Genetic Dissection of Sleep Homeostasis

Géraldine M. Mang and Paul Franken

Abstract Sleep is a complex behavior both in its manifestation and regulation, that is common to almost all animal species studied thus far. Sleep is not a unitary behavior and has many different aspects, each of which is tightly regulated and influenced by both genetic and environmental factors. Despite its essential role for performance, health, and well-being, genetic mechanisms underlying this complex behavior remain poorly understood. One important aspect of sleep concerns its homeostatic regulation, which ensures that levels of sleep need are kept within a range still allowing optimal functioning during wakefulness. Uncovering the genetic pathways underlying the homeostatic aspect of sleep is of particular importance because it could lead to insights concerning sleep's still elusive function and is therefore a main focus of current sleep research. In this chapter, we first give a definition of sleep homeostasis and describe the molecular genetics techniques that are used to examine it. We then provide a conceptual discussion on the problem of assessing a sleep homeostatic phenotype in various animal models. We finally highlight some of the studies with a focus on clock genes and adenosine signaling molecules.

Keywords Sleep homeostasis · Genetics · QTL · Polymorphism · Knockout · Sleep deprivation · EEG delta power · Sleep need · Clock genes · Adenosine

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1 Introduction

Despite decades of research aimed at elucidating sleep and wake regulation in mammals, little is known about the identity of genes that regulate sleep, a fundamental behavior that in humans occupies about one third of our lifespan. The various aspects of sleep differ in their regulation and interact with each other and with the environment, each of them being under the control of a multitude of genes. Therefore, each component of sleep must be considered a complex trait. Over the past 70 years, abundant evidence has accumulated demonstrating that many aspects of sleep and the electroencephalogram (EEG) are strongly determined by genetic factors (Andretic et al. 2008). In humans, the earliest observations came from twin studies showing that sleep patterns and sleep habits within monozygotic twins have a higher concordance than within dizygotic twin pairs or unrelated subjects with heritabilities ranging from 40 to 60 % (Geyer 1937; Linkowski 1999). More recent twin studies showed that quantitative EEG features, with heritability estimates of well over 80 % for specific frequency components, rank among the most heritable traits in humans (Van Beijsterveldt et al. 1996; Stassen et al. 1999; Ambrosius et al. 2008; De Gennaro et al. 2008). After these initial observations in humans, most subsequent genetic sleep and EEG studies have been performed in mice. In the 1970s, Jean-Louis Valatx introduced sleep genetics in the mouse at the Université

Claude Bernard and observed profound differences in sleep among inbred strains of mice (Valatx et al. 1972; Valatx and Bugat 1974). Like in humans also in the mouse additive genetic factors account for about half of the variance in the amount and distribution of sleep and >80 % for a variety of EEG traits (Andretic et al. 2008). Despite numerous reports on the genetic determinants of sleep and the EEG, remarkably little progress has been made in isolating the genes or gene pathways underlying these traits. The development of recent technologies and statistical tools has greatly facilitated genetic studies of sleep.

One important aspect of sleep, that will be the focus of this review, concerns its homeostatic regulation. Like other physiological variables that are homeostatically regulated (e.g., blood glucose levels, body temperature, food intake), the concept of homeostasis can be applied to sleep, since it seems essential for optimal functioning of the organism and lack of sleep is compensated by sleeping more and/or sleeping deeper. This sleep homeostatic process is thought to keep a level of sleep pressure within physiological range; i.e., sufficiently low to allow the brain and organism to operate in an appropriate manner. However, the neurophysiological function of sleep and the variables that are homeostatically defended still remain elusive, and is one of the main topics of current sleep research. Our modern 24/7 society, in which professional and social activities increasingly prevail to the detriment of sufficient good quality sleep, comes with a cost for health and well-being both to the individual and to society (Bixler 2009). Therefore, finding clues about the genetic and molecular processes defining and controlling the need for sleep could be beneficial to improve human's well-being and performance. Gaining insight into the molecular pathways underlying sleep homeostasis requires a multidisciplinary approach, using a combination of genetic and molecular genetic approaches making use of available animal models and analytical tools. This review focuses on the use of genetic and molecular techniques to study sleep homeostasis in three model organisms; i.e., human, mouse, and fly. Other aspects of sleep will not be discussed here. In the following, we first describe the concept of sleep homeostasis (Sect. 2), then we provide a background of the genetic approaches used to study sleep homeostasis in model organisms (Sect. 3). The subsequent sections present studies on some of the gene pathways that have been implicated in sleep homeostasis, with emphasizes on circadian clock genes and components of the adenosine pathway. Section 8 briefly discusses the molecular genetic screens that have highlighted potential homeostatic molecules and provides an example of a combination of several genetic techniques that led to the discovery of a now established marker of sleep homeostasis, *Homer1a*.

2 Sleep and its Homeostatic Regulation

The notion of homeostasis was first introduced by Claude Bernard in 1926 and further developed by Walter Cannon, as the property of a system to maintain its internal environment stable and constant allowing an organism to function

optimally over a broad range of environmental conditions (Canon 1915). Homeostatic regulation involves three essential parts: a sensor that measures the controlled variable, an integrator that processes information and compares the variable to a set point (or rather the optimal range within which the controlled variable can vary), and effectors that respond to the commands of the integrator when the level of the variable deviates from set point. None of these three parts are known with certainty in sleep because we do not know what function(s) sleep fulfills. Although we have identified variables that reliably track the prior sleep–wake distribution (see below), it is likely that these are not themselves the homeostatically defended variables but rather reflect an underlying process. Moreover, sleep homeostasis has distinct local and use dependent properties suggesting that sleep's function(s) is a property of neuronal assemblies rather than involving the whole organism or even the whole brain (Krueger et al. 2008). Therefore, the homeostatic control of sleep might, like that for circadian rhythms, operate at cellular-molecular level (Hinard et al. 2012).

2.1 Homeostatic and Circadian Control of Sleep

Homeostatic sleep pressure (*aka* sleep propensity or sleep need) accumulates during wakefulness and decreases during sleep. The increased need for sleep that accompanies sleep loss seems to be compensated by sleeping longer and/or intensifying sleep leading to the return to set point. Sleep homeostasis is primarily studied by performing sleep deprivation (SD) experiments although in a few studies the effects of a nap (Werth et al. 1996; Vienne et al. 2010) and the effects of spontaneous waking episode durations (Franken et al. 2001) on sleep pressure have been studied. The effects of SD can be assessed on a variety of sleep parameters.

The control of sleep cannot solely be explained by homeostatic control, as it involves at least one other main process: a circadian process that determines the time of day at which sleep occurs (Borbély 1982; Daan et al. 1984). In mammals, the circadian control is orchestrated by the suprachiasmatic nucleus (SCN) of the hypothalamus (Klein et al. 1991) which is considered the master circadian clock. The output of this clock gives time context to most physiological processes and behaviors including sleep, and ensures the proper entrainment of internal rhythms to the daily light–dark cycle. The interaction between the homeostatic and the circadian process was described first in the two-process model of sleep regulation (Borbely 1982; Daan et al. 1984). It now has become established that their close interaction determines the duration, the quality, and the quantity of sleep and that it enables us to stay awake and alert through the day despite an increasing need for sleep and to sleep throughout the night despite a decreasing need for sleep (Dijk and Franken 2005). Despite this important interaction between the two, evidence has shown that each of the processes develops independently (Trachsel et al. 1992; Dijk and Czeisler 1995; Easton et al. 2004; Larkin et al. 2004; for review see Franken 2013).

2.2 NREM Sleep Homeostasis

The homeostatic regulation of non-rapid eye movement (NREM) sleep has been extensively studied in mammals; it consists of an increase in both the duration and the depth or "intensity" of NREM sleep after extended periods of wakefulness. The most widely used index to quantify homeostatic sleep need relates to the prevalence and amplitude of EEG slow waves (or delta waves; 0.75–4.5 Hz), quantified by the Fourier transformation as EEG delta power (*aka* slow-wave activity or SWA). Changes in EEG delta power follow the sleep–wake distribution (Tobler and Borbély 1986; Dijk et al. 1987; Franken et al. 1991a, 2001). EEG delta power in NREM sleep is high at the beginning of the sleep episode, and decreases with the duration of time spent asleep. The declining trend of EEG delta power over the course of a sleep episode is thought to reflect the homeostatic decline of sleep propensity. After an episode of extended wakefulness, EEG delta power in subsequent NREM sleep is high and its magnitude depends on the duration of prior wakefulness. The changes in EEG delta power are highly reliable and can be predicted mathematically solely based on the sleep–wake distribution both under baseline conditions and after SD (Franken et al. 1991b, 2001, 2006; Huber et al. 2000; Achermann and Borbely 2003; Deboer 2009). The fact that most of the variance in EEG delta power can be attributed to the sleep–wake distribution contributed to the notion that this variable reflects a homeostatic process related to NREM sleep. In a functional context, NREM sleep with high levels of EEG delta power is considered especially recuperative not only for the brain but also for the body (Tasali et al. 2008).

Besides its intensity, also the time spent in NREM sleep seems to be homeostatically regulated as it tends to increase after SD. However, sleep duration also importantly depends on the circadian phase at which sleep occurs, in contrast to EEG delta power of which the dynamics are little affected by the circadian process (Dijk and Czeisler 1995). Another difference between the homeostatic regulation of EEG delta power and NREM sleep duration is that the dynamics of the former are fast (i.e., hours), while rebounds in the latter can be delayed and span over several days (e.g., Franken et al. 1991a). The homeostatic regulation of NREM sleep duration has received very little attention thus far. This is also reflected by current hypotheses on sleep function that focus exclusively on the sleep–wake dependent dynamics of EEG delta power while ignoring time spent in sleep (e.g., Tononi and Cirelli 2006). Interestingly, while EEG delta power or other electrophysiological correlates of sleep need have not been identified in *Drosophila melanogaster* and although sleep after enforced wakefulness in the fly also deepens judged on increased arousal thresholds and increased consolidation, the homeostatic regulation of sleep is derived mainly from the time spent asleep (Huber et al. 2004).

