Genetics of Sleep

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Section 3

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Introduction

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Chapter 11

The inclusion for the first time of a specific Section on genetics in *Principles and Practice of Sleep Medicine* is a heralding event as it signifies that the study of the genetic control of the sleep–wake cycle is becoming an important approach for understanding not only the regulatory mechanisms underlying the regulation of sleep and wake but also for elucidating the function of sleep. Indeed, as genetic approaches are being used for the study of sleep in diverse species from flies to mice to humans (see Chapters 13 to 16), the evolutionary significance for the many functions of sleep that have evolved over time are becoming a tractable subject for research, as many researchers are bringing the tools of genetics and genomics to the sleep field. The complexity of the sleep–wake phenotypes and the difficulty in collecting phenotypic data on a large enough number of animals and humans to begin to unravel genetic mechanisms has in part been responsible for why few comprehensive attempts have been made to identify "sleep genes" beyond the circadian clock genes regulating the timing of sleep (see Chapter 12).

As noted by Landolt and Dijk (Chapter 15), sleep is a rich phenotype that can be broken down into a wide variety of sleep–wake traits based on the electroencephalogram (EEG) and the electromyogram (EMG).[1] Furthermore, the genetic landscape for regulating multiple sleep–wake traits is clearly going to involve hundreds of genes and integrated molecular neurobiological networks.[2] The fact that the environment also can have major effects on sleep–wake traits, particularly sleep duration in humans, also makes it difficult to uncover the underlying genetic control mechanisms. Indeed, while a large number of genome-wide association studies in humans have identified multiple genetic loci and genes involved in regulating a wide variety of physiologic systems and disease states, only a single relatively small and inconclusive genome-wide association study has been undertaken for sleep–wake traits in humans.[3]

A great deal of progress has been made in elucidating a number of circadian clock genes that regulate the diurnal timing of the sleep–wake cycle as well as most, if not all, of 24-hour behavioral, physiologic, and cellular rhythms of the body. The simplicity and ease of monitoring a representative "output rhythm" of the central circadian clock from literally thousands of rodents in a single laboratory, such as the precise rhythm of wheel running in rodents, was a major factor in uncovering the molecular transcriptional and translational feedback loops that give rise to 24-hour output signals.[4,5] There is now substantial evidence demonstrating that deletion or mutations in many canonical circadian clock genes can lead to fundamental changes in other sleep–wake traits including the amount of sleep and the response to sleep deprivation.[6] Indeed, as discussed in the Chapter 14, one can argue that many circadian clock genes are also "sleep genes."

Another reason for the successful identification since the 1990s of the core clock genes in mammals is the remarkable conservation of the major clock genes from flies to mice to humans. Thus, core clock genes identified in flies, which involved mutagenesis and the screening of thousands of flies for mutant phenotypes, eventually led to finding the same genes in mice and humans. The relatively recent search for sleep genes in flies has been an active area of study since only about the turn of the century, and this approach is expected to uncover new sleep-related genes that will have mammalian orthologues. Indeed, the recent finding that different alleles of a core circadian gene, *per*, first indentified in flies, can affect the homeostatic response to sleep deprivation and the amount of slow-wave sleep (Chapter 15) argues that uncovering sleep genes in flies will directly lead to the genes underlying the changes in sleep–wake traits in mammals.

Early studies by Valtex and colleagues on inbred strains of mice[7] and early human twin studies[8] provided considerable evidence for a strong genetic basis for some sleep–wake traits, but little has been done to unravel the complex network of genetic interactions that must underlie this universal behavior in mammals. Tafti, Franken, and colleagues pioneered the use of quantitative trait loci in

recombinant inbred mouse strains, which has led to the identification of a small number of genes that are associated with specific sleep–wake properties.[9] More recently, the first attempt to record sleep in a large genetically segregating population of mice revealed considerable complexity to the genetic landscape for multiple sleep–wake traits.[2] Using 2310 informative single-nucleotide polymorphisms, and assessing 20 sleep–wake traits in 269 mice from a genetically segregating population, led to the identification of 52 significant quantitative trait loci representing a minimum of 20 distinct genomic regions.[2] Because this study involved only two inbred mouse strains contributing to the genetic diversity, it can be expected that many other loci will be identified once sleep is recorded in the offspring of different crosses of inbred and outbred mouse strains. Uncovering these loci, and understanding how interactions between genes and environment contribute to different sleep–wake states, is expected to not only reveal the molecular events underlying the sleep–wake cycle but also to yield new targets for drug discovery. New therapies based on the genetic control of sleep may be particularly important for treating genetic-based disorders of sleep (Chapter 16).

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Circadian Clock Genes

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Chapter 12

Abstract

Circadian (near 24-hour) rhythms can be produced by individual mammalian cells in a self-sustaining manner. These rhythms result from coordinated daily oscillations in the transcription and translation of several clock-component genes. In mammals, central to the generation of these cycles are the levels of the proteins PER and CRY, which feed back to inhibit transcription of their own genes. This inhibition is exerted on the enhancement of transcription that results from binding of the CLOCK and BMAL1 proteins to E-box elements of the promoter regions of the Per and Cry genes. These four genes are thought to form the core feedback loop of the time-keeping mechanism.

Additional interactions between the protein products of these genes, as well as other proteins, appear to add to the complexity of the circadian system. The phosphorylation of PER by casein kinase α (CKIα) can lead to its degradation, and the association with BMAL1 appears needed for CLOCK to be present in the nucleus. Rhythmic transcription of Bmal1 appears to result from regulation via the protein REV-ERBα, its transcription regulated by CLOCK-BMAL1 binding to E-box elements. Furthermore, Bmal1 and other clock genes are involved in the transcriptional regulation of important metabolic genes, such as Rev-erbα, providing direct molecular links between the circadian clock and metabolic regulatory pathways.

Finally, it appears that rhythms in histone acetylation contribute to the circadian expression pattern of some core circadian genes, raising the intriguing possibility that circadian rhythms at other functional levels may contribute to the genetic clockwork. Additional genes have been identified based on altered circadian rhythms in mutants, although the precise roles of these genes in the circadian system remain to be determined.

Since the 1980s, remarkable progress has been made in elucidating the molecular substrates that underlie the generation of 24-hour rhythms in mammals. A major finding that has arisen since the turn of the 21st century is that most cells and tissues of the body contain and express the core 24-hour molecular clock mechanism. Although the circadian rhythm is normally coordinated, individual tissues and cells are capable of producing sustained rhythms in isolation. These rhythms are the result of oscillations of expression of a core set of interrelated circadian genes. This chapter describes the genes expressed in cells of the hypothalamic suprachiasmatic nucleus and other oscillators and our understanding of the roles they play in this daily rhythmicity. In addition, in view of the homology of mammalian clock genes with those in the fly, where appropriate, a discussion of the discovery of fly and mammalian genes is provided.

THE MAMMALIAN CELLULAR CIRCADIAN CLOCK

Several lines of evidence pointed to the suprachiasmatic nucleus (SCN) as the site of the master circadian pacemaker beginning in the 1970s. Destruction of the SCN abolishes circadian oscillations in the plasma concentration of cortisol[1] and in locomotion and drinking.[2] These oscillations are independent of inputs from the eye,[1] although an autonomous circadian clock has been demonstrated to exist within the eye that controls, among other functions, the shedding of rod outer segment disks.[3] Normal circadian rhythms can be restored to an SCN-lesioned animal by transplantation of fetal SCN tissue but not by transplantation of fetal tissue from other regions of the brain.[4] Transplant into an SCN-lesioned animal of fetal tissue from the SCN of a circadian mutant animal confers the short period of the donor,[5] indicating that the properties of the rhythm are determined by the SCN rather than other tissues or brain regions. Thus, several lines of evidence point to the SCN as the site driving or controlling circadian behavior for mammals.

Recent studies of rhythms in gene expression have indicated that persistent rhythms can be observed in tissues throughout the organism, even in tissue explants kept in culture for extended periods of time.[6,7] The phase of these peripheral tissue rhythms differs from that of the SCN, but nonetheless it appears to be coordinated by the SCN. In SCN-lesioned animals, these peripheral rhythms persist but no longer exhibit the consistent phase seen in unlesioned animals.[7] Some environmental manipulations, such as temperature cycles or restricted feeding, can alter the phase of peripheral rhythms.[8,9] In addition, studies confirm the presence of oscillations in gene expression throughout the body, with different phases in different tissues.[10-13] Loss of many of these rhythms is reported with SCN lesions. However, these studies cannot discriminate between a loss of rhythmicity by individual animals and a loss of synchronicity among the individuals. Thus the roles of the SCN and of the peripheral oscillators in the mammalian circadian system continue to be defined.

CIRCADIAN CLOCK PROPERTIES AND CLOCK GENES

Half a century ago, the formal properties of the circadian clock function had been well defined. Included among the 16 "empirical generalizations about circadian rhythms" defined by Colin Pittendrigh[14] were that circadian rhythms are ubiquitous in living systems; they are endogenous; they are innate, they are not learned from or impressed by the environment; they occur autonomously at both cell and whole-organism levels of organization; the free-running period of circadian rhythms are so slightly temperature-dependent that it is proper to emphasize their near independence of temperature; and they are surprisingly

intractable to chemical perturbation. These six observations already suggested what we now know to be the case: A cell-autonomous program of gene expression makes up the mammalian circadian clock that produces these rhythms.

How do individual cells generate rhythmic activity with a period of about 1 day? Many pacemaker neurons generate oscillatory activity, such as rhythmic patterns of action potentials, and these relatively rapid oscillations can be explained by the concerted action of a small number of ion channels. However, the much slower oscillations of the individual SCN neurons are not likely to involve the same mechanisms. Pittendrigh's observation that circadian rhythms are not sped up or slowed down by changes in temperature and that they are relatively impervious to chemical perturbations would similarly argue against such a neuronal mechanism underlying the generation of 24-hour oscillations. In fact, the finding that nonneuronal tissues (Pittendrigh's cell–autonomy) can produce sustained circadian rhythms, as well as the prevalence of circadian rhythms in plants and unicellular organisms (Pittendrigh's ubiquity), argue against a neural process underlying generation of circadian rhythm.

Indeed, it appears that the synthesis of proteins by each oscillatory cell is central to the mechanism for the generation of 24-hour rhythms. The initial evidence for this is that application of protein synthesis inhibitors in the region of the SCN shifts the circadian phase of activity of animals by an amount and in a direction that depends upon the time when the inhibition is imposed.[15,16] A similar shift in the phase of vasopressin release from explanted SCN also results from inhibition of protein synthesis.[17] Thus, gene expression is central to the generation of circadian oscillations.

Gene expression profile studies, in which expression levels are sampled at regular time points in constant darkness (free-running conditions) focusing on the SCN and on peripheral tissues reveal that approximately one third of the transcriptome is rhythmically expressed, even in peripheral tissues.[10-13] Reporter gene constructs combining genes known to be rhythmically expressed with luciferase (thus allowing visualization of expression in culture via the luciferase's glow) demonstrated in rats and in mice that peripheral tissues are capable of self-sustained rhythms.[6,7] With so many genes exhibiting circadian expression, and competent oscillators present in such a variety of tissues, one cannot assume that either rhythmic expression or expression in the SCN are valid criteria for a clock gene. Identification of which genes are central to the generation and maintenance of circadian cycles thus represents a challenge.

A potential solution to the challenge of identification of clock genes (as distinct from clock-controlled genes) is to refocus attention on the formal properties of the circadian system. Arnold Eskin and others promoted this conceptual framework in the late 1970s by characterizing rhythm properties as arising from the input pathway, the clock itself, or the output pathway (Fig. 12-1).[18] As articulated for pharmacologic approaches (but equally valid for genetic ones), manipulations that produce phase-dependent shifts in the rhythm (a phase-response curve), or changes in the phase-response curve or the free-running period are likely to be affecting the clock, or at least not affecting an output process.[19] Applying this logic, a genetic perturbation that alters the free-running period in constant darkness, or the phase-response curve to light pulses, or the persistence of rhythmicity in constant conditions is likely to be a perturbation in a clock gene, although no one alteration alone is necessarily a clock change (rather than input or output).

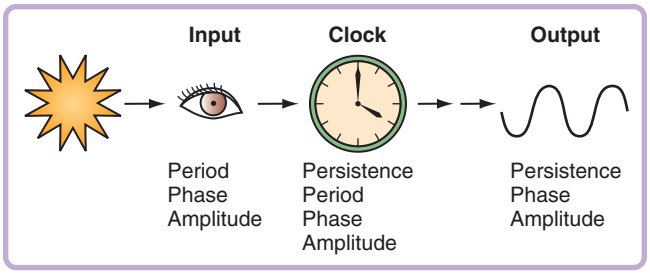

Figure 12-1 Classic view of circadian system and circadian clock properties. At minimum, the circadian clock system would have an input pathway by which entraining signals are received (light is illustrated), a clock mechanism, and output pathways. No single property of observed circadian rhythms is necessarily determined by the clock mechanism; these properties may be affected by changes in the input or output. However, when a mutation is observed to affect multiple properties of circadian rhythms, then that genetic change may most parsimoniously be attributed to a change in the clock mechanism itself.

Zatz and others[20,21] proposed more restrictive criteria: Null mutations should abolish rhythmicity, the gene's protein product level or activity should oscillate and be reset by light pulses, changes in amount or activity should result in phase shifts, and prevention of oscillation of protein levels or activity should result in loss of rhythmicity.[21] In the parlance of the field, these criteria would define a state variable—a rate-limiting element that itself defines the phase of the core oscillation. A self-sustaining clock would require at least two state variables,[22] although more are clearly possible. To date, no single gene in the mammalian system has satisfied all the criteria for a state variable. Indeed a hallmark of the mammalian circadian clock seems to be the multiple homologues of many genes that appear to play related but nonredundant roles (Fig. 12-2). It may thus be that "state variable" status is actually shared by related groups of genes in the mammalian system.

POSITIVE ELEMENTS

Clock

In the early 1990s, no genes in mammals had been identified as even possible candidate circadian clock genes, leading us to undertake a mutagenesis and screening in mice in an effort to identify mammalian circadian clock genes. For this, we used the C57BL/6J mouse strain, in which wild-type mice show robust entrainment to a light–dark cycle and have a circadian period between 23.6 and 23.8 hours under free-running conditions in constant darkness (DD). In a screen for mutations of more than 300 progeny of mutagen-treated mice, we found one animal that had a free-running period of about 24.8 hours, more than six standard deviations longer than the mean.[23] In the homozygous condition this mutation results in a dramatic lengthening of the period to about 28 hours, which is

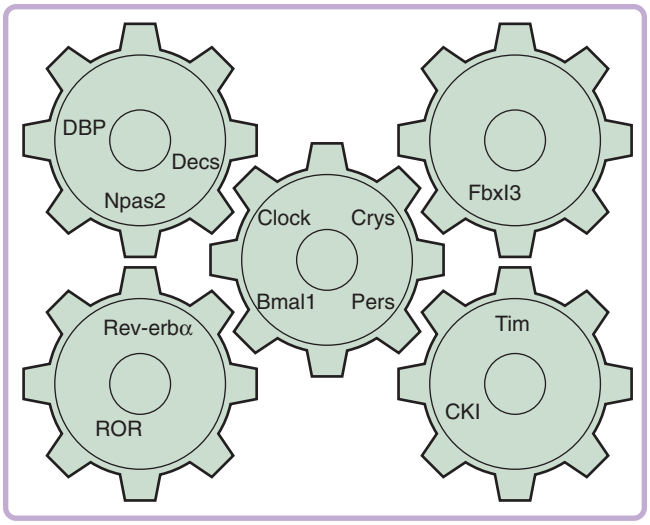

Figure 12-2 Multiple gears are required to build a genetic clock. The central gear, composed of the positive elements *Clock* and *Bmal1* and the negative elements *period* and *Cryptochrome,* constitutes a clear feedback oscillator, but several genetic elements have been identified that interact with each of these and are critical for determining the functional properties of the circadian clock.

usually followed by the eventual loss of circadian rhythmicity (i.e., arrhythmicity) after about 1 to 3 weeks in DD. The affected gene was mapped to mouse chromosome 5 and named *Clock*.[23,24]

We cloned the *Clock* gene by a combination of genetic rescue and positional cloning techniques. *Clock/Clock* mutant mice were phenotypically rescued by a bacterial artificial chromosome (BAC) transgene that contained the *Clock* gene, allowing functional identification of the gene.[25] The *Clock* gene encodes a transcriptional regulatory protein having a basic helix-loop-helix DNA-binding domain, a PAS dimerization domain, and a Q-rich transactivation domain. The mutant form of the CLOCK protein (CLOCK Δ19) lacks a portion of the activation domain found in wild-type protein, and thus, although it is capable of protein dimerization, transcriptional activation is diminished or lost. The PAS domain is so named because of the genes originally identified with this protein dimerization domain, ***p****er*, ***A****RNT,* and ***s****im. Clock* mRNA is expressed in the SCN as well as other tissues, but it has not been found to oscillate in a circadian fashion.[26]

Bmal1

The presence of the PAS dimerization domain in CLOCK protein suggested that it might form a heterodimer similar to that of PER and the protein product of another *Drosophila* clock gene, TIM.[27] A screen for potential partners for the CLOCK protein using the yeast two-hybrid system revealed that a protein of unknown function, BMAL1 (Brain and Muscle ARNT-Like 1), was able to dimerize with the CLOCK protein.[28] Creation of mice harboring a null allele of *Bmal1* (also referred to as *MOP3*) demonstrated the critical role of this gene in generating circadian rhythm. These mutant mice, which display light–dark responsive differences in activity level, become arrhythmic immediately upon release in constant darkness.

Additional actions of the CLOCK:BMAL1 heterodimer have become clear. Although *Clock* mRNA does not oscillate, its protein's nuclear versus cytoplasmic localization does.[29] By studying the intracellular localization of CLOCK and BMAL1 in fibroblasts of mouse embryos with mutations in different clock genes, and ectopically expressing the proteins, it was found that nuclear accumulation of CLOCK depended on formation of the CLOCK:BMAL1 dimer, as was phosphorylation of the complex and its degradation.[29] Other PAS domain–containing proteins failed to affect the localization of CLOCK, indicating that these posttranslational events are specific to the CLOCK:BMAL1 dimer.

NEGATIVE ELEMENTS

Period Genes

As described in Chapter 13, the first identified gene (defined as a mendelian gene as opposed to a sequenced, cloned gene) that encodes a clock component, *period*, denoted with the symbol *per*, was discovered in 1971 in *Drosophila* using a forward genetic approach consisting of chemically inducing random mutations in the genome and then detecting the mutations that affect circadian rhythms by screening the progeny of the mutagenized individuals for altered rhythmicity.[30] This approach has the advantage that no assumptions are made about the nature of the genes or gene products involved, but it is based on the presumption that there exist genes that, when mutated, will alter rhythms in a detectable manner. At the time, this presumption of the existence of genes that regulate a complex behavior was considered radical, but it has proved to have been a field-defining moment.

Initially, three alleles of the *per* gene were identified by the process of mutagenesis and screening. Flies carrying these alleles had either no apparent rhythm in eclosion (emergence from the pupal case) or locomotion, or they had either long (e.g., 29 hours) or short (e.g., 19 hours) periods for the rhythms of eclosion and locomotor activity.[30] The finding of three alleles with three different phenotypes made it possible to have confidence in the conclusion that the *per* gene encodes a protein that is a clock component. Had only an arrhythmic mutant been found, then the alternative explanation could be proposed that the lack of circadian behavior was secondary to another primary defect that did not lie in a clock component. The approach of mutagenesis and screening has also been successful in identifying circadian clock genes in other organisms such as *Neurospora crassa*,[31] plants[31] and cyanobacteria.[33]

Confirmation of the importance of the *per* gene as a central circadian clock component was the rescue of the mutant phenotype after introduction of the wild-type allele of the *per* gene into mutant flies.[34,35] The level of the mRNA transcript encoded by the *per* gene was shown to oscillate in a circadian fashion[36] as a result of transcriptional regulation,[37] and the levels of the PER protein were shown to lag the *per* mRNA levels.[38] In fact, shifts in the circadian phase can be evoked by the induction of PER protein under the control of a noncircadian promoter.[39] Thus, many lines of evidence indicate that the *per* gene encodes a protein that is a clock component.

Three orthologues of the *per* gene—*mPer1*, *mPer2*, and *mPer3*—have now been identified in the mouse, and the levels of their mRNA have also been shown to oscillate with a circadian period.[40-44]

Following the identification of CLOCK:BMAL1 dimerization, the ability of this heterodimer to regulate transcription was tested using a reporter construct based on the upstream regulatory elements of the *per* gene. The *per* gene of *Drosophila* contains an upstream regulatory element, the clock control region, within which is contained a sequence needed for positive regulation of transcription, the E-box element (CACGTG).[45] CLOCK-BMAL1 heterodimers were found to activate transcription of the *mPer* gene in a process that requires binding to the E-box element.[28] However, CLOCK Δ19 mutant protein was not able to activate transcription, consistent with the finding that exon 19, which is skipped in *Clock* mutant animals,[26] is necessary for transactivation. Thus, CLOCK protein interacts with the regulatory regions of the *per* gene to allow transcription of the *per* mRNA and eventual translation of PER protein. A similar activation of transcription of the *tim* gene by the CLOCK-BMAL1 heterodimer also occurs in flies.[46] However, this positive regulation alone does not produce an oscillation in *per* mRNA levels, which is known to be responsible for the oscillation in PER protein levels.[37]

Findings that the *Clock* mutation dramatically decreases *per* genes' expression also confirms the positive regulation of CLOCK:BMAL1 on *per* transcription in situ.[47,48] Mice with null mutations of *mPer1*, *mPer2*, or *mPer3* alone display altered circadian periods,[49,50] and mice with both *mPer1* and *mPer2* null mutations lose rhythmicity. *mPer3* null mutant mice exhibit only a subtle alteration in rhythmicity, and *mPer1/mPer3* or *mPer2/mPer3* double mutants are not substantially distinct from the *mPer1* or *mPer3* single mutants. These findings suggest there may be some compensation of function among the different mammalian *per* genes, and raise the question of the significance of *mPer3* for the generation of mammalian circadian rhythms.

Cryptochromes

Cryptochromes are blue light–responsive flavoprotein photopigments related to photolyases, so named because their function was cryptic when first identified. In mammals, two cryptochrome genes, *Cry1* and *Cry2*, have been identified. They were found to be highly expressed in the ganglion cells and inner nuclear layer of the retina as well as the SCN,[51] and their mRNA expression levels oscillate in these tissues. Targeted mutant mice lacking *Cry2* exhibit a lengthened circadian period, and mice lacking *Cry1* have shortened circadian period; mice with both mutations have immediate loss of rhythmicity upon transfer to constant darkness.[52-54] Thus, like the mammalian *period* genes, the *cryptochrome* genes appear to have both distinct (given their opposite effects on circadian period) and compensatory (given that either gene can sustain rhythmicity in the absence of the other) functions.

Because of their expression pattern, the *cryptochromes* were thought to be the long-unidentified mammalian circadian photoreceptors (see later), and thus light responses were examined in characterizing the null mutants. *Cry2* mutant mice exhibit altered phase shifting responses to light pulses.[52] *Cry1/Cry2* double mutants exhibit impaired light induction of *mPer1* in the SCN, but light induction of *mPer2* in double mutants remains.[53,55] Neither *mPer1* nor *mPer2* exhibit persistent oscillations in expression in the SCN in constant conditions in *Cry1/Cry2* double mutants.[53,55] Thus, although the *cryptochromes* are not the mammalian circadian photoreceptor, they do appear to play a central role in the generation of circadian signals.

Further evidence for a central clock function is the finding that the cryptochromes appear to share a number of regulatory features with the *period* genes. In *Clock* mutant mice, the mRNA levels of *Cry1* and *Cry2* are reduced in the SCN and in skeletal muscle,[56] suggesting that the cryptochromes also are induced by CLOCK:BMAL1 transactivation. Using mammalian (NIH 3T3 or COS7) cell lines, CRY1 and CRY2 were found by co-immunoprecipitation to interact with mPER1, mPER2, and mPER3, leading to nuclear localization of the CRY:PER dimer as indicated by co-transfection assays with epitope-tagged proteins.[56] Luciferase assays indicate that CRY:CRY or CRY:PER complexes were capable of inhibiting CLOCK:BMAL1 transactivation of *mPer1* or vasopressin transcription.[56] Thus, the CRYs as well as the PERs are capable of a negative feedback function, inhibiting CLOCK:BMAL1-induced transcription.

MODULATORS OF *Period*

Timeless

How is the level of the PER protein regulated by the circadian clock? The first hint came from the identification of the *timeless* gene *tim*, which when mutated produces abnormal circadian rhythms in *Drosophila*.[57] The levels of the mRNA encoded by the *tim* gene oscillate with a time course that is indistinguishable from those of *per* mRNA.[58] The levels of the TIM protein lag behind those of *tim* mRNA by several hours,[59] similar to the finding with *per* mRNA and PER protein. The PER and TIM proteins form heterodimers[60] that are transported to the nucleus.[61] The finding that the heterodimer is transported to the nucleus suggested that it might be involved in the regulation of transcription of the *per* or *tim* genes. Indeed, experiments have shown that the transcription of the *per* and *tim* genes is repressed by the PER-TIM protein heterodimer.[46] This finding is very important because it demonstrates that the production of mRNA encoded by a clock component gene, the delayed accumulation of the encoded protein, and later feedback to the clock gene's promoter in the nucleus can explain the basic features of the circadian clock in *Drosophila*.

However, interactions between PER and TIM are not sufficient, and the basic mechanism does not become clear until one adds interactions with other clock genes. In experiments using a luciferase reporter assay, the luminescent luciferase protein was expressed under the control of the promoter regions of the *Drosophila per* and *tim* genes. It was found that the fly homologue of *Clock*, *dClock*,[62] was capable of driving expression of luciferase[46] in cells that have high endogenous levels of the *Drosophila* homologue

of BMAL1, CYC (*cycle*). The effect of the PER-TIM heterodimer on the ability of the dCLOCK-CYC heterodimer to drive the transcription of the *per* and *tim* genes was tested by co-transfecting the encoding genes into the cells that expressed the luciferase reporter gene. Indeed, it was found that the expression of both the *per* and *tim* genes were reduced by their own protein products. This negative feedback has recently been found for a mammalian heterodimer consisting of homologues of the TIMELESS and mPER1 proteins.[63]

Whether the mammalian *tim* homologue identified[63,64] actually represents an orthologous gene has been called into question.[65] This issue has been difficult to resolve, because gene targeting to create a null mutant resulted in early embryonic lethality. Differences in results obtained by different groups examining the oscillation of *mTim* expression could result from both a full-length and a truncated protein being expressed, with only the full-length form oscillating.[66] Using antisense oligodeoxynucleotides directed against *Timeless* in rat SCN slice preparations results in a disruption of neuronal oscillations in vitro, suggesting a role in rhythmicity might exist.[66] However, true functional homology of *Timeless* in mammals remains to be demonstrated.

Casein Kinase 1

The *tau* mutation of the hamster arose spontaneously in a laboratory stock.[67] The mutation is semidominant and shortens the period from 24 to 22 hours in heterozygotes and to 20 hours in homozygotes. This mutation has been of great importance for several reasons. The mutation predated the *Clock* mutation and demonstrated that single-gene mutations could profoundly alter the circadian clock in mammals, just as in flies and *Neurospora*. *Tau* mutants display several other physiologic phenotypes, such as alteration of the responses of males to photoperiod length[68] and effects of the estrous cycles in females,[69] which gave further insights into the importance of the circadian clock for other biological cycles. The evidence that the SCN is indeed the site of the master circadian oscillator (see earlier) was demonstrated unequivocally using transplantation of the SCN that employed the *tau* mutation. These manipulations also gave rise to the evidence necessary to conclude that the *tau* mutation encodes a protein that is a clock component. Unfortunately, the genetic tools needed for cloning this important and interesting gene were not available for the hamster, and thus its molecular identity could not be determined by conventional genetic mapping or positional cloning approaches.

Lowrey and colleagues identified a genomic region of conserved synteny (a grouping of genes together on a chromosome) in hamsters, mice, and humans that encompassed the *tau* mutation.[70] *Tau* was thus identified as being a mutation in the *Casein Kinase 1 epsilon* (*CK1ε*) gene, the mammalian orthologue of the *Drosophila doubletime* gene. Sequencing of the gene identified a point mutation that leads to altered enzyme dynamics and autophosphorylation state. In vitro assays demonstrated that CKIε can phosphorylate PER proteins and that the *tau* mutant enzyme is deficient in this ability. Thus, CKIε can lead to degradation of PERs, slowing the accumulation of PER in the nucleus and thus repression of CLOCK:BMAL1. Casein Kinase 1 delta (CKIδ) has also been implicated in mammalian circadian rhythmicity.

