The Control of the Biological Clock Beyond Transcription

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Summary

The master regulator of the biological clock, located in the suprachiasmatic nucleus, synchronizes all physiological processes to the environment. The basis of the molecular circadian clock is a transcription-translation feedback loop where certain proteins inhibit their own transcription. The molecular mechanisms underlying this circadian rhythm go beyond transcription because they are regulated via post-transcriptional mechanisms and epigenetics as well. Involved post-transcriptional mechanisms are splicing events, RNA interference via micro RNAs, and the controlling of poly(A) tail lengths of clock gene transcripts. Besides that, NOC and SR proteins play a role in the regulation of the circadian rhythm. Furthermore, epigenetics is crucial for generating a circadian rhythm via RNA methylation and histone acetylation. All these mechanisms should be linked to each other in order to get a full understanding of the biological clock and to make it of clinical relevance.

Introduction

Almost all organisms behave according to the daily cycle of daylight and darkness (Bear et al., 2015). This daily cycle of approximately 24 hours is called the circadian rhythm, where the term circadian is latin meaning “approximately a day” (Halberg., 1959). Besides behaviour, most biochemical and physiological processes also follow the circadian rhythm (Bear et al., 2015). For example, the body temperature, blood flow, hormone levels and metabolic processes have a certain rhythmicity as well. The timing of food intake and the utilization of nutrients is critical to health and is also regulating according to a certain rhythm (Stubblefield et al., 2018). All these processes are coordinated by the organism’s biological clock. This is an intrinsic clock, meaning that it is regulated biologically in the brain and not astronomically, i.e. by the sun and Earth. The first evidence revealing the intrinsic clock in organisms was found in the Mimosa plant. In 1729 the French scientist de Mairan showed a circadian rhythm in this plant even in the absence of light (Mairan., 1729). At this time, it was not know yet how the circadian clock was generated exactly.

The Suprachiasmatic Nuclei is the master regulator of the circadian rhythm

Almost all mammalian cells contain an intrinsic clock (Bear et al., 2015). These cells are all coordinated by one master regulator called the suprachiasmatic nucleus (SCN) which was discovered in 1972 (Moore & Lenn., 1972). The nuclei in mammals are bilaterally paired and are located within the hypothalamus, right above the optic chiasm (suprachiasmatic) and next to the third ventricle (Fig. 1) (Moore & Lenn., 1972). The SCN regulates the circadian clocks throughout the rest of the body. The cells of the SCN are synchronized to day-night cycles by input from light. The light comes in via a light-sensitive ganglion cell in the retina. Through the retinohypothalamic tract this signal is sent to the SCN directly (Bear et al., 2015). Subsequently, the SCN sends synchronization signals to the other cells of the body via hormone secretion or via the autonomic nervous system (ANS). Indirect cues like body temperature, activity rhythms and feeding time can be used as well to send signals to the cells (Bozek et al., 2015; Buijs et al., 2003). Lesion of the SCN abolishes circadian rhythms in an organism. This was proven in a study by Eastman et al, where they removed both nuclei in rats resulting in an absence of circadian rhythms in constant dim light (Eastman et al., 1984). This observation shows that the SCN is the basis of the biological clock and it is required to create the circadian rhythm.

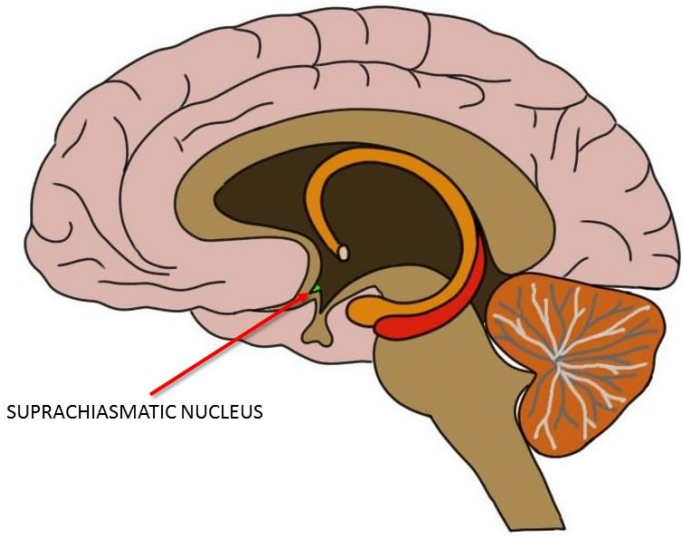

Fig. 1: The location of the SCN in the human brain. The SCN is indicated in green. (Retrieved from https://www.neuroscientificallychallenged.com/blog/know-your-brain-suprachiasmatic-nucleus)

Primary Molecular Mechanisms of the Biological Clock

The SCN contains a timekeeping molecular mechanism which depends on a self-sustained transcriptional negative feedback loop. The loop contains a built-in time delay in feedback inhibition (Buijs et al, 2016). Both the SCN as all the other cells have clock genes involved with this feedback system (Partch et al., 2014). The first genes involved in the circadian rhythm were discovered in 1971 where researchers showed that mutant flies with a disturbed rhythm all have a mutation involving the same functional gene on the X chromosome. They named this gene *period* (Konopka & Benzer., 1971). Further research has found multiple genes involved in the molecular mechanism underlying the clock. Like mentioned above, the basis of the molecular circadian clock is a transcription-translation feedback loop (TTFL). This loop takes about 24 hours to complete (Patke et al., 2019). Genetic and biochemical studies revealed a TTFL with two transcription factors that function as inhibitors, Period (PER) and Timeless (TIM). PER and TIM can bind to each other forming a dimer. As a dimer, the two proteins are able to enter the nucleus of the cell. In the nucleus they block the *period* gene thus blocking the transcription of its own gene. The mRNA of *period* and the PER protein oscillate, where the oscillation of the *period* gene results from the feedback inhibition of the PER protein (Fig. 2A).

