AN INTEGRATED SOFTWARE SYSTEM FOR EEG/EMG-BASED FORWARD GENETIC SCREEN OF SLEEP/WAKE ABNORMALITIES IN ENU-MUTAGENIZED MICE

APPROVED BY SUPERVISORY COMMITTEE

Masashi Yanagisawa, M.D., Ph.D.

Benjamin Tu, Ph.D.

Joseph Takahashi, Ph.D.

Robert Greene, M.D., Ph.D.

Dedicate to my parents, my wife, and my daughter.

AN INTEGRATED SOFTWARE SYSTEM FOR EEG/EMG-BASED FORWARD GENETIC SCREEN OF SLEEP/WAKE ABNORMALITIES IN ENU-MUTAGENIZED MICE

by

MAKITO SATO

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

May, 2014

ACKNOWLEDGEMENTS

First of all, I would like to thank my advisor, Dr. Masashi Yanagisawa for outstanding mentoring and for enormous support and understanding. He always thoughtfully considered my opinions and gave me extraordinary freedom to pursue this project. Also, I would thank the members of my dissertation committee: Dr. Benjamin Tu, Dr. Joseph Takahashi, and Dr. Robert Greene, for their guidance and encouragement.

I would also like to thank all the Yanagisawa lab members from past to present: Toshiyuki Motoike, Hidetoshi Kumagai, Yuichi Ikeda, Ayako Suzuki, and Taizo Matsuki have taught me various experimental techniques and scientific way of thinking. Inik Chang, Alex Chang, Abdullah Shaito, Allen Tsai, and Ivan Lee have provided me a clear guidance on how to survive as a graduate student. Stephanie Baldock and Shelley Dixon have seamlessly orchestrated our lab like concertmistresses to let us fix our eyes on science. Amber Skach has performed many histology works for our previous publications. And, last but not least, Kathleen Godwin, Marcus Thornton, Randal Floyd, Manabu Murakami, Ummay Marriam, Yu Ogawa, Nina Yangisawa, John DuBose, and Kim Trang have worked directly with me to tackle this big project. Even though this project would not go along smoothly without any one of them, I would like to express my special thanks to Kathleen and Marcus for their continuous efforts to perform our experiments with wonderful teamwork.

I would further like to thank all the ENU-group members at Yanagisawa lab in the University of Tsukuba, especially, Hiromasa Funato, Chika Miyoshi, Kei Taniguchi and Yuto Goto for their kind support and beautiful management to conduct the large-scale screening overseas.

Finally, I would like to express my special gratitude to friends and families for their continuous encouragement and support. I would particularly thank Mio Yanagisawa for proofreading my dissertation, Yasu Morita for allowing me, then a high school kid, to see his exciting biomedical research more than a decade ago at Johns Hopkins, and Hiromi Yanagisawa for inviting my families to the Yanagisawas almost every week for a delicious dinner. And, at the end of this acknowledgement, I would like to express my deepest gratitude to my parents and my wife for their unconditional love and unceasing prayers that gave me the strength and motivation to pursue my dreams.

AN INTEGRATED SOFTWARE SYSTEM FOR EEG/EMG-BASED FORWARD GENETIC SCREEN OF SLEEP/WAKE ABNORMALITIES IN ENU-MUTAGENIZED MICE

Publication No._____

MAKITO SATO

The University of Texas Southwestern Medical Center at Dallas, 2014

Supervising Professor: Masashi Yanagisawa, M.D., Ph.D.

The executive neural circuitry and chemistry for sleep/wake switching mechanisms have been increasingly revealed in recent years. However, the very fundamental mechanism of sleep regulation remains a mystery, for example, with the question of what is the neural substrate for "sleepiness" still unanswered. My project tackles this challenging but highly interesting question through forward genetics in mice. We have initiated a dominant screen of ENU-mutagenized mice, in which a comprehensive set of sleep parameters is measured via EEG/EMG-based somnography analysis in basal light/dark periods as well as in the recovery period following forced sleep deprivation. A major obstacle for such large-scale genetic studies of sleep/wakefulness in mice has been the low throughput of EEG/EMG-based sleep analysis. In

order to break this bottleneck, I have developed an automated sleep-scoring program, which adapts itself to the EEG/EMG variability between and within individual mice through a simple pattern-matching algorithm. This new software, combined with highly streamlined surgical procedures as well as a custom database software for administering large-scale experimental logistics, have enabled us to sustain a throughput of up to 112 mice fully sleep-scored per week. Importantly, quantitative parameters of sleep behavior provide relative standard deviations as small as 5-10% in isogenic cohorts of mice, enabling a robust screen. We have so far screened ~7,000 ENU-mutagenized mice, and established >10 heritable phenodeviant pedigrees, including one exhibiting markedly increased NREM sleep amounts, and one with reduced episode durations and amounts of REM sleep. The causal mutations for these two pedigrees have been positionally cloned through the whole-exome sequencing in combination with a classical linkage analysis.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

Ikeda Y, Kumagai H, Skach A, **Sato M**, and Yanagisawa M. Modulation of Circadian Glucocorticoid Oscillation via Adrenal Opioid-CXCR7 Signaling Alters Emotional Behavior. Cell. 2013 Dec 5;155(6):1323-36.

*Funato, H., ***Sato, M.**, Sinton, C. M., Gautron, L., Williams, S. C., Skach, A., Elmquist, J. K., Skoultchi, A. I., & Yanagisawa, M. Loss of Goosecoid-like and Dgcr14 in interpeduncular nucleus results in altered regulation of REM sleep. Proc. Natl. Acad. Sci. USA, 2010 Oct 19;107(42):18155-60. Epub 2010 Oct 4.

*These authors contributed equally to the work.

LIST OF FIGURES

LIST OF TABLES

LIST OF DEFINITIONS

5-HT	Serotonin
ACh	Acetylcholine
BF	Basal forebrain
cM	centi-morgan
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DA	Dopamine
DAT	Dopamine transporter
DEC2	Differentially expressed in chondrocytes protein 2
Dps1	Delta power in SWS QTL 1
EEG	Electroencephalography
EMG	Electromyography
EOG	Electrooculography
ENU	N-ethyl-N-nitrosourea
fmn	fumin
GABA	γ-aminobutyric acid
GB	Giga-bytes
GSCL	Goosecoid-like
GUI	Graphical User Interface
His	Histamine
IVF	In Vitro Fertilization
LC	Locus coeruleus

LDT	Laterodorsal tegmental nucleus
LOD	Logarithm of the odds
MB	Mega-bytes
mns	minisleep
MUSDB	Mutagenesis Universal Support Database
NA	Noradrenaline
NREM	Non-rapid-eye-movement
ORX	Orexin
PPT	Pedunculopontine nucleus
QTL	Quantitative trait loci
REM	Rapid-eye-movement
RSD	Relative standard deviation
SD	Standard deviation
SNP	Single-nucleotide polymorphism
sss	sleepless
TMN	Tuberomammillary nucleus
VLPO	Ventrolateral preoptic nucleus
vPAG	Ventral periaquductal gray
WT	Wild type
ZFN	Zinc finger nuclease

CHAPTER ONE

INTRODUCTION

Historical background

Why do we have to sleep? What is the neural substrate for "sleepiness"? How do we have dreams? We are still unable to give clear answers to these simple questions, yet an estimated 50 million plus Americans suffer from sleep problems (NHLBI, 2003), and nearly 9 million adults take prescription sleep aid in the United States (Chong et al., 2013). In fact, sleep is a mysterious behavior also in terms of its evolution, since sleep is the only behavioral state when a healthy animal reversibly loses consciousness; during sleep, the animal is completely unproductive and highly vulnerable to various hazards. Nevertheless, in mammals, there is no species known that does not sleep for at least a few hours a day. Sleep has to provide some significant evolutionary advantage surpassing these risks, but there is no convincing explanation as to what is that advantage.

For a long period of time, sleep, a ubiquitously-observed behavior in animal species with a well-developed central nervous system, had been considered as a passive state with diminished brain activities and abandoned sensory stimuli. In the late 1940s, Moruzzi and Magoun offered new insight into sleep research through the observation that a sleeping cat became awaken when electrical stimulation was applied on the reticular formation in brain stem, while damage to the reticular formation made the cat continuously sleep, suggesting that the reticular formation is a central regulator of sleep/wake behavior (Moruzzi and Magoun, 1949; Starzl et al., 1951).

In 1953, Kleitman and his colleagues introduced the following observations: 1) Sleep comprises two distinct phases, a state with rapid eye movement (REM sleep) and one with non-rapid eye movement (NREM sleep). 2) Strong electroencephalographic activation, of which the characteristic is similar to wakefulness, is observed during REM sleep (Aserinsky and Kleitman, 1953). These findings are some of the biggest discoveries in the history of sleep research, drastically impacting the fundamental concept of sleep.

As the idea of the active regulation of sleep/wake became widely accepted, more detailed neural mechanisms have been gradually revealed, especially with the advancements of molecular biology in late 20th century. For example, the monoaminergic (catecholaminergic, serotonergic, histaminergic) systems (Aston-Jones and Bloom, 1981; Fornal et al., 1985; Steininger et al., 1999; Ko et al., 2003; John et al., 2004), the cholinergic system (Lee et al., 2005), and the GABAergic system (Sherin et al., 1998; Chamberlin et al., 2003) were found to be closely yet complexly linked to each other to modulate sleep, mainly in the brain stem and hypothalamic regions (Figure 1-2).

Our group has contributed to the field by finding an additional, important modulator of sleep. We identified the orexin neuropeptides, exclusively expressed in the lateral hypothalamus (Sakurai et al., 1998). We subsequently discovered their essential function in sleep/wake regulation through the observation that the orexin-deficient mice exhibited multiple behavioral arrests, which were similar to the sleep attacks and cataplexy observed in patients with narcolepsy (Chemelli et al., 1999). Indeed, electroencephalography (EEG) and electromyography (EMG)-based studies unveiled that the orexin-deficient mice frequently exhibit abnormal, direct transitions from wakefulness to REM sleep.

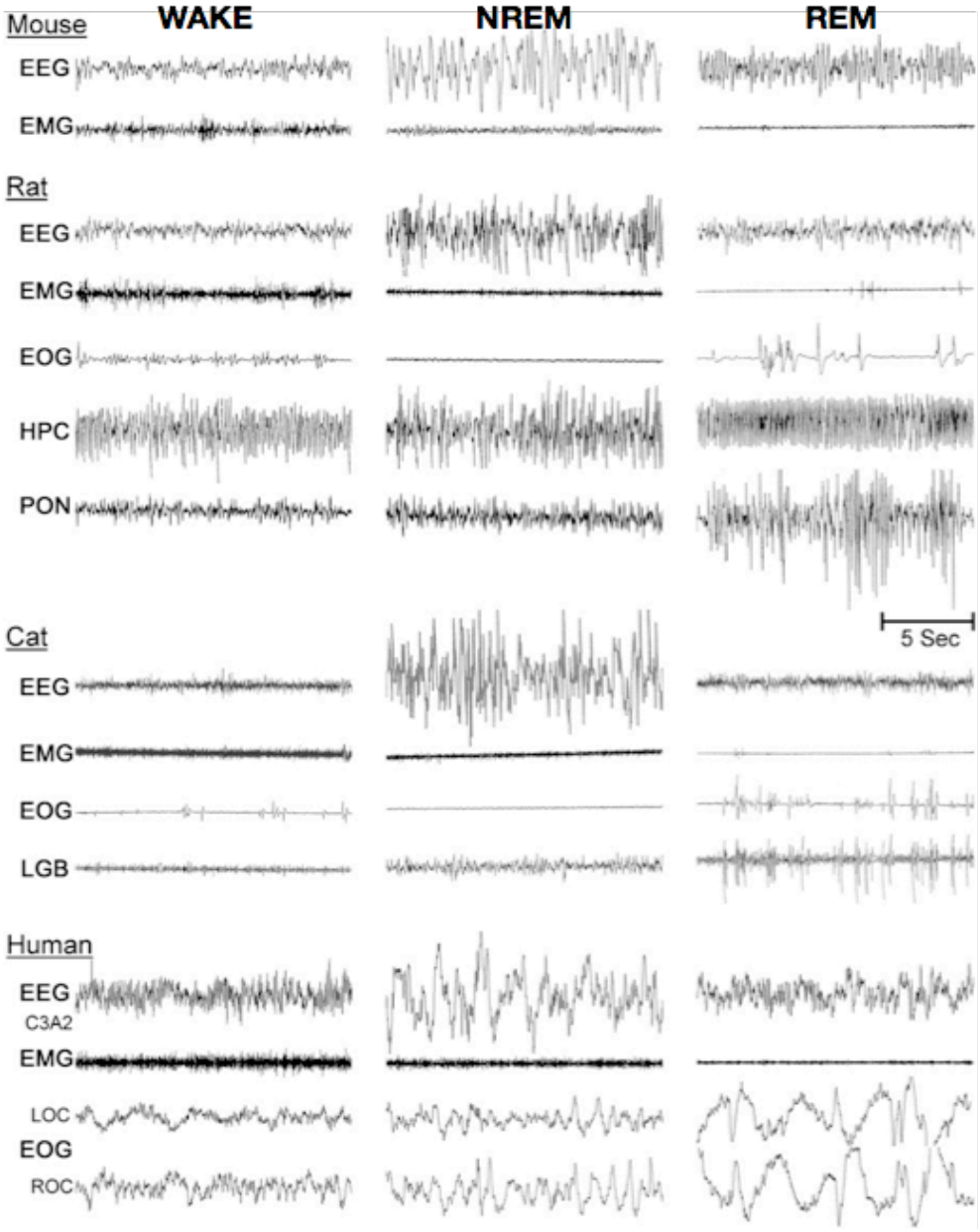


Figure 1-1 Characteristics of polysomnographic signals in different mammalian species. In humans, vigorous eye movements are observed during REM sleep as seen in EOG signals. During NREM sleep, a low-frequency component of EEG, delta waves, becomes dominant, while EEG oscillates faster with high theta waves during REM sleep in all mammalian species shown in this figure. (Adapted from Datta and Maclean, 2007)