2.3 REM Sleep Homeostasis

Even though, at least in rodents, the amount of REM sleep is usually more accurately regulated than the amount of NREM sleep, the question whether REM sleep is homeostatically regulated remains a topic of debate. Support for a homeostatic regulation of REM sleep comes from studies in cats, rats, mice as well as humans (Benington et al. 1994; Endo et al. 1997; Rechtschaffen et al. 1999; Franken 2002; Amici et al. 2008). Although in all species listed REM sleep pressure, quantified as the number of attempts to enter REM sleep, does increase when REM sleep is selectively deprived of (Benington et al. 1994; Endo et al. 1998; Ocampo-Garcés et al. 2000), an increase in REM sleep time during recovery sleep that is proportional to the loss of REM sleep during a preceding deprivation, is only consistently observed in some animal studies (see Franken 2002 for a review). Unlike NREM sleep, a loss of REM sleep seems to be primarily compensated by spending more time in REM sleep, although EEG measures indicative of the depth of REM sleep have been proposed (Borbély et al. 1984; Roth et al. 1999). While such observations do suggest that REM sleep amount is homeostatically regulated, this has received little attention in genetic studies and unraveling the molecular underpinning of the NREM sleep homeostatic process has been a main focus. Nevertheless, several KO mouse studies found evidence for clock genes regulating the degree by which REM sleep increases after SD (see Sect. 5).

3 Approaches and Techniques to Study Sleep Genetics

3.1 Forward Genetics

To study the genetics of sleep, three complementary approaches are generally considered: forward, reverse, and molecular genetics (Fig. 1). Conceptually, the forward genetic approach is the most powerful strategy for the identification of novel genes and gene pathways involved in any biological process. Forward genetics is a classical genetic approach starting from the observation of a particular phenotype within an organism and comprises several means to map and identify the gene or set of genes that are responsible for this precise phenotype. Examples of forward genetic approaches in animals are mutagenesis screens and quantitative trait loci (QTL) analysis. QTL analysis has been proposed to dissect complex traits because with this approach, natural allelic variation of genes with small effect can be mapped (Lander and Botstein 1989; Darvasi 1998; Belknap et al. 2001; Flint and Mott 2001). QTL analysis has been used in several segregating populations including intercross and backcross, advanced intercross and backcross panels, and notably in genetic reference populations (GRP) such as recombinant inbred (RI) stock (Darvasi 1998; Talbot et al. 1999). The best characterized GRP is the BXD

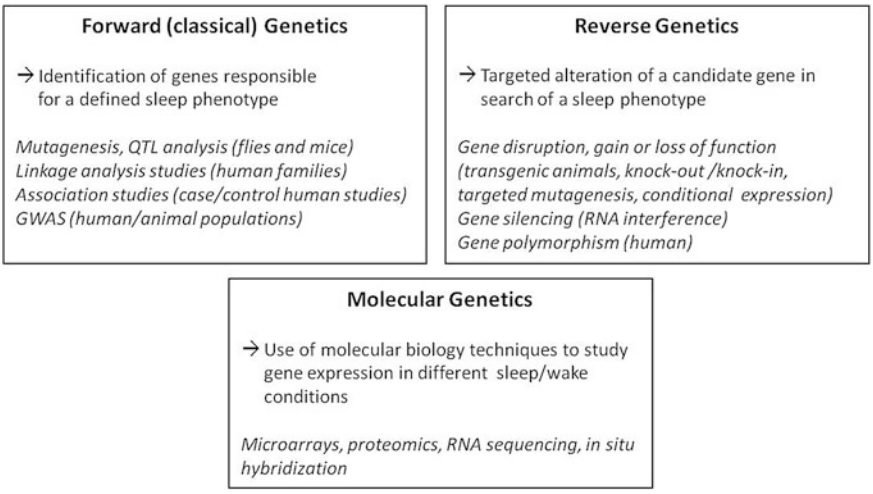

Fig. 1 Genetic approaches used in sleep homeostasis research. For each of these approaches, different techniques can be employed in different animal species to bring information about the involvement of genes in sleep and its homeostasis. The three approaches are complementary and should be combined to decipher the molecular pathways of sleep homeostasis

RI set derived from two inbred strains: C57BL/6 J and DBA/2 J. The BXD set has been used for decades to map the genetic basis of complex traits (see www.genenetwork.org for the accumulated data). The first forward genetic study that specifically assessed as a trait in the mouse was performed in the BXD set (Franken et al. 2001). Another example of GRP is the Collaborative Cross, a more recent mouse population with high allelic diversity that is being constructed using a randomized breeding design that systematically outcrosses eight founder strains, followed by inbreeding to obtain new RI strains (Philip et al. 2001). In *Drosophila* the first mutagenesis screen for sleep phenotypes was performed in 2003 (Cirelli 2003). Using DNA alkylating agents such as ethyl methane sulfonate, or DNA transposable elements, random mutations are induced throughout the genome. With high-throughput screening of thousands of offspring for dominant, semi-dominant, or recessive mutations, a major effect on a given trait can be identified. Thus, forward genetics can be used to establish causal relationship between the function of individual genes and otherwise complex phenotypes. However, genetic screens in this approach are for fully penetrant dominant and recessive mutations and therefore cannot identify small effect sequence variations that may turn out to be essential for some aspects of the phenotype. Thus because of advantages and disadvantages associated with each of these two approaches, QTL and mutagenesis should be viewed as complementary (Belknap et al. 2001).

In humans, traits of interest are generally examined by linkage analyses. Linkage studies investigate shared chromosomal fragments among members of a family who manifest the trait of interest or the disease. By analyzing the co-segregation of the trait and chromosomal markers, it may be possible to

statistically identify chromosomal loci "linked" to the trait. However, linkage studies are limited by the availability of family-based samples, and rely on the observation of heritability of the trait of interest. In comparison, association studies of genetically complex traits require larger samples and involve comparisons between cases and controls with respect to selected polymorphisms (Altshuler et al. 2008; Chiang et al. 2009). In genome-wide association studies (GWAS) the concept of association study is extended to the whole genome, with the idea to observe the segregation of many polymorphisms with the disease or the trait. Thus, the relative impact of different genomic regions can be assessed simultaneously in the same sample. Despite initial excitement the loci identified in GWAS studies have, in general, weak additive power, explaining only a small portion of the narrow-sense heritability reported for a given phenotype. This suggests that rare rather than the common variants interrogated in a GWAS, underlie the phenotype of interest. Moreover, most reported loci are noncoding and thus not immediately informative and GWAS results not always replicate across studies and populations leading to false positives (Ward and Kellis 2012).

The GWAS can also be applied to outbred mouse populations (Yalcin et al. 2010). Increased recombination in outbred populations is expected to provide greater mapping resolution than traditional inbred line crosses, improving prospects for identifying the causal genes for a trait. However, outbred populations are not a GRP and thus each individual is genetically unique and has to be genotyped individually. This also limits the number of phenotypes that can be obtained for a given genotype which is another advantage of using GRPs.

3.2 Reverse Genetics

In contrast to forward genetics, in which the strategy is to go from the observed phenotype to the underlying genotype, reverse genetics starts with a disrupted or altered gene of interest and examine its effect on a phenotype or phenotypes of interest. In humans, reverse genetic studies most often concern natural occurring functional polymorphisms while the use of animal models allows for targeting specific genes. Transgenic animals can be used to study the consequences of overexpression, ectopic expression, time- and tissue-specific expression, and gain or loss of function of a specific gene. To date, a large number of studies in sleep genetics have utilized reverse genetic approaches by knocking-out a gene of interest (see Sect. 5.1). The delineation between reverse and forward genetic approaches is not a formal one and depends on the scale at which they are used. Transgenic strategies can also been used in forward genetic studies, in which hundreds of mutated or knockout (KO) lines of mice (Knight and Abbott 2002), or flies (Cirelli 2003) are screened. The International Knockout Mouse Consortium is currently creating a collection of mouse in which all protein-coding genes are mutated and will therefore provide a population of animals for genome-wide screens (Ringwald et al. 2011).

3.3 Molecular Genetics

Molecular genetics is an approach that evaluates changes in gene expression related to the trait of interest; it is an unbiased method that makes use of several techniques such as DNA microarrays, proteomics, and RNA sequencing. Molecular approaches in sleep studies are based on the assumption that the expression of genes change as a function of time spent awake or asleep (Cirelli et al. 2004; Mackiewicz et al. 2009; Thompson et al. 2010). Rhyner et al. were the first to use a molecular approach to identify genes that change their expression after SD in the rat, and identified the protein neurogranin to be decreased in the rat forebrain after 24 h of SD (Rhyner et al. 1990). These methods have been helpful in identifying the brain expression of genes involved in sleep or modulated by sleep (see Sect. 8). However, these approaches cannot reveal a causal relationship between a gene or a set of genes and a particular behavior or phenotype. For example, a gene that does not show transcriptional modification may nonetheless play an important role in the process under investigation. On the other hand, a transcript that does increase with increased sleep need might merely be driven by the sleep–wake distribution while not playing a role in the homeostatic sleep process itself.

