REVIEW ARTICLE

Phenotypic effects of genetic variability in human clock genes on circadian and sleep parameters

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Abstract

Circadian rhythms and sleep are two separate but intimately related processes. Circadian rhythms are generated through the precisely controlled, cyclic expression of a number of genes designated clock genes. Genetic variability in these genes has been associated with a number of phenotypic differences in circadian as well as sleep parameters, both in mouse models and in humans. Diurnal preferences as determined by the selfreported Horne–Östberg (HÖ) questionnaire, has been associated with polymorphisms in the human genes *CLOCK, PER1, PER2* and *PER3*. Circadian rhythm-related sleep disorders have also been associated with mutations and polymorphisms in clock genes, with the advanced type cosegrating in an autosomal dominant inheritance pattern with mutations in the genes *PER2* and *CSNK1D*, and the delayed type associating without discernible Mendelian inheritance with polymorphisms in *CLOCK* and *PER3*. Several mouse models of clock gene null alleles have been demonstrated to have affected sleep homeostasis. Recent findings have shown that the variable number tandem polymorphism in *PER3*, previously linked to diurnal preference, has profound effects on sleep homeostasis and cognitive performance following sleep loss, confirming the close association between the processes of circadian rhythms and sleep at the genetic level.

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High degree of conservation of the major clock gene families across eucoelomate phyla

Animal clock genes were initially discovered in *Drosophila* (Konopka and Benzer 1971). Systematic genetic dissection of the circadian system of the mouse revealed that the circadian oscillator in mammals is not only driven by genetic components forming an interlocked feedback loop, as in *Drosophila*, but that practically all of the components are in fact orthologous to those of the *Drosophila* clock. Few if any physiological pathways display such a remarkable degree of conservation across this vast evolutionary distance, indicating a corresponding degree of conservation in the robust properties of the circadian system, despite the many other profound differences between insects and mammals (Kafka 1912). The mechanisms for vision, oxygenation, excretion, and many other functions have all been reinvented, or at least profoundly remodelled, during the half a billion years of evolution that separate us from arthropods, but the machinery underlying the circadian pacemaker has remained remarkably constant, as proven by the discovery of orthologous clock genes in intermediate phyla (Constance *et al*. 2002). Evolution clearly recognizes a winner when it sees one.

It is also worth noting that, apart from the more obvious behaviours directly related to circadian rhythms, scientists have also made the singularly surprising discovery during recent years that the process of sleep is not restricted to vertebrates, but is observed and shows considerable similarities in invertebrates such as *Drosophila* as well (reviewed in Cirelli and Bushey 2008). It may be that these processes emerged simultaneously in a common ancestor, for as we shall see in the following, genetic variability in mammalian clock genes can have a profound effect on sleep as well.

The main difference between the complement of clock genes in insects and mammals lies not so much in the properties of the components, as in their number. In mammals, each clock gene component forms a small gene family with two or three paralogues, which have only one counterpart in the *Drosophila* genome (Tauber *et al*. 2004). This

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reflects a common phenomenon, caused by the genome duplications that are believed to have occurred early in the emergence of vertebrates from their chordate ancestors (Ohno 1970). The resulting emergence of gene families where redundance between the initial duplication products allowed for the evolution of more or less novel functions has been proposed to be a major contributing factor in the success of the vertebrates, and no doubt the duplication of the clock genes has contributed to this success. Following gene duplication, selective pressure for retaining the original function is maintained on one of the duplication products, whereas the other paralogues may either accumulate mutations that render them nonfunctional pseudogenes which eventually disappear, or they might begin to assume altered functions by virtue either of their structural features or of their expression patterns.

The finer details about what we know, how the circadian clock work is put together at the molecular level are outside the scope of this review, and have been described well and often elsewhere (most recently in Takahashi *et al*. 2008). However, the main groups of genes generally recognized as core clock genes, which will be discussed in this review, are those which encode: (i) positive transcription factors containing PAS domains for protein dimerization and basic helix-loop-helix (bHLH) domains for DNA binding, (ii) negative feedback factors with an oscillating expression pattern approximating the 24-h cycle of a night and day, and (iii) protein kinases acting on these components. Other important clock genes have been discovered after these groups, such as the nuclear receptor Rev-erb α (Preitner *et al*. 2002), which forms an additional feedback loop. They may be at least as important as the others, and very likely there are additional ones remaining to be discovered. However, because there is no literature yet available describing the impact of their genetic variation in the human species, they will not be further discussed here.

