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Evolutionary conservations, changes of circadian rhythms and their effect on circadian disturbances and therapeutic approaches

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ABSTRACT

The circadian rhythm is essential for the interaction of all living organisms with their environments. Several processes, such as thermoregulation, metabolism, cognition and memory, are regulated by the internal clock. Disturbances in the circadian rhythm have been shown to lead to the development of neuropsychiatric disorders, including attention-deficit hyperactivity disorder (ADHD). Interestingly, the mechanism of the circadian rhythms has been conserved in many different species, and misalignment between circadian rhythms and the environment results in evolutionary regression and lifespan reduction.

This review summarises the conserved mechanism of the internal clock and its major interspecies differences. In addition, it focuses on effects the circadian rhythm disturbances, especially in cases of ADHD, and describes the possibility of recombinant proteins generated by eukaryotic expression systems as therapeutic agents as well as CRISPR/Cas9 technology as a potential tool for research and therapy. The aim is to give an overview about the evolutionary conserved mechanism as well as the changes of the circadian clock. Furthermore, current knowledge about circadian rhythm disturbances and therapeutic approaches is discussed.

1. Introduction

Circadian clocks present an evolutionary benefit for organisms. Low levels of conservation can even be seen in bacteria and eukaryotes. One mechanism which has been conserved between species is the transcriptional/translational feedback-loop of the circadian system. The circadian rhythm is essential for the interaction between a living organism and its environment. One of the most important so called "zeitgebers" is light. Zeitgeber are external influences which act on the internal clock, resulting in the synchronisation of circadian rhythms and the environment. Essential processes, including intricate metabolic pathways, as well as higher cognitive functions are influenced by the light-dark cycle. Other zeitgebers, besides light, are social interaction, particularly between humans. Chronotype and everyday circadian misalignment can result in social jetlag.

Disruptions in circadian rhythms are associated with a range of neuropsychiatric disorders, one of which is attention-deficit hyperactivity disorder (ADHD) (McGowan et al., 2016, 2020). Current studies, however, indicate that the association between circadian genes and neuropsychiatric disorders is not a direct one. The circadian genes present no risk genes in ADHD (Demontis et al., 2019). It has, however, been shown that ADHD medication influences both circadian genes and their corresponding circadian rhythms (Coogan et al., 2019).

In all species, misalignment of circadian oscillation with the environmental rhythm is deleterious to evolutionary fitness, resulting in a reduction of lifespan (Patke et al., 2019). Approaches to remedy the circadian misalignment could include eukaryotic expression systems, which produce recombinant proteins as therapeutic agents (Geisse et al., 1996). Another approach with high therapeutic potential in relation to circadian rhythm disturbances and associated neuropsychiatric disorders is the CRISPR/Cas9 technology (Korge et al., 2015; Bording et al., 2019; Ortiz-Virumbrales et al., 2017).

The aim of this review is to summarize current knwoldege about conserved mechanisms of the circadian clock as well as the influence of zeitgeber. Furthermore, this review will give an overview about circadian rhythm disturbances and current therapeutic approaches.

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2. Methods

The review will sum up the current knowledge and literature about conserved mechanisms and processes of circadian rhythms and the inner clock. Furthermore, consequences of circadian rhythm disturbances with regard to human neuropsychiatric disorders, especially attention-deficit hyperactivity disorder (ADHD), will be discussed. This review will also give an insight into current therapeutic approaches relating to disruptions in the circadian rhythmicity as well as neuropsychiatric disorders.

A search with following keywords using Pubmed database was performed: circadian clock, circadian rhythm, circadian genes, prokaryotes, cyanobacteria, eukaryotes, green algae, plants, fungi, mammals, neuropsychiatric disorder models, spontaneously hypertensive rat, dopamine transporter knockout mice, coloboma mutant mice, Grin1 mutant mice, eukaryotic expression system, CHO cells, COS cells, recombinant baculovirus, yeast expression system, CRISPR/Cas9, antisense therapy, zeitgeber (time cues).

3. Results

3.1. Circadian clock mechanisms in prokaryotes

A circadian clock exists in prokaryotes (e.g. Cyanobacteria, Proteobacteria, Bacteriodetes). Cyanobacteria depend on the light-dark cycle, especially for oxygenic photosynthesis during the day and oxygen-sensitive nitrogen fixation in the dark. The first circadian rhythms of Cyanobacteria were reported in *Synechococcus elongates* (Ishiura et al., 1998). In general, the machinery of the circadian system in Cyanobacteria is made up of a central oscillator generating the approximate 24 h rhythm. In case of *Synechococcus elongates* the core proteins of the oscillator are KaiA, KaiB and KaiC (Ishiura et al., 1998). The central oscillator protein is KaiC with a double KaiC domain and a Walker motif that can bind ATP in each domain (Nishiwaki et al., 2000). Additionally, KaiC shows enzymatic activity (Nishiwaki et al., 2007; Terauchi et al., 2007). KaiA forms a homodimer that interacts with KaiC and stimulates phosphorylation as well inhibiting the dephosphorylation process (Kim et al., 2008). In contrast, KaiB tetramers antagonize the phosphorylation effect of KaiA (Xu et al., 2003).

For environmental signals there exists an input pathway to ensure modification and synchronization of the oscillator to the daily rhythm. Key proteins of this input pathway are: CikA, LpdA and Pex (Kutsuna et al., 1998; Schmitz et al., 2000; Katayama et al., 2003; Ivleva et al., 2006). Additionally, an output pathway is necessary to relay information from the oscillator to corresponding downstream processes. Key proteins in the output pathway are: SasA, RpaA and LabA (Iwasaki et al., 2000; Takai et al., 2006; Taniguchi et al., 2007).

The genes *kaiB* and *kaiC* are conserved among prokaryotes and archea whereas *kaiA* is often missing in prokaryotic genomes with *kaiB* and *kaiC* (Dvornyk et al., 2003). *Prochlorococcus* only expresses *kaiB* and *kaiC*, however, it displays circadian rhythmicity (Holtzendorff et al., 2008). The assumption is that other prokaryotes with *kaiBC* could also contain clock mechanisms and display circadian rhythmicity. Another bacterium with a circadian-like timing mechanism, and the presence of *kaiB* and *kaiC* cyanobacterial homologues genes is the purple bacterium *Rhodobacter sphaeroides*. One explanation for the absence of *kaiA* could be different evolutionary functions of *kaiBC* compared to cyanobacterial homologues (Min et al., 2005). Additionally, *Legionella pneumophila,* an environmental bacterium and opportunistic human pathogen also contains *kaiB* and *kaiC*. Interestingly, the function of the genes in *Legionella pneumophila* are not known, unlike that of the *kai*-genes in Cyanobacteria (Loza-Correa et al., 2010).

In summary, the presence of *kai* genes have been reported in cyanobacteria, proteobacteria, archea, chloroflexi and bacteriodetes (Holtzendorff et al., 2008; Loza-Correa et al., 2010). All cyanobacteria species except *Prochlorococcus* encode KaiA in their genome. There are, however, no studies showing KaiA being present in other prokaryotes, even when they encode KaiB and/or KaiC. In contrast, KaiB was shown to be present in all cyanobacteria and bacteriodetes, but only in some proteobacteria (Loza-Correa et al., 2010). No homologues of bacterial Kai proteins have been found in mammals, insects or fungi (Ishiura et al., 1998; Glossop et al., 1999; Cheng et al., 2001).

3.2. Circadian clock mechanisms in eukaryotes

3.2.1. Unicellular hetero-autotroph Euglena gracilis

Euglena gracilis is a unicellular, hetero-autotroph flagellate, which simultaneously exhibits autotrophic nutrition, as seen in plants, as well as heterotrophic nutrition such as mammals. Kunne et al. (1997) reported that some polypeptide proteins are synthesized rhythmically by these species with a period of 26 h (Kunne et al., 1997). Schnabel (1968) showed that light-off is a dominant zeitgeber in autotrophic as well as in mixotrophic cultures of *Euglena*. It is noteworthy, that the entrainment range is limited to a period of 16 h by a light-dark ratio of 1:1 (Schnabel, 1968).