It is clear that the above approaches are complementary; e.g., the function of candidate genes identified with QTL or molecular genetics approaches have to be confirmed in KO animals (an example is provided in Sect. 8.1). Future efforts should therefore combine genetics approaches in animal models and GWAS in humans to facilitate uncovering the molecular pathways that underlie sleep homeostasis. However, other approaches and techniques will remain important in making progress in elucidating the complex physiology of sleep; genetic dissection of sleep can be used in conjunction with state-of-the-art electrophysiological, neuroanatomical and pharmacological techniques already used with great success in sleep research.

4 In Search for Sleep Homeostatic Genes: Defining the Phenotype

A sleep homeostatic gene could be defined as a gene that modifies the sleep–wake dependent dynamics of EEG delta power or a gene that affects the (compensatory) increase in NREM or REM sleep duration after SD. Establishing whether a gene variant affects a sleep homeostasis process is, however, not always straightforward and several considerations have to be taken into account in the analysis. Here we illustrate some of the general problems that can occur with interpreting the results of SD studies that should be considered before claiming a homeostatic phenotype is observed.

Although, sleep homeostatic processes could, in principle, be assessed and quantified under baseline conditions, usually sleep homeostatic responses are

studied after experimentally challenging sleep need through; e.g., keeping subjects awake for a certain duration at a time-of-day sleep is present under undisturbed baseline conditions. In most studies, sleep variables measured during recovery sleep after enforced waking are then contrasted to the individual levels reached in baseline. Subsequently, these relative changes are compared between genotypes to assess the effect of a disrupted or mutated gene of interest on sleep homeostasis (Fig. 2a). A slightly more elaborate variant of such analysis are the so-called "gain-loss" time course analyses in which the sleep loss incurred over the course of a SD is analyzed by accumulating the differences of sleep observed during the SD minus the sleep duration observed during the same time-of-day during baseline (Fig. 2b′). This accumulated sleep deficit then serves as the starting point of the sleep gained during the recovery period following a SD and is assessed in the same way (i.e., as relative differences from matching times during baseline). Genotype differences in 'gain-loss' dynamics are then taken as evidence of an altered homeostatic regulation of sleep. An important short-coming of such analyses is that they implicitly assume that the sleep obtained during baseline is the amount a subject "needs" or, in other words, the duration of sleep that is homeostatically defended. For instance, the deficit in sleep duration in the gain-loss analyses introduced above exclusively reflects the sleep duration obtained during the baseline period the SD took place (given that no sleep is obtained during the SD protocol). Also the recovery dynamics are obtained by contrasting recovery values to corresponding baseline values. In the hypothetical example shown in Figs. 2b and 2b′, the recovery dynamics of sleep is exactly the same; i.e., each hour the same duration of *extra* sleep is obtained making the recovery curves run in parallel. Nevertheless, a researcher can claim the discovery of a homeostatic phenotype because of statistical genotype differences that, in this case, are due only to a difference of sleep expressed during baseline at the time SD was performed. In another example, recovery was made to be exactly proportional to the sleep duration the animal was deprived of and this amount of *extra* sleep was linearly distributed of the 18 h depicted (example in Fig. 2b′). As a result, in this exercise, recovery is "complete" by hour 24 (i.e., after 18 h of recovery) reaching the zero deficit level in both genotypes. Nevertheless, a researcher can again claim that homeostatic regulation is different between genotypes because the SD resulted in different relative deficits and the slopes of the gain process differed, while it could equally be claimed that recovery of sleep time lost is perfect and not different in both because in one case more sleep was deprived of and thus a higher pressure for sleep was accrued leading to more extra sleep. Many other scenarios could be construed pointing out these or related problems such as the so-called "ceiling effect." This effect is sometimes alluded to account for a smaller increase attained during recovery at times-of-day when during baseline the subject already sleeps a lot. It is clear, and all will agree, that the level of a sleep variable expressed during baseline cannot be attributed to homeostatic need alone. For example, in a study examining sleep need in short and long sleepers that differed in habitual sleep duration by more than 3 h, it was concluded that the dynamics of the sleep

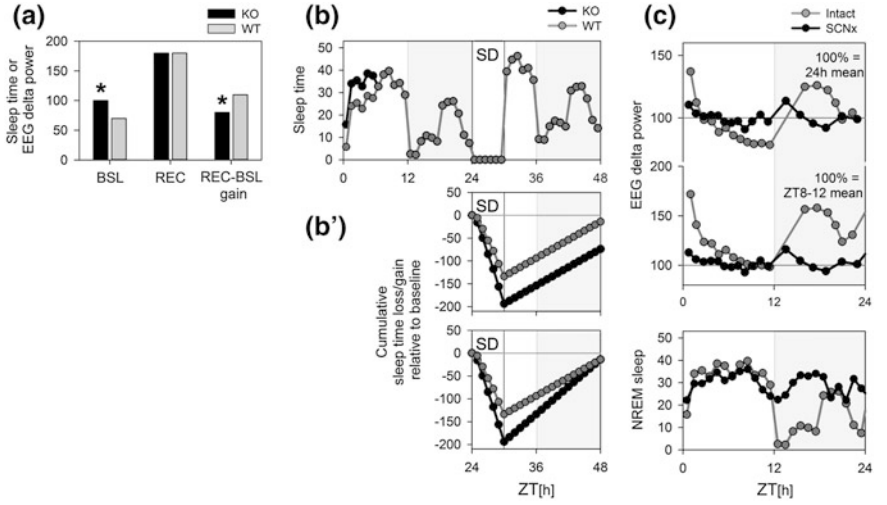

Fig. 2 Conceptual issues when assessing a sleep homeostatic phenotype. (*Panel a*) Sleep regulation in mice carrying a targeted deletion of a gene (i.e., knockout or KO) is compared to that in wild-type (WT) controls by submitting mice of both lines to a sleep deprivation (SD). Time spent asleep during the recovery period (REC) or the level of EEG delta power reached after the onset of recovery sleep was higher than in baseline (BSL) in both genotypes but levels reached did not differ between them. Nevertheless, by contrasting the values obtained during recovery to those obtained during baseline (REC-BSL gain), a significant (*) difference in gain was observed. The gain difference in this hypothetical example must, however, be ascribed to the differences in baseline. (*Panels b*) In another example, KO mice differed only from WT mice in the first 6 h of the baseline (ZT0-6; top panel) during which KO mice slept 1 h more. During the remainder of the experiment the two genotypes had identical amounts of sleep. The effect of SD was analyzed with a loss/gain analysis by accumulating the sleep time lost during the SD (−140 min in WT) and the extra sleep obtained during recovery from a 6 h SD (+200 min in both genotypes). Because of the baseline difference in sleep amount at the time the SD was performed the following day, KO mice start with a 60 min higher sleep deficit at the start of recovery sleep. Although the extra sleep obtained during recovery sleep did not differ (*parallel gain curves*) KO maintain a deficit. In (*Panel b′*) a scenario is depicted where both genotypes fully compensate for their differing sleep time lost during SD; i.e., reaching 0 levels within the 18 h of recovery. Does sleep homeostasis differ (*steeper gain curve*) or not (same end point)? Baseline and recovery data for b′ not included in *upper panel*. (*Panels c*) The sleep–wake distribution (*lower panel*) drives changes in EEG delta power such that during periods when NREM sleep is prevalent (light period ZT0-12) EEG delta power decreases in an exponential fashion while during period of wakefulness (first half of the dark period; ZT12-18) EEG delta power in subsequent NREM sleep is elevated. In SCN-lesioned (SCNx) animals that sleep more and in which the distribution of NREM sleep over the day is strongly reduced, EEG delta power can be expected to be low and its time course flat over a 24 h *baseline* recording. Taking the 24 h mean value of EEG delta power as a reference (100 % *upper panel*), as is often done, results in overall higher relative values of EEG delta power in SCNx mice such as in *baseline* (e.g., ZT6-12) and also after SD (not illustrated) compared to intact mice that can lead to erroneous conclusions concerning homeostatic sleep need. We propose to take the EEG delta power values reached during the last 4 h of the main rest phase (ZT8-12) as a reference (100 % *middle panel*)

homeostat did not differ and that short sleepers somehow could resist higher levels of sleep pressure (Aeschbach et al. 1997, 2001).

The problem becomes even more complex when the variable cannot be directly analyzed in absolute terms (i.e., in the units they are measured) because, due to a range of confounds (e.g., electrode placement, impedance) variability among individuals is large thereby decreasing statistical power. Quantitative EEG variables are therefore often expressed relative to an individually calculated reference value to reduce variability among subjects. Differences in absolute levels should also be corrected for variables for which only the sleep–wake dependent changes are considered informative. Our genetic analyses of the sleep–wake dependent relative changes in EEG delta power and of the contribution of EEG delta power to NREM sleep EEG revealed that these two aspects are under the control of different genetic factors (Franken et al. 2001; Maret et al. 2005). Similarly, EEG delta power contributes more prominently to the NREM sleep EEG in female compared to male mice; nevertheless, the sleep–wake dependent dynamics of the changes in EEG delta power differ with sex such that the build-up in females is slower than in males, and therefore higher absolute EEG delta power not necessarily reflect higher homeostatic sleep need (Franken et al. 2006). Studies in humans arrive at similar conclusions. EEG analyses in subjects carrying specific polymorphisms has demonstrated that large genotype effects on the prevalence and amplitude of EEG delta waves (absolute values) do not necessarily translate in differences in the sleep–wake dependent relative changes in EEG delta power (i.e., homeostasis; reviewed in Franken 2012). As a last example illustrating this duality between these two aspects of EEG delta power, the effect of benzodiazepines can be mentioned. These sleep-promoting drugs are known to reduce EEG delta power while leaving the sleep–wake dependent dynamics unaffected (Borbely and Achermann 1991). Therefore, one should be cautious with making claims on sleep homeostasis and sleep depth based on absolute EEG values. Assessing other, non-EEG indexes of NREM sleep depth or quality such as the fragmentation of NREM sleep (Franken et al. 1991a, 1999) or arousal thresholds (Neckelman and Ursin 1993; Wimmer et al. 2012) could be used to strengthen such claims.

