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GENETICS OF SLEEP AND SLEEP DISORDERS

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1. ABSTRACT

Sleep has been observed in all vertebrates studied and in several invertebrates, notably the fruit fly *Drosophila melanogaster*. In all species, a substantial portion of life is spent in this behavioral state and disturbed sleep or lack of sleep has immediate negative impacts on performance and health. Although it is agreed upon that sleep fulfills a fundamental biological need, the function of sleep remains an enigma. Because the expression and regulation of sleep and some sleep disorders have strong genetic components, the recent progress in human, mouse, and fruit-fly genome sequencing projects have given rise to the expectation that the molecular pathways underlying sleep disorders and sleep regulation or even function can now be more readily identified. We review here available genetic data both from basic sleep research and sleep disorders with emphasis on recent advances in our understanding of the molecular basis of the homeostatic regulation of sleep. Recent studies in the dog, the mouse, and the fruit-fly have begun to reveal exciting new molecular pathways that regulate sleep. This illustrates that only the continued use of multiple animal models and genetic approaches will ensure a rapid progress in the relatively new field of sleep genetics.

2. INTRODUCTION

Much progress has been made in our understanding of the mechanisms underlying different aspects of sleep physiology such as the neuronal substrates, the neuro-chemical messengers, and the dynamics of the regulatory processes involved in initiating and maintaining sleep. With the recent discovery concerning the link between the hypocretin (/orexin) system and narcolepsy in mind, we also begin to understand the basic patho-physiological mechanisms involved in sleep disorders. Nevertheless, despite this important progress, the identification of the neurobiological substrate of sleep function seems to remain as remote as ever. We do know that the expression and regulation of sleep and some sleep disorders have strong genetic components. We also know from the discovery of the hypocretin pathway and its involvement in narcolepsy and from examples from the field of circadian rhythm research, notably the identification of the circadian gene, *Clock*, in mice, that genetic approaches can be very successful in identifying novel proteins and pathways implicated in complex behaviors such as sleep. In addition, with the recent identification of sleep in the fruit fly, *Drosophila melanogaster*, an additional powerful and promising tool for the genetic dissection of sleep became available. For these reasons and because of the current progress in the human, mouse, and fruit-fly genome sequencing projects, expectations are high that the molecular pathways underlying several sleep disorders and sleep regulation or even function will be uncovered soon. Thus, the genetic dissection of sleep constitutes a viable alternative approach to address questions concerning the physiology of sleep and sleep function.

Sleep is a complex behavior that can be studied at many different levels. Depending on the questions being asked, sleep can be studied through behavioral observation, sleep/wake related changes in neuronal activity or in gene expression, subjective assessment of sleep, and waking

performance to name a few. In this review the homeostatic regulation is put central since we believe that the identification of the molecular pathways underlying this aspect of sleep regulation will bring insight into what it is that is depleted or accumulates during wakefulness and necessitates sleep. The review is structured according to the three main genetic approaches that can be taken to genetically dissect sleep followed by a brief overview of sleep disorders for which a genetic component has been established with special emphasis on narcolepsy.

3. SLEEP IS A HIGHLY COMPLEX PHENOTYPE

Sleep is a complex behavior both in its manifestation and its regulation. The various aspects of sleep differ in their regulation and interact with each other and with the environment. Each of these aspects is likely to be under the control of a multitude of genes and each component of sleep must therefore be considered a complex trait. To successfully apply genetic analysis to these traits it is of importance to precisely and quantitatively identify the phenotype to be analyzed. Although sleep is a behavior, in a laboratory setting, sleep in mammals is no longer studied through behavioral observation. Instead, sleep researchers almost exclusively rely on electrophysiological potentials measured from the cerebral cortex (EEG), muscle (EMG), and the eye (EOG) to determine sleep; i.e., polysomnography. Based on these electrophysiological signals, in birds and mammals one can distinguish the three main behavioral states: wakefulness, rapid-eye-movement sleep (REMS), and non-REMS (NREMS). With these measures the amount, distribution, and quality of sleep (indexed as sleep fragmentation, consolidation, or sleep episode duration) can be quantified. Apart from using the EEG signal to determine behavioral state, the signal itself contains information concerning neuronal activity of the structures that generate these signals and are thought to govern sleep. Specific phasic events such as EEG spindles, K-complexes, and the typical EEG patterns preceding REMS in rodents, can be extracted from the signal using pattern recognition algorithms. The amplitude and frequency of rhythmic EEG activity (e.g. delta, theta, and alpha oscillations) can be quantified using periodogram algorithms such as the Fast-Fourier Transform (FFT). The electrophysiological correlates of sleep have been thoroughly examined in the mouse thus forming the basis for the genetic dissection of sleep and the sleep EEG in this species (1-6). In invertebrates, sleep has to be established behaviorally according to criteria such as behavioral quiescence, increased arousal thresholds, and rapid reversibility (7). With these criteria two recent studies have reliably identified sleep in the fruit fly (8-9). Both the mouse and the fruit fly are now used as the model systems of choice to study the genetics of sleep (reviewed in 10).

The functions of sleep remain unknown. One step towards the elucidation of sleep function is to learn how sleep is regulated. By determining how the various aspects of sleep change is response to sleep loss (either spontaneous or induced) and how these responses vary with different mouse and fruit fly genotypes, might give important clues to the molecular substrates of sleep need or even function. In general, sleep is believed to be regulated by two main processes: a circadian and a homeostatic process (11-13). The circadian process is a self-sustained oscillation that is generated in the suprachiasmatic nuclei (SCN) of the hypothalamus in mammals (14) and in the small ventrolateral neurons (*s*-LN_vs) in the fruit fly (15). It gives time-context to most physiological processes and behaviors including sleep, and ensures proper entrainment between internal rhythms and the external alternations in photoperiod. Thus, the distribution of sleep over the 24-h day is strongly determined by the circadian process. The homeostatic process tracks sleep need which accumulates in the absence of sleep and decreases in its presence. Thus, with increased time-spent-awake the propensity or need to initiate sleep increases. As a consequence of the daily (circadian) distribution of sleep and wakefulness the homeostatic process also follows a 24-h oscillation. The important distinction being that this oscillation is driven by the sleep-wake distribution whereas the circadian rhythm is self sustained. The two processes develop independently but their interaction determines the timing, duration, and quality of both sleep and wakefulness (16-17).