Euglena presents circadian rhythmicity in photo- and gravitaxis. A free-running period of 24 h was observed for phototaxis, whereas Lebert et al. (1999) observed in synchronized cultures with no cell growth a distinct circadian rhythm of negative gravitactic orientation. The rhythmicity was lost after the transfer of the cultures into continuous light, whereas transferring cultures to permanent darkness also resulted in a persisting circadian rhythm with a progressively shorter cycle (Lebert et al., 1999; Edmunds and Laval-Martin, 1984).

3.2.2. Green algae Chlamydomonas reinhardtii

The green algae *Chlamydomonas reinhardtii* has two flagella and an eyespot apparatus on the edge of the chloroplast utilised as a visual system, and thus allowing phototaxis. Several circadian rhythms are described in green algae, such as chemotaxis, cell division, and starch metabolism (Harris, 2001; Byrne et al., 1992; Goto and Johnson, 1995; Ral et al., 2006). Several photoreceptors have been reported to influence the clock machinery (Schulze et al., 2010). In *Chlamydomonas reinhardtii*, the green light photoreceptors Channelrhodopsin-1 and -2 are localized in the eyespot. It is still unknown, whether Channelrhodopsin-1 and -2 are involved in the light-signaling pathways which encode the circadian machinery (Schmidt et al., 2006; Hegemann, 2008). Proteins such as Roc (Rhythm Of Chloroplast), Ck1 (Casein Kinase 1) and Chlamy1 have been demonstrated to be involved in the circadian rhythm. *Chlamy1* is involved in maintaining phase and period of the circadian rhythm (Iliev et al., 2006). *Ck1* has been shown shown to play a role in circadian phototactic signalling, hatching and flagella formation (Schmidt et al., 2006). Some *Roc* genes (e.g. *Roc15*, *Roc40*, *Roc66*, *Roc75*) appear to be under circadian control, whereas others (e.g. *Roc114*) are constantly expressed (Matsuo et al., 2008). Variations in the expression levels of these genes resulted in circadian rhythm defects such as phase and period alterations. *CikA* of *Synechococcus elongates* is conserved to some extent in *Chlamydomonas reinhardtii* (Mittag et al., 2005).

Homology searches have been carried out to compare the conservation of clock-relevant factors in *Chlamydomonas reinhardtii* and *Drosophila melanogaster*. These studies found no evidence for a putative *Per* (*Period*) or a *kaiC* homologue. Also, the similarities to clock components of *Arabidopsis* were limited and often restricted to conserved domains (Mittag et al., 2005). Two putative homologues of plant clock genes were identified with limitation to conserved domains. Both are key elements in the circadian machinery of the green microalga *Ostreococcus tauri* (Corellou et al., 2009). In *Chlamydomonas reinhardtii* Casein Kinase 1 and 2 (Ck1 and Ck2) were found to be homologues to clock components in *Neurospora*, *Drosophila* and/or mammals (Mittag et al., 2005). The enzymes N-Acetyl-Transferase and Hydroxy-Indole-O-Methyl-Transferase, which are involved in the last steps of melatonin synthesis, are conserved when comparing

Chlamydomonas reinhardtii, to vertebrates, non-vertebrates and plants (Mittag et al., 2005; Reiter, 1993; Goldman, 2001).

Blue as well as red light resulted in the reset of the circadian clock phase. In higher plants, red as well as blue light photoreceptors include Phytochromes and Cryptochromes (Cry) which both may play a role in establishing circadian rhythms (Harmer, 2009).

Phytochromes (Phy) were excluded as red light photoreceptors, because red/far-red reversibility was not found. Furthermore, homology searches showed no Phytochrome homologue between *Chlamydomonas reinhardtii* and higher plants (Mittag et al., 2005).

Two Cry proteins were identified in *Chlamydomonas reinhardtii* with homology to plant-like as well as animal-like Cry proteins (Mittag et al., 2005). Cry functions as a blue light receptor in *Drosophila*, whereas in mammals, two Cry proteins act as central components of the circadian rhythm by binding to Per proteins (Wijnen and Young, 2006).

3.2.3. Plants

Intrinsic clocks are needed to generate circadian rhythms. Organisms have developed mechanisms to anticipate changes such as day-night cycles as well as seasonal changes. Even under constant conditions the circadian gene expression keeps oscillation and drives circadian rhythms (e.g. leaf movement) (Greenham and McClung, 2015). Evolution of plant circadian rhythms are characterized by increased gene number and interactions through additional feedback loops (Linde et al., 2017).

The circadian machinery in plants is based on self-sustaining oscillation and results in an approximately 24 h rhythm (Harmer, 2009). The circadian rhythm reacts to environmental stimuli such as light and temperature, which in turn train the inner clock (Johnson et al., 2003). Circadian rhythms regulate diverse plant processes; such as stomatal opening, leaf movement, growth, metabolism, induction of flowering and response to stress (Greenham and McClung, 2015). Diverse genes are under circadian control (Covington et al., 2008; Michael et al., 2008) and disruption of the circadian rhythm as well as alterations in circadian genes result in fitness costs (Yerushalmi and Green, 2009).

In angiosperm, the circadian machinery can be described as an intricate network of interlocked transcriptional and translational feedback loops, which include a set of single Myb domain transcription factors, a family of Pseudo-Response Regulators (PRRs) as well as plant-specific genes with unknown biochemical function (Pokhilko et al., 2013; Fogelmark and Troein, 2014; De Caluwe et al., 2016).

The morning-phased genes *Circadian clock-associated 1* (*Cca1*) and *Late elongated hypocotyl* (*Lhy*) are Myb-like transcription factors, that inhibit day- and evening-phased genes (Fogelmark and Troein, 2014; Wang et al., 1997; Schaffer et al., 1998; Mizoguchi et al., 2002; Kamioka et al., 2016). The three evening-phased proteins are Lux arrhythmo (Lux) as well as Early flowering 3 (Elf3) and Early flowering 4 (Elf4) (Hicks et al., 2001; Doyle et al., 2002; Hazen et al., 2005; Nusinow et al., 2011). Other genes with circadian rhythm function are *Timing of cab expression 1* (*Toc1)*, *Gigantea* (*Gi)* and *Zeitlupe* (*Ztl)*. *Toc1* together with *Cca1* constituted the first conceptual model of the *Arabidopsis thaliana* clock (Alabadi et al., 2001). *Ztl* and *Gi* are reported to function in the circadian rhythm (Fowler et al., 1999; Somers et al., 2000). Gi interacts with Ztl, which in turn, regulates the stability of Toc1 and Prr5 (Mas et al., 2003; Kim et al., 2007a).

Cca1 and *Toc1* are conserved in different species. Both genes have putative homologues in *Chlamidomonas reinhardtii* and *Ostreococcus tauri*. However, other genes such as *Elf3*, *Elf4*, *Gi* and *Ztl* seem to have no homologues.

3.2.4. Circadian rhythm in thylakoid and mitochondria

Prokaryotes do not contain cell compartments. Consequently, fine-tuning of gene expression in these organisms is extremely difficult. Light regulated genes in eukaryotes, like plants, are activated by sunlight exposure and downregulated in darkness.

Genes involved in photosynthesis, such as genes of the light-harvesting complex (LHC), are light-inducible; but additional circadian regulation causes an increase in components of photosynthesis before dawn resulting in an immediate start of photosynthesis (Millar and Kay, 1996). Another example are the genes responsible for photoprotection. These genes are also expressed in Arabidopsis before dawn (Harmer et al., 2000).

The presence of a circadian clock allows organisms to anticipate changes in their environment instead of reacting to acute stimuli which results in increased efficiency (Langmesser and Albrecht, 2006).

