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Mgr. Vendula Lužná

Molekulární mechanismy synchronizace fetálních cirkadiánních hodin

Molecular mechanisms of entrainment of the fetal circadian clocks

Disertační práce

Vedoucí práce: Doc. PharmDr. Alena Sumová, CSc., DSc.

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Prohlášení:

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V Praze, 11.04.2021

Podpis

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Abstract

In order to adapt to changing external conditions, organisms developed the endogenous biological clock for predicting daily alterations. This so-called circadian system drives functions and processes in the whole body with an approximately 24h period. The central oscillator, located in hypothalamic suprachiasmatic nuclei (SCN), is synchronized by light and subsequently sends the information about the time of the day to the rest of the body. Even in the ontogenesis, the functional SCN clock is crucial for proper development as well as health later in life. Since the maturation of embryonic SCN is not completed before birth, maternal signals seem to play a fundamental role in setting and synchronizing the fetal clock.

During my PhD studies, we focused on elucidating the nature of maternal signals and their diverse impact on fetal SCN of rat and mouse models. We have revealed that developing SCN is able to sense distinct signals related to various maternal behavioral regimes. Importantly, we have discovered eminent role of glucocorticoids in synchronizing the fetal SCN, along with their ability to accelerate SCN development. These observations point out the importance of regular daily routine and noxious effect of stress during pregnancy.

Since the mother communicates with the fetus through placenta and there had been a lack of knowledge about the placental clock and its synchronization, we turned our attention to the impact of chronobiologically relevant maternal signals on placental clock. We have identified parts of placental tissue harbouring circadian oscillators and found out that glucocorticoids are significant players in their synchronization. On top of that, the role of dopamine and melatonin has been carefully studied and the complementary effect of these two hormones on placental clock has been confirmed by our experiments.

Altogether, our novel observations highlight the intricate and complex mechanisms of maternal signaling in setting the fetal clock, as well as the importance of necessary follow-up studies that would provide better understanding of how mother, placenta and fetus communicate during the pregnancy.

Abstrakt

Rytmicky se střídající světelné podmínky na Zemi vedly ke vzniku endogenních biologických hodin – evoluční adaptaci umožňující organismům tyto změny předvídat. Tento tzv. cirkadiánní systém řídí v těle velké množství rytmických funkcí a procesů s periodou přibližně 24 hodin. Centrálním oscilátorem jsou suprachiasmatická jádra (SCN) hypothalamu, jež jsou seřizována vnějšími světelnými podmínkami, následkem čehož vysílají silný synchronizační signál do ostatních buněk a tkání těla. Synchronizace SCN je nezbytná již v průběhu ontogeneze, neboť poruchy ve vývoji biologických rytmů mohou vést ke vzniku onemocnění v dospělosti. Jelikož prenatální SCN ještě nejsou plně vyvinuta, jejich rytmicita je pravděpodobně řízena především mateřskými signály.

Během mého doktorského studia jsme se zaměřili na objasnění podstaty těchto mateřských signálů a jejich vlivu na hodiny ve fetálních SCN u myši a potkana jakožto modelových organismů. Jedním z našich stěžejních zjištění je fakt, že fetální cirkadiánní hodiny jsou schopny specificky reagovat na různé změny v mateřské signalizaci. Následně jsme zkoumali funkci glukokortikoidních hormonů a objevili jejich potenciál působit jako silný synchronizační mateřský signál. Pozorovali jsme, že glukokortikoidy jsou schopny nejen nastavit, nýbrž také urychlit vývoj cirkadiánních hodin ve fetálních SCN. Naše výsledky mimo jiné poukazují na důležitost pravidelného denního režimu a zdůrazňují neblahý vliv stresu v průběhu těhotenství.

Komunikace matky s féty probíhá v těhotenství skrze placentu, avšak o hodinách v placentě a jejich synchronizaci nebylo dosud k dispozici příliš mnoho poznatků. Zaměřili jsme se proto na vliv vybraných, chronobiologicky relevantních molekul na cirkadiánní systém v tomto orgánu. Nejprve jsme identifikovali části placenty disponující funkčním hodinovým mechanismem a následně jsme zjistili, že, podobně jako u hodin ve fetálních SCN, jsou glukokortikoidy silným synchronizačním signálem i pro hodiny placenty. Dalšími hormony, jež ovlivňují hodiny v placentě, jsou na základě našich poznatků dopamin a melatonin. Efekt těchto dvou hormonů se vzájemně doplňuje v závislosti na denní době, v níž jsou schopny na tyto hodiny působit.

Naše výsledky tak odhalují komplexnost mateřských signálů nastavujících fetální cirkadiánní hodiny, a vedle důležitých nových poznatků zároveň přinášejí další otázky, které je třeba zodpovědět, abychom lépe pochopili složitou komunikaci mezi matkou, placentou a fétem v průběhu těhotenství.

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List of abbreviations

AVP	arginine vasopressin
cAMP	cyclic adenosine monophosphate
Ccg	clock-controlled genes
CK	casein kinase
CRE	cAMP response element
CREB	cAMP response element-binding protein
Cx	connexin protein
DEX	dexamethasone
DNA	deoxyribonucleic acid
DRD1a	D1a dopamine receptor
E	embryonic day
FBXL	F-box/LRR-repeat protein
GABA	γ-aminobutyric acid
GC	glucocorticoid hormones
GIT	gastrointestinal tract
GR	glucocorticoid receptor
GRE	glucocorticoid regulatory element
GRP	gastrin-releasing peptide
HAT	histone acetyltransferases
HSD2	11β-hydroxysteroid dehydrogenase
IF	impact factor
LD	light/dark
LL	light/light (constant light)
MR	mineralocorticoid receptor
mRNA	messenger RNA
NMS	neuromedin S
OC	optic chiasma
P	postnatal day
PAS	Per-Arnt-Sim domain
PER2::LUC	PER2 protein fused with luciferase in transgenic mouse
PRC	phase-response curve
pRGC	photosensitive retinal ganglion cells

RF	restricted feeding
RHT	retino-hypothalamic tract
RNA	ribonucleic acid
RT-qPCR	quantitative reverse transcription polymerase chain reaction
SCF	Spk, Cullin, F-box containing complex
SCN	suprachiasmatic nuclei
TTFL	transcriptional-translational feedback loop
VEH	vehiculum
VIP	vasoactive intestinal polypeptide
VPAC2	VIP receptor, alternative name VIPR2

Introduction

1.1 Circadian rhythms

Due to the rotation of the Earth around its axis, there are two main environmental states that affect life of almost all living organisms across our planet, which are alternating constantly – day and night, light and dark. Different light conditions during the 24 hours are one of the strongest evolutionary pressures to adapt to. It is also an opportunity for diverse life forms to specialize. Hence, there exist diurnal and nocturnal species as well as crepuscular ones – those active during day, night, or twilight, respectively. Such animals alter their sleep/awake phases and food consumption time to ideally fit in their ecological niche. We can observe flowers that open their blossoms during a specific phase of the day in accordance with a different composition of the light spectrum so as to match with their specific pollinators. Apart from rhythmical changes in sleep/awake cycle or feeding regime, there are daily alterations in core body temperature and hormonal synthesis in animals as well as interchange of photosynthesis and breathing in plants. These rhythms in behavior and physiological parameters have a period that corresponds with the daily solar cycle and is in close proximity (i.e. *circa*) to 24 hours; thus, they are called circadian.

Following many observations describing this phenomenon in nature, the fundamental question was whether these rhythms are just a reaction to the environment or if they are an inner feature of living organisms. Since these changes in environment are invariable, they are also predictable, which constitutes the crucial condition for inheritable adaptation. This was confirmed by two experiments on plant *Mimosa pudica.* In 1729, French polymath, Jean-Jacques d'Ortous de Mairan placed the mimosa into constant darkness, during which leaf movements, typical for this plant, continued with a period of around 24 hours **(Fig. 1)**; almost a century after that, in 1823, Swiss botanist Augustin de Candolle observed persisting periodical movements of mimosa leaves also in constant light (de Candolle, 1825). These experiments opened a new avenue to an extensive follow-up research based on which it was concluded that the above-described daily rhythms are governed by an internal mechanism serving to anticipate and therefore predict daily changes in the environment, which is very useful and “wise” for life on this planet. Besides this conclusion, protocols using constant conditions for studying the internal period of organisms are used until nowadays in experimental work.

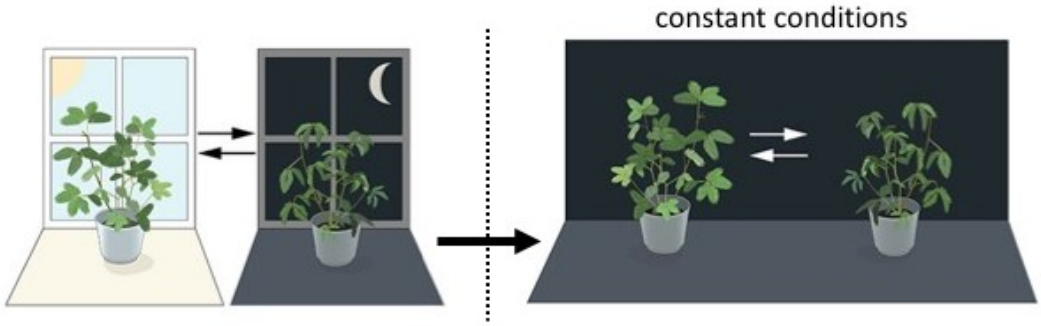

Figure 1. Experiment confirming the existence of internal biological clock.
Daily rhythms in leaf movements of *Mimosa pudica,* observed in changing light conditions, persist in constant darkness. This experiment proved the endogenous nature of biological clock (based on: www.nobelprize.org/prizes/medicine/2017/prize-announcement/).

Once the inner nature of circadian rhythms was confirmed, animal and, above all, mammalian clock has been brought into focus. The crucial question was: Where is "the clock" located within the body of mammals?

1.2 Central pacemaker

Researchers concluded that the central oscillator would most probably be placed in the hypothalamus, a brain region that can receive information about light and dark from the eyes (Stephan and Zucker, 1972). The structure connected with the retina via direct neuronal pathway is a pair of small nuclei sitting at the base of hypothalamus on both sides of the third ventricle, right above the optic chiasma – suprachiasmatic nuclei, SCN. The discovery that SCN is essential for determining the circadian period in mammalian body was made by trans-genetic SCN transplantation in hamsters (Ralph and Menaker, 1988; Ralph et al., 1990). In retina, there is a special subpopulation of cells sensitive to light – photosensitive retinal ganglion cells (pRGC) utilizing the blue light-sensitive photopigment, melanopsin. Axons of these pRGCs project to the SCN and mediate the entrainment of the circadian clock according to the light conditions of the environment (Berson et al., 2002). Since the genetic period of the circadian clock slightly deviates from 24 hours, regular setting of the inner clock to the outer light is crucial. The SCN also plays a role in rhythmical production of various neurotransmitters and neuropeptides and governs rhythmicity in the body at multiple levels; including the release of melatonin, insulin, and plasma glucose, maintaining sleep and reproductive functions and controlling drinking and feeding time (Van den Pol and Tsujimoto, 1985; Klein *et al.*, 1991;

Weaver, 1998; Lee *et al.*, 2013). Some of these features of SCN, mostly gained from the experiments on rodents, will be described in detail later.

1.2.1 Architecture of the SCN

Each of the rodent SCN is comprised of 10 000 highly packed neurons. Every one of them has its own molecular clock (Welsh *et al.*, 1995). The SCN neurons are synchronized: firstly, by external light to "tick" in accordance with the outer world, and secondly, by multiple types of connection between the neurons to "tick" in the same phase. As a result, SCN generates robust oscillations and is able to set the clock in other brain regions, organs and tissues in the whole body (Mohawk *et al.*, 2012).

Within the SCN, there are different populations of neurons, which can be roughly divided into the core (ventral part of the SCN) and the shell (dorso-medial part) (Moore and Silver, 1998). Core neurons receive information about light since they are directly connected with the retina via retino-hypothalamic tract (RHT), whereas shell neurons send the information about specific time of the day to the body by means of neuronal and humoral signals. However, neurons of both parts of the SCN project to downstream structures to set their phase (Watts *et al.*, 1987; Morin, 2007) **(Fig. 2)**. Within the SCN, core neurons send projections to the shell multiple times more than the other way round (Leak *et al.*, 1999; Leak and Moore, 2001). Thus, after light exposure, the changes in the gene expression are first detected in the core and only afterwards in the shell (Silver *et al.*, 1996). These regional differences in the response to light stimuli are important for adjusting to the photoperiodic changes of the environment, meaning that, across the planet, the clock has to adapt to various lengths of the light/dark phases of the day during the year (Sumová *et al.*, 2004). The prediction of the changing photoperiod is also crucial for animals to regulate their breeding time according to the length of their pregnancy. However, this convenient attribute of slight de-synchrony within the SCN circuit is causing difficulties in modern society when traveling across time zones. It is manifested as jetlag, because the core, which is connected with retina, is re-entrained to the new time faster than the shell (Nagano *et al.*, 2003; Davidson *et al.*, 2009; Rohling *et al.*, 2011).

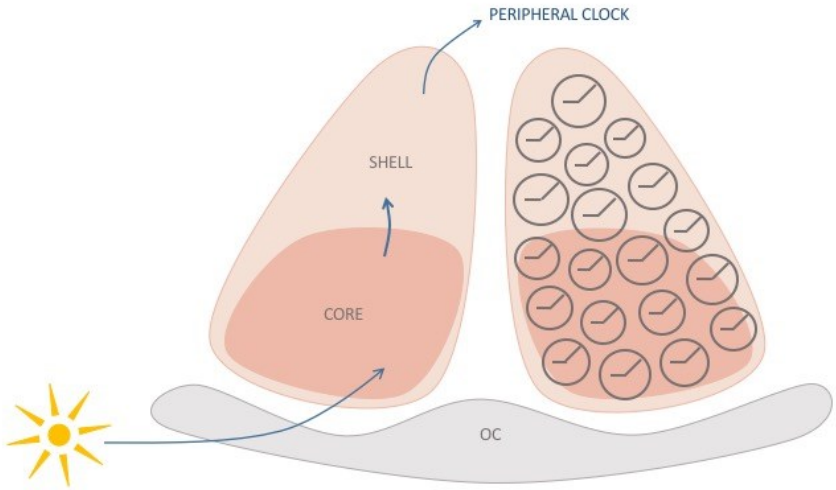

Figure 2. Suprachiasmatic nuclei – SCN
The SCN are composed of two main parts – core and shell. Every cell within the SCN contains its own molecular clock. The cells in the core are synchronized with the external environment via connection with retina and they send the signal to the shell. Rhythmical SCN further set the clocks in the other organs and tissues throughout the body. OC – optic chiasma

1.2.2 Communication within the SCN

In order to maintain the robust oscillations, neurons within the SCN have to communicate with one another, nicely demonstrated by de-synchrony of individual SCN neurons dispersed in the culture (Welsh *et al.*, 1995). The interconnection and communication is realized on several levels. One of them are synapses, whose importance is shown in the discord of the SCN rhythm after using tetrodotoxin, which blocks the Na^+-dependent action potentials and thus blocks the synaptic communication (Yamaguchi *et al.*, 2003). At the synaptic level, the information is mediated via neurotransmitters. Other level of communication is mediated via paracrine neuropeptide signaling, gap junctions and glial cells (Hastings *et al.*, 2018). Most of the communication pathways will be discussed in detail.