In addition to TIM, other proteins are required as well for this oscillation. The *doubletime* gene codes for the DOUBLETIME (DBT) protein kinase. This kinase phosphorylates PER which leads to a PER degradation. This contributes to the delay of several hours between the *period* mRNA and the PER protein accumulation. Where the PER protein accumulates after the peak of the *period* mRNA. Finally, the *clock* and *cycle* genes which code for the CLOCK (CLK) and CYCLE (CYC) proteins respectively, are transcription factors responsible for the activation of the *period* gene. CLK and CYC interact with each other to subsequently activate the transcription of *period*. In contrast, TIM and PER act as negative regulators of CLK activity. After the degradation of PER and TIM, the transcription of *per* and *tim* mRNAs is activated by CLK and CYC (Fig. 2B). This restarts the circadian rhythm (Patke et al., 2019).

So many genes involved in the circadian clock are regulated via activating or inhibiting transcription. The central transcriptional activators are CLC and CYC and the central transcriptional inhibitors are TIM and PER. Nevertheless, with only these genes the circadian rhythm would not be exactly 24 hours and eventually the cells would not function in synchronization with the environment anymore. To accomplish this a zeitgeber is required.

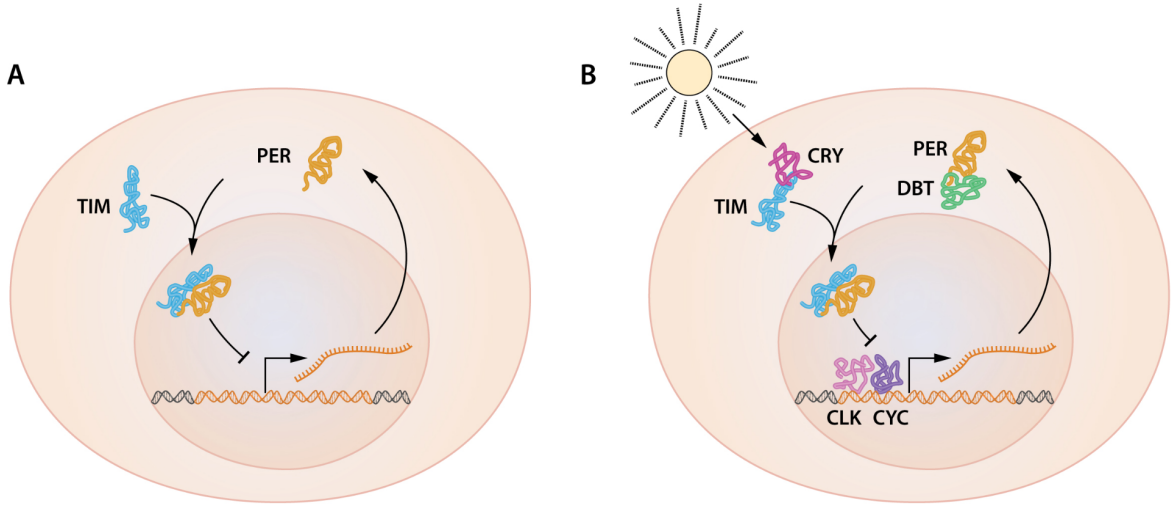

Figure 2: A simplified illustration of the feedback regulation of the period gene. Retrieved from Mattias Karlén, 2017 (https://www.nobelprize.org/prizes/medicine/2017/advanced-information/).

Light synchronizes the clock to the environment

As discussed above, this mechanism is a self-sustained TTFL, but it needs input for the environment. The intrinsic clock requires occasional resetting to synchronize with the day-night rhythm of the outside world because the cycle of the intrinsic clock is not exactly 24 hours. External stimuli help to synchronize the clock to the environment. These stimuli are for instance light and dark or daily temperature changes. All these environmental cues are called zeitgebers (German for 'time givers'), which entrain the organisms to the day-night rhythm (Asschof., 1960). This enables the organism to maintain a cycle of exactly 24 hours. In humans the zeitgeber is light. This input and subsequent synchronization occurs via the cryptochrome *cry* gene and its protein CRY. Light activates CRY, promoting its binding to TIM. This initiates the degradation of TIM, making DBT able to phosphorylate and degradate PER (Fig. 2B). In the sustained absence of sunlight, the output of physiological processes will still oscillate. However, these oscillations will not be exactly 24 hours because this requires a zeitgeber (Bear et al., 2015). Thus, a lot is already known about the molecular mechanisms underlying the circadian rhythm, including the self-sustained TTFL with the core clock genes TIM, PER, CLK and CYC and with the critical input from CRY. But a lot more is going on besides this TTFL.

Input beyond the self-sustained TTFL is crucial

The core clock genes and the TTFL are found to be fundamental to the circadian rhythm and most research has been done on these primary molecular mechanisms of the biological clock. However, multiple studies have shown the relevance of post-transcriptional modifications in maintaining a stable circadian rhythm of 24 hours (Asher et al., 2010; Bozek et al., 2009; Green et al., 2018; Lee et al., 2001). Since more and more evidence is showing that post-transcriptional regulation and epigenetics is critical to the biological clock as well, the present review aims to summarize known ways of post-transcriptional regulation of the circadian rhythm. Furthermore, it will describe the epigenetics of the biological clock.