Thylakoids are part of the chloroplast and play a key role in photosynthesis. The protein D1, that is involved in photosystem II reaction, has been reported to be under the control of an endogenous circadian rhythm. Under greenhouse conditions, the photoautotrophic higher plant *Spirodela oligorrhiza* displayed differences in the ratio of phosphorylated proteins compared to total D1 protein of photosystem II as a result of diurnal oscillation. These rhythms were out of phase with the period of maximum light intensity and are entrained by an external signal. Light interrupts the dark period and entraining of the circadian rhythm results in altered regulation of the D1 phosphorylation. The rhythmic oscillation of D1 phosphorylation was not observed in total darkness due to the fact that light is required for almost all chloroplast activities (Booij-James et al., 2002). Furthermore, the synthesis of the proteins involved in the light-harvesting complex (LHC proteins) is regulated by the circadian system. LHC proteins, such as D1 protein, are components of the thylakoid membrane of chloroplasts and part of the photosynthesis process. To execute the several steps of photosynthesis, precise coordination and timing of processes such as LHC protein accumulation is required and determined by the circadian clock mechanism (Piechulla, 1999).

Mitochondria, cell components of almost all eukaryotes and responsible for the respiration chain and energy balance, display interactions with the circadian clock. Mitochondrial dynamics is the process by which mitochondria continuously fuse and divide. This process contains all of the bioenergetics properties modulation according to the nutritional demands of the organism. Schmitt et al. (2018) presented evidence that the morphology of the mitochondrial network exhibits circadian oscillation in cultured fibroblasts. There is a change from a tubular mitochondrial network at 16 h after serum shock to highly fragmented network at 28 h post-synchronization. This results in rhythmic ATP content as well as oxidative phosphorylation. These circadian rhythms are lost in fibroblasts from PER1/PER2 deletion mice as well as the rhythmicity of ATP levels (Schmitt et al., 2018). Neufeld-Cohen et al. (2016) described altered circadian rhythms in mitochondrial respiration in PER1/PER2 lacking mice (Neufeld-Cohen et al., 2016). In cultured hepatocytes from BMAL1 lacking mice, mitochondrial morphology alterations were described (e.g. swollen mitochondria) (Jacobi et al., 2015). *Bmal1* knockout as well as *Clock*Δ*19* mutant mice showed a reduction in contractile muscle force and muscle mitochondrial volume as well as respiratory function. Furthermore, the expression of *Pgc1a* and *Pgc1b,* a marker of mitochondrial biogenesis in human muscle, was altered (Andrews et al., 2010).

Retinal ATP content and various signaling molecules related to metabolism are under circadian control. The mitochondrial dynamics in light-sensing retinal cells have been reported to be influenced by light as well as regulated by the circadian system (Chang et al., 2018). The interaction between mitochondria and circadian rhythms, including deacetylation mechanisms as well as AMPK activation and oxygen levels, were recently (2018) reviewed in Sardon Puig et al. (2018) and de Goede et al. (2018).

Several studies have demonstrated the interaction between the circadian rhythm system and thylakoids, as well as mitochondria. Dysregulations in these interactions may result in fitness loss of an organism.

3.2.5. Filamentous fungi Neurospora crassa

The filamentous fungus *Neurospora crassa* contains a circadian clock machinery that is comparable to other eukaryotic circadian clocks.

The main component is *Frequency* (*Frq*) that shows long, short and arrhythmic periods, some of which are altered by temperature compensation (Gardner and Feldman, 1980; Loros et al., 1986). The protein Frq is rhythmically expressed with a period of approximately 22.5 h under constant conditions (Aronson et al., 1994; Garceau et al., 1997). The circadian rhythm starts with the binding of White collar complex (Wcc) on the *frq* promotor. The interaction between Frq and Wcc facilitates phosphorylation of the Wcc, which then is inactivated (Crosthwaite et al., 1997; He et al., 2006, 2002; Belden et al., 2007; Schafmeier et al., 2008, 2005; Hong et al., 2008). Frh (Frq-Interacting RNA Helicase) forms a complex together with Frq that acts as part of the negative feedback loop (Guo et al., 2010). Additionally, one positive feedback loop exists. Wc-1 (White collar 1), a blue light photoreceptor, increases when Wcc is inactivated as indirect result of Frq's repressive activity on Wcc (Schafmeier et al., 2005; He et al., 2005; Shi et al., 2010). Wc-1 is responsible for all known light responses in *Neurospora crassa* (He et al., 2002; Ballario et al., 1996; Froehlich et al., 2002). Additional kinases are needed in the regulatory steps of the circadian rhythm. *Neurospora crassa* expresses the *Casein kinase 1* (*Ck1a*, *Ck1b*). Ck1a is more similar to the mammalian *Ck1ε* (He et al., 2006; Gorl et al., 2001).

In addition, to the role of Wc-1 in the circadian negative feedback loop, it also acts as a blue light receptor required for resetting and entrainment of the circadian rhythm (He et al., 2002, 2005; Froehlich et al., 2002; He and Liu, 2005; Heintzen and Liu, 2007; Guo and Liu, 2010).

It has been reported that light input is modified by the Vivid protein, that itself is clock regulated and can gate the light response to the clock (Heintzen et al., 2001). The *vvd* promotor has also been reported as a direct target of the Wcc and inhibits it. This interaction sets the circadian rhythm at the dusk transition as well as contributing to temperature condensation (Elvin et al., 2005; Hunt et al., 2007). Additionally, Vvd levels in the dark inactivate Wcc induced by moonlight and keep the circadian rhythm in phase during bright moonlight nights (Malzahn et al., 2010; Hurley et al., 2015).

Temperature has also been demonstrated to be involved in the entrainment of the circadian rhythm. Frq levels increase under elevated temperature conditions (Garceau et al., 1997; Hurley et al., 2015; Liu et al., 1997). The Frq is similar to the Per protein in *Drosophila* and vertebrates, and can be found in diverse complexes with other proteins required to execute a negative feedback loop. Animal Per proteins are also reported to be associated with Ck1 (Gallego and Virshup, 2007). Bmal1 shared sequence homology to Wc-1. Wc-1 plays the same role in *Neurospora crassa* as Bmal1 in mammals (Lee et al., 2003). To sum up, some proteins in *Neurospora crassa* are orthologues to the mammalian circadian proteins. But the functional machinery of the circadian rhythm seems to be conserved among species. Positive elements activate negative elements and negative elements inhibit the activation of the positive elements (Baker et al., 2012).

3.2.6. Circadian clock mechanisms in Drosophila melanogaster

Drosophila melanogaster mutants exhibited shorter (19 h) or longer (28 h) rhythm periodicity as well as completely eliminated rhythms (Konopka and Benzer, 1971). The first circadian genes discovered in *Drosophila melanogaster* were *Period* (*Per*) and *Timeless* (*Tim*). Both genes, with feedback loops of their own transcription, form the core of the circadian rhythm machinery. Other important genes included in the core transcriptional feedback loop are *Clock* (*Clk*), *Cycle* (*Cyc*), *Cryptochrome* (*Cry*), kinase *Double-time* (*Dbt*), *Shaggy* (*Sgg*) and *Vrille* (*Vri*) (Sehgal et al., 1994; Allada et al., 1998; Rutila et al., 1998; Emery et al., 1998; Stanewsky et al., 1998; Kloss et al., 1998; Price et al., 1998; Martinek et al., 2001; Blau and Young, 1999; Dubowy and Sehgal, 2017).

Tim stabilizes Per in the cytoplasm and is required for its transport into the nucleus (Zheng and Sehgal, 2012). Both genes regulate transcription by regulating their transcriptional activators, Clk and Cyc, which form a heterodimer complex. Clk-Cyc binds to E-box elements upstream of the promoters of *Per* and *Tim*. Additionally, Per recruits the kinase Dbt (CK1ε homologue) (Dubowy and Sehgal, 2017; Lee et al., 1999; Kim and Edery, 2006; Kim et al., 2007b; Nawathean et al., 2007). A second loop involving the Clk-Cyc complex as well as Vri as repressor has also been reported. This suggests that the second loop has a function in stabilizing the circadian rhythm machinery while providing it with greater precision (Cyran et al., 2003; Glossop et al., 2003). Pdf (Pigment dispersing factor) as well as Pdfr (Pdf receptor) are important for synchronization of the circadian rhythm, but the exact mechanisms are still being explored (Shafer et al., 2008; Klose et al., 2016). Detailed information about the transcriptional/translational feedback loops are given in Patke et al. 2019 (Patke et al., 2019).

