a previous results from study by Ohta \textit{et al.} studying \textit{PER1:GFP} mice (Ohta \textit{et al.} 2005) behaviour of which became desynchronized in LL. In our study, the locomotor activity rhythms were preserved in all our adult Per2\textsuperscript{LUC} mice even after a very long LL exposure (7-9 months). On the other hand, the rhythms were disrupted or completely absent in aged mice after either short term (40 days) or long-term (7-9 months) exposure to LL.

6.2.1 Effect of LL on circadian clock in adult and aged SCN

The Per2\textsuperscript{LUC} bioluminescence rhythms in SCN of explants from animals exposed to LL were also affected by aging in larger proportion of animals. The persistence of in vitro Per2\textsuperscript{LUC} rhythmicity in SCN slices was impaired; however, the effect was primarily present in the middle region of the SCN and the rhythmicity in other parts of SCN remained unaffected. SCN explants containing the middle region of SCN were either exhibiting a rhythm with an extremely long non-circadian period or they were completely arrhythmic. The middle region of the SCN contains neurons from the retinorecipient core cell sub population, which are likely more sensitive to LL. Most of the rostral and caudal regions of SCN, present in our other SCN slices, contain both shell and core, but are lacking some core subpopulations of neurons. Other than middle region SCN explants were from the most rostral or caudal margins of SCN likely containing mostly neurons from the shell, therefore, they maintained their rhythmicity (Yan \textit{et al.} 2007). Interestingly, the effect observed in the SCN was slightly more pronounced in aged than adult mice. Moreover, this arrhythmicity was not exhibited in explants from aged animals maintained in LD 12:12 or DD. Therefore, aging per se was clearly not the reason. The absence of the rhythmicity in all explants from SCN might be explained either by mutual desynchrony among the core and shell region of SCN (Ohta \textit{et al.} 2005) or by mutual uncoupling of individual oscillators (Yan and Okamura 2002; Yan \textit{et al.} 2007). Another study from 2012 also showed, that aging induced spatiotemporal differences in SCN responses to shifts in LD cycles due to increased desynchronization of sub regions of the SCN (Sellix \textit{et al.} 2012). Additionally, the possible desynchrony among the left and right nucleus of SCN cannot be excluded since it has been previously shown in mice (Ohta \textit{et al.} 2005) and in hamsters (de la Iglesia 2000).

Importantly, the effect of LL on the period disruption was not due to a low viability of the explants because repeated treatments of the arrhythmic explants restored their rhythmicity.
in vitro. Taken together, according to our findings, aging potentiates the effect of LL exposure mostly in the slices of middle sub-region.

6.2.2 Effect of LL on circadian clock in adult and aged peripheral organs

Our results showed the high resilience of circadian clock in pancreas to aging. This observation led us to explore whether and how the clock in pancreas is sensitive to a situation of compromised rhythmic inputs from central clock in SCN. It was previously shown, that the exposure to an environmental LD cycle may help to maintain the rhythmicity of peripheral clocks via SCN-independent mechanisms (Husse et al. 2014). Rhythmicity of the SCN in vitro could be disrupted by exposing the animals to LL and it could uncouple the SCN from peripheral clock. SCN and peripheral clock uncoupling may impair the rhythmicity of circadian clocks in the GIT tissues. In this thesis, we provide evidence, that exposure of mice to LL changes the properties of the peripheral clock in organ specific manner, thus, it affects the clock more robustly in pancreas than in the lungs. Exposure of mice to LL previous to explanting led to lengthening of bioluminescence rhythm period. LL induced effect in the lung explants was fully reversible by in vitro daily treatment simulating the regular rhythmic stimuli from the central clock present in vivo, demonstrating a high sensitivity of lung circadian clock to systemic rhythmic signals. Contrastingly, exposure to LL accelerated the dampening of the bioluminescence rhythms in pancreatic explants and the LL induced effect on rhythm periods was not reversible by the treatment. Therefore, the properties of the pancreatic clock were changed due to the previous exposure to LL regimen, rather than due to simple absence of rhythmic signals.
7 Conclusion

Aging is a process often linked with the deterioration of physiological functions; however the mechanism is still unclear. Circadian clocks form a vital part of well-functioning physiology of mammalian body, since they are at the crossroad of many metabolic processes.