Splicing of *per* transcripts regulates circadian clock

In the TTFL of the D. melanogaster, light is not the only zeitgeber. As PER being a core clock gene, a study conducted by Majercak et al. focussed on this protein using D. melanogaster. They aimed to find how changes in average daily temperatures influence the timing of activity rhythms. Temperature is found to be a key environmental modality that regulates the timing of circadian rhythms since seasonal changes like photoperiods are accompanied by predictable changes in the average temperature (Sweeney & Hastings., 1960). It was found that temperature regulates the levels of *per* and *tim* transcripts. This regulation acts in opposite directions, where levels of *per* transcripts are higher at colder temperatures and levels of *tim* transcripts are higher at higher temperatures. In this process, a splicing event in the three prime untranslated region (3'-UTR) of *per* RNA (dmpi8) plays an important role and is thermosensitive. The 3'-UTR is the part of the mRNA that immediately follows the translation termination codon and this section contains regions that regulate gene expression (Green., 2018). This splicing event upregulates the levels of *per* RNA a low temperatures. This shows a role for splicing of the dmpi8 in establishing the phase of the clock on days with colder temperatures. The upregulating of *per* transcript at lower temperatures leads to a preferential daytime activity of D. melanogaster on cold days.

From this study it is not clear how the cold-induced splicing of dmpi8 leads to an increase in *per* mRNA. Possibly this occurs via a stimulation of RNA 3'- end formation leading to an upregulation of *per* mRNA abundance (Majercak et al., 1999). However, they state that temperature-dependent changes in these transcripts are mainly due to changes in the levels of *tim* transcripts. TIM influences the stability of PER in the cytoplasm where PER is degraded in the absence of TIM (Vosshall et al., 1994). Besides that, TIM is a photosensitive protein and the levels of TIM rapidly decrease in response to light (Hunter et al., 1996). So long photoperiods increase the delay of TIM levels and short photoperiods lead to an advanced TIM accumulation. A long photoperiod then can partially counteract the increase of *per* mRNA levels since the accumulation of PER is dependent of TIM. This underscores a role for PER as an integrating point of information regarding temperature and day length (Fig. 3). The accumulation of per mRNA following a cold temperature can be neutralized by a long photoperiod (Majercak et al., 1999). In a later study by Majercat et al. they proposed that the degradation of TIM, induced by light, is crucial for the synchronization of the biological clock to the local time. And they proposed that the thermal regulation of the dmpi8 is crucial to anticipate the seasonal calendar information (Majercak et al., 2004). This study does not explain what exactly underlies this thermosensitivity of *per* mRNA, but later research aimed to reveal this.

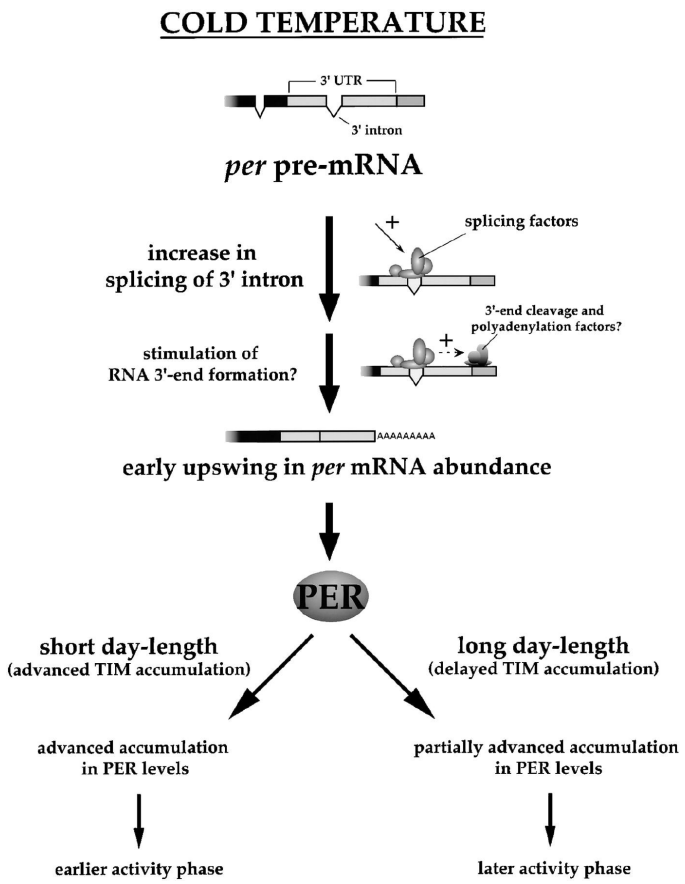

Figure 3: A model for how decreases in temperature and photoperiod regulate the D. melanogaster circadian clock. Cold temperate enhances *per* mRNA abundance and this information is combined with the day-length, to establish a proper activity phase. Retrieved from Majercak et al., 2017.

More recent research has revealed more on the underlying mechanism of this splicing event. It is found that weak splice sites underlie the thermosensitivity of the dmpi8 splicing and that higher splicing efficiency leads to higher *per* RNA levels (Low et al., 2009). The same lab has shown that not only weak splice sites underlie this thermosensitivity, but also multiple single nucleotide polymorphisms (SNPs) in the 3' UTR can modulate efficiency of the dmpi8 splicing (Low et al., 2012). However, the latter finding is mainly involved in the siesta of the D. melanogaster. The fly is a diurnal animal and exhibits a mid-day siesta (Rosato & Kyriacou., 2006). When the daily temperature increases, the onset of the evening is delayed and the mid-day siesta is more prominent (Ishimoto et al., 2012; Parisky et al., 2016). At lower temperatures, the splicing of dmpi8 is increased, which leads to a shorter mid-day siesta. Low et al. found that the least efficiently spliced version of the dmpi8 is associated with longer mid-day siesta, mainly at lower temperatures. These SNPs explains the natural variation in mid-day siesta levels in the D. melanogaster (Low et al., 2012). Expectedly, more is going on in the establishment of the mid-day siesta. Another important factor in the process of splicing, which is found in humans as well, is the highly conserved serine/arginine (SR) family of proteins. They can bind to the transcript RNA and suppress or enhance splicing events (Jeong., 2017). A study conducted by Zhang et al. aimed to find a SR protein that is involved in the mid-day siesta or the splicing efficiency of the dmpi8 intron. They found that the protein B52 is responsible for increases the efficiency of dmpi8 splicing. Other SR proteins are found to

have no effect (Zhang et al., 2018). RNA splicing is the first step in post-transcriptional regulation and obviously more regulation is occurring thereafter as well.