Homeostatic regulation implies that manipulations that increase sleep drive (e.g. sleep deprivation) ought to increase subsequent sleep intensity and/or duration. During sleep, sleep drive should again decrease. An easily quantifiable EEG measure of NREMS intensity, delta power (i.e., EEG power in the frequency range of delta oscillations: 1-4 Hz range), is a reliable physiological marker of homeostasis (11-13). Other aspects of sleep, such as amount of NREMS and amount of REMS are also homeostatically regulated but their dynamics differ from that of NREMS intensity (4,18-21). Both these aspects of homeostatic sleep regulation (duration and intensity) are also evident in the fruit fly (8-9). Sleep deprived fruit flies show compensatory increases in sleep time during recovery that is associated with increased arousal thresholds (8-9). In mammals, variations in arousal thresholds correlate with changes in EEG delta power (22-23).

4. STRATEGIES TOWARDS THE GENETIC DISSECTION OF SLEEP

Several (complementary) approaches can be taken to arrive at the genetic underpinnings of the various aspects of sleep, the sleep EEG, sleep homeostasis, and sleep disorders. Roughly, the approaches can be categorized as molecular genetic approaches, forward genetic approaches, and reverse genetic approaches. Progress achieved using each of those approaches will be reviewed below with special emphasis on the quantitative-trait-loci (QTL) approach since it is best suited to dissect complex traits like sleep and is the approach we adopted in our laboratories.

4.1. Molecular genetic studies

The molecular approach aims at identifying genes that change their expression as a function of time-spent-awake (or time-spent-asleep) as opposed to genetic approaches that try to identify genes of which mutated or

polymorphic alleles modify the expression of a given sleep phenotype. The molecular genetic approach is motivated in part by the fact that the temporal dynamics of sleep homeostasis, indexed as EEG delta power in NREMS (4,13,24), are compatible with the dynamics of gene expression. Both the accumulation of sleep need and processes involving transcription and translation that can lead to substantial changes in proteins, are relatively slow (many minutes to hours). In contrast, processes involved in sleep initiation and sleep-state transitions are fast (minutes and shorter); especially in the mouse, and therefore it seems unlikely that changes in gene expression play a role at this level of sleep regulation. The expectation of the molecular approach is to identify genes that are functionally relevant to the homeostatic regulation of sleep although it cannot be ruled out that the expressional changes of some of these genes are merely driven by the sleep-wake distribution instead of being functionally relevant. Rhyner and colleagues (25) were the first to use a molecular approach to identify genes that change their expression after sleep deprivation in the rat. With substractive hybridization several clones were isolated with increased or decreased relative expression, among which neurogranin and dendrin where later identified (26-27). Cirelli and Tononi and colleagues have continued this work to include changes in expression after spontaneous periods of sleep and wakefulness and both short- and long-term sleep deprivations (reviewed in 28-29). They employed a multitude of techniques to systematically screen brain gene expression such as mRNA differential display and cDNA microarrays. The total of their results indicates that only a minority (<1%) of the ~10,000 genes screened seem to be modulated by sleep and wakefulness in the rat; a finding similar to that obtained in the fly (8). In rats, modulated genes include heat-shock proteins, transcription factors, metabolic enzymes, growth factors, and neural plasticity-related genes (28-29).

One gene that changed its expression with wakefulness in the rat codes for the enzyme aryl-sulfotransferase (28). Compared to sleeping rats, the cortical expression of aryl-sulfotransferase increased as a function of time-spent-awake; i.e., >2-fold after 8-h of spontaneous or enforced wakefulness and >4-fold after long-term sleep deprivations (4-14 days). These findings were confirmed in the fruit fly; the expression of the gene arylalkyamine N-acetyl transferase (*Dat*), which is functionally closely related to aryl-sulfotransferase, was increased after sleep deprivation (8). Both enzymes are implicated in the catabolism of monoamines. It is suggested that these enzymes play a role in countering the waking related monoaminergic release (28). Impaired enzyme activity should therefore be associated with increased sleep need. Evidence to support this idea was obtained in fruit flies mutant for *Dat*. These flies showed an increased rebound of sleep amount after sleep deprivation (8). The degree by which the rebound after sleep deprivation was increased compared to wild-type controls varied with the degree by which the mutation affected DAT activity. Thus the wakefulness related accumulation of monoamines may be a factor contributing to sleep need. These findings illustrate that the molecular genetics approach, complemented with other genetic approaches, is powerful in identifying genes that might be implicated in sleep homeostasis and that the mouse, rat, and fruit-fly models should be used in parallel.

Although these data demonstrate that the molecular approach is valuable tool to find 'sleep genes', a gene that does not show transcriptional modification may nonetheless play an important role. Two examples from the field of circadian rhythm research include the circadian genes *Clock* in the mouse and *Cycle* in the fly (30-31), neither of which shows a robust circadian oscillation. Both genes were identified with mutagenesis, a forward genetic approach (31-32).

4.2. Forward genetic studies

Abundant evidence exists that many aspects of 'normal' sleep and EEG, and several sleep disorders have strong genetic components (reviewed by 33-35), suggesting that allelic variants or mutations of genes must exist that underlie them. Especially results from twin studies make this clear. Sleep and EEG patterns of monozygotic twins have a much higher resemblance than those of dizygotic twins or unrelated subjects (36-52), confirming that these complex traits are tightly controlled by genes and that environmental factors play a lesser role. Genetic studies of sleep in the mouse, pioneered by Valatx, yielded similar results. 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 REMS (1,53-58). However, until very recently, none of the genes underlying these sleep traits have been identified. The discovery that a mutation of the hypocretin-2 receptor gene underlies canine narcolepsy is the best example of a successful application of the genetic approach to finding 'sleep'-genes (59; see below).