When investigating the homeostatic regulation of sleep, the relative changes in EEG delta power related to the sleep–wake distribution are analyzed. The individual reference value for EEG delta power should be chosen such that effects of eventual differences in the sleep–wake distribution and sleep duration do not affect the result. As the sleep–wake distribution is the main determinant of the changes in EEG delta power, genotype differences in sleep duration as well as sleep distribution can greatly impact average levels of EEG delta power while not (necessarily) impacting the dynamics of the underlying homeostatic process. This can be illustrated in SCN-lesioned animals (Trachsel et al. 1992). In our hypothetical example (Fig. 2c), removing the SCN results in an increase in sleep and in a "flat" distribution of sleep over the 24 h day mainly due the lack of the consolidated period of wakefulness after dark onset. The bout of spontaneous wakefulness results in high levels of EEG delta power in subsequent NREM sleep in the intact animals, levels that are never reached in SCN-lesioned animals. Therefore, when

taking the 24 h mean value of EEG delta power as a reference, as is often done, this reference value will be higher in the intact animals compared to the same reference in the arrhythmic animals. As a result, the relative values calculated using this reference, will be lower in the intact animals and when compared to the lesioned animals could lead to erroneous statements concerning sleep homeostasis because the difference in the level in EEG delta power reached after SD are an artifact of selecting the reference. As an alternative, we have proposed to use the mean EEG delta power reached in the last portion (4 h) of the rest phase (or light phase in nocturnal species) as a reference (Franken et al. 1999). Levels reached at this time are less dependent on eventual differences in the sleep–wake distribution because EEG delta power decreases exponentially and EEG delta power differences at sleep onset gradually disappear over the course of the rest phase. In our hypothetical case of the SCN-lesioned animal, EEG delta power does not appreciatively deviate from this low level (Fig. 2c).

Another issue concerning the interpretation of EEG delta power seen in the literature is to contrast EEG delta power measured after SD to its value at corresponding clock time during baseline. Already minor genotype differences in the sleep–wake distribution during baseline can lead to significant differences in EEG delta power. Therefore, by expressing values reached after SD as a percentage of values reached during particular times during baseline can lead to wrong conclusions. To circumvent the problem of having to contrast values obtained after a SD to a baseline reference, "dose–response" experiments could be considered. In such studies, SDs of different durations could be used to quantify directly the dynamics of the relationship between the time animals were kept awake and the response variable (sleep duration, EEG delta power etc.; e.g., Tobler and Borbely 1986; Franken et al. 2001; Seugnet et al. 2006). To quantify the relationship between the sleep–wake distribution and EEG delta power, computer simulations can be used as an alternative approach (Franken et al. 2001).

In conclusion, the definition of an altered sleep homeostasis is not trivial. Besides some of the conceptual issues outlined above, differences in methods to perform SD such as the sleep time allowed during the SD, recording and analyzing the EEG further add to the problem of unambiguously establishing whether a genotype difference in the response to sleep loss qualifies as a homeostatic sleep phenotype.

Many different cells, molecules, and signaling pathways have been investigated through reverse genetic studies for their possible role in the control of sleep homeostasis (Table 1). In the Sect. 5 through 8, we give an overview of the pathways that have been linked to sleep homeostasis, with an emphasis on clock genes and adenosine. The list provided is, however, non-exhaustive and many other systems are currently under investigation. Even though these studies brought essential information in the understanding of sleep homeostasis, a unifying picture on its genetic control has yet to emerge.

Table 1 Genes investigated through reverse genetic studies for their involvement in sleep homeostasis

Family	Gene	Species	Homeostatic phenotype	References
Clock genes	*Clock*	Mouse	Yes	Naylor et al. 2000
		Fly	Yes	Shaw et al. 2002
	Bmal1	Mouse	Yes	Laposky et al. 2005
	Cycle	Fly	Yes	Shaw et al. 2002
	Npas2	Mouse	Yes	Franken et al. 2006
	Cry1, Cry2	Mouse	Yes	Wisor et al. 2002
	Dbp	Mouse	No	Franken et al. 2000
	Per1, Per2	Mouse	No	Kopp et al. 2002
		Mouse	No	Shiromani et al. 2004
		Fly	Yes	Shaw et al. 2002
	Per3	Mouse	No	Shiromani et al. 2004
		Mouse	Yes	Hasan et al. 2011
		Mouse	Yes	Hasan et al. 2012
		Human	Yes	Viola et al. 2007
	2-Dec	Mouse	Yes	He et al. 2009
Adenosine	A_1R	Mouse	No	Stenberg et al. 2003
		Mouse	Yes	Bjorness et al. 2009
		Mouse	Yes	Halassa et al. 2009
	A_2R	Mouse	Yes	Urade et al. 2003
	dAdoR	Fly	No	Wu et al. 2009
	Adk	Mouse	Yes	Palchykova et al. 2010
	CD73	Mouse	Yes	Zielinski et al. 2012
Neurotransmitters	*COMT*	Human	No	Bodenmann et al. 2009
		Human	Yes	Goel et al. 2011
	GABAR	Mouse	No	Winsky-Somerer et al. 2009
		Mouse	No	Vienne et al. 2010
Ion channels	*Sh*	Fly	No	Cirelli et al. 2005
	Sss	Fly	Yes	Koh et al. 2008
	Hk	Fly	No	Bushey et al. 2007
	Kcna2	Mouse	No	Douglas et al. 2007
Signaling pathways	*BDNF*	Human	Yes	Bachman et al. 2012
Stress and immunity	*BiP*	Fly	Yes	Naidoo et al. 2005
	Hsp83	Fly	Yes	Shaw et al. 2002
Synaptic plasticity	*Homer1*	Mouse	No	Maret et al. 2007
	Homer	Fly	Yes	Naidoo et al. 2012
	Homer1a	Mouse	No	Naidoo et al. 2012

The current list is non-exhaustive and presents only the studies that were discussed in the chapter (see Sects. 5–8). The observation of an altered homeostatic sleep phenotype (4th column of the table) is based on the conclusions of the authors of the cited publications. See text for details

5 Circadian Clock Genes

Although the genes referred to as clock genes are involved in many pathways they are best known (and named) for their role in circadian rhythm generation. Clock genes are transcriptional regulators engaged in negative feedback loops that underlie the molecular circuitry of the circadian clock machinery (Ko and Takahashi 2006). Positive elements of this feedback loop are three factors: CLOCK, NPAS2, and BMAL1. NPAS2::BMAL1 and CLOCK::BMAL1 heterodimers can drive transcription of many genes including the *Period* (*Per1* and *-2*) and *Cryptochrome* (*Cry1*, and *-2*) genes. PER::CRY protein complexes suppress CLOCK/NPAS2::BMAL1-mediated transcription and thus their own transcription thereby constituting the negative elements in the feedback loop. Additional interactions between these core clock genes and other actors (e.g., REV-ERBα, RORα) at the level of transcription, translocation back into the nucleus, post-translational modifications add further complexity and stability. In *Drosophila*, the molecular oscillator relies on very similar timing systems using orthologs to the mammalian clock genes (Benito et al. 2007). The central autoregulatory feedback loop is composed of the two factors CLOCK and CYCLE, which are the homologues of the mammalian CLOCK and BMAL1, respectively. The CLOCK::CYCLE heterodimers activates the transcription factors PERIOD and TIMELESS which act as negative regulators like the mammalian PER and CRY proteins, respectively (Tomioka and Matsumoto 2010).

The circadian process and the homeostatic process appear independent (for review see Franken 2013). Rendering animals arrhythmic through lesioning the SCN (Trachsel et al. 1992; Easton et al. 2004) or a light pulse (Larkin et al. 2004), did not affect the increase in EEG delta power after SD. Moreover, studies in humans showed that the sleep–wake dependent dynamics of EEG delta power are little affected by circadian factors (Dijk and Czeisler 1995). Analyses of sleep homeostasis in humans, mice, and flies carrying polymorphism or targeted disruptions of clock genes demonstrate, however, that at least at the molecular level, circadian rhythms and sleep homeostasis are difficult to dissociate (Shaw and Franken 2003; Franken and Dijk 2009). In the following, we will discuss the different clock gene mutants/polymorphisms that have been studied and suggesting a non-circadian role of clock genes in sleep homeostasis.

5.1 Reverse Genetic Studies for Clock Genes

Clock is the first circadian gene identified in a mammal (Vitaterna et al. 1994) and subsequently cloned (Antoch et al. 1997; King et al. 1997). The *Clock* mutation ($Clock^{\Delta/\Delta}$) affects numerous aspects of circadian rhythmicity, including a lengthened circadian period. *Clock* was also one of the first clock genes for which a role in sleep homeostasis has been claimed (Naylor et al. 2000). Under baseline conditions,

Clock$^{\Delta/\Delta}$ mice showed decreased NREM sleep, associated with a reduction in NREM sleep episode length. When challenged with a 6 h SD, *Clock*$^{\Delta/\Delta}$ mice showed a normal NREM sleep rebound but a reduced rebound in REM sleep. The authors suggested that NREM sleep homeostasis was affected by the *Clock* mutation, because NREM sleep delta energy was reduced both during baseline and during recovery. Because this measure is a function of the total time spent in NREM sleep, the difference has, however, to be largely attributed to the fact that these mice slept less. Moreover, when sleep homeostasis was evaluated according to the time course of EEG delta power, no genotype differences could be observed. Therefore, it remains unclear whether the *Clock* mutation affects NREM sleep homeostasis. The altered relative increase in REM sleep, which could not be explained by differences in REM sleep amount in baseline, points to an altered homeostatic regulation of REM sleep. In flies, the mutation of the *Drosophila Clock* homolog mildly affects baseline sleep as well as the response to SD (Shaw et al. 2002). It remains to be determined whether *Clock* KO mice have a sleep homeostatic phenotype.