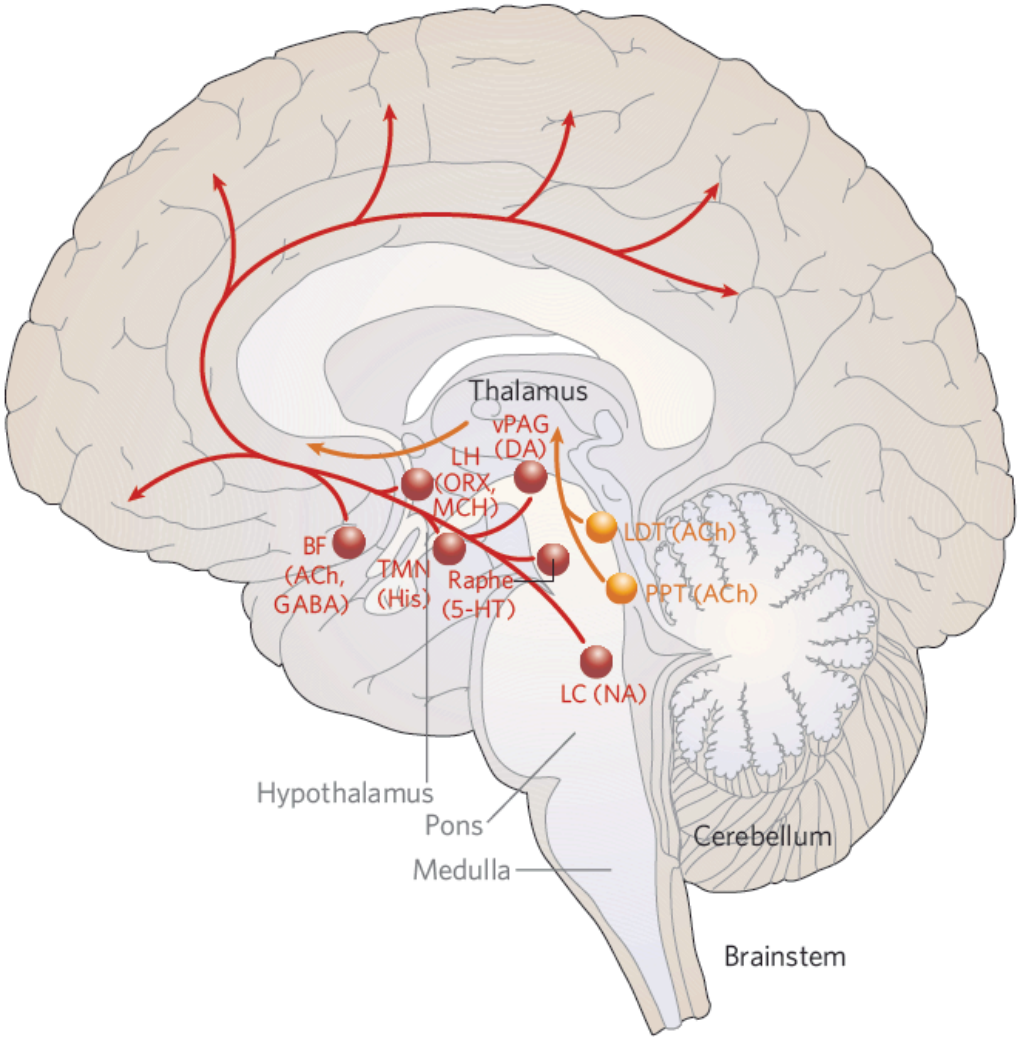


Figure 1-2 Major components of the ascending arousal system. Many different transmitters and modulators play a role in multiple pathways from the midbrain to the cortex via hypothalamic or thalamic regions. (Adapted from Saper et al., 2005)

Ascending projections of orexin neurons to the cerebral cortex as well as descending projections to the monoaminergic and cholinergic systems were also described (Peyron et al., 1998; Sakurai et al., 2005). Their role in sleep/wake regulation has been summarized as an indispensable, stabilizing and wake-inducing component of the flip-flop switching mechanism for sleep and wakefulness (Figure 1-3) (Saper et al., 2005).

While the executive neural circuitry for switching sleep/wake states has been extensively studied in recent years, more fundamental questions of sleep regulation have remained to be elucidated. A widely accepted hypothetical model of sleep regulation is the "two process" model, proposed originally by Borbély in 1982, which argues that two distinct components of "sleepiness" are at work: circadian and homeostatic (Borbély, 1982; Borbély and Achermann, 1999). While "process C," which is inverse of the circadian sleep drive, oscillates each day informing usual sleep time, the latter homeostatic sleep drive, or "process S," is increased during waking and dissipated during sleep (Figure 1-4). Indeed, the cortical EEG recording revealed that the amplitude of lower frequency region of EEG from 0.5 to ~5Hz during NREM sleep, called delta power, correlates with the process S as accumulating after sleep deprivation and quickly decreasing after the sleep onset. The delta power has been the only widely accepted surrogate readout available to measure subject's homeostatic sleep drive (Borbély, 1982; Franken et al., 2001; Allada and Siegel, 2008; Bjorness et al., 2009).

If we could answer what the neural substrate for homeostatic sleep drive is, or how the brain maintain relatively constant daily amounts of sleep, we may be able to open the black box of sleep mechanisms and eventually provide a significant benefit to our sleep-deficient society.

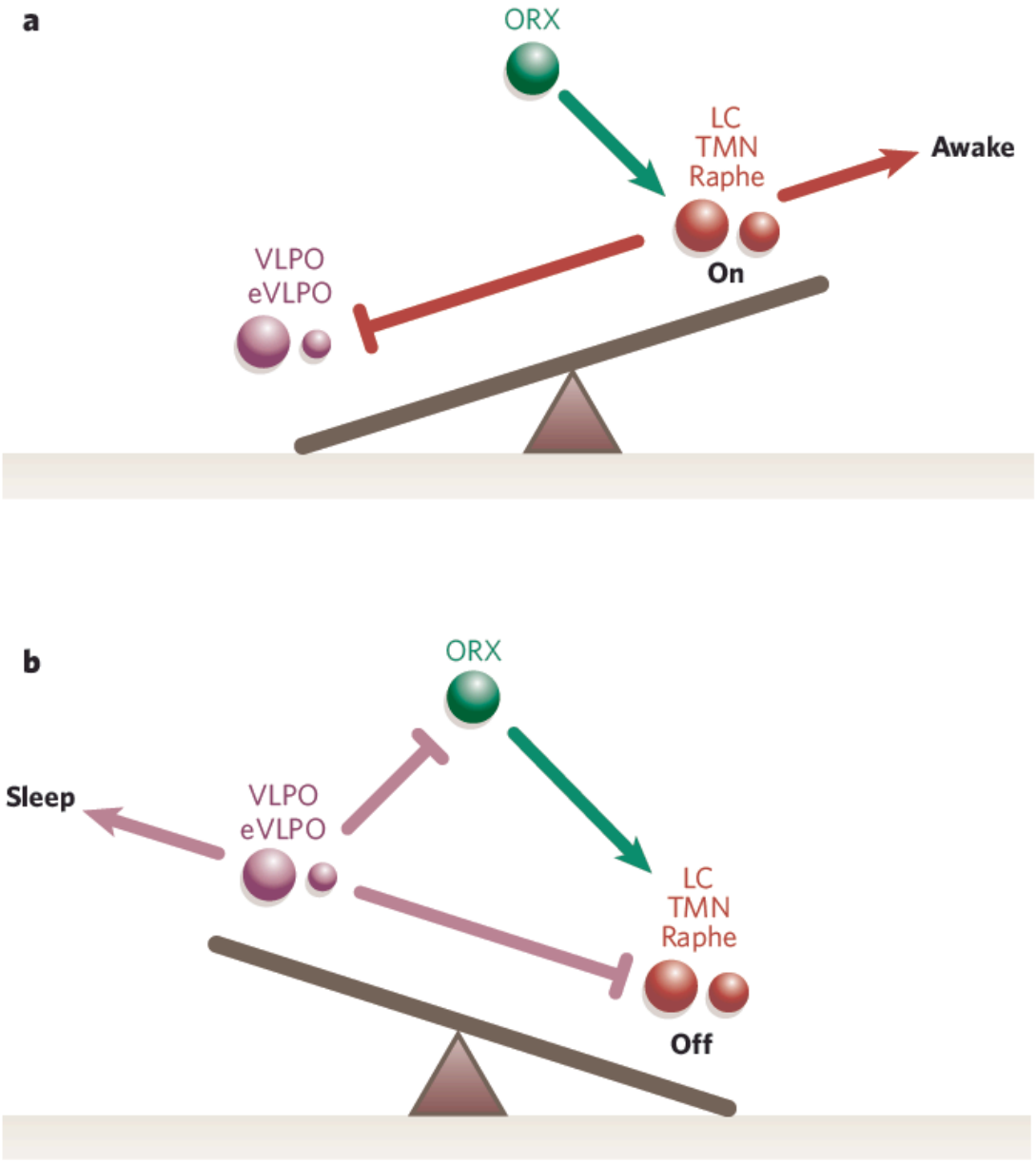


Figure 1-3 A flip-flop switch model for sleep/wake switching. During wakefulness (a), orexin neurons activate the monoaminergic neurons in the LC, TMN, and Raphe nuclei, w hich in turn inhibit the sleep-active VLPO neurons. During sleep (b), the VLPO neurons inhibit orexin neurons, as well as neurons in LC, TMN, and Raphe to prevent activation of the monoaminergic systems. (Adapted from Saper et al., 2005)

