CHAPTER 4

Genetic Mechanisms Underlying Rhythmic EEG Activity during Sleep

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INTRODUCTION

To unambiguously determine whether a mammal or bird is asleep or not and if so, in which sleep state, electroencephalographic (EEG) recordings are indispensable. The EEG captures the summed activity of millions of neurons. In humans, activity of neurons of the cerebral cortex are thought to contribute the most to the EEG signal while activity from deeper sources contribute less to currents measured with scalp electrodes. In laboratory animals, such as rats and mice, epidural electrodes are used and, since cortical thickness in these species is greatly reduced as compared to humans, activity of deeper structures such as the hippocampus importantly contribute to the EEG patterns observed. The EEG signal shows oscillations of specific frequency ranges representing synchronized and coherent activity over large networks of neuronal assemblies. For instance, thalamocortical and corticocortical networks underlie the slow oscillation (<1 Hz), delta oscillations (1–4 Hz), and spindle oscillation (11–15 Hz; i.e., the sigma frequency range), characteristic of non-rapid-eye-movement (NREM) sleep (McCormick & Bal, 1997; Steriade, 2003), while the septo-hippocampal/entorhinal-cortex system underlies the theta oscillations (5–9 Hz) that dominate the EEG during rapid-eye-movement (REM) sleep in rodents (Chrobak, Lorincz, & Buzsaki, 2000; Vertes & Kocsis, 1997). Based on the visual inspection of these state-specific EEG patterns, usually in combinations with the electromyogram (EMG) to quantify muscle tone, the states waking, NREM, and REM sleep can be readily discerned (Fig. 4.1) and measures such as the daily amount of sleep, its distribution over the day, the period of the NREM-REM sleep alternation, sleep continuity or quality, sleep-onset latency, and the changes in these variables in response to experimental interventions, can all be quantified (Mang & Franken, 2012).

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Besides its use in classifying sleep-wake state, the EEG signal itself contains a wealth of information not only important for the study of the neuronal processes that shape the EEG but also for gaining inside into the regulation, physiology, and pharmacology of sleep. The frequency components that contribute to the EEG within each state can be quantified using spectral analyses algorithms such as the widely used fast Fourier transform (FFT) (Borbely, Baumann, Brandeis, Strauch, & Lehmann, 1981) (Fig. 4.1). Such algorithms allow for the quantification of the activity or power over a broad frequency range or spectrum (e.g., 0.5–100 Hz) or in specific frequency bands such as those introduced above. Besides the pronounced state-dependent changes in the spectral composition of the EEG on which state classification is based, within each state EEG spectra vary as a function of circadian time and time-spent-awake (or asleep) (Dijk & Czeisler, 1995). The latter changes are especially clearly reflected by the sleep-wake driven or homeostatic changes in EEG delta power (Daan, Beersma, & Borbely, 1984; Franken, Chollet, & Tafti, 2001), a measure capturing both the amplitude and prevalence of EEG delta oscillations. The spectral changes in the sleep and waking EEG after drug administration are specific to the class of drug resulting in a recognizable "EEG fingerprint" (Hasan et al., 2009; Tobler, Kopp, Deboer, & Rudolph, 2001; Winsky-Sommerer, 2009). The term EEG fingerprint has also been used to describe the surprisingly stable and trait-like spectral composition of the sleep EEG within an individual over time contrasting the marked EEG differences that can be observed among individuals (Buckelmuller, Landolt, Stassen, & Achermann, 2006; De Gennaro, Ferrara, Vecchio, Curcio, & Bertini, 2005; Finelli, Achermann, & Borbely, 2001). Identifying the molecular basis underlying such EEG fingerprints and the homeostatic EEG changes has led to new insights and hypotheses (Krueger et al., 2008; Tononi & Cirelli, 2006). Since to date no systematic genetic studies have been performed in humans to identify the genetic variants contributing to the rhythmic EEG activity, this chapter will have to rely largely on available mouse data. In the context of this book, a further focus is that only the genetics of the EEG during physiological sleep will be considered and not the genetics of the manifestation of sleep *per se* (i.e., its duration, distribution, and so forth) or the genetics of sleep disorders that have been reviewed elsewhere (e.g., Franken & Tafti, 2003; Sehgal & Mignot, 2011).

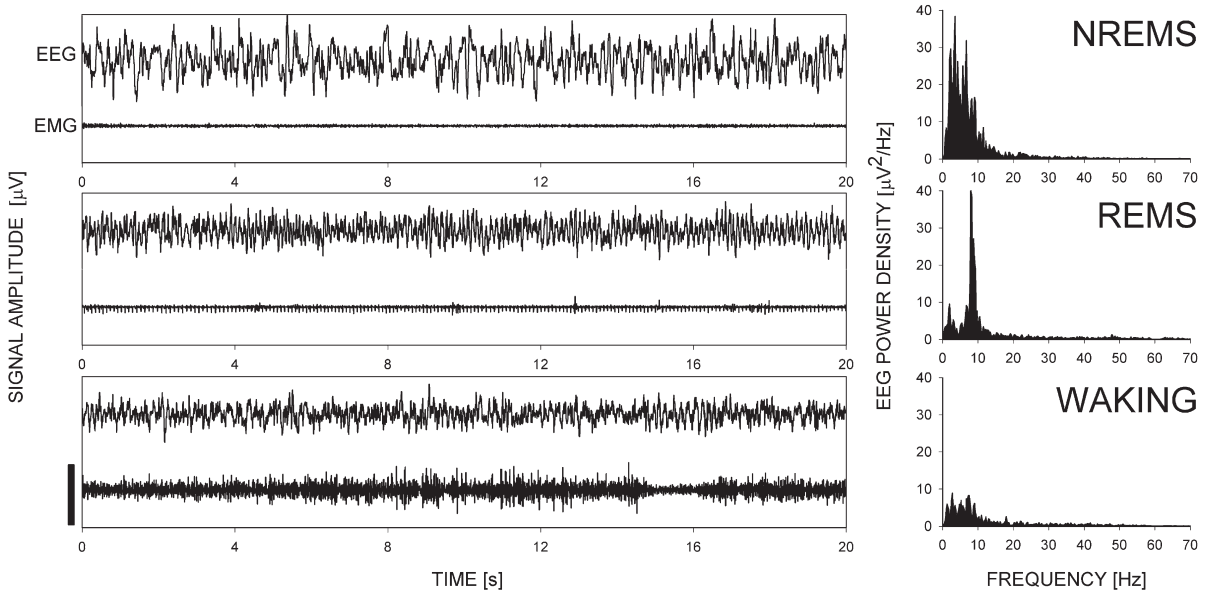


Figure 4.1 ***Sleep-wake state determination is based on EEG and EMG patterns.*** *Left panel:* The determination of the states NREM sleep (top), REM sleep (middle), and wakefulness (lower panel) in the mouse is based on the distinctive patterns of the EEG and EMG. NREM sleep is characterized by the prevalence of high amplitude slow waves in the EEG concomitant with a low and invariable muscle tone in the EMG. The REM sleep EEG is dominated by highly regular theta oscillations with muscle atonia with occasional twitches (note the 10 Hz heartbeat artifact in the EMG). EEG activity during wakefulness is generally of low amplitude and mixed frequency together with a high and variable EMG. Both signals vary depending on the animal's waking behavior. Sleep-wake states were annotated for consecutive 4 s epochs. Twenty-second traces were obtained in an individual male mouse of mixed genetic background (C57BL/6J x 129P/Ola) within an undisturbed 12 h light period. The vertical scale bar delimits 400 μV and applies to both signals. *Right panel:* Spectral content of the signals depicted on the left was quantified with the fast Fourier routine (FFT). Spectra are averaged over the five spectra calculated within each of the 4 s epochs (sampling rate 200 Hz; 0.25 Hz frequency bins). Examples clearly illustrate the dominance of delta activity during NREM sleep, that theta oscillations are the main (only?) rhythmic component during REM sleep, and that in the waking EEG in this particular example no clear oscillation can be visually discerned. *Reprinted from Xie et al. (2005) with permission.*