1.2.2.1 SCN neurotransmitters and neuropeptides

The best studied aspect of communication throughout the SCN is the transfer of neurotransmitters and neuropeptides. Almost all SCN neurons produce neurotransmitter γ-aminobutyric acid (GABA) (Moore and Speh, 1993), yet the subclasses of the SCN neurons can be also defined by clusters of specific neuropeptides that they release (Silver and Schwartz, 2005). The neurons in core communicate with each other mainly via vasoactive intestinal polypeptide (VIP) and gastrin-releasing peptide (GRP), while shell neurons express arginine vasopressin (AVP) (Maywood *et al.*, 2011a). The receptor for the VIP (VPAC2) is widely expressed in most of the SCN neurons, including those in shell; hence, VIP also serves to secure signaling between core and shell (Kalamatianos *et al.*, 2004). The localization of the expression of VIP and AVP is consistent across mammalian species (Cassone *et al.*, 1988). Quite recently, a third population of neurons which release neuromedin S (NMS) and D1a dopamine receptors (DRD1a) was described but its role is still not entirely clear **(Fig. 3)** (Mori *et al.*, 2005; Lee *et al.*, 2015; Grippo *et al.*, 2017). Although there have been new discoveries about the neuropeptides released throughout the SCN, and novel findings demand a more flexible perception of the SCN architecture, the division into core and shell is still a widely accepted model.

Looking closer at VIP, this peptide is crucial for maintaining synchronization within the SCN, because mice lacking the VIP or VPAC2 have impaired behavioral rhythms (Harmar *et al.*, 2002; Colwell *et al.*, 2003) and activation of VIP neurons in the SCN leads to phase-shifts of the clock *in vivo* and *in vitro*. VIP, due to its location within the SCN core, is also participating in photic entrainment of the SCN and light influences VIP expression in the SCN. VIP and VPAC2 are expressed rhythmically in the SCN *in vivo* and *in vitro*, hence impact of their signaling differs during the day (Reed *et al.*, 2001; Piggins and Cutler, 2003; Jones *et al.*, 2015, 2018; Mazuski *et al.*, 2018). Moreover, VIP can phase-shift rhythmical AVP release (Takahashi *et al.*, 1989; Watanabe *et al.*, 2000) and it can control daily rhythms in heart rate and corticosterone secretion, which links VIP production in the SCN with the downstream physiological processes (Paul *et al.*, 2020).

Neurons in the core also produce GRP and its receptors are present in the shell (Shinohara *et al.*, 1993; Karatsoreos *et al.*, 2006). Similar to VIP, GRP is able to mediate photic entrainment and application of GRP on asynchronous SCN can restore the rhythms (Aida *et al.*, 2002; Maywood *et al.*, 2006). But contrary to the VIP, GRP knock-out mice remain rhythmical

in behavior and clock genes expression. Blocking of GRP receptors *in vitro* also had no effect on the SCN rhythms (Aida *et al.*, 2002).

As the VIP is mainly responsible for synchronization of the SCN neuronal net, AVP, in accordance with its localization, has originally been considered as the output of the SCN, whose attenuation leads to a drop in the robustness of signals coming from the SCN (Brown and Nunez, 1989; Kalsbeek *et al.*, 2010). However, it turned out that AVP also plays role in coupling the SCN neurons and is crucial in determining the circadian period (Mieda *et al.*, 2015, 2016). When receptors for AVP are abolished in knock-out mice or blocked by antagonists of these receptors, the mice and their SCN are resistant to jetlag and they re-entrain to the new time almost immediately (Yamaguchi *et al.*, 2013). This discovery has also high potential to help manage jetlag or shiftwork problems.

By "turning off" individual neuropeptides and their receptors, we can establish their hierarchy within the SCN. The most robust is the VIP signaling, but without VIP, SCN is still able to oscillate due to AVP, the least strong is the GRP signaling. Even though the SCN itself has a number of backup mechanisms to sustain oscillations without mentioned neuropeptides, *in vivo* behavioral and metabolic processes are in most cases impaired, presumably due to missing output signals of these molecules (Li *et al.*, 2009; Maywood *et al.*, 2011a).

Concerning newly described NMS, cells releasing this neuropeptide include 40 % of all SCN neurons and are located in the central part of the SCN, thus spatially overlapping with VIP and AVP neurons. This neuropeptide is, contrary to VIP and GRP, a candidate for non-photic entrainment (Mori *et al.*, 2005). Lengthening the period of NMS neurons led to lengthening the period of behavior rhythms in mice, and moreover, without functional molecular clock within the NMS neurons, mice lacked the synchronous oscillations in behavioral rhythms. These findings suggest that NMS neurons are essential for SCN working as a pacemaker (Lee *et al.*, 2015). However, there is still much to be discovered, not only about NMS, but also about e.g. DRD1a expressing cells, which represent 60 % of all SCN neurons, mostly overlapping with VIP expressing cells, and very little is known about their function (Hastings *et al.*, 2018).

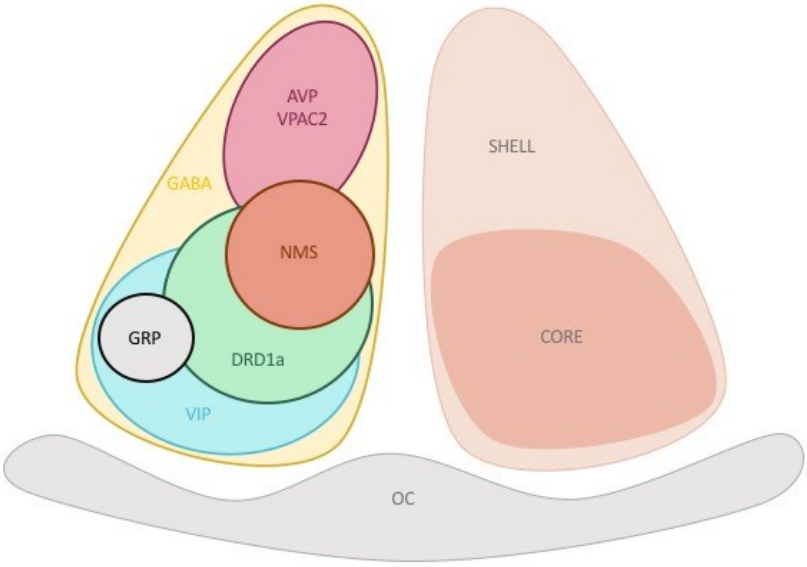

Figure 3. Subtypes of the SCN neurons producing different neuropeptides
All neurons within the SCN communicate via GABA. The core of the SCN releases VIP and GRP, in the shell is expressed AVP and receptors for VIP (VPAC2). The expression of NMS and dopamine receptors (DRD1a) have been described in the middle part of the SCN.

1.2.2.2 Other levels of communication

Based on the fact that SCN exhibit metabolic rhythms already at embryonic age before synaptogenesis is complete (Schwartz and Gainer, 1977; Bedont *et al.*, 2015), it is obvious that synapses are not the only way how SCN neurons communicate in generating synchronized signal. Another level of communication is carried out via paracrine non-synaptic signaling, nicely shown by co-culturing wild-type SCN graft with host SCN slice deficient in different neuropeptide release. Therefore, above described neuropeptides can be released on synapses as well as affect the SCN tissue in paracrine manner (Maywood *et al.*, 2011a; Hastings *et al.*, 2018). It is also speculated about the role of gap junctions which allow electrical communication between nearby neurons and glia (Bennett *et al.*, 1991; Connors and Long, 2004; Long *et al.*, 2005). Gap junctions are present in both SCN neurons and glial cells (Colwell, 2000). Interestingly, coupling mediated via gap junctions is rhythmical and blocking or modulating the release of small molecules from gap junctions resulted in phase-shifting, de-synchrony and arrhythmicity of the SCN (Prosser *et al.*, 1994; Shinohara *et al.*, 2000; Shirakawa *et al.*, 2001). It is possible that without functional gap junctions, the communication between neurons and glial cells is disrupted – hence SCN output is desynchronized (Prosser *et al.*, 1994). Because the location and exact function of the gap junctions in the SCN is not clear, deeper studies are needed. Generally, the amount of the gap junctions within the brain differs during

the ontogenesis (Belluardo *et al.*, 2000). Therefore, the number and the location of the gap junctions within the SCN present an interesting area for studying the development of circadian rhythms.

Despite the very dense net of small-body neurons within the SCN, it was lately discovered that long-time overlooked glial cells also play an important role in maintaining the functional SCN circuit. It is intriguing because their number is approximately three times lower than the number of neurons in the SCN, which is relatively low compared to other brain regions (Güldner, 1983; Azevedo *et al.*, 2009). When the glial metabolic inhibitor – fluorocitrate – was applied *in vitro*, it markedly shortened the period of SCN slices and phase-delayed the behavior of rats *in vivo* (Prosser *et al.*, 1994). This may be explained by diminished communication between SCN neuronal subpopulations, which is partly mediated via glia (Becquet *et al.*, 2008; Wang *et al.*, 2014). Moreover, it was recently described that glial cells and neurons in the SCN have a directly opposite phase in the rhythmical expression of clock genes. Glial cells are active during the circadian night and release glutamate to regulate the neuronal activation in the SCN (Brancaccio *et al.*, 2017). The exact role of the glia has yet to be elucidated, but it is clear from the abovementioned findings that they should no longer be neglected.

On top of what has been previously said, interesting and still not explained is the communication between both SCNs, because in normal physiological conditions, they are ticking in the same phase. The nature of the connection is not the same as within each of the individual SCN – a probable role of glutamatergic signaling has been described, however glutamate is not expressed by SCN neurons, thus other types of cells are likely involved in this SCN-SCN coupling (Strecker *et al.*, 1997; Michel *et al.*, 2013).

1.3 Molecular basis of circadian clock

As mentioned above, every neuron in the SCN has an individual molecular clock which has to be synchronized to the others. However, not only every SCN neuron, but almost every cell within the body has a molecular clock. These self-sustaining clocks are based on transcriptional-translational feedback loops (TTFL) **(Fig. 4)**, The nature of this mechanism is very similar in almost all living organisms – whether it is a mammal, an invertebrate, or a plant – only the names of the genes and proteins and their number sometimes differ (Dunlap, 1999; Harmer *et al.*, 2001; Gardner *et al.*, 2006; Lowrey and Takahashi, 2011). For elucidation of this core mechanism in *Drosophila*, the 2017 Nobel Prize for Physiology or Medicine was awarded.

In mammals, the core loop consists of four key players: genes *Per* (Period; in mammals there are *Per1, 2, 3*) and *Cry* (Cryptochrome; *Cry1* and *Cry2*) that are regulated by proteins CLOCK (and its paralogue NPAS2) and BMAL1 (also known as ARNTL). These positive factors in TTFL bind the regulatory element E-box (enhancer box) in promoters of *Per* and *Cry* – genes coding the negative factors of this loop. At the beginning of the circadian day, transcription factors CLOCK and BMAL1 create heterodimer by binding each other by their PAS domains and together translocate into nucleus, where they bind into E-boxes of *Per* and *Cry* and therefore initiate their transcription (King *et al.*, 1997; Shearman *et al.*, 1997; Gekakis, 1998; Hogenesch *et al.*, 1998; Kume *et al.*, 1999; Vitaterna *et al.*, 1999; Bunger *et al.*, 2000; Takahashi, 2017). This initiation is enabled by CLOCK-BMAL1 interaction with histone acetyltransferases (HATs) in order to provide accessible chromatin for transcription, and CLOCK itself has been shown to have HAT activity (Doi *et al.*, 2006). After translation, approximately in the middle of the circadian day when levels of the proteins are high enough, PER and CRY heterodimerize and translocate into nucleus, where they bind and repress the CLOCK/BMAL1 complex and as a result its own transcription. Consequently, PER and CRY levels decline (Takahashi, 2017). The output of this negative feedback loop is the rhythmical expression of *Per* and *Cry,* and other genes containing the E-box in their promoter, called "clock-controlled genes" (Bass and Takahashi, 2010; Asher and Schibler, 2011).

The core loop is stabilized by a second (positive) loop consisting of *RevErb (*α – also *Nr1d1*; and β *– Nr1d2*) and *Ror (A, B, C)* genes. The role of this additional loop is to drive the rhythmical expression of *Bmal1* by the binding of REVERB or ROR protein products into its promoter regulatory element RORE: REVERBs inhibit the *Bmal1* expression, whereas RORs activate it. This leads to an anti-phase rhythm of *Bmal1* to *Per* and *Cry* mRNA (Preitner *et al.*, 2002; Triqueneaux *et al.*, 2004; Akashi and Takumi, 2005).

The third loop consists of *Dbp* (D-box binding protein), which contains E-box and is thus regulated by CLOCK/BMAL1 complex, and of *Nfil3* (nuclear factor, interleukin-3 regulated; also known as *E4bp4*), which is regulated by REVERB/ROR at the RORE promoter site. DBP and NFIL3 interact at the D-box promoter site of *Ror, RevErb and Per* genes (*RevErb and Per* genes have both D-box and E-box in promoter) (Mitsui, 2001; Ueda *et al.*, 2005; Ohno *et al.*, 2007). Altogether, these three interlocking feedback loops generate transcription of clock genes and proteins with different phases of expression throughout the 24h day (Takahashi, 2017) **(Fig. 4)**.

Considering the usual time taken by the transcription and translation processes, it is obvious that there has to be some delaying mechanism to prolong the cycle into 24 hours. This is ensured by a set of posttranscriptional and posttranslational events. An important role in prolonging the period of expression rhythms is played by, on one hand, casein kinases 1 delta and epsilon (CK1δ, CK1ε) – serine/threonine kinases phosphorylating PER proteins and thus marking them for ubiquitylation by SCF (Skp, Cullin, F-box) E3 ligase complex (Akashi *et al.*, 2002; Eide *et al.*, 2005), and, on the other hand, AMPK (AMP-activated protein kinase) phosphorylating CRY proteins, which targets them for ubiquitin-dependent degradation by two paralogues: FBXL3 and FBXL21 (F-box and leucine-rich repeat proteins) - parts of SCF (Busino *et al.*, 2007; Godinho *et al.*, 2007; Siepka *et al.*, 2007; Lamia *et al.*, 2009). For the stability of PER and CRY, balance between all mentioned players is necessary (Lee *et al.*, 2011). When the proportion of PER and CRY is optimal, they create heterodimer and, together with both casein kinases, they translocate into nucleus. As long as the ratio of the two clock proteins is not ideal, they are degraded in proteasome (Reischl and Kramer, 2011). Supported by experiments with mutated CK1δ, CK1ε, FBXL3 and FBXL21, or their combinations, it seems most likely that the (in)stability of PER and CRY proteins is responsible for determining the period of the circadian mechanism (Lowrey, 2000; Busino *et al.*, 2007; Godinho *et al.*, 2007; Siepka *et al.*, 2007; Maywood *et al.*, 2011b). The same mechanism of PER and CRY degradation is employed at the beginning of the circadian day to free the CLOCK-BMAL1 proteins complex binding the E-boxes, and enables their further function as transcription factors (Takahashi, 2017). It is beyond doubt that other factors will be added into this intricate, yet fine-tuned mechanism **(Fig. 4)**.