In addition to abolishing circadian rhythms in overt behaviors, targeted disruption of *Bmal1* leads to profound differences in sleep (Laposky et al. 2005). Under baseline conditions, *Bmal1*$^{-/-}$ mice showed attenuated amplitude of the distribution of sleep and wakefulness across the 24 h day as well as elevated NREM and REM sleep amounts. Moreover, this increase in sleep time was accompanied by a more fragmented sleep. The authors found that sleep intensity, determined as the average of absolute levels of EEG delta power in baseline, was increased in *Bmal1*$^{-/-}$ mice, a surprising finding given that sleep fragmentation and intensity are usually inversely correlated (Franken et al. 2001). To assess genotype effects on the homeostatic regulation the relative, sleep–wake dependent changes in EEG delta power were analyzed in baseline and after a 6 h SD. As normalization the averaged 24 h baseline level was used. With this normalization higher than wild-type (WT), EEG delta power levels were reached in the light period and lower values in the dark period of baseline. This time course can serve as an example of the problem that exists with the use of this reference value (Fig. 2c), especially because *Bmal1*$^{-/-}$ mice sleep overall more and lack the sustained period of wakefulness in the first half of the dark period. As a result, levels of homeostatic sleep pressure can be expected to be constitutively low in *Bmal1*$^{-/-}$ mice, consistent with the lower levels of sleep consolidation observed. Nevertheless, the relative increase in EEG delta power was reduced pointing to slower build-up of homeostatic sleep need.

An extreme sleep homeostatic phenotype was observed in *Cycle* mutants, the fly homolog of *Bmal1*. *Cycle* mutant flies showed an exaggerated sleep rebound after SD and died after 10 h of SD (Shaw et al. 2002). This was the first study to show a vital role for sleep in flies. The combination of circadian disruption and alterations in the response to SD in *Bmal1*$^{-/-}$ mice and in *Cycle* mutant flies supports the notion that this clock gene plays a role in both circadian and sleep homeostatic processes.

In mammals, CLOCK is the main component of the circadian machinery in the SCN and peripheral organs, whereas in peripheral brain regions (i.e., peripheral to the SCN), it is substituted by its paralog NPAS2. CLOCK and NPAS2 are similar in amino acid sequence, share BMAL1 as an obligate partner, bind to the same DNA recognition element, are suppressed by CRY proteins, and commonly depend on favorably reducing ratio of NAD factors (Rutter et al. 2001). Because NPAS2 acts both as a sensor and an effector of intracellular energy balance, and because sleep is thought to correct energy imbalance incurred during waking, *Npas2* might be a candidate for a sleep homeostatic gene. In contrast to $Clock^{\Delta/\Delta}$ mice, which showed a lengthened circadian period (Vitaterna et al. 1994), $Npas2^{-/-}$ mice (Garcia et al. 2000) have a shorter period of activity (Dudley et al. 2003) whereas $Clock^{-/-}/Npas2^{-/-}$ double KO are completely arrhythmic (DeBruyne et al. 2007). This suggested that CLOCK and NPAS2 function as redundant regulators of circadian behavior. With regard to sleep, $Npas2^{-/-}$ mice were found to sleep less in the latter half of the baseline dark period, a time of day at which sleep need is high and WT mice showed a consolidated period of sleep (i.e., a nap) conceivably to discharge accumulated sleep pressure (Franken et al. 2006). After SD, these mice were incapable in initiating the appropriate compensatory behavior during the circadian phase in which mice are usually awake, i.e., the dark period (Franken et al. 2006). They regained less NREM sleep in the following hours after SD, and the EEG delta power after SD was smaller. Based on simulation analysis, the estimated rate at which EEG delta power increases during wakefulness tended to be slower in $Npas2^{-/-}$ mice. In conclusion, NPAS2 affects the homeostatic regulation of NREM sleep and, in contrast to $Clock^{\Delta/\Delta}$ mice, homeostatic regulation of REM sleep was not affected.

Mice lacking both *Cry1* and *Cry2* genes lack a functioning circadian clock and are behaviorally arrhythmic when kept under constant conditions (van der Horst et al. 1999; Vitaterna et al. 1999). Under baseline light–dark conditions, $Cry1,2^{-/-}$ mice spent more time in NREM sleep and sleep was more consolidated (i.e., longer uninterrupted episodes of NREM sleep). This increased consolidation of sleep was accompanied by a higher level of EEG delta power. In contrast to $Npas2^{-/-}$ mice, simulation analysis revealed that these higher levels of EEG delta power were due to a faster rate at which EEG delta power increases during wakefulness in $Cry1,2^{-/-}$ mice, compared to control mice (Wisor et al. 2002). The apparent higher sleep drive during baseline could also explain that after SD, $Cry1,2^{-/-}$ mice did not exhibit significant increases in NREM and REM sleep time, and only a brief and smaller increase in EEG delta power. These results were not observed in the $Cry1^{-/-}$ and $Cry2^{-/-}$ single KO mice (Wisor et al. 2008), consistent with the functional redundancy between the two CRY proteins observed for circadian rhythms (van der Horst et al. 1999; Vitaterna et al. 1999).

Albumin D-binding protein (DBP) is a PAR leucine zipper transcription factor that is expressed according to a robust circadian rhythm in the SCN. Mice lacking DBP display a shorter circadian period in locomotor activity and are less active (Lopez-Molina et al. 1997). Although DBP is not essential for circadian rhythm generation, it does modulate the expression of core clock components as well as

important clock outputs (Bozek et al. 2009). In particular, in vitro and in vivo studies have shown that the expression of the *Per* and *Cry1* genes is modulated through activation of the D-box in their promoter, an element that is bound by DBP (Vatine et al. 2009; Yamajuku et al. 2010, 2011; Ukai-Tadenuma et al. 2011; Mracek et al. 2012). Mice lacking the *Dbp* gene showed an altered sleep–wake distribution in baseline with reduced amplitude of the daily changes (Franken et al. 2000). These findings suggest that DBP, in addition to changing the period of the circadian clock, modifies the strength of the SCN output signal, which governs the distribution and consolidation of sleep and wakefulness over the day (Dijk and Czeisler 1995). In addition, $Dbp^{-/-}$ mice showed a decreased NREM sleep consolidation and EEG delta power amplitude, suggesting an overall lower sleep propensity. Computer simulations predicting the time course of EEG delta power demonstrated that the difference in EEG delta power was, to a large extent, due to a reduction in the circadian amplitude of the distribution of sleep and wakefulness and not to an altered dynamics of the homeostatic regulation of EEG delta power (Franken et al. 2000). This study demonstrated that DBP mostly affects those aspects of sleep that are known to be under direct circadian control but leaves the homeostatic regulation of NREM sleep unaffected. Nevertheless, similar to $Clock^{\Delta/\Delta}$ mice, $Dbp^{-/-}$ mice showed a reduced compensatory rebound in REM sleep pointing to an altered homeostatic regulation of REM sleep.

Per1 and *Per2* represent key element of the mammalian molecular clock in the SCN, and their disruption leads to gradual loss of rhythmicity under constant conditions. Two studies have investigated a possible role of *Per* genes in sleep homeostasis using mutant lines. In a first study, Kopp and colleagues observed that the main differences between genotypes occurred in the distribution of sleep and wakefulness over the day under baseline conditions (Kopp et al. 2002). *Per1* mutants slept less than WT in the dark period, whereas *Per2* mutants slept less before dark onset. This earlier decrease of sleep in *Per2* mutants is consistent with the earlier onset of the active phase in these mice. Although the authors concluded that both *Per1* and *Per2* mutants mice had intact sleep homeostasis, SD led to a larger increase in total sleep time during recovery in *Per2* mutants. Moreover, both mutant lines showed earlier onset of NREM and REM sleep rebound after the SD and *Per2* mutant mice showed a larger relative increase in sleep time in the recovery dark period. Finally, lower levels of EEG delta power were reached during recovery sleep immediately following the SD. Together these observations would argue for altered dynamics of the sleep homeostat. In a second study, Shiromani and colleagues recorded sleep in *Per1*, *Per2,* and *Per1,2* double mutant animals (Shiromani et al. 2004). Similar to the previous findings, they observed an altered sleep–wake distribution especially in *Per2* mutant and double mutant mice. More importantly, the authors observed that after 6 h SD, the rebound in EEG delta power was longer lasting in *Per1* and *Per1,2* mutant mice. Although the authors did not comment on the differences in the magnitude of the response, the relative increase in EEG delta power after SD seemed also larger in *Per1* and *Per1, 2* mutant mice. Thus in contrast to the authors' conclusion these clock genes do seem to alter the dynamics of the sleep homeostatic process albeit the genotype

effects on the increase in EEG delta power seem opposite to those reported by Kopp et al. (Kopp et al. 2002). Also *Drosophila Per* mutant flies exhibit a homeostatic phenotype, with an increased sleep rebound compared to WT flies (Shaw et al. 2002). Together, these data support a role for *Per1* and *Per2* signaling in sleep homeostasis although results in mice deserve further investigation.