GENETICS OF THE HUMAN EEG: TWIN, LINKAGE, AND CANDIDATE-GENE STUDIES

The earliest indications that genetic factors play a role in shaping sleep and the EEG come from studies comparing phenotypic variance between monozygotic (MZ) and dizygotic (DZ) twins. In these studies, a higher concordance in a phenotype between MZ twins as compared to DZ twins is taken as proof for a contribution of genetic factors because MZ twins are considered genetically identical, whereas DZ twins share, on average, only half of their segregating genes. Resemblance within twin pairs can also be due to shared environmental influences and an additional assumption is that this shared environment accounts equally for the similarity in MZ and DZ twins. With these assumptions twin studies provide information about the heritability of a trait, that is, the fraction of the total phenotypic variance that can be attributed to additive genetic factors (Boomsma, Busjahn, & Peltonen, 2002).

Sleep patterns and the duration of sleep within MZ twins have a higher concordance than within DZ twin pairs or unrelated subjects. For these sleep traits heritabilities between 40% and 60% can be estimated (Gedda & Brenci, 1979, 1983; Hori, 1986; Linkowski, 1999; Webb & Campbell, 1983). For aspects of the EEG activity within a behavioral state, twin studies revealed that additive genetic factors importantly outweigh environmental influences and heritabilities up to 90% have been reported (Christian et al., 1996; Juel-Nielsen & Harvald, 1958; Lykken, Tellegen, & Thorkelson, 1974; Stassen, Lykken, & Bomben, 1988; Stassen, Lykken, Propping, & Bomben, 1988; van Beijsterveldt & Boomsma, 1994; Vogel, 1970). Most EEG studies in twins concern the spectral composition of the EEG signal during wakefulness quantified with FFT. The contribution of the alpha rhythm (8–13 Hz) during waking has received much attention, but the activity in the delta, theta, and beta (15–35 Hz; the precise frequency range delimiting beta varies among studies) frequency ranges were also found to be highly heritable (>80%). Two more recent twin studies confirmed that the EEG spectral profile of NREM sleep, especially in frequencies below 16 Hz, also shows a heritability estimate of 96% (Ambrosius et al., 2008; De Gennaro et al., 2008), indicating that all of its phenotypic variance can be attributed to genetic factors. With these strikingly high heritabilities, EEG traits qualify as the most heritable traits in humans, matched only by heritabilities obtained for brain architecture such as has been demonstrated for the distribution of gray matter in the cerebral cortex (Thompson et al., 2001). It has been suggested that these two traits might well be interrelated in that common

genetic factors underlie functional brain connectivity as well as rhythmic brain activity (Posthuma et al., 2005). Contributing factors could concern the inter-individual differences in the rate at which the brain matures as revealed by the EEG (Buchmann et al., 2011).

The contribution of alpha activity to the waking EEG is a clear example of an EEG trait that is both highly variable among individuals and highly stable over time within an individual. Moreover, twin and family studies have demonstrated that its variation is determined mainly by genetic factors. In fact, the differences in alpha activity found for an adult studied on two different occasions are similar to the differences (or rather the lack thereof) observed within a MZ twin pair (Lykken et al., 1974). One relatively common alpha activity variant, "low voltage alpha," seems to follow a simple Mendelian mode of inheritance with high penetrance (Anokhin et al., 1992; Vogel, Schalt, Kruger, Propping, & Lehnert, 1979). Genetic linkage studies could map the low voltage alpha trait to chromosome 20q in some families (Anokhin et al., 1992; Steinlein, Anokhin, Yping, Schalt, & Vogel, 1992; Steinlein, Fischer, Keil, Smigrodzki, & Vogel, 1992). This remains, surprisingly, the only genetic study in humans aimed at uncovering the genes contributing to an observed EEG trait. We will have to await the results of several currently ongoing genome-wide association studies (GWAS) to learn about the allelic variants that shape the EEG in the general population.

Meanwhile, a handful of candidate gene studies do convincingly illustrate that known polymorphisms can importantly impact the spectral composition of the EEG in humans (Landolt, 2011). For example, a single nucleotide polymorphism at position 22 (22G > A) in the gene encoding the enzyme adenosine deaminase (*Ada*) affects the contribution of delta activity to the EEG of both NREM and REM sleep with higher levels reached in heterozygous G/A allele carriers than in homozygous G/G carriers (Bachmann, Klaus, et al., 2011; Retey et al., 2005). The G/A genotype is associated with lower ADA activity (Riksen et al., 2008) and thus could result in altered extracellular adenosine levels (Hirschhorn, Yang, Israni, Huie, & Ownby, 1994). Also a 1083T > C polymorphism in the gene encoding the adenosine A2A receptor (*Adora2a*) affects the EEG (Bodenmann et al., 2011; Retey et al., 2007). In the theta/low-alpha frequency range (7–10 Hz) subjects with the T/T genotype displayed higher EEG power than C/C subjects during waking as well as during sleep. These results are of interest in the context of a role of the adenosine signaling pathway in the homeostatic regulation of sleep (Benington & Heller, 1995;

Landolt, 2008; Porkka-Heiskanen, Kalinchuk, Alanko, Urrila, & Stenberg, 2003).

Another example is the gene encoding the catecholamine-metabolizing enzyme catechol-O-methyltransferase (*Comt*) for which a common polymorphism exists altering an amino acid in its protein at codon 158 from valine to methionine (Val158Met). Val/Val individuals show higher COMT activity and lower dopaminergic signaling in prefrontal cortex than Met/Met individuals (Akil et al., 2003; Chen et al., 2004; Slifstein et al., 2008) and EEG activity in the alpha/sigma (11–13 Hz) frequency range is lower in Val/Val compared to Met/Met carriers (Bodenmann & Landolt, 2010; Bodenmann, Rusterholz, et al., 2009; Bodenmann, Xu, et al., 2009). A fourth example concerns a nonsynonymous Val66Met polymorphism in the gene encoding brain-derived neurotrophic factor (*Bdnf*) that was also found to affect the EEG (Bachmann, Klein, et al., 2012). Alpha activity in wakefulness doubled in Val/Val subjects compared to Val/Met carriers. In REM sleep, besides alpha, EEG activity in the theta and sigma frequency bands was also higher in Val/Val carriers. In NREM sleep EEG activity in the entire delta and theta range was enhanced in the Val/Val compared to Val/Met carriers while power in the alpha and sigma frequencies was reduced. The Val66Met polymorphism of *Bdnf* is associated with a pronounced neuroanatomical phenotype affecting many brain areas including grey matter volume in hippocampus and prefrontal cortical areas (Toro et al., 2009). This again illustrates the possibility that developmental differences in brain maturation underlie the trait-like individual EEG "fingerprints." Finally, homozygous carriers of five copies of a tandem-repeat in the circadian gene Period-3 ($Per3^{5/5}$) display higher EEG delta power during NREM sleep, higher EEG alpha power in REM sleep, and higher theta/alpha activity compared to homozygous carriers of only four repeats ($Per3^{4/4}$) (Dijk & Archer, 2010; Viola et al., 2007). The molecular functional relevance of this tandem-repeat polymorphism remains to be identified.