Defining circadian phenotypes in human subjects

Circadian biology deals with what at least initially seems like very tidy behavioural phenotypes. Mutations in the *Period (Per)* gene in *Drosophila* (Konopka and Benzer 1971), and its three orthologues *Per1, Per2* and *Per3* in the mouse, all cause clear and reproducible effects in terms of free-running circadian period. It is probably fair to say that circadian geneticists are in the enviable position to deal with a much more straightforward genotype–phenotype relationship than most other behavioural geneticists. After all, most of the genes which were given (and have retained) the lable 'clock genes' were either previously undiscovered or orphan genes. It was probably always naive, however, to suppose that they would be exclusively connected with the clearly defined parameters of circadian rhythmicity. Perturbations in clock genes, both in humans and other animals, have also been associated with parameters such as psychiatric conditions (Lamont *et al*. 2007), addiction (Zou *et al*. 2008), obesity and metabolic syndrome (Scott *et al*. 2008), liver disease (Sookoian *et al*. 2007), and cancer (Zhu *et al*. 2005). It is a matter open for discussion, however, whether these findings are best described as direct pleiotropic effects, or whether they are manifestations of the profound effects of circadian rhythms on multiple aspects of physiology and behaviour. This review will almost exclusively deal with the phenotypic aspects that are most directly linked to the circadian oscillator, but it will include one aspect which is almost impossible to disentangle from the circadian oscillator: sleep.

With fruit flies and mice, measuring free-running circadian rhythms under conditions of constant darkness is perfectly straightforward. With human volunteers, it is barely feasible. Accordingly, different solutions more acceptable to potential participants have been employed. One of these is to recruit from a group of individuals who live their lives in a light-proof isolation unit created by nature, being completely blind and having lost not only the rods and cones that provide conscious vision, but also the photoreceptive ganglion cells that are primarily responsible for nonvisual photic responses (Hankins *et al*. 2008). These individuals have been shown to have free-running circadian rhythms in spite of being surrounded by the nonvisual *Zeitgebers* (time-cues) provided by their social environment (Lockley *et al*. 1997). Nonetheless, it cannot entirely be ruled out that their circadian rhythms are unaffected by their illness or by these *Zeitgebers*. Additionally, without more extensive and intrusive studies it cannot be determined for certain whether a blind patient whose physiology conforms to the 24 h day has functional nonvisual photoreceptors, is able to entrain by other means, or has a free-running period length of 24 h. An alternative method for studying sighted volunteers is to apply the forced desynchrony protocol, in which the subject is exposed to an artifical day/night cycle which is either too short or too long for the circadian clock to be able to entrain, causing it to give up trying, and simply free-run according to its intrinsic period (Czeisler *et al*. 1999). This approach has obvious advantages over locking somebody up in a dark room for a few weeks, but is still too intrusive and costly to be feasible for large-scale studies. This is the reason why our own and other groups have focussed on the tool of selfreported diurnal preference scales. The scores obtained from these scales have a more or less bell-shaped distribution, as one would expect from a trait mostly dependent on minor but common polymorphisms in multiple genes, with extreme morning (larks) and evening (owls) type at either end. Unlike the free-running circadian period (Czeisler *et al*. 1999), diurnal preference shifts towards increased morningness with increasing age (Taillard *et al*. 2004) owing to an altered relationship between circadian phase and sleep-wake timing (Duffy and Czeisler 2002), a factor which can be compensated for when selecting extreme phenotypes from a wider-age range (Robilliard *et al*. 2002). The one diurnal preference scale which has been successfully

applied in genetic studies to date is the Horne–Östberg (HÖ) questionnaire, which has been used extensively for over 30 years (Horne and Östberg 1976). When first viewed by the bench-top scientist, these questionnaires may at first appear uncomfortably similar to a quiz in a glossy magazine. However, they have been demonstrated to have a remarkable degree of association with physiological parameters, including circadian period (Duffy *et al*. 2001). Another interesting, more recently developed alternative is the Munich chronotype questionnaire (MCTQ) (Roenneberg *et al*. 2003). This questionnaire has been designed to measure habitual, rather than preferred, activity rhythms. MCTQ has been linked to longitude, so that individuals living further west in a time zone are more inclined towards a later sleep phase than those living further east (Roenneberg *et al*. 2007). On the other hand, no reports have emerged to date linking MCTQ score with genotype.