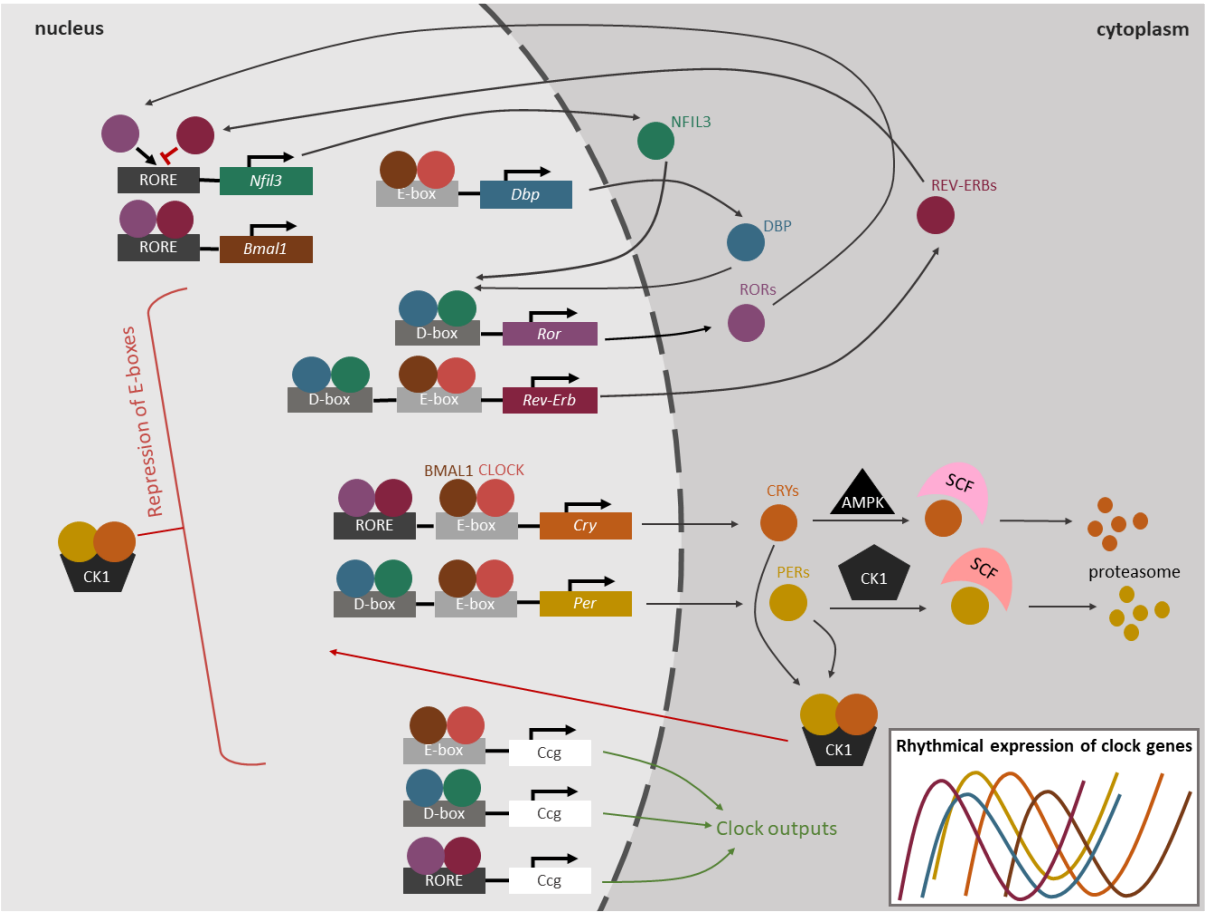

Figure 4. Molecular mechanism of mammalian biological clock

The molecular clock comprises three transcriptional-translational feedback loops (TTFL). The core loop consists of transcription factors BMAL1 and CLOCK, which bind to E-box regulatory elements in promoters of genes *Per* and *Cry.* After PER and CRY proteins are translated in cytoplasm, they bind to casein kinases 1 (CK1) and translocate as a heterodimer into nucleus to inhibit their own transcription by binding to BMAL1-CLOCK complex. If the amount of PER and CRY proteins is not sufficient and properly balanced to make the heterodimer, they are targeted for ubiquitin-dependent degradation in proteasome. In that case, PER proteins are phosphorylated by CK1, and CRY proteins by AMPK, after which are both ubiquitinated by SCF E3 ligase complexes. The second loop is composed of *RevErb* and *Ror* genes, which bind into RORE promoter site, and drive the rhythmical expression of *Bmal1*. *Dbp* and *Nfil3* genes form the third loop. They bind into the D-box promoter element of target genes. Diverse distribution of regulatory elements and their combinations in promoters of clock and "clock-controlled genes" (Ccg) create a complex mechanism of rhythmical expression with various phases during the 24h day (According to: Takahashi, 2017).

The TTFL machinery drives circadian rhythms in the expression of clock-controlled genes. The fascinating fact is that this group covers from 5 to 20 % of all genes and proteins in the mammalian body, depending on tissue (Akhtar *et al.*, 2002; Duffield *et al.*, 2002; Panda *et al.*, 2002). The circadian regulation happens on multiple levels, first of them is transcription, during which clock proteins are binding into E-box, D-box and RORE elements of concerned genes. The other levels of control cover splicing, polyadenylation, nuclear export, miRNA pathway, translation and RNA degradation (Lim and Allada, 2013; Kojima and Green, 2015). Above all, epigenetics is starting to be taken into account (covered in detail in Takahashi, 2017). Many of clock and clock-controlled genes are involved in various metabolic pathways, immune response or cell cycle control (Bozek *et al.*, 2009). Moreover, large number of drugs directly target the products of circadian genes (Zhang *et al.*, 2014). The aforesaid points out the importance of circadian system and also the necessity of understanding it.

1.4 SCN development

The synchrony of the SCN neurons is the crucial feature of the central pacemaker, as was described in previous chapters. During ontogenesis, many of key synchronizing properties are missing or are not fully finished, which is reflected in the gradual development of the clock genes oscillations in the perinatal and postnatal SCN (Shibata and Moore, 1987; Sládek *et al.*, 2004; Sumova *et al.*, 2012) **(Fig. 5)**. For example, synaptogenesis within the rodent SCN proceeds into postnatal period (Moore and Bernstein, 1989; Bedont and Blackshaw, 2015). The same applies to the appearance and maturation of glial cells, these are first detected only shortly before delivery (Botchkina and Morin, 1995; Munekawa *et al.*, 2000). The neurogenesis itself is finished in the second half of the pregnancy (Ifft, 1972; Shimada and Nakamura, 1973; Altman and Bayer, 1978; Kabrita and Davis, 2008). Consistently across rodent species, neurogenesis in ventral SCN core precedes the maturation of dorsal shell and it was proposed that core and shell neurons are derived from distinct progenitors. Interestingly, development of the shell neurons lasts longer compared to core neurons and this anteroposterior patterning is similar to photoperiodic encoding in adulthood (Altman and Bayer, 1978; Ralph *et al.*, 1990; Kabrita and Davis, 2008; Carmona-Alcocer *et al.*, 2020).

Timing of developmental processes depends on animal species. In mice, where embryogenesis lasts 19-21 days, neurogenesis occurs between embryonic (E) day 10 and 15, astrocyte development starts around E17 and is finished when pups open their eyes, around postnatal (P) day 13 (Shimada and Nakamura, 1973; Kabrita and Davis, 2008; Shimogori *et al.*, 2010; Bedont and Blackshaw, 2015). Synaptogenesis of the SCN was not studied in mouse model so far. *Avp* mRNA is first detected at E17.5 and protein postnatally (VanDunk *et al.*, 2011). *Vip* mRNA is expressed at E18.5 and protein also postnatally (Landgraf *et al.*, 2014). In rats, having gestational period about 21-22 days long, neurogenesis lasts from E12 to E18, synaptogenesis starts at E19 and is completed at P10, when light can reach the SCN, and astrocyte maturation occurs from E20 to P13 (Moore and Bernstein, 1989; Munekawa *et al.*, 2000; Cambras *et al.*, 2005; Bedont *et al.*, 2015). Expression of *Vip* mRNA is detected at E18, with its rhythm appearing at E19, similarly as the oscillating expression of *Avp* (Ban *et al.*, 1997; Houdek and Sumová, 2014). VIP protein was found in the fetal SCN at E20, whereas AVP protein occurs postnatally (Laemle, 1988; Isobe *et al.*, 1995).

Interconnection of the SCN with other parts of the brain and with retina happens mostly postnatally in both mice and rats (Bedont *et al.*, 2015), which makes, together with the aforesaid, the developing SCN vulnerable to external signaling, contrary to the extremely

resilient adult SCN (Nishide *et al.*, 2008). Despite all this, rat SCN show rhythmical utilization of glucose already at E19 (Schwartz and Gainer, 1977) and higher daytime firing at E22 *in vitro* (Shibata and Moore, 1987). Furthermore, the rhythmical expression of clock genes was detected already in the embryonic age (Shimomura *et al.*, 2001; Houdek and Sumová, 2014; Carmona-Alcocer *et al.*, 2020).

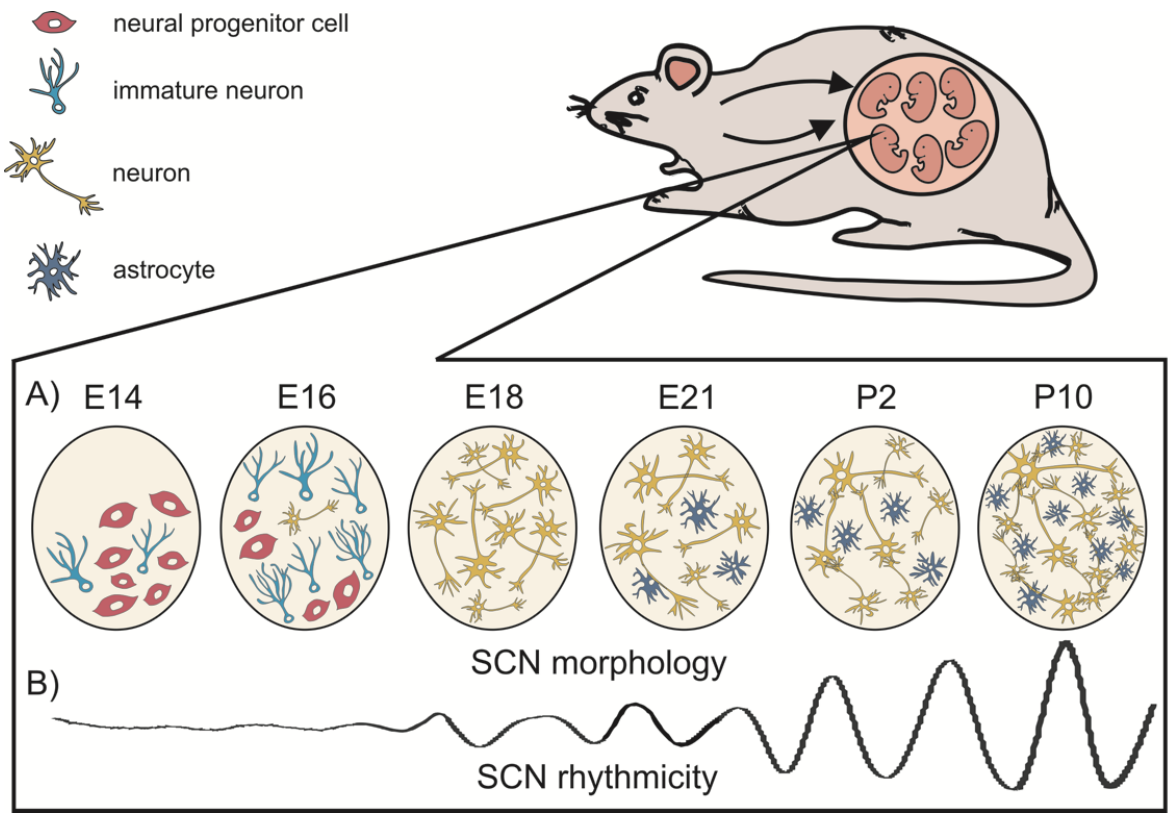

Figure 5. Gradual development of the SCN rhythmicity
As the ontogenesis proceeds, the rhythms of the developing SCN are becoming more and more robust. After the processes of neurogenesis, synaptogenesis and maturation of the glial cells are complete, the postnatal SCN oscillations are equal to that of the adults. In rats, this happens at postnatal day 10, which coincides with the opening of the pups' eyes (from Sumová and Čečmanová, 2020).

1.4.1 Development of molecular clock within the SCN

To ascertain the molecular mechanism within the fetal SCN, it is crucial to select, in the view of the fact that it is an extremely small portion of tissue, a highly sensitive approach. *In vivo* studies mainly use *in-situ* hybridization and laser dissection to detect rhythms in mRNA expression. In these experiments, using population of SCN to construct 24h rhythms is necessary. In rats, the first rhythmical gene is *RevErbα* at E19, followed by *Per2* and *Bmal1*, both rhythmical at E20/21. By this time, expression of the clock genes is in the right phase to one another. Even though mRNA was detected before birth, levels of PER and CRY proteins are not detectable in fetal stage. Interestingly, rhythm in immediate early gene *c-fos* was detected also by E19, accompanied by *Vip* and *Avp* (Houdek and Sumová, 2014). In mice, *Per1* daily oscillations were reported at E17, but *Per2* expression stays constitutive up to P3 (Shimomura *et al.*, 2001). Daily changes in some mouse clock proteins levels are detectable at P2 (Ansari *et al.*, 2009). As *in vivo* studies still meet some limitations, the exact point of initiation of the clock molecular machinery is still not known. The robustness and the right phasing of all core clock genes develops step by step postnatally, and state corresponding to adult SCN can be observed at P10, which is in line with finished morphological maturation of the SCN (Sládek *et al.*, 2004; Kováčiková *et al.*, 2006; Olejníková *et al.*, 2015) **(Fig. 5)**.

Another approach to examine embryonic rhythms is *in vitro* by using organotypic explants of the SCN. The most common are explants made from genetically modified mice, where *Per2* clock gene is fused with luciferase and the resulting bioluminescence signal can be detected in real time by adding luciferin into media. The advantage is the possibility of examining one SCN slice from each animal separately and without the influence of the external, mainly maternal, signals. These techniques revealed stable rhythmical expression of PER2 protein in mouse E15.5 SCN (Wreschnig *et al.*, 2014; Landgraf *et al.*, 2015; Carmona-Alcocer *et al.*, 2018). By this time, 70 % of neurons within the SCN oscillate, compared to E14.5, where only 10 % are rhythmical (Carmona-Alcocer *et al.*, 2018). Interestingly, these rhythms persist without GABA or VIP signaling (Wreschnig *et al.*, 2014; Carmona-Alcocer *et al.*, 2018). Even though SCN is rhythmical in culture prenatally, its immaturity leads to extreme sensitivity to culturing procedure manifesting in large responses to nonspecific stimuli (Nishide *et al.*, 2008). Reason for earlier *in vitro* detection of rhythmical clock genes compared to *in vivo* studies is unclear. Whether PER2-driven bioluminescence detection is more sensitive, or whether there is some bias hidden in *in vivo* population studies remains to be elucidated.

1.4.2 Maternal signals setting the developing clock

Due to the inability of the fetus to detect light changes directly, its clock has to be set via maternal signals. It was shown that pups are born with clocks entrained to their mothers' (El-Hennamy *et al.*, 2008). This mechanism is poorly understood but it seems that embryonic clock is synchronized in a similar way as the other peripheral clocks in maternal body. This includes behavioral, hormonal and metabolic signals (Duncan *et al.*, 1986; Reppert and Schwartz, 1986; Weaver and Reppert, 1989; Viswanathan *et al.*, 1994; Varcoe *et al.*, 2011) **(Fig. 6)**. The importance of synchronization between offspring and mother is obvious especially in animals with large litters, such as rodents. Also, maternal chronodisruption may lead to preterm births (Reschke *et al.*, 2018), and serious conditions in later life of the offspring (Amaral *et al.*, 2014; Smarr *et al.*, 2017).