MODULATORS OF *Bmal1*

Rev-erbα and ROR

The negative feedback of PER and CRY proteins on their own CLOCK:BMAL1-induced transcription constitutes a form of negative feedback, and it may be sufficient to explain the oscillations in expression of *mPer* and *Cry* genes, but the rhythmic expression of *Bmal1* with an opposite phase is not explained by this feedback. What regulatory elements produce the rhythmic transcription of *Bmal1*, with an antiphase relationship to the *Per*s? *Rev-erbα*, an orphan nuclear receptor, may act as the missing link. Its promoter region contains 3 E-boxes, and transcription is thus positively regulated by CLOCK and BMAL1.[71] Its transcription is negatively regulated by PER and CRYs and is at a minimum when mPER2 is at a maximum, and it is constitutively expressed at intermediate levels in *Cry1/Cry2* or *Per1/Per2* double knockouts.

REV-ERBα protein appears to drive the circadian oscillation in *Bmal1* transcription: The *Bmal1* promoter includes two RORE sequences (enhancer sequences that recognize members of the REV-ERB and ROR orphan nuclear receptor families), and *Bmal1* expression is drastically reduced in *Rev-erbα* null mutants.[71] Thus *Rev-erbα* might act to link the positive and negative regulatory signals of other clock genes to the transcription of *Bmal1*. Given the importance of orphan nuclear receptors in regulating cellular metabolic properties,[72] interaction with circadian clock genes might, at the molecular level, form the links between circadian clocks and metabolic regulation, with important implications for health and disease. See chapters in Section 5 for more detail.

The differences between the phase of *Cry1* mRNA rhythms relative to other clock genes whose transcription is enhanced by CLOCK:BMAL binding to E-boxes may also be attributable to *Rev-erbα*. The *Cry1* gene has three candidate REV-ERB/ROR binding sites[73]; in vitro assays indicate that REV-ERBα binds to two of these sites. Luciferase reporter assays indicate that REV-ERBα protein can inhibit transcription of *Cry1* through binding at these two sites. REV-ERBβ also appears to share some functional redundancy with REV-ERBα.[74]

MODULATORS OF *Cry*

In addition to REV-ERB modulation of *Cry*, other genes appear to modulate the activity of the cryptochromes.

Fbxl3

The *Overtime* mutation in mice was identified in a mutagenesis screen based on a lengthened free-running circadian period.[75] The responsible mutation was ultimately identified as being in a known gene encoding the F-box protein Fbxl3, but a gene previously unknown to be involved in circadian rhythmicity. *Fbxl3*^{OVTM} mutant appear to be functionally comparable to null mutants. FBXL3 protein leads to degradation of CRY1, but the OVTM mutant protein is less effective in this capacity. Thus, the period lengthening may be a direct result of a

delay in degradation of CRY, effectively preventing the core cycle from restarting.

Other bHLH-PAS Family Members

NPAS2 (neuronal PAS family member 2) shares the closest homology with CLOCK of all identified bHLH-PAS family members. Null mutants of this gene have altered circadian activity patterns, notably the absence of a "siesta" in later subjective night, but no dramatic alterations in circadian free-running period or persistence.[76] However, when null mutants of the *Clock* had less-dramatic phenotypes than the Δ19 mutant,[77] the role of NPAS2 was reexamined. In the absence of functioning CLOCK, NPAS2 appears to be able to partially compensate.[78]

The *DBP* gene has E-box elements in its promoter, and it exhibits robust oscillations in expression in the SCN and the liver. *DBP*-null mutant mice display alterations in circadian period as well.[79] *NPAS2* is another bHLH-PAS family member that forms heterodimers with BMAL1. Null mutant mice for this gene display alterations in the pattern of their activity rhythms.[80]

Dec1 and Dec2

Like other clock genes, *Dec1* and *Dec2* are basic helix-loop-helix transcription factors that bind to E-boxes. DEC1 and DEC2 have been found to inhibit transactivation of *Per* by CLOCK and BMAL1.[81] DEC1 and DEC2 form dimers.[82] The inhibition of CLOCK and BMAL1 transactivation may be related to interactions with BMAL1, but it can also be attributed to binding to (and thus possibly competition for) E-boxes.[83]

A human allelic variant in *Dec2* has been linked with total sleep time.[84] This functional relationship has been confirmed in transgenic mice expressing the human *Dec2* allele.

OUTPUT REGULATION

Clock

There also is evidence of regulation of clock gene transcription via rhythms in acetylation of H_3 histone: CRY proteins might inhibit H_3 acetylation,[73] the *Per1*, *Per2*, and *Cry1* promoters have rhythms in H_3 acetylation, and the *Per1*, *Per2*, and *Cry1* promoters have rhythms in RNA polymerase II binding. These promoter rhythms are in phase with mRNA levels. P300, a histone acetyltransferase, immunoprecipitates together with CLOCK in liver nuclear preparations, with a peak at CT (circadian time) 6 and minimum at CT 18.[73] P300 may be part of the CLOCK:BMAL1 coactivator complex; a *Per1* promoter-driven luciferase reporter assay indicates that CRY proteins can disrupt this. Hence, inhibition of histone acetylation by P300 provides a potential separate mechanism by which CRY proteins can preclude CLOCK:BMAL1 transactivation of *Per* and *Cry* genes.

Prokineticin 2

Prokineticin 2 (PK2) is rhythmically expressed in the SCN,[85] and infusion of PK2 into the cerebral ventricles inhibits locomotor activity. Mice with a null mutation in the PK2 gene exhibit dramatically reduced levels of activity[86] with reduced circadian amplitude. These mice also exhibit attenuated rebound in raid eye movement (REM) sleep, non-REM (NREM) sleep, and delta power following sleep deprivation.[87]

Transforming Growth Factor α

The peptide transforming growth factor α (TGF-α) was identified in a screen for SCN factors that might inhibit locomotor activity; when infused into the third ventricle, this peptide inhibits locomotor activity. Mice with targeted mutations of the epidermal growth factor receptor (the receptor likely to bind TGF-α) also display disruption of activity rhythms.[88] These effects are attributable to actions on the epidermal growth factor (EGF) receptors.

VPAC2

Mice that lack the peptide receptor VPAC2 show abnormal entrainment and disrupted rhythms, indicating that vasoactive intestinal peptide (VIP) signaling in the SCN may be necessary for normal expression and coordination of rhythms.[89]

Cardiomyotrophin-like Cytokine

Cardiomyotrophin-like cytokine (CLC) also is expressed in the SCN in a rhythmic manner in vasopressin neurons. Infusion of CLC into the third ventricle (near the SCN) dramatically inhibits locomotor activity, and infusion of antibodies to the CLC receptor increases activity.[90]

INPUT REGULATION

Melanopsin

The circadian rhythms of many humans who are blind, with no conscious perception of light, are nevertheless able to be entrained by light.[91] This intriguing observation led to studies of the circadian light-input pathway and the photoreceptors and photopigments in mammals.

In experiments employing mouse mutations that result in degeneration of rods[92] or both rods and cones,[93] light entrainment of the circadian rhythm was preserved.[94] However, the eye must be the site of the light-entraining pathways in mammals because enucleated mammals are not capable of light entrainment.[92] Indeed a morphologically distinct set of retinal ganglion cells projects to the SCN via the retinohypothalamic tract.[95] Ablation of the SCN abolishes circadian rhythmicity, and ablation of the retinohypothalamic tract abolishes light entrainment.[96] Thus, the light signal responsible for light entrainment enters the SCN via a unique axonal pathway from the eye.

Melanopsin, a member of the opsin family of photopigments, was first found in the inner retina[97] and later found to be expressed in the somata and dendrites of retinal ganglion cells of the retinohypothamamic tract.[98] Neurons that contribute axons to the retinohypothalamic tract were found to express the marker pituitary adenylate cyclase–activating polypeptide (PACAP)[98]; when PACAP was used as a marker for rat retinohypothalamic tract neurons, every PACAP-positive neuron was found to express melanopsin, and every melanopsin-positive neuron was PACAP positive.[98]

Further evidence confirming the role of melanopsin as the phase-shifting pigment has come from genetically engineered mice in which the gene encoding melanopsin

was disrupted.[99,100] Two behavioral measures of the circadian rhythm's responses to light were altered in these mice: the phase-shifting response to a discrete light pulse was of lesser amplitude in the knockout mice than in the wild-type mice, and the free-running periods of the knockout mice were lengthened less by exposure to constant light than the periods of wild-type mice. Hence, it appears that melanopsin represents a primary photopigment, with other photopigments also having input to the circadian system.

Rab3a

The *Rab3a* gene was identified in a mutagenesis screen *(earlybird)* based on an advanced phase angle of entrainment and shortened circadian period. Null mutant mice display a similar phenotype.[101] Further, both the *Rab3a* null and *earlybird* mutants exhibit alterations in the homeostatic response to sleep deprivation[101] as well as alterations in emotional behavior.[102]

CONCLUSIONS

The core circadian oscillator is autonomous to individual neurons of the SCN and is the result of the daily oscillation in the levels of several clock component proteins. The basis for this oscillation in mammals, as in other organisms, lies in rhythmic feedback regulation of transcription of the genes encoding these proteins. The levels of the PER and CRY proteins alter the rate of transcription of their own genes. This alteration is achieved by inhibition of the enhancement of transcription that results from binding of the CLOCK-BMAL1 heterodimer to the E-box element of the promoter region of the *Per* and *Cry* genes. Additional interactions between circadian clock proteins may slow the time course of this feedback, achieving the near-24-hour interval: The phosphorylation of PER by CKIα can lead to its degradation, and the association with BMAL1 appears needed for CLOCK to be present in the nucleus. Rhythmic transcription of *Bmal1* appears to result from regulation via REV-ERBβ, itself regulated by E-box elements. Finally, it appears that rhythms in histone acetylation contribute to the circadian expression pattern of some core circadian genes. Additional genes have been identified based on altered circadian rhythms in mutants, although the roles of these genes in the circadian system remain to be determined.

The majority of the core genes have been identified in mice or in flies by forward genetics, in which mutations were induced in the genome randomly, and the mutations that specifically affect the circadian oscillator were identified with carefully crafted circadian phenotypic screens. Now that these clock-component proteins have been identified, it will be easier to find the proteins that serve the input and output pathways of the circadian oscillator and to identify the components that are out of order in disease states that affect circadian rhythms. It is fortuitous that the unraveling of the molecular basis for circadian rhythmicity is occurring at a time when the general public is becoming aware of the importance of normal circadian time keeping for human health, safety, performance, and productivity.

REMAINING QUESTION: CLOCK GENES AS SLEEP GENES?

Are clock genes really sleep genes? As more and more circadian clock gene mutants are examined for other phenotypes, it becomes clear that the circadian system plays a role in the health and well-being of the organism in unanticipated ways. For example, the *Clock*^{d19} mutant mouse has now been shown to have alterations in emotional behavior,[103] response to addictive psychostimulant drugs,[103] metabolism,[104] tolerance of cancer chemotherapeutic agents,[105] reproduction,[106] and, notably, sleep.[107] Particularly noteworthy regarding the sleep phenotype is the finding that not only is the temporal pattern of sleep disrupted, but also the amount was drastically altered (Table 12-1). There may be additional sleep phenotypes not described in Table 12-1; many studies were focused on homeostatic sleep regulation only.

Studies in mice and flies have found that deletion or mutation in core clock genes induces unexpected alterations in sleep amount, sleep architecture, and compensatory responses to sleep deprivation.[107-115] These results have led to the hypothesis that circadian clock genes may also be central to sleep-regulatory processes beyond just the circadian timing of sleep. For example, mutation in the mammalian circadian gene, *Clock*, induces a decrease in NREM sleep time and an attenuated REM recovery following sleep deprivation.[107] A mutation in the fly homologue of this gene, *dClock*, reduces consolidated rest time and leads to alterations in the response to rest

Table 12-1 Clock Genes and Clock-Related Genes That Have Already Been Examined for Sleep Phenotype in Mice

GENE	CHROMOSOME	SLEEP PHENOTYPE	REFERENCE
Clock	5	↓ Sleep, ↑ wake (Δ19 mutant)	107
Per1	11	↓ Sleep, ↑ wake in dark (null)	108
Per2	1	↓ Sleep, ↑ wake in late light (PAS deletion)	108
Per3	4	No change reported, bouts and fragmentation not reported	109
Cry1	10	↑ NREM in double null	110
Cry2	2	↑ NREM in double null	110
Bmal1	7	↑ Total sleep time, ↓ response to sleep deprivation (null)	111
Npas2	1	↓ Sleep in dark (null)	112
Dbp	7	↓ Circadian amplitude, ↑ fragmentation (null)	113

deprivation.[114,115] Cryptochrome double-knockout (*Cry1/Cry2*) mice have increases in baseline amounts of NREM sleep and consolidation of NREM episodes and NREM delta power over wild-type control levels, and they lack the normal compensatory response in sleep amount and NREM delta power following sleep deprivation.[110] Interestingly, a mutation in the *Drosophila* gene *timeless*, which might function in a similar manner to the mammalian *CRY* gene, leads to an impaired recovery response to short-term rest deprivation in flies.[114,115] In addition, deletion of *Bmal1* in mice and the fly homologue, *cycle*, also leads to altered sleep–wake and rest–activity amounts under baseline conditions as well as after sleep deprivation.[111,114,115]

The finding that mutation or deletion of canonical circadian clock genes can induce major effects on the sleep–wake cycle that go beyond just the timing of this rhythm may be just the beginning of a new way of thinking about how the circadian clock regulates a multitude of physiologic and behavioral rhythms. It may well be that once the central circadian input to a particular output rhythmic system has been disrupted, that downstream rhythm may now be disrupted or altered in many different ways due to loss of normal temporal organization. Indeed, the recent discovery that *Clock*-mutant animals show a wide range of metabolic abnormalities supports the hypothesis that a disrupted molecular circadian clock can have far-reaching implications for physiology and pathophysiology.[72] Whether such disruptions in physiology and behavior are due to altered circadian information from the SCN or are due to local tissue-specific changes in the molecular circadian clock are not known. Regardless of the mechanisms, such results point to a very central role of circadian clock genes in regulating biochemical, metabolic, and physiologic processes at many different levels of organization.[116]

❖ Clinical Pearl

Circadian clock genes have a central role in regulating biochemical, metabolic, and physiologic processes throughout the body. They may play a pivotal role in the linkage between circadian misalignment (e.g., shift work) and the increased risk of developing metabolic and other abnormalities such as the metabolic syndrome.

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Genetics of Sleep in a Simple Model Organism: *Drosophila*

Ravi Allada

Chapter 13

Abstract

The complexity of sleep in mammals has led to a movement toward examining simpler model systems, such as nematodes, flies, and fish, that exhibit sleep (or sleeplike states) and harbor technical advantages not evident in more conventional rodent, feline, or primate models. One system is the fruit fly Drosophila melanogaster, best known for its use for genetic studies. Remarkably, the mammalian homologues of many Drosophila genes have been found to function in a manner similar to the way they do in flies. Indeed, most human disease genes have clear fly homologues. Given the notable similarity between Drosophila and mammals, it is not surprising that fruit flies exhibit many of the defining features of sleep, including immobility, reduced responsiveness to sensory stimuli, and homeostatic regulation. Furthermore, preliminary indications are that even the genetic and pharmacologic underpinnings of sleep are conserved between flies and mammals. Here I will address why the Drosophila model is used for sleep studies, information that has been garnered in the understanding the circadian regulation of behavior, and insights into sleep regulation. These insights could prove important in understanding the genetic basis of human sleep and ultimately in answering the question of why we sleep.

In contrast to studies of naturally occurring genetic variation, one can attempt to induce mutations in animal models to test whether a given gene is important for sleep. One strategy to understand the molecular basis of complex behavior such as sleep is classic or forward genetics.[1] Here a population of animals is randomly mutagenized using DNA alkylating agents such as ethyl methane sulfonate (EMS) or a mobile DNA transposable element. This mutagenized population is screened for a mutant phenotype of interest, such as altered sleep. Using molecular genetic approaches, one can then identify the mutant gene responsible for the mutant phenotype. Thus, forward genetics can be used to establish causal relationships between the function of individual genes and otherwise complex phenotypes. Forward genetics is unbiased and does not require any prior knowledge about the genetic basis of the phenotype of interest and is therefore an ideal approach for studying sleep. In contrast, reverse genetics starts with a disrupted gene in search of a phenotype. Nonetheless, the finding of a gene can provide insight into biochemical and cellular pathways that are important for sleep, perhaps even providing novel diagnostic tests or targets for drug development for sleep disorders.

DROSOPHILA AS A MODEL SYSTEM FOR GENETICS

Many of the model organisms in genetics, such as zebrafish (*Danio rerio*) and the nematode *Caenorhabditis elegans*, have been adopted for sleep studies because they are highly suited to the forward genetics approach.[2] Here I will focus on one of the premier model systems for genetics, the fruit fly *Drosophila melanogaster* (Fig. 13-1, Video 13-1). The fruit fly has been a workhorse for genetic studies since pioneering studies of Thomas Hunt Morgan in the early 20th century.[3] A major advantage of *Drosophila* over many alternative model systems is the ability to grow and handle large numbers relatively easily and cheaply.[1] A single female can produce hundreds of offspring. In addition, it has a short generation time, about 10 to 12 days from fertilized egg to fertile adult at room temperature. Because of these traits, *Drosophila* has been a model par excellence for high throughput screening of mutants with altered phenotypes. The facility of genetic mapping, gene disruption using transposable elements (mobile DNA), and the full genome sequence allows one to identify mutant genes responsible for mutant phenotypes.[4] Remarkably (to some), the mammalian homologues of *Drosophila* genes have been found to function in a similar manner to their *Drosophila* counterparts. Indeed, entire signaling pathways are shared between *Drosophila* and their mammalian counterparts. For example, most human disease genes have clear fly homologues.[5] Thus, genes identified in flies will likely serve comparable functions in more complex mammalian systems, including that of humans.

The conservation between flies and mammals extends to the nervous system. Although flies have only about 1/1,000,000 the number of neurons as humans (about 10^5 versus 10^{11}), the fly and human genomes are surprisingly similar in gene number (14,000 versus ~22,000), with differences largely due to gene duplication.[6] In fact, the fly brain uses comparable neuronal machinery, including neurotransmitters, ion channels, receptors, and signal transduction pathways. Consequently, flies have been used as valuable nervous system models for olfaction,[7] vision,[8] hearing,[9] sexual behavior,[10] synaptic transmission, axon guidance,[11] and learning and memory.[12] Flies have also been exploited as models for numerous human diseases including diabetes,[13] aging,[14] pain,[15] Alzheimer's disease,[11] Parkinson's disease,[11] epilepsy,[16] and fragile X mental retardation.[17] Finally, flies have been successfully used to study the response to clinically important drugs such as ethanol,[18] cocaine,[18] and general anesthetics.[19] In many of

Figure 13-1 *Drosophila melanogaster,* a genetic model organism. Shown here is a fruit fly attached to a tether with recording electrodes implanted for measurement of electrical correlates. (Photo courtesy B. Van Swinderen, Queensland Brain Institute, Australia.)

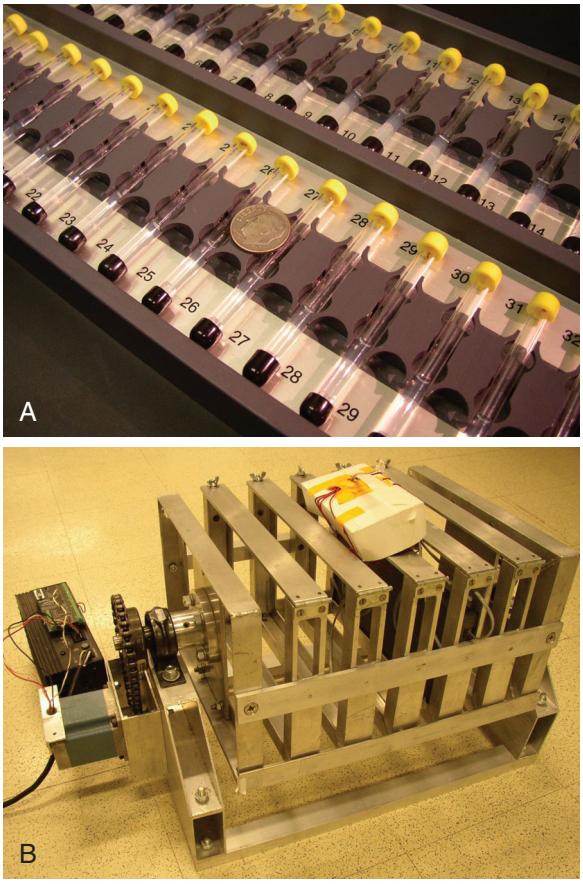

Figure 13-2 The *Drosophila* activity monitoring system and rotating sleep depriving box. **A,** The *Drosophila* Activity Monitoring (DAM) system. A U.S. dime (diameter = ~1.5 cm) is shown for scale and is placed over the location of infrared emitter/detectors. **B,** *Drosophila* sleep deprivation apparatus. A DAM monitor can be placed into a slot, and the box is rotated randomly to disrupt fly sleep. (Photo courtesy B. Chung, Northwestern University, Evanston, IL.)

these cases, genes identified in *Drosophila* serve similar functions in mammalian systems.

DROSOPHILA AS A MODEL FOR STUDIES OF SLEEP

Studies of *Drosophila* sleep have been predicated on a small but noteworthy literature examining sleeplike states in other invertebrate models such as mollusks[20] and in insects such as cockroaches[21] and honey bees.[22,23] These classical descriptions of sleep behavior formed the basis for pursuing similar studies in *Drosophila*.

Sleep in the fruit fly is typically measured behaviorally using the *Drosophila* Activity Monitoring (DAM) System developed by Trikinetics (Waltham, MA; Fig. 13-2). Single flies are placed into a small transparent glass tube plugged on one end by agar food and the other end by a porous cap, allowing air passage. Each tube is placed into a monitor that contains a series of 32 infrared emitter-detector pair, one for each tube. An awake fly moves and back and forth in the tube, periodically breaking the infrared beam. Independent methods indicate a close correlation between infrared beam breaks and overall activity. A five-minute period of inactivity (no beam breaks) has been found to be a reliable indicator of sleep.

Given the remarkable similarity between *Drosophila* and mammals, it is not surprising that fruit flies exhibit many of the defining features of sleep. Flies exhibit extended periods of behavioral quiescence that can last for hours, and the majority of sleep typically occurs in bouts greater than 30 minutes.[24] In addition, sleeping flies exhibit reduced responsiveness to sensory stimuli and exhibit homeostatic regulation.[2,24-27] Video-based monitoring has also been coupled to measurements of beam breaks to provide higher spatial resolution analysis of fly sleep behavior.[24,28]

Drosophila sleep studies do not solely rely on measures of spontaneous movement; they also rely on responsiveness to sensory stimuli. Arousal threshold is assayed by application of a stimulus and measuring a behavioral response, typically induction of locomotor activity. During periods of extended immobility, flies are less likely to respond to a range of sensory stimuli including social, mechanical, vibratory, thermal, and visual.[24,25,29,30] Although this responsiveness is typically measured behaviorally, it can also be uncoupled from movement using electrophysiologic measures.[31] The typical fly demonstrates an increase in arousal threshold reaching a plateau after 5 minutes.[25,30] The 5-minute criterion for sleep is in part based on this observation. Thus, one can distinguish quiet wakefulness from sleep by assessing arousal threshold.

Importantly, fly sleep is under homeostatic regulation: Flies deprived of sleep rebound the following day. Flies are typically deprived of sleep mechanically using automated

devices or by tapping the flies by hand (see Fig. 13-2).[24,25,30,32] The former must be programmed to vary the stimulus to avoid adaptation, and the latter allows one (with great patience and stamina) to deliver the stimulus selectively to sleeping flies. If a fly is deprived of sleep, it exhibits increases in sleep duration and intensity (the latter as measured by sleep bout length) the following day. Sleep rebound is not observed or is much less evident if similar deprivation protocols are applied to flies that are already awake, arguing strongly against nonspecific stress effects of mechanical disruption.[24,25,30] Continuous sleep deprivation ultimately results in premature death in about 2 to 3 days[32] as it does in some mammals.[33] Unperturbed flies can live for 1 to 2 months. Thus, sleep is essential for life in the fly.

Although flies do not display the precise electroencephalographic signatures of mammalian sleep—the synchronous changes in neural activity seen as slow waves by electroencephalogram that are diagnostic of mammalian sleep have not been observed in *Drosophila*[29]—they do exhibit electrical correlates of sleep behavior providing behavior-independent state markers. In vivo electrical correlates in behaving flies have relied on the development of novel approaches to study the fly brain. In this approach, recording electrodes are inserted into the center of the *Drosophila* brain and into the optic lobes of a tethered fly (see Fig. 13-1).[29] Local field potentials (LFPs) are measured, reflecting neural activity near the electrode. Leg movement in the tethered fly is simultaneously monitored. There is a general, but not perfect, correlation between spikelike potentials recorded from the central brain and waking movement. If flies are exposed to a rotating stripe, LFPs in the 20- to 30-Hz frequency are observed, reflecting attention to the stimulus, but these LFPs are reduced when the fly is asleep.[31] In addition, periods of poor correlation between LFPs and movement are associated with increased arousal threshold and precede behavioral quiescence.[31] These approaches clearly demonstrate that differences in arousal states can be characterized using electrophysiologic correlates as they are in more-complex organisms. The differences between fly and mammalian neuroanatomy could account for the differing electrical manifestation of sleep even if the underlying molecular and cellular mechanisms are similar. Studies of eye development provide some precedent for the idea that common genetic mechanisms can underlie anatomically distinct but functionally analogous structures.[34]

In addition to the core features of sleep, flies also display age-related changes in sleep architecture similar to those of aging mammals. Directly following emergence from the pupal case, young flies display elevated levels of sleep similar to their mammalian counterparts.[25] With increasing age, sleep becomes more fragmented and less consolidated.[35] In addition, drugs that increase oxidative stress can mimic these effects.[35] Thus, the fruit fly has the potential to become a valuable model for the analysis of aging effects on sleep.

Mammalian sleep has been demonstrated to influence various aspects of memory consolidation.[36] Similarly, flies also display sleep-loss–related deficits in learning and memory, using a number of different learning paradigms. For example, flies normally exhibit phototaxis but can be trained to avoid light using aversive stimuli. However, flies that have reduced sleep perform more poorly on this task.[37] In courtship conditioning, male flies learn to stop courting females that have already mated. Under the appropriate conditions, males can remember this experience for over 24 hours; however, if flies are subjected to sleep deprivation after training (i.e., during the period of presumed memory consolidation), they fail to retain this memory.[38] Finally, waking experience—in particular, social experience—can increase subsequent sleep amount, a process that might use dopamine and cyclic adenosine monophosphate (cAMP) pathways.[38] Taken together, these data implicate a reciprocal relationship between sleep–wake regulation and synaptic plasticity memory in *Drosophila*, as is proposed in mammals.

DROSOPHILA CIRCADIAN BEHAVIOR REVEALS CONSERVED MECHANISMS BETWEEN FLIES AND HUMANS

The best case for the argument that *Drosophila* genetics will illuminate the genetics of human sleep has emerged from studies of circadian behavior. As in many (but not all) organisms, sleep is under temporal control by a circadian clock in *Drosophila*.[24,25] The first identified fly circadian mutants displayed short and long period rhythms in constant conditions and phase advanced and delayed activity in light–dark conditions, analogous to human advanced and delayed sleep phase syndromes.[39,40] Cloning of the genes responsible for these fly phenotypes led to breakthroughs in our understanding of the core biochemical mechanisms of circadian timing. These studies also provide an experimental roadmap for elucidating basic mechanisms of sleep homeostasis. Although circadian clocks have often been viewed solely as timekeepers, both circadian genes and their accompanying neural circuits extensively regulate sleep–wake, perhaps independent of their timing functions (see later). Thus a deeper molecular understanding of the circadian system should provide insights into the control mechanisms for sleep.

Most aspects of the fly molecular clockwork are conserved with mammals, including humans (Table 13-1).[41,42] Persons affected by familial advanced sleep phase

Table 13-1 *Drosophila* Clock Genes and Their Highly Conserved Mammalian Homologues

DROSOPHILA	MAMMALS
Period	*Period1,2,3*
Timeless	*Timeless*
Clock	*Clock, NPAS2*
Cycle	*Bmal1*
Doubletime	*CK1δ/ε*
CK2	*CK2*
Cryptochrome	*Cryptochrome 1,2*
Clockwork orange	*Dec1, 2*
Slimb	*β-TRCP*

syndrome (FASPS) exhibit an advanced phase of sleep–wake rhythms and shortened circadian period that is inherited in a mendelian dominant manner.[40] Mutations in the human *PER2* and *CK1delta* genes, orthologues of fly circadian genes *period* and *doubletime*, respectively, are responsible for this advanced sleep phase.[43,44] These data argue that the basic architecture and core components of circadian clocks can be traced back to the shared ancestor of flies and humans hundreds of millions of years ago. Given the close association of circadian to sleep behavior, this suggests that the basic mechanisms of sleep homeostasis might also be conserved with flies.

CELLULAR AND MOLECULAR BASIS OF *DROSOPHILA* SLEEP

As described earlier, considerable evidence indicates that fruit flies display the core characteristics of sleep. Substantial progress has been made in identifying discrete molecular and neural circuits that convey signals to time sleep and wake behavior. Here the neural circuits that contribute to sleep–wake behavior are described and the genes that are regulated by and that regulate sleep are discussed. The emerging picture (and take-home message) is that the molecular mechanisms governing *Drosophila* sleep may be shared with animals that have more complex nervous systems.

