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Review

Genetic basis of human circadian rhythm disorders

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ABSTRACT

Circadian rhythm disorders constitute a group of phenotypes that usually present as altered sleep–wake schedules. Until a human genetics approach was applied to investigate these traits, the genetic components regulating human circadian rhythm and sleep behaviors remained mysterious. Steady advances in the last decade have dramatically improved our understanding of the genes involved in circadian rhythmicity and sleep regulation. Finding these genes presents new opportunities to use a wide range of approaches, including *in vitro* molecular studies and *in vivo* animal modeling, to elevate our understanding of how sleep and circadian rhythms are regulated and maintained. Ultimately, this knowledge will reveal how circadian and sleep disruption contribute to various ailments and shed light on how best to maintain and recover good health.

Contents

Introduction

Behavioral studies using model organisms are stimulating and relatively approachable since the researchers can, for the most part, control both external and internal variables. These studies have provided us novel insights into the genetic nature of many interesting behaviors. Predictably, gaps remain when trying to translate what scientists learn in model organisms to something applicable or useful for human health conditions. In contrast to model organisms, studying behavior in humans such as sleep requirement and preferred sleep–wake times is a daunting task due to the complexity of confounding co-morbidities, environmental factors, and the polygenic nature of behavioral phenotypes.

Like most organisms, humans exhibit daily behaviors that are regulated in a circadian (24-hour) manner. Early pioneers of circadian

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biology research, such as Jurgen Aschoff, observed that human behaviors under constant conditions exhibited rhythms with an approximately 24-hour periodicity (Aschoff et al., 1971). Aschoff's studies in humans revealed the existence of an endogenous biological time-keeping mechanism. Decades later, studies from model organisms uncovered a molecular clock comprised of many genetic players that governs the circadian oscillation of physiology and behaviors through complex methods of regulation (Dunlap et al., 2004; Hastings et al., 2008). Not surprisingly, there are shared molecular mechanisms for the molecular clock among diverse organisms, including *Neurospora*, *Drosophila*, rodents and humans. However, there are also salient differences. For instance, core clock mechanisms are presumably more widely integrated with other forms of physiological regulation in metazoans compared with unicellular organisms due to the inherent complexity of intercellular interactions. In addition, while molecular differences between vertebrates and invertebrates cannot be overlooked (Hardin, 2011; Lowrey and Takahashi, 2011), differences between closely related species, such as rodents and humans, are also expected. For instance, mice are nocturnal and with circadian period average approximately 23.5 h under constant darkness (Lowrey and Takahashi, 2011), whereas humans are diurnal with an average slightly longer than 24 h (Czeisler et al., 1999). More importantly, the highly polyphasic nature of sleep where dozens to hundreds of sleep–wake transitions occur every hour in mice directly contrasts with the single 5–9 hour block of consolidated sleep in most modern adult humans with only a few sleep to wake transitions.

Identification of the underlying genetic basis of human circadian rhythm behaviors was first advanced with the characterization of the first Mendelian circadian trait, familial advanced sleep phase (FASP), in 1999 (Jones et al., 1999). This study began with meticulous phenotypic characterization of a 69 year old woman who had life-long early sleep–wake onset, which led to the identification of a large family segregating this behavior. This story pioneered the field of human sleep genetics at the molecular level, including the search for rare Mendelian single gene/mutation forms and genome-wide association studies aimed at discovering novel variants in larger populations. Since the initial FASP findings, other human circadian/sleep phenotypes have been attributed to underlying genetic components, such as familial natural short sleep (FNSS) (He et al., 2009; Zhang et al., 2011). Therefore, studies of rare and extreme Mendelian behavioral traits have established a foundation for identifying human genetic components for circadian rhythms and sleep behaviors, which then provide further opportunities for understanding the molecular mechanisms of these behaviors. This review will outline the current understanding of the field of circadian rhythm disorders.

Human circadian rhythm sleep disorders

Human alertness demonstrates a circadian rhythmicity with a seemingly paradoxical nadir of sleepiness at the end of the day (the "Maintenance of Wakefulness Zone") (Strogatz et al., 1987), followed by a peak in difficulty sustaining wakefulness in the second third of the sleep period (approximately 3–5 A.M.) and then a gradual increase in alertness until the next evening. Pineal release of melatonin is stimulated by the suprachiasmatic nucleus of the hypothalamus (SCN) starting about 1–2 h before habitual sleep onset time and continuing through the night, unless such stimulation is masked by light of more than 50–100 lx intensity (Lewy et al., 1980).