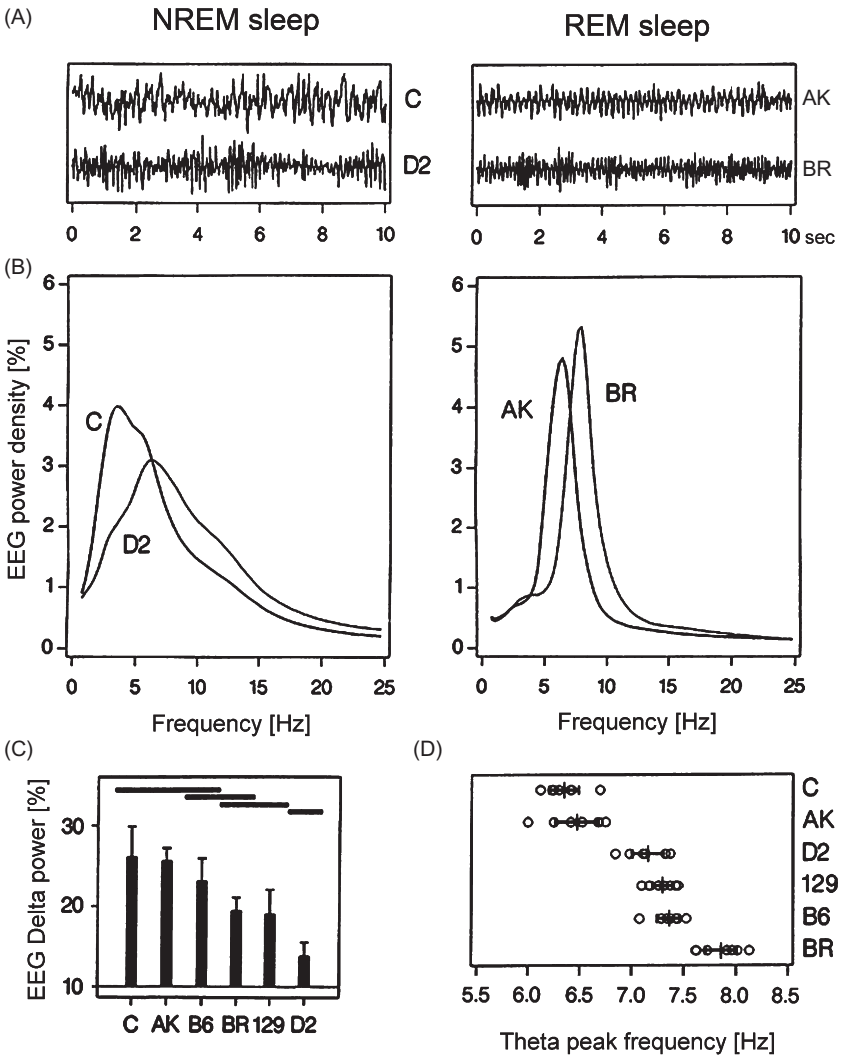
GENETICS OF THE EEG: MOUSE STUDIES

In genetics, the segregation of a trait of interest is followed in recombinant offspring and subsequent mapping aims at identifying the underling genes using polymorphic markers spanning the entire genome. At present, so-called single nucleotide polymorphism (SNP) arrays, which contain several hundred thousands of SNPs, are currently the norm to genotype individual mice or mouse lines and guide mapping of genes or genomic

regions (i.e., quantitative trait loci or QTL) co-segregating with the traits of interest. In such genome-wide genetic screens no *a priori* assumptions are made on the gene systems involved allowing for the discovery of novel genes and gene pathways. QTL analysis is used to genetically dissect complex traits such as sleep and the EEG because it allows mapping of multiple naturally occurring allelic variants or gene mutations each contributing with small effect to the phenotypic variance (Abiola et al., 2003; Darvasi, 1998; E. S. Lander & Botstein, 1989). QTL analysis can be performed in a variety of segregating mouse populations including inter- and backcross and recombinant inbred (RI) strains of mice. Two or more inbred mouse strains differing for the trait of interest 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. RI sets are generated by inbreeding F2 mice until homozygosity, thereby "fixing" a unique set of recombinations in several inbred lines. A promising set of RI lines, the "collaborative cross" (CC), is derived from eight inbred lines of mice in a balanced breeding design (Churchill et al., 2004). Inbreeding and genotyping of several hundred CC lines has been finalized and awaits phenotyping of EEG traits (Philip et al., 2011). With this number of lines, number of variable alleles, and available high-density SNP markers, mapping small QTLs (explaining 5% of the variance) at a 1-cM resolution is thought to be feasible (Valdar, Flint, & Mott, 2006). QTL analyses can also be performed in populations of outbred mice requiring the genotyping and phenotyping of each individual mouse (Yalcin et al., 2010). As such, using outbred mice is equivalent of a GWAS study in humans.

Besides QTL analysis, which aims at identifying naturally occurring allelic variants or gene mutations affecting a trait of interest, in mutagenesis the implication of specific genes in a phenotype is assessed by randomly inducing point-mutations using mutagens like N-ethyl-N-nitrosurea (ENU). 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 feasibility of this approach in the mouse was demonstrated by the isolation of the circadian gene *Clock* (King et al., 1997; Vitaterna et al., 1994). Thus far, no attempts have been made to use this approach for EEG traits although, through phenotyping for abnormal locomotor activity and gait, mutagenesis screens identified a mutation in the gene encoding the neuronal voltage-gated sodium channel *Scn8a* to cause EEG spike-wave discharges (Papale et al., 2009).

Genetic studies of sleep in the mouse were pioneered by Valatx in the early 1970s (Valatx & Bugat, 1974; Valatx, Bugat, & Jouvet, 1972). Initial comparisons of inbred strains of mice revealed that many aspects of sleep greatly differed among strains and, as in humans, heritabilities for sleep durations and its distribution between 40% and 60% were obtained. The EEG spectral profiles also greatly differed among inbred strains. In a panel of six strains of mice we identified large differences in the relative contribution of EEG delta oscillations to the NREM sleep EEG and



in the prevailing frequency of EEG theta oscillations during REM sleep (Franken, Malafosse, & Tafti, 1998) (Fig. 4.2). Like human twin pairs, mice of a particular inbred strain can be considered genetically identical clones that differ from other inbred strains. Unlike humans, mice are amenable to detailed genetic and subsequent functional analysis, and different approaches can, and have been, successfully employed to find genes implicated in sleep and the EEG.

Almost 20 years after Valatx's group collected sleep data in a panel of CXB RI mice, we used these data to report the first QTL sleep study (Tafti et al., 1997). CXB RI mice are derived from the inbred strains BALB/cBy (C) and C57BL/6By (B). Due to the small number of strains (i.e., 7 lines) the results had no power in yielding significant QTLs. Two years later, in a larger CXB RI set consisting of 13 lines, Toth and Williams identified several QTLs related to both NREM and REM sleep duration, none of them significant at a genome-wide level (Toth & Williams, 1999). Subsequent mapping of sleep-related QTLs in a set of BXD RI lines was more successful (Andretic, Franken, & Tafti, 2008). BXD RIs are derived from the inbred strains C57BL/6J (B) and DBA/2J (D). When