In the genome-wide search for genes affecting a particular phenotype, no *a priori* assumptions on the gene systems involved are made. Although this approach may lead to already known physiological mechanisms, its strength is that systems previously unknown to be involved in sleep may be uncovered. Therefore, a genome-wide search is the method of choice if we are to discover 'sleep'-genes. To arrive from phenotype to genotype the following steps are usually followed (60-62). First, a highly reliable phenotype has to be defined that describes the trait of interest. Subsequently, the mode of inheritance of this trait has to be determined in segregating offspring. Then the localization of the gene has to be mapped. Initial mapping entails a genome-wide search using polymorphic markers (e.g. RFLPs, SSLPs, SNPs) tagging the entire genome at regular intervals. In the recombinant offspring the trait will co-segregate with the markers most closely linked to the underlying gene(s). This step will yield large genomic regions (usually 10-30 cM) containing the genes of interest. Subsequent fine mapping should reduce this to a sub-cM interval. At this point confirmation in other crosses or pedigrees is desirable before committing to the final steps. Unless, data-base searches yield a convincing candidate gene, positional cloning techniques must then be applied to

find and characterize the gene sequence. Once the gene is identified its function can be verified by gain- or loss-of-function in knock-out and transgenic mice models. All these steps were followed in the discovery of the 'narcolepsy'-gene (see below) and basically are also followed in the other two main genetic approaches: mutagenesis and QTL analysis.

QTL analysis is the method of choice to genetically dissect complex traits like sleep since with this approach naturally occurring allelic variations or gene mutations with small effect can be mapped (61-66). QTL analysis can be performed in several segregating mouse populations including inter- and backcross, advanced inter- and backcross, recombinant inbred (RI), and heterogeneous stocks (61,67). Usually, two inbred mouse strains differing in a trait of interest (although not a prerequisite for identifying meaningful QTLs) are crossed and their F1 offspring are then either intercrossed to generate F2 offspring or backcrossed to one of the progenitor strains to generate backcross populations. Further random intercross or backcross generations can be performed to generate advanced inter- and backcross populations. To generate RI sets, F2 mice are inbred by brother-sister matings until full homozygosity thereby 'fixing' a unique set of recombinations in several inbred lines. Heterogeneous stocks are generated by intercrossing several inbred mouse strains over many generations and therefore represent higher rates of recombination and polymorphism useful for fine mapping (67-68). Although RI sets are usually not suitable for QTL mapping due to their limited number of strains, for QTLs of large effects, they may provide significant mapping accuracy because of the fourfold increase in recombination as compared to a F2 population (61). In QTL analysis, mapping entails finding statistical significant associations between variation in a quantitative trait and variation in genotypes at particular genomic loci, typically ~100 well-distributed markers polymorphic between the progenitor strains. QTLs then refer to genomic regions 10-30cM in size that contains gene(s) with functionally polymorphic alleles that affect the trait of interest. Further gene identification follows the basic steps listed above.

Whereas the QTL analysis aims at identifying 'naturally' occurring allelic variants or gene mutations that modify sleep, in mutagenesis, gene function is assessed by randomly inducing mutations. A mutagen like *N*-ethyl-*N*-nitrosurea (ENU) is used to mutate spermatogonia at an average rate of 0.001 mutations/locus/gamete (60). With high-throughput screening of several hundreds of offspring for either dominant or recessive mutations, a major effect on a given trait can be identified. The individual mouse or fruit fly for which an aberrant phenotype has been recorded then has to be crossed 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* (32,69). The success and choice of each of the approaches is determined by the gene effect. Although some mutations can induce remarkable phenotypic changes, others produce only subtle effects that, in addition, can be confounded by gene-gene interactions (epistasis) and genetic background (modifier genes) (70). Mutagenesis will therefore be more successful for fully penetrant dominant or recessive mutations whereas the QTL is more powerful in detecting natural allelic variations controlling complex traits (63). This is not to say that QTL analysis cannot identify major effect genes (genes that explain a large portion, > ca. 25% of the genetic variance of a trait) or single genes. In fact, in an initial QTL screen these will be the genes/QTLs that will be identified first, if present. In addition, with currently available technologies, recording and analyzing sleep of thousands of mice in a mutant screen does not seem feasible, at least not in a single academic laboratory.

Controversy exists concerning the efficacy of the QTL approach in identifying genes and currently forward genetics by genome-wide mutagenesis is being favored (71). Nonetheless, 29 genes underlying polygenic traits have thus far been identified using QTL mapping (72). Furthermore, the QTL approach has several clear advantages for evaluating complex traits where gene effects are small (pointed out above). QTL and mutagenesis should be viewed as complementary approaches (65). A good example of this was again provided by Takahashi's group for the circadian behavior in mice (73). Although most of the circadian genes that constitute the molecular circadian clock have been discovered mainly by direct molecular techniques and mutagenesis, these genes do not explain the complexity of the observed circadian behavior, i.e., none of the known circadian genes has been found to be involved in the difference in circadian period length between BALB/c and C57BL/6 inbred mouse strains. Instead, QTL analysis in a BALB/c x C57BL/6 intercross panel revealed several new loci with epistatic interaction (73).

4.2.1. QTL analysis of sleep and the sleep EEG

Many aspects of sleep and the sleep EEG differ dramatically among different inbred strains of mice (1-6,74). Because mice of a particular inbred strain can be considered genetically identical clones that differ from other inbred strains, genetic factors are likely to underlie these strain differences. The segregation of these sleep traits in recombinant offspring of the strains for which a trait differed can be used for mapping of the genes involved. As stated earlier QTL analysis was designed to dissect complex traits such as sleep that are presumably under the control of many genes. QTL analysis is most readily explained in sets of recombinant inbred (RI) strains for which all individuals are homozygous at all loci for one of the two parental alleles and individuals of one RI strain have an identical recombination pattern. Therefore, in the mapping analysis only one genotype has to be considered for each marker/gene and genotyping has to be done only once for each RI-strain. In its most simple form this mapping entails point-correlations between the strain-distribution pattern (SDP) of the phenotype and the SDP of the genotype at each maker as illustrated in figure 1. It is assumed that the gene(s) responsible for the variations in the phenotype are linked or have co-segregated and must be localized in the proximity of markers that give the lowest *p*-values (or highest LOD-scores). The *p*-values of the