Interestingly, the feedback loop of the circadian rhythm in *Drosophila melanogaster* is conserved. Cyanobacteria as wells as *Neurospora crassa* also display a negative feedback loop, which is generated in *Neurospora* by cyclic expression of *Frequency* (Ishiura et al., 1998; Aronson et al., 1994; Hardin et al., 1990; Sehgal et al., 1995).

Feedback loop as well as gene functions are also conserved in insects and mammals. For example, *Per2* and *Ck1δ* as well as *Clk* and *Cyc* (*Bmal1* in mammals) are homologues with similar functions (Dubowy and Sehgal, 2017; Toh et al., 2001; Xu et al., 2005; Partch et al., 2014). Interestingly, *Cry* does not act in mammals as a photoreceptor. Instead *Cry* takes the function of *Tim*. Mammalian light input is mediated by photosensitive retinal ganglion cells (Dubowy and Sehgal, 2017; Guler et al., 2008). The core circadian components are co-expressed in a restricted set of approximately 150 neurons, which serve a similar function as the suprachiasmatic nucleus (SCN) in mammals (Dubowy and Sehgal, 2017).

3.2.7. Circadian clock mechanisms in mammals

The inner clock contains a circadian rhythmicity of approximately 24 h, and this circadian rhythm influences the physiology and behaviour of mammals. The clock is influenced by the environment such as light-dark cycles as well as social interaction (Hastings et al., 2003; Curtis and Fitzgerald, 2006). Several studies have reported that disruptions in the circadian clock result in a variety of pathologies including both metabolic and psychiatric disorders (Klerman, 2005; Levi and Schibler, 2007). The master pacemaker in this process is the suprachiasmatic nucleus (SCN) of the hypothalamus. Most tissues in mammals are reported to contain autonomous peripheral oscillators. These peripheral clocks are regulated and entrained by the SCN and different physiological signals such as glucocorticoid production, as well as cAMP input (Kornmann et al., 2007; Schibler and Sassone-Corsi, 2002; Stratmann and Schibler, 2006; Yoo et al., 2004).

In general, the circadian rhythm is regulated by transcriptional/translational feedback loops. The core genes are *Clock* (*Circadian locomotor output cycles kaput*) and *Bmal1* (*Brain and Muscle ARNT-Like 1*), which form a heterodimer complex, that in turn activates *Per1-3* (*Period*) and *Cry1-2* (*Cryptochrome*). *Per* and *Cry* inhibit the Bmal1-Clock complex and thus their own expression. This degradation of Per-Cry allows the initiation of a new cycle (Schibler and Sassone-Corsi, 2002; Koike et al., 2012). An additional feedback loop involves the nuclear orphan receptors REVERBα and RAR-related orphan receptor A (RORA) that modifies the transcription of *Bmal1* (Preitner et al., 2002; Sato et al., 2004). Changes in *Per* and *Cry* gene expression result in a faster or a slower clock in *Drosophila* as well as mammals (Shigeyoshi et al., 2002; Yang and Sehgal, 2001; Maywood et al., 2011).

3.2.7.1. Mice. Hughes et al. (2009) presented a comparison of oscillating transcripts between mouse hepatocytes, NIH3T3 (standard fibroblast cell line) and U2OS cells (bone osteosarcoma epithelial cells). They reported that the majority of cycling transcripts from liver cells as well as those from NIH3T3 and U2OS cells show a period length of approximately 24 h. About 260 transcripts showed a period length of around 12 h and 63 transcripts a period length of around 8 h. The

authors reported that 12 h oscillatory transcripts occur in several tissues, which is lost *ex vivo* and under restricted feeding conditions (Hughes et al., 2009).

Alterations in circadian genes result in disruption of the circadian rhythm: Milit et al. (2016) described the importance of *Per2* PAS domain for circadian pacemaking in *Early doors* mutant mice (*Edo*). The circadian rhythm of *Per2* in *Edo/Edo* mice is accelerated by 1.5 h and the vulnerability to degradation of *Per2* mediated by *Casein kinase 1 ε* (*Csk1E*) is increased (Militi et al., 2016).

Several studies have reported that the absence of *Bmal1* results in a loss of circadian rhythm as well as an acceleration of ageing, a shortened life span, reduced body weight, and neurodegeneration in mice (McDearmon et al., 2006; Kondratov et al., 2006; Musiek et al., 2013; Yang et al., 2016). Additionally, Park et al. (2015) have reported that mutant mice with a C-terminus truncated *Bmal1* (*Bmal1*$^{GT}\Delta^{C}$) allele either lost circadian behaviour rhythms under constant darkness (homozygous mutant mice) or displayed a gradual loss of rhythm (heterozygous mutant mice). Homozygous mutant mice showed arrhythmic mRNA and protein expression in both the SCN and the liver. Interestingly, overexpression of *Bmal1*$^{GT}\Delta^{C}$ was unable to activate *Per1* (Park et al., 2015).

3.2.7.2. Human. Previous studies concerning the circadian rhythm have suggested that human circadian genes may have more important roles in the circadian machinery than in mice.

Matsumura et al. (2019) used *Per3* deficient human bone osteosarcoma cells (U2OS) to illustrate phase advance in circadian transcription compared to wildtype cells. Additionally, the authors reported that the period length in *Per3* deficient cells was significantly shorter than in wildtype cells. These observations are consistent with studies concerning mouse *Per3* and indicate, that the Per3 protein functions similarly in both mice and humans (Matsumura and Akashi, 2019; Bae et al., 2001; Shearman et al., 2000).

Matsumura et al. presented the following possible explanation for the shorter period length of *Per3* in mice and human: the nuclear translocation of mCry1 and mCry2 is dependent on complex formation with mPer1 and mPer2 as well as phosphorylation by mCKIε. Furthermore, mPer3 can bind to mCry1 and mCry2, but the binding process is slower (Lee et al., 2001, 2004; Lee et al., 2009, 2011). *mPer3* may contribute to elongation of the period length by delaying the transcription repression phase in competition with *mPer1* and *mPer2* (Matsumura and Akashi, 2019).

In summary, the molecular mechanisms of the circadian rhythm share similarities, regardless of the species, and include enhancer/repressor elements as well as phosphorylation-dephosphorylation, methylation and acetylation reactions (Hardin, 1998; Dunlap, 1999; Charrier et al., 2017). The molecular circadian clock of both insects and mammals consists of interlocking transcription/translation feedback loops driving by a heterodimeric complex; either Clk-Cyc in flies or Clock-Bmal1 in mammals. The activation of *Per* and *Tim* in *Drosophila* or *Per* and *Cry* in mammals is through binding of the heterodimeric complex on E-box elements in the promotor region of these genes. Cry proteins are a notable difference between the circadian rhythm in flies and mammals. Cry of *Drosophila* is not a component of the transcription/translation feedback loop, whereas the mammalian Cry acts as the main repressor of the Clock-Bmal1 complex and takes over the function of Tim (Patke et al., 2019).

3.3. Circadian rhythms and psychiatric disorders

Mice lacking *Cry1* and *Cry2* display cognitive dysfunction, anxiety-related behaviour and sensitivity to psychostimulant drugs (De Bundel et al., 2013) and *Per3* deficient mice display alterations in sleep-wake timing and sleep homeostasis (Hasan et al., 2011). Mutations in human circadian genes are associated with sleep disorders (Ebisawa et al., 2001; Viola et al., 2007; Hirano et al., 2016; Patke et al., 2017) and thus may influence cognition, mood, anxiety, and reward-related behaviours (Wulff et al., 2010). Interestingly, single nucleotide polymorphisms of *Clock* are associated with several psychiatric disorders (attention-deficit hyperactivity disorder, alcohol use disorder, bipolar disorder, delayed sleep phase syndrome, major depressive disorder, mood disorders, and schizophrenia) (Schuch et al., 2018). Demontis et al. identified 12 significant risk loci in a genome-wide association studies (GWASs), but found no association with circadian genes (Demontis et al., 2019). Additional GWASs studies reported associations between chronotype and genotype, particularly, in circadian genes (Lane et al., 2016; Jones et al., 2016; Hu et al., 2016; Kalmbach et al., 2017; Gaspar et al., 2017).