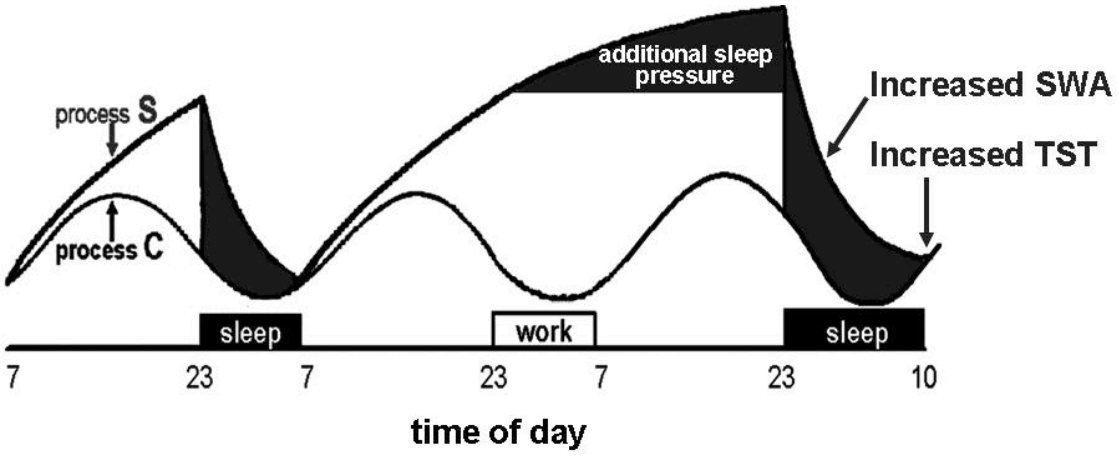


Figure 1-4 Two-process model of sleep regulation. The figure represents scenario for humans. Processes S and C represent the hypothetical degrees of the homeostatic and circadian sleep drive, respectively. The individual's sleep drive at a given time is represented by the difference between process S and process C; the sleep drive reaches maximum at the beginning of the sleep period at a regular night. Additional sleep pressure accumulates as process S further increases after a night without sleep, and the ensuing recovery sleep is longer and "deeper" than usual in order to repay the sleep debt. SWA, EEG slow wave activity (delta power); TST, total sleep time. (Adapted from http://www.scholarpedia.org/article/File:2-process.JPG and Borbely AA, 1982)

Forward genetics of high-order behaviors in mice

In conjunction with the development of techniques in molecular biology, genetic analysis also attracted attention as a potent tool for elucidating unknown biological mechanisms in multiple model organisms, especially when combined with the whole-genome analyses. Among the genetic analyses, forward genetics utilizing drug-induced mutagenized mice in particular has made substantial contributions to uncovering diverse mechanisms, such as within the immune (Sandberg and Ljunggren, 2005; Tabeta et al., 2006; Hoebe and Beutler, 2008; Theodoratos et al., 2010) and metabolic systems (Lloyd et al., 2005; Wilkes et al., 2009). Meanwhile, as this mutagenesis approach became widely used, it also became obvious that positional cloning of causal mutations associated with "complex behaviors" takes more effort than originally expected because phenotypic variances between subjects or trials. Nevertheless, several large-scale behavioral screenings were already done in different institutions, and candidate genes were successfully isolated in areas such as epilepsy (Frankel et al., 2009), ataxia (Sharkey et al., 2009), and locomotor activities (Furuse et al., 2010). In the field of circadian rhythm research, findings by Takahashi and his colleagues, such as the discovery and identification of $Clock^{\Delta 19}$ and *Overtime* mutations in N-ethyl-N-nitrosourea (ENU) mutagenized mice have had a significant impact (Vitaterna et al., 1994; Siepka et al., 2007) (Figure 1-5). In their review, Takahashi et al describe how these accomplishments were rigorously derived by choosing less variant readouts such as circadian period length, tau, and maximizing the number of mice that are screened at once (Takahashi et al., 1994; 2008). Thus, in conducting a new genetic screening for a complex behavior in mice, it is crucial

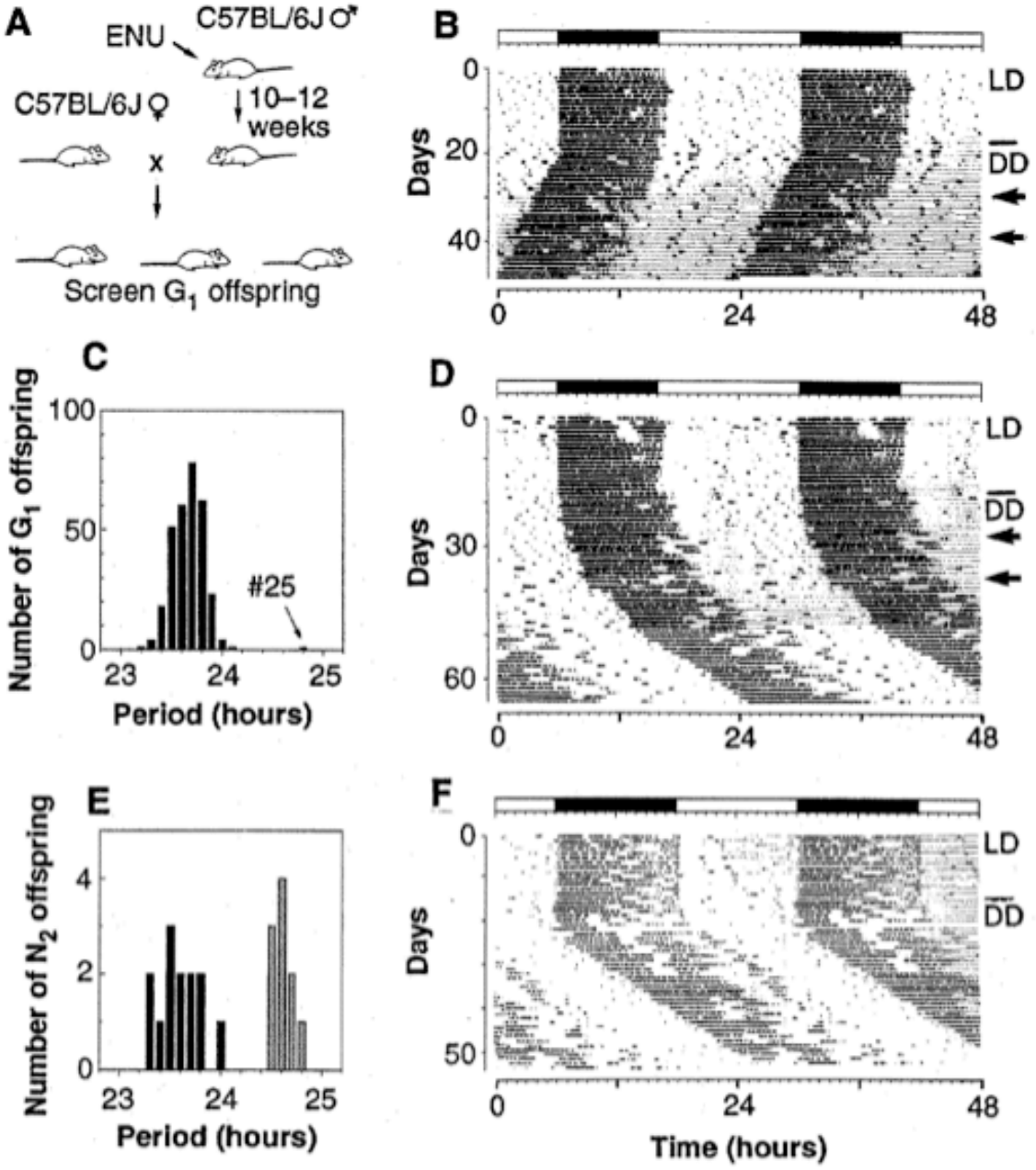


Figure 1-5 Identification of the clock mutant through ENU mutagenesis screen. The Clock mutant mice with a Clock$^{\Delta 19}$ allele has prolonged circadian period length on constant dark environment. (Adapted from Vitaterna, et al., 1994)

either to construct an experimental strategy that allows isolation of subtle behavioral differences, such as the second-generation method (which utilizes statistical power of multiple mice in the second generation that carries the same mutations with 50% chance, although it requires significantly greater time and resources) (Kumar et al., 2011), or carefully select a phenotype that employs readouts that are robust and tight enough for conducting a first-generation screening.

Genetic analysis of sleep/wake in fruit flies

There is a consensus that "sleep" in lower animal species (where EEG is not applicable) can be defined behaviorally with the following criteria: 1) absent or reduced movement; 2) elevated sensory threshold for external stimuli; 3) homeostatic rebound following deprivation; 4) regulation by the circadian clock; 5) Relatively quick, reversible state transitions.

Using these criteria, there are several reports of large-scale forward genetic screening of sleep phenotype in flies. One of the representative findings through genetic screening in flies is the line Cirelli et al. introduced, named "*Minisleep*" (*mns*) (Cirelli et al., 2005). The *mns* flies exhibit markedly reduced number of sleep episodes, and the authors determined a point mutation in the *shaker* gene, encoding a voltage-gated potassium channel subunit. Sehgal and her colleagues isolated a fly mutant that also has a long wakeful time through screening of transposon-based library stocks, and named the mutant "*Sleepless*" (*sss*) (Figure 1-6) (Koh et al., 2008). Subsequently, the authors claimed that a protein encoded by *sss* regulates expression and activity level of the shaker channel (Wu et al., 2010). Another line of mutant

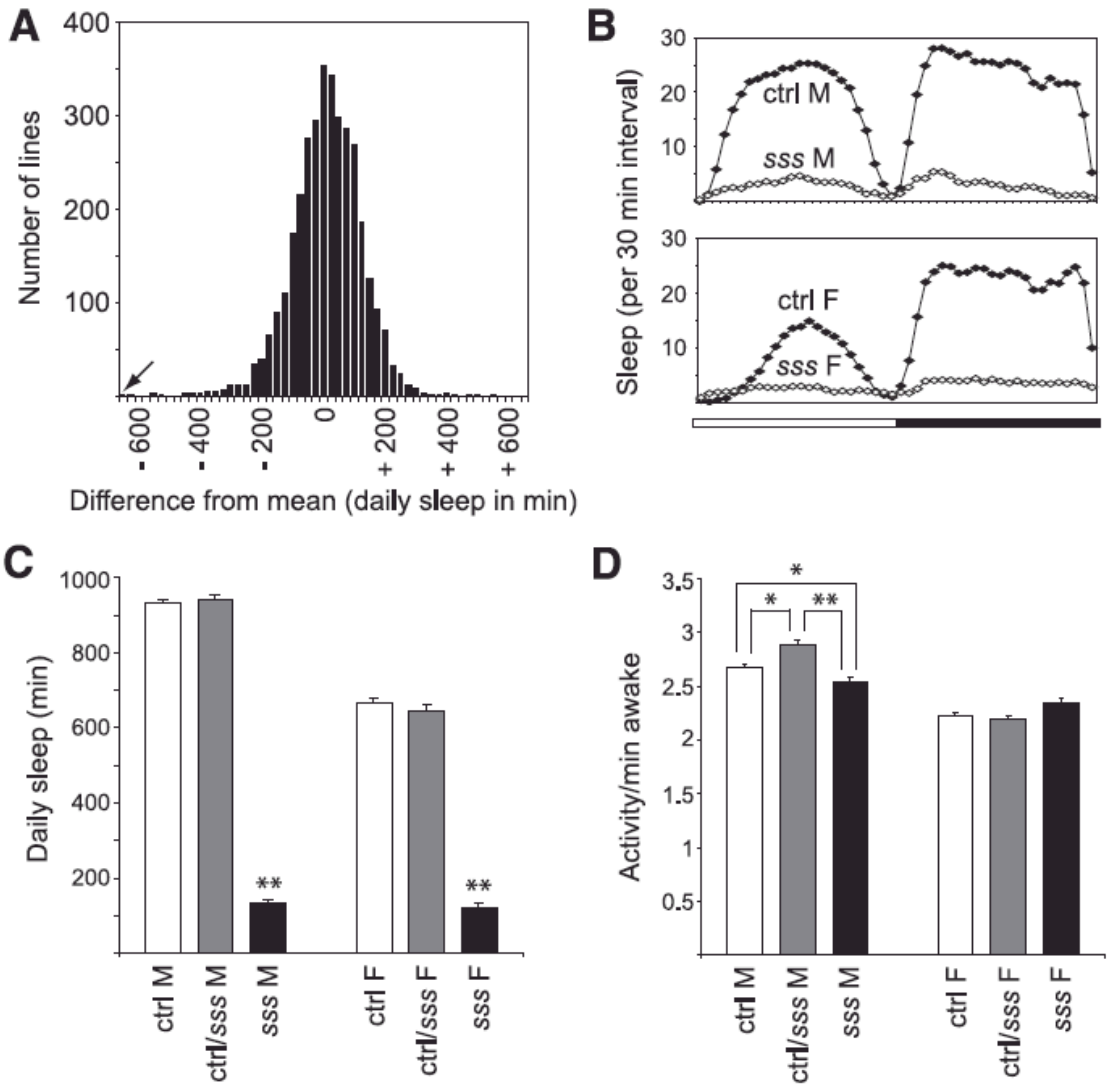


Figure 1-6 Prolonged wakefulness of a Sleepless (sss) mutant in flies. Total daily sleep time and mean sleep duration are significantly reduced on both mutant males (M) and females (F). (Adapted from Koh et al., 2008)

flies with reduced sleep is called "*Fumin*" (*fmn*), caused by a loss-of-function mutation in the dopamine transporter gene (Kume et al., 2005).