While, *Per1* and *Per2* are widely considered to be integral part of the core circadian clock machinery, the role of the third *Per* homologue, *Per3*, in maintaining circadian rhythmicity is controversial. This controversy comes from observations that in mice, the absence of *Per3* has only a subtle effect on circadian rhythm phenotype (Shearman et al. 2000; reviewed in van der Veen and Archer 2010). Disruption of *Per3* in mice seems to alter (non-circadian) light-sensitivity which in turn could result in some of the circadian phenotypes reported such as a shortening of the free-running period under constant dark conditions (Van der Veen and Archer 2010). In the aforementioned study Shiromani and colleagues also recorded sleep in *Per3* mutant mice but did not note any differences in the increase of time spent asleep or EEG delta power during recovery from SD (Shiromani et al. 2004). However, in a more recent paper homeostatic sleep phenotypes were reported for $Per3^{-/-}$ mice (Hasan et al. 2011). Differences in the rebound in REM sleep were found during recovery from SD. These differences must, however, be attributed to baseline differences at the time the SD was performed because the recovery dynamics in REM sleep time as well as the levels reached in recovery did not differ. The resulting gain–loss curve resembles the hypothetical example presented in Fig. 2b′. Also the levels of EEG delta power reached after SD did not differ between $Per3^{-/-}$ and wild-type mice suggesting that the rate of increase of homeostatic sleep pressure during the SD was similar. Nevertheless, EEG delta power during the dark periods of baseline and recovery was significantly higher in $Per3^{-/-}$ mice, a difference that could not be explained by alterations in the amount or distribution of EEG delta power between the two genotypes. Thus like its role in circadian rhythms, a critical role for *Per3* in the homeostatic regulation of sleep in the mouse remains questionable.

In humans, a primate-specific variable number tandem repeat (VNTR) polymorphism in the *Per3* gene was investigated a few years ago for its role in circadian rhythmicity (Jenkins et al. 2005). A 54-nucleotide coding-region segment of the gene is repeated either 4 or 5 times, leading to different alleles. Initially, an association study revealed a higher frequency of people homozygous for the 5-repeat in morning types than in evening types suggesting possible functional role for *Per3* in sleep and circadian behavior (Archer et al. 2003). In follow-up studies investigating sleep phenotypes in *Per3* 4/4 and 5/5 carriers, it was found that this polymorphism affected electrophysiological and behavioral markers of sleep homeostasis such as sleep latency, EEG delta power, and the decrement in waking performance (Viola et al. 2007, 2012), executive function (Groeger et al. 2008), and neurobehavioral performance after sleep restriction (Rupp et al. 2012). This was the first evidence in human of a non-circadian role of clock genes in sleep regulation (Dijk and Archer 2009). While the human *Per3* VNTR polymorphism has been linked with differences in sleep homeostasis,

cognitive vulnerability to sleep loss, and differences in functional MRI-assessed brain activity in response to sleep loss (Dijk and Archer 2009), none of these studies has shown any association between the *Per3* VNTR and any circadian phenotype. Thus, while other core clock proteins may have overlapping roles in both the circadian and sleep systems, PER3 phenotypes from human and animal studies point toward a more prominent role for PER3 in the regulation of sleep homeostasis. A new transgenic mouse line carrying the human *Per3* polymorphism in the mouse *Per3* gene is currently under investigation, and preliminary analyses seem to highlight similarities with the human sleep homeostatic phenotype (Hasan et al. 2012).

DEC2, a member of the basic helix-loop protein family of transcription factors, by repressing CLOCK::BMAL1 acts as a negative component of the circadian clock. In a family-based candidate gene resequencing study a point mutation in the *Dec2* gene (P385R) was found to be associated with extremely early wake-up times and reduced sleep time (He et al. 2009). To examine the effect of DEC2 on sleep, several animal models were constructed. In transgenic mice carrying the human P385R *Dec2* gene, both NREM sleep and REM sleep were reduced and sleep was more fragmented in baseline compared to mice carrying the wild-type human *Dec2* thus recapitulating the human short sleep phenotype. A 6 h SD in P385R *Dec2* mice resulted in a smaller rebound in both NREM sleep and REM sleep, and a smaller relative increase in EEG delta power, compared to the control mice. The loss–gain analyses of the effect of SD on REM sleep duration revealed, however, a dynamics resembling our example depicted in Fig. 2b, indicating that the apparent difference in REM sleep homeostasis might be due to differences to REM sleep in baseline. Also the analyses of the rebound in EEG delta power do not allow for a careful evaluation of genotype differences in the sleep–wake dependent dynamics; given the poor time resolution over which EEG delta power was calculated (6 h intervals), it is impossible to establish whether the reported smaller increase after SD is due a slower build-up of sleep need during the SD, a faster decrease of sleep need during recovery sleep, or to differences in NREM sleep during the initial 6 h of recovery sleep. In both P385R *Dec2* transgenic mice and in *Dec2* KO mice the compensation of NREM sleep duration lost during the 6 h SD was compromised pointing to a role of *Dec2* in this aspect of sleep homeostasis specifically. In line with the human and mouse short sleep phenotype, transgenic flies expressing the murine *Dec2* gene carrying the human mutation P385R slept less than control flies (He et al. 2009). Unfortunately, the response to a homeostatic challenge (i.e., SD) was not assessed in these flies nor in humans. Although more data are needed, these data suggest that the P385R genotype shortens sleep independent of species background.

5.2 Clock Gene Expression Changes as a Function of Sleep

In line with the findings that several clock genes are involved in the control of sleep homeostasis, several studies have shown that expression of some clock genes in the mouse brain varies as a function of sleep propensity. SD results in a constellation of changes in gene expression in the brain (Cirelli et al. 2006; Terao et al. 2006; Maret et al. 2007; Thompson et al. 2010) that are sleep–wake related and thus, can serve as biomarkers for sleep loss and recovery (see Sect. 8). Among the transcripts that exhibit sleep-related changes in the cortex are the circadian genes *Per1*, *Per2,* and *Dbp* (Wisor et al. 2002; Franken et al. 2006, 2007; Mongrain 2010; Curie et al. 2013). The expression of *Per1* and *Per2* increases according to a linear function of the duration of the time mice are kept awake, whereas the expression of *Dbp* decreases (Wisor et al. 2002; Franken et al. 2006, 2007). These SD-induced changes were, however, strongly dependent on the time of day at which the SD was performed (Curie et al. 2013). Moreover, the SD-associated increase in corticosterone proved to be an important contributor to these increase in clock gene expression such that the expression of *Per1* did no longer increase after SD in adrenalectomized mice (Mongrain et al. 2010). In addition to stress, SD seems to be able to alter the clock gene expression through directly modifying DNA-binding of the transcription factors CLOCK, BMAL1, and NPAS2 to specific E-boxes in clock gene promoters (Mongrain et al. 2011).

Together, the data from human, mice, and flies have contributed to the notion that, at the molecular level, sleep homeostasis and circadian rhythms are not independent, and that clock genes participate in both aspects of sleep regulation. According to the fact that several clock genes belong to a class of PAS transcriptional regulators that can act as sensors of environmental signals (Gu et al. 2000), we proposed that clock genes and their protein products act as molecular sensors and translate homeostatic sleep need into transcriptional signals at the cellular level, independent of the circadian machinery (Franken and Dijk 2009). Especially, the sensitivity of the clock gene machinery to redox state and metabolism (Bass and Takahashi 2010) is of interest in the context of sleep homeostasis as maintaining metabolic balance is often mentioned as a potential key function of sleep.

6 Genes of the Adenosine Pathway as Homeostatic Regulators?

Adenosine is an inhibitory neuromodulator that has been proposed to act as a homeostatic regulator of sleep and to link humoral and neural mechanisms of sleep–wake regulation (Porkka-Heiskanen et al. 1997, 2000; Basheer et al. 2004; Kalinchuk et al. 2011). In mammals, four subtypes of G-protein coupled receptors mediate the effects of adenosine: A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R and two of them have been investigated for their role in sleep homeostasis (Fredholm et al. 2001).

It is thought that A_1R are responsible of the sleep effects of adenosine (Rainnie et al. 1994; Benington and Heller 1995), but a careful study performed in A_1R KO mice showed that the homeostatic aspect of sleep regulation was unaltered in animals lacking A_1R (Stenberg et al. 2003). This study revealed that mice lacking A_1R had normal baseline sleep–wake distribution and responded normally to sleep pressure, with NREM sleep rebound and EEG delta power rebound being similar to WT animals. More recently, a conditional central nervous system KO of this adenosine receptor was created. $A1R^{-/-}$ mice were found to have reduced EEG delta power in NREM sleep during baseline, as well as during sleep restriction (Bjorness et al. 2009) whereas the sleep–wake distribution and amount were preserved. More importantly, when the mice were allowed to sleep for 2 h following 4 h of sleep restriction, the relative increase in EEG delta power during NREM sleep above baseline levels was smaller in the KO mice although the amount of time spent in NREM sleep was similar to WT. The authors suggested that the elevated sleep need signaled by adenosine is, at least in part, mediated through the A_1R. However, the implemented sleep restriction protocol allows mice to recover during the 2 h sleep opportunity windows between the 4 h SDs, importantly affecting the level of EEG delta power. Moreover, the authors observed a general decrease in EEG delta power when calculated over all states (wakefulness, NREM and REM sleep) which might indicate a specific effect of the gene on general electrical brain activity rather than an effect on sleep homeostasis. Given the contradictory findings, the role of A_1R in sleep homeostasis remains unclear. It might be interesting to generate inducible KO animals to study the loss of A_1R in the adult stage only, thereby circumventing several potential confounds such as developmental compensation. The accumulated evidence indicates that besides A_1R also $A_{2A}R$ contributes to the effects of adenosine on sleep. A preliminary report indicated that $A_{2A}R$ KO mice do not show a NREM sleep rebound following 6 h SD, revealing an altered sleep homeostasis in these mice (Urade et al. 2003). Changes in EEG delta power were not reported in these mice. Along the same line, a human study revealed that a distinct polymorphism in the gene encoding the $A_{2A}R$, *Adora2*, modulates individual sensitivity to subjective and objective effects of caffeine on sleep (Rétey et al. 2007). It would be of interest to test the effect of this polymorphism on the homeostatic process by submitting individuals carrying the different polymorphism to a SD. In *Drosophila*, one single adenosine receptor gene has been identified, *dAdoR*, that is most closely related to the mammalian *Adora2* gene. Study in *dAdoR* mutant flies revealed that sleep was not affected by the mutation, neither in baseline, nor in recovery from SD (Wu et al. 2009). These results suggest that in flies, adenosine receptors are not required to maintain sleep homeostasis.