The International Classification of Sleep Disorders lists approximately 60 disorders of human sleep (ASDA, 1997) including circadian rhythm sleep disorders (CRSD). CRSD usually present as a social problem in a person's sleep/wake timing. The most common complaints for CRSD are difficulty initiating or ending sleep at appropriate social times.

Circadian rhythm sleep disorders are classified into the following types according to the American Academy of Sleep Medicine (AASM, 2012):

1. Advanced sleep phase disorder (ASPD)

Individuals with ASPD have earlier sleep/wake times than a majority of the population, whereby the entire sleep–wake cycle of ASPD is shifted up to several hours earlier with respect to solar time (Fig. 1) (AASP, 2005). These people are considered "morning larks". People with ASPD fall asleep during the "Maintenance of Wakefulness Zone" for conventional sleepers and tend to wake up alert and energetic in the early morning hours when most people are the sleepiest. ASPD patients usually present with both difficulty in staying awake to satisfy domestic responsibilities in the evening and an obligate early morning awakening before others are active. This can result in significant sleep deprivation if social responsibilities keep the patient awake late and their biological clock wakes them up early. ASPD is more common in the elderly. It is notable that many people have extremely early sleep and wake times and in whom it is *not* considered a disorder. Such individuals may in fact feel virtuous for being the 'early bird' that gets the worm. Thus, perception of waking early may be positive or negative and is really in the eye of the beholder, underscoring the fact that these clinical classifications are truly behavioral phenotypes.

The International Classification of Sleep Disorders criteria for the clinical diagnosis of ASPD are based on the report of a patient who seeks medical attention specifically because of a complaint of a sleep time that is earlier than his/her "desired" sleep schedule (ASDA, 1997). No (solar) clock times of the habitual morning wake up are specified, and no measurements of melatonin, temperature, or other rhythms are required.

2. Delayed sleep phase disorder (DSPD)

DSPD individuals are "night owls" and routinely have later sleep/wake onset compared to the rest of the population (Fig. 1) (AASP, 2005). Their entire sleep–wake cycle is shifted later with respect to solar time, and DSPD patients feel wide awake, energetic and motivated until late in the night. Depending on the severity, sleep onset may be delayed until 01:00 to 06:00 A.M., and the circadian "morning" increase in alertness does not occur until approximately 10:00 A.M. to 2:00 P.M. DSPD individuals are often sleep deprived because sleep onset is delayed by the biological clock and morning waking time is enforced by the alarm clock and social responsibilities. DSPD is common in adolescents and young adults with an estimated prevalence of 7–16%. Again, it is important to point out that there are many night owls who do *not* consider it a disorder.

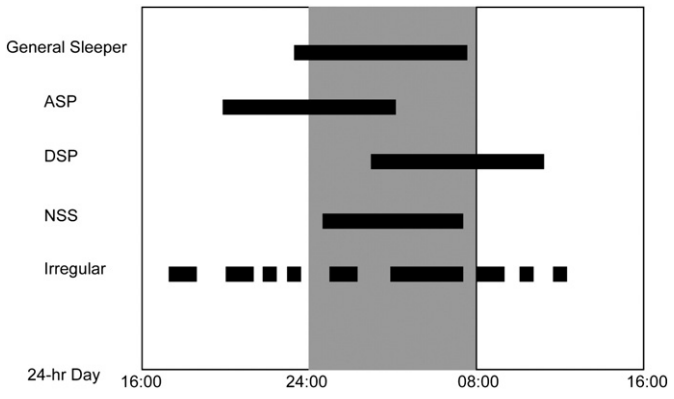

Fig. 1. Schematic sleep schedules for ASP, DSP, NSS, and ISW in comparison to general sleepers during each 24 hour period. Black-filled bars represent time spent in sleep.

3. Free-running sleep disorder (FRSD)

People with free-running disorder have a variable but predictable sleep–wake cycle that shifts approximately 1 h later every day (AASP, 2005). FRSD is usually associated with a history of either total retinal blindness or sleep phase delay.

4. Irregular sleep–wake disorder (ISWD)

These individuals have undefined sleep–wake cycles and their sleep is fragmented into a series of naps that occur throughout the 24-hour day (Fig. 1) (AASP, 2005). A mild symptom of irregular sleep–wake rhythm is sometimes seen in many neurological disorder patients (*e.g.* dementia) and in mentally retarded children. Individuals with this disorder suffer from excessive sleepiness, chronic insomnia, or both.

