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24H TIMING MOLECULES: IMPLICATIONS FOR CIRCADIAN RHYTHMS PHENOTYPES IN HUMANS

Mario Pedrazzoli, Danyella Silva Pereira

Departamento de Psicobiologia – Universidade Federal de São Paulo

Corresponding author:
Dr. Mario Pedrazzoli
Rua Napoleão de Barros, 925.
Vila Clementino – São Paulo – SP 04024-002
Brazil
Tel: +55-11 5539-0155
Fax: +55- 11 5572-5092
E – mail: pedrazzo@psicobio.epm.br

ABSTRACT

The history of the understanding of the molecular regulation of circadian rhythmicity is a history of successful and quickly scientific research. Since the first weak evidences, in 1971 by Konopka and Benzer, that the circadian rhythms could be regulated genetically, many discoveries of circadian rhythm-regulating genes have been made; we have now a much clearer picture of the mechanism by means this regulation occurs in mammals. Several genes are involved in this regulation and their interaction occurs through feed back loops. These loops include protein–protein interactions, activation and inhibition of gene transcription. Mutations in these genes lead to alterations in animals' free running period resulting in short or long free running periods. These period variations are associated with shifts in phase angle entrainment by light, a phenomenon akin to morningness–eveningness tendencies.

Molecular studies in mammals and population studies in humans have been shown that variations in one or more of these genes can modulate trends to morningness or eveningness and also generate circadian rhythms disorders. These evidences indicate that even small alterations in the clock genes may cause abnormal regulation of the circadian rhythm in animals and humans leading to different phenotypes in the morningness – eveningness spectrum. A better comprehension of this molecular regulation of the 24h-timing will be helpful to treat circadian disorders.

INTRODUCTION

The history of the molecular basis of circadian rhythmicity is a history of a quickly and successful scientific investigation. In thirty years the research in the field went from some vaguely indications of a genetic regulation of rhythmic behavior in flies to a wild view of the molecular control of these rhythms in rodents and man. The question now is: how long to its applicability in human healthy? In this mini review we will summarize the main events and rationales in this 30 years pathway.

The first report indicating genetic regulation of circadian rhythmicity appear in the early 70´s, Konopka and Benzer (1971) have described that some mutant flies present abnormal circadian rhythmicity of the locomotor activity and eclosion. These authors have found mutants with absolute no 24h rhythms or with free running periods ranging from 19h to 28h, while normal flies had an average free running period of about 24.4h. Impressive was the capacity of Seymour Benzer to preview how the genetics of flies could be applied in humans; according his own words from 1971 about flies that present short or long free running periods: " *In a normal word, these mutants would appear to wake up too early or too late. One need not look far to find human analogs of these types, and perhaps a*

better appreciation of genetic factors would lead to more sympathetic understanding of such idiosyncrasies ". As he could see a picture of the field today (Benzer, 1971).

In spite of this discovery in the early 70's, reports about genes regulating circadian rhythmicity in vertebrates or mammals did not appear until 1988, when by serendipity, Martin Ralph, during his PhD work, found a hamster in his colony that presented an abnormal circadian rhythm regulation with a shorter free-running period of locomotor activity and an abnormal entrainment to 24h light/dark cycle (Ralph and Menaker, 1988). Breeding this animal with females in the colony, he found a pattern of inheritance that suggested a mutation in a single autosomal locus that followed as a Mendelian trait in a partially dominant fashion, named the *tau* mutation. While the normal animals exhibited a free-running period of about 24h, the heterozygous presented a period of about 22h, whereas *tau* mutant homozygous animals had rhythms with periods of about 20h. At the time of this discovery molecular biology tools were not wild available to further characterize the mutation, but this study was a landmark in the molecular chronobiology of vertebrates and pointed out that the same features found in flies by Konopka and Benzer could be seem also in mammals and however pointed the way to humans.