miRNAs regulate PER2 levels in the cytoplasm

After splicing, the mRNA leaves the nucleus where it can be translated into a protein. This translation can be controlled by microRNAs (miRNAs). MiRNAs are small noncoding RNAs of approximately 20-30 nucleotides that base pair with the 3' UTR of the target gene mRNA to inhibit translation. This binding is called RNA interference (RNAi) (Carthew & Sontheimer., 2009). It is likely that miRNAs are involved in the regulation of the biological clock since one-third of human genes are regulated by them (Stefani & Slack., 2008). The exact mechanisms underlying the time delay between the production of PER and the feedback inhibition by this PER protein remains unclear. However, miRNAs are found to be involved in this (Lee et al., 2001). To investigate this, a study by Chen et al. used *Dicer*-deficient cells and mice in which miRNA processing is affected. The biogenesis of mature miRNAs is distubed in these mice. They showed that RNAi mediated by miRNAs is a crucial mechanism underlying the time delay between the transcription of *per* and the PER accumulation in the cytoplasm. Moreover, they found that the only affected clock gene was *per2*, a member of the *period* family genes. The Dicer mutant mice have a shorter period caused by the increased build-up of PER2 which inhibits its own transcription. So this revealed that the absence of miRNAs does not disturb the circadian rhythmicity but it shortens the period (Fig. 4) (Chen et al, 2013).

Complementary to these findings, other research has found miRNAs that lengthen the circadian period. In a study by Yoo et al. they found candidate miRNAs involved in the post-transcriptional modification of *per2* that do so. They used Per2::LucSV mice in which the *per2* 3'-UTR was replaced by a SV40 late poly(A) signal, this enables the miRNAs to bind to the *per2* gene and regulate it. Thus, in this transgenic animal, the specific modulatory functions of miRNAs on the expression of the *per2* gene is blocked. After analysis of the 3'-UTR, Yoo et al. found two miRNAs, miR-24-3p and miR-30-5p, to be associated with the modification in mice. PER2 protein translation is suppressed by miR-24 and to a lesser extent by miR-30 (Yoo et al., 2017). These two results suggest a role of miRNAs on the expression of PER2 and the period of the rhythm. It shows that miRNAS suppress the translation of PER2 which prevents the accumulation of PER2 in the cytosol. PER2 is inhibiting its own transcription to a lesser extent when it is not piling up in the cytoplasm, making the period longer thus lengthening the circadian rhythm. Using cell culturing another cluster of miRNAs are uncovered. A study identified miR-192 and miR-194 as regulators of the circadian clock as well where they target the transcription of all three *per* genes, by downregulating them. This was obtained by exogenously overexpressing these two miRNAs (Nagel et al., 2019).

However, a study in 2020 showed a role of a miRNA where the period length was shortened after overexpressing it. Using mice models, they found miR-25-3p to be responsible for this. When the miR-25-3p was overexpressed, the period length shortened and when it was inhibited, the period length became longer and the levels of PER2 became higher (Park et al., 2020). This shows that miRNAs can have dual roles in regulating the circadian clock, depending on the type of miRNA. Besides that, it suggests that PER2 has a dual role in regulating the period length since in the latter study higher PER2 levels led to a longer circadian period as opposed to the findings from Chen et al. and Yoo et al. where it

led to shorter periods. These observations are all focussed on the PER2 protein, which is a core clock gene. However, a lot more gene products in the cell have a rhythmic expression.

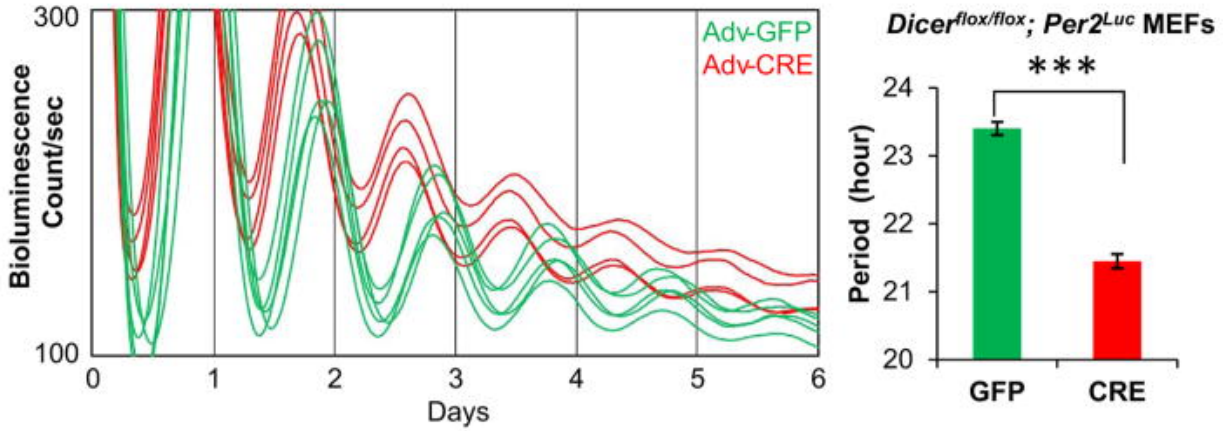

Figure. 4: Circadian period is shortened in Dicer-deficient cells and mice. CRE-expressing (= Dicer-deficient) cells show a dramatically shorter period (by ~2 hours) than GFP (=WT) cells. Retrieved from Chen et al., 2013.