3.3.1. Attention-deficit hyperactivity disorder

Attention-deficit hyperactivity disorder (ADHD) is characterised by the core symptoms inattention, impulsivity and hyperactivity (Polanczyk et al., 2007; Kooij and Bijlenga, 2013; Kaiser et al., 2015). ADHD shows a high frequency of comorbidity with other psychiatric disorders (Matthews et al., 2014) and is associated with sleep disturbances (Hvolby, 2015). The circadian rhythm modulates alertness as well as cognitive performance (e.g. learning, memory). Circadian phase disruptions are responsible for retrograde amnesia and memory recall deficits that may explain inattentiveness in ADHD (Fisk et al., 2018; Krishnan and Lyons, 2015; Tapp and Holloway, 1981; Fekete et al., 1985; Devan et al., 2001; Cho et al., 2000; Philipsen et al., 2006).

Several studies have reported that circadian oscillations are altered in ADHD. This includes the dampening of circadian rhythms as well as an increase of eveningness chronotype (Coogan and McGowan, 2017; Baird et al., 2013; Rybak et al., 2007; Van Veen et al., 2010). Furthermore, genome-wide association studies (GWASs) showed an association between circadian genes and chronotype (Lane et al., 2016; Jones et al., 2016; Hu et al., 2016; Kalmbach et al., 2017; Gaspar et al., 2017; van der Meer et al., 2017; Lane et al., 2017; Lasky-Su et al., 2008). Lane et al. (2016) reported an association of 12 loci with such chronotypes with some of these loci in or near circadian genes. Using the same biobank Jones et al. (2016) reported 16 loci associated with the morningness chronotype; also involved in circadian rhythm or photoreception. Of the 15 loci associated with morningness in Hu et al.'s (2016) study, 7 loci were near to circadian genes (Hu et al., 2016). Nine loci were were also included in the studies of Lane et al. and Jones et al. (Kalmbach et al., 2017).

3.3.2. Models of neuropsychiatric disorders with focus on ADHD

Early-life-insults are related to increased appearances of neuropsychiatric diseases in adulthood. Therefore, genetic factors are thought to play a major role in the aetiology of ADHD. Further, disrupted circadian rhythms are indicated to developmental models of neuropsychiatric disorders (Marco et al., 2016). Models of neuropsychiatric disorders focusing on ADHD are valuable tools for investigating causal determinants as well as neural development and key molecular, cellular, and behavioural mechanisms (Arime et al., 2011). Animal models of ADHD presuppose the genetic and neuropathological abnormalities along with the three core clinical symptoms of ADHD (inattentiveness, hyperactivity, impulsivity) that should be improved by pharmacological agents. Several animal models for ADHD have been proposed.

3.3.2.1. Spontaneously hypertensive rat. The spontaneously hypertensive rat (SHR) was developed by inbreeding rats of the Wistar-Kyoto (WKY) strain (Okamoto and Aoki, 1963) and shows several major ADHD-like symptoms, such as impulsivity, hyperactivity, and poor sustained attention (Sagvolden et al., 2009). Adult SHRs are prone to the development of hypertension (Arime et al., 2011). In order to investigate this effect, crossbreeding of SHR with WKY developed the WKHA model. These rodents are normotensive but hyperactive and hypersensitive to

stress. In their 2012a study Drolet et al. observed that stimulant medication with methylphenidate does not decrease hyperactivity in WKHA compared to WKY and outbred albino Wistar rat strains (Drolet et al., 2002) and WKHA does not appear to represent a useful model of ADHD.

SHR remains the most widely studied animal model for ADHD, expressing high activity, inattention and impulsive behaviour during operant and task tests (Sagvolden et al., 2009; Sagvolden, 2000). The impulsivity and hyperactivity in SHR develops over time (Sagvolden et al., 2009; Sagvolden, 2000) and is ameliorated by methylphenidate and D-amphetamine (Sagvolden et al., 1992; Natsheh and Shiflett, 2015). One study indicated that the α-2A-adrenoceptor agonist guanfacine improves the core symptoms of ADHD in SHR (Sagvolden, 2006).

Additionally, the SHR model exhibits a disturbed norepinephrine transmission, impaired dopamine release in the prefrontal cortex, nucleus accumbens systems and striatum as well as abnormalities in dopamine transporter activity (DAT) (Russell et al., 2000; Heal et al., 2008; Leo et al., 2003). In summary, SHR is a valid animal model for ADHD, particularly as a model of ADHD in children, due to the fact that young SHRs are normotensive. SHRs as well as ADHD-affected children both show response re-engagement deficits (Sagvolden et al., 2009).

3.3.2.2. Dopamine transporter (DAT) knockout mice. The Dopamine transporter (DAT) is a plasma membrane protein that belongs to the large family of NaCl-dependent transporters responsible for the clearance of released extracellular dopamine. DAT-1 gene has been associated with ADHD (Cornish et al., 2005; Hasler et al., 2015; Pineau et al., 2019). DAT protein is a major target for stimulant medication, such as methylphenidate and amphetamine.

DAT knockout mice lack the DAT gene, expressing high extracellular dopamine levels in the striatum and nucleus accumbens, and exhibiting spontaneous hyperactivity, cognitive deficits and sleep dysregulation (Gainetdinov and Caron, 2000; Spielewoy et al., 2000; Li et al., 2010). Pharmacological treatment with methylphenidate and amphetamine is effective in reducing hyperactivity in DAT knockout rat models (Gainetdinov and Caron, 2000). It seems, however, that DAT knockout mice have alterations in mesocortical circuitry, suggesting additional mechanisms are implicated (Zhang et al., 2010). More limitations are reflected in significantly elevated dopamine levels in the striatum and nucleus accumbens and the lack of DAT protein (Arime et al., 2011; Shen et al., 2004).

Using zinc-finger nucleases (ZFN) technology, Leo et al. (2018) developed a strain of rats with a disrupted DAT gene. DAT knockout rats exhibit pronounced hyperactivity, cognitive dysfunctions, neurotrophin BDNF dysregulation and persistently increased dopaminergic transmission (Leo et al., 2018). Additionally, Cinque et al. (2018) observed alterations in decision-making processes and in motivational states in DAT knockout rats, suggesting an improved model of ADHD (Cinque et al., 2018). To elucidate this, more studies are required, for example with other therapeutic agents such as the selective norepinephrine reuptake inhibitor atomoxetine, and the α-2A -adrenoceptor agonist guanfacine.

3.3.2.3. Coloboma mutant mouse. Coloboma mutant mice exhibit a variety of behavioural, neurophysiological and developmental deficits, which may be compared with those found in ADHD children (Wilson, 2000).

The coloboma mutant mouse was developed using neutron irradiation causing a mutation in the synaptosomal-nerve-associated protein with a size of 25 kDa (SNAP-25) and phospholipase C beta-1 (Hess et al., 1996). SNAP-25 is part of the trans SNARE (soluble N-ethylmaleimide-sensitive-factor attachment receptor) complex responsible for synaptic vesicle exocytosis and exocytotic release of neurotransmitters during synaptic transmission. The SNAP-25 mutation leads to alterations in neurotransmitter release. Several studies indicated an association between the SNAP-25 and ADHD as well as other neuropsychiatric disorders (Barr et al., 2000; Thapar et al., 2005).

Coloboma mutant mice are extremely hyperactive, with spontaneous locomotor activity, that is reduced by amphetamine (Hess et al., 1996, 1992). Hess and colleagues (1996) observed that injection of amphetamine significantly reduced the locomotor activity in Coloboma mice compared to the activity of control mice, whereas methylphenidate injection increased locomotor activity in both groups (Hess et al., 1996).