In 1994 in a mutagenesis study specifically planed to screen circadian rhythms mutants Vitaterna and co-workers found a mouse that presented a longer free-running period of activity and an abolished persistence of rhythmicity. At that time with more molecular biology tools available, they have localized the mutated gene in the midportion of mouse chromosome 5 (in a syntenic region to human chromosome 4) and found that it segregate as a single gene in a semidominant fashion. They name the gene *Clock* (standing for **C**ircadian **L**ocomotor **O**utput **C**ycle **K**aput). Now with that deepen analysis of such a mutation that affects the circadian clock system surprisingly, it became clear that a single gene could affect a very complex physiological process that involve several variables ranging from hormonal secretion and temperature regulation to behavior.

After these first insights into the molecular regulation of circadian rhythmicity, new studies start to come out showing the role of other genes controlling the circadian clock system in mammals.

The *Bmal1* gene also known as *Mop3* was first identified as a molecular partner of the *Clock* gene, it was demonstrated that the heterodimer CLOCK-BMAL1 drive the expression of the *Per* genes (Gekakis et al., 1998). The genetic ablation of *Bmal1* gene resulted in immediate and complete loss in circadian rhythmicity in constant darkness, leading also to an impaired locomotor activity and activity levels in normal light/dark cycle (Bunger et al., 2000).

Conserved sequences of the *Drosophila* per locus were found to be expressed in mammal's suprachiasmatic nucleus in 1992 (Maler et al., 1992). The full description and characterization of the mammalian *Period* gene (*Per1*) appear in 1997 (Tei et al., 1997). In

1998 Takumi et al. isolated a second *mPer* gene (*Per2*) in mammals, with high homology to *Per1* gene and finally a third mammalian period (*Per3*) was isolated (Zylka et al.; Takumi et al., 1998). Different from *Drosophila* clock the mammalian molecular clock has three PER proteins with probably no redundant role (Dunlap et al., 1999; Vitaterna et al., 2001), *Per1* and *Per2* have different responses to light pulses in the suprachiasmatic nucleus (Zylka et al., 1998) while *Per3* has a light independent oscillation pattern of expression (Takumi et al., 1998). Knockout animals to *Per1, Per2* or *Per3* genes present a shorter free-running period, but this effect is less evident in *Per3* knockout animals that present just a 30 minutes shortened period (Bae et al., 2001; Shearman et al., 2000)

The cryptochromes (CRY1 and 2) are members of the family plant blue light receptors (Todo et al., 1996; van der Spek et al., 1996; Hsu et al., 1996; Kobayashi et al., 1998). In mammals they were first placed as possible candidates to circadian photoreceptors, possibly resembling their function in the *Drosophila* circadian system (Stanewsky et al., 1998), but further analysis demonstrated that they have a light-independent role in the circadian system (Griffin et al., 1999). A knockout mice study of *Cry1* and *Cry2* genes have revealed that they are instead essential molecular components of the master clock and are involved in the regulation of the period length and entrainment (van der Horst et al., 1999). Mice lacking *Cry1* gene present a shorter free running period of activity while mice *Cry2* knockout have a longer one, double knockout (*Cry1 and Cry2)* are completely arrhythmic in the dark (van der Horst et al., 1999).

The *Timeless* gene is the most controversial component of the molecular pacemaker system in mammals. In *Drosophila* is well established that it is the partner of the *Per* gene in the negative limb of the clock molecular loop (Sehgal et al., 1994; Rosbash et al., 1996), but in mammals, this picture is not so clear.

The *Timeless* (*Tim*) gene was first identified in mammals (mice and man) in 1998 (Koike et al., 1998; Sangoram et al., 1998; Zylka et al., 1998). In these studies the researchers have shown that the *Tim* was expressed in the suprachiasmatic (SCN) nucleus in mice, however they could not demonstrate any oscillation pattern as expected for a circadian clock gene (Takumi et al., 1999) Although not strong oscillation of TIM protein was found, its interaction with *Per* genes indicated at that time the TIM protein as an important component of the molecular circadian clock. Gotter et al. (2000) have proposed that *mTim* is essential for embryonic development, but does not have a substantiated circadian function. Although Barnes et al. (2003), using a conditional knockdown TIM protein, have replaced its role as a central component of the mammalian molecular clockwork.