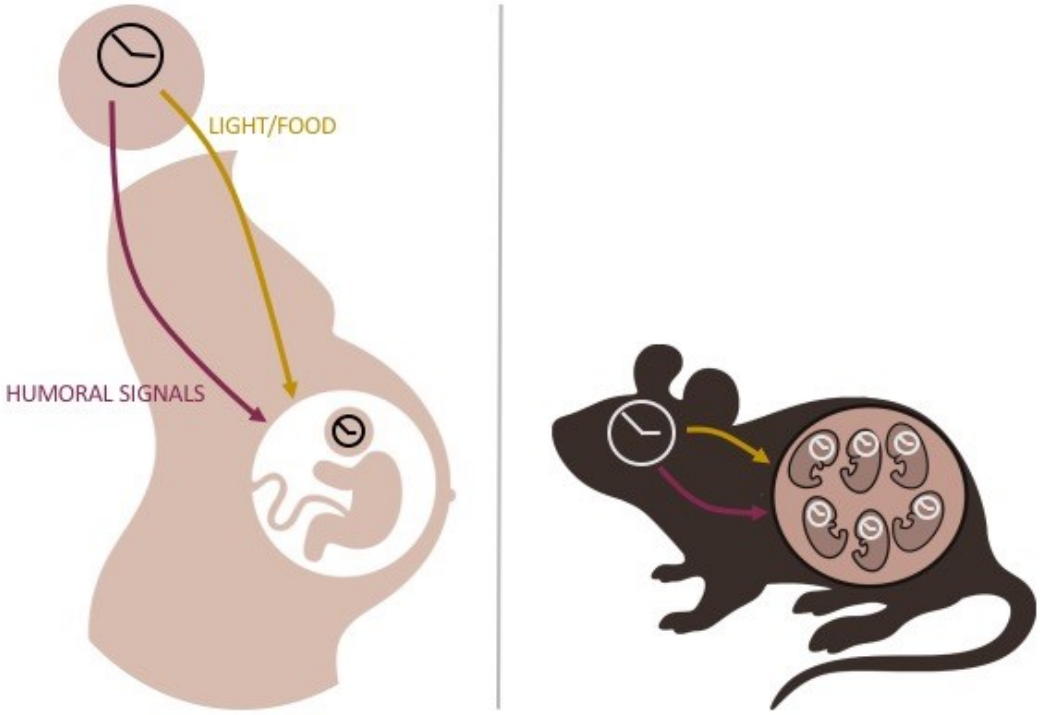

Figure 6. Maternal synchronization of the fetal SCN
Mother has to synchronize its embryos with the external conditions to prepare them for the changing environment and inform them about the time of the day. Some of these signals were already described, others still await their discovery. Humoral signals, such as melatonin, dopamine, and glucocorticoids, as well as behavior and metabolic cues are probably participating in the entrainment of the developing clocks. In the animals with large litters, mother has to set the clocks in all embryos, to assure their mutual synchrony as well.

We know quite a lot about the architecture and response of the mature SCN to the external stimuli, however, to study the fetal SCN clock and their responses to various inputs, it was necessary to establish more sensitive techniques and modern approaches. Thus, much of the previous works focusing on maternal signaling were done postnatally. A very effective way to influence pups' clock seems to be to change maternal behavior by manipulating her light schedule. Pregnant rats exposed to shift of the light/dark (LD) regime were able to entrain the clock of their newborns (El-Hennamy *et al.*, 2008). Moreover, arrhythmic mother, due to exposure to constant light (LL regime) or to SCN lesion, leads to desynchronized pups (Reppert and Schwartz, 1986; Shibata and Moore, 1988; Weaver and Reppert, 1989; Nováková *et al.*, 2010). Other way how to examine the role of maternal behavior is to change her feeding time. For example, when rats were kept on LD regime combined with restricted feeding (RF) during non-active phase of the day it had no effect on newborns' SCN clock; unlike in mothers kept on LL regime combined with RF (when their activity corresponded to food availability), which led to newborns' SCN adjusting to the maternal RF regime (Nováková *et al.*, 2010; Olejníková *et al.*, 2015). Apart from food intake, stimulating maternal behavioral changes can be achieved also by cross-fostering pups within two different rat strains, which leads to changes in pups' clock as well (Olejníková *et al.*, 2018).

Above-mentioned processes are multifactorial and are probably guided by a mixture of particular humoral and metabolic pathways, so for a deeper understanding of these and other synchronizing mechanisms, impacts of the individual hormones were also examined. One of the most prominent hormones in circadian field is melatonin. It is elevated in maternal blood during dark period of the day (Kivelä, 1991; Nakamura *et al.*, 2001), and melatonin receptors are expressed already in the fetal SCN (Reppert *et al.*, 1988). Prenatal administration of melatonin to SCN-lesioned mothers set the phase of behavioral rhythms in P20 hamsters (Davis and Mannion, 1988; Viswanathan and Davis, 1997) and injection of melatonin to pinealectomized pregnant rats kept on LL, established rhythms in *c-fos* and *Avp* genes in the SCN of their P1 pups (Houdek *et al.*, 2015).

Dopamine is referred to as a strong regulator of the developing circadian circuit, with its maximal levels in blood during active phase of the day, and its D1 receptors present in the fetal SCN (Bender *et al.*, 1997; Duffield *et al.*, 1999). It was proposed that it has a complementary function to melatonin (Sowers and Vlachakis, 1984; Hirst *et al.*, 1991; Viswanathan and Davis, 1997; Yujnovsky *et al.*, 2006). Sensitivity of the SCN to dopamine is decreasing during development, as it is being more and more synchronized by the light (Weaver and Reppert, 1995). Injection of dopamine receptor agonist to hamster mothers has a significant

influence on pups' SCN (Viswanathan *et al.*, 1994; Viswanathan and Davis, 1997). And prenatal exposure to dopamine can influence the light response later in life (Ferguson and Kennaway, 2000).

Another hormone with strong circadian variation in blood stream and breast milk, as well as with receptors in the fetal and postnatal SCN is corticosterone, the glucocorticoid steroid hormone that is secreted from the adrenal glands, also known as a stress hormone (Cheifetz, 1971; Rosenfeld *et al.*, 1988; Olejníková *et al.*, 2018). It plays an important role in control of homeostasis, differentiation and development (Seckl, 2001). Glucocorticoids were also described as a strong synchronizer in peripheral clock (Balsalobre, 2000; Torra *et al.*, 2000; Cheon *et al.*, 2013), but adult SCN is resilient to their signaling (Balsalobre, 2000; Tahara *et al.*, 2015). It was demonstrated that stress can affect SCN clock in blinded rats (Ohta *et al.*, 2003). Changes in feeding regime can elevate plasma corticosterone levels of mothers and thus influence the fetal/neonatal SCN clock (Krieger, 1974; Olejníková *et al.*, 2018). Whether these or other hormones may affect the clock before birth remains to be confirmed.

1.5 Peripheral clocks and their synchronization

SCN communicate with the rest of the body via both neuronal and humoral signals in order to synchronize peripheral organs, which harbour their own circadian clocks (Sakamoto *et al.*, 1998; Balsalobre *et al.*, 2000; Nagoshi *et al.*, 2004; Yamamoto *et al.*, 2004; Yoo *et al.*, 2004; Wu *et al.*, 2010). Describing clocks and their properties in all peripheral organs as well as brain regions is beyond the scope of this thesis; hence, only nature of their synchronization and communication with the central clock will be roughly mentioned here. By synaptic connections, SCN sets the clock in regions of hypothalamus, thalamus and forebrain (Watts, 1991; Kalsbeek and Buijs, 2002). Hormones described to be employed in setting the phase of peripheral clock include glucocorticoids, dopamine, melatonin, and insulin (Balsalobre, 2000; Pevet and Challet, 2011; Baba *et al.*, 2017; Crosby *et al.*, 2019). More recently, a role of peptides ghrelin and leptin has been suggested (LeSauter *et al.*, 2009; Arble *et al.*, 2011). The peripheral clocks, which drive release of hormones, can be set not only by SCN-driven signals, but others as well (Damiola *et al.*, 2000; Brown *et al.*, 2002). This can be an advantage as well as an obstacle because more synchronous signals make the clock more robust, but discrepancies in timing of these signals may lead to its de-synchrony and consequently cause serious health problems (Axelsson *et al.*, 1989; Schernhammer *et al.*, 2001; Maury *et al.*, 2010). In other words, internal synchrony in the whole body is crucial for proper functioning of the organism. Although the circadian clocks in most of the peripheral organs are quite well described and understood, there is one particular temporary organ, in which its function from the circadian point of view is still shrouded in mystery.

1.5.1 Placental clocks and their synchronization

One of the peripheral organs probably accommodating circadian clock is placenta. Placenta plays a crucial role in pregnancy, providing a functional interface between mother and fetus, supplies embryo with nutrients (oxygen, sugars, lipids, proteins), and assures protection against detrimental compounds (toxins). It also modulates the influx of substances from maternal blood (hormones) and in addition, synthesizes many of them on its own.

Placenta develops concurrently with the embryo and consists of many types of cells. In general, placenta derives from two types of tissue: fetal and maternal. These two parts are tightly connected in hemochorial type of placenta which can be found almost exclusively in humans and rodents, determining rats and mice as an ideal animal models for placental studies (Fonseca *et al.*, 2012; Soares *et al.*, 2012). Hemochorial type is the most invasive form of

placentation. In more detail, fetal part of the rat placenta is comprised of labyrinth, where oxygen and nutrients are exchanged, because fetal and maternal blood are here in close proximity; junctional zone producing hormones; and chorionic plate. Maternal part of placenta consists of decidua basalis derived from endometrial tissue (Fonseca *et al.*, 2012) **(Fig. 7)**. Abnormalities in placentation can lead to pathological conditions such as preeclampsia (Kaufmann *et al.*, 2003; Geusens *et al.*, 2010; Fonseca *et al.*, 2012; Soares *et al.*, 2012).

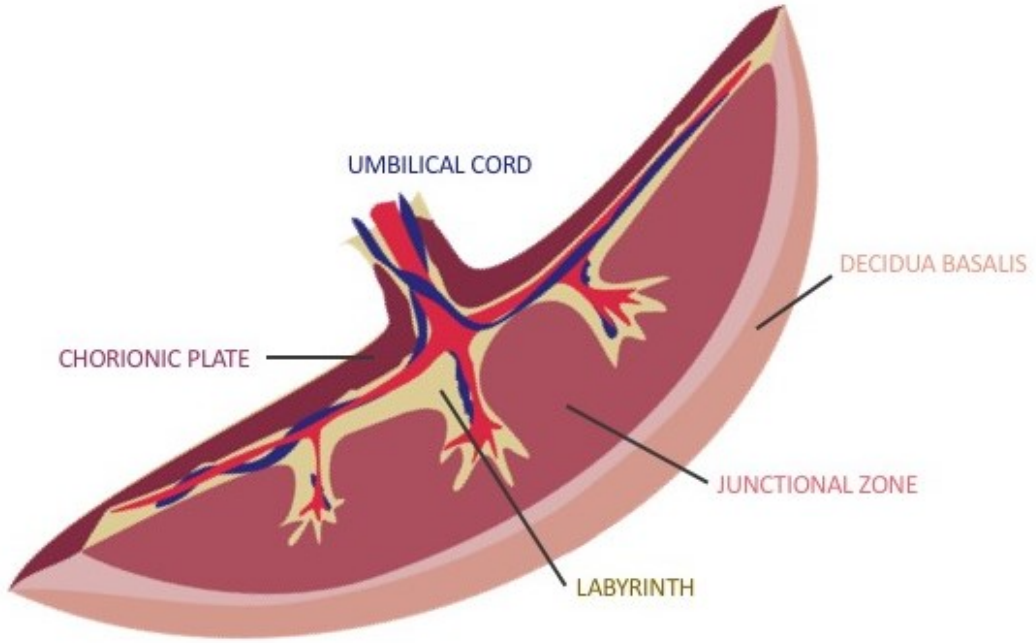

Figure 7. Rodent placenta
Rodents and humans share the hemochorial type of the placenta, in which the maternal and fetal blood are in close proximity. Maternal part of placenta (decidua basalis) is derived from maternal tissue, fetal part of placenta originates from trophoblast and comprises of junctional zone, labyrinth and chorionic plate. Umbilical cord comes out from fetal side of placenta and connects it with the fetus.

Studies of human placental clock are rare and arguable, because it is hard to have a consistent and comparable set of placenta samples collected at the same stage of the pregnancy, over the entire day, and moreover, from mothers of the same age and babies of the same sex. Nevertheless, there are chronobiological studies providing evidence of rhythmical gene expression in human full-term placenta (Pérez *et al.*, 2015), and data describing abnormalities in clock mechanism, if maternal daily rhythm is disrupted by shift-work (Clarkson-Townsend *et al.*, 2019). For ethical and experimental reasons, most of the work examining the placental clock was performed on rat and mouse models (Akiyama *et al.*, 2010; Ratajczak *et al.*, 2010;

Wharfe *et al.*, 2011; Crew *et al.*, 2018) or alternatively on human trophoblast cell lines cultured *in vitro*, where rhythms in clock genes were detected (Frigato *et al.*, 2009). In rats, the expression of clock genes was shown in labyrinth and junctional zone *in vivo*, but the circadian variation was very shallow (Wharfe *et al.*, 2011; Crew *et al.*, 2018). It seems that the metabolic state of the mother is an important aspect of placental clock, since obese Wistar rats in the last week of pregnancy show down-regulation of *RevErbα* gene expression (Crew *et al.*, 2018). *In vitro* placental explants made from rats at the day of their delivery show rhythms of PER1 protein only in the maternal part of the placenta (Akiyama *et al.*, 2010). In mice, *in vitro* explants from the whole placenta, prepared a week before and one day before delivery, show rhythmical expression of PER2 protein, and mRNA from these explants reveal rhythms in genes *Per* and *Cry* as well as *Dbp* (Ratajczak *et al.*, 2010). In comparison to the other peripheral clocks, placental rhythms are not so robust and overall expression levels of clock genes is changing during pregnancy (Akiyama *et al.*, 2010; Dibner *et al.*, 2010; Ratajczak *et al.*, 2010; Crew *et al.*, 2018).

Because of the mentioned maternal influence on the embryonic clock, and the fetal origin of some placental parts, it is crucial to better understand the reason for having a circadian system in such a temporal organ as the placenta, as well as its function. It is of utmost importance to examine the effect of hormones and other substances, which have been shown to play some role in pregnancy and in maternal signaling, on placental circadian clock. Likely candidates for synchronizing signal are glucocorticoid hormones, since they are rhythmically released into maternal bloodstream (Cheifetz, 1971), placenta possesses the glucocorticoid receptors and regulates their influx to the fetus by synthesizing 11β-hydroxysteroid dehydrogenase (HSD2) enzyme, whose role is to degrade glucocorticoids, and thus protect the fetus from their noxious excess (Waddell *et al.*, 1998; Burton and Waddell, 1999). Other candidates are melatonin and dopamine, which share maternal rhythmical release and presence of their receptors in placenta with glucocorticoids. Moreover, both of these hormones are transported almost unmetabolized across the placenta to reach the fetus (Watanabe *et al.*, 1990; Kim *et al.*, 1997; Naitoh *et al.*, 1998; Okatani *et al.*, 1998; Vaillancourt *et al.*, 1998; Lanoix *et al.*, 2008; Gratz *et al.*, 2018). Melatonin is mainly synthesized in pineal gland, but, during pregnancy, this hormone is also produced by placenta itself, where it up-regulates antioxidant enzymes and thus protects the fetus from oxidative stress as well as other pregnancy-related pathologies (Lanoix *et al.*, 2008; Tamura *et al.*, 2008; Richter *et al.*, 2009; El-Malkey *et al.*, 2021). Dopamine is produced in neurons of substantia nigra, and its main role in placenta is inhibiting secretion of placental lactogen from trophoblast cells (Lee *et al.*, 1999). Since

decreased maternal lactogen is linked with preeclampsia (Letchworth and Chard, 1972), the impact of dopamine on placental tissue should be further examined. It has been shown that many peripheral tissues are entrainable by feeding schedule (Damiola *et al.*, 2000; Schibler *et al.*, 2003, 2015), therefore the influence of hormones and peptides connected with food consumption needs to be also elucidated. All presented facts underline the importance of taking placenta into account in chronobiological developmental studies.