3.3.2.4. Grin1 mutant mouse. *Grin1* mutant mice are a heterozygous mutant strain developed in the RIKEN Mutagenesis project using N-methyl N-nitrosourea (ENU) technology (Umemori et al., 2013). ENU is a chemical mutagen that introduces single base pair changes into genomic DNA. Grin1 is an essential subunit in the heterodimeric complexes of NMDA receptors, that plays an important role in neurotransmission and brain development (Monyer et al., 1994). Grin2B has been previously associated with ADHD (Dorval et al., 2007), and thus *Grin1* has been considered a potential candidate risk gene for ADHD. *Grin1* mutant mice have the homozygous missense mutation R844C and are exhibiting novelty-seeking behaviour towards objects and decreased social interactions and hyperactivity, which both are ameliorated by methylphenidate (Furuse et al., 2010). Cognitive functions have not yet been studied in this animal model.

3.3.3. Eukaryotic expression systems

Eukaryotic expression systems produce recombinant proteins as therapeutics. When selecting an expression system, the biochemical and biological properties as well as the required quantity of the protein of interest should be considered (Geisse et al., 1996). Most commonly used expression systems are based on stably transfected adherent Chinese Hamster Ovary (CHO) cells and other mammalian cells, e.g. monkey kidney tissue (COS) cells, along with alternatives such as insect cells which have been infected with baculovirus, and yeast or filamentous fungi expression systems (Geisse et al., 1996).

3.3.4. Stably transfected CHO cells

The most widely used host cells for eukaryotic expression systems are mammalian CHO cells. CHO cells are safe, have low specific productivity, efficient post-translational protein modification and are easily adapted to growth in serum free suspensions (Kim et al., 2012). Optimal cell cultivation and maintenance in bioreactors, as well as the choice and composition of media are important factors to consider for successful high titre expression of recombinant proteins in industry (Geisse et al., 1996).

Using derivatives of the CHO cell line, CHO-K1 and CHO pro-3 as the hosts, two specific cell lines, DUKX-X11 and DG44 have been produced by lipofection (Lee et al., 2010). Due to chemical mutagenesis, these CHO cell lines are deficient in dihydrofolate reductase (DHFR) activity, and thus not being able to grow in a medium without nucleosides. DHFR catalysis the conversion of folate to tetra-folate, playing an important role in glycine, thymidine and purine biosynthesis (Zheng and Cantley, 2019). An early method of producing recombinant proteins is gene amplification by selective agents, such as the folic acid analogue methotrexate. Methotrexate binds and inhibits dihydro folate reductase (DHFR) stoichiometrically, thus triggering gene rearrangements and amplification for survival (Kaufman et al., 1985). However, gene amplification followed by an extensive screening is time-consuming and labour-intensive (Kim et al., 2012).

More recent approaches, such as vector engineering through gene targeting by site-specific integration and cis-acting element for augmenting gene expression are more convenient for CHO cell line development (Kito et al., 2002; Girod et al., 2005; Kameyama et al., 2010). These methods are advantegeous by enhancing the expression and stability of protein production. To improve CHO cell growth and foreign protein production, and to increase the time integral of viable cell concentration, numerous cell engineering strategies have been

developed. This includes anti-apoptosis, anti-autophagy, proliferation and cell cycle engineering, as well as secretion and metabolic engineering (Kim et al., 2012). Furthermore, the use of miRNA in cell engineering can regulate global gene expression (Muller et al., 2008; Barron et al., 2011).

Although significant improvements in therapeutic protein production of CHO cells have been achieved, there is still substantial genomic information about CHO cells that remains to be elucidated.

3.3.5. COS cells: Production of smaller quantities of proteins

COS cells are easy to maintain in culture and easy to transfect using transient expression by means of extrachromosomal replication. In COS cells, transient expression of heterologous genes is driven by SV40 large T-antigen expression (Geisse et al., 1996). Small quantities of cell supernatants containing the protein of interest are produced after which the functional integrity of genes and plasmids can be evaluated (Trill et al., 1995). Moreover, cell supernatants are produced rapidly and expression cloning occurs (Dietsch et al., 1993; Hamann et al., 1993).

Using origin defective SV40 genome, Gluzman and colleagues (1981) developed three African Green Monkey CV-1 cell lines (COS-1, -3, -7). These cell lines constitutively express SV40 large T antigen and support the replication of SV40 mutants that encode non-functional large T antigens (Gluzman, 1981). Plasmid replication in COS cells is highest at around 48 h post-transfection, when cells slowly shed a high amount of plasmid copies and show signs of cell death (Gerard and Gluzman, 1985). Recombinant protein expression in COS cells reaches its maximum after 72 h post-transfection and continues for 5–10 days (Edwards and Aruffo, 1993). This allows to use the COS system as an extended batch.

3.3.6. Insect cells by recombinant baculovirus

Geisse et al. (1996) observed that stably transfected cell lines, CHO, Sp2/o and MEL cells produced fully glycosylated leukaemia inhibitory factor (hu-LIf) at variable product titres. Moreover, transient expression in COS cells or baculovirus-mediated infection of insect cells rapidly generated the incompletely glycosylated hu-LIF (Geisse et al., 1996).

Baculoviruses are a large family of invertebrate-specific large circular, double-stranded DNA-viruses. Initial use of baculoviruses focussed on their use as safe microbiological insecticides infecting mainly the order *Lepidoptera* (Jehle et al., 2006). Afterwards, it was observed that during the late phase of infection, baculoviruses produces large amounts of protein. Smith et al. (1983) observed that the baculovirus prototype, *Autographa californica Nuclear Polyhydrosis Virus* (AcNPV), produces two gene products, the polyhedrin and p10 proteins involved in polyhedra formation (Smith et al., 1983). The formation of viral particles is not inhibited by the mutation of these genes and the replacement of these genes by heterologous DNA sequences leads to high level expression of the transgenes (Geisse et al., 1996).

In contrast, their use in expression of secreted mammalian proteins is far more limited. Compared to the mammalian cells lines, the amount of secreted proteins produced by baculovirus is lower (Jarvis, 2009). Viral production and amplification is a time-consuming process. Once established, the baculovirus have to be titred for an optimal expression and stocks have a limited shelf life (Dalton and Barton, 2014). The baculovirus expression vector system is, however, effective for production of cytosolic proteins that are unable to be synthesized in prokaryotic hosts (Unger and Peleg, 2012).

In summary, for research and biomedical purposes, baculoviruses represent a versatile eukaryotic vector (Martinez-Solis et al., 2019).

3.3.7. Yeast expression system

Yeasts are useful organisms for the production of functional recombinant proteins. The biotechnological advantage of yeast is their ability to accomplish post-translational modification, fast growth, simple genetic manipulation, scalable fermentation, high bio mass concentration, as well as pathogen-free production (Kim and Kim, 2017; Han and Yu, 2015; Nielsen, 2013). The best known and often preferred yeast expression system is *Saccharomyces cerevisiae*. This nonpathogenic yeast has been used in the production of biopharmaceuticals as well as other useful products (e.g. Hepatitis B surface antigen, insulin) (Llopis et al., 2014; Celik and Calik, 2012). Its limitations include low protein yield and plasmid instability (Xie et al., 2018). These limitations have resulted in the development of alternative expression systems including other yeasts and fungi, as well as the mammalian CHO and COS cells (Celik and Calik, 2012).

Saccharomyces cerevisiae strains for protein production include wildtype and mutants. Important strains for lab research are S288c and A634A (reference/control, cell cycle studies). The strain BJ5464 is mostly used for recombinant protein production (Munoz et al., 2005; Schacherer et al., 2007; Young et al., 2013; Baghban et al., 2019).

Another yeast *Pichia pastoris* is an excellent system for production of recombinant proteins including trypsin and human serum albumin (Irani et al., 2016). Most expression strains are derived from NRRL-Y 11430 strain (Cregg et al., 2000). Common vectors for recombinant protein expression are pPinkα-HC and pPICZα-E (Baghban et al., 2019). Expression vectors of *Pichia pastoris* include multiple cloning site (MCS), alcohol oxidase promotor (*AOX1*), secretion signal sequence (SIG), *pGKL* killer protein and alpha-MF, transcription termination site (TT), selection sequence (e.g. Ampicillin), *HIS4* (marker for selection using hydroxyhistidinase) and *ColB1* (replication element for plasmid proliferation in *E. coli*) (Baghban et al., 2019; Ahmad et al., 2014).