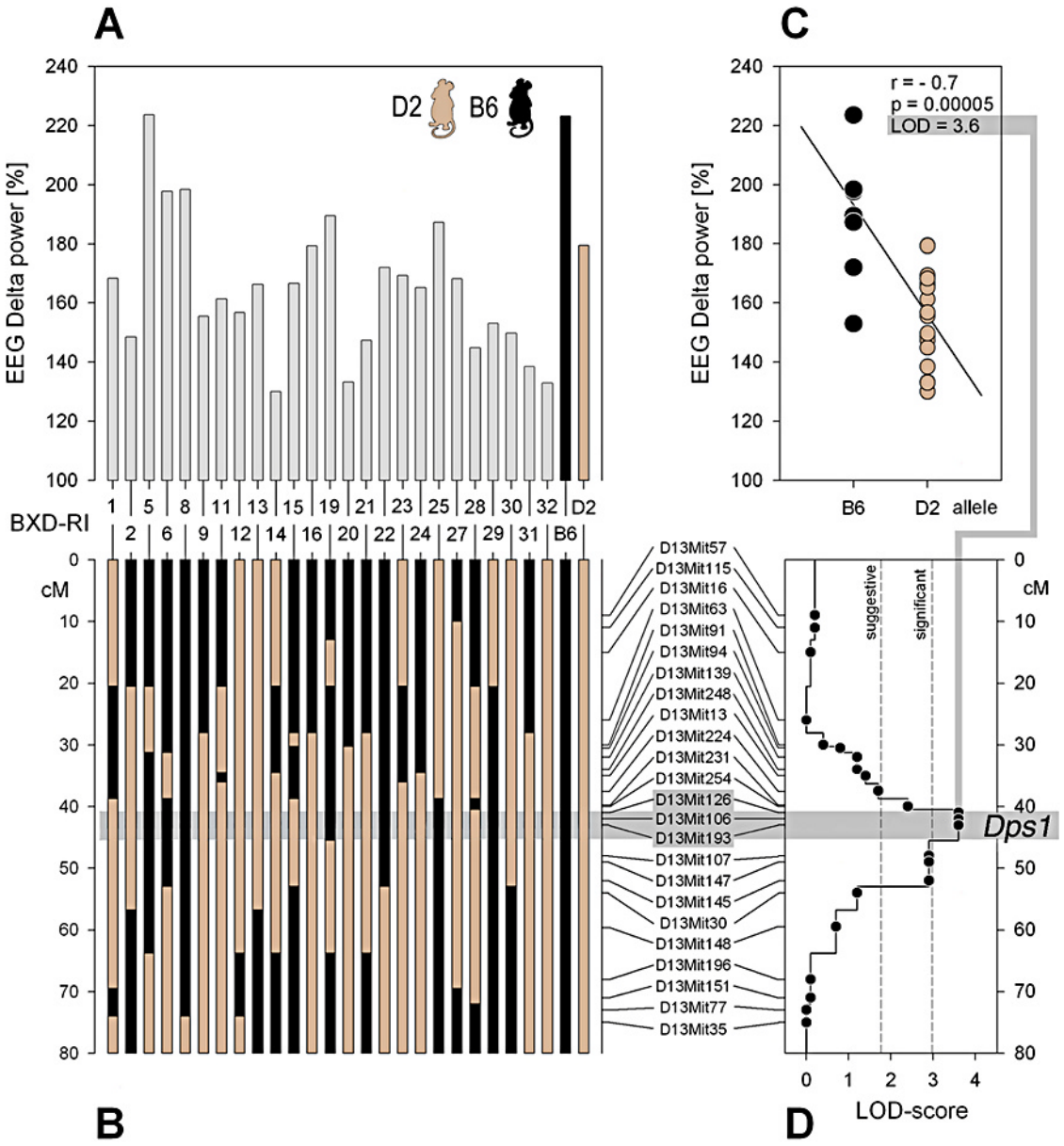

Figure 1: Quantitative-Trait-Loci (QTL) analysis illustrated for the chromosome 13 *Dps1* QTL (see text for details). **A.** The strain-distribution pattern (SDP) of the phenotype. In 25 BXD-Ty Recombinant Inbred (RI) strains (BXD-1 to BXD-32) and their parentals C57Bl/6J (B6; black) and DBA/2J (D2; brown) the rebound in EEG delta power was measured after 6-h sleep deprivation (bars indicate mean strain values; n=128; 4-7/strain). **B.** BXD-RI recombination pattern for chromosome 13 (B6 alleles in black; D2 alleles in brown). 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 centiMorgan (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 grey horizontal bar in panel B) yielded the best correlation coefficient (r), which was highly significant (*p*) and translated into a 3.6 LOD-score. **D.** Among all 788 Mit-markers used, only for these three markers a genome-wide 'significant' level ($p<0.05$; see text) was obtained. We named this QTL *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(s) 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 (4) and refined mapping is in progress.

individual correlations have to be corrected to avoid type-1 errors; i.e., obtaining significant correlations by chance, which can be quite substantial when hundreds of markers are being tested. To correct for this, genome-wide probability (*p*) levels can be established using a permutation algorithm in which trait data are randomly

assigned among the RI strains for e.g. 10,000 times (75). For each permutation the single best p-value is recorded yielding an empirical probability function from which genome-wide probability levels for linkage are derived with designations such as 'suggestive' (genome-wide *p<0.65*), 'significant'(*p<0.05*), and 'highly significant' (*p<0.001*) (76). QTL analysis of inter- and backcross panels follows these same principles. The use of these panels have disadvantages since each individual's recombination pattern is unique and thus has to be established individually and also one phenotype can be correlated to that genotype instead of the mean of several in RI-strains. In addition, the rate of recombination is four-fold lower compared to RI-strains yielding less precise localizations. These disadvantages are however outweighed by the fact that RI-lines are limited in number (and therefore unable to detect small effect QTLs) and cannot be easily generated whereas inter- and backcross offspring can be generated easily and unlimited.

We followed this protocol to identify QTLs for the homeostatic regulation of NREMS in BXD-RI mice (figure 1). The trait of interest was defined as the level of EEG delta power in NREMS reached after a 6-h SD. Large inter-strain differences were observed in this trait and ca. 37% of the total variance could be attributed to additive genetic factors (i.e., heritability). We have identified a genome-wide 'significant' QTL on chromosome 13 (genome-wide *p<0.01*; nominal correlation *p<0.00005*) and a 'suggestive' QTL on chromosome 2 (4). 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. Analysis of EEG delta power at the onset of their major rest period (after an extended period of spontaneous wakefulness) yielded a suggestive QTL that overlapped with the QTL obtained for EEG delta power levels reached after enforced wakefulness. We termed this QTL *Dps1* for delta-power-sleep-1. We identified two additional significant QTLs (*Dps2,3*) for sleep need at sleep onset in baseline but since 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 prior to sleep onset than to the homeostatic regulation of sleep itself. A QTL on chromosome 2 could not be confirmed in baseline probably because it's relative contribution had become too small or, alternatively, because its effect on EEG delta power after enforced wakefulness was related more to the experimental condition needed to keep the animals awake ('stress', increased activity) than to the wakefulness itself. The basic assumption underlying the QTL analysis is that the identified chromosomal regions contain genes with functionally different alleles. Although a mere speculation at this point (the identified ~15cM region for *Dps1* could easily contain 200 genes), the following candidate genes previously implicated in sleep regulation could underlie *Dps1*: the dopamine transporter, neurotrophic tyrosine kinase-2 receptor, and corticotropin releasing-hormone binding-protein.