SPECIFIC NEURAL CIRCUITS ARE IMPORTANT FOR SLEEP–WAKE REGULATION

A theme of mammalian sleep studies is the notion that discrete neural circuits are important for initiating and maintaining sleep and wake states. Using gene-based tools to study neural circuit function in live animals, distinct neural circuits have been found that regulate sleep in *Drosophila*. Thus far, three anatomically defined loci have been implicated in sleep–wake regulation: the mushroom bodies (MBs), the pars intercerebralis, and the circadian pacemaker neurons: the large ventral lateral neurons (lLNv) (described earlier) (Fig. 13-3). In addition to these loci, additional circuits have been defined based on their transmitter identity (e.g., dopamine). These are discussed further later. To discover novel circuits involved in sleep regulation, an approach akin to forward genetics has been employed with the modification that, instead of screening for genes, circuits are screened in an unbiased manner for behavioral functions.

A cornerstone of this approach is the binary GAL4/UAS system.[45,46] In one parental strain, the yeast transcription factor GAL4 is placed under the control of a tissue-specific promoter. In the second strain, the upstream activating sequence (UAS) bearing GAL4 binding sites is fused to an effector gene of interest. In the progeny of these two strains, the effector gene is expressed in the distribution specified by the tissue or circuit-specific promoter driving GAL4. Fortunately for *Drosophila* geneticists, there is a plethora of GAL4 lines available that provide a nearly limitless display of temporal and spatial expression patterns. In addition to the multitude of GAL4 lines, numerous UAS effector lines have been generated, including

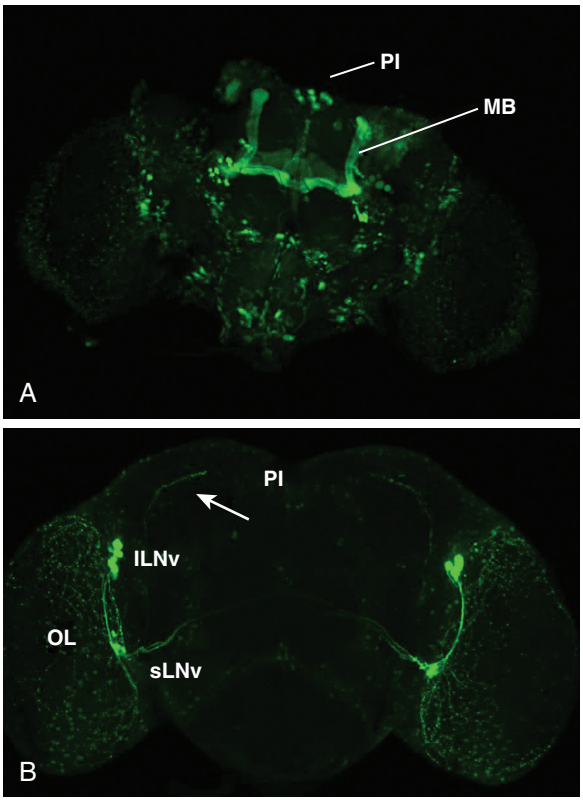

Figure 13-3 Neuroanatomy of *Drosophila* sleep–wake circuits. **A,** The sleep-regulatory mushroom bodies *(MB)* and pars intercerebralis *(PI)* neurons are labeled with green fluorescent protein (GFP). **B,** Large and small ventral lateral neurons (*lLNv, sLNv*, respectively) are labeled with GFP. The arousal-promoting large subset sends projections to the ipsilateral and contralateral sLNv and optic lobes *(OL)*. sLNv sends projections to the PI.

those that have been engineered to alter specific cellular properties such as membrane excitability or synaptic transmission. While these approaches are well developed in *Drosophila*, they have inspired even more sophisticated strategies in mammalian models.[47]

A number of cellular effectors have been successfully used for *Drosophila* sleep studies. One tool that has been used to conditionally block synaptic transmission is the UAS-driven *shibire*^{ts1} (*shi*^{ts1}) transgene.[48] *shi* encodes for a multimeric GTPase homologous to mammalian dynamins. SHI is required for vesicle scission, a process that is required for synaptic vesicle recycling and thus the maintenance of fast synaptic transmission. UAS-driven expression of the *shi*^{ts1} allele in a wild-type neuron can block synaptic transmission at an elevated restrictive temperature (e.g., 29°C) but not at the permissive temperature (e.g., 21°C). Using the GAL4/UAS system and given the fact that flies are not homeotherms, one can specifically manipulate synaptic transmission in discrete neural circuits in a live behaving animal, using temperature acting as a remote control, and then assay the behavioral consequences of circuit modulation. Tools to manipulate cellular excitability have also been developed and applied. For example, a bacterial depolarization-activated sodium channel, NaChBac, has been used to increase cellular excitability.[49] On the other hand, a non-inactivating mutant

form of the voltage-gated Shaker potassium channel, termed *electrical knockout* (*eko*) has been used to silence neuronal activity.[50]

The use of these transgenic tools in combination with various GAL4 drivers led to the discovery of a sleep-regulatory role for the mushroom bodies, a bilateral neuropil well known for its role in learning and memory.[12,51,52] To discover novel sleep-promoting circuits, a series of adult neural GAL4 lines were crossed with UAS-driven *shi*^{ts1}.[51] Their progeny were exposed to 12-hour cycles at restrictive (29° C) and permissive (21° C) temperatures, and the sleep behavior was examined. Reduced sleep at 29° C could be attributed to reduced synaptic transmission in the relevant neural circuits. Of nearly 100 lines tested, only a handful exhibited this reduced-sleep phenotype, suggesting that perturbation of neural function did not generally affect sleep behavior and that there are specific circuits in the fly devoted to promoting sleep. All of these lines displayed MB expression.

An independent method, chemical ablation of the MBs with hydroxyurea, was also used to assess MB sleep function. Hydroxyurea fed to larvae during the appropriate developmental time (first-instar) selectively ablates the neuroblasts that give rise to the large majority of the MBs.[53] Like their *shi*^{ts1}-expressing counterparts, flies fed hydroxyurea exhibited reduced sleep,[51,52] and in both cases the reduced sleep was largely due to reduced sleep bout length—in other words, an inability to maintain sleep.[51] In addition, flies with impaired or absent MBs exhibited a reduced lifespan consistent with a loss of restorative sleep.[51] Thus, these flies are analogous to insomniac humans who are unable to maintain sleep and suffer adverse consequences as a result.

Although these observations suggest a sleep-promoting role for the MBs, other data using a different MB-GAL4 line suggest that the MBs might also promote wakefulness. These studies made use of a modified version of GAL4 called Gene Switch, in which the GAL4 is fused to the ligand-binding domain (LBD) of the progesterone receptor.[54] In the absence of the ligand RU-486, the LBD retains GAL4 in the cytoplasm, rendering it inactive. In the presence of ligand, the LBD-GAL4 fusion is released to the nucleus, where the GAL4 binds its target UAS DNA sites and transcription can be initiated. RU-486 can be selectively delivered to adult flies by feeding to avoid any developmental effects of GAL4-driven gene expression. Using a version of Gene Switch that is driven by an MB-specific promoter,[55] the silencing transgene *eko* resulted in increased sleep while expression of the activating *NachBac* transgene reduced sleep, suggesting that MB neuron activity increases wake.[52] One possibility is that different subsets of MB neurons play opposing roles in sleep regulation. The finding that a part of the brain important for learning and memory is also important for sleep further supports the idea that sleep and memory consolidation are closely linked in the fly.

Flies that lack MBs demonstrate both spontaneous sleep (albeit reduced) and a robust homeostatic response, indicating that other brain loci promote sleep. One such locus is the pars intercerebralis (PI), a neuroendocrine cluster considered genetically analogous to the mammalian hypothalamus.[56] Targeted decreases in epidermal growth factor (EGF) function in the PI result in reduced sleep.[57] The PI may be a direct target of circadian pacemaker neurons. The dorsal projections of a subset of circadian pacemaker neurons, the small ventral lateral neurons (sLNv), terminate in close proximity to the PI neuron soma (see Fig. 13-3), and loss of the key peptide transmitter of these neurons, pigment dispersing factor (PDF), affects molecular circadian rhythms in PI neurons.[58]

In addition to sleep–wake circuits in the MBs and PI, the circadian pacemaker lLNv neurons promote wakefulness (see Fig. 13-3). Excitation of the lLNv using NaChBac or a novel tool, TrpA1, reduces sleep, especially at night.[59-61] TrpA1 encodes for a cation channel that is activated at elevated temperatures, allowing conditional temperature-dependent regulation similar to the *shi*^{ts1} system.[62] Selective ablation of the lLNv results in increased sleep.[60] The vigilance affect is similar to that observed in animals in which the mammalian circadian pacemaker, the suprachiasmatic nucleus, is ablated.[63] An important molecular effector of PDF^{+} pacemaker neuron functions in sleep is the transcription factor ATF-2, a member of the ATF/CREB (activating transcription factor/cAMP response element binding) family.[64] The activity of these arousal-promoting neurons appears to be inhibited by gamma-aminobutyric acid (GABA),[61] a relationship that is reminiscent of a similar organization of mammalian sleep circuits.[65]

GENETICS AND PHARMACOLOGY OF SLEEP: WHICH MOLECULES REGULATE SLEEP?

The power of the *Drosophila* system lies in the ability to identify genes whose function is important for sleep. One strategy is to manipulate the function of genes and assay the consequences on sleep. In addition, traditional pharmacologic approaches have complemented genetics to identify pathways whose function is important for sleep. Finally, genomic approaches have been applied to identify genes whose expression is regulated by sleep–wake state. The discovery of novel sleep genes in *Drosophila* using genetics has relied on a combination of candidate-gene approaches and classical forward genetics. Unbiased large-scale screens are especially powerful because they tend to identify the strongest contributors to a process among thousands of mutagenized candidates. Not surprisingly, identified genes are involved in various aspects of neural function, including genes involved in stress and immune responses, signal transduction, neurotransmitter or neuromodulator function, and cellular excitability. These studies suggest that many sleep pathways are conserved between flies and mammals and support the notion that studies in *Drosophila* should yield insights into the molecular basis of sleep in more complex systems.

Circadian Clock Pathway

As in many mammalian species, sleep is under the control of a circadian clock in *Drosophila*.[24,25] Mutations in the core clock transcription factors *Clock (Clk)* and *cycle (cyc)* result in reduced sleep.[66] However, in certain arrhythmic mutants, such as *per*^{01}, sleep homeostasis is intact consistent with the two-process model.[24,25] Some of these clock gene effects

are likely mediated by the PDF neuropeptide through its function in the arousal promoting lLNv (see Fig. 13-3).[60,61]

Stress and Immune Pathways

Studies of the role of the circadian clock gene *cyc* led to the discovery of a role for heat-shock stress-response genes in sleep homeostasis. Female, but not male, *cyc* mutants display an exaggerated sleep rebound.[32,66] In addition, *cyc* mutants, both male and female, are hypersensitive to the lethal effects of sleep deprivation.[32] These phenotypes are likely due to a noncircadian *cyc* function, because other clock mutants fail to display these phenotypes.[32] They do not reflect a general impairment in stress response because *cyc* flies are not sensitive to other stressors. These effects appear to be due to inadequate expression of heat-shock proteins (HSPs), protein chaperones important in the cellular response to stresses, such as elevated temperature. Heat shocking flies before sleep deprivation, which induces *hsp* transcription, rescues the premature lethality due to sleep deprivation.[32] Moreover, mutants of the heat-shock protein 83 (*hsp83*) gene also display hypersensitivity to the lethal effects of sleep deprivation.[32] This study suggests that the heat-shock stress response is an important pathway for defending against the adverse consequences (i.e., death) of sleep loss.

In addition to the heat-shock stress pathway, endoplasmic reticulum stress responses might also be crucial for sleep homeostasis. The endoplasmic reticulum (ER) stress pathway both is regulated by and regulates sleep. The ER chaperone, BiP, is upregulated during wake and during sleep deprivation.[25,67] In addition, the amount of rebound sleep following sleep deprivation is dependent on BiP levels.[67] BiP plays an important role in the stabilization and translocation of newly synthesized secretory proteins from the cytosol to the ER. BiP is also upregulated as part of the unfolded protein response (UPR), which is activated in response to an abundance of unfolded proteins in the ER. The UPR is also upregulated in mammals in response to sleep deprivation.[68] These data suggest that the UPR response, and subsequent BiP activation, might occur as a consequence of extended wakefulness and suggest a central role for ER stress pathways in sleep homeostasis.

Another important regulator of *Drosophila* sleep is the immune response. Infection resistance and immune-response genes, including the immune system master regulator NFκB Relish, are upregulated in response to sleep deprivation.[69] Reductions in Relish function in the fat bodies, a key immune-response tissue in *Drosophila*, results in reduced sleep.[69] Notably, the immune-related cytokines are important regulators of sleep in mammals.[70]

Membrane Excitability

The role for membrane excitability in sleep regulation is evident from studies using engineered heterologous transgenes, such as *eko* and *Nachbac* (see earlier); however, these studies leave open the question of which specific channels normally underlie sleep function. Studies in *Drosophila* have highlighted the function of the voltage-gated potassium channel *Shaker* (*Sh*). The *Sh* mutant was discovered several decades ago as a mutant whose legs shake under ether anesthesia.[71] Positional cloning of this mutant led to the identification of the first voltage-gated potassium channel and subsequently several similar channels in mammals, highlighting the similarity in fly and mammalian nervous system components.[72-74] Independent unbiased mutagenesis screens identified mutants in the SH potassium channel and a novel SH regulator, called SLEEPLESS (SSS), that exhibit dramatically reduced sleep amounts, losing as much as 80% of total sleep in an *sss* mutant.[75,76] *sss* encodes a glycosylphosphatidyl-linked membrane protein that is potentially released into the extracellular space to promote SH expression.[76] In addition, mutants of an SH regulatory subunit *Hyperkinetic* (*Hk*), also exhibit a reduced sleep phenotype.[77]

Both *Sh* and *sss* mutant flies fail to exhibit large changes in waking locomotor activity, suggesting a primary role in regulating the transition between sleep and waking states.[75,76] Both *Sh* and *sss* mutants display reduced lifespan, although it is not clear if this is a primary consequence of their reduced sleep.[75,76] *Sh* and *sss* mutant differ in that *Sh* mutants display intact sleep rebound after sleep deprivation but *sss* mutants exhibit reduced sleep rebound.[75,76] It is not yet clear what the basis for this difference is. Short-sleeping *Sh* and *Hk* mutants display reduced memory in a short-term memory paradigm, providing a genetic link between reduced sleep and cognitive function.[77] SH sleep function is highly conserved as genetic inactivation of mammalian SH orthologues also results in reduced sleep.[78,79] The magnitude of the sleep-duration phenotypes in both *Sh* and *sss* mutants, coupled to their independent isolation in unbiased screens and conserved functions in mammals, highlights the central role of SH and membrane excitability in sleep regulation.

Growth Factors and Signal Transduction

Two other gene classes that have been implicated in sleep-wake regulation are growth factors, which activate intracellular signaling pathways, and components of intracellular signal-transduction pathways. The growth factor most strongly and broadly relevant is epidermal growth factor (EGF). EGF, like other growth factors, is processed and then secreted to activate cell-surface receptors, which act as tyrosine protein kinases that autophosphorylate, leading to activation of intracellular signaling cascades.

The function of EGF was uncovered by the combination of the GAL4/UAS system and RNA interference (RNAi).[64,80] UAS-driven RNAi transgenes are constructed to express inverted repeats corresponding to a gene of interest. The inverted repeat RNAi base pairs with itself, forming a double-stranded hairpin RNA (hpRNA). The hpRNA is recognized by the cellular RNAi machinery and processed into small interfering RNAs (siRNAs). siRNAs in turn base pair with endogenous transcripts of a given gene, targeting them for degradation by the RNAi machinery. Genome-wide libraries of UAS-RNAi transgenic flies have been established, allowing one to knockdown virtually any gene in the *Drosophila* genome in a spatially and temporally targeted manner.[81]

rho and *star* encode two processing proteins important for EGF action.[57] *rho* and *star* induction increases sleep, and *rho* knockdown using RNA interference reduces sleep.[57] EGF appears to be released by the neuroendocrine cells of the pars intercerebralis, a potential fly analogue of the hypothalamus, to promote sleep.[57] EGF and its related

set of ligands (e.g., transforming growth factor α) appear to serve similar functions in promoting sleep in *C. elegans*[82] and mammals,[83,84] suggesting an ancient sleep function for EGF.

Components of the cAMP signaling pathway also play conserved roles in sleep regulation. Various neurotransmitters act at cell-surface G protein–coupled receptors (GPCR) to activate intracellular signal transduction cascades via metabotropic receptors (e.g., dopamine). Activation of the G protein–coupled receptors, such as dopamine receptors, leads to an increase or decrease in adenylate cyclase, which modulates cAMP levels. cAMP in turn activates protein kinase A (PKA), which phosphorylates a number of targets, including the transcription factor CREB (cAMP response element binding protein). Mutants affecting this pathway increase activity (e.g., increase cAMP or PKA activity) resulting in increased wake while mutants that decrease cAMP flux generally reduced wake.[52,85] Furthermore, CREB activity is linked to sleep homeostasis as a CRE reporter gene is upregulated in response to sleep deprivation and reduced CREB activity results in an elevated sleep rebound.[85] In addition to a wake-promoting role for this pathway in *Drosophila*, the cAMP pathway serves similar functions in both nematodes and mice.[86,87] Many of the mutations that affect the cAMP pathway were originally isolated in unbiased genetic screens for mutations that disrupt learning and memory. Thus, signaling important for sleep and memory might intersect at cAMP pathways.

Arousal Neurotransmitters: Monoaminergic Arousal Pathways

A number of different neurotransmitters and neuromodulators have been shown to play important roles in sleep regulation. Although the anatomic organization of the *Drosophila* brain is distinct from that in mammals, flies use similar neurotransmitters and receptors. Indeed, it appears that flies use transmitters to regulate sleep similar to those used in mammals. In mammals, monoamine transmitters, such as dopamine, histamine, and norepinephrine, generally promote wake or arousal.[88] Similarly in flies, these monoamines or their fly counterparts also promote wake, suggesting that the nervous system of the common ancestor of flies and mammals used similar arousal transmitters.

The monoamine most strongly linked to arousal in *Drosophila* is dopamine. Mutants of the dopamine transporter *fumin* exhibit dramatically reduced sleep duration (about 50%).[89,90] Mutants in this gene were also identified in a large-scale unbiased genetic screen for sleep mutants.[90] The psychostimulant methamphetamine, which is thought to increase dopaminergic neurotransmission, also reduces sleep.[91] When awake, flies treated with methamphetamine display increased spontaneous locomotor activity as well as hyperresponsiveness to mechanosensory stimuli, suggesting that these flies were hyperaroused.[89-91] In addition, although its mechanism of action remains unclear, the clinically prescribed wake-promoting drug modafinil might operate by enhancing dopaminergic neurotransmission.[92] Importantly, modafinil has similar wake-promoting properties in *Drosophila*.[93]

Dopamine, or other monoamines, might also play a role in the homeostatic response to sleep deprivation. The arylalkamine acetyltransferase gene (*Dat*), involved in monoamine catabolism, is upregulated in response to sleep deprivation, and *Dat* mutants exhibit an exaggerated sleep rebound.[25] However, dopamine transporter mutants exhibit either normal[90] or reduced rebound.[89]

Dopamine might exert its wake-promoting effects by acting at the MBs, a major locus for sleep–wake regulation (see earlier). Dopaminergic neurons densely innervate the MBs.[94] In addition, dopamine D1 receptor (dDA1) activation in the MBs might mediate the wake-promoting effects of caffeine[95] and can mitigate the effects of sleep loss in an MD-dependent learning paradigm.[37] One possibility is that sleep and memory functions overlap in the MBs, an important site for the function of adenylate cyclase in short-term memory[96] and PKA activity in sleep.[52] The connection between sleep and memory is particularly intriguing given the proposed function of sleep in regulating synaptic plasticity. Taken together, these data indicate that dopamine is an important transmitter for arousal and cognitive function in *Drosophila*.

Two other monoaminergic transmitters implicated in fly arousal are octopamine and histamine. Octopamine is thought to serve as a functional homologue of mammalian norepinephrine. Genetically reduced octopamine synthesis or octopamine neuron activity results in increased sleep, which can be rescued in the former case by pharmacologically restoring octopamine.[97] These effects require PKA but do not operate through the MB.[97] Histamine has been implicated principally by pharmacology. The H1 receptor antagonist hydroxyzine induces sleep and reduced sleep latency in flies,[25] suggesting conserved functions for histamine. Not all monoamines promote arousal in *Drosophila* (e.g., serotonin[98]). In addition, the arousal-promoting orexin neuropeptide, which is important in human narcolepsy, has not yet been reported in *Drosophila*. Nonetheless, the role for monoamines in promoting arousal in mammals is largely preserved in *Drosophila*.

Sleep Neurotransmitters: GABA and Adenosine Sleep Pathways

A crucial neurotransmitter for sleep promotion in both flies and mammals is the inhibitory neurotransmitter GABA. Many of the most commonly prescribed hypnotics act at inotropic GABA receptors, promoting GABAergic neurotransmission.[99] To test the role of GABAergic neurotransmission in *Drosophila* sleep, the hyperpolarizing Shaw potassium channel was expressed in GABAergic neurons to silence their activity.[100] Targeted expression was accomplished using a GAL4 driven by the promoter glutamic acid decarboxylase, a key enzyme in GABA biosynthesis (GAD-GAL4). Shaw expression resulted in reduced sleep, consistent with a sleep-promoting role for these neurons.[100] In *Drosophila*, a mutation in one GABA(A) receptor subunit gene is responsible for resistance to the dieldrin insecticide, hence its name: *r*esistant to *diel*drin (RDL). These receptors rapidly desensitize upon GABA activation, and this process is reduced in insecticide-resistant *Rdl* mutants, prolonging GABA-activated currents.[100] Flies typically fall asleep soon after transfer from light to dark. In these *Rdl* mutants, this latency to sleep is reduced, consistent with an important role for GABA in promoting sleep in *Drosophila*.[100] *Rdl* might promote sleep in part by

reducing the activity of PDF arousal-promoting neurons (see earlier, and see Fig. 13-3).[61] Given the conserved role for inotropic GABA receptors in sleep regulation, it will be interesting to see if flies respond to many clinically used hypnotics that target this receptor class.

Adenosine is thought to play a critical role in sleep homeostasis in mammals.[101] Adenosine has been implicated in the promotion of sleep in *Drosophila*. Adenosine, a metabolic product of ATP, acts through specific G protein–coupled receptors.[101] The stimulant effects of caffeine are thought to operate by antagonizing adenosine receptors. Flies fed caffeine exhibit reduced sleep, and flies administered cyclohexyladenosine, an adenosine agonist, exhibit increased sleep.[24,25] As mentioned earlier, these effects may be mediated by dopamine receptor function in the MBs.[95] The conservation of these central sleep-promoting pathways suggests an ancient function for GABA and adenosine.

WHICH GENES ARE REGULATED BY SLEEP–WAKE?

Although the focus of classic genetics is to identify genes whose function is important for a process of interest, it is also of interest to determine how sleep–wake might in turn regulate gene function or expression. A number of sleep–wake-sensitive changes in gene expression have been described, including the activity of a CRE reporter,[85] upregulation of the ER chaperone, BiP,[67] and immune-system genes.[69] The most extensive attempts at identifying sleep–wake-regulated genes have used DNA microarrays to assess genome-wide gene expression under conditions of sleep, wake, and sleep deprivation.[102,103] To control for circadian regulation of gene expression, sleep-deprived and spontaneously asleep animals are analyzed at the same circadian time. By identifying the genes that correlate with a behavioral state, one is presumably identifying factors that sustain or reflect that state. In addition, one can monitor gene expression linked to homeostatic drive by screening for genes whose expression increases with the duration of wakefulness. These changes in gene expression might provide clues to the molecular processes occurring during sleep and thus might reveal its underlying function.

SUMMARY

Since the turn of the 21st century, the fruit fly model of sleep has been validated as an important model for the study of sleep. The fly has many of the core features of sleep in common with mammalian systems. Many features of *Drosophila* sleep are independent of changes in spontaneous movement and include elevated arousal threshold, homeostatic regulation, electrophysiologic correlates, and conserved responses to sleep–wake regulatory drugs. In addition, genetic screens have identified shared genes and pathways of sleep control with their mammalian relations. Future work exploiting the power of genetics in this model organism promises to reveal the underlying molecular and cellular mechanisms of sleep regulation and ultimately provide some clues to the function of sleep.[104]

❖ Clinical Pearl

Chronic sleep loss can lead to adverse health consequences. Indeed, sleep deprivation in the fruit fly leads to premature death.

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Genetic Basis of Sleep in Rodents

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Chapter 14

Abstract

This chapter explores the progress and various approaches toward identifying genes important for an understanding of sleep and sleep-related traits in rodents. Until the functions of sleep are more clearly defined, it may be difficult to uncover the core genetic basis for sleep and the core molecular events that underlie this fundamental behavior in most, if not all, animal species. However, similar to other areas of medicine, an understanding of the underlying genetics will become increasingly important in sleep medicine. At this time, rodents represent the best animal models for such studies, especially the many genetically modified mouse lines that have been created and the mouse strains that form important genetic reference populations for a wide range of biomedical research. Given the similarities in physiology and genetics across eutherian mammals, it is likely that genes influencing sleep traits in mice also influence sleep traits in humans, or they can at least help identify the most critical molecular pathways and networks.

As discussed throughout the genetics section of this volume, genes and their allelic variants are important for an understanding of sleep for various reasons. For example, genetic analyses can allow researchers to identify critical functions and the core molecular pathways involved in normal sleep or in sleep disorders. This includes genes that play a role in the possible functional restorations of sleep such as energy state, protein levels, or some types of synaptic optimization (including, but not limited to, learning and memory). Genes and gene regulation may or may not be directly critical to the regulation of sleep–wake transition traits; nevertheless, genes still code for the proteins that are critical to all biological functions, and genetic analyses might still provide the keys for finding these functions. For example, state-to-state transitions between non–rapid eye movement (NREM) sleep, rapid eye movement (REM) sleep, and wake are probably too rapid to be regulated directly by gene expression but more likely involve posttranslational changes in ion channels or other proteins that alter membrane potential. Such proteins and the kinases and other proteins that alter their status are encoded by genes, and these genes most likely have allelic variation in murine and human populations that contribute to variations in sleep behavior. As a remarkable example of this, see the story of the sleepless mutation in *Drosophila*[1] discussed in Chapter 13, which supports the idea that hyperpolarization of neurons and ion channel regulation are a fundamental feature of sleep–wake regulation in all animals.

This chapter addresses various approaches to finding genes and gene alleles that influence sleep behavior. The genes that regulate sleep might or might not be different from those that are involved in the functional restorations that sleep may provide. Similarly, some genes that alter electroencephalogram (EEG) traits might or might not be central to the understanding of the function of sleep. In addition, a large group of genes vary their expression across sleep and wake, some of which are probably important to downstream functions relevant to sleep and wake even if they themselves are not core sleep-regulating genes.

To help understand the current state of the art in these various approaches towards understanding sleep, this chapter is divided into sections based on the most important general methodologies used to identify genetic mechanisms:

- Changes in gene expression, or more precisely changes in steady-state specific messenger RNA (mRNA) levels, during sleep and wake. The majority of these genes might simply be responding to arousal state, but some of these genes are likely to be critical components in the regulation of sleep–wake traits.
- Identification of naturally occurring allelic variants that underlie individual differences or strain differences in sleep-related traits. Some of these genes are likely to be important in sleep regulation or functional aspects of sleep, although many might represent pleiotropic genes that only indirectly alter sleep. Nevertheless, such genes and associated gene networks might still be important for understanding the underlying physiologic and pathophysiologic mechanisms that underlie normal sleep as well as sleep disorders. We particularly emphasize the quantitative trait locus (QTL) approach in uncovering the allelic variants influencing sleep–wake phenotypes.
- Identification of genes that regulate sleep or influence sleep-related traits using mutagenesis and transgenic strategies. These approaches artificially alter or knockout genes to find those that influence sleep.

GENE EXPRESSION, mRNAs, AND MICROARRAY STUDIES

The first class of genes that was clearly shown to vary across sleep and wake were rapid response genes, often called immediate early genes (IEGs) such as *Fos*.[2-4] Changes in expression of IEGs are of interest for at least two reasons. Because most IEG mRNAs and proteins increase with neuronal activity, they can be used to identify brain regions activated by changes in arousal state. Second, because most IEGs are transcription factors, they might represent

master-switch genes that initiate a complex of molecular signaling cascades.[5] These pathways may be important for longer-term homeostatic control or restorative functions critical to sleep and wake.