As can be seen from the examples above, genetic screenings in flies are considered to be established approaches in the sleep field; however, it is already known that the approaches carry two potential drawbacks (Shaw and Franken, 2003; Cirelli and Bushey, 2008; Freeman et al., 2013): 1) While candidate gene approach in flies can be a powerful tool to validate mutations started in mammals (see below), mutations found in forward genetic screening in flies do not necessarily lead to the same phenotype in mammals; 2) It is difficult to reach the fundamental mechanisms underlying sleep regulation in higher vertebrates as we see two distinct sleep phases, NREM and REM sleep. This complexity is a stark contrast to the "sleep" in flies. Futhermore, all the above-mentioned criteria defining the "sleep" behavior are solely based on locomotor activity as the only proximal readout. This makes the distinction between "quiet wakefulness" and "sleep" arbiturary. Indeed, all reported sleep mutants in flies have been those with reduced sleep or increased waking.

Drosophila is also one of the experimental species that has been extensively utilized for validating mutations that we have observed in human sleep abnormalities. For example, a mutation in the human circadian clock gene *DEC2* has been reported to cause a short sleep phenotype, with the corresponding mutation induced in mice and Drosophila producing the same phenotype (He et al., 2009). Similarly, recent report shows that abnormalities in sleep regulation in human subjects caused by a mutation in the *ABCC9* gene were reproduced in flies by artificially inducing the same mutation in the fly genome (Allebrandt et al., 2013).

Mapping sleep-related genes by inter-strain QTL analyses in mice

In order to elucidate mechanisms of complex phenotypes, quantitative trait loci (QTL) analysis has been widely and classically used in genetic studies of multiple organisms, including mice and humans. QTL is a DNA region in chromosomes that affects specific quantitative phenotypic characteristics. In QTL analysis, the region is mapped through linkage analysis, and eventually we are able to identify a causative genetic polymorphism. While it requires a large number of subjects to identify QTLs in humans due to genetic variability, it has become an effective approach for mice, especially by using two different established isogenic strains. Several groups have conducted sleep analyses in mice using this method.

The first group to perform sleep QTL analysis in mice was Tafti and Franken, and they reported multiple QTL regions in the mouse CXB line, which is generated by crossing C57BL/6J and BALB/cBy strains, that causes differences in sleep parameters (Tafti et al., 1997). Subsequently, they performed the same experiment with BXD line, from C57BL/6J and DBA/2J, and successfully identified QTL regions that affect delta power, which is a lower frequency comportment of EEG wave prominent during NREM sleep (Franken et al., 2001). They were only able to narrow the QTL region down to 45cM, which consists of over 200 genes, even though they utilized BXD mapping panels, which was the most fine panel available due to whole-genome sequence data on both of two parent strains (C57BL/6J and DBA/2J) (Table 1-1). More recently, they reported a candidate gene through bioinformatics analysis, *Homer1a*, from one of the regions named “Delta power in sleep 1” (*Dps1*) (Maret et al., 2007; Mackiewicz et al., 2008). Homer1a is a splicing variant of Homer1, an immediate

Chromosome	cM	Vigilance state	Candidate genes
1	48–77	PS	Acetylcholine receptor delta and gamma
			Interleukin 10
			Prostaglandin synthase 2
2	65–74	PS	Adrenergic α2b receptor
			Interleukin 1 α and 1 β
			Arginine vasopressin
			Oxytocin
			Prion protein
4	48–55	TST, SWS	Tyrosine kinase receptor 1
			Glycine transporter 1
			Histocompatibility 15
5	28–53	PS, TST, SWS	Cholecystokinin α
			GABA-Aα and β subunit
			Clock
7	5–26	PS	$H^+ K^+$ and $Na^+ K^+$ ATPase
			Voltage gated Na^+ channel β polypeptide
			Interleukin 11
			Tryptophan hydroxylase
9	31–37	TST, SWS	Acetylcholine receptor α5
12	8–24	PS	Histocompatibility 34 and 38
			Thyroid peroxidase
			Somatostatin receptor 1
15	6–30	TST, SWS	Prostaglandin E receptor EP2 and EP4
			TRH receptor
17	8–47	PS	Major histocompatibility complex
			TNF
19	0–16	PS	Glutathione S-transferase pi1 and pi2
			Galanin

Centimorgan location of the loci from the centromere (cM) and the candidate genes were retrieved from the Mouse Genome Database (MGD); URL: http://www.informatics.jax.org (1998).

Table 1-1 Candidate genes located in the region of sleep QTL. Even though several genomic regions that alter sleep phenotype are identified, all regions contain hundreds of genes. (Adapted from Tafti et al., 1999)

early gene product that binds to group 1 metabotropic glutamate receptors. *Homer1a* mRNA is indeed upregulated after 6 hours of sleep deprivation, while its expression is back to basal level during the recovery period after deprivation, suggesting *Homer1a* is involved in homeostatic sleep regulation. However, its constitutive function in sleep remains to be determined, since they could not validate a relevant phenotype in *Homer1a* knockout mice (Naidoo et al., 2012).

Taken together, it is obvious that the mapping resolution becomes a bottleneck when executing inter-strain QTL analyses of complex behavior such as sleep, because the density of inter-strain polymorphisms is very high, usually more than 1 per kilobase. In order to solve this problem, there is a method combining expression level analysis such as microarrays to pinpoint a candidate gene from a list of several hundred genes in the QTL region; however, it could still be difficult to prove that the changes in expression level induce abnormal sleep phenotype. Moreover, there is no guarantee that we can isolate a single candidate gene from the list by just observing changes in expression levels.

Feasibility and obstacles for conducting forward genetic screen of sleep/wake in mice

From these previously published observations, we determined that forward genetic screening of sleep/wake behavior by true EEG/EMG measurements in ENU-mutagenized mice is the best choice to reveal the mystery of sleep, particularly in mammals. However, several practical obstacles become huge barriers when conducting the large-scale sleep screening. The first one is the variability of sleep parameters as described previously. As Takahashi et al. mentioned in their review, isolating any gene through the genetic screening

becomes highly difficult if readouts have high relative standard deviation (RSD) values, such as in fear conditioning with typical RSD values over 100% (Takahashi et al., 2008). However, as Franken et al. reported in their QTL analysis in sleep, the actual RSD value of total sleep time is around 8% on C57BL6 strain (Franken et al., 1998), which is second in parameter tightness only to the circadian tau (less than 1%). This suggests the feasibility of finding phenodeviant mice through a forward genetic approach if we are able to establish standardized methods with enough throughput for surgery and recording. Another and the most significant obstacle is a labor-intensive nature of sleep staging from the raw EEG/EMG data, which can be rate limiting for executing the screening. Therefore, we aimed to increase the throughput of sleep screening by developing a novel automated sleep scoring software specialized to maintain screening throughput of >100 mice per week, as described later in Chapter 3.

CHAPTER TWO

DEVELOPING STREAMLINED TECHNIQUES FOR LARGE-SCALE SLEEP RECORDING IN MICE

Introduction

Forward genetic screen requires a highly uniform and consistent phenotyping assays, while maintaining a high throughput. In order to achieve this, we must continuously make an effort to standardize protocols for implanting surgery and data acquisition; the multitude of researchers/technicians involved requires assurance of same accurate and reliable output regardless of the experimenter. Also, hundreds of mice are handled by multiple people at any give time, further emphasizing the requirement to develop a centralized database system encompassing the entire screening flow. In this chapter, I will discuss streamlined surgery techniques, a data acquisition software, and a database software, which were all newly developed for our forward genetic screening.

Designing custom-made EEG/EMG electrode implants

The first thing we tackled was standardization and outsourcing of pre-fabricated EEG/EMG electrodes for mice. In most sleep research laboratories, EEG is recorded through small screws blindly twisted into the skull (Wu et al., 2009). While this method is relatively inexpensive, the stability of the screws depends on the thickness of the skull, and more importantly, the depth of implanted screws varies depending on the surgeon, which ultimately causes variability of EEG characteristics. To avoid these issues, we have been

using custom-made electrodes since we started sleep recording *orexin*-null mice in 1999 (Chemelli et al., 1999). These electrodes are made from connecters for electrical circuit boards, called “female pin headers”, and they have the advantage of more direct tip insertion. On the other hand, this electrode has two drawbacks: 1) Time needed to construct it by hand from a pin header; and 2) Electrode can be inserted too deeply, increasing the risk of surgery-related complications such as bleeding.

Thus, we meticulously reviewed the electrode and decided to outsource the production to a manufacture company (Unique Medical Inc., Tokyo, Japan). When we designed the new electrode, we also incorporated 4 stoppers on the electrode base (Figure 2-1), enabling us to insert the electrode more simply and precisely.

Refining techniques of stereotaxic surgery

With dominant genetic screening, a screening with n=1, it is crucial to avoid accidental loss of mice during surgery and surgical complications that can introduce additional noise into resulting sleep parameters. We improved our implant surgery protocols to allow shorter operation time without sacrificing overall preciseness and consistency. For example, instead of using a conventional injectable anesthetic such as Nembutal or Ketamine/Xylazine, we switched to gas anesthesia, a mixture of isoflurane/oxygen. Gas anesthesia lessens the impact of anesthesia by enabling a full intraoperative control of the depth of anesthesia, and a much shorter recovery time from anesthesia.

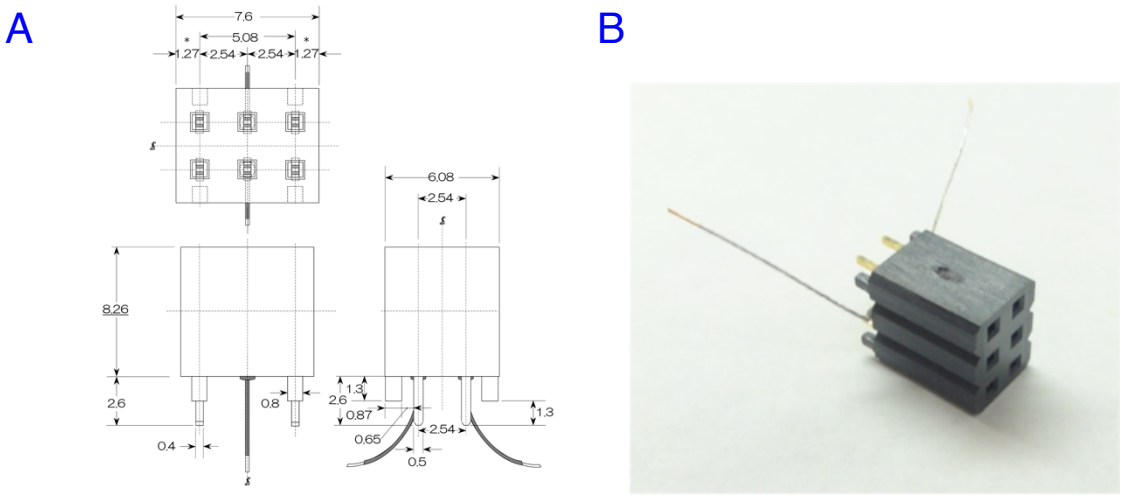


Figure 2-1 A custom-designed EEG/EMG module. The current design was drawn based on the handmade module that was previously used (A). A picture of the mass-production model is shown with newly designed stoppers in each corner at the bottom of the module (B).

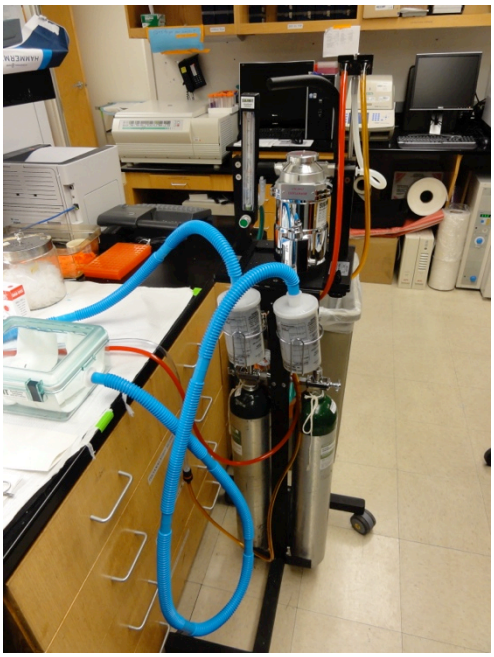
In addition to the gas anesthesia, we also adopted a new stereotaxic table with 3-dimentional digital gauges, enabling us to precisely insert EEG/EMG electrodes with 1/100 mm precision and eliminate any technical variability between surgeons.