5. Jet lag disorder

This happens when the sleep/wake times of the new location for a traveler are misaligned with the traveler's internal body clock derived from his/her original place of abode. The severity increases with the number of time zones crossed; symptoms are temporary.

6. Shift work disorder

When an individual's work hours are during their optimal sleep time, this causes sleepiness during the work shift and difficulty remaining asleep during the new required sleep time. Depending on the timing of the shift, a person's inherent sleep preference can influence his/her ability to adjust to the shift work. For example, morning larks will be able to adjust to early shift work easier than the rest of the population.

Phenotyping CRSD

The mammalian circadian rhythm system is complicated (Saper et al., 2005) and whole-organism circadian behavior is further confounded by strong interactions between the organism and its environment, especially the perceived photic regime (Takahashi et al., 2001). That the core circadian "clock" mechanism is relatively inaccessible inside the central nervous system creates a further investigative challenge to measuring intrinsic or "biological" time relative to the timing of the perceived light–dark cycle. Under laboratory confinement, it is possible to measure the timing of objective markers of human biological circadian time (*e.g.* core body temperature or plasma melatonin levels) relative to the timing of the most recently-imposed light–dark cycle (the so-called "phase angle of circadian entrainment") in a "constant routine" protocol (Mills et al., 1978). The more prolonged "forced desynchrony" protocol has been successfully employed in many human studies to measure the intrinsic period length, or speed, of the hypothalamic circadian clock (Czeisler et al., 1986). These laboratory procedures are expensive for the investigator and time-consuming for the participant. Therefore, many human circadian studies are ambulatory and report measurements of various phase angles of entrainment for either self-reported phase markers (*e.g.* times of first falling asleep and final awakening) or objective phase markers (*e.g.* the time of saliva melatonin secretion onset) (Burgess and Fogg, 2008) or the onset time of the daily rhythm of wrist movement (Littner et al., 2003). Questionnaires such as the Morningness–Eveningness Questionnaire (Horne and Ostberg, 1976) and the Munich ChronoType Questionnaire (Roenneberg et al., 2003) also have moderately strong correlations with objective assessments (Duffy et al., 2001; Horne and Ostberg, 1976) of phase-advanced or delayed sleep–wake rhythms (*i.e.* morning or evening "chronotype") (Roenneberg, 2012). However, inferring intrinsic circadian physiology from observed rhythms in daily behavior outside the laboratory is confounded in humans by self-imposed artificial (*i.e.* non-solar) photic regimes and by the circadian influence of idiosyncratic developmental, psycho-social, physical/medical, and non-circadian sleep characteristics. Therefore, careful individual human chronotyping includes consideration of these multiple categories of information.

Familial advanced sleep phase disorder

Molecular genetic studies of CRSD began from familial advanced sleep phase disorder (FASPD, formerly familial advanced sleep phase syndrome or FASPS) since it is the first CRSD with identified mutations. FASPD is an autosomal dominant human sleep trait characterized by stable entrainment of sleep and wakefulness to early solar times. In the mid-1990s, FASPD was first genetically characterized in a Utah family, due to its very dramatic advancement of sleep phase in the proband (Jones et al., 1999). The proband's sleep phase was advanced ~4–6 h, in comparison to controls; however, FASPD subjects' sleep quality and quantity were measurably normal. We have since then identified a large number of families that exhibit the FASPD phenotype segregating as an autosomal dominant trait. Identification of these families afforded the opportunity to search for underlying genes causative for the advanced sleep phase phenotype.

PER2

The first FASPD family was large enough to allow for linkage analysis, which localized the mutant allele to the chromosome 2q telomeric region (Toh et al., 2001). Positional cloning, cDNA identification, and mutation screening of genes within the critical region identified a single base change in the human *Period 2* gene (h*PER2*). *PER2* is one of the three homologs of the *Drosophila per* gene, and the mutation substitutes a serine with a glycine residue at amino acid position 662 of PER2.