Figure 4.2 ***Spectral composition of the sleep EEG is determined by genetic factors.*** *Panels A:* Visual inspection of the EEG signals in a panel of six inbred strains of mice readily identified qualitative differences in the main contributing frequency components; the prevalence and amplitude of EEG slow waves during NREM sleep (left panel) is visibly reduced in DBA/2J (D2) mice as compared to Balb/cByJ (C) mice rendering the EEG "faster" in D2 mice. Also the frequency of the theta oscillations that characterize REM sleep (right panel) is visibly faster ("denser") in C57BR/cdJ (BR) mice as compared to AKR/J (AK) mice. *Panels B:* These qualitative EEG differences can be quantified with spectral analyses [FFT; average spectra for all 4 s epochs scored as NREM sleep (left) or REM sleep (right panel) in a 24 h baseline recording; n = 7/strain] and clearly show the reduction of the contribution of delta activity in D2 mice compared to C mice during NREM sleep and the right shift of the theta peak in BR mice compared to AK mice during REM sleep. *Panel C:* Quantification of the relative contribution of EEG delta power to the EEG spectrum during NREM sleep in all six strains. The horizontal bars on top group strains for which EEG delta power similarly contributed to the NREM sleep EEG. The B-D strain difference led to the mapping of a polymorphism in the promoter of the *Rarb* gene in a panel of BXD recombinant inbred mice (Maret et al., 2005). *Panel D:* Individual and mean (±2SEM) values of theta peak frequency for all six inbred strains. Three significantly distinct groups can be distinguished for which individual theta peak frequency values did not overlap: slow theta (AK and C; <6.8 Hz), fast theta (D2, C57BL/6J (B6), and 129P/Ola (129); 6.8–7.6 Hz), and "very" fast theta (BR; >7.6 Hz) mice. The B-C strain difference led to the mapping in inter- and backcross panels derived from B and C mice of a spontaneous deletion mutation in this C substrain (i.e., Balb/cByJ) in the gene *Acads* (Tafti et al., 2003). *Original data published in Franken et al. (1998).*

we initiated these experiments we had access to 25 BXD RI lines. In the meantime, this resource has been substantially augmented and a total of some 90 lines have become available making this a powerful resource for mapping QTLs with moderate to large effects (Peirce, Lu, Gu, Silver, & Williams, 2004). In the BXD RI set, we identified a genome-wide significant QTL on chromosome 14 for the contribution of EEG delta activity to the NREM sleep EEG. Subsequent haplotype mapping in several unrelated inbred strains and the candidate-gene approach identified retinoic-acid receptor beta (*Rarb*) as the underlying gene thereby implicating retinoic-acid signaling in modulating cortical synchrony during NREM sleep (Maret et al., 2005).

Another example of a successful QTL EEG study concerned the frequency of theta oscillations characteristic of REM sleep. Inbred strains vary greatly for this trait and "slow" and "fast" theta strains could be distinguished with 6.75 Hz as the frequency separating the two groups (Franken, et al., 1998; Tafti et al., 2003) (Fig. 4.2). The segregation of this trait was followed in a panel of inter- and backcrosses between BALB/cByJ, a "slow-theta" strain, and C57BL/6J, a "fast-theta" strain. A single gene was identified on chromosome 5 that was tightly linked to theta frequency. Subsequent fine mapping through selective phenotyping revealed short-chain acyl-coenzyme A dehydrogenase (*Acads*) as the responsible gene. BALB/cByJ mice acquired a deficiency in *Acads* that slows theta (Tafti et al., 2003). The isogenic substrain, BALB/cBy, which does not carry this spontaneous mutation, has "fast" theta oscillations, further confirming *Acads* as the gene causing this EEG trait.

These two examples demonstrate that QTL analysis, as a "genetics" approach, can be successful in identifying signaling pathways previously not thought to play a role in the generation of neuronal oscillations. Retinoic-acid receptors are important in brain development (Jacobs et al., 2006; Lane & Bailey, 2005) further supporting the idea that the EEG spectral profile could be used to index brain maturation. Alternatively, retinoic-acid receptors are implicated in several signaling pathways in the adult brain (Bremner & McCaffery, 2008; Krezel et al., 1998) and, like the *Comt* polymorphism mentioned above (Bodenmann, Rusterholz, et al., 2009), could have effects on EEG delta power through altered dopaminergic neurotransmission (e.g., Dimpfel, 2008; Kitaoka et al., 2007). The role of fatty-acid beta oxidation, in which *Acads* plays a key role, in determining the frequency of hippocampal theta oscillations is also unexpected because this pathway was not thought to be of importance in the adult brain.

Candidate-gene approaches also referred to as "reverse genetics" (i.e., from "genotype-to-phenotype"), are, strictly speaking, not genetic approaches in the sense that they do not allow for the identification of allelic variants or mutations underlying a trait. Since the introduction of the term reverse genetics, genetic approaches have sometimes been dubbed "forward genetics" (i.e., from "phenotype-to-genotype"). In the mouse, reverse genetics entail genetically altering a gene of interest and studying the effects of this intervention on a trait under investigation. Mice can be engineered to either lack a functional gene, overexpress a gene product, have an altered gene construct inserted, or express genes from other species. Extensive use of these techniques has been made in sleep research, which has been adequately reviewed elsewhere (Franken & Tafti, 2003; Wisor & Kilduff, 2005). Most concern genes involved in neurotransmitter signaling, endocrine and para-/autocrine signaling, and ion channels. Not surprisingly, disruption of ion channels can result in striking differences in the EEG spectral profiles as observed in mice lacking the small conductance calcium-activated potassium channel *Kcnn2* (Cueni et al., 2008). Although transgenic animals are mostly used to confirm the implication of existing pathways in sleep and the EEG complementing pharmacological and neuroanatomical approaches, sometimes unexpected results are obtained that lead to new insights. An example of this is the discovery that the transcription factors involved in circadian rhythm generation (or clock genes) also play a role in the homeostatic regulation of sleep (see below).

REGULATION OF SLEEP: TIME-OF-DAY VERSUS TIME-SPENT-AWAKE

Two main processes are generally considered when studying the regulation of sleep: a circadian process that sets internal time-of-day and assures proper entrainment of behavior and physiology to the daily light-dark cycle, and a homeostatic process that tracks and signals the propensity or need for sleep (Borbely, 1982; Daan et al., 1984). In mammals, a self-sustained circadian oscillation is generated in the suprachiasmatic nucleus (SCN) of the hypothalamus (Klein, Moore, & Reppert, 1991), which is considered the master circadian clock. The oscillating output of this clock gives time-context to most physiological processes and behaviors including sleep. Thus, the distribution of sleep over the 24-hour day is strongly determined by the circadian system. The homeostatic process tracks sleep need such that the need for sleep and the propensity to initiate sleep increases

while awake and decreases as a function of time-spent-asleep. The two processes are thought to be generated independently but their interaction determines the timing, duration, and quality of both sleep and wakefulness (Dijk & Franken, 2005). So-called forced-desynchrony protocols have been especially instrumental in quantifying the interaction between the two processes (Dijk & Czeisler, 1994, 1995). These studies demonstrated that the circadian system is more than a device dictating when to sleep; it actively generates a sleep-wake propensity rhythm that is timed to oppose homeostatic changes in sleep drive, enabling us to stay awake and alert throughout the day despite an accumulating need for sleep, and asleep during the night despite a waning of sleep need (Dijk & Franken, 2005).

Of the various aspects of sleep that are homeostatically regulated, EEG delta power during NREM sleep most reliably varies as a function of time awake and asleep (Dijk, Beersma, & Daan, 1987; Franken et al., 2001; Franken, Tobler, & Borbély, 1991; Tobler & Borbely, 1986; Werth, Dijk, Achermann, & Borbely, 1996). In mouse, rat, and human, changes in EEG delta power are so predictable that its time course can be mathematically calculated in detail solely based on the sleep-wake distribution both under baseline conditions and after sleep deprivation (Achermann & Borbely, 2003; Franken et al., 2001; Franken, Tobler, et al., 1991; Huber, Deboer, & Tobler, 2000a) (Fig. 4.3), further supporting the view that changes in EEG delta power mainly reflect a sleep-wake dependent, homeostatic process. Studies in animals rendered arrhythmic by lesioning the SCN or through a shift of the light-dark cycle show that the sleep-deprivation induced increase in EEG delta power is unaffected and thus does not depend on a functioning circadian system (Easton, Meerlo, Bergmann, & Turek, 2004; Larkin, Franken, & Heller, 2002; Trachsel, Edgar, Seidel, Heller, & Dement, 1992). Results from the above-mentioned forced desynchrony experiments in humans confirmed that the circadian contribution to the time course of EEG delta power during NREM sleep is very small albeit not zero (Dijk & Czeisler, 1995). These and other observations let to the established notion that sleep homeostasis and circadian rhythm generation are separate processes.