With these techniques, we are now capable of performing the same implant surgery within 20 minutes per mouse, which is ~1/3 of time than before, enabling us to perform more surgeries per day (Figure 2-3).

SleepyDAQ: software for simultaneous multi-channel EEG/EMG data acquisition

Continuous recording of EEG/EMG data from one mouse for 72 hours entails 500 samples per every second for both EEG and EMG; consequently, the recorded file becomes about 250MB. In order to maintain a throughput of >100 mice per week, we must have a stable system that digitizes more than 25GB of data seamlessly without error every week. Thus, we developed a modified system for EEG/EMG recording. The hardware for the system consists of Grass Model LP511 amplifier (Grass Instruments, West Warwick, RI) or Nihon-Koden Model AB-611J amplifier (Nihon-Koden, Tokyo, Japan) with appropriate filter settings (EEG: 0.3-300 Hz, EMG: 30-300 Hz), a NI PCI-6220 analog-to-digital converter (National Instruments, Austin, TX) at 250 Hz sampling rate, and a regular tower-type PC (OS: Windows7 64-bit version). Each PC is able to simultaneously record up to 16 mice until storage media becomes full. SleepyDAQ, the recording software, was specially designed for EEG/EMG recordings based on LabView (National Instruments, Austin, TX), where all the EEG/EMG data are processed and displayed on a monitor in real-time. In addition to basic

A



B

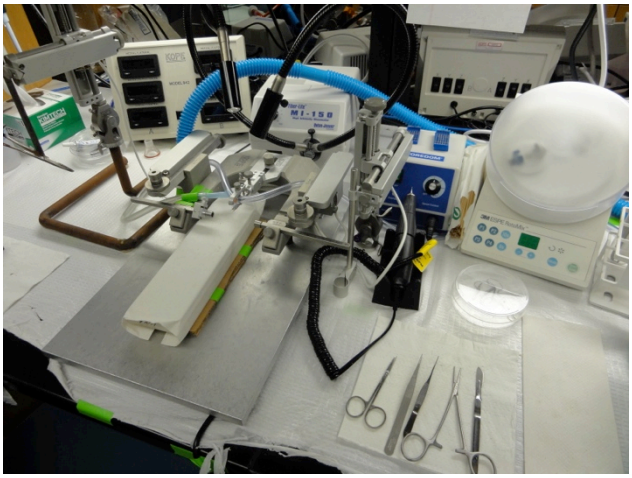


Figure 2-2 Isoflurane vaporizer and stereotaxic table for mouse implant surgery. Flow rate of isoflurane/oxygen mixed gas and isoflurane concentration is precisely adjusted using the vaporizer (A). The electrode position is continuously monitored through digital gauge shown on the top left corner of the picture (B).

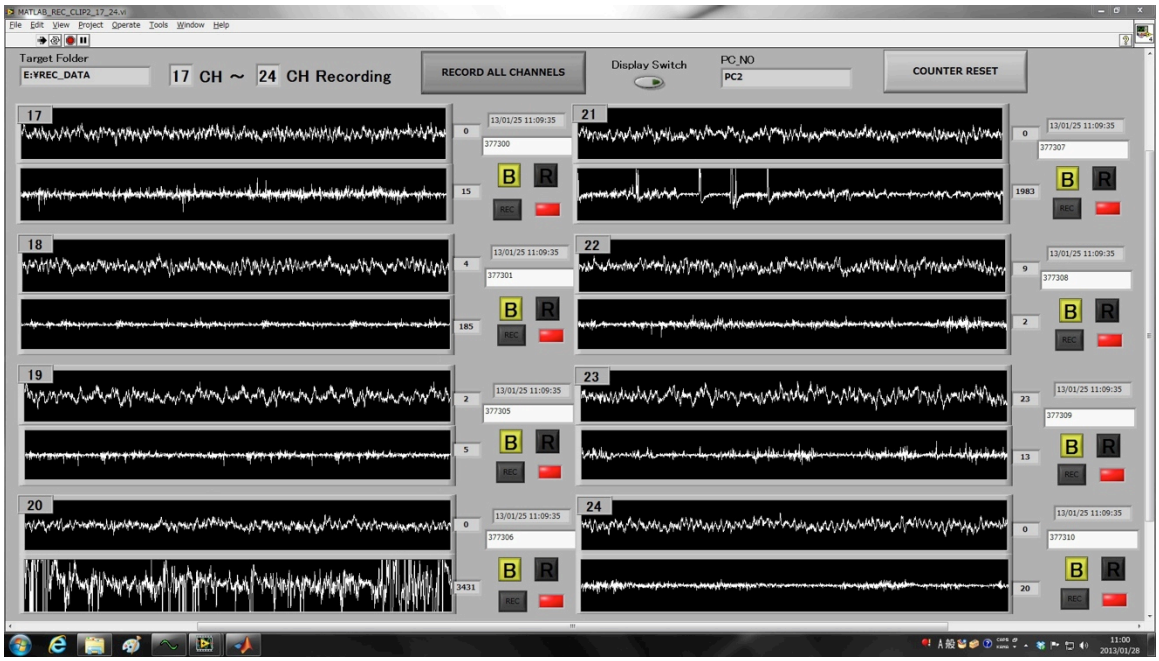


Figure 2-3 A screenshot of the SleepyDAQ data acquisition software. Each display is capable of simultaneously show live EEG/EMG signals from 8 mice.

A

B

Figure 2-4 Recording setups for EEG/EMG data acquisition. The large-scale EEG/EMG recordings are currently ongoing at UT Southwestern (A) and University of Tsukuba (B). Each PC is connected with up to 16 mice via signal amplifiers and analog-to-digital convertors to record EEG/EMG. The recording room is subjected to 12/12-h light/dark cycles with controlled humidity (40-60%) and temperature (24˚C, thermoneutral for mice).

functions, we also added a feature to inform users if there is any clipping of data during recording sessions.

SleepyDB: a database system for performing large-scale sleep screening

In our forward genetic screening, there are 5 major events and daily health checks for every mouse (Figure 2-5). Data is entered with a laptop computer and a portable barcode reader that can be taken to any vivarium room. In order to manage and integrate the large-scale volume of data most accurately and efficiently, we also developed a custom made database software, named SleepyDB. The software is coded in Filemaker (Filemaker inc. Santa Clara, CA), and all the data analyzed by users are automatically imported to this database. The database also periodically calculates mean and SD on each sleep parameters, based on strain, generation, and pedigrees. Thus, all users are able to easily find appropriate data for specific mice, compare sleep parameters while avoiding false negatives and minimizing significant mistakes, and recognize important information such as which mice need to be bred or performed IVFs. In addition, since we are collaborating with other laboratories for this research, SleepyDB is specially designed for smooth importing and exporting of data, enabling intensive communication between different databases; for example, we routinely import basic data from other databases such as MUSDB (Masuya et al., 2004) before transferring mice from collaborators, allowing us to carefully inspect all the mice at the time of acceptance.

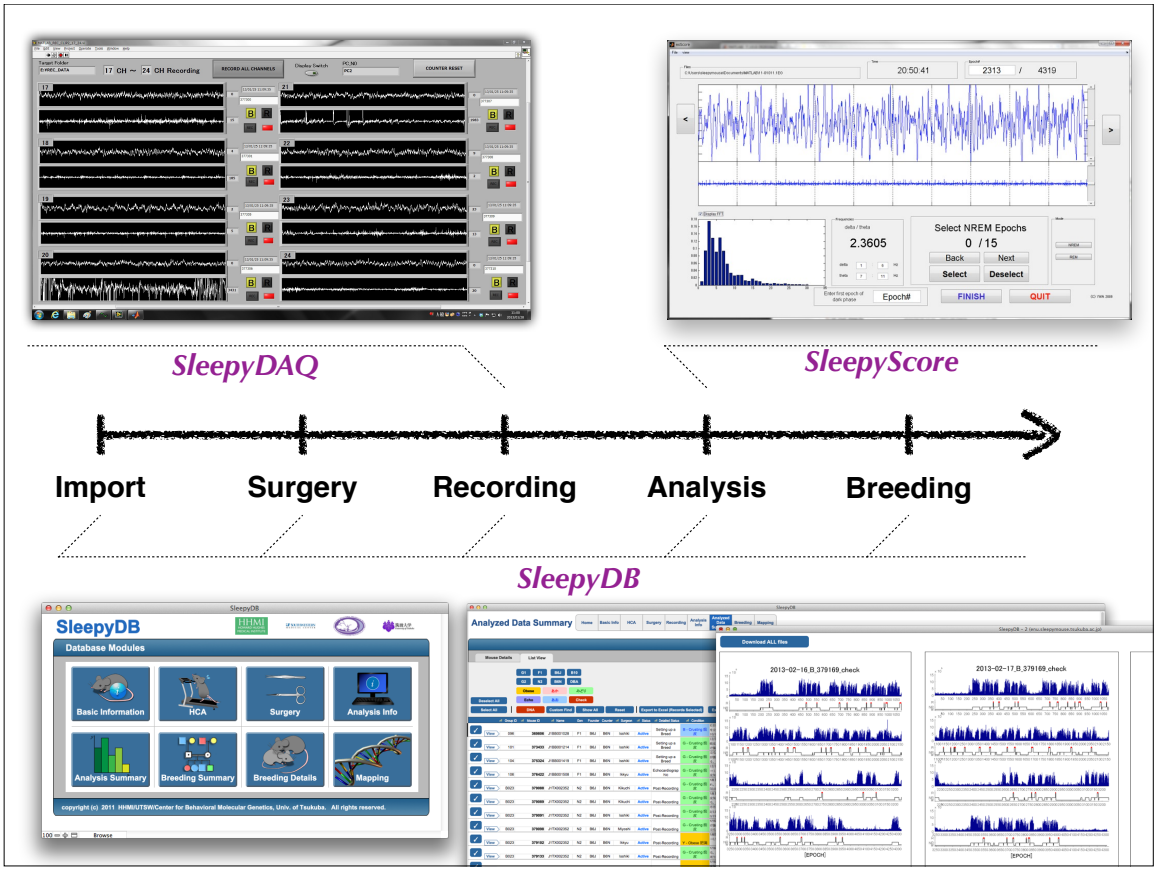
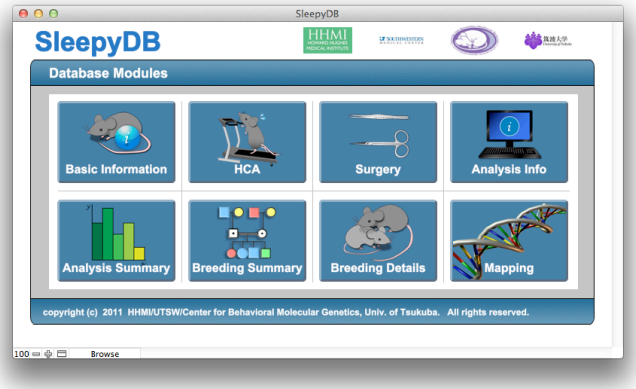
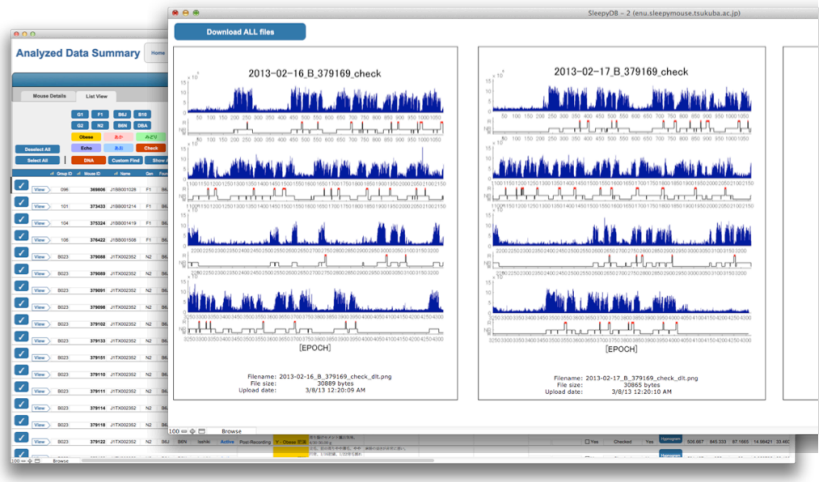


Figure 2-5 Major events and associated software in the flow of large-scale sleep screening. SleepyDB integrates all sleep data and occurrences throughout this screening in order to manage these data most accurately and efficiently.