Apart from the above mentioned study, surprisingly few other QTL studies have investigated 'natural' sleep and EEG traits. We performed the first sleep QTL study in 1997 (77). In that study, amounts of baseline sleep were determined in seven CXB (BALB/cBy and C57BL/6) RI-strains and only QTLs related to REM sleep were identified. Due to the small number of strains the results had only limited power in yielding significant QTLs. In an extended CXB set consisting of 13 lines Toth and Williams, identified several QTLs related to both NREM and REM sleep (78). One QTL for the amount of REM sleep on chromosome 17 was present in both studies but for none of these QTLs a genome-wide 'significant' level was reached. In an accompanying paper the same authors in the same CXB set found a 'significant' QTL for the variation in the viral-infection induced increase in NREM sleep on chromosome 6 (79). A promising candidate gene located in the QTL region codes for the growth-hormone-releasing-hormone (GHRH) receptor. GHRH promotes NREM sleep (80) and mice lacking a functional GHRH-receptor fail to mount the normal increase in NREM sleep in response to an influenza challenge (81). The identification of a functional GHRH-R polymorphism between BALB/cBy and B6 mice (the two progenitor strains of this RI set) should provide further confirmation. Baseline sleep has also been studied in BXD RI mice and significant QTLs has been reported for the amount of REM sleep in the light period on chromosome 1 and for the light-to-dark-period difference in total sleep amount on chromosome 19 (82-83). Finally, it is important to emphasize the results of our most recent QTL study (84) since it is the first study that found positive genetic evidence for a gene underlying a sleep related trait using the quantitative genetic approach. Inbred strains vary greatly for their frequency of EEG theta oscillations (5-9 Hz) during REM sleep (2). The frequency difference amounts to 1 Hz between inbred strains with slow (5.75-6.25 Hz) and strains with fast theta oscillations (6.75-7.75 Hz). The segregation of this trait was followed in inter- and backcross and RI panels between BALB/cByJ (slow theta) and C57BL/6J (fast theta; 84). With the QTL approach one single gene was identified on chromosome 5 that was tightly linked to theta frequency (LOD score >30). This example proves that QTL analysis can be successful in identifying new genes underlying sleep and EEG traits although in this particular case the trait difference between the progenitor strains was found to be a single gene effect.

4.3. Reverse genetic studies

As opposed to genetic approaches ('from phenotype to genotype') reverse genetic approaches test the involvement of a candidate gene in the expression of a trait of interest ('from genotype to phenotype'). To further emphasize this distinction, genetic approaches are now being referred to as forward genetics. Reverse genetic have been made possible by the development of gene targeting techniques that directly manipulate a specific gene of interest at the level of the DNA producing transgenic animals. By homologues recombination in embryonic stem cells, an altered gene construct replaces the existing gene (85). If the inserted gene-construct translates into a non-functional protein, the animals homozygous for this construct are often referred to as 'knock-out' animals. With non-homologues (illegitimate) recombination one or

Table 1. Effects of gene manipulation on sleep in mice

Gene	Main Effect	Ref.
Knock-Out		
Albumin D-binding protein	Decreased sleep continuity, Decrease daily amplitude in EEG delta power No increase in REMS after SD	97
c-fos	Increased wakefulness, decreased NREMS	96
Cryptochromes-1 and -2	Increased NREMS consolidation, amount, & EEG delta power, Decreased response to SD in these variables	100
Dopamine transporter	No response to amphetamines and modafinil	136
fos B	Decreased REMS	96
GABA-A receptor ß3	No response to Oleamide	137
Histidine-decarboxylase	Increased REMS, decreased wakefulness at dark-onset, NREMS EEG power redistributed to faster frequencies	138
Histamine H_1 receptor	No response to hypocretin 1	139
Interleukin-1 type I receptor	Decreased TST during the dark period No response to IL-1 beta	140
Interleukin-10	Increased NREMS during the dark period, Altered response to lipopolysaccharide challenge	141
Period-1, Period-2	Altered distribution of sleep amount in baseline Reduced response to SD in frontal EEG delta power	142
Prepro-hypocretin	Narcolepsy	130
Prion protein	Decreased sleep continuity, Longer lasting EEG delta power increase after SD	91
Prion protein	Longer lasting EEG delta power increase after SD	119-120
Rab3a	Increased NREMS, Reduced response to SD in NREMS amount	101
Serotonin 1A receptor	Increased REMS, no REMS increase after SD	103
Serotonin 1B receptor	Increased REMS, no REMS increase after SD	102
Serotonin 2C receptor	Less NREMS in baseline, Increased rebound in NREMS and EEG delta power after SD	99
TNF receptor-1	Decreased TST in L-period, No response to TNF alpha No change in TST	143 144
TNF receptor-2	Decreased REMS in L-period	144
TNF/Lymphotoxin-a	Decreased REMS in L-period Increased NREMS consolidation & EEG delta power.	144
Voltage-gated K-channel	Decreased 'high' EEG delta power	145
Point-mutation		
GABA-A receptor a1	No effect on Diazepam-induced sleep changes	146
Mutagenesis		
Clock	Decreased NREMS, no REMS increase after SD	98
'earlybird' (Rab3a)	Reduced response to SD in NREMS	101
Transgenic		
ß-amyloid precursor protein	Decreased REMS, increased sleep fragmentation	147
Growth hormone	Decreased NREMS in mice with a somatotropic deficiency Increased REMS when overexpressed	92 95
Hypocretin/ataxin-3	Narcolepsy	134
Insulin	Non-specific background effect	148
Prostaglandin D synthase	Increased NREMS after tail clipping	149

REMS = Rapid-Eye-Movement sleep; NREMS = non-REMS; SD = sleep deprivation

more gene copies are inserted into the genome at undefined locations (86). Animals carrying these constructs are often referred to as transgenics although with both homologues and non-homologues recombination techniques transgenic animals are generated. Animals produced with this method usually are gain-of-function mutants since they express a novel gene product or misexpress a normal gene. Thus, with gene-targeting techniques one can create model organisms to study the effects on sleep of a change in protein levels (from overexpression in transgenics, to non-functional protein in knock-outs), and altered, or novel proteins (knock-in, transgenic). These models are also useful in confirming the role of genes that were identified by forward genetic approaches. Advantages and problems of these techniques have been addressed in several reviews (87). The main issues concern developmental compensation (e.g. other molecules could compensate for the lacking protein; 88), non-specificity (the relevant protein is absent in all the cells of the organism in stead of the tissue of interest), and genetic back-ground (genes that co-segregate with the introduced gene might differ between the back-ground strain, usually C57BL/6, and the strain in which the altered ES cells were introduced, usually a 129-strain, and might affect phenotype; 89). Some of these issues could be overcome by developing (tissue-specific) conditional or inducible knock-out models where the acute effects of loss-of- function can be studied in structures of interest (90).