The proband was willing to participate in a time-isolation (under an environment without any time cues) experiment for three weeks and showed an ~1 h shortening in her circadian period ("tau"). Following the identification of the mutation, mouse models carrying the h*PER2S662G* variant were engineered and period length for these mice was measured under laboratory conditions (Xu et al., 2007). Under standard "free-running" constant darkness (DD), S662G mutant transgenic mice exhibited a near-2-hour shorter free-running period (tau) and a ~4–6 hour phase advancement of the behavioral phenotype, which recapitulates the behavioral phenotype seen in human subjects.

Mechanistic insights regarding the human clock regulation *via* h*PER2* were partially revealed using molecular and biochemical approaches (Xu et al., 2007). PER2 serine 662 is followed by four additional serines, each separated by two amino acids (a recognition motif for CKIδ/ε). The S662G mutation renders PER2 hypo-phosphorylated by blocking phosphorylation by CKIδ/ε (Toh et al., 2001; Xu et al., 2007). The mutation also affected transcript levels for both endogenous mouse *Per2* and the human *PER2* transgene in the transgenic mice. Both showed a reduced level with phase advancement compared to wild-type control mice, indicating that PER2S662G is a stronger transcriptional repressor than wild-type PER2. To further enhance our understanding of the role of serine 662, parallel investigations with S662D in addition to S662G were performed. Importantly, S662D transgenic mice demonstrated a longer tau and exhibited increased m*Per2* and h*PER2* transcript levels with delayed phase in relation to wild-type controls, indicating that PER2S662D is a weaker transcriptional repressor than wild-type PER2. Together, these results demonstrate the direct impact of S662 phosphorylation on *PER2* transcriptional activity, which then plays a critical role in setting the speed of the molecular clock.

PER2 S662G and S662D transgenic mice were also crossed with *CKIδ* wild-type transgenic (with 4–5 copies of *CKIδ*) and *CKIδ* heterozygous knock-out mice (one copy of *CKIδ*) to investigate the genetic

interactions between *PER2* and *CKIδ*. The copy number of wild-type *CKIδ* does not have any effect on the tau of wild type mouse behavior. However, a lower copy number of *CKIδ* led to longer tau for mutant *PER2* transgenic mice, and a higher copy number of *CKIδ* caused the tau of *PER2* mutant transgenic mice to be shortened. These results implied that phosphorylation by CKIδ on another region(s) of PER2 can modulate tau independently from the S662 region. The phosphorylation status of S662 also influenced PER2 stability; PER2S662G is less stable and PER2S662D is more stable than wild type PER2 (Vanselow et al., 2006). Hence, the stronger repressor is less stable and the weaker repressor is more stable. This finding raised the possibility that the suicide model of transcriptional factors is a likely mechanism for regulating PER2 (and possibly for other circadian repressors), and this mode of regulation can also provide a highly sensitive system to track the timing of the clock (Fu, 2008).

Casein kinase I delta (CKIδ)

In the early 2000s, with a growing number of human genome sequences in the public databases, it became reasonable to search for causative mutations using a candidate gene approach since the number of candidate genes had dramatically increased. A second causative mutation for FASPD was identified in human *CKIδ*; the mutation substitutes threonine at amino acid position 44 by alanine (T44A) (Xu et al., 2005). The conservation of threonine at amino acid 44 and the mutation-induced decrease in phosphorylation of alpha-casein and PER protein substrates by *in vitro* kinase assays strongly suggested the biological relevance of this genetic variant. A BAC transgenic mouse model carrying *CKIδT44A* was generated and the "free-running" period (tau) was shorter than control mice by ~20 min. The mutant transgene on a *CKIδ* knock-out background demonstrated a much shorter tau (22.7 h) in addition to rescuing the lethal phenotype of the *CKIδ* knock-out. Intriguingly, expressing the human *CKIδT44A* mutation in *Drosophila* generated flies with a significantly longer period than control flies (Xu et al., 2005), which highlighted the underlying differences between mammals and invertebrates.

CKIδ is a critical core component of the molecular clock. To further explore the clock composition through CKIδ, proteomic studies were conducted using CKIδ and CKIε. These studies were carried out at different circadian time points to reveal the dynamics of CKI proteomes, and distinctive potential interacting partners and/or substrates were found for these enzymes at different circadian times. Importantly, different pools of proteins were identified for CKIδ and CKIε, though with some overlap, supporting the partially redundant roles for these enzymes. *Prohibitin2* (*PHB2*) was chosen after the initial *in vitro* screening process for further characterization. Interestingly, PHB2 protein levels were regulated by CKIε, yet its RNA levels were modulated by CKIδ. Characterization revealed that *PHB2* is a novel clock component and acts as a transcriptional repressor for clock controlled genes in a CLOCK/BMAL1 independent manner (Kategaya et al., 2012).