Aims of the thesis

The main goal of this thesis was to understand the mechanisms of entrainment of the fetal circadian system. To elucidate these processes, partial aims were defined:

1**) To summarize recent findings about the emergence of circadian oscillations in the fetal SCN, and to point out the knowledge gaps and necessity of new approaches to fill them.**

2) To determine the influence of changes in maternal behavior on expression of clock genes within the fetal SCN.

3) To describe the effect of glucocorticoids on the placental and fetal SCN clock.

4) To investigate placental clocks in rodents and examine their sensitivity to different hormones, which might play the role in placental clock synchronization.

List of publications

Publications discussed in this thesis

1) Sumová A, Čečmanová V. Mystery of rhythmic signal emergence within the suprachiasmatic nuclei. Eur J Neurosci. 2020 Jan;51(1):300-309. doi: 10.1111/ejn.14141. Epub 2018 Sep 27. PMID: 30188597. IF: 3.115

2) Lužná V, Houdek P, Liška K, Sumová A. Challenging the Integrity of Rhythmic Maternal Signals Revealed Gene-Specific Responses in the Fetal Suprachiasmatic Nuclei. Front Neurosci. 2021 Jan 7;14:613531. doi: 10.3389/fnins.2020.613531. PMID: 33488354. IF: 3.707

3) Čečmanová V, Houdek P, Šuchmanová K, Sládek M, Sumová A. Development and Entrainment of the Fetal Clock in the Suprachiasmatic Nuclei: The Role of Glucocorticoids. J Biol Rhythms. 2019 Jun;34(3):307-322. doi: 10.1177/0748730419835360. Epub 2019 Mar 11. PMID: 30854919. IF: 3.122

4) Lužná V, Liška K, Sládek M, Sumová A. Hormonal fine-tuning of clock in decidual region of mouse placenta by dopamine, melatonin, insulin, leptin and ghrelin. Placenta. 2021 Mar 27;108:55-63. doi: 10.1016/j.placenta.2021.03.015. Epub ahead of print. PMID: 33819862. IF: 3.177

Publication not related to this thesis

5) Liška K, Sládek M, Čečmanová V, Sumová A. Glucocorticoids reset circadian clock in choroid plexus via period genes. J Endocrinol. 2021 Feb;248(2):155-166. doi: 10.1530/JOE-20-0526. PMID: 33350982. IF: 4.041

Summary of the publications

For deeper understanding, detailed information and figures, please see the attached publications in Supplement. The postulated aims of this thesis correspond with following publications:

Publication 1

Sumová A, Čečmanová V. Mystery of rhythmic signal emergence within the suprachiasmatic nuclei. Eur J Neurosci. 2020 Jan;51(1):300-309. doi: 10.1111/ejn.14141. Epub 2018 Sep 27. PMID: 30188597.

My contribution to this publication was proofreading the manuscript, reviewing the current literature, and creation of the illustration depicting the gradual development of the fetal SCN clock.

In this review, we focused on fetal circadian clock, and put emphasis on elucidation of the reasons why it is hard to detect the exact time of the emergence of circadian clock within the fetal SCN. We also discussed issues that have to be solved to answer this research question.

Firstly, we described the hierarchy of the circadian system in mammals, molecular mechanism of the clock and complexity of the SCN network. We further focused on morphological development of the SCN, with emphasis on rodents as model organisms. Based on recent knowledge, it seems that rhythmical expression of the clock genes begins during late prenatal stage, and the robustness and synchrony of the whole system develops gradually up to postnatal period. This progressive process is probably due to neurogenesis, synaptogenesis and astrocytes occurrence in different stages of the ontogenesis. The reason for the detection of the rhythmical signals, before all mentioned processes are completed, are probably maternal signals. We summarized all the experiments that support this hypothesis and put the accent on maternal feeding regime as a time-cue signal synchronizing the fetal circadian clock in the absence of fully functional maternal SCN.

We also described the development of a molecular clock in the fetal SCN. This happens on multiple (single cell, cellular network, and systemic) levels – but none of these are fully understood yet. Turning point in the investigation was the implementation of new sensitive techniques into research. Currently, there are new *in vivo* and *in vitro* approaches available.

Nevertheless, information gained by both is somewhat contradictory, which may be caused by limitations in these two types of studies. In *in vivo* experiments it is necessary to construct 24h profiles of clock genes expression from multiple animals (typically at least 4-5 animals per time point, sampled in 3-6h intervals). Major advantage of this approach is that gene expression levels reflect natural situation including the influence of maternal signals. On the other hand, detecting the signal from one animal is possible in *in vitro* studies, but without the natural environment of the tested tissue. We tried to solve this issue in the last part of our publication, in which we postulated a hypothesis on the origin of the SCN rhythmicity.

Based on our own as well as other observations, *in vitro* culturing of the fetal SCN explants presents a challenging problem because tissue without proper synaptic and other connections is highly sensitive to culturing procedure *per se*. Thus, the described early emergence of the circadian rhythms may be just a consequence of the medium/temperature change accompanying the explanting process, which can set the phase of individual uncoupled cells. According to published data, the number of rhythmic cells within the SCN is gradually increasing during development. Thus, in *in vivo* experiments, during which we dissect fetal SCN and isolate mRNA from the whole tissue to construct expression profiles, it may cause inconsistent results and blur the fact that some parts of the SCN are already oscillating. What is clear so far is that some genes (namely *Vip* and *Avp*) and also outputs from the fetal clock show rhythms with higher amplitude compared to clock genes in the same developmental stage. As previously hypothesized (Sumova *et al.*, 2012), current knowledge is in favor of dominant role of maternal signals in synchronizing the cellular clocks in the fetal SCN as these may potentially set the rhythms of output signals from the fetal SCN, independently of the clock genes. Gradually, maternal signals are replaced by more and more robust clock machinery of the pups' SCN and, above all, their ability to sense the light directly. In the final part of our review, we lay stress on combining both *in vivo* and *in vitro* approaches, together with the introduction of new techniques to shed light on the origins of the fetal clock oscillations.

This study was supported by the Czech Science Foundation grant 16-03932S (to A.S.), the OPPK BrainView CZ.2.16/3.1.00/21544 and the Research Project RV0: 67985823.

Publication 2

Lužná V, Houdek P, Liška K, Sumová A. Challenging the Integrity of Rhythmic Maternal Signals Revealed Gene-Specific Responses in the Fetal Suprachiasmatic Nuclei. Front Neurosci. 2021 Jan 7;14:613531. doi: 10.3389/fnins.2020.613531. PMID: 33488354.

My contribution to this publication: experiments – mating the animals, measuring the body weights, manipulation of light-dark and feeding regime, collecting tissue samples, sectioning the frozen brains in two out of four experimental groups, laser dissecting of the SCN in all experimental groups; RNA isolation, reverse-transcription and RT-qPCR of all samples, analyzing the data, creating the graphs; manuscript – writing drafts of introduction, material and methods, results and figure legends; constructing the figures, proofreading the final text.

In this publication, we tested whether and how the fetal SCN clock will be influenced by various manipulations with maternal regime. In all experiments we used pregnant Wistar rats whose fetuses were sampled at embryonic day (E) 19 in 3h intervals during 24 hours, to cover one circadian cycle. We analyzed mRNA expression of nine chosen genes in laser-dissected SCN of their embryos. In addition, we recorded locomotor activity of the mothers, and weighed them and their food consumption during the experiment, along with weighing embryos and placentas at the end of the study.

We performed two types of experimental setups, both with two groups of pregnant rats. The first experiment consisted of shifting the LD regime by 6 hours in the direction of phase delay to one group of mothers at E14. These were compared with the control group, which remained on the original LD 12:12 regime for the whole 19 days of the experiment. Therefore, animals exposed to phase delay had 4-5 days (depending on time of their sampling) for adaptation to the new light regime. This manipulation imitated a situation of disturbed circadian system, which can be an analogy to shift work or just the irregular modern life style. We analyzed expression of mRNA from the fetal SCN from both groups of mothers using RT-qPCR. As a result, we found out that the expression levels of clock genes from delayed embryos were mostly lower compared to the control group. On the other hand, the expression of *Vip* and *Avp* genes was not affected by delayed LD regime in any way. An interesting discovery was that only the expression of gene *c-fos* was shifted in accordance with delayed light/dark regime, indicating that this immediate early gene is sensing the maternal behavioral state and is able to adjust to the new regime.

In the second set of experiments, we exposed two groups of rats to constant light (LL) from the beginning of their pregnancy to desynchronize their circadian system and thus eliminate rhythmical cues to fetuses. One group of rats was kept on LL regime and fed *ad libitum*, while the other group was exposed to LL combined with restricted feeding (RF), with food availability limited to 6 hours a day. Since it is known that exposure of pregnant rats to RF can restore rhythms in SCN of newborn pups in the absence of rhythmical signals from the maternal SCN (Nováková *et al.*, 2010), we tested whether this applies to the fetal SCN as well. We can conclude from the locomotor activity that RF was able to restore behavioral rhythms of mothers maintained on LL because their activity was restricted to the time of food presence. Firstly, we examined the effect of RF on maternal and fetal weight. Even though mothers exposed to RF gained less weight compared to LL *ad libitum* and LD control groups, it did not affect fetal or placental weight or size of the litter. Nevertheless, LL regime in general led to bigger weight of embryos and smaller weight of placentas, compared to LD. Secondly, we analyzed gene expression levels in the fetal SCN. LL generally led to smaller amplitudes or completely abolished rhythms, but RF was able to reinstate oscillations in *Nr1d1* gene and increase amplitudes of *Per1* and robustly in *Vip* gene. In this set of experiments, *c-fos* expression was arrhythmic in both groups, pointing out the differences between both types of experimental setups.

Taken together, we have shown that the fetal SCN clock respond differently to various manipulation procedures with maternal regime, probably because of the employment of diverse signaling pathways. We can conclude that the central embryonic oscillator is sensitive to the state of maternal circadian system.

The study was supported by the Czech Science Foundation grant 19-01845S (to A.S.) and the Research Project RV0: 67985823.

Publication 3

Čečmanová V, Houdek P, Šuchmanová K, Sládek M, Sumová A. Development and Entrainment of the Fetal Clock in the Suprachiasmatic Nuclei: The Role of Glucocorticoids. J Biol Rhythms. 2019 Jun;34(3):307-322. doi: 10.1177/0748730419835360. Epub 2019 Mar 11. PMID: 30854919.

My contribution to this publication: in vivo experiments – mating rats, injections of DEX/VEH to rats, collecting tissue samples, laser dissection, RNA isolation, reverse-transcription, RT-qPCR; preparation of organotypic explants from placenta, most of the in vitro treatments, data analysis, graphs construction; manuscript – writing drafts of introduction, materials and methods, results and figure legends, preparing figures, proofreading the final text.

In this publication, we tested the hypothesis that glucocorticoids have the ability to synchronize the fetal SCN clock. This hypothesis was based on the fact that the fetal SCN is, unlike that of adult ones, sensitive to glucocorticoids (GCs) because it contains GC receptors (GR). Moreover, GCs are released rhythmically into maternal bloodstream, their excess or shortage have negative developmental effects and their amount is therefore tightly regulated by placental enzyme 11β-hydroxysteroid dehydrogenase (HSD2), catalyzing the conversion of rodent corticosterone into its inactive form. This implies that to ascertain the influence of GCs on the fetal SCN clock, we need to elucidate its role in the placental clocks as well. Since these were only sparsely studied, we first examined the rhythmical expression of the chosen clock genes in two distinct parts of the placenta. To achieve this, we kept pregnant Wistar rats on light/dark (LD) regime 12:12 and sacrificed them at E19 in 3h interval over 24h period. We collected samples of five placentas from one mother at each time point and divided them into maternal and fetal part. After RNA isolation and reverse transcription, we measured mRNA expression of chosen genes in both parts by RT-qPCR. We found that both parts exhibit the rhythmical expression of *Per2* and *Nr1d1* clock genes, with the maternal part showing higher amplitude. Furthermore, we detected rhythm in GR and *Hsd11b2* mRNA, but only in the maternal part of placenta. The result suggested that circadian clock in maternal placenta drives rhythm in local GC metabolism. Besides that, we prepared placental organotypic explants from PER2::LUC mice and observed the rhythms in bioluminescence, and thus in PER2 protein expression, in maternal part of the placenta only. Organotypic explants were prepared from mice at gestational day 17 because it roughly corresponds to E19 in rats. These findings provide evidence that the

maternal part (decidua basalis) of the placenta contains autonomous clock, and its regulation of GC levels is under circadian control.

Thereafter, we tested if these clocks are entrainable by the acute GC influx. We applied GC analog dexamethasone (DEX) into cultivation media of placental explants at different phases of PER2 expression. By using this approach, we were able to construct so called "phase-response curve" (PRC), classic circadian tool to manifest time-dependent response to some stimuli. Results have shown that DEX was able to increase the amplitude of the PER2 rhythms, suggesting the placental clocks are more robust after exposure to GCs − and, most importantly, GCs are able to set the phase of the clock in the placenta in a time-dependent manner. This effect is mediated via GR, because after blockage of these receptors by specific antagonist (mifepristone), the effect of DEX on placental explants was abolished and it was comparable to the effect of vehiculum (VEH). Moreover, in non-rhythmical fetal part of the placenta, oscillations after DEX treatment emerged temporarily.

To test if GCs may affect the clock in the fetal SCN, we first confirmed GR mRNA expression in both mouse and rat fetal SCN, and in the second case also its rhythmical pattern. To examine acute response to GCs, we applied DEX into media of the E17 mice SCN explants, as in the case of placental ones. DEX also increased the amplitude of PER2 bioluminescence rhythms and influenced the phase of the rhythm in fetal SCN explants, but, unlike in the placenta, the DEX-induced shifts of clock in SCN explants differed to those induced by VEH administration only during narrow time window. Even though this effect was restricted to a tight phase of PER2 rhythm, it was blocked by mifepristone and therefore mediated via GR.

As some studies had shown inconsistent data about the exact time of the emergence of *in vitro* SCN rhythmicity, we further tested the possibility that GCs – which are often part of the cultivation media – might accelerate the beginning of the oscillations. As a first step, we explanted the fetal SCNs from nine mothers at E15 and left them undisturbed in culture. The start of PER2 rhythmical expression was very heterogeneous, even between SCNs from the same litter. Contrary to that, in almost all fetal SCNs cultivated with DEX in the media, oscillations emerged immediately after explanting. This suggests that GCs are necessary for the development of circadian clock.

To explore the mechanism underlying these effects, we injected pregnant Wistar rats with DEX or VEH and collected fetal SCNs and placentas 1, 2, 4 and 8 hours after the injection. Our aim was to see acute changes in mRNA levels. Since we have detected no difference between the effects of VEH and DEX injections on expression of clock and other genes containing GRE element in their promoter, we can exclude the employment of this pathway.