An attempt has been made to compile a complete list of published studies in which sleep was recorded in transgenic and mutagenized mice (table 1). A short statement on the most prominent sleep changes observed is added but for details the reader is referred to the original reference. The first sleep studies using transgenic mice appeared in 1996 (91-92). Most studies focused on pathways already known to be involved in sleep regulation. For example the monoamines including serotonin,

dopamine, histamine were among the first compounds to have been suggested to play a role in sleep regulation (93) and knock-out models for their receptors and transporters have been studied (table 1). Another pathway studied and implicated in sleep is the cytokine pathway (94). Transgenic models for this pathway include Il-1β, Il-10, Tnf, and the Tnf receptors-1 and -2 (table 1). A last group concerns genes that are regarded as canonical circadian genes [*Clock*, period-1 and -2 (*Per1*, *Per2*), cryptochromes-1 and -2 (*Cry1*,*Cry2*)] and genes known to alter circadian rhythms [albumin-D-binding protein (*Dbp*), ras-associated binding protein 3a (*Rab3a*); table 1]

In view of the relevance of identifying genes that are involved in the homeostatic regulation of sleep, studies in which sleep deprivations were performed are of special interest. Mice over-expressing growth hormone show more REM sleep under baseline conditions but show normal recovery patterns following sleep deprivation (95). Disruptions of *c-Fos* and *Fos-B* modify baseline sleep but *c-Fos* KO mice respond to sleep deprivation primarily with an increased latency to sleep onset (96). In mice lacking DBP the distribution of sleep is altered and sleep is more fragmented but the response to sleep deprivation in NREMS amount and delta power did not differ from wild-type (97). Also in *Clock*-mutant mice the relative increase in NREMS after sleep deprivation was the same as in wild-type mice although NREMS amount in baseline was reduced (98). These examples demonstrate that altered expression of sleep under baseline conditions can not be taken as evidence of altered homeostatic regulation of sleep. This, however, 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 (99), *Cry1* and *Cry2* (100), and *Rab3a* (101) all were reported to have an altered NREMS rebound after sleep deprivation. In these cases the difference was, however, attributable to a large extend to NREMS differences in baseline since no differences in recovery 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 effect of spontaneous and enforced periods of wakefulness on a regulated variable are quantified (see 4 for both approaches). Furthermore, especially where the regulation of NREMS is concerned, one cannot rely on only one aspect because changes in both duration and intensity or consolidation have to be taken into account. Changes in REMS homeostasis have been observed in several knock-out models. In mice lacking serotonin-1A or -1B receptors (102-103), *Dbp* (97), *Cry1*,*Cry2* (100), and in *Clock*-mutant mice (98), loss of REMS was followed by a compensatory increase in REMS that was smaller than in wild-type animals or lacking all together. Apart from the serotonin-1A and -1B receptor knock-out mice that displayed increased REMS during baseline (102-103), these changes in the REMS response after sleep deprivation could not be attributed to genotype differences in REMS during baseline.

One rational for using knock-out mice for genes critical for circadian rhythm generation such as *Clock*, *Per1*, *Per2*, *Cry1*, and *Cry2* (104) is that they provide a model in which sleep homeostasis can be studied in the presence of an altered or absent circadian modulation. Several studies that examined circadian and homeostatic influences on sleep including studies where circadian rhythms were eliminated by lesioning the SCN (105-108) and studies where subjects followed a forced-desynchrony protocol (16-17), revealed that direct circadian effects on the sleep homeostatic process were small, if at all present. However, loss of circadian genes does not only affect circadian rhythms, it seems also to affect the homeostatic regulation of sleep. A clear demonstration of this was observed in *Cry1*,*Cry2* double knock-out mice that under baseline conditions showed all the hallmarks of high NREMS pressure, including a higher amount of NREMS, increased NREMS consolidation, and higher NREMS delta power compared to wild-type controls (100). After 6-h sleep deprivation, there was no further increase in NREMS time and consolidation and a reduced rebound in EEG delta power. This suggests that apart from their role in regulating circadian rhythms, cryptochromes or genes regulated by cryptochromes, play a role in sleep homeostasis. This suggestion is strengthened by observations in fruit flies where strains carrying loss-of-function mutations for the fruit fly canonical circadian genes *Per*, *Timeless*, *Clock*, or *Cycle* all show a more pronounced sleep rebound after sleep deprivation than wild-type fruit flies (109). *Cycle*-mutant flies were exceptional in this respect since 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 h and longer. Thus clearly, lack of circadian genes in mice and flies do not affect circadian rhythms only. In retrospect this might not be too surprising because circadian genes are expressed throughout the body and not only in the suprachiasmatic nuclei of the mouse or the small ventrolateral neurons of the fruit fly and because of the inherent pleiotropic nature of genes (110). Nevertheless, the involvement of circadian genes in sleep homeostasis remains a new and intriguing molecular pathway (10).

A final strategy that qualifies as a reverse genetic strategy is antisense targeting. With this strategy one can selectively, locally, and transiently down-regulate the expression of a gene product at the level of the RNA or DNA (111-113). The basic idea is to induce translational arrest through sequence specific hybridization of the mRNA to synthetic oligodeoxynucletides. This technique has been applied to study the effects on sleep of c-Fos protein expression in the medial pre-optic area (114), of glutamic-acid decarboxylase (115), and hypocretin-2 receptor (116) in the pontine reticular formation, and of the serotonin transporter in the dorsal-raphe nucleus (117).