Our results demonstrated the robustness of molecular circadian clock in aged SCN from mice maintained in LD 12:12 and DD at least at the cell population level. Therefore, the impaired regulation of the output rhythms (in our case rhythms of behaviour) likely may not be a result of an inability of clock to generate rhythmic signals, but rather from a worsened ability to convey the signals further to the body. Furthermore, our results have shown that the SCN of aged mice are slightly more prone to disruption by constant light than SCN of adult animals. This suggests that adverse lightning conditions might be more harmful for the circadian clock in the elderly. It has been previously demonstrated that chronic circadian disruption in aged animals markedly increases mortality (Davidson et al. 2006). We detected the impact of aging on the SCN sensitivity to constant light in a period starting from late adulthood, which is analogous to the humans. Additionally, we demonstrated that exposure to LL together with aging compromises the rhythmicity of SCN only in specific sub-regions of the SCN, leaving cell oscillators in other parts of SCN well synchronised. Thus, resulting disruption of behaviour in aged animals subjected to LL seems to be also a result of the loss of mutual coupling among the specific SCN sub-regions. Each sub-population of cells in SCN may differ in their regulatory output pathways, therefore contributing to age-related pathologies.

Aged Per2LUC mice in our study also exhibited signs of hyperinsulinemic hypoglycaemia, indicating profound changes in their pancreatic functions and glucose homeostasis.

By comparing the daily profiles of the clock gene expression in various peripheral tissues collected around the clock, we did not detect gross suppression of any of the studied clock gene rhythm amplitude due to aging. Instead, we were able to detect only selective gene and tissue-specific changes, such as downregulation of expression of CLOCK in the pancreas and BMAL1 in the colon and lungs, and upregulation of BMAL1 in pancreas. Taken together, the lack of an effect of aging on deterioration of the inner mechanisms of pancreatic clock in vitro, suggest that the age-dependent modulation of the expression of BMAL1 found in vivo, might be a part of compensatory clock-independent mechanisms triggering signalling pathways involved in Pclo-mediated insulin secretion. Comparing the in vitro properties of the pancreatic clock with the clock in lungs, that is a tissue not involved in glucose homeostasis, we provided evidence for high resiliency of the core molecular clock mechanism in the pancreas of aged animals.

Our data also demonstrate that although the molecular mechanism of circadian clock in pancreas is not altered by aging, it seems highly sensitive to disturbances in external lightning conditions. The properties of pancreatic clock in vitro did not improve by providing the rhythmic signal, therefore, they were changed due to the previous exposure to LL regimen, rather than due to simple absence of rhythmic signals. Higher sensitivity of pancreas might provide a basis for previously demonstrated disorders in glucose homeostasis in human subjects exposed to shift work (Bescos et al. 2018).

The results discussed in this thesis added valuable information and helped to broaden our knowledge of the regulation of circadian clock in aging. They could help us to understand the mechanisms of aging better and might possibly provide a basis for the amelioration of some symptoms of senescence.
8 List of publications

Publications with results discussed in this thesis:

1. Identification of functional properties of adult and aged circadian clock in pancreas

*Zuzana Novosadová, Lenka Polidarová, Martin Sládek, Alena Sumová* (2018): Alteration in glucose homeostasis and persistence of the pancreatic clock in aged mPer2^{Luc} mice, *Scientific Reports*, 8: 11668
   DOI: 10.1038/s41598-018-30225-y

2. Characterization of circadian clock in the SCN of adult and aged animals

*Lenka Polidarová, Martin Sládek, Zuzana Novosadová, Alena Sumová* (2016): Aging does not compromise in vitro oscillation of the suprachiasmatic nuclei but makes it more vulnerable to constant light, *Chronobiology International*, 34: 105-117
   DOI: 10.1080/07420528.2016.1242491

Other publications:

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