Two conditions that may described as pathological extremes of morning or evening preference are referred to (following a restlessly transforming nomenclature) as advanced or delayed sleep phase syndrome (ASPS/DSPS), advanced or delayed sleep phase disorder (ASPD/DSPD), or, most recently, circadian rhythm sleep disorder, advanced/delayed sleep phase type (ASPT/DSPT). In these conditions, the patient's preferred sleep and wake timing differs from the societal norm by an advance or delay of 3 h or more. ASPT is less frequently reported, very possibly because many sufferers do not actually feel that they are suffering (Reid and Zee 2005). A number of pedigrees have been described in which ASPT is transmitted with a Mendelian inheritance pattern (Jones *et al*. 1999). The HÖ scores in these patients are consistent with an extreme morning preference, and the circadian period in a member of the most well-characterized pedigree has been calculated to 23.3 h. DSPT is a more commonly diagnosed complaint, which may reflect at least in part that it is more likely than ASPT to cause professional or social problems (Reid and Zee 2005). In one single case, the free-running circadian period has been determined in temporal isolation (although with indoor lighting), and found to be 25.38 h, nearly an hour longer than that of normal controls (Campbell and Murphy 2007). It has also, however, been postulated that the phase abnormalities in DSPT patients may be caused by altered timing of light exposure (Uchiyama *et al*. 2000). A heterogeneity within the disorder is certainly possible. It is worth noting that, unlike ASPT, no DSPT pedigrees have been described where DSPT is inherited according to a classical Mendelian pattern, in spite of some evidence for more complex modes of familial inheritance (Ancoli-Israel *et al*. 2001).

Positive transcription factor genes

A *trans*-activating factor dimer acts on the promoters of clock-controlled genes, most prominently those of the negative feedback factors described below, which themselves generate the near-24-h rhythm in circadian gene expression. The dimer consists of one paralogous member each of two groups. These members appear to be more or less interchangeable in terms of their ability to bind with each other, and to interact with their target *cis*-acting elements. One of the binding partners is either CLOCK or NPAS2, and the other one is either BMAL1 (also known as ARNTL or MOP3) or BMAL2 (alias ARNTL2 or MOP9). The most common combination is CLOCK (which also has histone acetyltransferase activity (Doi *et al*. 2006)) and BMAL1. With CLOCK and its paralogue NPAS2, there appears to be an at least partially complementary tissue distribution, with the former dominating in the forebrain (Reick *et al*. 2001). A similar arrangement may be in place with the BMAL paralogues, although surprisingly little is known about BMAL2 in spite of it being a significantly more efficient transactivating partner of CLOCK than BMAL1 (Hogenesch *et al*. 2000).

Clock was the first mammalian clock gene to be discovered in an ENU-induced mutagenesis screening programme (Vitaterna *et al*. 1994). The *Clock* mutant strain carries a dominant-negative mutation (King *et al*. 1997), which causes a lengthened (26–28 h) and ultimately unstable free-running circadian period (Vitaterna *et al*. 1994). *Clock* mutants also have alterations in sleep homeostasis, including a sleep duration shortened by 1–2 h (Naylor *et al*. 2000). However, homozygous *Clock* null allele mice have a much less dramatic phenotype than the dominant-negative mutant, with a period length i.e., only 20 min shorter than the wild-type (Debruyne *et al*. 2006).

The first clock gene polymorphism that was reported to be associated with phenotype in humans was a single-nucleotide polymorphism (SNP) in the 3′-untranslated region of *CLOCK* (T3111C), where the minor allele (C) was reported to be associate with an increased evening preference, as indicated by HÖ score, in a mostly Caucasian American populations (Katzenberg *et al*. 1998). By contrast, three other studies in British (Robilliard *et al*. 2002), Japanese (Iwase *et al*. 2002), mixed European (Johansson *et al*. 2003) and Brazilian (Pedrazzoli *et al*. 2007) populations did not find associations with diurnal preference, DSPT, or in a cohort of blind free-runners; nor did the polymorphism convey any significant effect on translatability in a reporter gene assay (Robilliard *et al*. 2002). Possible reasons for these differences include ethnic differences, or linkage to another polymorphism.