Poly(A) tails of mRNAs exhibit daily oscillations

Another important post-transcriptional mechanism for regulating the circadian rhythm is the control of the length of the poly(A) tail of several different mRNAs in the cell. The length of 2.3% of the tails of all expressed mRNA in the liver of mice cycle daily (Kojima et al., 2012). The poly(A) tail consists of numerous adenosine monophosphates. So it is a region on the RNA with only adenine bases. Their function is to stabilize the RNA molecule and to contribute to the nuclear export and translation of the molecule (Kühn & Wahle., 2004). Research has been done on the involvement of the circadian rhythm on the post-transcriptional regulation of the poly(A) tail showing that the length of the poly(A) tail of some mRNAs exhibits daily variations. These are said to be poly(A) rhythmic (PAR) mRNAs and the peak tail lengths can be at all phases of the circadian cycle. For example, by means of northern blotting a study discovered that oscillations in the tail of the Fabp7 mRNA is dependent on the time of the day. This PAR mRNA codes for the fatty acid binding protein 7 found throughout the whole brain of both mice and human. Fabps bind small hydrophobic lipids and they act as transporters (Gerstner et al., 2012). The levels of Fabp7 mRNA are highest during the sleeping period and the levels of Fabp7 protein peak at the early morning (Fig. 5). So there are oscillations in the levels of these genes products. Overexpression of this gene in the transgenic flies leads to both increased sleep as an increased long-term memory (LTM). So this protein is also found to be involved in memory formation. This enhancement of LTM occurs during the consolidation period of learning. This study suggests the oscillations of Fabp mRNAs and proteins is implicated in sleep/ wake cycles and also memory (Gerstner et al., 2011).

The rhythmic deadenylation is the strongest aspect in controlling the rhythmicity of the PAR mRNA. It is suggested that the same deadenlases in a cell are used in order to synchronize all the genes around the clock (Yao et al., 2020). To generate a tissue-specific circadian expression of genes, the

expression of deadenylases differ in different tissues (Pizarro et al., 2013). Ultimately the rhythmicity in the regulation of the poly(A) tails by polyadenylation and deadenylation is mediated by the rhythmicity of the molecules controlling these processes, for example transcription factors, polymerases and deadenylases (Yao et al., 2020). As previously stated, the core clock genes form the fundament of the biological clock. This makes it interesting to focus on one of these genes products.

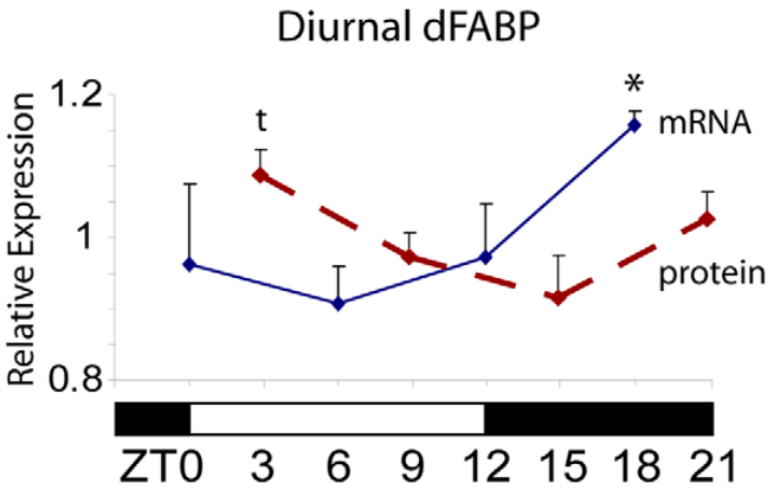

Figure. 5: Relative expression of Fabp mRNA (blue solid line) and protein levels (red dashed line) in D. melanogaster. Open bare = lights on (wake), filled bar = lights off (sleep). Retrieved from Gerstner et al., 2011.

A study by Grima et al. also looked at PAR mRNAs in the D. melanogaster. Here, they focussed on the mRNA of *timeless*, one of the core clock genes (Grima et al., 2019). POP2 is a deadenylase and is a key part of the CCR4-NOT deadenylation complex (Timme et al., 2014). The study by Grima et al. showed that the POP2 is responsible for altering behavioral rhythms. It does not control the oscillation of *clock* and *period* mRNA directly. It specifically contributes to the oscillation of *tim* mRNA and the TIM protein by shortening the poly(A) tail (Grima et al., 2019). The latter unstabilizes the transcript and increases translation (Lima at al., 2017). In the experiment of Grima et al. they found that a POP2 downregulation in flies leads to a lengthening of the *tim* mRNA and thus POP2 is responsible for the shortening of the poly(A) tail. Besides that, increased levels of *period* mRNA were found in flies with a downregulation of POP2, probably because of a lack of negative feedback from the PER/TIM dimer. So POP2 is necessary for keeping the poly(A) tail of *timeless* transcript very short, activating its translation. The study suggests that the activity of POP2 is higher in the evening based on their result that POP2 downregulation is less efficient in the evening. This complements the data that TIM activity is higher at nighttime and inhibited during daytime via CRY binding (Fig. 2A and 2B). However, the POP2 is active at all circadian times (Grima at al., 2019). As stated above, a short poly(A) tail is found to be associated with highly expressed genes and with more mRNA degradation. Thus, *tim* mRNA is unstable, which is expected for PAR mRNA and probably is crucial for the oscillation of *tim* mRNA (Lück et al., 2014).

As mentioned in the introduction, the biological clock uses zeitgebers as a cue to synchronize the clock to the environment. Besides light as a zeitgeber, also the temperature of the body is a zeitgeber in mammals. Two proteins, called Cirbp and Rbm3, control polyadenylation by binding the mRNA, thus

they are RNA-binding proteins (RBP). They use alternative polyadenylation, meaning that multiple transcripts can be produced containing different 3'ends. This expands the diversity of mRNAs and the encoded proteins (Zhang et al., 2021). The control of polyadenylation by Cirbp and Rbm3 is a crucial process in directing the amplitude of the temperature entrained circadian rhythm (Liu et al., 2013). Not only temperature gives information to the biological clock, also food and the nutritional status of a cell can act as a zeitgeber.