5. GENETICS OF SLEEP DISORDERS

Sleep disorders are highly frequent and have dramatic health, social, and economic impacts. Their

Table 2. Genetics of human sleep disorders

Sleep Disorder	Mode of Inheritance	Genetic Evidence	Ref.
Fatal familial insomnia	Autosomal Dominant	Mutation at codon 178 of the prion protein gene	150
Primary nocturnal enuresis	Autosomal Dominant	Linkage to chromosome 13	151
		Linkage to chromosome 8	152
		Linkage to chromosome 12	153
		Linkage to chromosome 22	154
Familial advanced sleep-phase syndrome	Autosomal Dominant	Mutation at codon 662 of the period2 gene	155
Familial restless legs syndrome	Autosomal Recessive	Linkage to chromosome 12	156
		Association with MAO-A	157
	Autosomal Dominant	Segregation analysis	158
Familial sleep paralysis	Autosomal Dominant	Family analyses	159,160
Sleep apnea syndrome	Autosomal Dominant Or Unknown	Family and segregation analyses	161,162
Sleepwalking	Autosomal Dominant	Family and twin analyses	163,164
	Or Unknown	Association with HLA-DQB1*05/04	121
Sleep talking	Autosomal Dominant Or Unknown	Family and twin analyses	165,166
Bruxism	Autosomal Dominant Or Unknown	Family and twin analyses	167
Night terrors and nightmares	Autosomal Dominant Or Unknown	Family, twin, and segregation analyses	163,168
Kleine-Levin syndrome	Unknown	Association with HLA-DQB1*0201	169
REM-sleep disorder behavior	Unknown	Association with HLA-DQB1*05/06	170
Narcolepsy	Autosomal Dominant Or Unknown	Family, twin, and segregation analyses, Association with HLA-DQB1*0602	171

treatments remain largely symptomatic owing to our ignorance of their molecular patho-physiology. A large number of sleep disorders run in families suggesting that genetic factors might play an important role. The genetic dissection of well-characterized sleep disorders might also provide fundamental insights into the underlying neurobiological bases of normal sleep and wakefulness. Again, the availability of animal models is crucial as was elegantly demonstrated by Mignot and colleagues using the canine model of narcolepsy (see below).

5.1. Overview of sleep disorders with a genetic component

Here we list and briefly comment on the sleep disorders for which a genetic component in their etiology has been demonstrated or suggested. These disorders are summarized in table 2 and for the relevant references the reader is directed to that table. The first sleep disorder for which a gene mutation has been identified is fatal familial insomnia (FFI), first described by Lugaresi and colleagues in 1986 (118). This neurodegenerative disorder is caused by a point mutation in the Prion-protein gene and is responsible for a degeneration of specific thalamic nuclei. Although the condition is very rare and the associated insomnia might be secondary to other causes, some specific aspects such as the disappearance of slow-wave sleep, confirm the critical role of the thalamus in sleep and sleep EEG generation. The normal function of the protein is unknown but it is well established that the prion protein is involved in several disorders referred to as spongiform encephalopathies. Mouse models of Prion-protein mutations behave normally and have limited sleep abnormalities (91,119-120), suggesting yet unknown mechanisms of the prion protein action on brain development and neuro-degeneration.

Primary nocturnal enuresis is a highly common disorder affecting up to 10% of children under 7 years old. Not a single gene mutation has been identified but several linkage studies established the presence of at least 4 loci. Genetic heterogeneity is observed with loci on different chromosomes inducing the same condition. So far no specific phenotypic difference could be evidenced among kindred with different linkage results.

Familial advanced sleep phase syndrome (ASPS) is a rare condition with significant advance in sleep and circadian rhythm timing (up to 4 hours). It has been shown that ASPS can be highly familial with an autosomal dominant mode of inheritance. A point mutation in a casein kinase-epsilon phosphorylation site of the circadian gene *Per2* has been found in an ASPS family.

Restless legs syndrome (RLS) is associated with periodic leg movements and thus seriously affects sleep quality. RLS is highly frequent (2-5 %) and in a significant number of families presents an autosomal dominant mode of transmission even if its expressivity may be variable. A positive association between RLS and the MAO-A gene has been recently reported. A linkage study also indicated that a susceptibility locus might be localized on chromosome 12. However, in this study an autosomal recessive allele with high frequency was assumed.

The sleep apnea syndromes are common and complex disorders. In a few cases a familial trend has been identified. However, this complex disorder can be broken down to simpler endophenotypes that might be controlled by single genes. Candidate phenotypes include chemosensitivity to CO_2 and O_2, craniofacial morphology, and obesity (also a complex phenotype).

Sleepwalking (SW) is a highly frequent sleep disorder of childhood affecting up to 20% of children under 12 years of age. Although it has been proposed that SW generally disappears at adulthood, recent studies in twins indicated that adult cases are found in a proportion of 1 to 3% and that most of them have suffered from SW since childhood. Familial clustering of SW has been repeatedly reported but its mode of transmission remains unknown. In a recent study, we have found a strong association between SW and the Human Leukocyte Antigen (*HLA*) gene, *DQB1*0501* (121). Moreover analysis in familial cases of SW indicated an even stronger association and a preferential transmission of both *DQB1*05* and **04* alleles.

Three other sleep disorders have been found to be associated with *HLA* genes. In a single study, REM-sleep disorder behavior (RBD) has been associated with the *HLA-DQ1* (*B1*05* or **06*). Replication studies are needed and should be facilitated by the increasing number of patients diagnosed with RBD. The Kleine-Levin syndrom (KLS) is a highly rare disorder mainly affecting adolescent boys, and combines a severe periodic hypersomnia and behavioral abnormalities. Although the condition is not familial, many symptoms of KLS are consistent with an underlying autoimmune disorder and thus might involve genetic factors. We have found a significant association with *HLA-DQB1*0201* in thirty unrelated KLS patients. Together with narcolepsy, so far at least four sleep disorders have been associated with polymorphisms in the *HLA*-gene complex, confirming a close interaction at the molecular level between sleep and the immune system.

Other common sleep disorders such as sleep paralysis, sleeptalking, bruxism, night terrors, and nightmares have also been investigated in twins and families and substantial evidence indicates that genetic factors might play an important role.