A



B



C

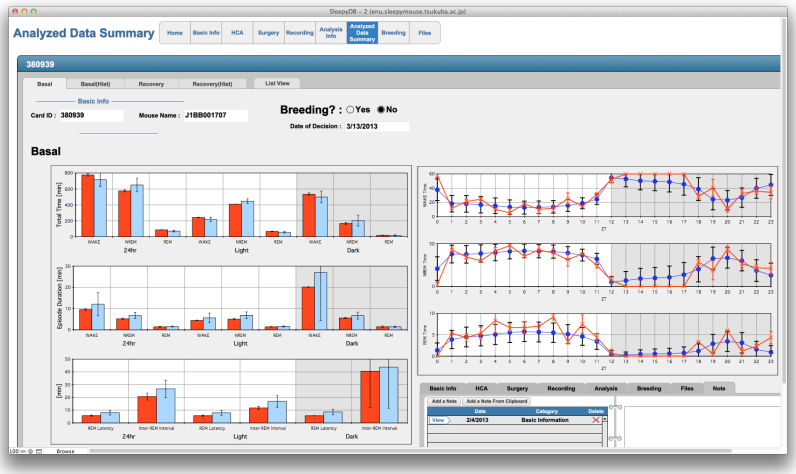


Figure 2-6 Screenshots of the SleepyDB database software. The database has multiple modules to enter different categories of data for each mouse or for groups of mice (A); Users are able to instantaneously check the screening status, health-related data, and sleep data for any of the thousands of mice through this database (B and C).

Discussion

We have developed a custom made electrode, streamlined surgery procedures, SleepyDAQ that simultaneously records EEG/EMG up to 16 mice per station, and SleepyDB, a centralized online database that integrates all the events associated to this entire project. With these enabling technologies and software, we are now allowed to conduct sleep recordings without relying on individual abilities. It also makes the entire project even more seamless by instantaneously sharing all the data among members in our group. However, while these improvements are necessary to conduct the screening, they alone are not sufficient; in the next chapter, I will describe the most significant bottleneck in conducting sleep screening in mice: sleep staging.

CHAPTER THREE

SLEEPYSCORE: A NEWLY DEVELOPED SEMI-AUTOMATED SLEEP STAGING SOFTWARE

Introduction

The most significant obstacle in executing large-scale genetic analysis of sleep in mice is the low throughput of EEG/EMG-based analysis. The rate-limiting step for sleep analysis in mice is the manual scoring of long hours of EEG/EMG recordings by human eyes; the average time a skilled scorer spends for scoring 24 hours of EEG/EMG data is over 2 hours. Even though several automated sleep-scoring systems for rodents are already published (Doman et al., 1995; Veasey et al., 2000; Berthomier et al., 2007; Brankack et al., 2010; Sunagawa et al., 2013), these programs are not capable of efficiently and robustly handling a large number of mice and so are essentially useless for our purposes. For example, in a commercial software called "SleepSign", the user has to set certain numerical thresholds for EEG/EMG parameters for each mouse, and then the user has to repeat modifying these thresholds until the computed results meets his satisfaction – a chicken-and-egg situation (Figure 3-1) (Kohtoh et al., 2008). Moreover, these threshold-driven types of pattern recognition often do not work even on one mouse due to inherent variability of EEG/EMG parameters across several days of recording time. Here, I describe the newly developed MATLAB (Mathworks)-based EEG/EMG scoring software, named "SleepyScore," which enables us to conduct the large-scale sleep screening in mice more efficiently.

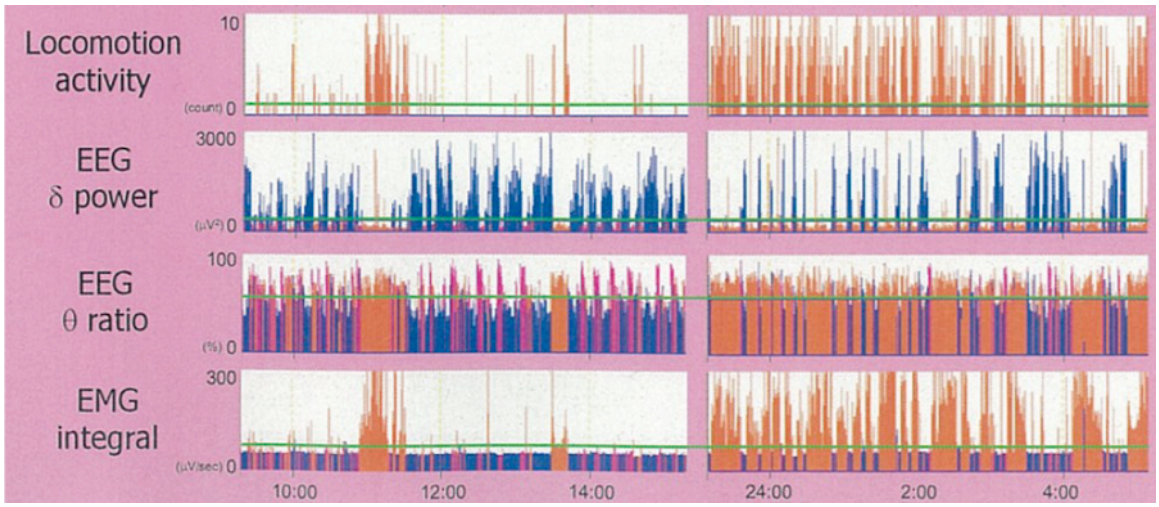
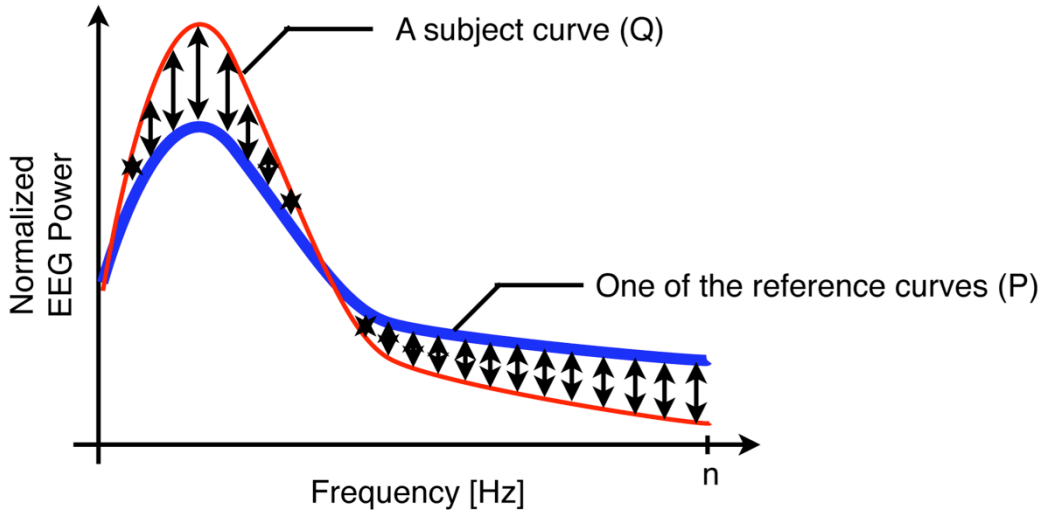


Figure 3-1 **Sleep Stages are selected based on the threshold-based algorithm in the SleepSign software.** Users must repeatedly select certain thresholds until scored result becomes satisfactory. (Adapted from Kohtoh S, et al., 2008)

Core algorithm: Non-parametric fitting of EEG spectra to user-selected reference epochs

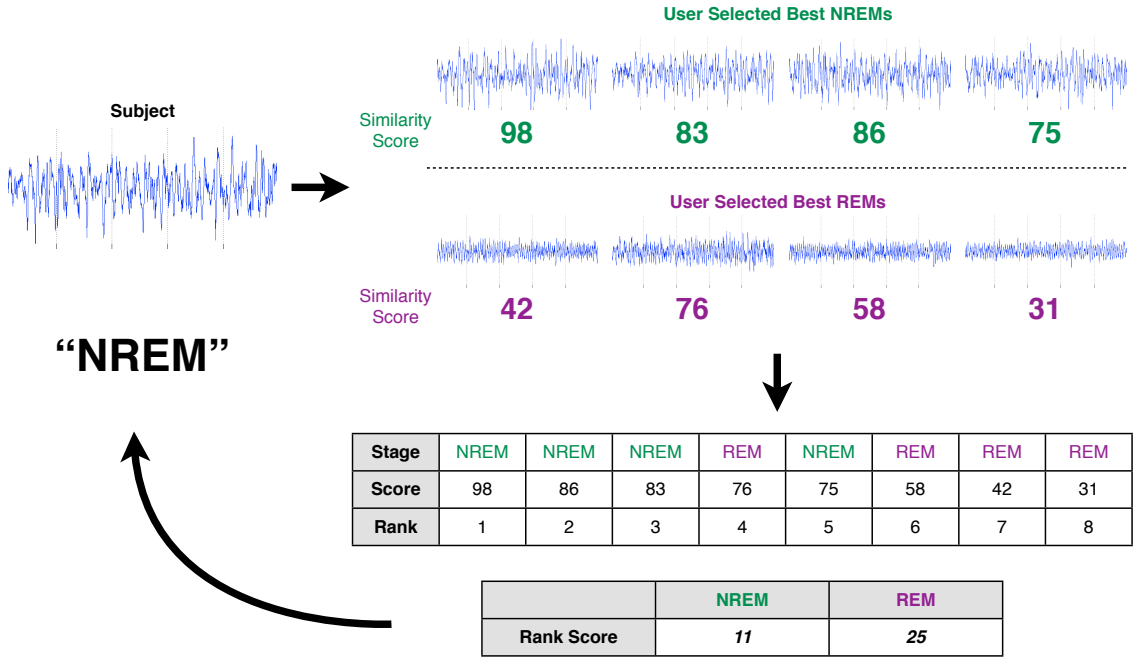
Since EEG signals are intrinsically very weak, they are easily interfered by external factors. From our extensive experiences in sleep recordings, we have recognized that EEG characteristics of each mouse are slightly different, even after standardization of surgical and recording procedures. Therefore, it is very important that the automated scoring algorithms are able to correctly discriminate three different sleep stages through the noise of subtle individual differences.

The software utilizes a simple pattern-matching algorithm, in which the human scorer is asked to randomly select, for each mouse, 15 representative (reference) epochs of NREM and REM sleep episodes from 3 days of EEG/EMG recording. Then, the software calculates the "similarity" of a given epoch to these reference epochs in the frequency domain, using Euclidean distance described in Figure 3-2. In this Euclidean distance calculation, we use the entire 0-30Hz band to make a score and do not limit to specific bands of the EEG spectrum, such as delta or theta, for the score calculation, so that the algorithm is able to grab the specific characteristics of EEG on particular mice. Lastly, the software evaluates these scores in non-parametric fashion to determine the final sleep stage. These calculations are actually performed on every 4-second sub-epoch, and the software determines the final score on 20-second epochs by incorporating the scores of adjacent two 4-second sub-epochs before and after the 20-second epoch (using the scores from a total of 9 sub-epochs), allowing the algorithm to take bold and flexible



$$\Rightarrow \quad d(P,Q) = \sqrt{\sum_{i=1}^{n}(P_i - Q_i)^2}$$

Figure 3-2 Calculating Euclidean distance on SleepyScore. The similarity between a subject EEG spectrum and every reference curves are calculated using the formula. If two curves are almost identical, the distance value, *d(P,Q)*, becomes close to zero.