Rhythmically expressed nocturnin regulates metabolism

Just like POP2, nocturnin (NOC), encoded by the gene *Nocturnin* (*Noct*), is a circadian protein. NOC provides a link between the circadian clock and metabolism via NADP(H) regulation. It acts as a the key regulator of metabolic amplitude and its expression depends on nutrient status and the circadian clock. NOC is rhythmically expressed in multiple tissues of the mouse (Wang et al., 2001). The amplitude of the rhythm is of relevance since the amplitudes of the core clock genes and the output has a massive influence on metabolic health. Thus a proper functioning of the biological clock is crucial for maintaining the metabolic health (Kessler et al., 2019). A study by Stubblefield et al. looked specifically at hepatic *Noct* mRNA in wildtype (WT) mice. However, they also confirmed that this mRNA is rhythmically expressed in several other tissues. Besides that they found that the rhythmicity and amplitude of the hepatic mRNA can be modified by a chronic High Fat Diet (HFD). After a HFD of 3 weeks the *Noct* mRNA levels in the mouse liver begins to rise earlier during the day and the expression is increased at its peak (Fig. 6). In response to fasting and refeeding the levels are acutely altered. After a fasting period of 10 hours, the expression level of *Noct* mRNA is decreased. Subsequently the mice were exposed to a 4 hour refeeding which elevated the expression.

This study also examined key metabolic enzymes regulating triglyceride (TG) and cholesterol (CHOL) synthesis. Knockout (KO) mice lacking the *Noct* gene were used and the results were compared to WT mice. In KO mice the amplitudes of the levels of mRNA expression of hepatic genes that code for key metabolic enzymes regulating TG and CHOL synthesis is increased. Mice lacking the *Noct* gene have increased plasma TG levels and increased hepatic CHOL levels, which are produced by the liver. The amplitude of resulting metabolites are increased as well. So the normal function of NOC is to promote the breakdown of the mRNAs in the TG and CHOL pathways. This decay specifically occurs during the night since the normal peak of NOC levels is present during the nighttime. Thus, the control of NOC post-transcriptionally is responsible for regulating the amplitudes metabolic pathways of TG and CHOL, especially during nighttime. The loss of NOC activity in regulating the amplitude leads to an increased metabolic flux and reduced obesity. This suggests that an increased amplitude functions to protect the mice from e.g. obesity and hepatic steatosis (Stubblefield., 2018). Gene expression involves multiple steps and almost all steps can be regulated. The regulation by NOC is a post-transcriptionally process, meaning it controls gene expression via RNA levels, between the points of transcription and translation. Epigenetics also affects gene expression but this occurs before transcription. The biological clock is also affected by epigenetic changes.

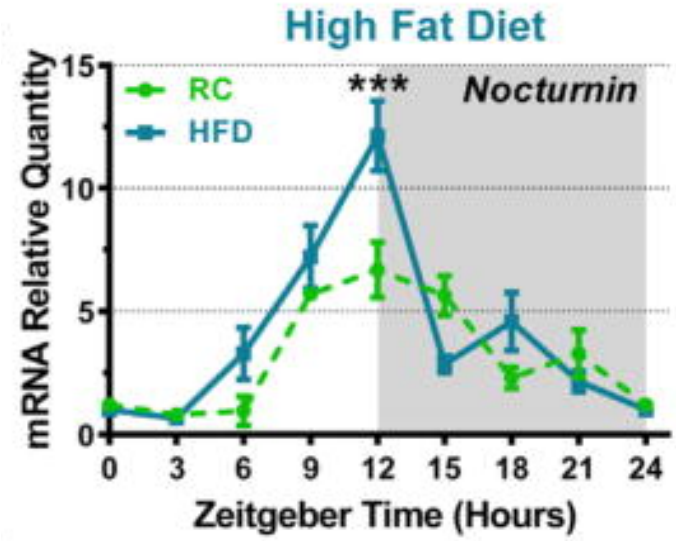

Figure. 6: Feeding condition alters NOC mRNA expression. RC = regular chow, i.e. normal diet. HFD = High Fat Diet. Retrieved from Stubblefield., 2018.

RNA Methylation changes the circadian period of PER2

In addition to post-transcriptional regulation as described above, epigenetics also plays a role in the regulation of the circadian clock. Two important epigenetic mechanisms are DNA methylation and histone acetylation. The circadian clock of mammals is sensitive to methylation. Inhibition of methylation in humans and mice cells leads to a longer period length (Fig. 7). This effect can be seen in both the SCN cells as the peripheral cells and the underlying mechanism involves both inducing as repressing certain genes. When separating those genes, it is found that the induced genes have more enrichment in RNA metabolic processing, like folding, modification or degradation. The repressed genes have enrichment translation processes. This latter is caused by high numbers of translation initiation factors and ribosomal proteins among these genes (Fustin et al., 2013). In a later study it is found that the inhibition of methylation also disrupts the circadian clock in plants and algae (Fustin et al., 2020). As stated previously, epigenetics involves two important mechanisms where histone acetylation will be discussed next.