3.3.8. Filamentous fungi

Filamentous fungi are used for the expression of recombinant proteins (e.g. primary metabolites such as organic acids, and secondary metabolites such as penicillin), due to their excellent capacity to produce proteins on a large scale at relatively low costs as well as their eukaryotic post-translational processing machinery. The main host strains are *Aspergillus niger*, which is able to produce 25−30 g/l of homologous secreted glucoamylase, as well as *Trichoderma reesei*, which can produce up to 100 g/l of extracellular protein in a controlled bioreactor cultivation. These yields are higher than those obtained from other expression systems such as yeasts as well as CHO and COS cells (Ward, 2012).

Expression vectors of fungi contain of a gene of interest and a transcription terminator linked to a transformation selection marker (e.g. nutritional markers like *pyr4* and *trp2*) under control of a suitable promotor. The main promotors in the expression cassette of fungi are *cbh1* (promotor of the gene encoding major cellulose), *pdc* (pyruvate decarboxylase) as well as *eno* (enolase). The cultivation and growth medium depends on the fungus strain and the promotor, which needs to be induced under cultivation conditions (Nevalainen et al., 2018).

A disadvantages of fungi as expression systems is the rather low transformation frequency. Protein modifications due to protease activity and low pH as well as potential for morphological defects also have been observed (Ward, 2012). A possible solution may be the use of new transformation strategies (e.g. piezoelectric shock waves) (Magana-Ortiz et al., 2013).

Although being a new technology, CRISPR/Cas9 already is useful in a broad range of fungi with high significance for industrial processes. This technology, alongside tuneable promoters, provides a remarkable expression control of genes as well as their protein products (Nevalainen et al., 2018).

3.4. Therapy approaches for neuropsychiatric disorders

3.4.1. CRISPR/Cas9

The technology “clustered regularly interspaced palindromic repeats (CRISPR)/ CRISPR-associated protein (Cas)” enables removal, addition, and alteration of DNA sequence sections. Single-guide RNA (sgRNA) recruits the endonuclease Cas9 to a desired region on the DNA sequence, where it induces a double strand break. The repair of such a break often

results in random insertions or deletions that possibly could result in a frameshift in the open-reading-frame and eventually loss of gene function (Williams and Warman, 2017). Korge et al. (2015) designed *Fbxl3* (*F-box and leucine-rich repeat protein 3*) knockouts in human U2OS cells. The modifications triggered by CRISPR/Cas9 causes loss of gene function by introducing premature STOP-codons in the open-reading-frame. The authors reported that *Fbxl3* knockouts displayed long period lengths and low amplitude of the *Bmal1-luciferase*-reporter as well as increased Cry1 stability causing higher Cry1 protein levels than normal. *Cry1* and *Cry2* mRNA levels were decreased as a consequence of their increased repressor function (Korge et al., 2015).

Bording et al. (2019) used CRISPR/Cas9 technology to generate *Cry1* knockout cells, *Cry2* knockout cells as well as double knockouts (human U2OS cells). *Cry1* knockouts displayed a short period length and low amplitude whereas *Cry2* knockouts showed long period lengths. Double knockouts displayed arrhythmic phenotypes. Cry1 protein was detected in wildtype cells and *Cry2* knockouts, but not in *Cry1* knockouts and double knockouts. The same applies for Cry2 protein: Cry2 was not detectable in *Cry2* knockouts, but Cry2 was present in wildtype and Cry1 knockouts (Bording et al., 2019).

CRISPR/Cas9 also was used to correct $PSEN2^{N141I}$ mutation in iPSC-derived basal forebrain cholinergic neurons (BFCNs) of patients diagnosed with Alzheimer's disease. Cells with the $PSEN2^{N141I}$ mutation displayed increased Aβ42/40 levels compared to healthy controls. Ortiz-Virumbrales et al. (2017) found that increased Aβ42/40 levels were normalised after CRISPR/Cas9 correction of the $PSEN2^{N141I}$ mutation (Ortiz-Virumbrales et al., 2017).

CRISPR/Cas9 probably thus opens the avenue for upcoming therapeutic approaches in the field of neuropsychiatric disorders associated with circadian rhythm disturbances like ADHD as well as neurodegenerative disorders like Alzheimer's disease.

3.5. Monoamine function – Comparison between plants and mammals

Monoamines such as norepinephrine, dopamine or serotonin have key functions in the neurotransmission in the central nervous system of mammals. In humans, disturbances in the monoamine neurotransmission are associated with the pathophysiology of neuropsychiatric disorders such as major depression. Antidepressants for example inhibit the reuptake of norepinephrine, dopamine or serotonin, act on the antagonism of inhibitory presynaptic norepinephrine or serotonin receptors, or inhibit monoamine oxidase reversibly or irreversibly. Norepinephrine deficiency is associated with decreased alertness, low energy, and inattention as well as cognitive deficits. Dopamine dysfunction is related to motivational problems, pleasure and reward and serotonin deficiency is associated with anxiety, intrusions, obsession as well as compulsion (Moret and Briley, 2011).

Norepinephrine also is associated with attention-deficit hyperactivity disorder (ADHD). Inattentiveness, as well as disturbances in alertness and executive function are hallmarks of ADHD. Norepinephrine (NE) neurotransmission deficiency as well as polymorphisms in the *NET* (*norepinephrine transporter*) gene link NE and ADHD (Biederman and Spencer, 1999; Beane and Marrocco, 2004). NE is reported to act as a main synchronizer of the circadian rhythm. It is, for example, responsible for the nocturnal stimulation of melatonin as well as the regulation of circadian gene expression (Simonneaux and Ribelayga, 2003; Terbeck et al., 2016; Akiyama et al., 2003; Durgan et al., 2005; Terazono et al., 2003; Li and Cassone, 2015; Chalmers et al., 2008; Andrade-Silva et al., 2014).

Dopamine plays a role in the pathophysiology of schizophrenia. Patients with a diagnosis of schizophrenia showed increased presynaptic dopamine concentrations in the striatum. Antipsychotics are useful in the treatment of schizophrenia. They attenuate psychotic symptoms by, among others, normalising excessive dopamine D2 receptor signaling and restoring the balance between dopamine D1 and D2 receptor pathways. The disadvantage of antipsychotics is their failure to improve other symptoms such as cognitive dysfunction (Kesby et al., 2018; Abi-Dargham et al., 2000; Cazorla et al., 2014).

Dopaminergic neurons from the ventral tegmental area play a role in the excitation of the mesocortical pathway and thus are relevant in health and disease. Particularly the dopamine D2 receptor signaling pathway has been reported to modulate fear and anxiety (Brandao and Coimbra, 2019).

Serotonin dysfunctions have also been associated with psychopathological disorders such as anxiety, as well as schizophrenia. It is noteworthy, that several drugs which display activity on the serotonin pathway are effective therapeutic agents in different neuropsychiatric disorders. For example, Selective Serotonon Reuptake Inhibitors (SSRIs) are used in the treatment of depression, anxiety, and schizophrenia, as well as eating disorders and impulse control disorders. Unfortunately, the non-response rate to SSRIs is significant (Marazziti, 2017; Trivedi et al., 2008).

NE and dopamine are the major bioactive components in *Portulaca oleracea* – a traditional herbal medicine in China (Yue et al., 2005). In plants, monoamines play a role in growth processes. Plants are able to release organic compounds (secondary metabolites) into the environment, where they influence the growth and development of neighbouring plants. This so called allelopathy is an ability of plants to protect themselves through natural allelochemicals (Inderjit and Duke, 2003). Dopamine is an allelochemical and effects the growth and cell viability as well as enzyme activity of soybean (*Glycine max*.) roots (Guidotti et al., 2013). It also may have a role as a precursor for various alkaloids and benzylisoquinolines (Lundstrom and Agurell, 1971). Dopamine function in plants is associated with defence against herbivores, nitrogen fixation, flowering as well as prevention of IAA oxidation (auxin, indole-3-acetic acid), intercellular regulation of ion permeability and phosphorylation of chloroplasts (Guidotti et al., 2013; Allen, 2003; Van Alstyne et al., 2006; Khurana et al., 1987).