Most brain regions are more active during wake than during NREM sleep, and they thus show higher levels of expression of IEGs during wake.[2-4] This includes virtually all of the cerebral cortex, although even here there is an interesting exception. A small subset of gamma-aminobutyric acid–ergic (GABAergic) neurons that express nNOS (neuronal nitric oxide synthase) increase *Fos* expression during NREM sleep.[6] Consistent data (for the increased *Fos* expression) were obtained in three different rodent species (mouse, rat, and hamster). These sleep-active cortical neurons might play a critical role in some aspect of sleep homeostasis, such as the synchronous firing that underlies EEG delta power (slow-wave activity).

Another interesting region where *Fos* is higher in sleep than wake is in the ventrolateral preoptic area (VLPO), consistent with the VLPOs being a sleep-active region.[7] Neuroanatomic tracings linked this sleep-active region containing inhibitory GABA neurons to wake-active structures, including histaminergic neurons in the posterior hypothalamus, suggesting a reciprocal interaction between these two structures.[8] Later, other hypothalamic nuclei were integrated into a model indicating that several hypothalamic regions combine to play a critical role in sleep–wake regulation.[9] Among these other regions are the hypocretin-orexin neurons that are central to our growing understanding of narcolepsy. The exciting breakthrough that led to the elucidation of a critical role for the hypocretins in narcolepsy came from a combination of dog genetics,[10] mouse knockouts,[11] human pathology and pathophysiology,[12,13] and, earlier, the use of subtractive hybridization and rat neuroanatomy.[14] This research is described in detail in the last chapter of this section.

Outside the hypothalamus, expression of IEGs has been used to support and extend our understanding of the dorsolateral pontine region in the control of REM sleep[15] and the locus ceruleus in wake.[16] Other studies have shown interesting regional differences throughout the brain for the expression of specific IEGs during sleep deprivation and recovery sleep,[17] and similarly for some of the heat-shock or stress-response genes.[18] The results from studies in rats and mice are consistent with one another,[19] suggesting an important generalizability across rodents and probably all mammals. In addition, diurnal squirrels have peaks of IEG expression and heat-shock protein 70 (*Hspa1b*) during the day (instead of during the night, as in nocturnal rodents), consistent with high levels of mRNA correlating with wake.[20,21]

Although it is still unclear what role these IEGs and heat-shock mRNAs and proteins play, their consistent increase during periods of wake and neuronal activity suggest the possibility of some restorative role or, more specifically with IEGs, the need for transcriptional activation during wake. It is possible that transcription is preferentially activated during wake and translation into proteins is increased during sleep. This is supported by some earlier protein work using ^{14}C-Leu autoradiography in rat and monkey, which showed that the rate at which labeled leucine was incorporated into the brain was positively correlated with the occurrence of slow-wave sleep, suggesting the exciting possibility that one function of sleep might be the restoration of proteins through increased synthesis.[22,23] In general, protein data across sleep and wake periods have been limited due in part to the greater difficulty of identifying and quantifying proteins (see reference 24 for review) compared to measuring mRNA levels. For example, microarray technology allows for hundreds or thousands of mRNAs to be compared across conditions, and has generally supported these results.[19,25]

The first large-scale microarray study investigating sleep versus wake was done in rats.[25] Several interesting observations were made in this extensive survey that compared transcripts derived from sleeping (undisturbed) versus sleep-deprived rats killed at 6 PM (sleep deprivation was for 8 hours), as well as from rats sacrificed at 6 AM that had spent the majority of the night awake. More than 15,000 transcribed sequences were found to be present in the cerebral cortex. Of these 15,000, about 10% differed between day and night, and about half of these (5% of the total) varied between sleep and wake regardless of time of day. The cerebellum, a structure not generally associated with sleep, had similar changes, and about 5% of the detectable transcripts were differentially expressed between sleep and wake. Although the cerebellum does not display the electrographic signs of sleep comparable to EEG recordings of the cerebral cortex, this result is consistent with the idea that all or most neurons in the brain might require similar cellular restoration during sleep. The few areas of the brain that have higher activity during sleep, such as the VLPO, could potentially perform these tasks during wake. Different brain regions are likely to express a different constellation of genes. As discussed earlier, the hypothalamus alone has many different nuclei that appear to play central and counterbalancing roles in sleep and sleep regulation during sleep and wake. In keeping with this diversity, the hypothalamus appears to be the most different in gene expression changes across sleep and wake (at least relative to the cerebral cortex and basal forebrain)[19] and to have fewer significant mRNA changes,[26] perhaps due to its functional diversity.

The nature of the restorative or other useful processes occurring during sleep are not well understood. During wake, there is a notable increase in certain categories of mRNAs, including those involved in oxidative phosphorylation (e.g., mitochrondrial genes), and other energy-related processes (e.g., *Glut1*). Other genes showing increased activity during wake involve transcription factors such as the IEGs noted earlier and some of the clock-related genes (e.g., *Per2*, discussed in detail later), stress response factors (e.g., heat-shock proteins such as *Hsp5*), glutamatergic neurotransmission-related genes (e.g., *Nptx2*, *Homer1*), and mRNAs related to activity-dependent neural plasticity and long-term potentiation (e.g., *Arc*, *Bdnf*).[25] In contrast, during sleep, different categories of mRNAs appear to be upregulated (or downregulated with wakefulness), including those involved with translational machinery (e.g., *Eif4a2*), membrane trafficking (e.g., members of the *Rab* and *Arf* gene families), promoting hyperpolarization (potassium channels such as *Kcnk2*, *Knck3*), and synaptic plasticity related to memory consolidation (e.g., *Camk4*, *Ppp3ca*).

An issue in this and many other studies is how to define the sleep–wake state for each group of animals from which tissues are collected for microarray analysis. For example, although mice and rats spend the majority of the day sleeping and the majority of the night awake, they are often awake one third of the day and sleeping one third of the night (with sleep and wake periods typically alternating over periods of minutes or even seconds). In rats and mice one cannot assume that the animals are sleeping during the light or daytime, or are awake during the dark or nighttime at the time of sample collections. Also, some mRNAs can change rapidly, in which case the prior hour or two is paramount, whereas other mRNAs may be influenced over many more hours. Lastly, it is not clear in studies of sleep-deprived versus control animals whether a gene is sleep-induced or goes down with sleep deprivation. Incorporating recovery sleep periods following sleep deprivation can partially address this issue, especially if multiple recovery time points are examined (1 hour, 2 hours, 4 hours after sleep deprivation). This has been done for several clock-related genes such as *Per1*, *Per2*, and *Dbp*,[27,28] which generally return to baseline levels following recovery sleep. *Per1*, *Per2*, and other clock genes are central to circadian pacemaker function in the SCN, as discussed in Chapter 12. However, in other brain regions, these genes appear to be more responsive to sleep, wake, and activity state rather than their internal clock time.[27-29] Mutations and knockouts of these genes in mice produce not only the expected circadian disturbances but also, unexpectedly, fundamental changes to sleep homeostasis. This is discussed in detail later.

One microarray study examined sleeping versus sleep-deprived mice across the entire day and found a large number of mRNAs involved in biosynthesis and transport to be higher in the sleeping condition.[26] This was most apparent for genes involved with cholesterol synthesis and lipid transport. In general, this finding supports the hypothesis that one function of sleep may be the restoration of certain molecular components that are perhaps depleted during wake. Another microarray study compared sleep-deprived and control mice of three different inbred strains at four different time points over the 24-hour day.[30] Out of more than 2000 brain transcripts found to vary as a function of time of day under control conditions, fewer than 400 remained rhythmic when mice were sleep deprived, suggesting that most diurnal changes in gene expression are, in fact, sleep–wake-dependent instead of being under direct circadian control. This study also demonstrated that many of the changes in gene expression were strain specific, and only a relatively small number of transcripts changed consistently in all three strains. Of these, *Homer1a*, showed the most consistent and dramatic changes. *Homer1a* is a truncated form of *Homer1* which is involved in glutamate neurotransmission and probably in intercellular calcium homeostasis. Thus, it may be important for neuronal recovery or optimization following periods of wakefulness. The *Homer1* gene is also in the middle of a chromosome region shown to influence recovery from sleep deprivation[31] (see later), a finding that might lead to more definitive evidence of a role for this gene in sleep.

In summary, gene-expression studies have suggested that at least 5% of mRNAs vary across sleep and wake, but many of these changes might not be consistent across strains and species, and they may be dependent on the specific conditions of each experiment. It seems likely that an even higher percentage of transcripts vary slightly with arousal state, but the present methods are generally not sensitive enough to pick up small changes in steady-state mRNA levels. The roughly 5% of mRNAs with measurable changes over the sleep–wake cycle code for many different proteins that are likely to reflect specific functions of sleep and wake, but more data from different levels of organization are needed to document these specific functions.

One concern raised by this kind of approach is the time-course issue. This is perhaps most relevant with the changes in transcription factor mRNAs, which are among the most consistent and reliable changes documented thus far. Presumably, in order to produce a functional change in the brain, the mRNAs must first be made into proteins, return to the nucleus, activate or inactivate transcription of their target genes, and then, if activated, these mRNAs must be made into proteins and perform some function that ultimately alters a neuronal property (such as resting membrane potential) before there is any fundamental change in sleepiness or other sleep-related variables. In most cases, this will take several hours. Of course, other molecular changes such as phosphorylation of proteins can take place in seconds and alter many neuronal properties. However, this does not elucidate how changes in mRNA levels ultimately affect sleep and wake.

As discussed earlier, sleep pressure can build up over many hours and days, and certainly these long-term processes could be both monitored and regulated by these relatively slow transcriptional and translational changes. However, it has been shown in mice that sleepiness begins to accumulate after as little as 1 hour of wake,[31,32] raising the question of whether these slow mechanisms are fast enough to underlie the physiology of sleepiness. It is of course possible that there are both slower and faster homeostatic responses to different arousal states, as has been suggested for REM sleep,[32] and changes in gene expression and mRNA levels might underlie only the longer ones. It is also important to consider that most changes in steady state mRNA levels are probably driven by sleep–wake changes and not the other way around.

A final caveat in extrapolating from changes in gene expression across arousal state to a functional role of these genes in sleep–wake regulation is the finding that sleep deprivation results in more changes in mRNA levels in the liver than in the brain.[30] If sleep is "of the brain, by the brain, and for the brain,"[33] then how does one account for this observation? Perhaps the brain is protected in some way from changes in transcription during sleep deprivation, or perhaps the liver in fact needs these greater changes to respond to increased wake. Is it even possible that the liver or other tissues are in fact sleeping or awake in any meaningful way? Or, it could be a matter of statistics, with more probe sets reaching significance levels in the liver because it is a less heterogeneous tissue as compared to the brain, which is composed of many anatomically and functionally different structures and cell types expressing

different genes according to specific times of day (see the earlier example for sleep-active neurons in cerebral cortex and VLPO). Until we understand the functions of sleep with more certainty, it is difficult to define what sleep might mean for peripheral tissues. In any case, the numerous findings that now link sleep deprivation to profound changes in metabolism and the expression of genes in many peripheral tissues indicates that sleep is more than just for the brain and that sleep–wake states and durations have pronounced effects on gene expressions throughout the body.[34]

IDENTIFICATION OF ALLELES THAT INFLUENCE SLEEP OR SLEEP-RELATED TRAITS

Although gene-expression studies are likely to lead to a better understanding of certain aspects of sleep, as noted earlier, there are many limitations to this approach, especially the issue of causality. Therefore, it is important to use genetic approaches to better understand sleep and wake as complements to gene-expression studies. There is substantial evidence that allelic differences in sleep-related genes exist, and identifying these alleles, and the genes themselves, will likely lead to identifying important sleep-related functions.

Abundant evidence exists that many aspects of normal sleep as well as several sleep disorders have strong genetic components (reviewed in references 35 to 38). Results from twin studies make this especially clear (Fig. 14-1). First, brain architecture and regional activity are much more similar in monozygotic than in dizygotic twins (see Fig. 14-1A).[39] Second, EEG patterns of monozygotic twins have a much higher concordance than those of dizygotic twins, with the patterns in monozygotic twins being nearly as similar as in the same subject recorded on two different occasions (see Fig. 14-1B and C).[40-42] These results support the hypothesis that complex EEG traits are largely controlled by genes and that environmental factors play a lesser role. In fact, for many EEG traits, more than 80% of the variance appears to be accounted for by genetic factors, whereas for other sleep traits such as the amount or timing of sleep, the relative importance of genes and environment is probably closer to 50% each.[35-38]

Like twin studies in humans, genetic studies of sleep in the mouse, pioneered by Valatx, yielded substantial support for the genetic control of sleep. In the early 1970s, Valatx's group initiated a series of crossing experiments and recorded sleep in hundreds of inbred, recombinant inbred, and hybrid mice mainly to follow the segregation of REM sleep.[43-45] However, until very recently, none of the genes underlying these, or any other, sleep traits had been identified. The first significant breakthrough in the field occurred in 1999, with the discovery that a mutation of the hypocretin-2 receptor gene underlies canine narcolepsy[10] (see Chapter 15). This gene was certainly not one that would have been predicted to have a role in narcolepsy, highlighting the strength of the genetic approach.

In a genome-wide search for genes affecting a particular phenotype, no a priori assumptions on the gene systems involved are made. Although this approach might lead to already known physiologic mechanisms, its strength is that systems previously not known to be involved in sleep may be uncovered. Going from variability in a reliable sleep-related phenotype to the underlying genotypic variability of differing alleles (sometimes called *forward genetics* or *traditional genetics*) usually involves a generally standard approach.[46-49]

In the first step, the mode of inheritance for a trait of interest is determined in segregating offspring, although for most complex traits this does not appear as simple mendelian inheritance patterns. Next, the localization of the underlying gene or genes is mapped by examining the entire genome at regular intervals using polymorphic markers (e.g., RFLPs, SSLPs, SNPs). Traits generally co-segregate with the markers most closely linked to the underlying gene(s). For simple mendelian traits, initial mapping in a few hundred offspring can yield sub-centimorgan (cM) resolution (typically on the order of 1 million base pairs, with perhaps 10 genes in the defined genetically linked region). However, for non-mendelian complex traits, this initial step usually narrows the region to about 10 to 30 cM. Subsequent fine mapping, if feasible, can further reduce these regions. Positional cloning techniques can then be applied to find and characterize candidate gene sequences, with potential follow-up studies using gain- or loss-of-function knockout and transgenic mouse models to confirm the functional involvement of the candidate gene in affecting the trait under study (see later).

This approach has been most successful with mendelian monogenic traits such as for canine narcolepsy, as discussed earlier, and for studies involving mutagenesis, as discussed later, where a single mutated gene usually accounts for the phenotype. However, positional cloning is becoming increasingly successful in even the more common and difficult cases where multiple genes and environmental influences interact to produce a wide phenotypic range of a quantitative trait, such as EEG power spectrum differences among inbred strains of mice.

The standard approach toward mapping chromosomal regions that underlie quantitative traits is QTL analysis. QTL analysis is a good method to genetically dissect complex traits, like sleep, because naturally occurring allelic variations or gene mutations with smaller effects can be mapped.[46-49] QTL analysis can be performed in segregating mouse populations that involve intercrosses, backcrosses, recombinant inbred (RI) strains, or heterogeneous stocks. Often, two inbred mouse strains differing in a trait of interest are crossed and their F1 offspring are then intercrossed to generate F2 offspring. To generate RI sets, F2 mice are inbred by brother-sister matings for 20 generations until essentially full homozygosity is achieved, thereby fixing a unique set of recombinations in each RI strain.

Controversy exists concerning the efficacy of the QTL approach in identifying genes, and in the past, forward genetics by genome-wide mutagenesis has been favored due to greater ease of identifying the underlying gene once a genomic region of interest has been indentified.[50] Nonetheless, more than 2000 QTLs have now been mapped in rodents with a high level of confidence, and although only about 100 of these have been identified at the gene level, improved methods are making QTL cloning tractable, even for genes that only mildly affect the quantitative phe-

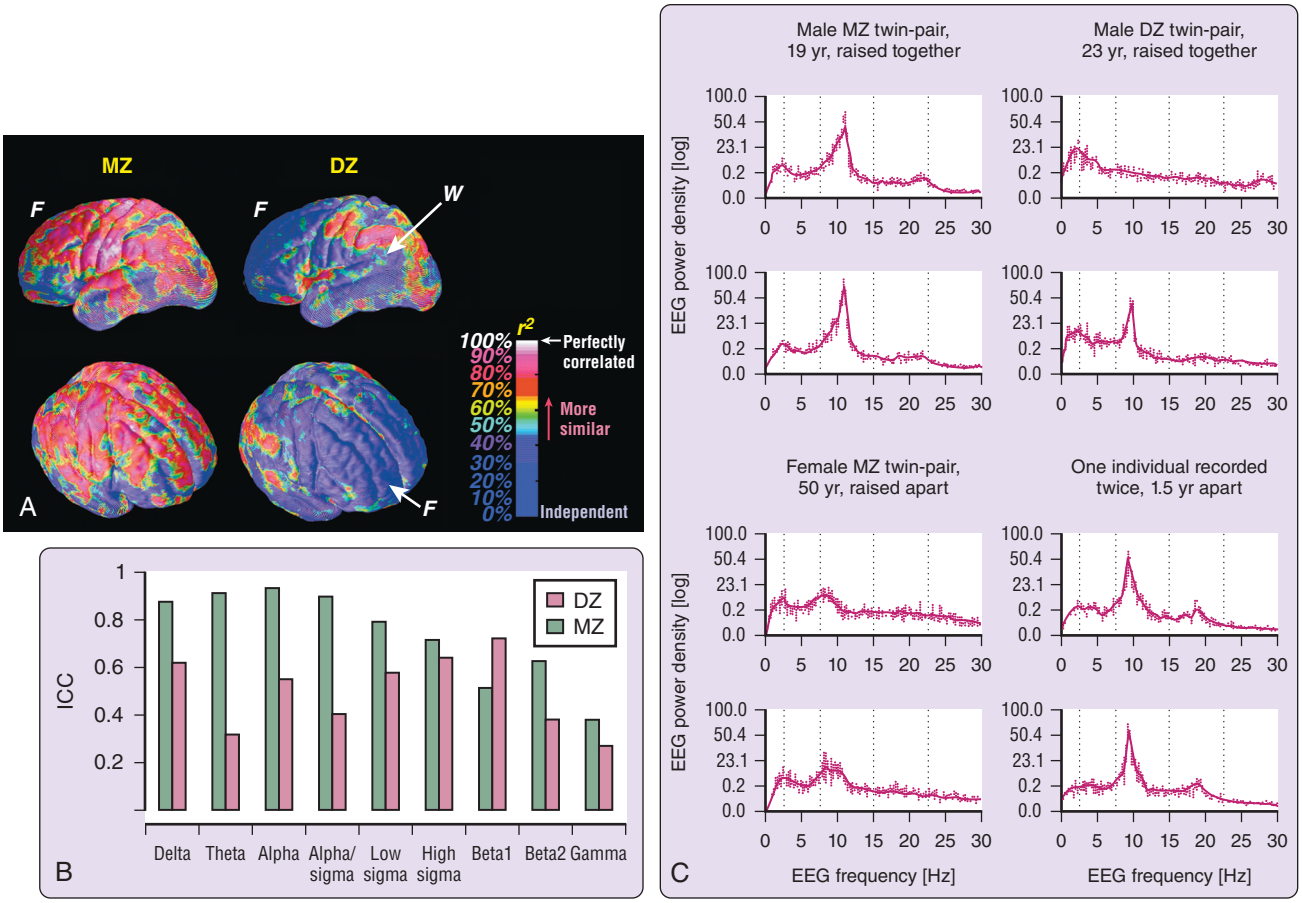

Figure 14-1 Basic brain structure and electroencephalogram (EEG) patterns are among the most highly heritable complex traits. **A,** Brain architecture is highly genetically determined. Genetically identical (monozygotic; MZ) twins are almost perfectly correlated in their gray matter distribution. Fraternal (dizygotic; DZ) twins are significantly less alike in frontal (F) cortices but are 90% to 100% correlated for gray matter in the perisylvian language-related cortex, including supramarginal and angular territories and Wernicke's language area (W). The significance of these increased similarities, visualized in color, is related to the local intraclass correlation coefficents (r). (From Thompson PM, Cannon TD, Narr KL, et al. Genetic influences on brain structure. Nat Neurosci 2001;4(12):1253-1258.) **B,** The spectral composition of the EEG during non–rapid eye movement (NREM) sleep is highly genetically determined. Intraclass correlation coefficients (ICCs) of EEG power in various frequency bands indicate a much higher concordance in MZ compared to DZ twin pairs, especially for lower frequencies (delta, theta, and alpha). (From Ambrosius U, Lietzenmaier S, Wehrle R, et al. Heritability of sleep electroencephalogram. Biol Psychiatry 2008;64(4):344-348.) **C,** The spectral composition of the waking EEG is equally under strong genetic control, and twin studies identified heritabilities of up to 90%, indicating that 90% in the variance of the phenotype can be accounted for by additive genetic factors. Note the higher similarly in MZ versus DZ twins even when raised apart. MZ twins are nearly as similar as the same subject recorded on two different occasions. (From Stassen HH, Lykken DT, Propping P, Bomben G. Genetic determination of the human EEG. Survey of recent results on twins reared together and apart. Hum Genet 1988;80(2):165-176.)

notype. QTL approaches can find different sets of genes than those uncovered in mutagenesis experiments and these procedures are thus complementary.[51] This has been shown to be the case in the circadian field.

Although most of the circadian genes that constitute the molecular circadian clock have been discovered via direct molecular techniques and mutagenesis (see Chapter 12), these genes alone do not explain the complexity of the observed circadian behavior. For example, none of the known circadian genes have been shown to regulate the differences in circadian period length or other circadian variables between BALB/c and C57BL/6 or other inbred mouse strains. By contrast, QTL analysis in a BALB/c x C57BL/6 intercross panel revealed several new loci that influence variables such as the free-running period, phase angle of entrainment, and the amplitude of the circadian rhythm of activity, as well as the total amount of activity.[52] In addition, the first QTL studies of circadian period length used BXD RI strains (derived from C57BL/6 and DBA/2) and CXB RI lines (derived from BALB/c and C57BL/6) to identify multiple loci that contribute to this trait.[53,54] To confirm these QTLs and identify new QTLs that influence circadian period, this same research group took one of the BXD strains (BXD19 with a period of 24.26 hours) and one of the CXB strains (CXB07 with a short period of 23.12 hours) and performed a standard intercross that did indeed confirm a major QTL on chromosome 1 and identified three additional QTLs.[55] Future identification of the gene alleles that underlie the QTLs from all of these studies should provide new information regarding the regulation of circadian period, activity levels, and other clock variables.

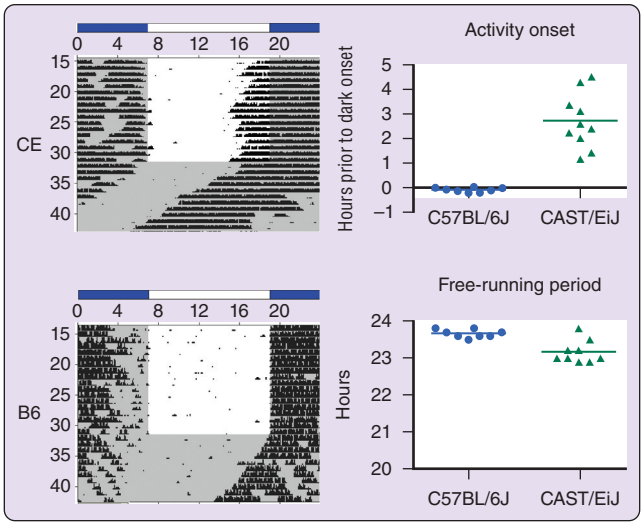

Figure 14-2 CAST/EiJ (CE) mice are early runners, with an advanced phase of activity relative to the light–dark cycle, and could thus provide a model for advanced sleep phase syndrome (ASPS). Typical mice such as C57BL/6J (B6) begin activity very close to dark onset *(blue circles)*. Ten out of ten CE mice *(green triangles)* have this advanced phase, suggesting a strong genetic component. However, the variability in the activity onset suggests important environmental or random factors as well. Note that CE mice have a shorter free-running period than B6, but this does not account for the magnitude of the advance (see Wisor JP, Striz M, DeVoss J, et al. A novel quantitative trait locus on mouse chromosome 18, "era1," modifies the entrainment of circadian rhythms. Sleep 2007;30(10):1255-1263).

Another example is the case of early-runner mice that show activity traits similar to those of humans with advanced sleep-phase syndrome (ASPS).[56] Virtually all normal mice begin their peak activity very close to dark onset. In contrast, early-runner mice begin their activity 2 to 6 hours before dark onset (Fig. 14-2), similar to ASPS humans who wake up several hours earlier than normal. Early runner mice also have shorter free-running periods than B6 mice, but this does not fully account for the several-hour phase advance in activity onset observed in these mice. Some cases of human ASPS are clustered in families and are called familial ASPS or FASPS (see Chapter 15 for more details). Inheritance is in a close to mendelian fashion, with mutations in specific genes such as *Per2*[57] or *CKI-delta*.[58]

Alternatively, more subtle allelic variations, such as those occurring in human *Per3*, might influence morningness and eveningness[59,60] along with environmental factors. At some point, extreme morning or evening preference can become ASPS or delayed sleep-phase syndrome (DSPS). The dramatic cases with known mutations are quite rare, and therefore are referred to as mutations rather than alleles. In contrast, the *Per3* alleles are common. Early-runner mice are more like this latter example in humans in that the primary QTL on chromosome 18 accounts for only about 10% of the total variance in this trait,[56] although it contributes a higher percentage of the genetic variance and the total variance in general daytime activity. Such mice may be useful in testing new pharmaceuticals or nonpharmaceutical approaches to treatment of ASPS.

Like circadian variables, many aspects of sleep and the EEG parameters measured during sleep differ dramatically among different inbred strains of mice,[31,44,61-65] and these differences are likely due to genetic factors. QTL analysis can be simplified in sets of RI strains that are derived from two parental inbred strains. In its simplest form, this mapping entails point correlations between the strain-distribution pattern (SDP) of the phenotype and the SDP of the genotype at each marker (Fig. 14-3 is an example of this approach). Polymorphic markers at a specific chromosomal locus that correlate significantly with the quantitative trait are presumably linked and co-segregate with the actual gene (or genes) whose differing alleles, derived from the two parental strains, contribute to the phenotypic variance.

Quantitative trait locus analysis of inter- and backcross panels also follows these same principles, but with two primary disadvantages. First, each offspring from these crosses is unique and must be genetically mapped to determine contributions from each parent—unlike RI strains where the recombination pattern is fixed by inbreeding and has already been mapped. Also, because each individual offspring is unique in traditional crosses (and cannot be made again), it is not possible to average over multiple mice. By contrast, in RI strains, observations from multiple identical mice can be averaged. However, a major advantage of traditional crosses is that a large number can be generated, unlike the very limited number of RI strains derived from a particular strain combination such as C (BALB/c) and B (C57BL/6). The limitation in numbers of RIs is partially compensated by the increased number of recombinations in each strain during the inbreeding process that produces improved mapping power per animal (approximately fourfold).

The limitations in the number of RIs are being addressed by extending existing RI panels by adding more strains, including a very large effort that could revolutionize complex genetics in mice for the study of almost any trait of interest, including sleep.[66] This major project is referred to as the Collaborative Cross because it will necessarily involve many research scientists. The cross includes eight different parental strains chosen for both their high genetic diversity and high diversity in almost every tested phenotype from cancer susceptibility to behavior. With plans for more than 1000 strains, and each strain having a very large number of historical recombination events fixed by inbreeding, it should be possible to map many QTLs at millimorgan resolution (essentially down to individual genes). Because RI lines only have to be mapped once, in theory, one can look at any trait in these mice such as total sleep time, analyze the correlations with allelic distributions, and have a very good idea of the gene alleles contributing to this trait. In a few years, when these mice are available, complete sequences for all eight founder strains are likely to be available as well, and it might even be possible in some instances to immediately predict which nucleotide difference among strains is responsible for a given QTL (sometimes referred to as a QTN, for quantitative trait nucleotide).