5.2. Narcolepsy

Narcolepsy is characterized by excessive daytime sleepiness and emotionally-triggered muscle atonia, called cataplexy. Genetics of narcolepsy is the best studied in the field of sleep disorders and recently substantial progress has been made owing to a valuable canine model. Up to 10% of narcolepsies are familial. Familial studies have also shown that besides the typical phenotype, attenuated forms of the condition characterized by isolated excessive daytime sleepiness do exist at much higher rates; 10-40% of first degree relatives of narcoleptics may be affected.

Polymorphisms of *HLA*-genes within the major histocompatibility complex were first investigated in narcoleptics in early 1980s and a significant increase of *HLA-Bw35* and a decrease of *Bw52* were observed in Japanese narcoleptics (122). Soon after a 100% association between narcolepsy and the *HLA-DR2/DQw1* haplotype was reported in Japanese patients (123), an extraordinary finding immediately confirmed in Caucasians. This serological haplotype was further characterized at the genomic level indicating that 4 specific alleles corresponding to *DRB1*1501*, *DRB5*0101*, *DQA1*0102*, and *DQB1*0602* constitute the susceptibility haplotype associated with human narcolepsy in Caucasian populations (reviewed in 124). However, shortly after this striking finding, *DR2*-negative patients with typical narcolepsy, *DR2*-negative familial cases, and narcoleptic siblings with *DR2*-positive in one and *DR2*-negative haplotype in the other were reported. It was also demonstrated that the *DR2* association is actually dependent on the ethnic origin with African American narcoleptics presenting a weaker (60-65%) association (125). In this ethnic group however, the strongest association is found with the *DQA1*0102*, *DQB1*0602* haplotype typically in linkage desequilibrium with *DRB1*1503*, a finding strongly suggesting that the susceptibility gene should be closer to the *DQ* loci (126). However, these putative susceptibility genes have been sequenced and no mutation was identified indicating, together with the presence of *DR2*-negative cases and the relatively high frequency of this haplotype in the general population (20 to 30 %), that these genes are neither necessary nor sufficient to trigger narcolepsy. Several studies sought for association between narcolepsy and non *HLA*-genes with potential patho-physiological interests (5-HT2A, TpH, ApoE4, MAO-A, MAO-B, TNF-alpha) but the results remain controversial. However, the possibility that the polymorphism of non *HLA*-genes might be associated with some narcoleptic symptoms or their severity cannot be excluded. A genome-wide linkage analysis was also performed in Japanese narcoleptic families and a suggestive localization was found on chromosome 4 (127) but this study was neither replicated nor followed up by any candidate gene analysis in the identified region.

Narcolepsy is also found in dogs and is clinically and electro-physiologically similar to the human disease. Canine narcolepsy, like human narcolepsy is not a simple genetic disease though in Dobermans and Labradors, narcolepsy is transmitted as a single autosomal recessive trait with full penetrance (128). Previous studies had shown that canine narcolepsy in Dobermans is not linked with the Dog Leukocyte Antigen (*DLA*) system as also significant number of human familial cases are not linked to the *HLA* system. After intensive work over the past 15 years on the genetics of canine narcolepsy at Stanford University, Mignot's group identified, through linkage analysis and positional cloning, mutations in the hypocretin-2 receptor as the cause of canine narcolepsy (59). Hypocretin-1 and -2 are hypothalamic neuropeptides acting on two receptor subtypes and first found to be involved in feeding behavior (129). Independently and almost simultaneously, Yanagisawa's group interested in the role of hypocretins in feeding behavior discovered in the mouse a phenotype similar to canine and human narcolepsy after a targeted deletion of the prepro-hypocretin gene (130). More recently, the hypocretin system has also been implicated in the etiology of human narcolepsy; narcoleptics have undetectable hypocretin-1 levels in their CSF and in a small number of postmortem cases a dramatic reduction in the number of hypocretin-containing neurons is observed in the hypothalamus (131-133). Also transgenic mice carrying the promoter of the human prepro-hypocretin gene ligated to a truncated human ataxin-3, a gene that can induce apoptosis of hypocretin

containg neurons, present symptoms similar to human narcolepsy (134). However, the gene defect in human narcolepsy remains unknown. Among fourteen polymorphisms identified in genes encoding the prepro-hypocretin and its two receptors, none segregates with human narcolepsy. Only a single atypical patient (*HLA-DQB1*0602* negative, very young age at onset, and severely affected) was identified with a *G*-to-*T* substitution resulting in a Leu-to-Arg change in the signal peptide of the prepro-hypocretin gene, suggesting that human narcolepsy is not caused by mutations in the hypocretin gene system. Therefore, together with the tight association with the *HLA* antigens, the most likely cause of hypocretin deficiency in narcolepsy might be an autoimmune process resulting in acute or progressive degeneration of hypocretin containing neurons. Because over 90% of narcoleptics have no family history of narcolepsy and monozygotic twins are mostly discordant, environmental factors might play an important role. The environmental factors might trigger narcolepsy by inducing an autoimmune reaction that targets hypocretin neurons. Although these new developments revolutionized our understanding of both human and canine narcolepsy, a great gap still needs to be bridged between the hypocretin system and what we know about the neurochemistry, pharmacology, and genetics of narcolepsy.

6. SUMMARY AND PERSPECTIVE

The results of the studies reviewed here give an overview of what has been achieved thus far in the emerging field of sleep genetics. A picture emerges that in order to progress, multiple approaches using different animal models and genetic techniques have to be used in parallel. The successful identification of a mutation in the hypocretin-2 receptor underlying canine narcolepsy is the single best example of the feasibility of this approach. Twenty years ago the study of an animal model of human narcolepsy and cataplexy began at Stanford University. Initial efforts were focused at the pharmacology of cataplexy but true progress was made using the genetic approach that culminated with the discovery of the responsible mutation. Once the pathway was identified in canines, its implication in human narcolepsy could be quickly demonstrated by the discovery of hypocretin deficiency. Future breakthroughs in the quest for the discovery of 'sleep' genes are likely to come from the parallel use of mouse and fruit-fly models as was the case for revealing the genes that make the circadian 'clock' tick. Thousands of fruit-fly and mouse mutant strains await sleep phenotyping and these numbers are rapidly increasing (135), possibly saturating the genome within the decade. Genome sequencing will become more efficient and soon bearers of heritable diseases could have their complete genome scanned enabling rapid mapping of candidate genes. Nevertheless, we should proceed cautiously especially with respect to correctly interpreting differences in sleep physiology that accompany changes in the genotype.

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