Serotonin in plants play a key role in growth regulation, flowering, xylem sap exudation, ion permeability as well as plant morphogenesis and antioxidant activity (Ramakrishna et al., 2011, 2009; Kang et al., 2007, 2009). It is found in different parts of plants, such as leaves, stems, roots, fruits and seeds, however the levels found in these tissue widely vary (Ramakrishna et al., 2011).

In summary, in mammals monoamines play a key role in neurotransmission and are associated with several neuropsychiatric disorders, whereas in plants, monoamines are related to regulation in growth and flowering processes as well as in defence reactions and antioxidant activity.

3.6. Role of environment and "Zeitgeber" in the circadian rhythmicity

One possible reason for disturbances in the circadian rhythm in the modern society is shift working, while at present there is no strong evidence that people fully adapt to shift work. Shift workers are predisposed to develop several chronic diseases. For improvement of the health of shift workers, understanding how to alter shift work and zeitgeber (time cue) schedules is necessary. The most important zeitgeber is light exposure, but other time cues like diet and food intake also play a role for the entrainment of the circadian clock (Vetter et al., 2015; van de Ven et al., 2016; Potter and Wood, 2020).

Large differences exist between individuals and their chronotypes. Chronotype appears to modify the association between shift work schedules and risk of health problems. Shift workers, whose work schedules match better to their chronotype sleep better than those without this synchronicity. For example, late chronotypes have problems working morning shifts but appreciate night shifts (Potter and Wood, 2020; Damiola et al., 2000).

Several studies suggested, that the nutrition cycle may be the primary time cue for some peripheral clocks in the circadian system. The nightshift takes place during the biological sleep time of the workers and snacking during shift works often occurs. The following digestion and

metabolism thus, mostly occur in the biological night time is a risk for health problems (Potter and Wood, 2020; Panda, 2016).

Seasonality of conception is reported to be related to psychiatric disorders as well as to the development of specific behavioural traits. Longer photoperiod at conception is associated with less depression in adulthood (Lucock et al., 2014; Chotai et al., 2003; Eisenberg et al., 2007). Furthermore, winter-born males showed more sensation seeking compared to non-winter-born males. However, interactions of the season of birth and dopamine gene *Drd4* resulted in increased venturesomeness (Eisenberg et al., 2007).

Other studies showed that also pandemics may influence the chronope, sleep quality as well as the circadian rhythmicity. Salehinejad et al. (2020) showed that the time to fall asleep as well as to get-up in the morning are significantly delayed in participants during home quarantaine. Sleep quality was reported to be significantly poorer in all participants and chronotypes (Salehinejad et al., 2020). Another study showed that the majority of participants during quarantaine and working in home-office shifted toward eveningness (66.8 %) when self-selected sleep was possible and 16.3 % participants were completely desynchronized to the end of stay-at-home. The authors suggested that human sleep habits may change according to existing living conditions (Team et al., 2020). In general, quarantaine caused by pandemic results in negative consequences for psychological health and well-being (Brooks et al., 2020; Pfefferbaum and North, 2020).

Alongside with the discovery of fire for cooking and lightning, the environmental light-dark cycle has been losing its importance in human life and underwent some evolutionary changes. Putilov et al. reported about 1665 polymorphisms in 36 chronobiology-related genes and suggested that such evolutionary changes took place as a part of general adaptation (Putilov et al., 2018).

4. Discussion/Conclusion

Cyanobacteria, proteobacteria as well as archea contain *Kai* genes, which regulate the circadian rhythm. Neither mammals nor insects or fungi have homologues of *Kai* genes. However, in *Chlamydomonas reinhardtii*, two Cry proteins homologeous to plant-like and animal-like proteins have been identified. The morning-phased genes *Circadian clock-associated 1* (*Cca1*), as well as the *Timing of cab expression 1* (*Toc1*) are conserved in different species, for example between plants and *Chlamydomonas reinhardtii*. Moreover, fungi like *Neurospora crassa* contain proteins which share homology with mammalian proteins. One example is Wc-1 (White collar 1), which is associated with the mammalian *Bmal1* (Brain and Muscle ARNT-Like 1). In addition, fungi exhibit a negative feedback loop in which positive elements activate a negative element, that in turn inhibits the expression of the positive elements of the reaction. These feedback loops, as well as several gene functions, have been conserved in both insects and mammals.

In conclusion, the molecular mechanisms and processes of the circadian clock share many interspecies similarities. One characteristic is the transcriptional/translational feedback loop, as well as the enhancer and repressor elements. There are also some important differences, such as the function of Cry proteins in flies and mammals.

Animal studies demonstrate that circadian gene deletions can lead to cognitive dysfunction and anxiety-related behaviour. Furthermore, disturbances in the human circadian rhythm are frequently associated with neuropsychiatric disease, sleep disorders, as well as cognitive impairment. One such example is the neurodevelopmental disorder ADHD, associated with sleep disturbances and cognitive deficits as well as alterations in circadian oscillation.

Current therapeutic approaches include the production of recombinant proteins through eukaryotic expression systems, as well as the CRISPR/Cas9 technology and pharmacological treatment.

For recombinant proteins the posttranslational modification is of utmost importance to avoid unwanted responses of the recipients immune system. This is best accomplished in mammalian cell lines, which can industrially utilised in modern bioreactors. The best known yeast expression system for recombinant proteins is *Saccharomyces cerevisiae,* already used in the biopharmaceutical industry. Its limitations, foremost low protein yield, have lead to the development of alternative mammalian expression systems such as CHO or COS cells.

CRISPR/Cas9 as an adaptation of the so called bacterial “immune system” has significant research and therapeutic potential. It already has been used to generate *Cry*-knockout cells as well as to correct mutations responsible for Alzheimer’s disease in iPSC-derived basal forebrain cholinergic neurones. In summary, the novel technologies discussed above, bear the possibility to change the way that physiology and genetics of both circadian rhythms and their neuropsychiatric sequelae are understood, researched, and treated in the near future.

However, environment significantly influences the circadian system. The most prominent zeitgeber (time cue) is light. But also social zeitgeber play an important role for the circadian system and as a consequence for the general well-being. Knowledge about the interaction between zeitgeber as well as environment with the circadian genes will be useful to improve, e.g. sleep, well-being as well as finding a cure for neuropsychiatric disorders.

Also a pandemic with quarantaine, home office as well as restrictions on social contacts effects the circadian rhythmicity and sleep quality as well as sleep behaviour and chronotype. Furthermore, the health of shift workers especially in pandemic times should be in focus. Many healthcare professionals for example, whose work is essential during pandemics (e.g. COVID-19), are working long shifts. To mention this in the current public discussion often overlooked important issue improvement of the health of shift workers should become a key part of current and future pandemic preparedness.

In conclusion, the circadian rhythm is adaptive and disturbances to this well balanced machinery on the biopsychosocial level in many cases influence human well being. On the one hand, the discovery of fire and artificial light made humans more independent from the environmental light-dark cycle and resulted in evolutionary changes. On the other hand, humans are still dependent on light, e.g. shift workers have a higher risk getting psychiatric disorders. Moreover, social interactions have a high influence on the circadian system and sleep behavior.

Declaration of Competing Interest

Johannes Thome has received financial support from pharmaceutical companies (Actelion, Astra Zeneca, Bristol-Myers Squibb, EVER Neuro Pharma GmbH, Janssen-Cilag, Lilly, Lundbeck, MEDICE, Merz, Novartis, Pfizer, Roche, Servier, Shire, Trommsdorff) some of which manufacture medication used in the treatment of ADHD patients.

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