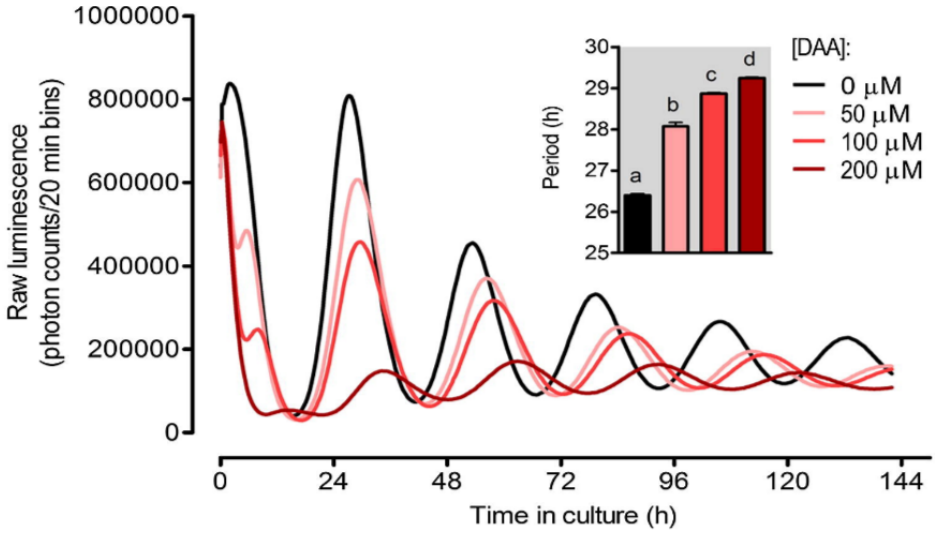

Figure. 7: Global methylation inhibition elongates the circadian period of PER2 in peripheral cell types. DAA = methylation inhibitor. Retrieved from Fusten et al., 2013.

Histone Acetylation during nighttime

Another epigenetic mechanism is histone tail modification, which is an important step in transcriptional regulation. Among these modifications, it is found that phosphorylation of the histone H3 is induced by retinal illumination. This is found in a study using mice where they applied a light pulse to mice during the subjective night (Fig. 8). The histone phosphorylation of H3 was only found in the SCN and no phosphorylation was found in other brain areas. This effect is gated by the circadian clock since the phosphorylation only occurs during the subjective night (Crosio et al., 2000). Another study found that the H3 histone acetylation in the liver also shows circadian rhythms. The circadian rhythm is found in the acetylation of the promoter regions of the *Per1*, *Per2* and *Cry* genes. Besides that, they showed that this acetylation is a potential target of the inhibitory action of CRY (Etchegaray et al., 2003).

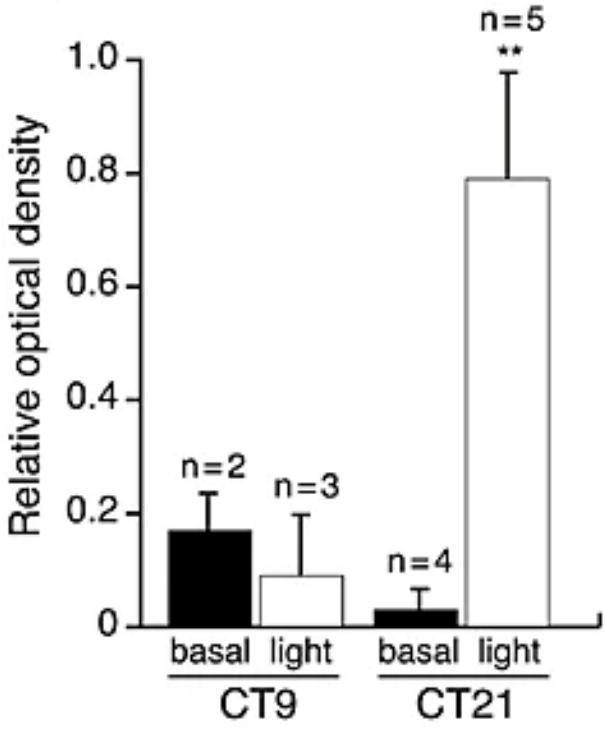

Figure 8: Time- and light-dependent phosphorylation of histone H3 in the SCN. Phosphorylation after a light pulse (light) or not after a light pulse (basal), where CT9 is daytime and CT21 is nighttime. Retrieved from Crosio et al., 2000.

Discussion

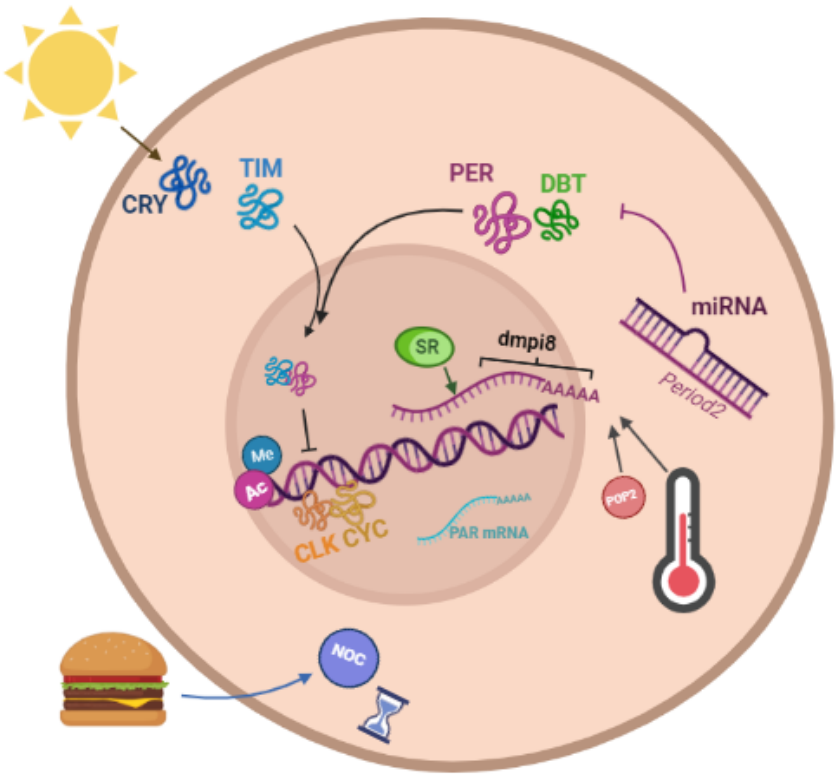

Figure 9: Summarizing Illustration. Showing splicing at the dmpi8, RNAi with miRNA, PAR mRNA and poly(A) tail control of period mRNA (e.g. via POP2), NOC proteins and epigenetic regulation via methylation and acetylation. Self-made, using BioRender.com.