Currently, although only small sets of RI strains are available, these strains have nonetheless contributed significant advances to the understanding of genetic influ-

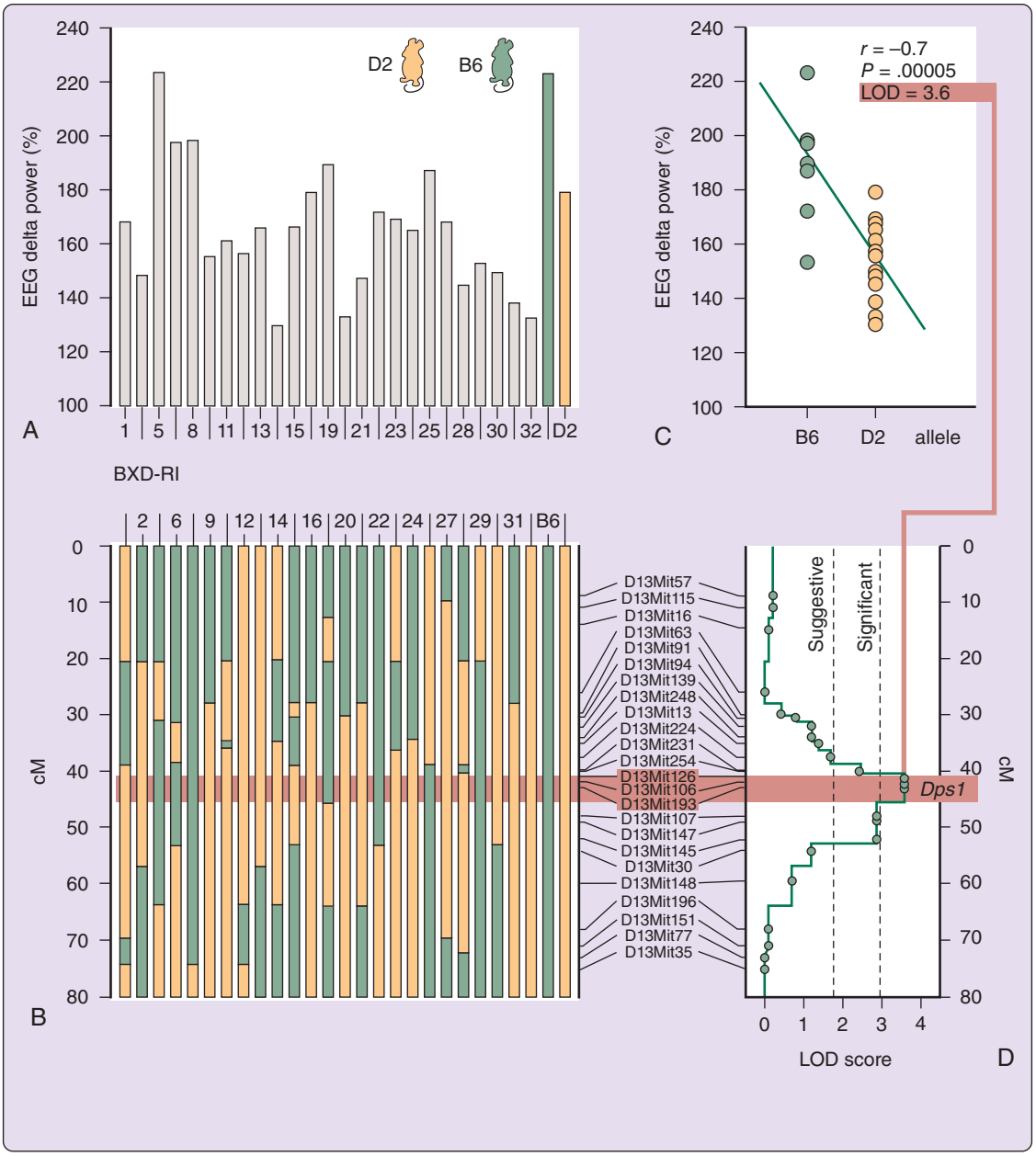

Figure 14-3 Quantitative trait locus (QTL) analysis for a sleep recovery trait in BXD recombinant inbred (RI) mice. QTL analysis is illustrated for the chromosome 13 *Dps1* QTL (see text and reference 31 for additional details). **A,** The strain-distribution pattern (SDP) of the sleep phenotype. In 25 BXD RI strains (BXD-1 to BXD-32) and their parentals C57Bl/6J (B6; *green*) and DBA/2J (D2; *orange*) the rebound in electroencephalogram (EEG) delta power was measured after 6 hours of sleep deprivation (*bars* indicate mean strain values; $n = 128$; 4-7/strain). **B,** BXD RI recombination pattern for chromosome 13 (B6 alleles in *green*; D2 alleles in *orange*). This pattern is based on the 24 Mit-markers polymorphic between B6 and D2 that were genotyped in the BXD RIs. Relative map positions in centimorgans (cM) from the centromere. For QTL mapping for each marker, the genotype SDP is correlated with the SDP of the phenotype. **C,** The SDP for markers D13Mit126, 106, and 193 (along the *red horizontal bar* in **B**) yielded the best correlation coefficient *(r),* which was highly significant *(P)* and translated into a LOD score of 3.6. **D,** Among all 788 Mit-markers used, only for these three markers was a genome-wide significant level ($P < .05$) obtained. This QTL was named *Dps1* (*Delta power in sleep-1*). The underlying assumption of the QTL approach is that a gene (or genes) within the *Dsp1* region segregated with the three D13Mit-markers in this BXD RI panel and that the B6 and D2 alleles for this gene are functionally different and modify the rebound in delta power after sleep deprivation. *Dsp1* has been confirmed for EEG delta power at sleep onset under baseline conditions, and refined mapping is in progress.

ences on sleep homeostasis. For example, the BXD RI strains were examined for sleep-related traits by EEG analysis to identify QTLs for the homeostatic regulation of NREM sleep.[31,36] The trait of interest was defined as the level of EEG delta power in NREM sleep reached after a 6-hour sleep deprivation (see Fig. 14-3A for variance in this trait among the two parental strains D2 and B6 and the 25 BXD RI strains). Large interstrain differences were observed in this trait and about 37% of the total variance could be attributed to additive genetic factors

(i.e., heritability). The contribution of the chromosome 13 QTL to the total genetic variance amounted to 49%, suggesting the presence of a major gene. Confirmation of the chromosome 13 QTL was obtained in baseline recordings of the same animals. This QTL was designated *Dps1* for delta-power-sleep-1. Two additional significant QTLs (*Dps2,3*) for sleep need at sleep onset in baseline were also identified. However, because EEG delta power is driven by the sleep–wake distribution, these genes presumably are more likely related to genotype-specific differences in the distribution of sleep and waking before sleep onset than to the homeostatic regulation of sleep itself. The basic assumption underlying the QTL analysis is that the identified chromosomal regions contain genes with functionally different alleles that somehow influence or regulate sleep homeostasis. As discussed in the prior section, *Homer1* is an excellent candidate gene, whose allelic variants might account for this QTL.

The same BXD RI lines also led to another QTL on chromosome 14 that contributes to the delta oscillations (1 to 4 Hz) that mark NREM sleep.[67] Although the study began as a QTL analysis, the data suggested that a single locus accounted for the majority of the variance in this trait. In this study, to verify that a single gene was responsible for the EEG phenotype, a panel of 30 different inbred strains was analyzed with 10 Mit-markers in this region of chromosome 14 and identified a unique biallelic marker D14Mit78 such that all 30 strains had either the B or D type allele. Next, a subset of highly divergent unrelated strains with the D allele were analyzed to see if they also had the predicted D EEG spectral properties (specifically, the power in the theta band, 6 to 7 Hz, divided by the power in the delta band). By using other polymorphic markers across many of these strains, a 350-kb region was identified as the smallest genomic region associated with this trait.

Within this region was retinoic acid receptor beta (*Rarb*), which contained a restriction fragment length polymorphism that co-segregated 100% with D14Mit78. *Rarb* has two different promoters that produce four different transcripts. Targeted deletion of these different transcripts, coupled with gene sequencing and real-time reverse transcriptase polymerase chain reaction (RT-PCR), clearly documented that alleles of *Rarb* did in fact underlie this EEG phenotype. Retinoic acid receptors and retinoid-X receptors are nuclear receptors that form heterodimers, are highly expressed in the brain, and are implicated in neuronal functions such as control of locomotion, long-term potentiation, and effects on dopaminergic and cholinergic neurotransmission.[68] These latter effects might underlie the role of *Rarb* in regulating the cortical synchrony that determines this EEG phenotype.

This same research group was able to use similar methods to identify another major gene, acyl-coenzyme A dehydrogenase (*Acads*), which, when mutated, dramatically alters the frequency of theta oscillations during REM sleep.[69] Microarray analysis of gene expression in mice with mutations in *Acads* also implicated *Glo1* (*glyoxylase 1*) as a key factor. This work suggested the surprising involvement of a metabolic pathway involving fatty acid β-oxidation in regulating theta oscillations during sleep. Since this work began as a QTL analysis, it would be fair to say that it represented the first successful identification of a gene that underlies a sleep-related QTL, and it is one of the first for any brain or behavior phenotype.

Apart from the above-mentioned studies, surprisingly few other QTL studies have investigated natural sleep and EEG traits. Despite decades of research in defining sleep-wake properties in mammals, little is known about the nature or identity of genes that regulate sleep, a fundamental behavior that in humans occupies about one third of the entire lifespan. Although genome-wide association studies in humans and QTL analyses in mice have identified candidate genes for an increasing number of complex traits and genetic diseases, the resources and time-consuming process necessary for obtaining detailed quantitative data have made sleep seemingly intractable to similar large-scale genomic approaches.

However, a study of natural sleep and EEG and EMG activity analyzed 20 different sleep–wake traits in 269 mice from a segregating population. The study revealed 52 significant QTLs, representing a minimum of 20 genomic loci, as being involved in the regulation of multiple and diverse sleep–wake traits.[70] Although many (28) QTLs affected a particular sleep–wake trait (e.g., amount of wake) across the full 24-hour day, other loci only affected a trait in the light or dark period, and some loci had opposite effects on the trait during light versus dark. Analysis of a dataset for multiple sleep–wake traits led to previously undetected interactions (including the differential genetic control of number and duration of REM bouts), as well as possible shared genetic regulatory mechanisms for seemingly different unrelated sleep–wake traits (e.g., number of arousals and REM latency). Construction of a bayesian network for sleep–wake traits and loci led to the identification of sub-networks of linkage not detectable in smaller data sets or limited single-trait analyses. Taken together, the results from this QTL study revealed a complex genetic landscape underlying numerous sleep–wake traits, and they emphasize the need for a systems biology approach for elucidating the full extent of the genetic regulatory mechanisms of this complex and universal behavior.[70]

MUTAGENESIS AND KNOCKOUTS

The QTL analysis aims at identifying naturally occurring allelic variants or gene mutations that modify sleep; in mutagenesis studies, however, gene function is assessed by randomly inducing mutations. Because mutagenesis studies involve a progression from phenotype to genotype (finding the mutation that alters the screened phenotype), it is also considered a forward genetics approach, similar to attempts to identify QTL associated with a particular phenotype or trait. In contrast, beginning with a gene of interest and using targeted deletion in mouse embryonic stem cells to knock it out, is a reverse genetics approach because the progression is from gene to phenotype (see later). As with other approaches, these distinctions can blur; for instance, if a study is initiated on a large collection of knockout mice, without identified genotypic effects, one might begin with phenotypic assessment, an approach that would have similarities to mutagenesis and forward genetics approaches. This is now becoming

feasible with the increasing number of knockout and other transgenic lines of mice and with high-throughput sleep analysis systems (see later). Whether it is forward or reverse genetics depends on whether one is screening more or less randomly or is selecting only genes with suspected roles in the traits under study.

Mutagenesis has been a successful technique for identifying genes that regulate circadian rhythms. A mutagen like *N*-ethyl-*N*-nitrosourea (ENU) mutates spermatogonia at an average rate of 0.001 mutations per locus per gamete.[49] With high-throughput screening of several hundreds or thousands of offspring for dominant, semidominant, or recessive mutations, a major effect on a given trait can be expected to be identified.[71,72] The individual mouse, fruit fly, or other organism for which an aberrant phenotype has been recorded then has to be crossed (usually with wild-type animals) to establish the mode of inheritance of this trait. The feasibility of this approach in the mouse was demonstrated by the isolation of the canonical circadian gene *Clock*.[73] Although some mutations produce dramatic phenotypic changes, as was the case for the mutant *Clock* gene, others produce only subtle effects that, in addition, can be confounded by epistatic interactions and genetic background (i.e., modifier genes).[74] In general, mutagenesis is probably more successful for fully penetrant dominant or recessive mutations, whereas the QTL approach is more powerful in detecting natural allelic variations controlling complex traits.

High-Throughput Screening

The sleep field has been fortunate that gene alleles with very large effects on sleep–wake traits are present in the common inbred strains of mice. However, the challenge for the future is to identify more typical QTLs that might contribute 1% to 5% of the genetic variance. Whether using QTL analysis or mutagenesis, both techniques require large numbers of mice to be screened to cover a majority of the genome (i.e., to produce functional alterations in a majority of the estimated 30,000 or so genes).[71] Although screening a thousand mice is currently quite cumbersome with traditional EEG recording, high-throughput methods to monitor sleep and wake in mice and other rodents might alleviate this problem.[75,76]

One high-throughput noninvasive technology uses piezoelectric films covering the cage floor that act as an extremely sensitive motion detector. During sleep, the primary movement is breathing and thus the system records a consistent periodicity of about 3 Hz, a rate representative of the respiratory rate in mice. During wake, a variety of movements produce a more erratic signal, because even during quiet wake, mice are grooming or making many more postural adjustments than during sleep. It might eventually be possible to distinguish REM versus NREM sleep because respiratory variability increases during REM sleep, but this has not yet been achieved.[75] This method could make mutagenesis screening for sleep traits more feasible and could take advantage of the very large collaborative crosses planned for mice.[66] It could also be used as an initial screen for sleep-promoting or wake-promoting drugs or could be used to screen the increasing number of available transgenic mice. Video methods for scoring sleep–wake traits are also showing increasing promise as a high-throughput method,[77] either in isolation or in combination with the piezo method.

Targeted Gene Deletion

Targeted gene deletion and other transgenic approaches have been exciting areas for sleep research and have been reviewed elsewhere,[36,78] but here we will discuss a few of the highlights. As discussed earlier, the use of these mouse models generally begins with a gene of interest that might influence some sleep phenotype, and so this is generally considered a reverse genetics approach (from gene to phenotype), as opposed to mendelian or QTL genetic approaches or mutagenesis approaches, which assay phenotype first with the goal of identifying the salient genes.

Reverse genetics approaches in mice have been made possible by the development of a range of techniques over the past several decades.[79,80] The field of sleep and circadian biology has benefited from knockout technology in which the insertion of a DNA construct into an exon results in a nonfunctional protein in mice, which are then bred to homozygosity. Advances have also come from transgenic methodology or nonhomologous (illegitimate) recombination, in which one or more DNA-construct copies are inserted into the genome at undefined locations, typically following injection of naked DNA into one of the two pronuclei at the one-cell stage.[79] These two techniques generally produce loss- or gain-of-function mutations, respectively. These models are also useful in confirming the role of genes that were identified by forward genetics approaches. Advantages and problems of these techniques have been addressed in other reviews.[81]

One important concern with respect to sleep regulation is developmental compensation, whereby other molecules, perhaps from the same gene family, could compensate for the lacking protein.[82] Other concerns involve nonspecificity (the relevant protein is absent in all the cells of the organism instead of the tissue of interest) and genetic background (genes that co-segregate with the introduced gene might differ between the background strain, often C57BL/6, and the strain in which the altered embryonic stem cells were introduced, usually a 129 strain) that might affect the phenotype.[83] Some of these issues can be overcome by developing (tissue-specific) conditional or inducible knockout models where the acute effects of loss-of-function can be studied in structures of interest[84]; however, most studies rely on the easier-to-construct general knockout mice or simple transgenic mice.

Despite these limitations, considerable knowledge has been gained from the study of knockout and other genetically altered mice, implicating genes in sleep and sleep-related traits in often unexpected ways. The first sleep studies using transgenic mice appeared in 1996.[85,86] Most knockout studies focus on pathways with previously described roles in sleep regulation such as monoamine neurotransmitters,[87] their receptors, and their transporters (reviewed in references 36 and 78). Additional studies have supported a role for cytokine pathways in the regulation of sleep, including interleukin-1, interleukin-10, tumor necrosis factor, and their receptors.[88] Finally, considerable information on sleep effects has been discovered about genes that are regarded as canonical circadian genes such as *Clock*, *Bmal1*, *Per1*, *Per2*, *Cry1*, *Cry2*, and *Npas2* (a

homologue of *Clock* expressed in the forebrain but rare in the suprachiasmatic nucleus [SCN]) and genes known to alter circadian rhythms such as *Dbp* and *Rab3a*.

Genetic Regulation of Homeostasis

The identification of genes involved in the homeostatic regulation of sleep has been advanced by the comparisons between baseline sleep and the responses to sleep deprivation. For instance, mice overexpressing growth hormone show more REM sleep under baseline conditions but show normal recovery patterns following sleep deprivation.[89] Disruptions of *Fos* increase wakefulness, while *Fos b* deletion reduces REM sleep. In addition, *Fos* knockout mice respond to sleep deprivation primarily with an increased latency to sleep onset.[90] In mice lacking the transcription factor *Dbp*, the circadian distribution of sleep was flatter and sleep was more fragmented. The NREM sleep response and the delta power response to sleep deprivation, however, did not differ from that in wild-type mice.[91] Also, in *Clock*-mutant mice the relative increase in NREM sleep after sleep deprivation was the same as in wild-type mice, although NREM sleep amount in baseline conditions was significantly reduced.[92]

Although a change in baseline sleep might reflect a change in sleep homeostasis, the lack of change following sleep deprivation raises the fundamental issue of what does qualify as a true change in the homeostatic response. For instance, mice lacking functional genes for the *serotonin-2C receptor* (*Htr2c*),[93] or *Rab3a*[94] all were reported to have an altered NREM sleep rebound after sleep deprivation. In these cases the difference was, however, attributable largely to NREM sleep differences under baseline conditions, because no differences in recovery sleep were observed.

Ideally, claims regarding an altered homeostatic regulation should be substantiated by quantifying the relationship between wake duration and the subsequent response of the regulated variable. This can be achieved by either establishing a dose-response relationship, in which the duration of the sleep deprivation is varied, or by mathematical means where the effects of spontaneous and enforced periods of wakefulness on a regulated variable are quantified.[31] Furthermore, especially where the regulation of NREM sleep is concerned, one cannot rely on only one aspect because changes in the duration and intensity or consolidation of sleep have to be taken into account.

Changes in REM-sleep homeostasis have also been observed in several knockout models. In mice lacking serotonin-1A or -1B receptors (*Htr1a*, or *-1b*),[95,96] *Dbp*,[91] *Cry1*, or *Cry2*[28] and in *Clock*-mutant mice,[92] loss of REM sleep was followed by a compensatory increase in REM sleep that was smaller than in wild-type animals or that was lacking altogether. Apart from *Htr1a* and *Htr1b* knockout mice that displayed increased REM sleep during baseline conditions,[95,96] these changes in the REM sleep response after sleep deprivation could not be attributed to genotype differences in REM sleep during baseline.

Genetic Regulation of Circadian Rhythm

One rationale for studying sleep in mice with modified or deleted genes critical for circadian rhythm generation, such as *Clock*, *Per1*, *Per2*, *Cry1*, or *Cry2*,[97] is that they provide a model in which sleep homeostasis can be studied in the presence of an altered or absent circadian modulation of sleep–wake time. Previously, the interactions between circadian and homeostatic influences on sleep were studied after circadian rhythms were eliminated by lesions of the SCN[98-101] or in studies in which subjects followed a forced-desynchrony protocol.[102,103] SCN lesion studies in rats revealed that direct circadian effects on the sleep homeostatic process were small, if present at all.[104] However, SCN-lesion studies in mice[105] and in monkeys[106] have found that SCN lesions can influence not only the timing of sleep but also the amount of sleep.

Deletions or mutations in circadian genes also influence sleep–wake traits beyond just the timing of sleep, including alterations in the homeostatic regulation of sleep. A clear demonstration of this was observed in *Cry1*,*Cry2* double knockout mice that under baseline conditions showed all the hallmarks of high NREM sleep pressure, including a higher amount of NREM sleep, increased NREM sleep consolidation, and higher NREM sleep delta power compared to wild-type controls.[28] After 6 hours of sleep deprivation, there was no further increase in NREM sleep time or consolidation or in the reduced rebound in EEG delta power. This suggests that apart from their role in regulating circadian rhythms, *Cry* genes or genes regulated by CRY (such as *Per1* and *Per2*) play a role in sleep homeostasis. Because *Per1* and *Per2* mRNA levels are responsive to sleep deprivation,[25,28,107] knockouts of these genes might be expected to have effects on sleep homeostasis. In two independent sets of experiments examining mice with nonfunctional *Per1*, *Per2*, or *Per3* and double knockouts of *Per1* and *Per2*, the authors suggested that there were no substantial alterations in sleep homeostasis.[108,109] However, in our view, these data might actually support an important role for *Per* genes in sleep homeostasis, because a number of sleep related changes were found in these mice.

Results from these studies showed *Per* mutations affected total sleep time, the timing of sleep, as well as the effects of light and dark on sleep patterns, REM sleep, delta power during sleep recovery following sleep deprivation, and certain other parameters. The authors noted that the most clear-cut differences from wild-type animals were in sleep distribution, consistent with circadian alterations, with *Per1* knockouts sleeping more at the dark-to-light transition and *Per2* knockouts sleeping more at the light-to-dark transition. However, other sleep perturbations were also observed that do not appear to be primarily circadian in nature. *Per1*-knockouts and *Per1/Per2*-double knockouts spent a much longer time in elevated delta power following sleep deprivation, from 5 to 12 hours compared to 3 hours in the wild-type mice. This contrasts with results found in *Cry1/Cry2*-double knockouts, which seem to have a greater overall sleep drive under baseline conditions, including a higher delta power, but that have elevated delta power for only 1 hour following sleep deprivation. One possibility suggested for the reduced increase in delta power in sleep-deprived *Cry1,2* knockout mice is that delta power is already so high it cannot be increased any further.

Taken together, these results are consistent with the possibility that the *Per* genes are involved in the process that ultimately modulates the sleep–wake dependent changes in EEG delta power. The absence of a more dramatic change in sleep in response to deletion of the *Per*

genes is perhaps not surprising because there are three *Per* genes, and each may be able to compensate for a loss in one or two of these genes.

Other genes in the molecular circadian clock network that perform similar functions might also be able to compensate for the loss of one member. This compensation might be even greater in regions of the brain outside the SCN where these genes may play greater roles in other processes. Further evidence that clock genes play a role in sleep homeostasis comes from work on *Npas2*-deficient mice. *Npas2* is a paralogue of *Clock* that can also bind with *Bmal1*, widely expressed in the forebrain but difficult to detect in the SCN. *Npas2*-deficient mice have normal circadian rhythms, although a very small amount of *Npas2* expression in the SCN appears to compensate for a complete loss of the *Clock* gene.[110]

In any event, the alterations in sleep behavior in *Npas2*-deficient mice are not the result of a disruption in circadian rhythmicity. *Npas2* knockout mice lack the typical nap found in the latter half of the dark period that is invariably present in wild-type mice of this strain.[29] Following sleep deprivation, however, these mice were also incapable of initiating the appropriate compensatory sleep response during the circadian phase when mice are usually awake. Similarly, these mice do poorly or even die when food availability is restricted to the light portion of the diurnal cycle.[111] These results suggest that the clock genes in the forebrain integrate behaviors such as sleep, wake, and feeding with physiologic cues, and when these cues are disrupted, the SCN-dependent circadian drive might dominate, limiting the expression of adaptive behaviors that can differ from the normal circadian peak for that behavior. These *Npas2* knockout results may be of particular interest because NPAS2/BMAL1 and CLOCK/BMAL1 heterodimers are redox sensitive, which might tie this gene network to basic energy metabolism.[112,113] Because restoration of an optimal neural energy state has long been considered a possible function of sleep, as discussed earlier,[114] this gene network might underlie a fundamental energy restorative aspect of sleep homeostasis.

The suggestion that clock genes are fundamental to sleep homeostasis (in addition to their role in the circadian pacemaker) is strengthened by observations in the fruit fly, *Drosophila*, where mutants carrying loss-of-function mutations for the canonical circadian genes *Per*, *Timeless*, *Clock*, or *Cycle* (the *Bmal1* orthologue) all show a more-pronounced sleep rebound after sleep deprivation than wild-type flies[115] (as discussed in Chapter 13). *Cycle*-mutant flies were exceptional in this respect because they clearly overcompensated for the amount of sleep lost, and this increase in sleep seemed permanent. In addition, *Cycle*-mutant fruit flies died after sleep deprivations of 10 hours or more, whereas wild-type flies typically survive for about 50 hours.

It has become increasingly accepted that rest and activity in flies share many features with sleep–wake states in mammals.[116-123] Mice homozygous for the *Bmal1* deletion showed an attenuated rhythm of sleep and wake distribution across the 24-hour period. In addition, these mice showed increases in total sleep time, sleep fragmentation, and EEG delta power under baseline conditions and an attenuated compensatory response to acute sleep deprivation.[124] Therefore, lack of circadian genes in mice and flies do not only affect circadian rhythms. In humans, as discussed in Chapter 15 and elsewhere, a simple repeat polymorphism in *Per3* has been shown to significantly affect performance following sleep deprivation[59,125,126] (in addition to the morningness-eveningness trait mentioned earlier). In retrospect, this might not be too surprising, because circadian genes are expressed throughout the brain and the body, not only in the SCNs of mammals or the small ventrolateral neurons of the fruit fly. In addition, many of the circadian clock genes are pleiotropic and affect a number of physiologic processes, not just timing. Therefore, the involvement of circadian genes in sleep homeostasis represents a new and intriguing molecular pathway for future research.[116]

❖ Clinical Pearl

An understanding of the genes and gene variants that influence sleep and wake quality and the susceptibility to sleep loss might suggest novel targets or approaches to improve sleep and wake, as well as for the treatment of sleep disorders. As in other areas of medicine, allelic differences in these genes might suggest different treatments for different patients with the same disorder, because the pathophysiology may be distinct. The combination of rodent studies using molecular, forward, and reverse genetic approaches, in combination with human studies, are likely to lead to novel insights into the regulation and function of sleep, which can then be translated into improved treatments for sleep–wake disorders as well as other mental and physical disorders associated with disrupted sleep.

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Genetic Basis of Sleep in Healthy Humans

Hans-Peter Landolt and Derk-Jan Dijk

Chapter 15

Abstract

Sleep is a very rich phenotype, and many aspects of sleep differ considerably in the population of healthy individuals even when only a very narrow age range is considered. Inter-individual variation in diurnal preference, sleep duration, and sleep structure, and variation in the electroencephalogram (EEG) during rapid eye movement (REM) sleep, non-REM sleep, and wakefulness, have all been shown to have a genetic basis. The response to challenges of sleep regulatory processes such as sleep deprivation and circadian misalignment has also been shown to vary between individuals. Some of the polymorphic variations in genes contributing to variation in sleep characteristics have now been identified. They include variations in genes associated with the circadian system (CLOCK, PER1, PER2, PER3), *the adenosine system* (ADA, ADORA2A), *and the catecholaminergic system* (COMT, AA-NAT). *For some of these genes, associations with only one aspect of sleep have so far been reported (e.g., for* PER2 *and sleep timing). Variations in other genes have been shown to affect multiple aspects of sleep and wakefulness, as well as the response to sleep loss or pharmacologic interventions. For example,* PER3 *and* ADA *affect the EEG and performance during prolonged waking, whereas* ADORA2A *and* COMT *modulate EEG and response to the stimulants caffeine and modafinil. All currently known polymorphic variations explain only a small part of the variation in healthy human sleep phenotypes, and many more genetic contributions remain to be discovered.*

EVIDENCE FOR GENOTYPE-DEPENDENT DIFFERENCES IN DIURNAL PREFERENCE, SLEEP TIMING, SLEEP DURATION, SLEEP ARCHITECTURE, AND SLEEP EEG

Many aspects of sleep and sleep–wake regulation are highly variable between individuals, yet highly stable within individuals. Uncovering genetic factors contributing to these traitlike individual differences in healthy humans constitutes one of the most promising avenues for fostering our understanding of the neurobiology of sleep in health and disease. This chapter will summarize the current evidence for genotype-dependent differences in duration, timing, and structure of sleep, as well as the sleep EEG, in healthy individuals. Table 15-1 summarizes the genetic variations in candidate genes that have been investigated to date to determine whether they contribute to genotype-dependent differences in diurnal preference, sleep timing, sleep duration, sleep structure, and sleep EEG. We will also review how these differences may relate to the homeostatic and circadian regulation of sleep. Several sleep characteristics differ between the sexes and between ethnic groups, but these differences will not be discussed here.

The manifestation and regulation of sleep and the sleep EEG reflect different aspects of complex behaviors. Each of these aspects is likely to be under the control of multiple genes, which may interact, and which are also influenced by the environment and other factors such as age. In humans, very little is currently known about the genes that contribute to the traitlike, individual sleep phenotypes. Similarly, very little is known about the genes that contribute to individual circadian-phenotypes, although a considerable number of genes that contribute to circadian rhythmicity have been discovered in animals.