Interestingly, we observed changes in expression of immediate early gene *c-fos*: its mRNA level was highly up-regulated in both parts of the placenta and down-regulated in the fetal SCN. This indicates a possible involvement of CREB-regulatory pathway in observed response to GCs.

In this paper, we have shown for the first time the ability of the GCs to accelerate the fetal SCN rhythmicity development, as well as their significant role in synchronization of the placental and fetal SCN clock. The effect is mediated via GRs in both tissues, probably by the involvement of a CREB-dependent pathway.

The study was supported by the Czech Science Foundation grant 16-03932S (to A.S.), the OPPK BrainView CZ.2.16/3.1.00/21544 and the Research Project RV0: 67985823.

Publication 4

Lužná V, Liška K, Sládek M, Sumová A. Hormonal fine-tuning of clock in decidual region of mouse placenta by dopamine, melatonin, insulin, leptin and ghrelin. Placenta. 2021 Mar 27;108:55-63. doi: 10.1016/j.placenta.2021.03.015. Epub ahead of print. PMID: 33819862.

My contribution to this publication: experiments – preparing all the placental explants, performing most of the treatments, data analysis, graphs construction; manuscript – reviewing the literature, writing drafts of introduction, materials and methods, results, figure legends, and parts of discussion; preparing figures, proofreading the final text.

Encouraged by our previous findings, we wanted to examine possible effect of other hormones, apart from glucocorticoids, on the placental circadian clocks. We hypothesized that the general hormonal milieu, rather than one particular player, set the proper phase of the placental clock. In this study, we therefore tested five hormones for their ability to entrain clock in maternal part of placenta *in vitro*. We employed a proven method of preparing organotypic placental explants from transgenic PER2::LUC mice. Regarding the hormones, we chose dopamine, melatonin and insulin for their previously described influence on other clocks – and leptin and ghrelin for their link with food consumption and metabolism. All of these substances had earlier been shown to have an impact on circadian system, whether it was on central or peripheral clock, *in vivo* or *in vitro*.

Based on our previously published data, we decided to focus only on the clock in maternal part of the placenta, as we concluded that fetal parts do not possess a self-sustained circadian clock. Nevertheless, we wanted to confirm these findings by the employment of newly acquired luminescence microscope (LV200, Olympus) in this study. Using this novel device, we were able to see individual placental regions within the explants and thus distinguish the source of the bioluminescence. We observed a strong rhythmical signal from decidua basalis, which is a part derived from maternal uterine tissue. In fetal derived parts, we detected a very weak signal coming from the junctional zone, whereas no signal from the labyrinth, and a strong, even though constitutive signal from the chorionic plate. As a conclusion, we can say that the maternal part of placenta is rhythmically expressing the PER2 protein, while the fetal part is showing strong non-rhythmical PER2 expression selectively in the chorionic plate.

After the confirmation of the origins of the detected signal within the placental explants, we used LumiCycle apparatus for further experiments as it is more appropriate for testing a large number of explants simultaneously. We monitored several parameters of placental PER2 bioluminescence rhythms: amplitude (reflecting the robustness of the oscillations), period (reflecting the length of one cycle), mesor (reflecting the amount of produced PER2), and phase. To elucidate the effect of each substance on the phase of the clock, we constructed PRCs to visualize the impact of examined agent at specific part of a circadian cycle (in minimum and maximum as well as on the rise and the decline of PER2 rhythm). In each parameter, we compared the effect with corresponding vehiculum. We observed that dopamine has the strongest influence of all five tested hormones on placental clock. It increased the amplitude of the PER2 driven bioluminescence and significantly decreased its mesor. Moreover, it changed the phase of the rhythm, especially when applied on its decline. On the contrary, melatonin changed the phase of oscillations when administered on the rise of PER2 expression. This may indicate their complementary role in setting the placental clock. It was interesting to find out that the players connected with metabolism have shown rather negligible effects, besides their mild ability in increasing the amplitude and shifting the phase on decline portion of the PRC. Finally, we confirmed sensitivity of placental tissue to the treatment procedure *per se*, which may blur the somewhat gentle effect of studied substances.

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Discussion

1.6 Maternal behavioral changes and their ability to influence the fetal clock

To be synchronized with the external environment is the key adaptation for organisms to survive in the periodically changing world. This ability is important even for newborns, which is manifested by serious impairments occurring in individuals born to the mothers with desynchronized biological clock (Amaral *et al.*, 2014; Mendez *et al.*, 2016; Smarr *et al.*, 2017; Richter *et al.*, 2018; Salazar *et al.*, 2018; Varcoe *et al.*, 2018). This fact indicates that maternal signals are crucial for setting the clock of the offspring (Sumová and Čečmanová, 2020), most of the knowledge, however, was gained from postnatal observations (El-Hennamy *et al.*, 2008; Nováková *et al.*, 2010; Mendez *et al.*, 2016; Olejníková *et al.*, 2018). To elucidate whether maternal signals can set the clock in the SCN already before birth, we employed the sensitive technique of SCN laser-dissection and detection of mRNA expression by qRT-PCR from prenatal Wistar rats, by which we have previously shown the rhythmical expression of several genes in the SCN of embryos three days before birth (E19) (Houdek and Sumová, 2014). Thus we wanted to deepen our knowledge of the mechanisms involved in setting of these rhythms in the mRNA expression by testing the effect of manipulation with the maternal regime, and using the same methodological approach.

Firstly, we exposed pregnant rat mothers to a phase shift in the light/dark (LD) cycle at E14 and compared the results with a control group of animals kept on unchanged light schedule **(Fig. 8)**. In line with previous results, in the control group, the same non-clock genes have shown rhythmical expression with the equal phase, namely *Vip, Avp* and *c-fos.* Additionally, we also found rhythmical expression of *Nr3c1* – the gene for GR, detection of which was not included in the original publication (Houdek and Sumová, 2014). On the contrary, the expression of the clock gene *Nr1d1* was different in both studies. The explanation may lay in the fact that at the examined embryonic age, the rhythms of the clock genes are quite instable, because the SCN is not fully developed; and/or the detected rhythms may vary throughout the litter, which results in their low amplitudes. Nevertheless, fetal SCN from mothers exposed to the delayed LD regime showed robustly suppressed expression levels of all studied clock genes as well as *Nr3c1*, probably due to the inability of the mothers to fully adapt to this shift. Indeed, analyses of locomotor activity confirmed that behavioral rhythms most of the mothers were in a transient state at the time of sampling, meaning their SCN clocks were not aligned with the new LD cycle yet. Since we sampled the animals throughout the fifth day of the LD shift, they

had only four full days for the adaptation. The adult SCN is able to entrain to 6h phase shift within three days (El-Hennamy *et al.*, 2008), but from our non-published data it is clear that pregnant rats have a different period of their endogenous rhythms, compared to non-pregnant ones, and their biological clock may therefore react diversely. Three days are also not sufficient for an early postnatal SCN to shift its *Avp* and *c-fos* expression according to the new light regime established prenatally; five days were, however, enough to align the SCN with the new cycle (El-Hennamy *et al.*, 2008). Interestingly, in the current study the expression of genes encoding neuropeptides VIP and AVP was not changed by maternal transient state, and, remarkably, *c-fos* was identified as the gene possibly responsible for sensing the maternal clock, because its expression was shifted in accordance with the new phase. Hence, we can speculate that four days may be enough to shift rhythm in expression of *c-fos*, but *Avp* expression only re-synchronizes after at least five days. The timing of the LD shift in the course of the developmental period may be another explanation of the discrepancy between results in both studies.

Secondly, we exposed pregnant rat mothers to constant light (LL) during the entire course of pregnancy **(Fig. 8)**. Under LL, neuronal activity within the adult SCN is dampened and cells become desynchronized (Ohta *et al.*, 2005; Lucassen *et al.*, 2016), so by employment of this protocol, we wanted to imitate an arrhythmic environment for the developing embryos. Constant conditions led to gradual vanishing of the maternal behavioral rhythm, in agreement with previously published data (Houdek and Sumová, 2014). We revealed that LL causes increased body weight of the embryos and decreased weight of the placentas, compared to the LD conditions. This is in disagreement with other studies, in which maternal LL regime led to decreased body weights in embryos or pups (Mendez *et al.*, 2012; Amano *et al.*, 2020). Nevertheless, neither food consumption nor body weights of the mothers were altered by the LL in our experiment. Expression of genes *Vip* and *Avp* was not affected by the LL in its rhythmicity and phase, whereas the *c-fos* rhythm was abolished. This points out that the fetal SCN *c-fos* is, again, able to react in accordance with the maternal behavior.

Feeding regime of the mothers was proven, besides light, as the signal responsible for setting the fetal clock (Nováková *et al.*, 2010; Olejníková *et al.*, 2015). To study the influence of food intake, the restricted feeding (RF) protocol is often used, in which food availability is limited to just few hours a day, either in line with or in opposition to the light regime. Food is the strongest signal for the clock in the peripheral GIT organs, but subordinate to light for the adult SCN rhythms (Damiola *et al.*, 2000; Hara *et al.*, 2001; Stokkan *et al.*, 2001; Bray and Young, 2012; Crosby *et al.*, 2019). Previous studies performing RF together with either

reversed LD or LL regime have shown the impact of maternal RF on newborns' SCN only when it was accompanied with LL, confirming light as the main cue even for the developing central clock (Nováková *et al.*, 2010). Based on these observations, we combined LL regime with 6h of RF to examine if maternal feeding is strong enough to entrain the fetal SCN already three days before birth **(Fig. 8)**. Interestingly, exposure of pregnant rats to RF significantly increased the amplitude of the rhythmical expression of *Vip* and the mesor of *Per1* expression, and, moreover, it established low-amplitude, but significant rhythms in clock genes associated with metabolism such as *Nr1d1*, *Rorα* and *Dbp*. The same effect of RF on *Nr1d1* expression in SCN was observed in adult rats exposed to RF (Nováková *et al.*, 2011), pointing out that the *Nr1d1* may respond to metabolic changes in the SCN, and thus be the molecular link between central and peripheral clock.

Comparison of data obtained in both sets of experiments revealed the different response of the fetal clock to distinct maternal behavioral states. When mothers were exposed to the shift of their LD regime, at the time of the sampling they were slowly adapting to the new conditions, and so the expression of the tested genes within the embryonic SCN was reflecting this transient state. One gene was already synchronized with the new regime, while some were not affected, and most of them were dampened. This could be because they were caught on their way to change the phase. In the case of the LL regime, mothers, when sampled, were already arrhythmic for a couple of days, and so most of the fetal genes were arrhythmic as well. Three of the studied genes reacted differently in both sets of experiments: *c-fos*, *Vip* and *Nr3c1*. The first mentioned was the only gene able to shift its expression according to the new LD cycle, with its rhythm vanishing after maternal LL exposure, and not being restored by RF. Because of this, we may speculate about the role of the *c-fos* as a "light sensing" gene for the fetal SCN, as it was already described for the adult one (Kornhauser *et al.*, 1990; Earnest and Olschowka, 1993). *Vip*, on the other hand, changed its expression only after establishing maternal behavioral rhythms by the RF protocol, for which we have, regrettably, not found a sufficient explanation in published literature. VIP is known to affect the rhythmical release of GC (Nicol *et al.*, 2004; Loh *et al.*, 2008; Paul *et al.*, 2020), and RF is known to restore GC rhythmicity (Krieger, 1974; Krieger *et al.*, 1977). But whether the signaling can work in reversed manner, is not clear. Finally, any alteration of the maternal LD regime impaired the rhythmical expression of GR gene (*Nr3c1*). Since the irregular daily routine and stress accompanying it are commonly known to change the circadian oscillations in GC levels, GCs may be one of the signals lying behind these observed changes.

In our study, we have demonstrated the ability of the fetal SCN clock to sense changes in the maternal circadian system. These findings are of an utmost importance, because we, as a modern society, do not live in an ideally rhythmical world. Whether it is due to phase-shifting work, frequent traveling across the planet, or "just" city life full of artificial light pollution. To understand if and to what extent these phenomena have a serious impact not only on adult, but also on the prenatal period of our life is crucial.

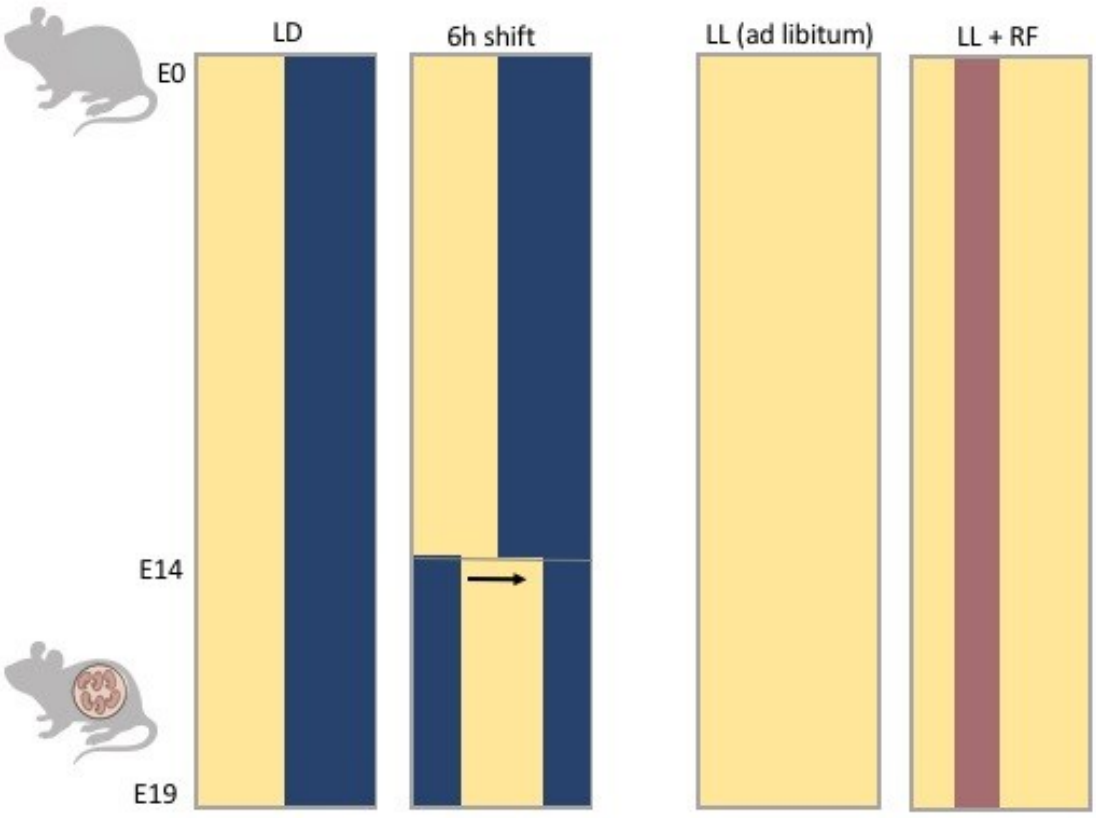

Figure 8. Experimental setup used to reveal the impact of maternal regime to the fetal clock
In the discussed publication, we used two sets of experiments to test the response of the fetal clock to various maternal behavioral regimes. Firstly, we shifted the light/dark (LD) regime of one group of the pregnant rats by 6 hours in the direction of delay in embryonic day E14 and compared these animals with the control group. Secondly, we kept mothers under constant light (LL) conditions. Two groups of animals with different feeding regimes were compared: the group fed *ad libitum* and group exposed to restricted feeding (RF), having the food available only 6 hours a day. Samples were collected during the 24 hours in 3h intervals at E19.