It should be noted, however, that because changes in EEG delta power are so predictable and salient, often the homeostatic regulation of sleep is equated with the sleep-wake dependent changes in delta power thereby overlooking the homeostatic regulation of other aspects of sleep such as the duration of sleep, especially that of REM sleep. This is also reflected in the prevailing hypotheses concerning sleep function that exclusively

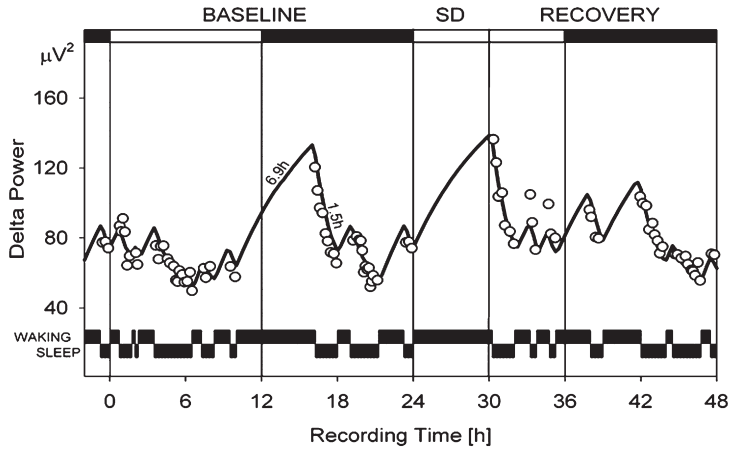


Figure 4.3 ***Changes in EEG delta power during NREM sleep can be accounted for by the sleep-wake distribution and index homeostatic sleep need.*** EEG delta power during NREM sleep depends on the prior sleep-wake distribution in that levels are high after periods of extended wakefulness; the longer the period of waking, the higher subsequent EEG delta power is. During NREM sleep, EEG delta power decreases exponentially. This is illustrated here for one individual male Balb/cByJ mouse. Open symbols depict absolute values of EEG delta power reached in individual NREM sleep episodes (>5 min) during a 24 h baseline recording followed by a 6 h sleep deprivation (SD) starting at light onset, and 18 h of recovery sleep. Both spontaneous (e.g., between 10–16 h) and enforced (24–30 h) periods of wakefulness of similar duration are followed by similar levels of EEG delta power. Note the rapid decline (recovery?) during periods of sleep. These dynamics suggest that EEG delta power reflects a yet to be identified homeostatically regulated recovery process, Process S (Daan et al., 1984). The dynamics of this process can be simulated iteratively by assuming that the need for NREM sleep increases during wakefulness and REM sleep (both states devoid of EEG delta oscillations) and decreases during NREM sleep according to exponential (saturating) functions. The simulated levels of Process S (solid line) predict the observed EEG delta power values in great detail and >80% of the variance in EEG delta power can thus be accounted for by the sleep-wake distribution. Optimal fit to empirical data in this mouse was obtained with 6.9 h and 1.5 h for the time constants of the buildup and decrease, respectively. The time constants quantifying the increase of Process S varied greatly among inbred strains (Franken et al., 2001). The black horizontal bars on top indicate the 12 h dark periods. *Graph reprinted from Franken et al. (2001) with permission.*

concern the dynamics of EEG delta power (Tononi & Cirelli, 2006). A clear dissociation of the homeostatic regulation of sleep amount and that of EEG delta power is illustrated with the following sleep-deprivation experiment carried out in the rat (Franken, Dijk, Tobler, & Borbély, 1991). Twenty-four hours without sleep resulted in the expected and immediate

increase in EEG delta power that quickly subsided over the first 4 hours of the recovery period. The duration of NREM sleep was also increased but this increase lasted for most of the 48 hours for which recovery was monitored. As a result of the increase in NREM sleep time, EEG delta power fell to values below those reached under baseline conditions (i.e., "negative rebound"). Although both the immediate positive and subsequent negative rebound in EEG delta power in this study could be readily explained based on the altered sleep-wake distribution (Franken, Tobler, et al., 1991), the following question presents itself: If EEG delta power indeed reflects homeostatic sleep need then why should animals continue to sleep more compared to baseline when sleep need is below baseline? Equally overlooked are the low predictive value of EEG delta power in modeling the effects of longer-term sleep restrictions on cognitive performance and attention (Van Dongen, Maislin, Mullington, & Dinges, 2003).

While variations in EEG delta power during NREM sleep measured over the day are mostly sleep-wake driven other frequency components of NREM sleep EEG are also influenced by circadian factors. Notably EEG activity in spindle or sigma frequency range reveals, besides a marked sleep-wake dependent variation, an equally marked circadian dependent variation (Dijk & Czeisler, 1995). For the waking EEG, modulation of alpha, theta, and beta activity according to circadian time were reported (Cajochen, Wyatt, Czeisler, & Dijk, 2002). Also in the rat a circadian modulation of EEG activity in specific frequency bands can be observed in all three sleep-wake states (Yasenkov & Deboer, 2011).

GENETICS OF THE HOMEOSTATIC REGULATION OF SLEEP

Comparisons of the sleep-wake dependent dynamics in EEG delta power among six inbred strains of mice revealed that the rate at which homeostatic sleep need increases during wakefulness varied greatly according to genetic background (Franken et al., 2001) (Fig. 4.4). In the BXD panel of RI lines introduced above we followed the segregation of the increase in delta power both after sleep deprivation and after sleep onset under baseline conditions. Both traits yielded overlapping QTLs on chromosome 13 that reached genome-wide significance. We named this QTL *Dps1* (for delta-power-sleep-1) (Fig. 4.4). The *Dps1* QTL is specific for the homeostatic regulation of EEG delta power in that the sleep-deprivation induced rebounds in NREM sleep and in REM sleep duration did not map to this region (our unpublished data). Moreover, the *Dps1* QTL differed from