Npas2 knockout mice display a marginally shorter free-running period than the wild-type. By contrast, the null allele has a significant effect on sleep homeostasis, and eliminated the rest periods normally taken by wild-type mice during the night (their main period of activity) (Dudley *et al*. 2003; Franken *et al*. 2006). No association between variability in the human *NPAS2* gene and circadian rhythm or sleep parameters have been published, although two reports describes a link between a coding-region polymorphism, seasonal affec-

tive disorder (Johansson *et al*. 2003) and winter depression (Partonen *et al*. 2007).

Bmal1 knockout mice completely lack circadian rhythmicity (Bunger *et al*. 2000), and have a high premature mortality rate owing to a syndrome which includes reduced body weight, progressive arthropathy (Bunger *et al*. 2005), haematological abnormalities (Sun *et al*. 2006), and also infertility (Alvarez *et al*. 2008). Whether a cause or an effect of the scant attention that has been paid to the *Bmal2* gene, no report of a knockout mouse model exists, although enhanced cell proliferation was observed in an antisense knockdown cellular model (Yeh *et al*. 2003). A recent publication reported a number of novel polymorphisms in these two genes, although association with phenotype were not investigated (Ciarleglio *et al*. 2008).

Negative transcription factor genes

Two groups of clock genes are expressed with a near-24-h rhythm, which is created by the controlled translocation into the nucleus of their protein products as heterodimers or polymers, where they inhibit the effect of the positive transcription factors, described above, until they are degraded, which in turn allows the start of another 24 h expression cycle. These are the three *Period (Per)* and the two *Cryptochrome (Cry)* genes. Knockout mice lacking any one of these single components have an altered and sometimes unstable free-running circadian period (van der Horst *et al*. 1999; Shearman *et al*. 2000; Bae *et al*. 2001). Double knockout experiments show that some, albeit abnormal, circadian rhythmicity is maintained in locomotion so long as either a functional gene for *Per1* or *Per2*, or *Cry1* or *Cry2*, respectively, is present. By contrast, in *Per1/Per2* double knockout mice, the *Per3* gene alone is not sufficient to maintain circadian rhythmicity. No effects of the *Per1* and *Per2* knockouts on sleep homeostasis were found (Shiromani *et al*. 2004), in contrast to *Cry1/Cry2* double knockouts, which have a profoundly altered sleep homeostasis (Wisor *et al*. 2002).

Only one report is available describing an effect of a *PER1* polymorphism on circadian parameters in humans. A silent polymorphism in exon 18, T2434C, confers a tendency towards morning preference on carriers of the C allele (Carpen *et al*. 2006). The mechanism for this polymorphism, which may be due to linkage disequilibrium with another polymorphism, remains unknown. No other *PER1* polymorphisms of potential functional importance have been reported, which may be an indication of a degree of selection pressure, although this has yet to be investigated.

By contrast, a mutation has been described in the *PER2* gene that fulfils all the criteria one could wish for—an unambigous phenotype, a classical Mendelian inheritance pattern, and a clearly demonstrated effect on the molecular level (Toh *et al*. 2001). This missense mutation has been found in one of the familial ASPT pedigrees described above (Jones *et al*. 1999), where it results in hypophosphorylation of the encoded protein by casein kinase I, which hastens the end of the negative feedback cycle. The mutation has not, however, been found in other families.

Two polymorphisms have been described which, although they do not alter the predicted amino acid sequence of the PER2 protein, associate with diurnal preference. One, C111G, was found in the 5′-untranslated region of the predicted *PER2* transcript, only 12 bases upstream from the transcription start codon (Carpen *et al*. 2005). The minor, G allele was found to associate significantly with extreme morning preference in a British population. No significant effect of this polymorphism was found in a reporter gene system. Nonetheless, this polymorphism does have intriguing features which may explain a functional significance. Modelling of the secondary structure of the encoded transcript suggested a radical predicted difference surrounding the transcription start site between the two alleles. Moreover, the same polymorphism was reported in patients in a small Japanese ASPT pedigree (Satoh *et al*. 2003). An additional, silent polymorphism (G2114A), has recently been described, where the A allele associated with eveningness in a Japanese population (Matsuo *et al*. 2007), although no model has been presented which could account for such an effect on the molecular level.