1.7 The role of glucocorticoids in setting the placental and fetal SCN clock

Based on our findings about GR mRNA levels response to maternal regime and also on the fact that stress is one of the abovementioned detrimental effects of the modern life style, we further focused on the role of GCs in circadian system during pregnancy. The rhythmical secretion of GCs into maternal bloodstream (Cheifetz, 1971) as well as the wide distribution of GRs throughout the body (Oster *et al.*, 2017), including the early postnatal SCN (Rosenfeld *et al.*, 1988; Olejníková *et al.*, 2018), draw our attention to GCs as one of the potential maternal synchronizing signals. GCs have higher affinity to mineralocorticoid receptors (MR) than to GRs; thus, MRs are saturated constantly throughout the day, while GRs are activated only during the GCs peak at the beginning of the active period or in cases of acute elevation of GCs due to stress stimuli (Kloet and Derijk, 2004). We have previously proved the impact of maternal stress caused by RF regime on the SCN clock of the neonatal pups (Olejníková *et al.*, 2015), and hence we wanted to further ascertain GC influence on the developing prenatal circadian clock.

In designing this follow-up study, we worked on the assumption that maternal hormonal signals to fetuses must overcome the placental barrier. This is even more important in the case of GCs because placenta synthesizes the enzyme HSD2 converting active corticosterone into its inactive form, and thus protecting the embryo from GC excess (Yang, 1997). The amount of *Hsd11b2* mRNA expression is decreasing in rat placenta towards the term, while the amount of corticosterone in placental tissue is increasing (Hundertmark *et al.*, 2001; Mark *et al.*, 2009), and overall levels of GCs are elevating in maternal blood throughout the pregnancy (Waddell and Atkinson, 1994). We found out that expression of the *Hsd11b2* and *Nr3c1* mRNA is rhythmical in the maternal part of the placenta collected from Wistar rats at gestational stage E19, and therefore we can speculate that activity of the HSD2 enzyme is under circadian control, as recently discovered in human placenta (Lamadé *et al.*, 2021). This information is crucial because collecting the samples once a day may misinterpret the results in the case of rhythmically expressed genes/proteins. Our findings favor the hypothesis that elevated levels of *Hsd11b2* during the end of the dark phase of the day protect rat fetuses from the undesirable influx of GCs at an improper time, as their highest physiological levels in maternal blood are at the opposite time of the day, i.e., at the beginning of the active period (Cheifetz, 1971; Crew *et al.*, 2016).

Furthermore, we described the rhythmical expression of some clock genes in both parts of the placenta (maternal and fetal). The amplitudes of *Per2* and *Nr1d1* were higher within the

maternal part. In fetal parts of the placenta, the shallow variation in the clock genes expression during the day has been shown by other researchers as well (Wharfe *et al.*, 2011; Waddell *et al.*, 2012). The rhythmical expression of clock genes was detected in whole placenta of humans and mice (Pérez *et al.*, 2015; Papacleovoulou *et al.*, 2017), but based on cited results, this is probably due to strong rhythmicity in the maternal part.

Next, we prepared placental organotypic explants from PER2::LUC mice at gestational stage E17 and monitored bioluminescence levels to confirm the presence of a self-autonomous circadian clock. This developmental stage was chosen due to the fact that E17 in mice roughly corresponds to the E19 in rats, as their gestational period length slightly varies. We detected rhythmical bioluminescence signal of PER2 protein expression, but again in the maternal part only. These findings are in accordance with rhythmicity in placental explants from rats (Akiyama *et al.*, 2010). Rodents are the only animals that share with humans the hemochorial type of the placenta, in which both parts, and therefore maternal and fetal blood, are tightly interconnected (Fonseca *et al.*, 2012). The difference in clock genes/proteins expression between these parts may be based on the fact that they differ in their developmental origin. The maternal part of placenta (decidua basalis) is originally derived from the maternal uterine tissue (Fonseca *et al.*, 2012), being mostly rhythmical throughout the body (Dibner *et al.*, 2010; Schibler *et al.*, 2015), and, on the other hand, the fetal part of the placenta is derived from trophoblast, i.e. fetal tissue (Fonseca *et al.*, 2012). In the prenatal peripheral organs, sustainable rhythms in the clock genes were described *in vitro* (Dolatshad *et al.*, 2010; Dan *et al.*, 2020), whereas *in vivo*, only *Nr1d1* rhythmicity – previously discussed as the gene linked with metabolism – was detected in GIT organs (Sládek *et al.*, 2007; Dolatshad *et al.*, 2010; Polidarová *et al.*, 2014). This points out that without a functional SCN as a central oscillator governing the rest of the body, other organs can hardly maintain their clocks mutually synchronized. Nevertheless, when DEX as a specific synthetic agonist of GRs had been added into cultivating media, even arrhythmic fetal placenta was able to initiate and sustain oscillations for approximately 2 days. Another proof of the impact of GCs on placental clock was a significant increase of the amplitude (i.e. robustness) in PER2 rhythms in maternal placental explants after DEX had been added into the media. Moreover, concluded from the constructed PRC **(Fig. 9)**, GCs' role is rather time specific, as the application of DEX during the various portions of the PER2 rhythm changed (and therefore synchronized) the phase of the maternal placental clock differently. The tissue is very sensitive to the treatment procedure *per se*, but DEX caused specific phase shifts during the interval of insensitivity to the treatment

procedure. All the observed effects were GR-dependent because their blockage by mifepristone (antagonist of the GR) prevented such changes.

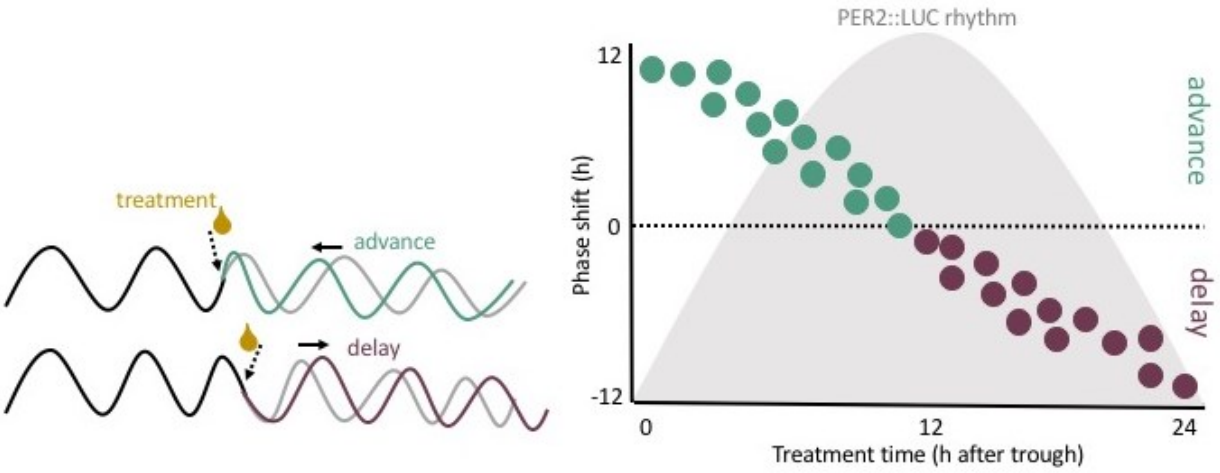

Figure 9. Constructing the PRC
Phase-response curve (PRC) is able to tell us, whether the application of some substance has time-dependent effect on clock in examined tissue. The administration of the tested substance in different phases of the PER2 rhythm can result in two outcomes – the clock will be advanced or delayed. The magnitudes of these effects are plotted as the PRC, which has to be further compared with the PRC of corresponding vehiculum.

To reveal the mechanism underlying the observed changes, we tested the acute response of the placental genes to GCs *in vivo* by injecting pregnant Wistar rats (E19) with DEX and then collecting placenta samples 1, 2, 4, and 8 hours following the injection. When activated by GCs, GRs are translocated into nucleus and bind to the GRE (glucocorticoid responsive) promoter elements of genes to modulate their transcriptional activity. Interestingly, the expressions of the examined clock genes containing GRE was not affected by DEX, compared to VEH. Hence, with no detected changes in transcription of these genes, the assumed involvement of the GRE in the observed GCs effect on the placental oscillations was disproved. The only effect was seen in significantly increased levels of *c-fos* expression in both parts of the placenta. Hence, the regulation of the molecular clock by GCs may employ CREB-dependent pathway instead, as GCs also interact with cAMP response element-binding protein (CREB) and promote its binding to DNA, as in the case of *c-fos,* containing CRE site in its promoter (Imai *et al.*, 1993; Haas and Pitot, 1999; Ratman *et al.*, 2013) **(Fig.10)**.

After confirming the role of GCs in entrainment of placental clock and circadian regulation of the placental barrier, we continued with elucidating their effects on the fetal SCN. For this purpose, we employed similar experiments and approaches. First of all, we confirmed

the presence of *Nr3c1* mRNA expression in both mouse and rat fetal SCN. We then prepared organotypic explants from the E17 fetal SCN of PER2::LUC mice to observe the influence of GCs on the fetal SCN *in vitro*, independent of other stimuli. We detected significant elevation of the rhythm amplitude after DEX treatment, unification of previously variable periods, and also phase-shifts at the specific time window. Again, this effect vanished after pretreatment with mifepristone. The biggest phase shifts of the fetal SCN were reached when GCs were applied around 3h later than in the case of placenta; thus, we may speculate that the influx of GCs from the maternal bloodstream into the fetal blood circulation is delayed by exchange and modification in the placenta, and because of this fact, both tissues are prepared to react to GCs at different time. Even though it is hard to convert *in vivo* time into *in vitro* timing of the PER2 expression, the sensitive time window of the fetal clock is approximately corresponding with their subjective night (Shearman *et al.*, 1997), meaning that fetal SCN are most susceptible to the influx of GCs at the inappropriate time of the day when their maternal levels should be low (Cheifetz, 1971; Crew *et al.*, 2016). This highlights the importance of avoiding desynchronization of the circadian clock in pregnant mothers.

As GCs are known to play an important role in the fetal development and maturation (Seckl, 2001), we wanted to find out whether GCs might accelerate the development of the fetal SCN rhythmicity. For this purpose, we prepared SCN explants from E15 embryos from litters of nine PER2::LUC mothers and left them undisturbed in the LumiCycle apparatus in order to detect the beginning of their rhythmicity. In contrast to E17 SCN explants – which were all rhythmical right after the explanting –, these younger ones greatly varied in the emergence of their oscillations. This diversity was present even among individual fetuses within the litters. As the next step, we added DEX into the media of another group of E15 SCN explants and observed presence of the rhythmicity in almost all of them immediately after explanting. This clearly shows the important role that GCs play in the circadian clock development. Both of the observed scenarios, rhythmical and non-rhythmical E15 SCN explants, had been previously described in literature (Wreschnig *et al.*, 2014; Carmona-Alcocer *et al.*, 2018), we may therefore speculate about the differences in the composition of the cultivating media, since some of them are supplemented with GCs automatically. The other possibility is that non-rhythmical explants are often considered dead or wrongly prepared and thus eliminated from the studies.

Alongside exploration of the acute changes in placental mRNA expression after injecting DEX to pregnant Wistar rats, we also inspected the gene expression profiles within the SCN of the same animals. Similarly, as in placenta, fetal SCN did not show any changes in clock genes mRNA, yet a significant decrease in *c-fos* expression was detected. Since *c-fos* is

one of the first rhythmical genes in the fetal SCN *in vivo* (Houdek and Sumová, 2014), DEX-induced changes in its expression might suggest the plausible mechanism of the fetal SCN-GCs interaction. The opposite changes in *c-fos* expression in the placenta and the fetal SCN could be explained by structural differences between CRE-binding complexes in both tissues (King and Nicholson, 2007). All things considered, further studies have to be performed to elucidate all the mechanisms of the GCs' influence on feto-placental system.

Our results allow us to conclude that GCs are able to accelerate the development of the circadian clock within the SCN, however, changes in the timing of their influx – caused by either impaired maternal clock or high amounts of stress, both of which are common phenomena in the modern society – may lead to the disruption of the GC signaling and subsequently to serious changes in placental and fetal clock.

1.8 Hormonal fine-tuning of the placental clock

Based on our discovery of GCs as very strong synchronizing stimuli for the placental tissue (Čečmanová *et al.*, 2019), we wanted to further investigate the role of other possible signals in providing the placental clock with the information about the time of day. We hypothesized that overall hormonal balance, rather than one molecule, is responsible for setting the placental clock. To satisfy our curiosity, we tested five selected hormones – namely dopamine, melatonin, insulin, ghrelin, and leptin – to address this issue. Both dopamine and melatonin levels are changing throughout the day in maternal blood, and they are easily passing through the placenta, in which their receptors are expressed (Watanabe *et al.*, 1990; Davis, 1997; Kim *et al.*, 1997; Naitoh *et al.*, 1998; Vaillancourt *et al.*, 1998; Lanoix *et al.*, 2008; Kasahara *et al.*, 2010; Soliman *et al.*, 2015; Gratz *et al.*, 2018). Moreover, both hormones were described to be able to shift the fetal SCN clock (Viswanathan *et al.*, 1994; Viswanathan and Davis, 1997). The selection of insulin, ghrelin, and leptin was based on the fact that these metabolism-linked substances are known to influence the clock in other peripheral tissues, and their receptors are expressed in the placenta as well (Desoye *et al.*, 1997; Hoggard *et al.*, 1997; Smith and Waddell, 2002; Zhao *et al.*, 2004; Nonoshita *et al.*, 2010; Ruiz-Palacios *et al.*, 2017). What is more, their signaling is affected by changes in food intake (Varcoe *et al.*, 2013; Ruiz-Palacios *et al.*, 2017). Regarding the methods, we employed an established approach of cultivating organotypic explants from E17 PER2::LUC mice, containing rhythmical decidua basalis, the maternal part of the placenta. This *in vitro* technique is convenient for eliminating the effects of other than examined substances.

Dopamine administration into the culturing media resulted in increased amplitude (i.e. robustness of the oscillations) and decreased mesor (i.e. level of expression) of PER2 rhythm. These two somewhat contradictory effects on the rhythm might be caused by the fact that the placenta accommodates two different types of dopamine receptors: D1-like receptors, which increase the activity of adenylyl cyclase; and D2-like receptors, which, on the other hand, reduce it (Kim *et al.*, 1997; Vaillancourt *et al.*, 1998; Gratz *et al.*, 2018). Adenylyl cyclase is an enzyme synthesizing the cAMP, and thus might link the dopamine effect with placental clock machinery, again via CREB-dependent pathway **(Fig. 10)**. This hypothesis is supported by observed increase of *c-fos* expression in the fetal SCN after maternal dopamine injections (Weaver *et al.*, 1992; Viswanathan *et al.*, 1994). The summation of activity of both receptors may, therefore, lead to the observed effect on PER2 expression. Additionally, to detect if the dopamine influence on the placental clock is time-dependent, we performed treatment at

various phases of PER2 rhythm to construct the PRC **(Fig. 9)**. As a result, we observed significant phase-delaying of placental clock when dopamine was applied on the decline of PER2 rhythm, when compared to VEH. This is in agreement with detected phase-shifts in mice retinal explants after dopamine and D2-like receptor agonist administration (Baba *et al.*, 2017). Since dopamine is considered to be a signal of the daytime, the observed sensitive phase of PER2 *in vitro* rhythm likely falls, as described above, into the light phase of the day. Hence, the elevated levels of dopamine during the non-active phase of the mice, i.e. in an inappropriate time, will lead to resetting of the clock. Melatonin PRC, on the other hand, manifested a quite opposite pattern: PER2 expression was phase-advanced after the treatment on the rise portion of its rhythm, and thus the placental clock sensitivity falls into the active part of the mouse day. Although the observed phase-shifts were less robust than those after DEX administration, we can conclude that both hormones complementarily "inform" the placenta about the time of day and play a rather delicate role in fine-tuning the placental clock. Interestingly, similar effect of the two hormones was previously described in setting the phase of fetal SCN clock (Viswanathan and Davis, 1997), and thus, they may be considered as other maternal signals, besides GCs, with the ability of synchronizing the whole feto-placental system.