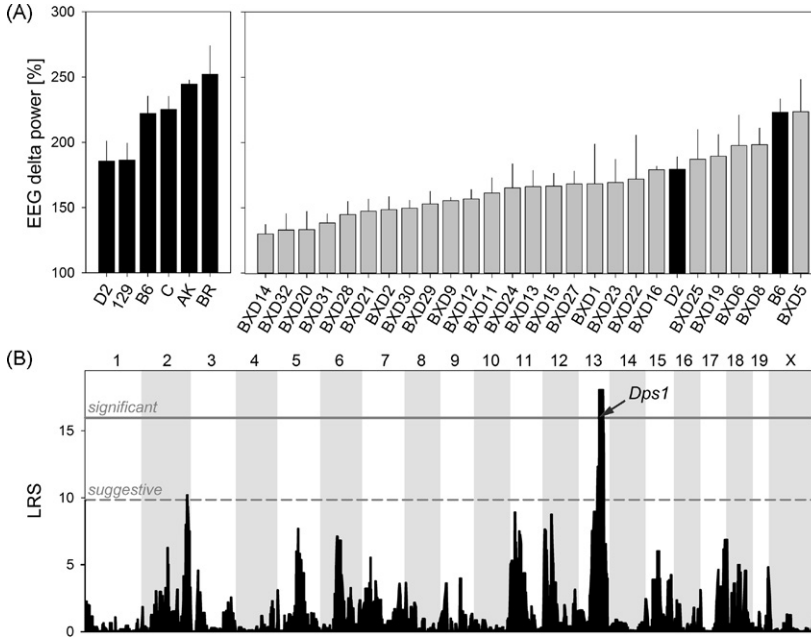


Figure 4.4 ***The homeostatic regulation of NREM sleep EEG delta power is under genetic control.*** *Panels A:* Inbred strains greatly differ for the level of EEG delta power reached after a 6 h sleep deprivation (SD). An initial screen of 6 strains (left panel; n = 7/strain, 10–12 weeks old males) showed that in DBA/2J (D2) and 129P/Ola (129) mice lower EEG delta power values were reached in the first 30 min of recovery sleep after SD as compared to AKR/J (AK) and C57BR/cdJ (BR) mice while in C57BL/6J (B6) and Balb/cByJ (C) intermediary levels were reached. In 25 recombinant inbred (RI) strains of mice derived from the B and D parental strains (i.e., BXD1-31; n = 4–7/strain; right panel) an even larger variability in this increase in EEG delta power after SD was observed surpassing the parental differences (black bars), especially towards lower range values. Values were expressed as % of individual values reached in the last 4 h of the preceding baseline light period (=100%), that is, the time the lowest levels are reached in the baseline. *Panel B:* QTL mapping of the segregation of the EEG delta power level reached after SD in the BXD RI panel (parentals included) yielded two loci; one "suggestive" on chromosome 2 and one "significant" on chromosome 13 (the *Dps1* QTL, see text). Significant and suggestive thresholds (horizontal grey lines) refer to likelihood ratio statistic (LRS) values corresponding to genome-wide probability levels of 5% and 63%, respectively, of falsely rejecting the null hypothesis of no linkage (E. Lander & Kruglyak, 1995). LRS quantifies the association between trait differences and differences in a particular DNA sequence and is calculated as the ratio of the probability of an association versus no association (e.g., an LRS of 3 indicates a 1000 times more likelihood of an association). The *Dps1* QTL explained 49% of the variance in the SD trait suggesting the presence of a "major" gene governing the increase of the homeostatic need for sleep during wakefulness. *Data in panels A taken from Franken, Malafosse, & Tafti (1999) and Franken et al. (2001), respectively. Association data depicted in panel B were generated with the use of WebQTL (www.genenetwork.org; Record ID: 10143).*

the chromosome 14 QTL obtained in the same mice for the contribution of EEG delta power to the NREM sleep EEG (see above under *Rarb*). This indicates that different aspects of the same variable can be governed by different genes. This also indicates that differences in EEG delta power observed among individuals do not necessarily equate to functional differences in the sleep homeostat.

In an extensive set of micro-array analysis experiments we then searched for genes for which mRNA levels in the brain paralleled the sleep-wake dependent changes in EEG delta power; that is, their expression should decrease (or increase; we did not impose an *a priori* direction-of-change as long as changes were consistent with the sleep-wake distribution) over the course of the light period when mice predominantly sleep, and increase during the active or dark period. In addition, these changes in gene expression should be increased with sleep deprivation dependent on the duration of the deprivation but independent of the time-of-day the deprivation was performed. Finally, changes in expression should be genotype dependent such that inbred strains that responded with a larger increase in EEG delta power after sleep deprivation should also have a larger increase in mRNA. The transcript *Homer1a* matched all these requirements (Maret et al., 2007) (Fig. 4.5). Of great interest is that *Homer1a* is also the best candidate gene in the *Dps1* region (Mackiewicz, Paigen, Naidoo, & Pack, 2008; Maret et al., 2007). Formal genetic proof confirming the involvement of this isoform of *Homer1* in the *Dps1* QTL could come from mice lacking *Homer1a*, specifically. Interest in *Homer1a* comes from its role in homeostatic synaptic scaling (Hu et al., 2010) and neuroprotection (Szumlinski, Kalivas, & Worley, 2006), both suggested as possible functions of sleep (Mongrain et al., 2010; Tononi & Cirelli, 2006).

This example demonstrates that only the combination of several approaches can yield new and meaningful insights. The combination of genome, transcriptome, and phenotypic data at multiple levels of organization to understand complex traits has been referred to as "systems genetics." With respect to sleep such approaches have only now begun to be implemented (Millstein et al., 2011; Winrow et al., 2009).

A NON-CIRCADIAN ROLE FOR CLOCK GENES IN SLEEP HOMEOSTASIS

Although sleep homeostasis and circadian rhythm generation are considered separate processes (see above), several examples indicate the presence

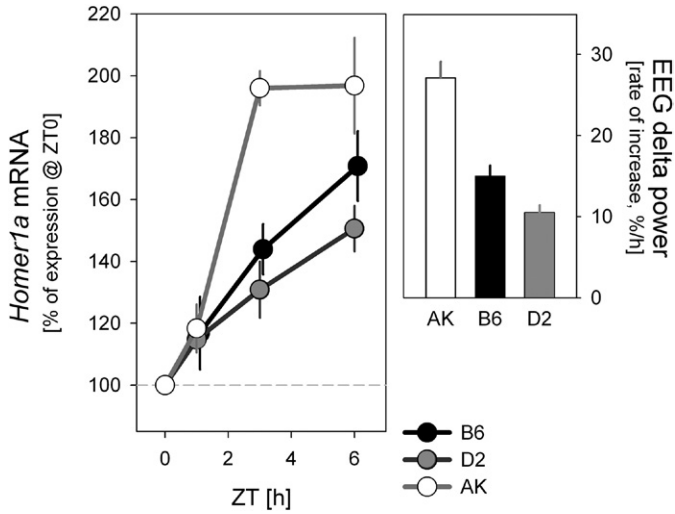


Figure 4.5 ***The genotype specific rate of increase in Homer1a expression matched the dynamics of EEG delta power.*** *Left panel:* The time course of *Homer1a* mRNA levels in the forebrain of male mice of three inbred strains after 1, 3, and 6 h of sleep deprivation starting at light onset (0 h; n = 4/strain/time-point). *Homer1a* expression in AKR/J (AK) mice accumulated at a faster rate than in C57BL/6J (B6) and DBA2/J (D2) mice. In AK mice after 3 h of sleep deprivation maximal levels of mRNA were already reached while in the remaining two strains this increase was more gradual reaching lower levels. *Right panel:* These strain differences in the increase rate of *Homer1a* match the strain specific increase rate in EEG delta power during wakefulness (n = 7/strain). The sleep-wake dependent changes in EEG delta power during NREM sleep are thought to reflect changes in sleep need, thereby implying that *Homer1a*, which is localized within the *Dps1* QTL (Fig. 4.4), is, at least, a reliable correlate of homeostatic sleep need. *Data taken from Franken et al. (2001) and Maret et al. (2007). Figure reprinted from Andretic et al. (2008), with permission.*

of at least some "cross talk" between the two; for example, sleep deprivation is able to phase shift the circadian clock in hamsters (Antle & Mistlberger, 2000) and firing rates of SCN neurons decrease during NREM sleep and are negatively correlated with the levels of EEG delta power attained during this state (Deboer, Detari, & Meijer, 2007; Deboer, Vansteensel, Detari, & Meijer, 2003). Our finding that circadian clock genes play a role in sleep homeostasis further obscures this distinction and suggest that the same molecular circuitry used to set internal time-of-day might be equally utilized to track and anticipate sleep need (Franken & Dijk, 2009).