The *Per3* gene, as described above, is the one *Per* paralogue that is not essential for maintaining circadian rhythmicity (Bae *et al*. 2001). A study in the Japanese population described a haplotype, defined by two missense SNPs, which significantly associated with DSPT (Ebisawa *et al*. 2001). One of these SNPs was reported to be associate with morning preference in a mixed European population (Johansson *et al*. 2003). Our own group studied another polymorphism described but not further studied in this paper, in which an 18-amino-acid motif is repeated either four or five times in the encoded protein. This variable number tandem repeat (VNTR) polymorphism is unique to primates, being present in different numbers in both old and new world primates (Jenkins *et al*. 2005). In a UK-based population sample, we found that the shorter allele (*PER3*^{4}) associated significantly with evening preference and DSPT, whereas the longer one (*PER3*^{5}) associated with morning preference (Archer *et al*. 2003). In a larger sample, subdivided by age group, we found that this association was strongest in individuals aged between 18 and 30 years (Jones *et al*. 2007). The same association with diurnal preference was reported independently in a Brazilian population, although the association with DSPT was the reverse (Pereira *et al*. 2005). The eveningness allele, *PER3*^{4} is the major allele in all investigaged populations with the exception of Papua New Guinea, where the *PER3*^{5} allele predominates (Nadkarni *et al*. 2005). In a prospective study, where homozygotes for each allele, matched for age, gender, and ethnicity, were studied, no significant difference in diurnal preference or sleep time was recorded. However, *PER3*^{5} homozygotes had a shorter sleep latency, and

displayed profound differences in sleep homeostasis, spending 50% more of their time asleep in deep sleep (Viola *et al*. 2007), as well as differences in waking EEG. When sleep deprived, *PER3*^{5} homozygotes exhibited much greater deficit in cognitive function in the early morning hours following a night's sleep deprivation than *PER3*^{4} homozygotes (Groeger *et al*. 2008).

There are no reports of any genetic variability in either one of the human *CRY* genes associating with circadian or sleep parameters, or indeed with any factors other than an increased cancer risk found to associate with a *CRY2* polymorphism (Chu *et al*. 2007). As with *PER1*, this may indicate the possibility of a higher selection pressure.

Protein kinase genes

Although there is no reason to assume that the functions of casein kinases Iδ and ϵ are specific to the circadian systems, mutations in both have been described in humans and in hamsters displaying very similar phenotypes. A mutation in *CSNK1D*, the gene encoding casein kinase Iδ, was found in a pedigree in which ASPT was inherited in an autosomal dominant fashion, very similarly to the mutation in the gene encoding its substrate PER2 (Xu *et al*. 2005). A very similar phenotype is found in the *tau* hamster mutation, where the mutation was located to the gene encoding the ϵ isoform of the same enzyme (Lowrey *et al*. 2000), causing a circadian period shortened by ~2 h in the heterzygote and 4 h in the homozygote (Ralph *et al*. 1990). The same phenotype was replicated in a knock-in mouse model carrying a mutation in the same amino acid, which was found to accelerate the degradation of PER, but not CRY proteins (Meng *et al*. 2008).

Concluding remarks

As would be predicted from the phenotypic effects in knockout mouse models, polymorphisms and mutations in a number of human clock genes cause effects on phenotypic parameters related to circadian rhythms. The near-bell-shaped distribution of diurnal preference is consistent with a polymorphic trait created by genetic polymorphisms that are neither rare nor severe. The well-established Horne–Östberg scale has proven very useful indeed for genetic studies as a proxy for circadian period, a much less feasible measure in the human species. The progress made so far gives reason to hope that we may, in the not too distant future, be able to define a number of morningness and eveningness alleles which together form the major determinants of diurnal preference.

The discovery of two clock gene mutations that cosegregate with the diagnosis of ASPT in an autosomal dominant fashion are suggestive of a more homogeneous and precisely defined disorder than DSPT, for which risk factors rather than causative mutations have been reported. One of these is the *PER3* VNTR polymorphism, which has also been associated with extreme diurnal preference. There is no inherent contradiction in the fact that no significant such association was found in the 24 homozygous subjects investigated in the prospective study. The association with diurnal preference in a much larger population sample is significant and reproducible, but modest. The tendency towards morning preference associated with the *PER3*^{5} allele may simply be a reflection of a higher sleep pressure in *PER3*^{5} homozygotes. Thus, as suggested by the observations made in several clock gene knockout mouse models, there is often no clear distinction between clock genes and sleep genes.

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