Stage	NREM	NREM	NREM	REM	NREM	REM	REM	REM
Score	98	86	83	76	75	58	42	31
Rank	1	2	3	4	5	6	7	8

	NREM	REM
Rank Score	*11*	*25*

Figure 3-3 Novel pattern-matching algorithm for automated sleep scoring. Assisted by the software, the user randomly selects several representative NREM and REM epochs for an individual mouse as reference epochs. Similarity scores (Figure 3-2) are calculated in the frequency domain for a given 4-sec sub-epoch from the same mouse, against each of the reference NREM and REM epochs. The similarity scores are compared to determine the sleep stage of the subject sub-epoch by non-parametric ranking. Actual software uses 15 reference NREM and REM epochs, instead of 4 epochs as shown in this schematic diagram. This algorithm adapts itself to the inter- and intra-individual variability of EEG/EMG parameters, ensuring very high accuracies in sleep scoring.

measures to determine the final sleep stages, including transitional epochs that are difficult to score even for humans.

Temporal EMG variance with median filter, a powerful algorithm distinguishing wake and sleep states

While the core algorithm described in the previous section is for distinguishing between NREM sleep and REM sleep by using their distinct EEG spectra, the software distinguishes between wakefulness and sleep (either NREM or REM) by using information contained in the EMG. One practical problem in EMG measurements that we have recognized is that a constant background noise can easily contaminate EMG signals depending on EMG wire positioning during surgery. This suggests difficulty of classification between wakefulness and sleep by simply comparing EMG powers on particular epochs. Therefore, we developed a novel algorithm that specifically detects only wake epochs, utilizing variances between short time bins of EMG power in a single epoch. First, EMG power within every 0.5-second bin is calculated, and then its variance is determined over 8 bins (over a 4-second sub-epoch). The final variance values are calculated after a total of five variance values within one 20-second epoch are filtered by median function. The median filter is very effective in excluding false calculations on epochs with micro-muscle twitches during sleep episodes as in Figure 3-4.

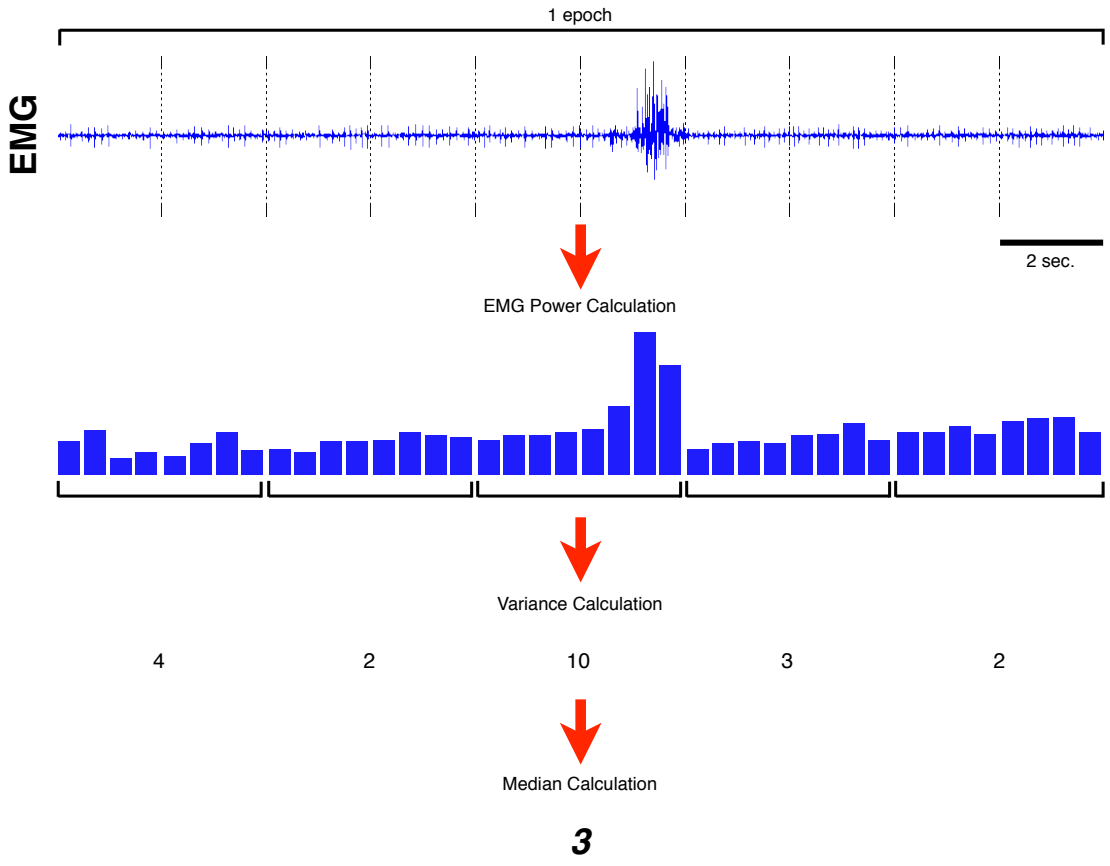


Figure 3-4 Calculating EMG variance to determine sleep/wake status. The EMG power on every 0.5-sec bin is used to calculate EMG power variance value for each 4-sec sub-epoch. The EMG variance value for the 20-sec epoch is then calculated through a non-parametric median filter as shown in this schematic diagram.

After the program calculates a final EMG variance value on all the epochs, the user selects, through a graphical user interface, one threshold that discriminates between wake and sleep.

User-friendly interfaces enabling fast sleep-staging corrections

Although pursuing perfect sleep scoring with automated algorithms is a critical step, building user-friendly interfaces plays an important practical role when a large number of sleep recordings are analyzed. When we execute SleepyScore, we are able to score intuitively, requiring no manuals, since the software uses plenty of graphical user interfaces (GUI) from selecting date of analysis and recording files, to the finishing window. In particular, a window for manually modifying sleep stages helps us identify and correct unavoidable mis-staging by the software. On this window, a graph of delta power or theta/delta ratio is shown right above the corresponding hypnogram, and with a simple click on a epoch of interest, the raw EEG/EMG data will be instantaneously displayed, enabling us to swiftly verify or correct the sleep stage (Figure 3-5).

When the sleep scoring is over, files with sleep stages, hypnograms, and values of all the sleep parameters are automatically generated. As I mentioned earlier, these files are imported automatically to our database, SleepyDB, for our forward genetic screening, and we can almost instantaneously compare the sleep parameters with that of the entire cohort.

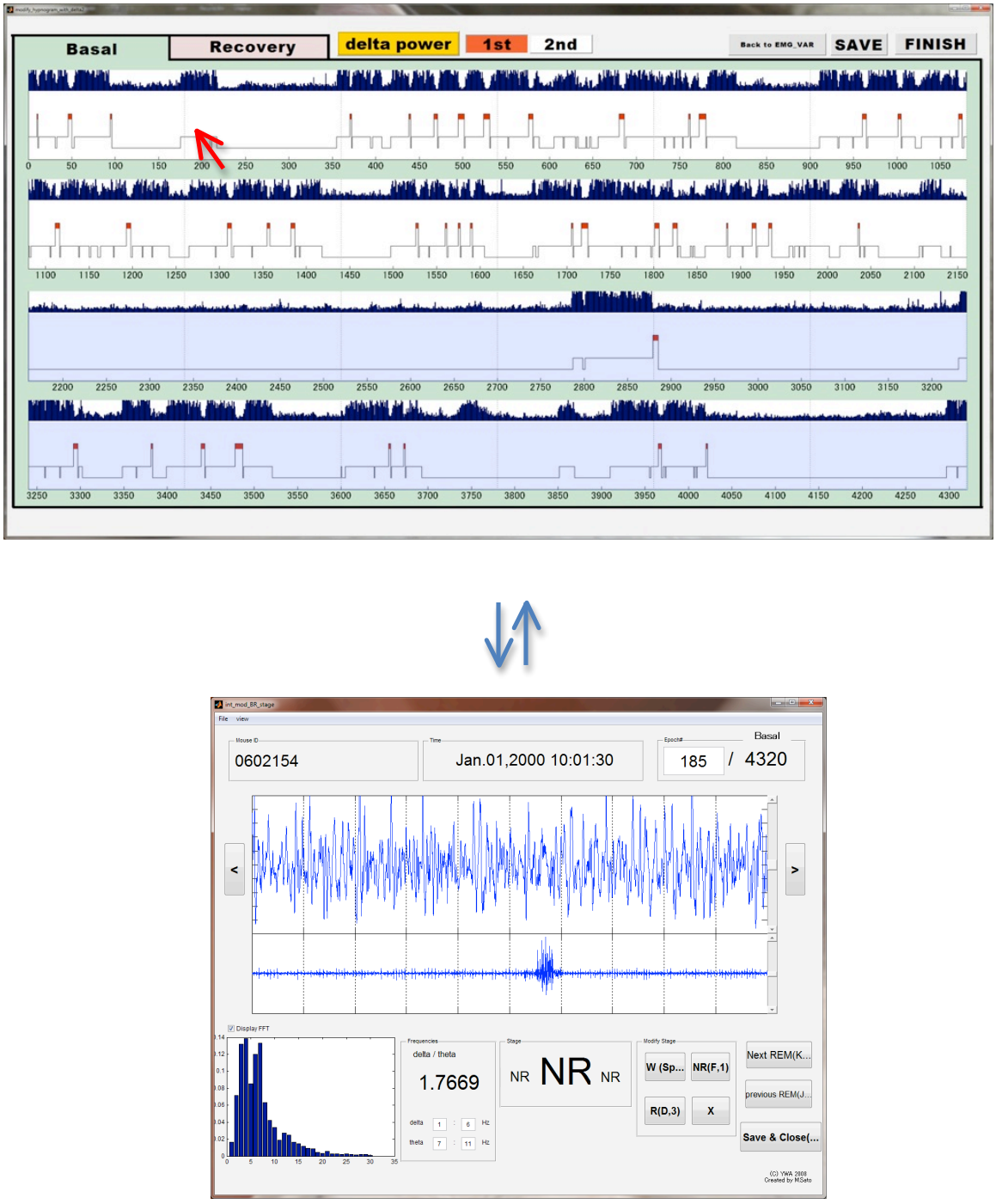


Figure 3-5 Instantaneously switching between two windows in SleepyScore to seamlessly manual-correct sleep stages. By examining sleep stages with corresponding delta power graph (or delta/theta ratio) above the hypnogram, users can easily find suspect epochs where the software may have potentially made a mistake. Clicking on that portion of hypnogram instantaneously displays the second window,which displays raw EEG/EMG data and allows re-staging as needed.

Example 1: Wildtype C57BL/6J mice

To examine and validate usability of SleepyScore on sleep screening, accuracy, specificity and sensitivity of each sleep stages are determined using EEG/EMG data from 8 male C57BL/6J mice.

As shown in Table 3-1, SleepyScore maintains about 92% in overall accuracy on fully automated scoring, which is slightly less than ~95% variability between two skilled human scorers. However, after a user spends maximum of 10 minutes modifying obvious computer errors, we observed significant increases in all the parameters; in particular, accuracy now improves to 98%, suggesting the software is functional for our purpose of scoring sleep data to perform accurate and robust comparisons between mutagenized mice.

Example 2: *Orexin* null mice - abnormal transitions with preserved micro-architecture

In addition to the ability to score sleep data accurately and precisely, the software is obviously required to detect sleep abnormalities in our forward genetic screening. EEG/EMG data for two distinct mouse strains with known sleep abnormalities, *orexin*-null and *goosecoid-like* (*Gscl*)-null mice, are available in our laboratory, and we utilized these recordings for further validating our software.