Mutagenesis screens in *Drosophila* and the mouse identified the genes *Period* and *Clock*, respectively (King et al., 1997; Konopka & Benzer, 1971; Reddy et al., 1984; Vitaterna et al., 1994). Subsequent studies revealed that these transcriptional regulators engage in molecular negative feedback loops that are thought to underlie circadian rhythm generation at the cellular level (Lowrey & Takahashi, 2004). The core of this self-sustained molecular oscillation in mammals consists of the positive elements *Clock*, *Npas2*, and *Bmal1* and of the negative elements *Period1* and *-2* (*Per1, Per2*) and *Cryptochrome1* and *-2* (*Cry1, Cry2*). BMAL1 can form transcriptionally active heterodimers by partnering with either CLOCK or NPAS2. Both dimers initiate the transcription of the *Per* and *Cry* genes. After translation and reentry into the cell nucleus, PER and CRY protein complexes suppress CLOCK:BMAL1- and NPAS2:BMAL1-mediated transcription thus providing the negative feedback enabling the initiation of a new cycle of transcription-translation. The involvement of these clock genes in circadian rhythm generation has been demonstrated by constructing mice carrying targeted disruptions for one or a combination of these genes. Thus mice lacking *Bmal1*, *Clock* and *Npas2*, *Cry1* and *Cry1*, or *Per1* and *Per2* all lack circadian organization of overt behavioral rhythms when kept under constant dark conditions (Lowrey & Takahashi, 2004).

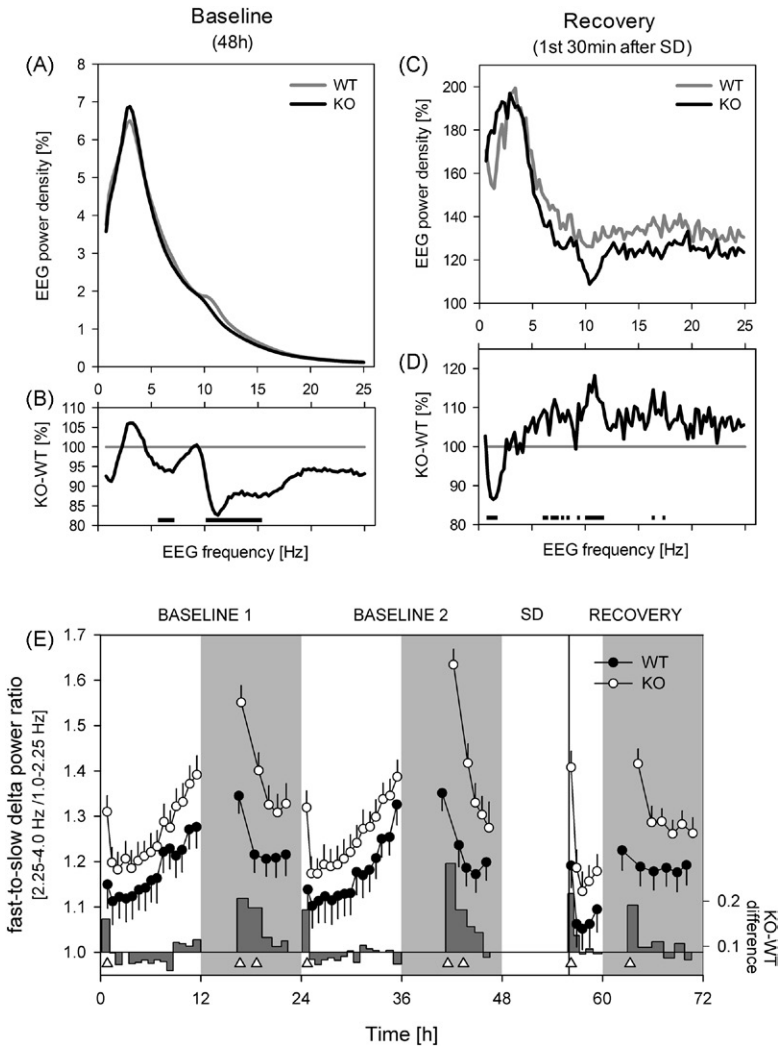
In an attempt to further establish the independence of circadian and sleep homeostatic processes we examined sleep in *Cry1,2* knockout mice (*Cry1,2*$^{-/-}$) (Wisor et al., 2002). As expected, these mice are behaviorally arrhythmic under constant conditions (van der Horst et al., 1999; Vitaterna et al., 1999). Besides this anticipated circadian phenotype these mice also had an unexpected and distinct homeostatic phenotype with more time spent in NREM sleep, more consolidated sleep, and higher EEG delta power during baseline while the rebound in EEG delta power after sleep deprivation was attenuated (Wisor et al., 2002). Aberrant sleep homeostatic phenotypes have been observed for other mouse lines carrying targeted disruptions for one or two clock genes. Mice homozygous for the *Bmal1* deletion showed increases in total sleep time, sleep fragmentation, and EEG delta power under baseline conditions, and an attenuated compensatory response to sleep deprivation (Laposky et al., 2005). For *Clock* mutant mice decreases in NREM sleep time and consolidation were reported under baseline conditions (Naylor et al., 2000). Mice lacking *Npas2* slept less during baseline and the compensatory response in NREM sleep time and EEG delta power (0.75–2.0 Hz) after an 8 hour sleep deprivation was reduced (Franken et al., 2006) (Fig. 4.6). Sleep regulation was also assessed

in mice lacking *Per1* and *Per2* although results were inconclusive; single *Per1* and *Per2* knock-out mice showed a smaller increase in EEG delta power after sleep deprivation (Kopp, Albrecht, Zheng, & Tobler, 2002), while in *Per1,2* double mutant mice EEG delta power seemed enhanced to a greater extent after sleep deprivation compared to wild-type mice (Shiromani et al., 2004). Evidence of clock genes being implicated in the homeostatic regulation of sleep was also found in *Drosophila* (Hendricks et al., 2003; Hendricks et al., 2001; Shaw, Tononi, Greenspan, & Robinson, 2002) and in humans (Viola et al., 2007) pointing to a possible evolutionary conserved pathway.

Another type of observation in support of a role for clock genes in sleep homeostasis is that in the forebrain expression of both *Per1* and *Per2* is increased after enforced waking (Franken et al., 2006; Franken, Thomason, Heller, & O'Hara, 2007; Maret et al., 2007; Mongrain et al., 2010; Wisor et al., 2002). *Per1* and *Per2* expression increased linearly as a function of the duration of the time mice were kept awake (Franken et al., 2007) and one study reported a positive correlation between the mRNA changes in *Per1* and *Per2* and EEG delta power (Wisor et al., 2008). High forebrain levels of *Per* expression seem also to be associated with increased sleep need under baseline conditions and under conditions where the sleep-wake distribution was altered (Abe, Honma, Namihira, Masubuchi, & Honma, 2001; Abe et al., 2001; Dudley et al., 2003; Masubuchi et al., 2000; Mrosovsky, Edelstein, Hastings, & Maywood, 2001; Reick, Garcia, Dudley, & McKnight, 2001; Wakamatsu et al., 2001).