Two main techniques for the genetic dissection of normal human sleep are available. The first is to examine the impact of candidate genes—that is, genes for which evidence exists that they are involved in sleep and sleep–wake regulation. With this method, individuals with distinct genotypes of known genetic polymorphisms are prospectively studied in the sleep laboratory. This approach precludes discovery of novel "sleep genes," but it may help us understand the consequences of these polymorphisms for sleep physiology. By contrast, genome-wide association studies may lead to the identification of novel "sleep genes," which may lead to the discovery of novel sleep regulatory pathways. These studies, however, require very large sample sizes and multiple replications. The weaknesses and strengths of these strategies were recently discussed in detail.[1]

Large interindividual differences are observed in preferred time of day for completion of distinct cognitive tasks, sleep timing, sleep duration, sleep structure, and sleep EEG. Genes contribute to each of these phenotypes, and a high degree of heritability (i.e., the percentage of variance explained by overall genetic effects) has been demonstrated for these variables. For some of these variables, the magnitude of interindividual differences exceeds by far the size of the effects of manipulations of sleep regulatory processes, such as sleep deprivation.[2]

GENES CONTRIBUTING TO HUMAN MORNINGNESS–EVENINGNESS AND TIMING OF SLEEP

Candidate Genes

The timing of the peaks and troughs of daytime alertness and the timing of nocturnal sleep (i.e., diurnal preference) are highly variable among healthy individuals. Some of us go to sleep when others wake up. Self-rating scales such as the Horne-Östberg morningness–eveningness questionnaire (MEQ) and the diurnal type scale show normal distribution along a morningness–eveningness axis,[3,4] indicating the contribution of additive, small effects of multiple genes in combination with the environment. Recent studies of large numbers of monozygotic (MZ) and dizygotic (DZ) twin pairs and of population- and family-

Table 15-1 Genes Investigated to Determine Their Contribution to Genotype-Dependent Differences

NCBI SNP-ID NUMBER	CHROM.	GENE	BASE CHANGE	AMINO ACID SUBSTITUTION	ALLELE FREQUENCIES* (%)
rs1801260	4	*CLOCK*	c.3111T/C	n/a	73 : 27
rs2070062	4	*CLOCK*	c.257T/G	n/a	73 : 27
	17	*PER1*	c.2548G/A	n/a	85 : 15
rs2735611	17	*PER1*	c.2434T/C	n/a	82.5 : 17.5
	2	*PER2*	c.1984A/G	Ser662Gly	100 : 0
rs2304672	2	*PER2*	c.111G/C	n/a	84 : 16
rs57875989	1	*PER3*	del(3031-3084 nt)	del(1011-1028 aa)	66 : 34
	17	*AANAT*	c.619G/A	Ala129Thr	100 : 0
	17	*AANAT*	c.-263G/C	n/a	51 : 49
	20	*ADA*	c.22G/A	Asp8Asn	94.5 : 5.5
rs5751876	22	*ADORA2A*	c.1083T/C	n/a	40 : 60
rs4680	22	*COMT*	c.472G/A	Val158Met	51 : 49
rs1799990	20	*PRNP*	c.385A/G	Met129Val	—

NCBI SNP-ID number, National Center for Biotechnology Information single nucleotide polymorphism reference number; *chrom*, human chromosome number; *gene*, NCBI gene symbol; *base change*, nucleotide substitution at indicated position of coding DNA; amino acid substitution, amino acid substitution associated with base change; *n/a*, no amino acid substitution (silent polymorphism); (√), possible contribution to phenotypic variation was investigated and reported; (*), allele and genotype frequencies may vary considerably among different ethnic populations. Values refer to published data from mainly white populations (except the values for c.263G/C polymorphism of *AANAT*, which are derived from a Japanese population).

based cohorts revealed roughly 50% heritability for diurnal preference[5] and 22% to 25% for habitual bedtime.[6,7]

Morningness–eveningness and timing of sleep are thought to be determined in part by circadian oscillators. At the molecular level, these oscillators consist of a network of interlocked transcriptional or translational feedback loops, which involve several clock-related genes including the transcription regulators *CLOCK*, *BMAL1*, *PER1-3*, *CRY1-2*, *TIM*, and other genes. This knowledge has provided a rational basis for the search for associations between these genes and morningness–eveningness and altered sleep timing.

The effect of a single nucleotide polymorphism (SNP) in the 3′-untranslated region (UTR) of the human "Circadian locomotor output cycles kaput" gene *(CLOCK)*, located on chromosome 4, on diurnal preference was first studied in middle-aged adults. This SNP may affect the stability and half-life of messenger RNA,[8] and thus alter the protein level that is finally translated. Katzenberg and colleagues[9] reported that homozygous carriers of the 3111C allele have increased evening preference for mental activities and sleep, with delays ranging from 10 to 44 minutes, when compared with individuals carrying the 3111T allele. A similar association with diurnal preference was found in a Japanese population, and MEQ scores were significantly correlated with sleep onset time and wake time.[4] By contrast, studies in healthy European and Brazilian samples failed to confirm an association between genetic variation in *CLOCK* and diurnal preference.[10,11] Interestingly, an almost complete linkage

GENOTYPE FREQUENCIES* (%)	DIURNAL PREFERENCE	SLEEP TIMING	SLEEP DURATION	SLEEP STRUCTURE	SLEEP EEG
T/T = 40 T/C = 53 C/C = 7	√	√	√		
T/T = 40 T/C = 53 C/C = 7	√				
G/G = 72 G/A = 27 A/A = 1	√				
T/T = 69 T/C = 27 C/C = 4	√				
A/A = 100 A/G = 0 G/G = 0		√			
C/C = 70 C/G = 28 G/G = 3	√	√			
PER3$^{4/4}$ = 43 *PER3*$^{4/5}$ = 48 *PER3*$^{5/5}$ = 9	√	√		√	√
G/G = 100 G/A = 0 A/A = 0	√				
G/G = 28 G/C = 47 C/C = 25		√	√		
G/G = 89 G/A = 11 A/A = 0				√	√
T/T = 13 T/C = 54 C/C = 33				√	√
G/G = 25 G/A = 53 A/A = 22	√			√	√
A/A = 37 A/G = 51 G/G = 12					√

disequilibrium was shown between the 3111T→C and the 257T→G polymorphisms located in the other extremity of this gene.[11] Full-length analysis of secondary mRNA structure revealed no interaction between the two polymorphisms.

Mouse *Per1* and *Per2* are importantly involved in maintaining circadian rhythmicity,[12] and possible associations between variation in these genes and diurnal preference were thus also investigated in humans. Screening for missense mutations and functional or synonymous polymorphisms in promoter, 5′- and 3′-UTR, and coding regions of the period-1 gene (*PER1*) in volunteers with extreme diurnal preference and patients with delayed sleep-phase syndrome (DSPS) remained initially unsuccessful.[13,14] By contrast, the distribution of the C and T alleles of a silent polymorphism in exon 18 was found to differ between extreme morning and evening types.[14] Thus, the frequency of the 2434C allele was roughly double in subjects with extreme morning preference (24%) compared with subjects with extreme evening preference (12%). This polymorphism may be linked to another functional polymorphism, or it may directly affect *PER1* expression at the translational level.[14]

A missense mutation in the human period-2 gene (*PER2*) currently provides the most striking example of a direct link with between genetic variation in a clock gene and changed circadian rhythms. Linkage analyses in families afflicted with familial advanced sleep-phase syndrome (FASPS) revealed associations with functional polymorphisms of *PER2* that cause altered amino acid sequences in regions important for phosphorylation of this protein[15] and a mutation in casein kinase delta (CKδ), which plays an important role in phosphorylation.[16] The subsequent finding in a transgenic mouse model express-

ing the human FASPS mutation that casein kinase I delta (CKIδ) can regulate circadian period through *PER2* provided further evidence that this gene is importantly involved in the mechanisms of circadian rhythm regulation in humans.[17] In accordance with this notion, a C111G polymorphism located in the 5′-UTR of *PER2* modulates diurnal preference in healthy volunteers.[18] Thus, the 111G allele is significantly more prevalent in subjects with extreme morning preference (14%) than in individuals with extreme evening preference (3%). Computer simulation predicted that the 111G allele has a secondary RNA structure different from that of the 111C allele, and that the two transcripts may be differently translated.[18]

Findings in mice suggest that *Per3* functions outside the core circadian clock work.[12] Nevertheless, a variable-number tandem-repeat (VNTR) polymorphism in the human period-3 gene *(PER3)* also appears to modulate morning and evening preference. A 54-nucleotide sequence located in a coding region of this gene on human chromosome 1 is repeated in either four or five units. This difference may alter the dynamics in PER3 protein phosphorylation. The longer five-repeat allele was associated in European and Brazilian populations with morning preference, and the shorter four-repeat allele with evening preference, respectively.[19,20]

The gene encoding arylalkylamine *N*-acetyl-transferase *(AANAT)* is located on human chromosome 17q25. This enzyme plays a key role in melatonin synthesis and thus may be important for diurnal preference and circadian rhythm disturbances. Comparison in a Japanese population between 50 outpatients diagnosed with DSPS and 161 unrelated healthy controls suggested that the frequency of a seldom-occurring threonine allele at codon 129 is significantly higher in patients than in controls.[21] This association was not confirmed in a Brazilian population, where virtually no allelic variation at this position was found.[22] In a small study conducted in Singapore, it was suggested that a commonly occurring, silent 263G→C polymorphism of *AANAT* modulates sleep timing and sleep duration (also see later) in healthy students.[23]

Genome-Wide Association Study

Only one genome-wide association study of sleep-related phenotypes is currently available in humans.[6] In the Framingham Heart Study 100K Project, phenotypic and genetic analyses were conducted in 749 subjects and revealed a heritability estimate for habitual bedtime of 22%. This small study suggests that a nonsynonymous polymorphism in a coding region of the gene encoding neuropeptide S receptor 1 *(NPSR1)* is a possible modulator of usual bedtime as obtained from a self-completion questionnaire. This polymorphism leads to a gain-of-function mutation in the receptor protein by increasing the sensitivity for neuropeptide S receptor 10-fold.[24] Although a possible association of NPSR1 to weekday bedtime is interesting, the statistical power of this pilot study is limited, and the necessary replication of this finding in independent samples is lacking. A recent analysis of a larger sample of the Framingham Offspring Cohort did not parallel the prior result.[7]

GENES CONTRIBUTING TO HABITUAL SLEEP DURATION

Habitual sleep duration, like diurnal preference, shows large variation between healthy individuals, and the physiologic sleep and circadian correlates of habitual short and long sleepers have been identified in small groups of subjects.[25-27] The temporal profiles of nocturnal melatonin and cortisol levels, body temperature, and sleepiness under constant environmental conditions and in the absence of sleep suggest that the circadian pacemaker programs a longer biological night in long sleepers than in short sleepers.[27] Individual differences in this program may contribute to the large variation in habitual sleep duration, which shows a perfect normal distribution in the general population.[28,29] Such a distribution is consistent with the influence of multiple, low-penetrance polymorphisms. Several older-twin studies and one recent genome-wide association study reported for sleep duration moderate heritability estimates of 17% to 40%.[6,30-32]

The Framingham Heart Study 100K Project revealed a linkage peak to usual sleep duration on chromosome 3 including the gene encoding prokineticin 2 (*PROK2*).[6] This neuropeptide may be an important output molecule from the suprachiasmatic nucleus (SCN), in particular in defining the onset and maintenance of the circadian night.[33,34] Because the danger of false-positive inferences from small genome-wide association studies is high, the methodological limitations of this work discussed earlier also apply to this potential association. It was not corroborated in a larger sample of the Framingham Cohort.[7]

GENES CONTRIBUTING TO SLEEP ARCHITECTURE

Many variables characterizing sleep architecture demonstrate large variation between individuals and high stability within individuals.[2,35-37] For example, the intraclass correlation coefficients, which estimate the intraindividual stability of a variable across conditions (e.g., baseline versus sleep deprivation), was reported to be 0.73 for slow-wave sleep (SWS) and 0.48 for REM sleep.[2] This observation suggests the presence of traitlike, interindividual differences in sleep physiology, which have a genetic basis. Indeed, twin studies show striking similarity and concordance in visually defined sleep variables in MZ twins, yet not in DZ twins. Already the first polysomnographic sleep studies in MZ twins have revealed almost complete concordance in the temporal sequence of sleep stages.[38] Subsequent work showed that in particular those variables that most reliably reflect sleep need are under tight genetic control. Apart from total sleep time, they include duration of non-REM sleep stages, especially SWS, and density of rapid eye movements in REM sleep.[39-41] Linkowski[41] estimated that the heritability of REM density is up to 90%.

Slow-Wave Sleep and REM Sleep

A few studies have conducted polysomnographic assessment in defined genotypes. The *CLOCK* genotypes that were associated with diurnal preference[9] did not signifi-

cantly affect sleep variables derived from nocturnal polysomnography.

Only two polymorphisms have been identified to date as affecting sleep architecture in humans. First, in one study, homozygous carriers of the long-repeat genotype in *PER3 (PER3$^{5/5}$)*, which associates with morningness, fell asleep more rapidly (about 9 versus 18 minutes) and showed more SWS (about 23% versus 16% of total sleep time) compared with homozygous four-repeat individuals.[42] In addition, during recovery sleep from sleep deprivation, REM sleep was reduced in *PER3$^{5/5}$* individuals.

Second, a functional variation in the gene on chromosome 20 encoding the enzyme adenosine deaminase *(ADA)* was found to have a profound impact on sleep architecture.[43] Adenosine and adenosine receptors are thought to be involved in regulating distinct aspects of human sleep,[44] and ADA plays an important role in regulating extracellular adenosine levels. Healthy individuals with the G/A genotype, associated with lower ADA activity in erythrocytes and leucocytes than in individuals with the G/G genotype, showed significantly more SWS in a baseline recording than subjects with the G/G genotype (about 93 versus 63 minutes). This difference is comparable to the difference between a baseline night and recovery night after one night without sleep. All other sleep variables were similar in both genotypes.

The Sleep EEG: Among the Most Heritable Traits in Humans

Visual sleep-state scoring relies on arbitrarily defined criteria and can reveal only limited information about sleep physiology. To obtain more detailed insights, quantitative analyses of the EEG signal recorded during sleep have to be performed. A powerful approach to quantify amplitude and prevalence of EEG oscillations with distinct frequencies is power spectral analysis based on fast Fourier transform (FFT).[36,45,46] Recent studies strongly suggest that especially the sleep EEG, but also the waking EEG, are highly heritable traits in humans. All-night sleep EEG spectra derived from multiple recordings in healthy individuals show large interindividual variation and high intraindividual stability.[36,37] Buckelmüller and colleagues[37] recorded in eight young men two pairs of baseline nights separated by 4 weeks. Although the spectra in non-REM sleep differed largely between individuals, the absolute power values and the shape of each subject's spectra were impressively constant across all nights (Fig. 15-1). The largest differences among the subjects were present in the theta, alpha, and sigma (i.e., about 5 to 15 Hz) range. Hierarchical cluster analysis of Euclidean distances based on spectral values as feature vectors demonstrated that all four nights of each individual segregated into the same single cluster.[37] Similar results were obtained in REM sleep, and by other researchers in men and women of older age.[36] These data strongly suggest that the sleep EEG contains systematic and stable interindividual differences that are at least in part genetically determined. This conclusion is supported by two recent studies comparing for the first time the spectral composition of the sleep EEG between MZ and DZ twin pairs. In non-REM sleep, the within-pair concordance in spectral power in the

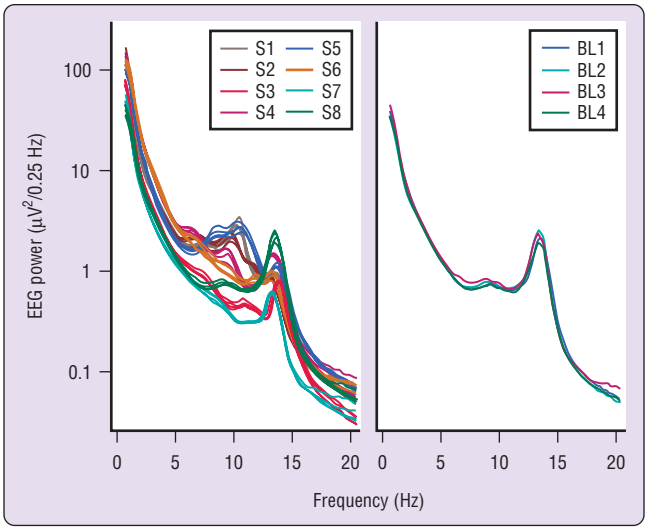

Figure 15-1 High *inter*individual variation *(left)* and high *intra*individual stability *(right)* in all-night EEG power spectra in non-REM sleep in 32 baseline nights of 8 young men (S1-S8). The largest interindividual variation is observed in theta, alpha and sigma frequencies (about 5-15 Hz). The spectra of all 4 baseline nights (BL1-BL4) of one individual (S8) are virtually superimposable. (Adapted and modified from Buckelmüller J, Landolt HP, Stassen HH, Achermann P. Trait-like individual differences in the human sleep electroencephalogram. Neuroscience 2006;138:351-356.)

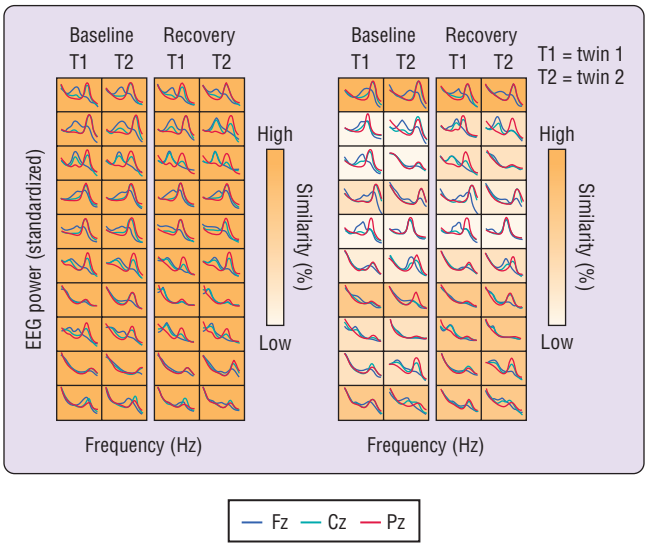

Figure 15-2 Heritability of non-REM sleep EEG is over 90%. Panels show color-coded similarity indices of 8- to 16-Hz activity in monozygotic *(left)* and dizygotic *(right)* twin pairs. The similarity index in each twin pair was scaled between minimal (0% similarity, *white*) and maximal (100% similarity, *dark orange*). *Black lines,* derivation Fz; *blue lines,* derivation Cz; *red lines,* derivation Pz (unipolar derivations referenced to averaged mastoid). (Modified from De Gennaro L, Marzano C, Fratello F, et al. The EEG fingerprint of sleep is genetically determined: a twin study. Ann Neurol 2008;64:455-460.)

2 to 13 Hz range is significantly higher in MZ twins than in DZ twins.[47] Especially alpha/sigma frequencies appear to reflect particularly strong genetic influences. Heritability in this frequency range may be as high as 96% (Fig. 15-2).[48]

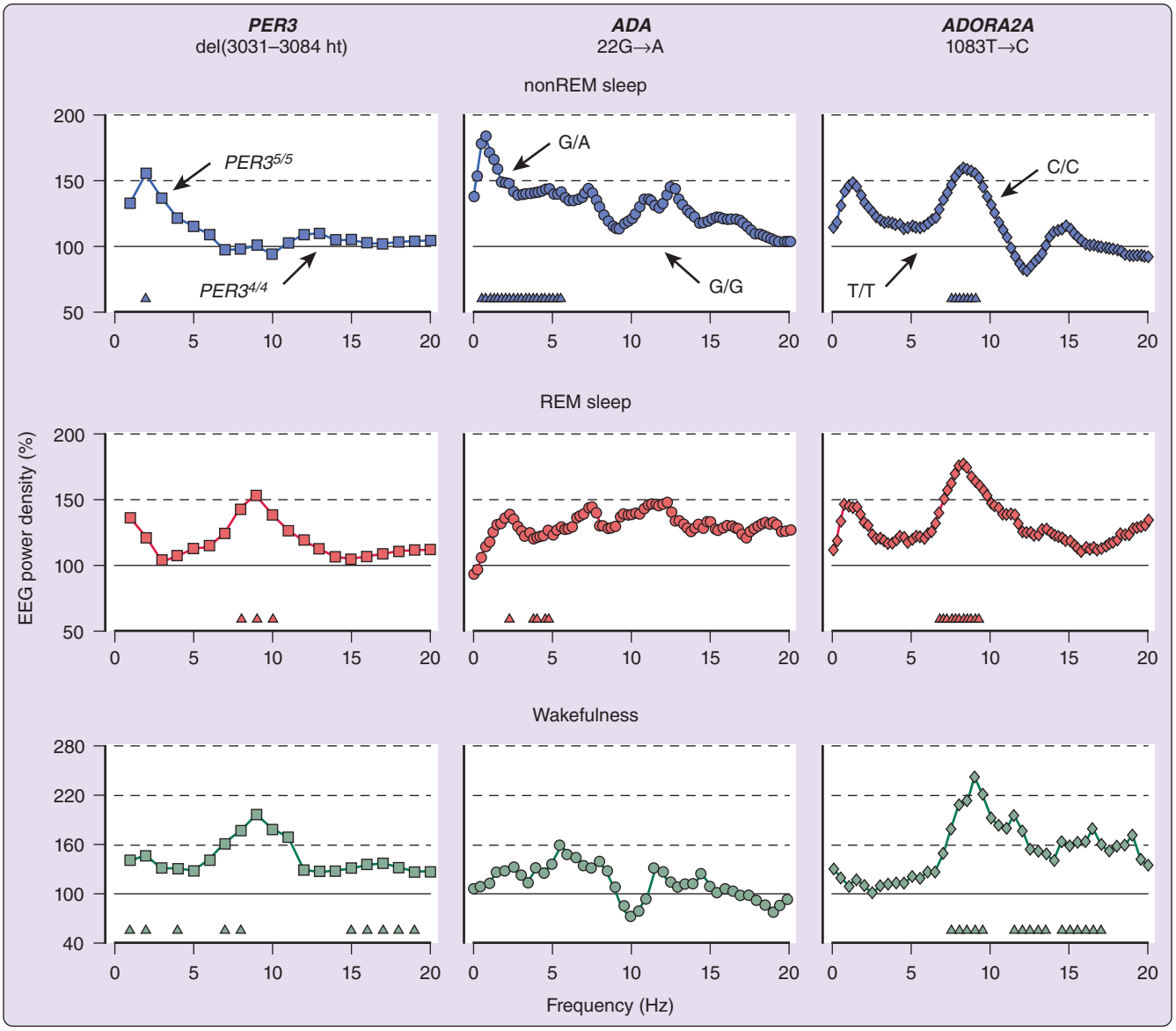

Figure 15-3 The period-3 *(PER3)* variable-number tandem-repeat polymorphism (insertion of nucleotides 3031 to 3084), the adenosine deaminase *(ADA)* c.22G/A polymorphism, and the adenosine A_{2A} receptor *(ADORA2A)* c.1083T/C polymorphism modulate the EEG in sleep and wakefulness. Relative EEG power density spectra (C3A2 derivation) in non-REM sleep (stages 2 to 4), REM sleep, and wakefulness. *Triangles* at the bottom of the panels indicate frequency bins, which differed significantly between the genotypes ($P < .05$, two-tailed paired *t* tests). (Adapted from Viola AU, Archer SN, James LM, et al. PER3 polymorphism predicts sleep structure and waking performance. Curr Biol 2007;17:613-618, and Rétey JV, Adam M, Honegger E, et al. A functional genetic variation of adenosine deaminase affects the duration and intensity of deep sleep in humans. Proc Natl Acad Sci U S A 2005; 102:15676-15681.)

GENES CONTRIBUTING TO THE SLEEP EEG

Healthy carriers of the $PER3^{5/5}$ genotype have not only more SWS but also more delta activity in non-REM sleep, as well as higher theta/alpha activity in REM sleep and wakefulness, than individuals with the $PER3^{4/4}$ genotype (Fig. 15-3).[42,49]

Like the *PER3* polymorphism, the 22G>A polymorphism of ADA not only affects duration of SWS but also the spectral composition of the EEG. Thus, in non-REM sleep and REM sleep, EEG activity within the 0.5 to 5.5 Hz range is higher in G/A allele carriers than in homozygous G allele carriers (see Fig. 15-3).[43] On the basis of data in animals, it may be expected that G/A and G/G genotypes behave differently during prolonged wakefulness. More specifically, quantitative trait locus (QTL) analyses in inbred mouse strains revealed that a genomic region including *Ada* modifies the rate at which non-REM sleep need accumulates during wakefulness.[50]

A synonymous 1083T→C polymorphism located in the coding region of the human adenosine A_{2A} receptor gene *(ADORA2A)* also affects the EEG in non-REM sleep and

REM sleep.[43] This polymorphism is linked to a 2592C→T_{ins} polymorphism in the 3′-UTR of *ADORA2A*. The latter may modulate protein expression.[51] Rétey and colleagues[43] observed that EEG spectral power in the range of approximately 7 to 10 Hz is higher in subjects with the 1083T→T genotype than in individually-matched subjects with the 1083C→C genotype (see Fig. 15-2). Because the difference is not sleep or wakefulness specific, this polymorphism may modulate EEG generating mechanisms rather than sleep–wake regulation. Moreover, the same genetic variation in *ADORA2A* contributes to subjective and objective responses to moderate caffeine intake on sleep.[52]

The gene encoding the important catecholamine-metabolizing enzyme catechol-*O*-methyltransferase (*COMT*) is located on human chromosome 22q11.2, in proximity to *ADORA2A*. Human *COMT* contains a common functional variation that alters the amino acid sequence of COMT protein at codon 158 from valine (Val) to methionine (Met).[53] Individuals homozygous for the Val allele presumably show higher COMT activity and lower dopaminergic signaling in prefrontal cortex than Met/Met homozygotes.[54-56] Sleep variables and their response to sleep deprivation do not differ between male carriers of Val/Val and Met/Met genotypes.[57,57a] In contrast, EEG power in non-REM sleep, REM sleep, and wakefulness is consistently lower in the upper-alpha (11 to 13 Hz) range in Val/Val compared with Met/Met homozygotes.[58] This difference is present before and after sleep deprivation, and it persists after administration of the stimulant modafinil. The data demonstrate that a functional variation of the *COMT* gene is associated with robust interindividual differences in the sleep EEG. This polymorphism profoundly affects the efficacy of modafinil after sleep deprivation in young healthy men.[57] Thus, two-time 100-mg modafinil potently improved vigor and well-being, and maintained baseline performance of executive functioning and vigilant attention throughout 40 hours of prolonged wakefulness in 10 Val/Val homozygotes, yet the same dosage was virtually ineffective in 12 Met/Met homozygotes. Interestingly, an opposite relationship between Val158Met genotype of *COMT* and measures of daytime sleepiness may be present in patients suffering from narcolepsy (see Clinical Pearl).

A point mutation at codon 178 (in rare cases also a mutation at codon 200) of the prion protein gene *(PRNP)* has been identified as the cause underlying the devastating disease, fatal familial insomnia (FFI).[59,60] Interestingly, although healthy relatives of FFI patients appear to have normal sleep EEG,[61] the polymorphic codon 129 of the *PRNP* gene may influence EEG activity during sleep.[62] Subjects with Met/Val genotype showed lower slow-wave activity and higher spindle frequency activity than individuals with the Val/Val genotype, independent of codon 178.

GENETIC BASIS OF SLEEP–WAKE REGULATION: INTERACTION BETWEEN HOMEOSTATIC AND CIRCADIAN SYSTEMS

Many of the traits and genes described here concern sleep–wake characteristics as assessed under baseline conditions. How these alterations in sleep characteristics relate to sleep–wake regulation and how they may lead to functional consequences remains largely unexplored. The available data, however, already indicate that the effects cross boundaries between sleep and wakefulness, and between homeostatic and circadian aspects of sleep–wake regulation. For example, the polymorphisms in *PER3*, *ADORA2A*, and *COMT* affect the EEG in non-REM sleep, REM sleep, and wakefulness.

To investigate whether these changes reflect changes in EEG generating mechanisms with or without a relationship to sleep regulatory processes requires these processes to be challenged by, for example, sleep deprivation. Comparing the response to sleep deprivation in $PER3^{5/5}$ individuals with that in $PER3^{4/4}$ individuals revealed that the increase in theta activity in the EEG during wakefulness was more rapid, and furthermore that the decline of cognitive performance was more rapid.[42] This genotype-dependent differential susceptibility to the negative effects of sleep loss on waking performance was particularly pronounced in the second half of the circadian night and on tasks of executive functioning.[63] One interpretation of these data is that the VNTR polymorphism in *PER3* affects the dynamics of the homeostatic process, which then through its interaction with the circadian regulation of performance leads to differential susceptibility to the negative effects of sleep loss. Indeed, an fMRI study has shown that the changes in brain responses of $PER3^{5/5}$ individuals to a working memory task during sleep deprivation is very different from the changes in $PER3^{4/4}$ individuals. Whereas $PER3^{4/4}$ individuals maintained activation and recruited new brain areas to the task, brain responses were greatly diminished in $PER3^{5/5}$ individuals.[64] These laboratory data suggest that clock and sleep genes could contribute to individual differences in tolerance to shift work and jet lag, which are highly prevalent in society.

CONCLUDING REMARKS

Sleep is a complex behavior, and any functional genetic variation associated with changes in one of the many neurotransmitter or neuromodulator systems can be expected to affect sleep and the sleep EEG. Polymorphic variations in a number of genes have now been shown to affect several characteristics of sleep, and some of these genes may indeed be involved in sleep regulatory processes. Elucidating the signaling pathways that are affected will aid in our understanding of individual differences in sleep–wake behavior.