Familial natural short sleepers

While screening genes for the "extreme early bird" phenotype, a mutation was identified in human *DEC2* (h*DEC2*). Variant carriers in this small family were further evaluated and found to possess a natural short sleeper (NSS) phenotype with a lifelong daily sleep time requirement of approximately ~6 h (He et al., 2009). Similar to the FASP human subjects, the FNSS individuals woke up at an extremely early hour in the morning; however, their sleep onset time was comparable to conventional sleepers, thus shortening their total sleep duration by ~2 h each day compared to conventional sleepers (Fig. 1).

DEC2

Using a candidate gene sequencing approach, a point mutation that changed the amino acid at position 384 from proline to arginine in hDEC2 was found to co-segregate with the FNSS phenotype. *In vitro* luciferase reporter experiments showed that the mutation mitigated transcriptional inhibitory function of hDEC2. Transgenic mice carrying this P384R mutation displayed a similarly shorter total sleep time, including an activity period (alpha) ~1.2-h longer than wild-type mice. When the mutant transgene was bred onto a mouse *Dec2* knock-out background, the mice exhibited an even more dramatic activity phenotype with alpha lengthened by ~2.5 h.

Electroencephalography (EEG) and electromyography (EMG) were also performed on *DEC2* P384R mutant transgenic mice and their littermate controls to further characterize the sleep phenotype. Mutant transgenic mice were awake for significantly longer periods of time during the light phase (when mice are supposed to be sleeping). Consistently, they showed a significant reduction of both non-rapid eye movement (NREM) and REM sleep in the light phase (He et al., 2009). NREM sleep in the light phase was more fragmented in the mutant transgenic mice than control mice. In addition, the EEG study also demonstrated that mutant transgenic mice accumulated less recovery sleep after sleep deprivation.

The function of *DEC2* in sleep regulation was also tested in *Drosophila* to see whether it gives a conserved phenomenon across species. This was carried out by generating transgenic flies with either wild-type or P384R m*Dec2* expressed in either whole brain or the mushroom bodies. In agreement with the findings from mammalian systems, flies with mutant m*Dec2* expressed in their mushroom bodies demonstrated lengthened activity duration (*i.e.* a shortened sleep length) compared to equivalent control flies. All this evidence supported a role for h*DEC2* in regulating sleep homeostasis.

Other clock genes

Interestingly, some mouse models of clock genes have been shown to have effects on sleep architecture. In mice, the *Clock* mutation was shown to alter sleep homeostasis (Naylor et al., 2000) and mice that are deficient for both *Cry1* and *Cry2* sleep longer than wild-type mice by almost 100 min (Wisor et al., 2002). Similarly, *Bmal1* (Laposky et al., 2005) and *Npas2* (Franken et al., 2006) knock-out mice were reported to have altered sleep time and architecture. These findings raise the possibility that the molecular circuitry used to set internal time-of-day might also be utilized, at least in part, to track and anticipate sleep need (Franken and Dijk, 2009).

Genetic association studies

In addition to searching for genes with Mendelian forms of CRSD, the rapid advance in human genome sequencing and SNP genotyping during the last two decades allowed for association studies of human CRSD (or morningness/eveningness preference) to become possible.

Sleep phase

CLOCK

Association between a polymorphism in the 3′-untranslated region of *CLOCK* (3111C to T) and evening preference was first reported in 1998 using human volunteers (Katzenberg et al., 1998). Individuals with C/C or C/T genotypes showed an increased evening preference compared with individuals with the T/T genotype. This *CLOCK* 3111C/T polymorphism was later found not to be associated with eveningness by Robilliard et al. (2002). In addition, Robilliard et al. reported that *CLOCK* 3111C/T is neither associated with tau length, nor with delayed sleep phase. Their molecular study further demonstrated that this polymorphism does not affect *CLOCK* mRNA

translation, hence weakening the value of *CLOCK* 3111C/T as a marker for diurnal preference, tau, or delayed sleep phase disorder in humans. However, the *Clock* mutation mouse model was shown to demonstrate phase delays of the rhythm for body temperature, activity and wake duration in one study (Sei et al., 2001) and delayed locomotor activity under constant light and during lactation in a separate study (Wakatsuki et al., 2007). These results do support the possibility that *CLOCK* mutations/polymorphisms may be associated with evening preference.