In situ hybridization studies revealed that *Per1* and *Per2* expression after sleep deprivation was affected the most in brain areas where the delta as well as sigma/spindle oscillations characteristic of the NREM sleep EEG are generated, that is, the cortex and thalamus (Franken, et al., 2007; Steriade, 2003; Wisor et al., 2008). NPAS2, which seems important for coupling *Per2* expression to the sleep-wake distribution (Franken et al., 2006; Reick et al., 2001), is abundantly expressed in these areas (Zhou et al., 1997) (Fig. 4.7). The EEG during NREM sleep in *Npas2*$^{-/-}$ mice displayed an overall reduction in sigma power (Fig. 4.6). Also EEG activity in delta frequency range was affected with a shift from activity in the slow delta frequencies (1.0–2.25 Hz) to faster delta frequencies (2.25–4.0 Hz), which was especially pronounced during NREM sleep immediately following a prolonged period including an 8 hour sleep deprivation (Franken et al., 2006) (Fig. 4.6). Other studies also reported that slow and fast delta oscillations were differentially modulated by prolonged waking (Deboer, Fontana, &

Tobler, 2002; Huber, Deboer, & Tobler, 2000b). One type of oscillation that contributes to the activity in the delta frequency range originates from thalamocortical neurons (Amzica & Steriade, 1998). When the membrane potential of these neurons reaches levels of hyperpolarization characteristic of deep NREM sleep (stages 3 and 4 in humans, i.e., slow-wave sleep), their frequency becomes faster and their contribution to delta activity at the level of the EEG greater (Amzica & Steriade, 1998; Dossi, Nunez, & Steriade, 1992). This could have contributed to the transient shift to faster delta frequencies immediately after long periods of wakefulness, because



NREM sleep is then deepest, and hyperpolarization greatest (Dossi et al., 1992). Following this conjecture, the higher fast-to-slow delta power ratio in *Npas2*$^{-/-}$ mice suggests that membrane potential of thalamocortical neurons during NREM sleep, on average, is more hyperpolarized, which is consistent with the reduction in sleep spindles that predominantly occur at intermediate levels of membrane hyperpolarization (Steriade, McCormick, & Sejnowski, 1993). These observations suggest an unexpected role for a circadian transcription factor in the generation of EEG rhythms of thalamocortical origin. The mechanisms through which this NPAS2 affects thalamocortical and corticocortical activity deserve further investigation. These analyses also underscore that activity in the delta frequencies does not uniformly respond to prior wake duration. In addition, NPAS2 might play a role in postnatal thalamocortical developmental; *Npas2* is expressed first after postnatal week 1 (Zhou et al., 1997), immediately preceding the time at which, in rats, the first slow waves and spindles appear (Davis, Frank, & Heller, 1999).

Figure 4.6 ***The circadian clock gene NPAS2 modulates thalamocortical oscillations.*** *Panel A*: Mean spectral EEG profiles calculated over two baseline days (48 hours) in *Npas2*$^{-/-}$ (KO; n = 19) and wild-type (WT; n = 19) control mice averaged for all 4-second epochs scored as NREM sleep. EEG spectra were normalized to total EEG power. *Panel B*: Spectral differences as percent change for KO (black line) versus WT (grey line) mice. The largest genotype effect was observed in the spindle frequency range (11–15 Hz; significant differences indicated by the horizontal black bar). The concomitant decrease in EEG power in slow delta frequencies (1.0–2.5 Hz) and the increase in fast delta frequencies (2.5–4.0 Hz) resulted in a significant shift in power within the delta band towards faster frequencies (see panel E). *Panel C*: Spectral changes in the NREM sleep EEG in the first 30 minutes of recovery sleep after an 8-hour sleep deprivation (SD). Significant increases in EEG power were observed in both genotypes over a wide frequency range especially prominent in the delta frequencies. Values expressed as % of corresponding baseline values (=100%). *Panel D:* Genotype differences for the increase in EEG power during NREM sleep after SD. KO mice displayed a smaller increase in the slow delta frequencies specifically, while the fast delta activity did not differ thereby even further augmenting the fast-to-slow delta power ratio (see panel E). The increase in theta and low sigma activity was larger in KO mice. *Panel E:* Genotype- and time-dependent changes in the fast-to-slow delta power ratio during NREM sleep. KO values were, in general, higher than WT and ratios were high immediately after a period of sustained wakefulness (i.e., first values of the light period, first value of the sleep episode during the dark (i.e., the "nap"), and first value after SD). At these sleep-onset times the ratio was further increased in KO mice (dark grey areas) and genotypic differences became significant (triangles). Light-grey areas denote 12-hour dark periods. *Data in panels A–D were taken from Franken et al. (2006). Panel E reprinted from Supplementary data (Franken et al., 2006), with permission.*

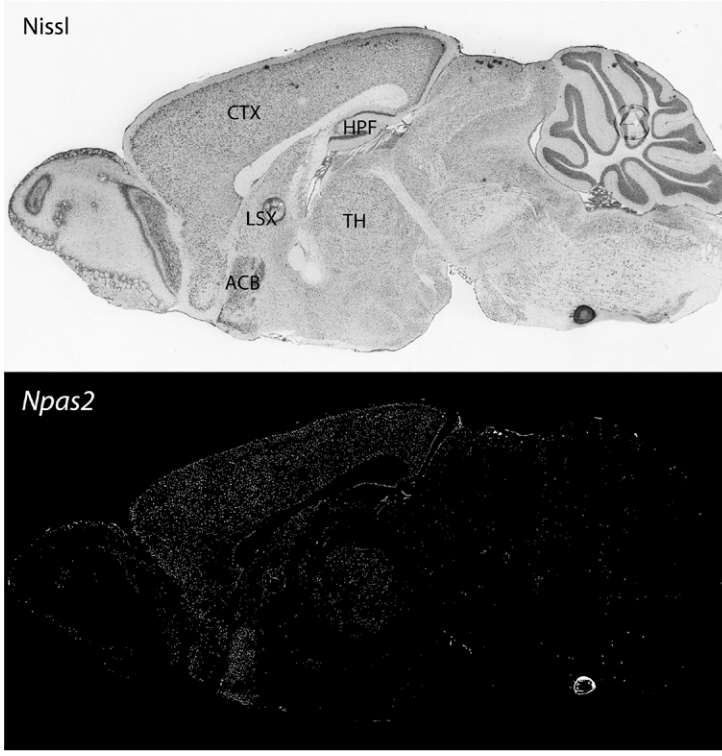


Figure 4.7 Npas2 ***in the mouse brain is most abundantly expressed in the cerebral cortex.*** *Upper panel:* Nissl stain for a sagittal section of the brain of a male C57Bl/6J mouse aged 8 weeks. *Lower panel: In situ* hybridization for *Npas2* in the same section. Expression was limited to the forebrain and was most abundant in the neocortex, nucleus accumbens, and thalamus confirming earlier work (Garcia et al., 2000; Zhou et al., 1997). Abbreviations: HPF = Hippocampal formation, LSX = Lateral septal complex, ACB = Nucleus accumbens, CTX = cerebral cortex (neocortex), TH = thalamus. *Images (ID: 70593327) were downloaded from the Allan brain atlas (www.brain-map.org) with permission.*

Twin studies in humans and comparisons of inbred strains of mice, candidate-gene studies in humans for natural occurring polymorphisms and in mice carrying genetically engineered allele constructs, and QTL and linkage studies all demonstrate that the spectral composition of the EEG both during sleep and wakefulness greatly depend on genetic factors. These factors are likely to underlie the surprisingly stable EEG spectra within an individual over time (EEG "fingerprint"). The genetically determined differences in rhythmic brain activity could be related to the

genetic programs in place for "wiring" the brain, that is, guiding functional neuronal connections during development and maturation (Buchmann et al., 2011; Mitchell, 2007). Genetically determined EEG differences need not be functional in the context of sleep-wake regulation in that, for example, differences in EEG delta power are not necessarily related to altered homeostatic regulation of sleep as was illustrated in a panel of RI mouse where one QTL affected the level of EEG delta power while another modulated the dynamics of the sleep-wake dependent changes in EEG delta power (Franken et al., 2001; Maret et al., 2005). Results of forward, reverse, and molecular genetic approaches applied to sleep homeostasis have contributed to hypotheses on sleep function as illustrated for *Homer1a* (Maret et al., 2007) and for clock genes (Franken & Dijk, 2009). This integrated or systems genetics approach will be instrumental in further unraveling the genes and gene pathways shaping the EEG in the mouse, human, and, possibly, *Drosophila* (Marley & Baines, 2011; Nitz, van Swinderen, Tononi, & Greenspan, 2002).

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