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❖ Clinical Pearl

Distinct alleles and genotypes in the genes of monoamine oxidase type A (MAO-A)[65]—but see Dauvilliers and colleagues[66]—and COMT[66] are thought to be associated with the clinical manifestation of narcolepsy. The Val158Met polymorphism of COMT exerts a sexual dimorphism and a strong effect of genotype on disease severity.[66] More specifically, women narcoleptics with high COMT activity fall asleep twice as fast during the multiple sleep latency test than those with low COMT activity. An opposite relationship, although less pronounced, is observed in men. Also the response to treatment with modafinil to control excessive daytime sleepiness differs between COMT genotypes. Patients (female and male) with the Val/Val genotype need an almost 100-mg-higher daily dosage than patients with the Met/Met genotype.[67] Intriguingly, in male healthy volunteers, the impact of the Val158Met polymorphism of COMT on modafinil's efficacy to improve excessive sleepiness after sleep deprivation is opposite to that in narcolepsy patients.[57]

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Genetics of Sleep and Sleep Disorders in Humans

Juliette Faraco and Emmanuel Mignot

Chapter 16

Abstract

Sleep is an evolutionarily old and vital process, and behavioral sleep has been observed in all vertebrates investigated, as well as some invertebrates. Although the precise physiologic functions remain elusive, the conservation of the behavior argues that sleep fulfils important biological needs. Despite the complexities, it is clear that genetic factors underlie the process of normal sleep, and they inevitably underlie sleep disorders. Recent work in animal models and in humans has begun to uncover the genetic underpinnings of various aspects of sleep, including circadian behavioral variation, homeostatic response, and numerous disorders affecting sleep. Many associations between observed human phenotypes and candidate loci have been published, but replications have been lacking. We present an overview of how genes regulate sleep and circadian processes, and we discuss what is known about how these relate to normal and disordered sleep in humans. We also provide a framework through which the numerous association studies published may be interpreted.

Behavioral sleep can be viewed as a complex phenotype. The process of sleep is initiated through interconnected drives responding to clock-dependent and sleep-debt–dependent cues. Two distinct forms of sleep, including five stages, are defined at the electrophysiologic level: non–rapid eye movement (NREM) sleep (stages 1 to 3) and rapid eye movement (REM) sleep. These stages are associated with distinctive physiologic changes affecting muscle tone, thermoregulation, endocrine function, gastrointestinal activity, and cardiorespiratory activity. Interaction with the environment at each of these stages adds additional complexity, and each of these aspects is potentially under the control of a wide variety of genes. A large number of studies have shown that specific set of neural systems, most notably aminergic and cholinergic systems in the brainstem and basal forebrain, as well as systems located in the posterior (hypocretin, histamine) and anterior hypothalamus (median and ventrolateral preoptic gamma-aminobutyric acid [GABA]-ergic systems), display changes across sleep stages and may be primarily important in orchestrating sleep stage and wake organization.[1,2]

Circadian rhythms drive a "clock-dependent" variation in sleep propensity, and they are observed throughout the animal kingdom, from unicellular organisms to mammals. These serve to coordinate the timing of important physiologic processes, including sleep, with the alternating photoperiod of the external environment. In mammals, circadian rhythms are primarily coordinated by the master clock in the suprachiasmatic nuclei (SCN) of the hypothalamus, and they are entrained by light through the retinohypothalamic tract, although it is now evident that many other tissues, notably peripheral tissues, have their own autonomous circadian clocks. The basis of these rhythms is a largely conserved transcriptional–translational feedback system plus a set of complex posttranscriptional regulatory steps (phosphorylation, targeted degradation) involving *Clock*, *Bmal1*, the *Period* genes, and the *Cryptochrome* genes.[3] The elaborate nature of these loops provides many distinctive points at which single gene mutations can manifest with clearly observable phenotypes such as altered period, phase angle, or loss of rhythm, which have been identified in models such as *Drosophila* and rodents, and in humans.

Sleep homeostasis, on the other hand, responds to accumulated sleep debt, increasing the intensity and propensity to initiate sleep according to cumulative time spent awake. Less is known about the genetic, neurochemical, and neuroanatomic bases of the homeostat than about the circadian process. The neuroanatomic basis is likely to be diffuse,[1] but slow-wave activity in the delta frequency range, quantified as delta power, varies in proportion to prior sleep and wakefulness and thus serves as one measure of homeostatic sleep need.[4,5] Changes in glutamatergic transmission in the cortex, possibly in reaction to synaptic plasticity changes that have occurred in wakefulness, and intracellular metabolic changes may be critical.[6] Studies in rodents have demonstrated that the rate of accumulation of this sleep need is also under genetic control.[7]

More generally, several electroencephalographic (EEG) features have been found to be highly heritable traits in humans, based on studies comparing monozygotic (MZ) and dizygotic (DZ) twins. Indeed, differences between MZ twins were not larger than successive recordings from the same individual. Autosomal dominant inheritance for an array of EEG variants has also been demonstrated through numerous family studies.[8] EEG frequency bands in the delta through sigma frequency ranges during wakefulness showed high heritabilities of 76% to 89% in a large study of MZ and DZ twins.[9] More recently, a twin study has also showed strong genetic control of spectral composition of NREM sleep, particularly in the delta, theta, alpha, and sigma frequencies.[10]

Thus, although the process of sleep is clearly under genetic control, the process is highly robust, making single gene mutations specifically abolishing sleep unlikely. The temporal dynamics of sleep are quite fast, indicating that gene expression per se is not likely to govern the state-to-state changes observed on the EEG. Rather, the roles of genes may manifest through differentially available stores

of neurotransmitters, or variations in activity of transmembrane channels.

GENETIC STUDIES OF HUMAN SLEEP: METHODOLOGICAL LIMITATIONS

Identification of single gene mutations in animal models has proved quite successful in the areas of circadian rhythms and narcolepsy, demonstrating that single gene mutations can have dramatic effects on sleep patterns, although in relatively rare situations. Linkage studies of sleep phenotypes in human families have often resulted in unreproduced or controversial results, probably because of a combination of small family size (resulting in limited power), phenotypes that are not fully penetrant or susceptible to phenocopy, rare occurrence of families, and allelic or locus heterogeneity. Even in the best case, linkage studies result in broad peaks containing many potential candidate genes.

Numerous association studies have also been published, based either on candidate gene screens or hypothesis-free genome-wide association (GWA) scans. Association studies in general have had a very poor replication rate, and one review showed that for 166 putative associations that were studied three or more times, only six were consistently replicated.[11] Keeping in mind that spurious or nonreproducible findings outnumber replicated findings in the published literature, it is critical to understand the inherent limitations of these studies (Box 16-1).

Case-control designs are popular and practical for association studies but are susceptible to population stratification, which causes problems when patients and controls are unknowingly drawn from different ethnic groups or subgroups. If the disease is more prevalent in one of these groups, it can be overrepresented in patients and underrepresented in controls, and any polymorphism marking the higher-risk subgroup will appear to be associated with the disease. A typical example of this problem is illustrated by previously reported studies of the dopamine D4 receptor gene *(DRD4)* in relation to personality traits and drug of abuse phenotypes, which were later shown to mostly represent differences in African-American admixture across samples.[12,13] In this particular case, hundreds of studies have been published, and even a recent meta-analysis could not clearly conclude whether this association was genuine or artifactual.[12] Indeed, even when strict meta-analyses are performed, it is impossible to exclude bias toward publishing positive replications that could inflate the number of positive reports. As an example, in 1999, we found that the Clock 3111C polymorphism was associated with delayed phase in 410 subjects of the Wisconsin Sleep Cohort,[14] a finding we could not replicate in 600 additional subjects and that was never published. When population stratification is a problem, alleles enriched in a sub-ethnicity will show differences for

Box 16-1 Common Issues in Published Association Studies

Population stratification: An association is observed because cases and controls do not have the same ethnic or sub-ethnic composition (it may not be apparent). This is most problematic in candidate gene associations.

Inclusion of phenotypes with poor test or retest reliability: In many cases, the phenotype under study is not robust or validated.

Studies of multiple phenotypes without correction for multiple testing: It is common for a study to start with a clear a priori hypothesis, but, despite nonsignificant results, to explore other phenotypes without properly stating that these were exploratory analyses. In a variation of this strategy ("phenotype dredging"), a borderline *P* value is improved by testing correlated sub-phenotypes (for example, studying amount of sleep after sleep deprivation rather than SWS power after sleep deprivation), or subdividing the groups (e.g., by sex). In general, these additional testing strategies are acceptable if they are clearly indicated as exploratory in reporting the study.

Pseudoreplication: Unfortunately, researchers often frown upon precisely replicating a protocol performed in a prior study, often using an "improved version." This, together with phenotype dredging, leads to multiple studies "replicating" a similar effect, where in reality this was not the case.

Report of gene–gene interactions or testing of multiallele differences without adequate control for multiple testing: The study of interactions, a reasonable idea by itself, is really problematic with respect to power. Most notably, the number of interactions that can be explored is infinite and difficult to control. Interactions should be studied only when there is a clear biological basis, and in general they should be presented as exploratory until replicated.

Testing of multiple genetic models without control for multiple testing: In the study of any genetic association, multiple models are frequently tested: allelic association, dominance, recessiveness, and haplotype analysis. The accepted standard to date is to conduct an allelic association test, and to explore further genotypic models only if the allelic test is significant.

Functional characterization: In the presence of weaker results, a standard in the field is to show functional effects of the associated polymorphism. This is especially important in cases with single occurrence of a mutation in a family when other families with a similar phenotype and a different mutation in the same gene are not available. Importantly, however, a high standard should be applied to these studies. It may, for example, be easy to find unrelated small changes in lymphocytic gene expression in relation to a polymorphism. Similarly, studies in animal models have limitations: A similar T44A mutation in CKIδ causes opposite circadian phenotypes in *Drosophila* and mice.

markers located across the entire genome, and they can thus be more easily detected in GWA-related designs (see later, notes on QQ plots, for example) than in candidate gene studies. For this reason, GWA studies are generally less susceptible to this problem.

False positives and nonreplications can also arise from a variety of other factors, including small study sizes, variable phenotype definition, insufficient correction for multiple testing, variable linkage disequilibrium (LD) between the polymorphism studied and the causal variant among different populations, and population-specific gene–gene or gene–environment interactions. Following the recent explosion of GWA studies, a white paper was published proposing best practices for conducting and publishing initial association reports and replication studies,[15] and it focused on assessment of validity of association reports and criteria for establishing replication (Box 16-2). Although recommendations were directed toward GWA designs, the key points also apply to candidate gene association studies. First, not only should a biologically meaningful report

Box 16-2 Key Recommendations for Finding and Replicating Associations

Design and Methodology

Case-Control Study Information

- The source of cases and controls, and recruitment information
- Method for determining and validating affected or unaffected status, and reproducibility of classification
- Case-control selection flow chart including exclusion points for missing or erroneous data
- Table comparing relevant characteristics (ethnic background, demographics, risk factors)

Genotyping Quality-Control Procedures

- Description of genotyping assays and genotype-calling algorithm
- Genotyping quality-control procedure described
- Analysis of call rates (by marker, by individual, by case-and-control status)
- Analysis of Hardy-Weinberg proportions (cases, controls separately) to identify poor assays
- Testing for cryptic relatedness in subjects
- Assessment of population heterogeneity including the following:
  - Average chi-square values and full distribution
  - QQ plots of chi-square analysis

Results

- Description of pre-analysis scheme for selection of results suitable for replication
- Evaluation of genotype clustering for key markers
- Description of specific statistical tests used and genetic models tested
- Discussion of choice of threshold for significance and statistical basis for multiple testing adjustment

Considerations Regarding Validity of Initial Report

- Suitably large sample size
- Description of the study's power to detect an effect
- Phenotypes assessed according to standard definitions, and specified in report
- Testing for underlying population structure differences between cases and controls
- Strength of observed effect
- Sufficiently stringent criteria for significance (small *P* values)
- Single-locus and multimarker haplotype analysis
- Significance of effect not dependant on altering established quality controls or inclusion criteria, or on unusual sub-phenotype
- Appropriate correction for multiple comparisons performed
- Description of local linkage disequilibrium; typing of markers in strong linkage disequilibrium (LD) shows similar results
- Biological or functional explanations firmly based on available data
- If replication not included, preliminary nature of report should be emphasized

Replication Studies

- Included in the initial association report
- Description equivalent in detail to original sample
- Sufficient size to distinguish between proposed effect and no effect
- Uses independent sample, but similar population group and same phenotype as used for initial replication
- Strong rationale for selection of additional markers to be studied in replication (from initial study, implicated through LD or function, implicated in published literature)
- Discussion of choice of threshold for significance
- Statistical significance obtained with same genetic model as initial study
- Joint analysis (if possible) yields smaller *P* value than seen in initial study
- Replication uses same marker allele or haplotype, shows similar effect in same direction as original study
- Summary of replication attempts by authors and summary of known replication attempts, including nonreplications

provide evidence of an association supported by a substantial odds ratio (OR) and a statistically significant *P* value, but it should also report a systematic phenotype criterion, demonstration of adequate sample size and lack of stratification, demonstration of quality of assays, description of multiple testing correction, and a declaration a priori of any weighting schemes, including which markers warrant a reduced multiple testing threshold.

Clean and well-defined phenotypes are more likely to result in robust associations, and phenotypic criteria need to be clearly described. Altering phenotype definitions to achieve greater statistical significance has resulted in unreplicated findings. Similarly, it is not uncommon to see reports of "pseudoreplications," where either the replication is in the opposite direction or it uses a more or less modified definition of the phenotype. Associations that are significant only after post hoc selection for unusual or highly specific sub-phenotypes, or phenotypes representing only a small proportion of a sample study, warrant cautious interpretation. Small studies pose problems because of lack of power, they are prone to large variation in risk estimates, and they are especially susceptible to cryptic stratification effects.

Multiple methods are available to demonstrate lack of stratification in GWAs, but most commonly QQ plots of chi-square analysis are used to demonstrate that the distribution of values obtained, with the exception of the positive hits, are close to those expected by chance. Systematic deviation from expected values is a measure of general difference between patients and controls. These methods rely on the availability of vast numbers of genotypes and are not applicable to candidate gene association approaches. Stratification may thus underlie the high rate of nonreplications in candidate gene studies. This will become less of an impediment as genotyping costs decrease and sets of ancestry informative markers (AIMs) become increasingly well characterized. Alternatively, it may be necessary to form collaborations, as combining multiple independent samples overcomes obstacles of insufficient power and improves the generalizability of the findings.

The enormous numbers of genotype–phenotype comparisons made in GWA studies lead to correspondingly large numbers of spurious hits. Without rigorous correction for multiple testing and filtering of artifacts, any real results can become obscured. Extremely small *P* values often result from technical artifacts, and it is important to examine Hardy-Weinberg equilibrium and genotype clustering quality for these genotypes. Methods for multiple testing correction are evolving. Although Bonferroni correction is accepted, it is overly conservative in GWAs because, as a result of LD, many markers are not independent. Genome-wide significance for a 900,000 single nucleotide polymorphism (SNP) chip is on the order of 10^{-8}, a daunting hurdle. Lowering the threshold for selected markers that may be anticipated to have a functional role is acceptable, but these will be rare, and they must be declared before analysis has begun, because there is considerable temptation to create credible biological hypotheses post hoc.

It is now recommended that any initial association report include a replication study, and this should be performed with the same phenotypic criteria and on an independent sample from a comparable population (preferably a collaboration, to avoid the temptation of splitting a well-powered study into two smaller samples). It is essential to replicate the same markers, and findings should show similar magnitude of effect and in the same direction. Patterns of LD may vary considerably among different ethnic groups. Studying other populations in subsequent replications can add substantial credibility and significantly narrow the region, but failure to replicate in different ethnic groups does not necessarily negate the initial finding. Where replication is not possible, functional analysis can be used to support the validity of the association.

In light of these issues, we have elected to primarily describe only findings that are supported by replication studies, or that have substantial functional biological evidence from model systems.

GENETIC FACTORS UNDERLYING THE CIRCADIAN CLOCK AND CIRCADIAN RHYTHM DISORDERS

A wealth of information is now known regarding the genetic basis of circadian rhythmicity, which is coordinated by a network of transcriptional–translational feedback loops that drive expression of a series of core clock components with approximately a 24-hour cycle. Analysis of circadian mutants has now led to the discovery of clock protein mutations in fungi, plants, *Drosophila*, and rodents.[1] There is an extensive body of work on the genetics, functional biology, and behavioral and metabolic phenotypic effects of circadian mutants in the mouse, which has been extensively reviewed (see reference 16). Animals carrying circadian clock mutations have phenotypes extending beyond alterations of rhythmic behavior (sleep homeostasis, response to sleep deprivation, metabolism, cancers), probably a reflection of the widespread distribution and activity of clock proteins and their targets.

Mutations in human clock-related genes are now well established in the etiology of familial advanced sleep phase syndrome (FASPS). This disorder was first described in a large pedigree from Utah that was segregating an autosomal dominant allele associated with a lifelong tendency to wake up and to go to sleep at very early times. Affected family members had normal sleep quality and quantity, but their preferred sleep and wake times, melatonin, and temperature rhythms were all advanced by 4 to 6 hours.[17] The free-running period of the proband was approximately 1 hour shorter than matched controls. The underlying mutation was a serine to glycine substitution (S662G) in the human *PER2* gene, and in vitro data suggested this to be a potential phosphorylation site by CK1ε.[18] A second FASPS pedigree was found to carry a threonine to alanine (T44A) mutation in the *CK1δ* gene, which reduced activity of the enzyme in vitro.[19] These findings in humans correspond well with identification of a CK1ε mutation in tau mutant Syrian hamsters[20] that leads to deficient phosphorylation of *PER*. Although these results demonstrate a key role for CK1δ/ε in the function of the clock, further studies in rodents have demonstrated complexity.[21,22] Surprisingly, CK1ε does not phosphorylate *PER2* at position 662 but instead acts to phosphorylate three serine residues nearby. Instead, phosphorylation at 662 by an unknown enzyme acts as a priming event leading to CK1ε activity elsewhere.

Furthermore, the S662G mutation does not decrease PER stability, as had been anticipated, but instead resulted in decreased transcription at *PER2*,[22] although this is debated.[21] The results support a model in which CLOCK timing is regulated by expression, degradation, and nuclear entry and retention of *PER2*. In addition, there is modulation through multiple states of *PER2* phosphorylation, some of which are not dependent on CK1δ/ε.[23] The search for this unknown kinase is ongoing.

Apart from the well-defined mendelian effect in FASPS, a study of 238 twin pairs found higher correlations for Horne-Ostberg (HO) diurnal preference scores among MZ twins, thus suggesting the presence of circadian factors in the general population.[24] A number of association studies have recently examined a connection of the *CLOCK* gene with diurnal preference. The initial study of Katzenberg examined 410 white individuals of the Wisconsin Sleep Cohort.[14] Individuals with the CLOCK 3111C allele in the 3′ untranslated region had lower HO scores, with a 10- to 44-minute delay in preferred timing of activity or sleep, suggesting that this SNP, or another SNP in tight LD, could underlie the effect. Further studies gave variable results, and the association was not found in a study of 105 normal subjects, 26 blind, or 16 delayed-sleep-phase patients,[25] but it was identified in a larger study of 421 Japanese subjects.[26] These results thus remain controversial, as indeed we could not replicate the association in an additional sample of the Wisconsin cohort (although overall results for the entire sample remain significant).

Similarly problematic results have been reported in the study of human *PER* gene polymorphisms. A purported association between the human PER3 locus and delayed sleep phase remains provisional. Although two groups have reported this general association,[27,28] the small samples (16 discordant sib pairs [DSPs], 48 DSPs) were from different ethnic groups (Japan, United Kingdom, and the Netherlands), and they found similar but not equivalent associations. In one case, a rare five-marker haplotype containing the major variable number of tandem repeat (VNTR) four-repeat allele (G647, P864, 4-repeat, T1037, R1158) but not the VNTR four-repeat allele alone was present on seven predicted DSPs chromosomes total (15% carrier frequency) versus 2% of control Japanese.[28] In the other study, in England, the VNTR four-repeat allele was associated with HO scores and delayed sleep phase[27] in 484 subjects, 75% of whom were homozygous, an effect later suggested to be significant only in younger subjects through the study of HO extremes in a bigger sample.[29] Similarly, a T2434C polymorphism in *HPER1* (rs2735611) was recently reported to be associated with extreme HO scores (80 individuals per group) drawn from 1590 British volunteers,[30] whereas in another, earlier 1999 study, G2548A (rs2253820) in *PER1* was not associated with HO in 463 individuals drawn from the Wisconsin Sleep Cohort.[31] The more recently published study, however, did not mention that these two *PER1* polymorphisms are located within 114 base pairs of each other and are in almost complete LD (r2 = 1), so that typing one is equivalent to typing the other. Considering the relatively small effect size and the broad spectrum of preferences reported in the general population, in contrast to the high penetrance and tight ranges of preferred activity in FASPS, a variety of combinations of different alleles at a number of circadian genes probably underline diurnal preference in the general population. Clearly, the next step is to greatly increase sample size (to several thousand subjects) to have power to exclude or confirm these prior studies. Resequencing of candidate genes in extremes, and finding familial clustering of the phenotype in relatives, to identify other rare strong effect alleles are also viable strategies. It is also likely that the HO, like any subjective assessment instrument, is less amendable to genetic analysis than more objective physiologic measures of circadian phase.

GENETIC FACTORS REGULATING EEG AND THE SLEEP HOMEOSTAT

The search for the genetic basis of selected EEG traits and the sleep homeostat is well underway using rodent models. Strong genetic effects are more clearly evident for spectral features of the EEG versus sleep architecture variability.[32] Power in the delta and sigma frequency bands in slow-wave sleep (SWS) is associated with genetic background, and a deficiency in a single enzyme (acyl–coenzyme A dehydrogenase) results in slowed theta activity during sleep.[33] Quantitative trait locus (QTL) and mapping studies demonstrate that strong genetic effects on SWS-need (measured as rate of accumulation of delta power after extended wakefulness)[7] may represent differences in synaptic plasticity mediated by the Homer1a gene through differential disruption of glutamatergic signaling complexes.[34,35] These same studies show that other genes have greater effects on sleep need depending genetic background, highlighting an issue of QTL models: one locus may represent a major gene in the context of two specific inbred strains, but the effect size in a more outbred population may be difficult to ascertain. These can complicate extrapolation to potential human phenotypes.

In humans, a *PER3* genotype was recently reported to be associated with differences in EEG markers of sleep homeostasis after sleep deprivation (and behavioral consequences) (10 $PER3^{5/5}$ versus 14 $PER3^{4/4}$ individuals respectively). This remains tentative, as the sample size was small and it has not yet been replicated by other groups.[36] It is also worth noting that multiple papers linking other phenotype differences to the $PER3^{5/5}$ genotype have all been made using the same initial sample[37,38] and thus cannot be considered replications. The finding is interesting as it suggests that circadian genes may be involved in regulating not only circadian timing but also sleep homeostasis, consistent with studies in mice and *Drosophila*.[1] We expect that GWA studies will explore the genetic basis of the EEG in the near future.

GENETICS OF NARCOLEPSY

Heritability

Narcolepsy affects the control of sleep and wakefulness, and it is characterized by excessive daytime sleepiness, symptoms of dissociated REM sleep (sleep paralysis, hypnagogic hallucinations), disrupted nocturnal sleep, and cataplexy (brief episodes of muscle weakness triggered by emotions). Although most of these symptoms appear in the general population in the context of sleep deprivation or

other sleep disorders, cataplexy is highly specific to narcolepsy.

Although narcolepsy is primarily sporadic, family and twin studies indicate a strong genetic basis for susceptibility to narcolepsy, as the prevalence of narcolepsy/cataplexy in first-degree relatives of probands is between 0.9% and 2.3%. Although this recurrence risk in siblings is low, it is considerably higher than the population prevalence and corresponds to a 20- to 40-fold increased risk.[39] MZ twins show a concordance rate of only 35%, indicating that narcolepsy/cataplexy results from an interaction of environmental factors on a susceptible genetic background.

Human Leukocyte Antigen in Narcolepsy

An association between narcolepsy and specific class II human leukocyte antigen (HLA) antigens (DR2 and DQ1) was first noted in the Japanese population.[40] HLA class II antigens are present on immune cells and function to present processed foreign peptides to T cells by engaging the T-cell receptor. The initial association was subsequently confirmed by many studies and further refined. DR2 and DQ1 are in complete disequilibrium in Japanese, but substantially less in African Americans. Using high-resolution mapping in different ethnic groups allowed refinement of the susceptibility region by examining the frequency of alternative haplotypes, and demonstrated that DQB1*0602 is the most specific marker for narcolepsy in all ethnic groups. Although 90% of narcolepsy cases are associated with DQB1*0602, this is a common allele across ethnic groups, ranging from 12% in Japanese, to 38% in African Americans, and thus is not sufficient for the development of the disease. Other HLA alleles also influence susceptibility to narcolepsy. A study of 420 narcolepsy-cataplexy patients and 1087 controls[41] identified additional predisposing alleles: DQB1*0301, DQA1*06, DRB1*04, DRB1*08, DRB1*11, and DRB1*12. Approximately 10% of narcolepsy-cataplexy patients are DQB1*0602 negative, but a large proportion of these carry the DQB1*0301 allele. Four protective alleles, DQB1*0601, DQB1*0501, DQB1*0603, and DQA1*01 (non-DQA1*0102), were also found. It is notable that whereas HLA DQB1*0602 confers susceptibility to narcolepsy, the very similar DQB1*0601 antigen is rather protective. Thus very minor changes in the peptide binding pockets of these molecules (where these differences localize) may determine disease risk. Protective DQA1 alleles[41-41b] may form transdimers, reducing formation of the susceptibility heterodimer DQα1*0102/DQβ1*0602.[41a] It is clear that non-*HLA* genes also contribute to susceptibility, as the proportion of recurrence risk attributable to *HLA* is well below the relative risk observed in first-degree relatives.[41]

The tight association with DQB1*0602, the typical peripubertal onset, and the low concordance in MZ twins all suggest an autoimmune mechanism for narcolepsy. The association of MHC proteins, particularly class II antigens, is well recognized in a variety of autoimmune diseases, although narcolepsy shows the tightest such interaction (reviewed in reference 39). The interaction of HLA proteins with processed antigens determines the resulting immune response. However, some features of narcolepsy are not as consistent with a typical autoimmune mechanism, as females are not at increased risk. Surprisingly, there is little consistent direct evidence for humoral or cellular immunity in narcolepsy. The disease has not been transferred through injection of serum into mice, and activity of T-cell subsets, and natural killer cells were not altered in patients with narcolepsy.[39,42,43] Increased autoantibodies against Tribbles homolog 2 (TRIB2) were recently identified by three groups, and were more prevalent close to onset of cataplexy.[41c-e]

Hypocretin Deficiency in Narcolepsy

The study of narcolepsy in canines led to our current understanding of the disease pathophysiology. As in humans, most cases of canine narcolepsy are sporadic, but the identification of dog pedigrees segregating an autosomal recessive form of narcolepsy with clear-cut cataplexy allowed identification of mutations of the hypocretin receptor-2 gene underlying the disease (Video 16-1).[44] Subsequent studies quickly demonstrated a profound reduction in hypocretin gene expression and peptide content in postmortem narcoleptic brains, and low or undetectable levels of hypocretin-1 in the cerebrospinal fluid (CSF) of patients with narcolepsy.[45-47] Mutation screening of the hypocretin ligand and receptor genes in narcolepsy cases was performed focusing on rare DQB1*0602-negative, and familial cases, which would be more likely to carry mutations. A single mutation was identified in a case of early-onset narcolepsy with cataplexy present at 6 months.[46] The mutation introduced a highly charged arginine residue into the signal peptide of the prepro-hypocretin protein. Functional analysis suggested abnormal trafficking of the mutant peptide precursor, resulting in toxicity to the cells, supported by undetectable hypocretin in the CSF. Further studies failed to find mutations in, or SNP associations with, the hypocretin system genes in more typical cases of sporadic DQB1*0602-positive narcolepsy.[46,48,49] Therefore, although narcolepsy-cataplexy is clearly associated with deficient hypocretin neurotransmission, this is not a result of hypocretin gene mutations or variants. The pattern of highly selective hypocretin cell loss together with the lack of identified mutations again points to an underlying autoimmune disease mechanism. Although hypocretin is only one of a number of neurotransmitters implicated in sleep regulation, it is notable that only deficiency in hypocretin leads such a highly specific sleep-related phenotype.

Non-HLA, Non-Hypocretin Genes in Narcolepsy

Several studies have attempted to map narcolepsy genes in human families through linkage, but regions of suggested linkage have not been replicated.[50,51] More recent efforts have focused on genome-wide association studies using high-density SNP platforms. The first such study[52] examined associations in a set of 222 narcolepsy patients and 389 Japanese controls. The top SNPS were submitted for replication in 159 narcolepsy patients and 190 controls. In each round, the smallest *P* value at rs5770917, an SNP located between *CPT1B* and *CHKB*, ranged from 1 to 6×10^{-4}. Additional genotyping in small samples from three ethnic groups—from Korea (115 versus 309), European Americans (388 versus 397), and African Americans (86 versus 98)—showed differences in minor allele

frequencies in the same direction. A significant replication *P* value was obtained in the Korean set (.03), but not in the American sets. Notably, this SNP had very unfavorable minor allele frequencies in white (4%) and African-American (2.6%) controls, underscoring the wide-ranging differences in allele frequencies among different populations.