Finally in 2000 the *tau* mutation locus identified first in 1988 in Syrian hamsters, was cloned in mice and showed to encode the Casein Kinase Iε (CKIε) gene, an enzyme involved in protein phosphorylation (Lowrey et al., 2000)

Almost all these genes described until now have several characteristics in common that characterize them as clock genes in mammals. First, they have a robust profile of 24h oscillation in the SCN, except *Clock.* Mutation in any of these genes, not only demonstrated for the *Tim*, causes abnormal circadian regulation with phenotypes ranging from short or long free-periods to arrytmicity and impaired entrainment by light. As if each of them take a place as a gear in the molecular clock and if one of them do not work well, the gears do not fits properly leading to an inadequate work of the clock machinery, delaying or advancing biological time.

Second, all of them coded proteins that have the PAS domain, a protein domain that allow protein-protein dimerization, demonstrated to be essential for the circadian clockwork function, the *Clock* and *Bmal1* have additionally the basic-helix-loop-helix domain that allow DNA interaction (Zheng et al., 1999; Shearman et al., 1999; Allada et al., 1998). A series of studies show that these gene products interact forming dimmers and moreover, that CLOCK and BMAl1 dimmer have the ability to bind DNA acting as a transcription factor (Lowrey et al., 2000; Shearman et al., 2000; Badiu, 2003; Gachon et al., 2004; Cermakian et al., 2003).

Based on these characteristics Lowrey (2000) have proposed a feedback loop involving these genes that would work as the molecular clock. This hypothesis proposed that a heterodimer formed by the products of CLOCK and BMAL11 proteins promotes the transcription of the *Per* and *Cry* genes while the PER-TIM, PER-PER, TIM-CRY and PER-CRY dimers block the effect of the BMAL1-CLOCK heterodimer, forming a transcription-translation feedback loop responsible for circadian rhythm control (Sangoram et al., 1998; Lowrey et al., 2000). The role of CKIε is to regulate *Per* activity by phosphorylation (Lowrey et al., 2000). However it is clear that this is a super simplified model that must have only a heuristic value.

These clock proteins, mainly the PER proteins, are quite complex. They comprise PAS domains associated with a cytoplasmatic localization domain, Casein Kinase binding sites, phosphorilation sites, and a nuclear localization domain. They have a very dynamic kinetics, including nuclear import and export that are not completely understood yet.

In the last three years some new genes involved in the regulation of circadian rhythmicity have been found. The orphan nuclear receptor *Rev-Erb*α has been described as a negative regulator of *Clock* and *Bmal1* genes (Preitner et al., 2002), and the *Dec* genes (*Dec1* and *Dec2*) are basic helix–loop–helix transcription factors that repress Clock/Bmal1-induced transactivation of the mouse Per1 promoter through direct protein–protein interactions with Bmal1 and/or competition for E-box elements (Homna et al., 2002). Also, it was reported recently the discovery of the melanopsin, a protein expressed in the eyes that is involved in photoreceptor-mediated entrainment (Panda et al., 2003), although the report shows no expression in SCN, it is likely to be involved in the light

information pathways to the molecular clock molecules in SCN. But as these genes participate in auxiliary loops of the major core loop of the clock, they will not be considered in details here.

The significance of the molecular clock to humans

An obvious consequence for those described studies in understanding the molecular regulation of the temporization system in mammals is they applicability in human regulation of the circadian rhythmicity. If mutations in the clock genes lead to abnormal regulation of circadian rhythmicity in animals would abnormal regulation of human circadian physiology (phase delays or phase advances) or at least the chronotype variation in human population be a consequence of genetic variability at these clock genes loci?