Food consumption has been repeatedly described as a strong synchronizing signal for peripheral and mainly GIT organs (Schibler *et al.*, 2003; Carneiro and Araujo, 2012), yet hormones involved in this signaling were studied only sparsely. One of the most prominent candidates linked with food consumption is insulin, described to be influencing the clock in kidney, liver, and fibroblasts (Tahara *et al.*, 2011; Crosby *et al.*, 2019). Two other peptides connected with food intake are leptin and ghrelin. Leptin is mainly produced by adipose tissue (with low levels during starving), and its receptors are expressed in the placenta (Hoggard *et al.*, 1997; Zhao *et al.*, 2004). Furthermore, its shortage or abundance is able to phase-shift the adult SCN clock (Prosser and Bergeron, 2003; Grosbellet *et al.*, 2015). Ghrelin is produced mainly in stomach and is widely known as the "hunger hormone", with its levels increasing during starving phase and decreasing after food consumption. The placenta is able to produce ghrelin on its own and, again, possesses its receptors (Gualillo *et al.*, 2001; Nonoshita *et al.*, 2010). Besides that, insulin and leptin also seem to be important for proper placental development (Bifulco *et al.*, 2003). In our study, all three hormones evinced only mild effects on bioluminescence rhythm in PER2 expression in placental explants. These effects were observed on the decline of PER2 rhythm, that is in the non-active phase of the day. The conclusion which may arise from these data is that the timing of food consumption is not crucial for the placental clock rhythmicity. On the other hand, as mentioned before, RF had been

described to restore the rhythmicity in corticosterone in SCN-lesioned mice (Krieger *et al.*, 1977) and corticosterone modulates the levels of secreted leptin and number of its placental receptors (Kronfeld-Schor *et al.*, 2000; Sugden *et al.*, 2001). This indicates GCs' interconnection with the feeding rhythms and their privileged place in the regulation of various rhythms in the whole body.

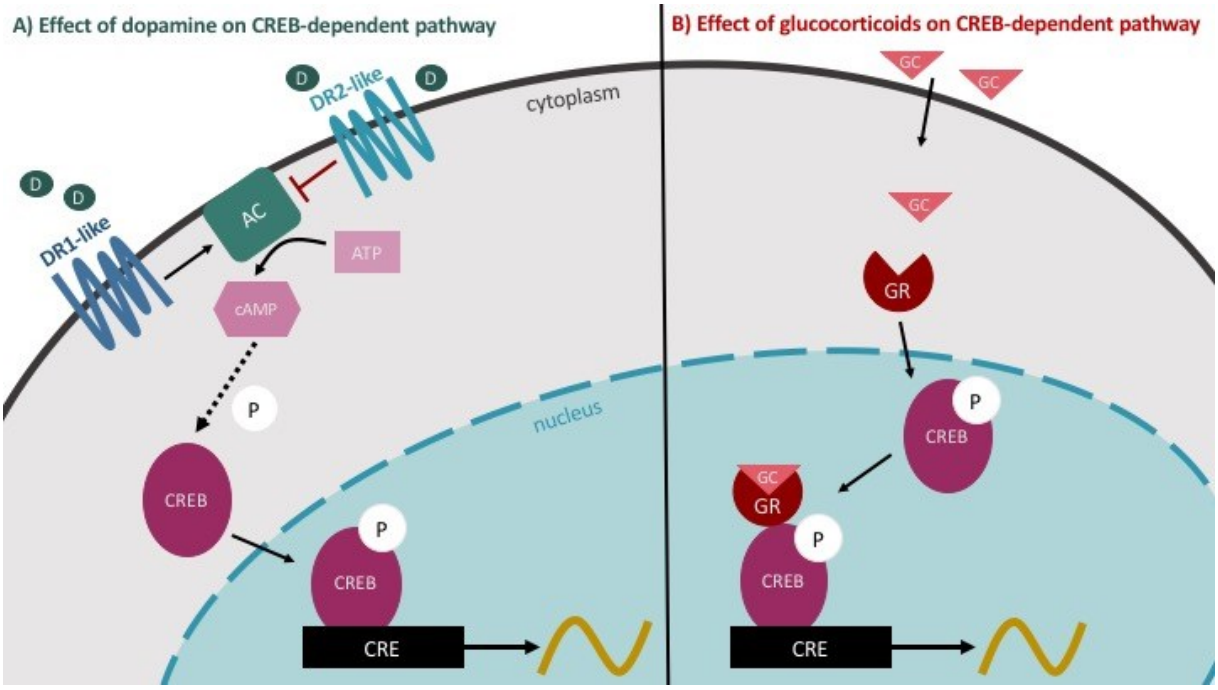

Figure 10. Simplified scheme of proposed employment of CREB-dependent pathways in synchronization of the fetal and placental clocks

We have discussed the employment of CREB-dependent pathway in our studies. The scheme provides simplified mechanism of this signaling. **(A)** After dopamine administration to placental explants, we have observed the changes in PER2 protein expression. Placenta expresses two types of dopamine receptors – D1-like and D2 like. Activation of these two types of receptors has opposite effects on adenylyl cyclase (AC), an enzyme converting the ATP into cAMP, which is necessary for downstream phosphorylation of CREB (cAMP response element-binding protein). Phosphorylated CREB then binds into the CRE promoter site of target genes, such as *Per* clock genes.

(B) Glucocorticoid treatment caused changes in the expression levels of *c-fos* gene in fetal SCN and placenta. The possible mechanism of this action may be following: Glucocorticoids (GCs) bind to their receptors (GR) and this complex translocates into nucleus, where it binds to phosphorylated CREB and enhances its binding into CRE element on *c-fos* promoter.

The journey leading to a full understanding of the placental rhythms, mechanisms of their synchronization, and the role of placental clock in pregnancy is certainly not over. There are still many questions that have to be addressed: firstly, how does the clock mechanism differ in various developmental ages? Secondly, as the tissue is highly sensitive to the procedure *per se*, how can the method be improved? And, in addition, what if some gentle effect of tested substances is overlapped by this sensitivity? Thirdly, is the fluctuating temperature, which accompanies the treatment procedure, also a synchronizing signal, as it has been shown in other tissues (Buhr *et al.*, 2010)? All together, these proposed questions offer an exciting direction for future studies.

1.9 The mystery of the fetal SCN rhythmicity – solved? Limitations and directions of future research

At the beginning of my PhD studies, we published a review paper in which we have raised important questions about the origin of the circadian rhythmicity of the fetal SCN (Sumová and Čečmanová, 2020). In the following publications, we tried to contribute to solving at least some of the mysteries. Two of them – the role and the nature of maternal synchronization, and mechanism of the emergence of circadian oscillations in fetal SCN – will be discussed here.

Essential role of maternal synchronization for gradually developing SCN, unable to maintain rhythmical signals, was one of our main postulated hypotheses. Yet the term "maternal synchronization" may be understood in two different ways: Firstly, as unifying the rhythm of cells within the individual SCN of each embryo, and secondly, as coordinating existing rhythms throughout the whole litter. Both of these roles have been demonstrated after birth – dopamine injections to mothers established behavioral rhythms of the individual pups (Viswanathan *et al.*, 1994), and the offspring from one litter born to the mother with impaired circadian rhythms had clocks out of the phase with each other (Reppert and Schwartz, 1986; Weaver and Reppert, 1989). Nowadays, we are trying to use more sophisticated techniques to study the fetal circadian system than just monitoring its behavior; however, to distinguish between the two functions of maternal synchronization is still challenging. Current *in vivo* methodological approaches, in order to construct the 24h expression profiles of clock genes/proteins, require collecting samples from multiple animals. If the detected expression is constitutive, we cannot tell whether it is due to non-rhythmical individuals or due to mutually desynchronized, even though rhythmical embryos. To just think about this issue from the evolutionary point of view, synchronization of the whole litter might be advantageous only for animals with a large number of offspring, such as rodents, but quite redundant for humans, primates, ungulates, etc. But even in the case of animals with small litters, it is convenient for a mother to prepare the fetus for the conditions in the outside world and to adjust offspring's clock to her own. It is therefore likely that maternal signals are able to synchronize neurons within the individual fetal SCN as well. Nevertheless, this theory has to be confirmed by future, more sensitive approaches that will be able to detect *in vivo* rhythms in a single SCN. Until these will be employed, we have so far focused on the nature of maternal signals and, most importantly, on the ability of embryos to distinguish between various types of stimuli.

By forcing pregnant rats to adjust to different light and food conditions, we have confirmed one of our proposed hypotheses about the different strength of the SCN-dependent and -independent pathways in resetting the fetal clock. We have also shown the ability of the

fetal SCN to react differently to various conditions. Moreover, our study has revealed the remarkable fact that even though the clock genes expression may be compromised by changes in maternal regime, the rhythm in SCNs' main neuropeptides VIP and AVP seems to be self-sufficient (Lužná *et al.*, 2021). These results are in accordance with previous findings that *in vitro* rhythmicity in prenatal neurons is developing gradually – from the core (expressing VIP) to the shell (expressing AVP) (Carmona-Alcocer *et al.*, 2018). Thus, even if the clock molecular mechanism is not yet fully developed, neurons are able to express VIP and, subsequently, AVP in rhythmical manner, and as it seems from our results, independently of the external environment. This has been confirmed by spontaneous development of the SCN oscillations *in vitro* (Wreschnig *et al.*, 2014; Čečmanová *et al.*, 2019). Nevertheless, the possibility that sustainable oscillations in VIP are sufficient, and hence responsible, for SCN rhythmicity *in vitro* has been tested, but unfortunately, with negative results (Wreschnig *et al.*, 2014; Carmona-Alcocer *et al.*, 2018).

In addition to light regime and feeding schedule, there are other maternal signals being discussed as serious candidates capable of setting the fetal clock, namely dopamine, melatonin, and – based on our novel results – also glucocorticoids (Viswanathan *et al.*, 1994; Viswanathan and Davis, 1997; Houdek *et al.*, 2015; Čečmanová *et al.*, 2019). However, as none of these are released from SCN itself, they are probably not the key for explanation of the spontaneous development of the oscillations in the fetal SCN *in vitro*. Even though we have shown for the first time the ability of GCs to accelerate the development of the SCN *in vitro* rhythms, we have also demonstrated that they would emerge on their own anyway (Čečmanová *et al.*, 2019), which indicates that SCN is able to mature *in vitro* without external stimuli. It was also observed that GABA, another chemical, besides VIP, released by SCN itself, is not necessary for the fetal oscillations, and thus can also be excluded as the molecule responsible for them (Carmona-Alcocer *et al.*, 2018). This leads to one of the biggest riddles of developmental chronobiology: What lies behind the earlier manifestation of self-sustained *in vitro* oscillations of the fetal SCN? To our knowledge, AVP has not been tested in this regard so far. If also excluded, the composition of cultivating media has to be scrutinized to find out if some of its components are not responsible for these rhythms, as one of the last options.

The discrepancy between *in vivo* and *in vitro* results in observing the first oscillations might be based on the fact that *in vitro* bioluminescence detection is more sensitive, and thus underlines the need of employing equivalent techniques in *in vivo* studies as well, such as single cell PCR or *in vivo* imaging. Besides that, since the fetal SCN explants are quite susceptible to any manipulation (Nishide *et al.*, 2008; Čečmanová *et al.*, 2019), e.g. changes in temperature –

a well-known synchronizing signal for pups' SCN (Yoshikawa *et al.*, 2013) –, it is also possible that *in vitro* oscillations are activated by the explanting procedure itself.

Even if we do not know the signal responsible for these early rhythms, we may speculate about the mechanism by which individual neurons cooperate without synaptic connections. Throughout the brain, neurons communicate with one another as well as with glial cells via gap junctions, that are constituted of transmembrane proteins connexins (Cx). Adult SCN neurons possess gap junctions composed of mainly Cx36, although with smaller amount than the rest of the brain (Jiang *et al.*, 1997; Colwell, 2000; Connors and Long, 2004). This protein has been found in different regions of the P1 rat brain, where it was more abundant than in the adult one (Belluardo *et al.*, 2000) – therefore, it could be present in the fetal SCN, however, this has yet to be confirmed. Small molecules such as cAMP can pass through Cx channels (Srinivas *et al.*, 1999), and cAMP/Ca2+ paracrine cytosolic signaling has been proposed to play a crucial role in synchronization of the SCN neurons, because its rhythms precede even those in *Per2* expression in the adult SCN (Hastings *et al.*, 2008, 2018; O'Neill and Reddy, 2012; Evans, 2016). As we have discussed multiple times the pivotal role of cAMP signaling in both the fetal SCN and the placental tissue, it is possible that rhythms in the fetal SCN may operate at gap junction level exclusively, as long as they lack the classical synapses. But where do these hypothetical rhythms in cAMP emerge from in the case of *in vitro* fetal SCN slices, still needs to be elucidated.

Regrettably, the mystery of rhythmic signal emergence within the fetal SCN still waits for being satisfactorily solved. Nevertheless, it definitely represents an attractive and up-to-date research question with the potential to move the circadian field forward for at least several years ahead.

Conclusion

The main focus of my PhD studies was aimed on the developing SCN clock and the mechanisms by which they might be synchronized. We proposed that maternal signals play a crucial role in this process and pointed out some knowledge gaps in a review paper. We then further proceeded to determine the nature of maternal cues involved in setting the fetal SCN clock. To achieve this goal, we designed and executed a series of experiments using rat and mouse models that resulted in important findings.

Firstly, we have described the ability of fetal SCN circuit to specifically react to different changes in maternal behavior. We have revealed, above all, the crucial role of immediate early gene *c-fos* in sensing the maternal signals, which could suggest that neuronal activation is an important part of the mechanism how the mother signals the time of day to fetus.

Secondly, our attention was drawn to GCs as a strong rhythmical maternal signal. We have revealed that these hormones can affect the fetal SCN clock *in vivo* as well as *in vitro*. They set the phase of the fetal clock depending on the time of day and, moreover, accelerate the development of the fetal SCN rhythmicity *in vitro*.

We further focused on the circadian clock in placenta as an important player in maternal-fetal communication. We have described the clock in two parts of this tissue that differ in their origin. In addition, we have ascertained the role of circadian system in placental regulation of GCs influx to the fetus. On top of that, we have revealed the significant impact of GCs on placental clock both *in vivo* and *in vitro*.

Thirdly, we extended our search for hormonal maternal signals capable of setting the placental clock. We have revealed a significant effect of dopamine and melatonin, and, on the other hand, a negligible effect of metabolism-connected hormones on the circadian clock in the maternal part of mouse placenta. Our results point at the complex, yet appealing mechanisms of maternal communication towards the fetuses and their clock.

Altogether, our findings provide a new insight into complicated signaling between maternal and fetal circadian systems and contribute to a better understanding of involvement of various cues in setting the developing SCN clock.

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Supplement: Publications

The publications attached bellow follow the order in which they are addressed in the Discussion.