A second GWA recently published by our group[53] identified an SNP variant strongly associated with narcolepsy across three ethnic groups, and was replicated in a third GWA focusing on Europeans.[41b] In our sample of 807 white narcolepsy patients versus 1074 HLA-DQB1*0602$^+$ controls, three SNPS in the T-cell receptor alpha (TCRA) locus were associated, with the highest significance at rs1154155 in the J segment region ($P = 1.9 \times 10^{-13}$).[53] This SNP showed significant association upon replication in whites (363 patients, 355 controls, $P = 3.67 \times 10^{-5}$) and Asians (from Japan and Korea; 561 patients, 605 controls, $P = 2.30 \times 10^{-7}$), and a similar OR but not formal significance in a small set of African Americans, in whom the allele is uncommon (133 patients, 144 controls, $P = .39$, allele frequency 10% in patients, 8% in controls). The homogeneity of OR and the strong replication in independent samples of multiple ethnic groups indicate that this is an important susceptibility factor for narcolepsy. In contrast to previous results in the Asian samples, the chromosome 22 locus showed no evidence for association in this large sample of whites, indicating that if real, that locus may have effects only in select narcolepsy subgroups. SNP rs1154155 was not identified in that study as it was not among the markers tested. Indeed the TCRA locus is an excellent biological candidate, as it interacts with HLA and a presented antigen, leading to an immune response. Like the immunoglobulin loci, the TCRA locus undergoes somatic cell recombination, which occurs between 46 functional variable (V) and 49 functional joining (J) segments. Precisely how a J segment region polymorphism (rs1154155C or another variant in tight LD) could increase the risk of narcolepsy is unknown, but it could involve nonrandom VJα choices in recombination. As narcolepsy is almost entirely associated with a single HLA allele, it may be hypothesized that the DQB1*0602/DQA1*0102 heterodimer could interact with a TCR idiotype with a specific VJα recombinant associated with the presence of rs1154155 (directly or indirectly). Unlike HLA, the TCR is expressed only in immune T cells, making it all but certain that narcolepsy is indeed an autoimmune disease. In the context of a selected antigenic trigger, this could then lead to further immune reaction, ending in the destruction of hypocretin-producing cells.

GENETICS OF RESTLESS LEGS SYNDROME

Heritability

Restless legs syndrome (RLS) is a quite common neurologic phenotype, with an age-dependent prevalence of up to 10% in individuals over 65 (reviewed in reference 54). The unique sensory–motor phenotype is characterized by an uncomfortable and intrusive urge to move the lower limbs. Symptoms typically manifest during rest or inactivity, are worse in the evening, improve with walking or moving the extremities, and often result in insomnia and severely disrupted sleep. Approximately 80% of patients with RLS also have periodic leg movements in sleep (PLMS), which are involuntary, highly stereotypical leg movements occurring with regular periodicity.[55] RLS has a strong genetic component with high concordance (83%) between MZ twins in one small study.[56] High heritability (0.6) was estimated in another large study of more than 4503 twins.[57] Up to 60% of idiopathic patients with RLS report that symptoms are present in at least one family member. Phenotypic variability can be quite large in families, ranging from severe and debilitating symptoms each night, with involvement of upper limbs, to mild symptoms experienced only occasionally. Familial cases are associated with an earlier age of disease onset (by 12 years), although clinical signs and symptoms were similar in familial and nonfamilial cases.[58]

RLS Linkage Studies

Despite the high familial recurrence risk and prevalence, attempts to identify RLS genes in families have identified neither specific mutations nor specific genes. Three genomic regions have been identified and replicated in additional studies of unrelated pedigrees (reviewed in reference 54). The RLS1 locus (Ch 12q) displayed recessive inheritance and has been replicated in a number of independent French-Canadian, Icelandic, and Bavarian families. The RLS2 locus was identified on chromosome 14q in a North Italian RLS family showing dominant transmission, and it was later replicated in an independent French-Canadian family. RLS3, mapping to chromosome 9p, has been somewhat controversial but has been implicated in two U.S. and two German families. In all of these studies, the presence of phenocopies, nonpenetrants, and stratification according to early-onset age has made identification of specific candidate genes or risk haplotypes difficult. Family studies have also presented mysteries regarding intragenerational risk and genetic effect size. A striking feature of a subset of pedigrees is the presence of disease in up to 100% of siblings, well above the expected rate of 50% for autosomal dominant transmission. Although not inconsistent with dominant mendelian inheritance, this may reflect other factors such as high phenocopy rate, multiple dominant alleles co-segregating, ascertainment bias, or overdiagnosis. It is also notable that loci identified in family linkage studies had logarithm of odds (LOD) scores much lower than had been predicted on the basis of family structure. Together, these results indicate that inheritance is probably complex and not explained by a simple monogenic mendelian trait.

Genome-Wide Associations

Significant progress in the understanding of the genetics of RLS has been based on two large GWA studies, revealing a surprising role for developmental regulatory factors in the pathophysiology. Winkelmann and colleagues[59] studied a large set of well-characterized RLS patients, totaling 1600 RLS patients and 2600 controls from Germany and Canada, in a two-stage design that included replication in two independent samples. Three genomic regions were identified that replicated, and that withstood genome-wide multiple testing correction, including Meis1 (Ch 2p), BTBD9 (Ch 6p), and a broad region between

MAP2K5 and LBXCOR1 (Ch 15q). An independent GWA study was concurrently published that studied a total of 429 Icelandic patients with RLS/PLMS versus 1233 controls, with replication in 188 patients versus 662 controls from the United States,[60] that found an association signal between RLS and the same BTBD9 variant.

MEIS1 as a Candidate and Its Symptomatology

The highest significance hit from the Winkelmann study was on chromosome 2 in the *MEIS1* homeobox gene, where the association is fully tagged by two SNPS (rs12469063, rs2300478) in an LD block containing exon 9. The region shows high interspecies conservation, and it has a striking effect, with a haplotype OR greater than 2. This association has subsequently been confirmed in an independent sample of 244 U.S. RLS patients and 497 controls,[61] but it was not detected in the Icelandic study, reflecting differences in phenotyping (identified through clinic visits and emphasis on familial cases versus self-report, and inclusion of patients with isolated PLMS), or potentially population-specific differences.

MEIS1 is a member of the *TALE* homeobox gene family and forms heterodimers with PBX and HOS proteins, which augment the specificity and affinity of DNA binding by HOX proteins. MEIS1 has broad-ranging functions in development, including a critical role in hematopoiesis and endothelial cell development, vascular patterning, and retinal development in vertebrates.[62-64] More relevant to RLS, MEIS1 is strongly expressed in dopaminergic neurons of the substantia nigra, where studies have reported lower iron levels in RLS patients, and is also high in the red nucleus, a region regulating coordination of limb movement as well as implicated in lower iron levels.[65,66] MEIS1 is essential for proximodistal limb formation during development.[67] MEIS1 is also part of a HOX transcriptional regulatory network that specifies motor neuron pool identity and thus the pattern of target-muscle connectivity, suggesting a key link to the pathophysiology of RLS. A growing body of evidence indicates a spinal origin of PLMS, potentially mediated by the dopaminergic A11 nucleus in the hypothalamus.[68] These neurons project to local hypothalamic centers and the neocortex, but the also descend and serve as the sole source of spinal dopamine. As these projections are long, they may be unusually susceptible to age-related damage or cell loss. Patients with RLS/PLMS display sleep-specific spinal sensorimotor hyperexcitability, manifesting with a lower threshold flexor reflex and a greater spread to adjacent muscles than seen in controls.[69] Thus MEIS1 is an excellent candidate in RLS, with potential roles in development and modulation of these circuits in adults.

BTBD9 as a Candidate and Its Symptomatology

Both genome-wide studies[59,60] identified an association between SNPS in a large LD block containing intron 5 of the *BTBD9* gene on chromosome 6p, with maximal association at rs3923809, and this was also confirmed in the subsequent U.S. study of RLS probands.[61] Again, the effect was quite strong, with allelic OR between 1.5 and 1.8. Notably, in the Icelandic cohort, there was no association of rs3923809A in subjects with RLS without PLMS, whereas the OR increased when subjects with PLMs with or without RLS were studied. These results imply that this locus may confer risk specifically for the motor component (PLMS), but because these individuals with isolated PLMS were relatives of patients with RLS, it is unknown whether this will also be true in the general population. In addition, serum ferritin levels were found to be decreased by 13% per A allele, potentially underlying the iron deficiency associated with RLS.

Little is known of the function of BTBD9 in mammals, but in *Drosophila*, proteins containing the BTB (POZ) domain have important roles in metamorphosis and limb pattern formation.[70,71] These proteins have wide-ranging functions, making assignment of a specific function to BTBD9 difficult.

LBXCOR as a Candidate and Symptomatology

The third locus identified by Winkelmann and associates was defined by seven SNPs on chromosome 15q. This large LD block contains both *MAP2K5* and *LBXCOR1* genes, and it was not possible to differentiate the origin of the association signal. *LBXCOR1* has appealing links to RLS, as it is expressed selectively in a subset of dorsal horn interneurons in the developing spinal cord, which relay pain and touch. Activity of the LBXcor1/Lbx1 pathway is essential to the generation of a gamma-aminobutyric acid (GABA)-ergic versus glutamatergic phenotype in these cells,[72] and this locus may contribute to the sensory component of phenotype by affecting modulation of sensory/pain inputs.

PTPRD and Nitric Oxide Synthase as Candidates in RLS

In addition to the three replicated loci identified in the initial study, a fourth locus showed suggestive association in the vicinity of RLS3 on chromosome 9p. After extending the sample size to a total of 2458 patients and 4749 controls and genotyping markers in this region, again using a two-stage design with replication, an association at protein tyrosine phosphatase receptor type delta (PTPRD) reached genome-wide significance, with signal identified in two separate haplotype blocks.[73] Surprisingly, no coding mutations were identified among patients from RLS3 families, and the relative risk to siblings attributed to SNPs at this locus could explain only a minor portion of the original RLS3 linkage signal. Nevertheless, PTPRD is an excellent RLS candidate, with established roles in axon guidance and termination of motor neurons during embryonic development in the mouse.[74]

Similar studies have implicated a role for neuronal nitric oxide synthase (NOS1) in RLS. An exploratory case-control study was initiated in a large area (366 genes, 21 Mb) in the chromosome 12 RLS1 region.[75] Three subregions were significant in the first tier of the study. After testing the most significant SNPs in a second sample, one SNP in the NOS1 locus showed significant allele frequency differences after multiple testing correction. These results remain provisional, however, as two SNPs showed significant association in the exploratory and replication samples, but the allele frequencies and direction of the effect differed in the two samples. In addition, no likely coding variants explaining the effect were identified in the *NOS1* coding sequence of a series of homozygous cases. The association results were not explained by stratification, or by lack of adequate SNP coverage, and further

testing in an even larger sample will be needed to confirm the finding. The *NOS1* gene is an appealing candidate in the symptomatology of the syndrome, acting as an "atypical neurotransmitter" in the central nervous system with roles in pain perception, control of sleep–wake regulation, and modulation of dopaminergic regulation.

GENETICS OF OTHER HYPERSOMNIAS, INSOMNIA, AND PARASOMNIAS

Many additional sleep disorders and parasomnias show genetic effects or familial clustering but so far have no established connections with specific genes. No studies have yet identified definitive loci selective for insomnia, and considering the frequency and the complexity of the phenotype, only using extremely large samples and a GWA approach, possibly using an EEG endophenotype,[76] is likely to succeed. Studies have shown that insomnia, like many other conditions, runs in families[77,78] and has higher concordance in MZ twins.[79]

Like insomnia, hypersomnia or isolated sleepiness in the general population is a poorly defined phenotype.[80] Candidate association loci have been reported,[81] using a low-density GWA study (100,000 markers, most of which were not significant after correction for multiple comparison), but they await confirmation. The distinction between narcolepsy without cataplexy (NWC) and idiopathic hypersomnia is difficult. Although presence (NWC) versus absence (hypersomnia) of two sleep-onset REM periods on the multiple sleep latency test is typically used to distinguish these, it is possible that they lie on a continuum with narcolepsy-cataplexy. There is evidence that this is the case for NWC, as rates of DQB1*0602 positivity (40%) are intermediate between the rates found in the general population (12% to 38%) and in narcolepsy cataplexy (70% to 100%). Although measures of cerebrospinal fluid (CSF) hypocretin are more typically within the normal range for NWC, some patients do show low levels in the narcolepsy-cataplexy range in association with DQB1*0602.[82] Hypocretin levels were within normal range for idiopathic hypersomnia, with rare exceptions that showed decreased levels.

Kleine-Levin syndrome (KLS) is a rare disorder characterized by recurring episodes of profound hypersomnia together with cognitive and behavioral changes. It affects primarily adolescent boys and typically mysteriously attenuates and disappears in adulthood. KLS was previously thought to be sporadic, but recent studies[83,84] indicate that there are likely to be genetic factors conferring susceptibility. An excess of Ashkenazi Jewish origin is reported in KLS cases compared with that expected on the basis of the general population, suggesting that a founder effect is potentially acting in this subset. In addition, five out of 105 patients with KLS reported KLS in a family member, indicating a substantially increased risk among relatives and suggesting the action of a major susceptibility gene. A proposed HLA association in KLS was not replicated in a second, larger study.[83,85]

Features of dissociated REM sleep are known to be highly heritable. Both sleep paralysis and hypnagogic hallucinations, representing intrusion of REM atonia and REM imagery into wakefulness, frequently occur in the general population, particularly in the context of insufficient sleep. Sleep paralysis is highly familial, with MZ twins showing higher concordance than DZ twins. Cases of autosomal dominant transmission have also been reported (reviewed in reference 86). There may be an increased prevalence in African Americans. In REM sleep behavior disorder (RBD), there is loss of inhibition of motor pathways in REM sleep, leading to robust and potentially dangerous motor activity in response to dream content. As with other features of dysregulated REM sleep, RBD is common in narcolepsy, but it also occurs in the general population and in patients with Parkinson's disease. There is increasing consensus that idiopathic RBD is an early sign of evolution toward a neurodegenerative disorder, with particular risk for Parkinson's disease.[87] The extent of heredity in RBD has not been established, but a variety of single gene defects are implicated in the development of Parkinson's disease.

Non-REM sleep parasomnias including sleepwalking, sleep talking, and night terrors typically occur during slow-wave sleep (stages III and IV). Prevalence of parasomnias in general is high in children, rarely requires medical intervention, and typically disappears during adulthood. Sleepwalking may be present in up to 20% of children, and it is present in up to 3% of adults,[88] who report that the disorder has been lifelong. If a parent was a sleepwalker, then children have a sixfold increased risk for the disorder. Twin studies show that sleepwalking, sleep talking, enuresis, bruxism, and night terrors have substantial genetic effects, and that they also co-occur, suggesting that they share some common genetic susceptibilities (reviewed in references 86 and 89).

AUTOSOMAL DOMINANT FRONTAL LOBE EPILEPSY

Nocturnal frontal lobe epilepsy (NFLE) can mimic parasomnia symptoms.[90-92] This distinctive disorder occurs in sporadic and autosomal dominant NFLE familial forms, with the clinical presentations having no significant differences. Three characteristic seizures associated with the disorder arise during NREM sleep (reviewed in reference 93). Paroxysmal arousals (PAs) are brief (<20 seconds), consisting of abrupt and frequently recurring arousal from sleep. They are associated with stereotypical movements, and patients raise their heads and sit up, often screaming or showing fright. Dystonic posturing may occur in the upper and lower limbs. A nocturnal paroxysmal dystonia (NPD)[91] type of seizure may develop from a PA and may last up to 2 minutes. These are associated with complex stereotypical movement sequences, with asymmetric tonic or dystonic posturing. Patients may also emit sounds, or nonsense words, and occasionally violent ballic movements with flailing of limbs. PA and NPD may recur with a periodic repetition at 30-second to 2-minute intervals. As observed in the EEG, these are often directly preceded by a K-complex, suggesting that they may trigger the epileptic seizures. Indeed, the periodic occurrence of K-complexes may act to trigger the onset of the seizures through modulating activity of frontal lobe foci. The third and longest form of seizure in NFLE is episodic nocturnal

wandering, which lasts up to 3 minutes. The associated somnambulism results in jumping from bed with agitated violent motor behaviors involving the head, trunk, and limbs. These are much less frequent but may cause severe injuries to the patient.

Differentiation of these from more typical NREM parasomnias can be difficult, requiring polysomnography with video monitoring to document the stereotypy of the attacks and abnormal motor aspects of the seizures. Although NREM parasomnias such as sleep terrors arise earlier in childhood, the age of onset of NFLE is more typically 14 years (but with wide variability). In addition, whereas episodes of sleep walking or sleep terrors are rare and isolated (once in 1 to 4 months), NFLE seizures occur nearly nightly, and with many repetitions through the night. Certainly, the extrapyramidal motor patterns (ballic movements, dystonic posturing) are distinctly different in their origin from simple partial sudden arousals. Finally, whereas parasomnias fade with ageing, NFLE persists into adulthood.

Mapping of autosomal dominant NFLE in families led to the identification of at least three loci causing the disease, with subsequent identification of dysfunction in alpha-4, alpha-2, and beta-2 subunits of the nicotinic acetylcholine receptor (nAChR) affecting the first, second, and third transmembrane domains of the receptor in familial and sporadic cases.[94-97] An involvement of rare variants in the promoter of the corticotrophin-releasing hormone gene have also been proposed.[98]

GENETICS OF OBSTRUCTIVE SLEEP APNEA

Obstructive sleep apnea syndrome (OSAS) is a complex and highly prevalent phenotype affecting 2% of middle-aged women and 4% of middle-aged men in developed countries[99] and more than 11% of older adults.[99,100] Family studies have demonstrated increased aggregation of OSAS for individuals with characteristic craniofacial features.[101] Incidence was not increased in families of very obese patients with OSA, suggesting that the obesity outweighed risk from morphologic predispositions. High rates of concordance were identified in MZ twins.[57]

Linkage was first performed using a set of 375 autosomal markers on a modest-sized set of 66 European-American families with probands, ascertained from a sample of 1349 subjects, and demonstrating an apnea-hypopnea index (AHI) of 20 or greater or severe symptoms warranting therapy. Families were further selected for members in the extremes of the sample's AHI distribution (mean pedigree size, 5.3; range, 4 to 14).[102] Variance component linkage analysis was performed using AHI and body mass index (BMI) as quantitative phenotypes, with several regions showing suggestive linkage to AHI and BMI after correction. An analogous study was also performed in 59 African-American families showing suggestion of linkage to different genomic regions.[103] Although extensively phenotyped, the relatively small numbers of pedigrees used in these studies had limited power to detect modest effects, and markers were spaced at 9-cm intervals, a low resolution in comparison with recently developed high-density platforms. The sample would be more amenable to study using a GWA design on a large scale. Indeed, this would also avoid the issue of the typically large physical distances underlying a linkage peak. Whereas linkage intervals are refined by the observation of recombinants on chromosomes in a family, associations reveal very small regions reflecting vast numbers of historical recombinations in the case-control sample. Associations thus often pinpoint a single gene, or even a subregion of a gene, as defined by the local haplotype block structure. A series of physiologic traits act together to determine the susceptibility to, and severity of OSA, through their individual or combined effects on the obstruction of the pharyngeal airway during sleep.[104] Thus it may be particularly useful to consider distinct separate sub-phenotypes in a genetic analysis of patients with OSA, potentially including pharyngeal anatomy, airway collapsibility (P_{crit}), upper airway muscle responsiveness during sleep, arousal threshold, reactivity of the airway, activation response of pharyngeal dilator muscles, and loop gain reactivity in response to hypoxia.

The only potential candidate that has been replicated is apolipoprotein E (*APOE*). This gene was first suggested as a candidate gene for OSA in a study of 791 middle-aged subjects from the Wisconsin cohort.[105] The probability of moderate to severe apnea significantly increased with dosage of the number of APOE ε4 alleles. This association was confirmed in 1775 subjects from the Sleep Heart Health Study, and an important effect of age was identified[106]; the effect was strongest in individuals younger than 65 years, and weaker in those older than 65. This association remains somewhat controversial, as two other association studies did not replicate the finding, although the samples studied differed by age, ethnicity, BMI, and screening methodology.[107,108] In addition, a recent study of 666 white and 545 African-American subjects in approximately 300 families also found no association between APOE ε4 and risk for OSA (AHI ≥ 15), even when restricted to a young or middle-aged sample.[109] Fine mapping suggested the presence of a susceptibility gene in the region but excluded an effect of APOE. These findings could suggest that susceptibility is conferred by another gene in LD with APOE. It is notable that this sample was multi racial, had a high BMI, and was ascertained through OSA-probands that were identified at clinic visits, potentially indicating more severe disease or more extensive comorbidity. Finally, two studies have suggested an association of APOE and linked polymorphisms with sleep apnea in children, although these findings were made in small samples.[110,111] How APOE ε4 could predispose to sleep apnea is unknown, but multiple mechanisms are plausible, including an effect through lowering levels of choline acetyltransferase and reducing neuromuscular activation of the upper airway dilator muscles, or differential lipid binding to beta amyloid or tau proteins leading to plaque formation in the respiratory center.

CONGENITAL CENTRAL HYPOVENTILATION SYNDROME

Congenital central hypoventilation syndrome (CCHS), formerly known as Ondine's curse, is a congenital and severe form of central sleep apnea resulting from abnormal autonomic control of breathing with decreased sensitivity

to hypercapnia and hypoxemia, particularly during sleep. In addition to the respiratory features, CCHS is also associated with Hirschsprung's disease, tumors of neural crest origin, and diffuse autonomic dysregulation (reviewed in reference 112). Although primarily sporadic, genetic factors are indicated by dominant transmission and by an increased prevalence of symptoms of autonomic nervous system dysfunction (although a considerably milder spectrum of features) among relatives of CCHS probands.[113] The recognition that the *PHOX2B* homeodomain protein is critical for the development of the autonomic system in mice, led to the identification of frequent heterozygous mutations in the *PHOX2B* (4q13) gene in individuals with CCHS.[114] The most common form (90%) of mutation identified is expansion of a polyalanine repeat (from the normal 20 alanine repeats to 25 to 33) in the protein, which probably acts as a spacer domain between functional elements of the protein. The remaining 10% of mutations consist of heterozygous missense, nonsense, and frameshift mutations at the same locus. Although the majority of patients have onset during the neonatal period, later onset cases, remarkably, have also been noted, with onset in later infancy, childhood, and even adulthood, and this has been linked to the presence of a 24-repeat alanine tract, which was also identified in nonpenetrant adult carriers.[115] Onset of symptoms in this form of the disease appears to require additional environmental factors (anesthetics, sedatives) to trigger symptoms in heterozygotes, but when it is homozygous, it may present in infancy. It is tempting to speculate that the variable autonomic nervous system (ANS) dysfunction in the extended families of patients with congenital CCHS is related to the presence of a "premutation" sized *PHOX2B* expansion (>20 and <24) with potential implications for diffuse ANS symptoms in the general population.

SLEEP DISORDERS IN ASSOCIATION WITH OTHER GENETIC DISORDERS

Sleep problems are commonly associated with genetic disorders, even when these are not strictly sleep disorders. Fatal familial insomnia (FFI) was the first "sleep disorder" with an identified single gene mutation, although the phenotype extends well beyond sleep abnormalities and includes dysautonomia and ataxia, as well as subsequent rapid decline in cognitive function. FFI results from the combination of two abnormalities in the prion protein gene: a mutation from asparagine to aspartic acid at position 178 in the context of the presence of methionine at codon 129 (a normal polymorphism).[116] In sporadic cases, the same phenotype can be induced through conformational changes in the prion protein that are not associated with detected mutations.[117] In FFI, there is degeneration in the anterior ventral and mediodorsal thalamic nuclei. As the thalamus and its cortical projections are important in the generation of cortical synchronization in slow-wave sleep and generation of sleep spindles, these thalamic lesions probably underlie the insomnia in FFI.[90]

Patients with Smith-Magenis syndrome have characteristic sleep problems including excessive daytime sleepiness but frequent awakenings at night.[118] The melatonin rhythm in these patients is severely phase shifted, and it is speculated that haploinsufficiency for a circadian-related gene in the characteristic chromosome 17 deletion is responsible.[119]

Obstructive sleep apnea is prevalent in Down syndrome,[120] very likely influenced by the typical flattened midface and upper airway dysmorphology as well as by low muscle tone and enlarged tonsils or tongue. There is major clinical relevance, as OSA could contribute to pulmonary hypertension in these individuals. OSA may also be uniquely tied to morbidity in Marfan syndrome (MS).[121] This connective tissue disease is associated with specific craniofacial abnormalities (high arched palate with dental crowding, retrognathia), which predispose to the development of OSA,[122] and a high rate of OSA was described in a consecutive series of patients with MS.[123] More importantly, OSA has been proposed to contribute to aortic root dilation in patients with MS,[124] which often progresses to aortic dissection, an important source of mortality. Neurodegenerative diseases are often associated with sleep abnormalities. Huntington's disease is associated with excessive daytime sleepiness, disrupted nocturnal sleep, insomnia, and various REM abnormalities.[125,126] Parkinson's disease is associated with significant sleep disturbances. REM behavior disorder may precede motor and dementia symptoms by several years.[127] Patients with Parkinson's disease develop secondary narcolepsy symptoms, and this is supported by evidence of significant hypocretin cell loss in the disease. Excessive sleepiness is a common and disabling symptom of myotonic dystrophy. Short sleep latencies and sleep-onset REM periods were commonly seen, and decreased CSF hypocretin-1 levels have been reported in some but not all cases.[128,129]

Cataplexy has been reported in a number of patients with genetic diseases, although it is highly selective and almost pathognomonic for narcolepsy. Niemann-Pick disease type C (NPC) is a recessive lysosomal storage disorder. Clinical symptoms and severity vary and include hepatosplenomegaly and neurologic findings, such as abnormalities of the EEG in sleep.[130] CSF hypocretin-1 levels in patients with NPC are repeatedly found to be significantly lower than in controls, suggesting that the lysosomal storage abnormalities have an impact on the hypocretin-producing cells of the hypothalamus. Norrie disease is primarily an X-linked eye disease, but other features are variably present depending on the size of the underlying chromosomal deletion, which can include deletion of the monoamine oxidase loci. Cataplexy and REM sleep abnormalities have been described in Norrie disease in association with absent monoamine oxidase activity and increased serotonin levels.[131] Narcolepsy-like symptoms, including excessive daytime sleepiness and cataplexy, have also been reported in Prader-Willi syndrome (PWS),[132] and several studies have reported reduced CSF hypocretin levels.[82] These results and others indicate that the excessive daytime sleepiness seen in PWS is not simply secondary to obesity and OSA in these individuals.

CONCLUSION

Thanks to the recently completed human genome sequence and the description of single nucleotide and copy number variation polymorphisms across the genome, gene

identification has progressed rapidly in many areas of medicine. Focus is now shifting from the identification of very rare familial single gene disorders using linkage analysis to genome-wide approaches using high-density marker sets. These are leading in some cases to the identification of common polymorphisms in entirely new pathways, such as the developmental genes that act as predisposing factors for RLS, and, in other cases in other areas of medicine, to the identification of novel rare high-penetrance mutations—for example, deleterious duplications or deletions that may cause autism. Additional studies are focusing on finding rare phenotypes and rare mutations in selected genes in the population, followed by family analysis—for example, in the circadian rhythm disorder area. It is likely that these approaches will increasingly be applied to sleep disorders research, a promising area, as sleep is an innate behavior with measurable physiologic variables. Importantly, however, as these studies are only nascent in our field, we suggest strict criteria for publication, including robust effects, adequately powered sample sizes, and independent replication of findings (for genetic association studies) or strong functional analysis data (for single-family reports and, if possible, in genetic association). The literature currently contains many studies with variable and sometimes conflicting results. When interpreting these, it is important to evaluate not only statistical confidence (*P* value), but also sample size, reliability of phenotype, and size of the effect (i.e., odds ratio). On one hand, some studies report rare mutations with very large effects and clear functional effects (as in familial circadian disorders, and the complex congenital hypoventilation syndrome phenotype). In other studies (typically association studies), effects are small and the proof primarily lies with statistical confidence and independent replication (for example, in RLS). In genome-wide association studies, it is likely that most of the identified polymorphisms will be risk factors only for sleep disorders, many with small effects, and will thus warrant further studies either in basic research programs to better understand the pathophysiology of the corresponding sleep disorder and associated phenotypes, or as tools to subdivide groups of patients if the polymorphisms are shown to have predictive outcome value or are helpful in guiding therapy.

❖ Clinical Pearl

A large number of publications have described associations between genetic polymorphisms and sleep-related phenotypes, but most have not been replicated, and results should be considered preliminary. Results using new methods such as genome-wide association studies have begun to flow in the sleep field, with exciting and robust results indicating novel pathways in sleep-related diseases.

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