Besides the receptors, adenosine metabolism has also been investigated in the context of sleep homeostasis. Intracellular adenosine levels are regulated by enzymes such as adenosine kinase (ADK) and adenosine deaminase. ADK is the key enzyme controlling adenosine levels, and the effect of its overexpression on sleep has been investigated in mice (Palchykova et al. 2010). ADK transgenic mice (*Adk-tg*), which have an increased enzyme activity, are thought to have lower adenosine tone in the brain (Fedele et al. 2005). In *Adk-tg* mice, sleep–wake

baseline distribution is altered, the mice being more active and sleeping less than the WT controls, especially during the dark period. After 6 h SD, these mice compensated with a lower EEG delta power in NREM sleep than the WT, despite a larger NREM sleep rebound. The authors suggested that *Adk-tg* mice have a reduced capacity to intensify sleep, and that adenosine metabolism plays an important role in maintaining sleep homeostasis. However, the increased amount of NREM sleep obtained during recovery sleep in *Adk-tg* mice could underlie the lower EEG delta power levels reached. In addition, the recovery time course of EEG delta power of *Adk-tg* and WT mice ran largerly in parallel which might point a problem with the choice of the baseline reference chosen to normalize the individual data. As outlined in Sect. 4, the baseline amount and distribution of NREM sleep can affect the level of the reference when the 24 h average EEG delta power is used. *Adk-tg* mice have less NREM sleep in the baseline period which can be expected to be accompanied to higher levels of EEG delta power in baseline and thus a higher reference value and to reduced relative levels during recovery. Unfortunately, the authors did not show the baseline time course of EEG delta power to counter this concern. Several other enzymes are involved in the conversion of adenosine nucleotides to adenosine. Extracellular AMP is converted into adenosine by the 5′ectonucleotidase enzyme CD73. A recent study of mice lacking CD73, that are thought to have a reduced capacity to enhance extracellular adenosine levels, has shown that CD73 KO mice have more spontaneous NREM sleep time, although less consolidated (Zielinski et al. 2012). After 6 h of enforced waking, KO mice had a smaller NREM sleep rebound and a smaller increase in EEG delta power over baseline levels, compared to WT. However, the time course of EEG delta power in recovery sleep is very similar in WT and KO mice and the relative differences between genotypes reported after SD have to be attributed to differences in the baseline time course of EEG delta power, suggesting that the homeostatic response is unaltered.

An important general concern of the KO studies dealing with adenosine signaling is the lack of information about the adenosine levels in the brain. In vivo measurement of extracellular adenosine levels is critical and controversial, because of difficulties in performing correct local adenosine measurement in brain tissue and because of large variations according to the methods used (Delaney and Geiger 1996; Latini and Pedata 2001). Thus, many studies are based on the assumption that adenosine levels are altered by the genetic manipulation in the synthesis/metabolism pathway. Some authors did make use of indirect measure of adenosine levels, by evaluating the activity of adenosine receptors using electrophysiology, without direct evidence that adenosine levels are effectively altered in the mice (Fedele et al. 2005). Therefore, it should be kept in mind that the phenotypes observed are not necessarily directly linked to an alteration of adenosine levels, and could be due to other deficits resulting from the genetic manipulation of the pathway.

Besides the receptors and metabolic pathway described above, other components seem to act in concert with adenosine in modulating sleep homeostasis. Prostaglandin 2, thought to be one of the most powerful sleep-promoting substances (Urade and Hayaishi 2011 for review), modulates adenosine levels in the

brain (Mizoguchi et al. 2001), and is believed to be indirectly involved in sleep homeostasis through its receptors and producing enzymes (Mizoguchi et al. 2001; Hayaishi et al. 2004). However, it remains unknown if the role of Prostaglandin 2 in sleep regulation can be dissociated from the adenosine signaling pathway, but it seems clear that these two molecules contribute to the sleep-wake control and probably to sleep homeostasis (Huang et al. 2007 for review).

Moreover, recent studies have highlighted a role for glial cells, and in particular astrocytes, in modulating the accumulation of sleep pressure through a pathway involving adenosine receptors (Halassa et al. 2009; Schmitt et al. 2012). Together, these data strongly suggest an involvement of adenosine and the associated pathways in the modulation of sleep homeostasis.

7 Other Signaling Pathways and Sleep Homeostasis

Under this section, we present only some examples of the signaling pathways in the brain that have been studied in the context of sleep homeostasis using forward and reverse genetic techniques.

7.1 Neurotransmitters

Many neurotransmission systems have been involved in the control of sleep and wakefulness, revealing that the neurobiology of sleep relies on the interaction of wake and sleep-promoting centers in the brain (Brown et al. 2012 for review). Among these systems, some of them have been shown to impact the homeostatic control of sleep as well. In flies, genetic manipulation of the dopamine system was found to impact on sleep homeostasis (Andretic et al. 2005; Kume et al. 2005; Wu et al. 2008; Qu et al. 2010); similar results were obtained with genes involved in monoamine catabolism (Shaw et al. 2000). In the mouse, several studies have suggested a role for serotonin in the control of sleep homeostasis (Frank et al. 2002; Popa et al. 2006). Along the same lines, human polymorphism in the *Catechol-O-methyltransferase* gene, which encodes for the principal enzyme involved in catecholamine's degradation, has been linked to EEG differences during sleep loss and differential homeostatic response to SD (Bodenmann et al. 2009; Goel et al. 2011). Together, these data from flies, mice and humans suggest a role for catecholamine system in sleep homeostasis in addition to its well established role in sleep-wake regulation.

Besides the previously mentioned wake-promoting molecules, the inhibitory neurotransmitter GABA has also been investigated for its role in sleep homeostasis. In mice, several reverse genetic studies have investigated the contribution of the GABA receptors in sleep homeostasis but it appeared that this major component of the sleep–wake gating control does not play a major role in sleep homeostasis (Winsky-Sommerer et al. 2009; Vienne et al. 2010).

In summary, although the role for neurotransmitters in sleep–wake regulation has largely been demonstrated in mammalian and non-mammalian species, their involvement in sleep homeostasis is less obvious. This strongly supports the notion that the maintenance of a proper homeostatic sleep balance involves other factors than those implied in the regulation of the behavioral states alternation and requires independent mechanisms.

7.2 Ion Channels

In both mammals and flies, potassium currents play a major role in the control of membrane excitability and transmitter release. One of the first large-scale forward screens in *Drosophila* has highlighted the function of the voltage-gated potassium channel Shaker (*Sh*) in sleep (Cirelli et al. 2005). Subsequent 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 a *sss* mutant (Koh et al. 2008). In addition, mutants of an SH regulatory subunit, Hyperkinetic (*Hk*) also show a reduction in sleep time (Bushey et al. 2007). These mutagenesis studies in the fly highlighted the central role of membrane excitability and subsequent control of neurotransmitter release in sleep regulation, in particular in the homeostatic aspect of sleep. In mice, a mutation in *Kcna2*, the closest homolog to *Drosophila Sh,* produces a reduction in sleep amount (Douglas et al. 2007). The response to SD could, however, not be assessed in these mice because of seizures and premature death. Together, the fly and mouse studies are indicative of the importance of ion channels, and in particular potassium channels, in the control of sleep and its EEG correlates.

7.3 Cytokines and Neurotrophic Factors

Cytokines represent another group of signaling molecules that have been linked to sleep and its homeostatic regulation. One particular cytokine, Tumor Necrosis Factor alpha (TNFα) is considered as a sleep-promoting factor and was found to affect sleep homeostasis (Clinton et al. 2011; Krueger et al. 2011 for review). In human, plasma TNFα levels are correlated with EEG delta power (Darko et al. 1995), and manipulating TNFα concentration in animals result in changes in NREM sleep time and EEG delta power levels (Yoshida et al. 2004; Taishi et al. 2007). Several transgenic mouse lines carrying targeted mutations of the TNFα signaling pathway have been investigated for a sleep phenotype (for review, see Krueger 2008). For example, mice lacking the TNF 55 kDa receptor fail to increase the amount of NREM sleep in response to TNFα treatment (Fang et al. 1997). Another study showed that the deficiency of one or two of the TNF receptors, or the deficiency of the ligand to the receptors reduces the amount of REM sleep and increases

EEG delta power after 6 h SD. In receptor 2 and ligand KO, the increase in EEG delta power concerned the faster delta frequencies (2.75–4.0 Hz) whereas in receptor 1 KO this increase was limited to the slower frequencies (0.75–2.5 Hz) (Deboer et al. 2002). More recently, the TNFα receptor double KO mice were investigated for a sleep homeostatic phenotype and showed shorter sleep latency and an altered rebound in both NREM sleep and REM sleep after a sleep fragmentation protocol (Kaushal et al. 2012). In sum, these data support a role for this cytokine in sleep homeostasis.