There are at least three sleep disturbances characterized by abnormalities of the circadian system well described. The Delayed Sleep Phase Syndrome (DSPS) is a disorder in which the main sleep episode is delayed, resulting in insomnia–like symptoms and difficulties to wake-up in the desired time in the morning. The Advanced Sleep Phase Syndrome is characterized by an early sleep episode, close to the beginning of the night and an arousal earlier than the desired time in the morning. The non-24h-Sleep-Wake Syndrome is a chronic pattern of daily delays of one or two hours of sleep onset and offset resulting in a non-constant schedule of sleep and wake time (American Sleep Disorders Association, 1997).

Moreover besides these pathological conditions of the circadian system, a normal condition of the human 24h pacemaker is the diurnal preference, a preferred time of the day to perform activities. Part of the population prefers to wake up early in the morning and is more active at this time of the day; they are the so-called morning type. Part is evening type, i.e., wake up later and are more active during evening; most people, however, is intermediary. Recently, these chronotypes have been suggested to have a hereditary component.

The firs report about the influence o a clock gene associated with a circadian rhythm phenotype in humans appeared in 1998, in this study Katzenberg report that a polymorphism in the 3´ flanking region of the *Clock* gene (C3111T) was associated with diurnal preference. They found that the presence of the C allele at the position 3111 is associated with increased eveningness preference. Robiliard et al., (2002) have not found this same association in a population sample in UK, and we have not confirmed this data in a sample of Brazilian population (data not published).

Archer et al. (2003) and Pereira et al. (in press) have found that a length polymorphism in the *Per3* gene is associated with morningness-eveningness tendencies, both research groups have demonstrated, in different latitudes (London and São Paulo)

that the frequency of the five-repeat length polymorphism is higher in morning group than in evening group.

Studies in families have been shown that the phase advance or delay may be an inherited character. The identification of families with circadian disturbances is an important tool to identifying genes related to circadian rhythm regulation of the sleep/wake cycle in humans.

Reid et al. (2001) reported a familial case of Advanced Sleep Phase Syndrome (ASPS) in which there was at least one member affected in each generation, following a mendelian pattern of autossomal dominant inheritance. Ancoli-Israel (2001) have reported a pedigree with Delayed Sleep Phase Syndrome. Toh et al. (2001) in a molecular approach of a familial case of ASPS have found that a mutation in the *Per2* gene was present in all affected members of the family.

Other polymorphisms in clock genes have been studied in non-familial cases of circadian disorders. Ebisawa et al. (2001) have reported that polymorphisms in the *Per3* gene could be associated with DSPS. Archer et al. reduced the region of influence of the *Per3* gene in DSPS to a length polymorphism localized in the exon 18 and found that the allele with four repeat in tandem was associated with DSPS. Pereira et al., (in press), reported also that the length polymorphism in the *Per3* gene was associated with DSPS, but curiously they have found that the opposite allele, i.e., the five-repeat allele was associated with the disease. As the two studies were done in populations with a Caucasian European genetic background living in very different latitudes, it suggests that latitude can affect the expression of clock genes.

More recently Takano et al. (2004), reported that a variation in the human *Casein Kinase I Epsilon* gene is associated with DSPS and non-24h-Sleep-Wake Syndrome.

In general animal studies have demonstrated that variations in the clock genes cause abnormalities in the regulation of circadian rhythms, mainly by changing the period length, leading to abnormal phase angle of entrainment by light. Very similar circadian phenotypes seen in animals are perfectly recognizable in humans; up to date the research in the field has been able to find gene variations that are associated with these phenotypes. Probably in next years more variations will be found to influence circadian regulation in humans and we will be able to build a kind of a map of variations in the clock genes that will help to deal with healthy problems related to abnormal regulation of circadian rhythms.

The discovery of these genetic variations regulating circadian phenotypes may have a significant therapeutic impact on preventive medicine. It may prove helpful in furthering understanding and treatment of sleep and circadian rhythm disturbances, as well as in the prevention of health hazards caused by transcontinental flights and night and shift work.

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