PER3

A length polymorphism in *PER3* was reported by Archer et al. (2003) to be linked to delayed sleep phase disorder and extreme diurnal preference. This length polymorphism is due to a variable-number tandem repeat (VNTR, 4 or 5 repeats) in *PER3*. The shorter 4 allele was found to have a strong association with delayed sleep phase disorder. They followed up this study and reported that polymorphisms in the *PER3* promoter are also associated with diurnal preference and delayed sleep phase disorder (Archer et al., 2010). However, a recent Norwegian study with 432 subjects was not able to reproduce these findings (Osland et al., 2011).

Sleep duration

Several genetic association studies for sleep duration have been conducted. The first report was for more than 700 participants from the Framingham Heart Study Offspring Cohort using the Affymetrix 100K SNP GeneChip. This family-based study was able to confirm significant heritability of sleepiness, usual bedtime, and usual sleep duration (Gottlieb et al., 2007). Allebrandt et al. applied a more focused approach using high-density markers from 19 clock genes to perform a two-stage design association study on two European populations, South Tyrol and Estonia. In this study, variants in the *CLOCK* gene were found to have an association with sleep duration for these two independent populations (Allebrandt et al., 2010). Another genome-wide association study was carried out for seven European populations, totaling 4251 subjects. The meta-analysis for this study revealed a genome-wide significant signal in the *ABCC9* locus that encodes a pore-forming subunit of an ATP-sensitive potassium channel (K_{ATP}) involved in energy metabolism and in the etiology of cardiomyopathies (Allebrandt et al., 2011). Interestingly, the authors also reported that knocking down *Drosophila ABCC9* caused shortened nighttime but not daytime duration of sleep-like behavior in the fly.

Concluding remarks

Identification of mutations that are responsible for various human diseases has revolutionized the field of biomedical research during the last twenty years. Many of these are rare diseases where large families are used to map and clone the causative genes/mutations. Knowledge of genes and pathways contributing to the pathophysiology of rare diseases often opens new areas for studying more common and non-Mendelian forms of the disease. Genes causing rare familial Alzheimer's and Parkinson's disease encode proteins that are central to the pathophysiology of the common sporadic forms of these diseases (Bertram et al., 2010; Hardy, 2010). A logical extension of this kind of work in disease genetics is to apply this approach to even more complex issues such as human behavioral phenotypes which are subject to large influences of environmental and social factors. Using human genetics to identify specific genes/proteins that are critical for specific behavioral phenotypes (especially those that are associated with human health) is a window into novel biological pathways relevant to regulation of human behaviors.

As with genetic studies in all organisms, the ultimate proof of functional mutations *vs.* polymorphisms lies in establishing causality of the phenotype by the mutation. Many Mendelian human genetic disease mutations were shown to be causative with multiple unrelated families co-segregating the DNA variant and disease phenotype. However, with human behavioral phenotypes, it is inherently difficult to obtain multiple families that have the same mutations. One way to circumvent this is by engineering the DNA variant into model organisms and to observe whether it produces a similar phenotype in the model organism. It is also difficult to demonstrate the causality of a mutation for a behavioral phenotype if the mutation is found in multiple random subjects with no family relation and does not produce a similar phenotype in model organisms. Conversely, cloning a variant found in only a few individuals but that replicates the phenotype in every single animal of the model organism (mouse, fly, etc.) proves causality with no reservations.

Although genetic association studies have provided new insights into human phenotypes, the inconsistency of the results remains a major difficulty. This complication may stem from the obstacle in finding large enough cohorts of subjects with proper phenotyping data which renders the replication for association study arduous. Also, the effect sizes are often quite small which calls to question the cost of such studies *vs.* the information learned. Rare heritable CRSD families have so far proven to be useful and offer new genetic and mechanistic information. However, one persistent challenge in studying these families is the amount of effort and difficulty in finding large families to define the potential mutation region with relative ease. This was one of the major factors limiting researchers to use the candidate gene approach in the last decade. With the advancement of sequencing technology and the decreasing cost, whole exome sequencing and whole genome sequencing are becoming more realistic approaches today. However, new challenges present themselves with these new technologies such as the enormous effort required to sort through the large number of SNP variants in order to identify true functional mutations. Regardless, these new methods do offer hope of finding novel human circadian/sleep genes that were previously impossible.

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