This review summarizes different post-transcriptional mechanisms that are found to be crucial for maintaining a circadian rhythm in mammals. Furthermore, it goes into the epigenetics of this rhythm (Fig. 9). The first mechanism is a splicing event at the 3'-UTR of the clock gene *period*. Splicing of the dmpi8 is thermosensitive, where splicing events are upregulated at low temperatures. This leads to higher levels of PER in the cytoplasm and shows a role for establishing the phase of the clock on days with colder temperatures. PER is an integration point of information about temperature and daylength, enabling the D. melanogaster to determine the peak of activity. The clock gene *tim* is necessary for this integration since it is photosensitive and the accumulation of PER is dependent on TIM. Weak splicing sites are found to be the underlying mechanism of the thermosensitivity of the dmpi8 splicing (Hunter et al., 1996; Majercak et al., 1999; Vosshall et al., 1994). Splicing can be suppressed or enhanced by SR proteins. SR proteins can bind to the mRNA and regulate dmpi8 splicing efficiency (Zhang et al., 2018).

After splicing, mRNA can be translated into proteins. This process is regulated by miRNAs which interfere with the mRNA via RNAi. RNAi underlies the time delay between the transcription of *per2* and the PER2 protein accumulation in the cytoplasm because it prevents the translation of *per2*. The absence of miRNAS shortens the period of the circadian rhythm of PER2. (Chen et al., 2013). Multiple miRNAs are discovered and they probably all inhibit the translation of *per2*, however some counteracting

effects were found. More research on the different miRNAs is required to establish the exact role of miRNAs in regulating PER levels. Since there is extensive literature showing the binding of miRNAs to *per* transcripts, this suggests they have a role in regulating other clock genes as well. This should be focussed on as well to get a full understanding of the impact of miRNAs.

Controlling the length of poly(A) tails of clock transcripts is another post-transcriptional mechanism involved in maintaining a circadian rhythm. PAR mRNAs have variable lengths, depending on the time of the day. For example the levels of the PAR mRNA called Fabp7 is highest during the night and the translation product is the highest at the early morning. This oscillation is controlled by the length of its tail (Gerstner et al., 2012). Shortening poly(A) tails is done by a deadenylase. POP2 is an example of a deadenylate involved in the circadian clock. It is responsible for the oscillation of PER protein by shortening *per* mRNA. The activity of POP2 is dependent on the circadian time (Grima et al., 2019).

Another circadian protein is the NOC protein. This protein links the circadian clock to the metabolism of a cell since it depends on the nutrient status and the circadian clock. NOC is rhythmically expressed and it promotes the breakdown of mRNAs in the TG and CHOL pathways of metabolism in the liver. NOC peaks during the night and thus this breakdown specifically occurs during nighttime (Stubblefield., 2018). This enzyme is involved in metabolism and the absence increases metabolic flux. Besides that, it is found that the absence reduces obesity, thus making it relevant and beneficial for health. However most evidence has been done on mice, so to make it applicable to humans this should be studied further.

Lastly the epigenetics are discussed in this review. RNA methylation and histone acetylation are found to be important in circadian rhythmicity. Inhibition of methylation leads to a longer period length, suggesting that the function of methylation is to shorten period length. Typically, methylation functions to repress gene transcription and translation. However, it is found that in the mechanisms of the biological clock, genes can be both induced and repressed (Fustin et al., 2013). Besides that, research has shown that histone phosphorylation of H3 is involved in the biological clock. The phosphorylation shows a circadian rhythmicity in the SCN (Crosio et al., 2000). Another study showed that this rhythmicity is also found in the liver (Etchegaray et al., 2003).

There are many post-transcriptional mechanisms responsible for generating a rhythmicity in the cells functioning. This has an impact on numerous physiological processes in humans, for example the circadian rhythm is involved in regulating sleep patterns, hormone release and blood pressure (Bear et al., 2015). The biological clock has an influence on the immune system just like that the immune system has an influence on the clock (Bozek et al., 2009). A study has shown that a polymorphism near PER1 is involved in the timing of rhythms in human behaviour and it has found evidence that PER1 is associated with time of death. The study suggests this is mediated by differential expression of PER1. These findings may assist the progress of scheduling of medical treatments or monitoring of vulnerable patients populations on an individual level (Lim et al., 2012). Another study found that during acute systemic inflammation, the expression of clock genes in peripheral blood leukocytes is altered dramatically. Possibly this is caused by the misalignment of this peripheral clock expression with the the SCN. The synchronization of these clocks may aid in the recovery of diseases, making it of clinical relevance (Haimovich et al., 2010). However, the connections between all the different levels of gene expression and transcriptional regulation are not fully understood. More and more molecular mechanisms are being uncovered but all the findings discussed in this review are not applicable to healthcare yet. So to make it of clinical relevance these should be linked together with the core molecular mechanism. This review

only focused on the post-transcriptional mechanisms and epigenetics. To get a greater insight, also post-translational processes should be taken into account.

It should be considered that this review assumes the TTFL to be the fundament of the biological clock. However, it can be questioned whether the TIM, PER, CLK and CYC are the actual core clock genes. For future research it should be investigated which regulations are the basis of the clock and which are consequences or result of certain regulations. Perhaps the core clock genes are just the result of other oscillations in the cell. It should also be noted that multiple observations discussed in the review are obtained from studies on the D. melanogaster. The retrieved information might not be applicable to human. For example, the D. melanogaster performs a mid-day sieste, which is not seen in humans.

The complexity of the biological clock suggests there is a lot more going on besides the aspects discussed in this review. So more research is needed to elucidate all the components of the clock and to integrate all the information. All the recent findings, together with data conducted from research in the future, should be put together or should be linked to make sense of all the components of the molecular system of the biological clock, to eventually make it of clinical relevance.

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