Brain-derived neurotrophic factor (BDNF) has been proposed to regulate sleep need in several models. Studies in the rat provided evidence for a causal role of BDNF secretion in sleep homeostasis (Huber et al. 2007; Faraguna et al. 2008). In humans, one functional polymorphism has been found in the gene encoding for the BNDF located on chromosome 11 (Egan et al. 2003). A recent study evaluated the effect of this polymorphism on sleep intensity and found a difference in NREM sleep amount and EEG delta power in both baseline and recovery from 40 h of SD (Bachmann et al. 2012). To better understand whether BDNF plays a causal role in regulating sleep homeostasis it might be interesting to evaluate sleep and response to SD in BDNF KO animals.

Converging observations pointed out a bidirectional interaction between sleep and the endocrine system; many hormonal secretions are correlated to sleep-wake distribution, and the secretion of several hormones is modified during extended wakefulness and recovery sleep (Takahashi et al. 1968, 1981; for review see Obal and Krueger 2004). Several KO studies in mice have investigated the involvement of the somatotropic axis, and in particular growth hormone in modulating sleep need (Obal et al. 2001, 2003; Hajdu et al. 2002). In conclusion of these studies, whereas the role of the somatotropic axis in sleep promoting is established, its involvement in the homeostatic control of sleep seems less evident.

Another growth factor pathway that has been discovered to affect sleep is the one involving epidermal growth factor receptor (EGFR). When the EGFR ligands Rho or Star are induced in flies, they lead to an increase sleep level and sleep consolidation (Foltenyi et al. 2007). More importantly, modulation of EGFR signaling in flies affects not only sleep amounts, but also recovery sleep. In mammals, the functional consequences of EGFR/ERK activation on sleep are unknown; however, a report from sleep deprived rats suggests a link between ERK activation, sleep and memory (Guan et al. 2004).

8 Molecular Changes Associated to Sleep Loss: Insights from Molecular Genetics Studies

As mentioned earlier, molecular genetic methods led to the discovery that SD results in a variety of changes in gene expression in the brain. Using microarray analysis, studies performed in rats, mice, and flies showed that several classes of

genes are up- or down-regulated after spontaneous waking or during SD relative to sleep. These classes include immediate early genes and transcription factors, genes related to energy metabolism, growth factors and adhesion molecules, chaperones and heat shock proteins, vesicle- and synapse-related genes, neurotransmitters, transporters and hormone receptors, and different types of enzymes (Cirelli and Tononi 2000; Cirelli 2005; Terao et al. 2006; Mackiewicz et al. 2007; Maret et al. 2007). Interestingly, among the many transcripts that change following SD, a class of small non-coding RNA molecules, the micro-RNAs, was discovered in microarray screens (Davis et al. 2007; Mongrain et al. 2010).

More recently, the microarray-based profiling methods have been used in conjunction with immediate early genes-based activity-mapping or high-throughput in situ hybridization (Terao et al. 2006; Lein et al. 2007; Thompson et al. 2010) to determine changes in specific brain regions that are associated to extended wakefulness and therefore provide an anatomical map of the SD effects.

Although transcriptome studies offer a first insight into the changes associated with sleep loss, the challenge remains to find genes, or classes of genes, that are causally linked to sleep need, and distinguish them from transcripts that change due to secondary effects of sleep loss or to the SD method. An example of such an effect is the surge in corticosterone associated with SD. By comparing the SD-induced changes in brain gene expression between sham-operated and adrenalectomized mice in which corticosterone levels do not change when sleep deprived it was found that corticosterone importantly amplifies the SD induced changes (Mongrain et al. 2010). By combining these results with the changes in gene expression after spontaneous sustained periods of wakefulness during baseline, genes could be selected for which the expression was affected mostly by increased sleep need. The resulting exclusive list of 78 might be regarded as candidate molecular components of the sleep homeostat as exemplified by the transcript *Homer1a* (see next section below) present on this list (Mongrain et al. 2010).

To add causality, transcriptome studies can be complemented with reverse genetic studies. As an example of the involvement of heat shock protein in sleep homeostasis, mutant flies for heat shock proteins showed an altered homeostatic response to sleep loss, whereas heat shocking flies before SD rescues the premature lethality that is due to SD in *Cycle* mutant flies (Shaw et al. 2002). A similar example is provided by the immunoglobulin binding protein, indicative of stress, which is increased in the mouse cerebral cortex as well as in *Drosophila* heads in response to sleep loss (Cirelli and Tonini 2000; Shaw et al. 2000; Mackiewicz et al. 2003; Naidoo et al. 2005). In flies overexpressing immunoglobulin binding protein, the response to SD is altered compared to the control line (Naidoo et al. 2007). These studies provide good examples of a combination of several genetic techniques to uncover the genetics of sleep homeostasis.

8.1 *The Identification of* Homer1a *as a Molecular Correlate of Sleep Loss*

Homer proteins constitute a family of scaffolding proteins localized in the post-synaptic density of excitatory synapses that function as molecular adaptators by binding to specific prolin-rich sequence in the C-terminus of metabotropic glutamate receptors and other proteins that play a role in calcium signaling. The vertebrate genome includes three *Homer* genes (*Homer1, -2,* and *-3*). *Homer1* is a complex gene with multiple splice variants among which: *Homer1a*, *-b,* and *-c*. Interest in *Homer1a* comes from its role in homeostatic synaptic scaling (Hu et al. 2010) and neuroprotection (Szumlinski et al. 2006) both suggested as possible functions of sleep (Tononi and Cirelli 2006; Mongrain et al. 2010). *Homer1a* is a short form that is up-regulated with neuronal activity and antagonizes the activity of the full length HOMER1b and -c proteins by competing for binding to the glutamate receptors. In contrast, *Drosophila* possesses a single *Homer* gene that encodes a cross-linking HOMER protein but no *Homer1a* homologue. In a study that involved in-depth phenotyping of sleep in recombinant inbred mice, a QTL for sleep homeostasis was identified (Franken et al. 2001). A genome-wide significant QTL for the increase of EEG delta power after SD was identified on chromosome 13, referred to as delta power in slow-wave sleep 1 (*Dps1*). This QTL accounted for 49 % of the variance in this trait between C57BL/6 J and DBA/2 J strains. Further in silico and transcriptome analyses using microarrays identified *Homer1a* as a potentially credible candidate gene for *Dps1* (Maret et al. 2007). A parallel study confirmed this finding by identifying genes that were both located in the *Dps1* region and differentially expressed between sleep and wakefulness in the brain of C57BL/6 J mice (Mackiewicz et al. 2008). These findings were concordant with previous expression analyses in flies showing that *Homer* expression is changed during sleep and extended wakefulness in flies (Zimmerman et al. 2006). However, it remained unknown whether changes in expression play a causal role in sleep-wake control or are simply a correlate of these behavioral states. A careful study recently investigated sleep and its homeostatic regulation in mutant animals for the *Homer* genes, and showed that these proteins play a role in sustaining sleep-wake behavioral states in both *Drosophila* and mice (Naidoo et al. 2012). In *Drosophila*, lack of *Homer* leads to an alteration in the ability to sustain both sleep and wakefulness but the effect on sleep consolidation is greater; moreover, the *Homer* null flies have an altered response to SD with a longer recovery period, although less consolidated, suggesting that the recovery period does not efficiently dissipate the drive for sleep. In contrast, in mice the major effect of *Homer1a* absence is their inability to sustain long bouts of wakefulness. Interest in *Homer1* was based primarily on it being a candidate for *Dps1* QTL for the rebound in EEG delta power after SD. No altered response to SD was, however, observed for the increase in EEG delta power in these *Homer1a* KO mice confirming the lack of a homeostatic phenotype in total *Homer1* (i.e., *Homer1a*, *-b*, and *-c*) KO mice (Maret et al. 2007). The authors concluded that HOMER1 scaffolding proteins are required for maintenance of behavioral state and that

consolidation of sleep and wake is governed by molecules other than traditionally known neurotransmitters. They also noted that in a model of competitive actions of *Homer1a* versus cross-linking forms of *Homer*, the up-regulation of *Homer1a* is functionally equivalent to down-regulation of cross-linking *Homer*. Thus, despite evolutionary changes in *Homer* gene structure and copy number, *Drosophila* and mice share the functional consequence of reduced *Homer* cross-linking during wakefulness and increased during sleep. Although the causal role of *Homer1a* in the control of sleep homeostasis remains unclear, it is to date the best know molecular marker of sleep need. Further studies using inducible KO in the adult animal will probably help to by-pass issues such as developmental compensation. The *Homer1a* discovery is one of the few examples of a successful combination of forward, molecular, and reverse genetic approaches.

9 Conclusion

Sleep homeostasis is a complex mechanism of control, and defining an alteration in this process as a genetic trait is obviously not simple or straightforward. In this review, we discussed the variables and parameters used to evaluate sleep homeostasis in animal models. Because different studies focus on different aspects of sleep homeostasis using different methods and analyses, comparisons among studies are difficult. In search for the molecular basis of sleep homeostasis, several genetic methods have been applied, and the combination of forward, reverse, and molecular genetic approaches across species offered promising results. Within the past few years, the development of microarray technologies has enabled to study the expression of genes that are changed with SD, and could highlight many pathways and molecules that are linked to sleep homeostasis such as the molecular circadian clock, metabolism, synaptic plasticity, immune response, and others. With the use of transgenic animals, researchers have tried to reveal causal relationship between these candidate genes and homeostatic process. However, controversial results in the various animal models confirm that sleep homeostasis is indeed complex and that many different pathways are likely to be involved. Here we have tried to give a critical overview of candidate genes that have been tested as regulator of sleep homeostasis, but the list is not exhaustive. Moreover, although not mentioned in our review, sleep homeostasis has been found to differ between males and females in some species, which suggests the possible involvement of other pathways related to hormones. In the future, the use of a combination of system genetic approaches in the mouse or other animal models, and GWAS in humans will probably be our best bet to uncover the molecular actors central to sleep homeostasis.

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