Even though *orexin*-null mice show essentially normal micro-architecture on both EEG and EMG, these mice exhibit frequent abnormal direct transitions from wakefulness to REM sleep mostly during dark phase, called cataplexy, whereas wildtype mice never exhibit these transitions (Chemelli et al., 1999). Since we do not include any algorithm that prohibits the transition from wakefulness to REM sleep, the software correctly detects cataplexy

			Automated Scoring						
			Wake		NREM		REM		
Genotype	Strain	Number of mice	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity	Accuracy
WT	C57BL/6J	8	91.85 ± 3.50%	92.67 ± 3.77%	92.72 ± 4.48%	92.14 ± 3.18%	93.45 ± 7.11%	92.26 ± 2.56%	92.84 ± 1.57%
Orexin$^{-/-}$	C57BL/6J	3	97.55 ± 1.35%	87.68 ± 7.11%	88.26 ± 7.13%	96.11 ± 1.88%	87.06 ± 7.68%	93.45 ± 3.97%	93.14 ± 3.95%
GSCL$^{-/-}$	C57BL/6J	3	99.27 ± 0.17%	89.32 ± 2.72%	88.55 ± 2.86%	99.01 ± 0.30%	96.25 ± 1.36%	94.46 ± 1.67%	94.72 ± 1.69%

			Automated Scoring with maximum of 10 minutes modifying obvious computer errors						
			Wake		NREM		REM		
Genotype	Strain	Number of mice	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity	Accuracy
WT	C57BL/6J	8	97.65 ± 2.48%	97.47 ± 2.13%	97.66 ± 2.19%	97.48 ± 2.29%	96.08 ± 4.75%	97.72 ± 2.27%	97.93 ± 1.33%
Orexin$^{-/-}$	C57BL/6J	3	98.29 ± 0.59%	92.81 ± 3.52%	92.49 ± 4.31%	97.96 ± 0.41%	95.54 ± 2.43%	95.71 ± 2.31%	95.66 ± 2.03%
GSCL$^{-/-}$	C57BL/6J	3	99.37 ± 0.12%	94.57 ± 0.52%	94.32 ± 0.47%	99.16 ± 0.25%	96.94 ± 1.35%	97.13 ± 0.43%	97.18 ± 0.43%

Table 3-1 Summary of the SleepyScore performance. 24-h basal EEG/EMG recordings with 12:12-h light/dark cycle were scored using the SleepScore. Each value represents the mean ± SD. The top table shows total accuracy, sensitivity and specificity of each sleep stages, using the automated scoring algorithm of SleepyScore. The bottom table shows final performance after maximum 10 min/recording of manual corrections.

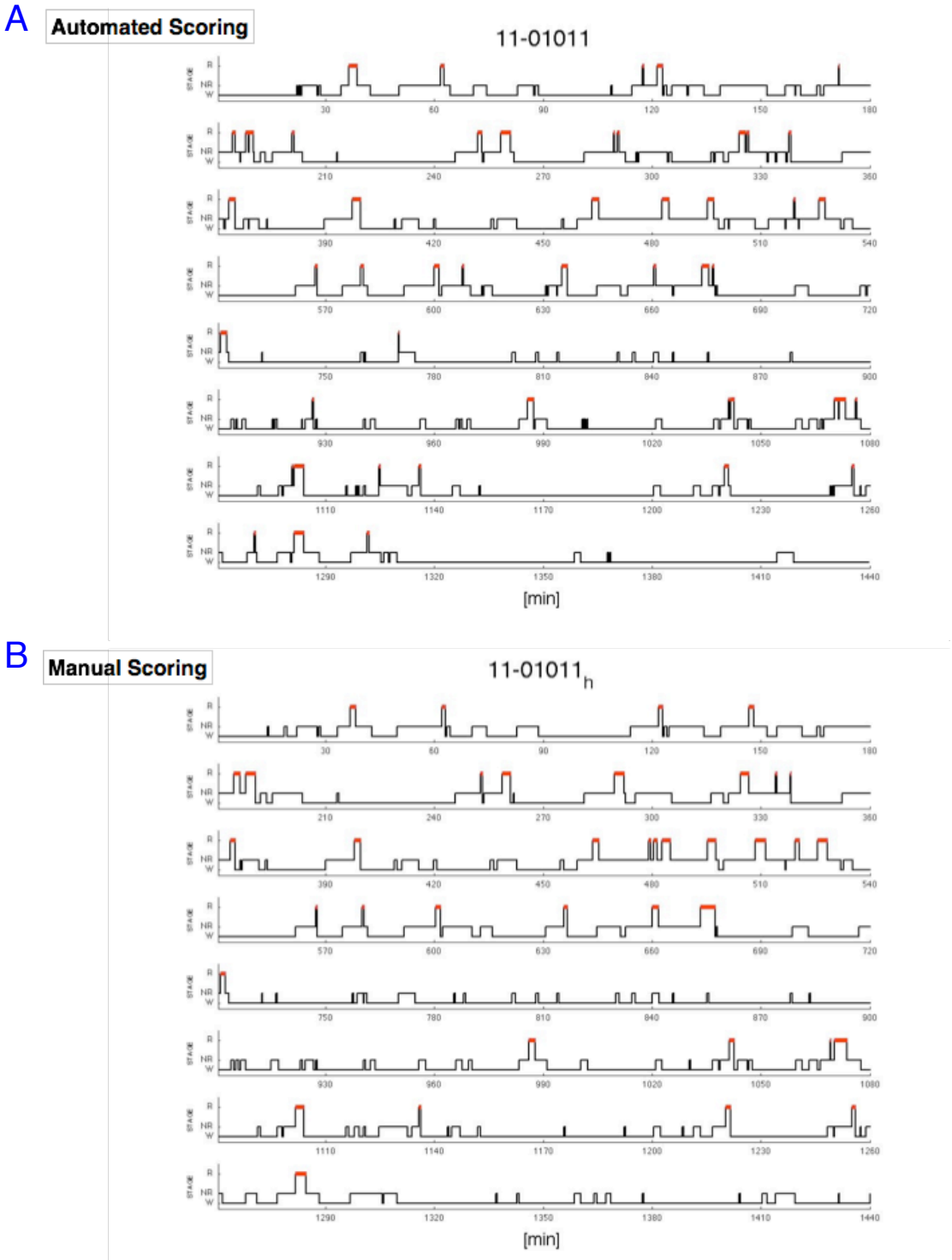


Figure 3-6 Sample hypnograms with automated (SleepyScore) and manual scoring using the same 24 hours EEG/EMG recording of a wild type mouse. W represents a period of Wakefulness; NR, NREM sleep; R, REM sleep.

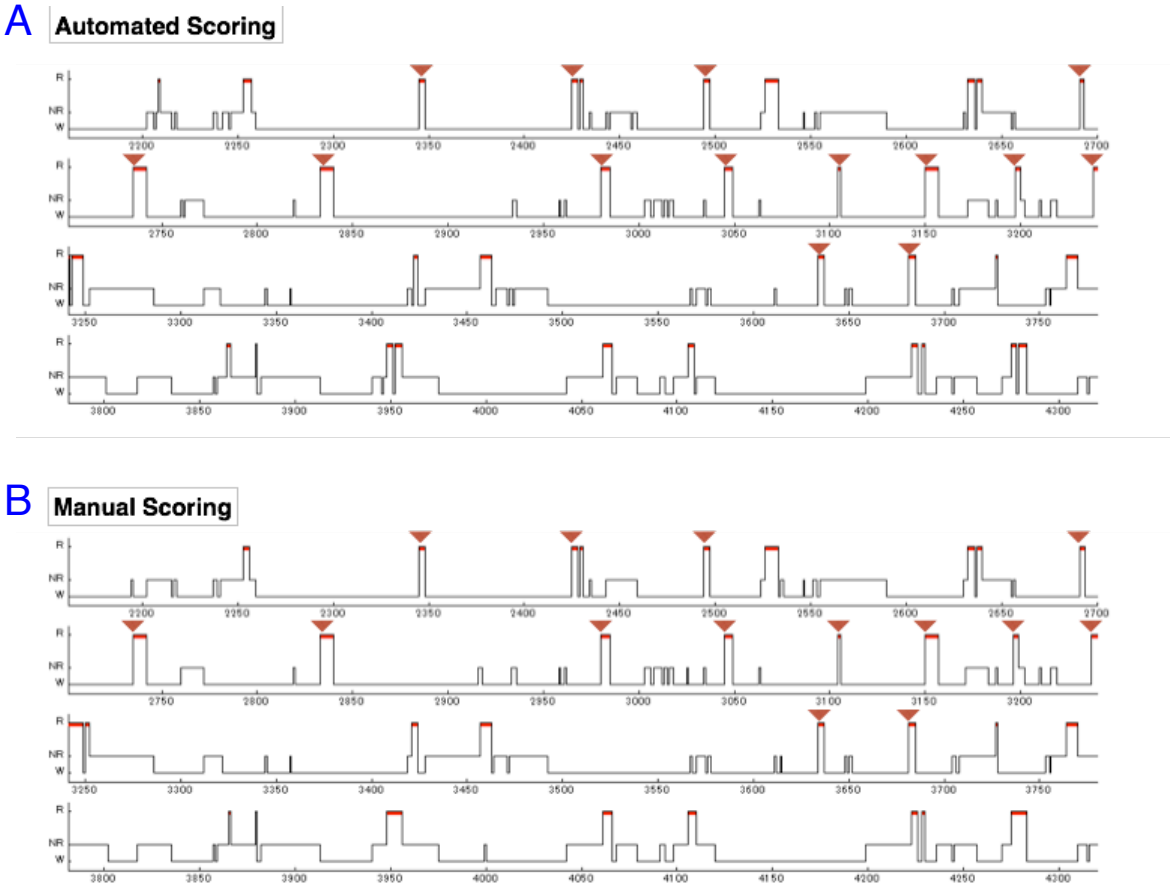


Figure 3-7 SleepyScore correctly stages cataplectic attacks of *orexin*-null mice. Note episodes of cataplexy during dark phase, marked by arrowheads, on both automated and manual scoring results.

attacks as in Figure 3-7. Also, overall accuracy and REM sleep sensitivity are also maintained at over 95%, enabling robust screen on forward genetics.

Example 3: *Gscl* null mice - abnormal REM micro-architecture

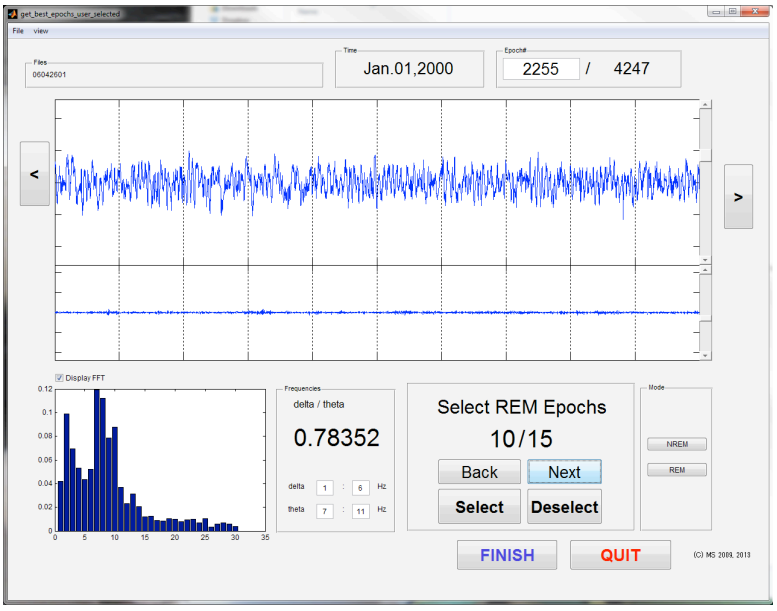
In contrast to *orexin*-null mice that have transitional abnormality, *Gscl*-null mice show abnormal EEG power spectra in REM sleep (Funato et al., 2010). Indeed, the abnormality in EEG micro-architectures tends to be overlooked in large-scale screenings without a double-checking system through visual inspection in place. SleepyScore also has difficulty detecting this type of anomalies through its automated scoring algorithm, since the software compares subject epochs with 15 reference NREM and REM epochs from the same individual mouse. However, unlike other fully automated sleep scoring programs, the opportunity to visually inspect raw EEG characteristics during REM sleep is preserved in SleepyScore, typically when the 15 REM sleep references are initially selected as shown in Figure 3-8. Therefore, in a sense, we are forced to perform additional inspection of the sleep characteristics of each mouse before elimination.

Discussion

As mentioned above, SleepyScore allows us to score many long sleep recordings in a significantly shorter time than manual scoring by incorporating novel algorithms and intuitive graphical user-interfaces. The overall accuracy is always over 90% just using the automated algorithm, and the accuracy increases to 98% when a user performs modifications for no more than 10 minutes. Also, validation using *orexin*-null and *Gscl*-null mice shows that SleepyScore has the ability to detect sleep/wake abnormalities in state transitions as well

as in micro-acchitectures. An updated version of SleepyScore will implement additional functions to quantitatively assess the markers of homeostatic sleep need by extracting decay characteristics of NREM delta power as well as cumulative NREM episode durations (NREM consolidation), particularly during the recovery recordings after sleep deprivation.

A



B

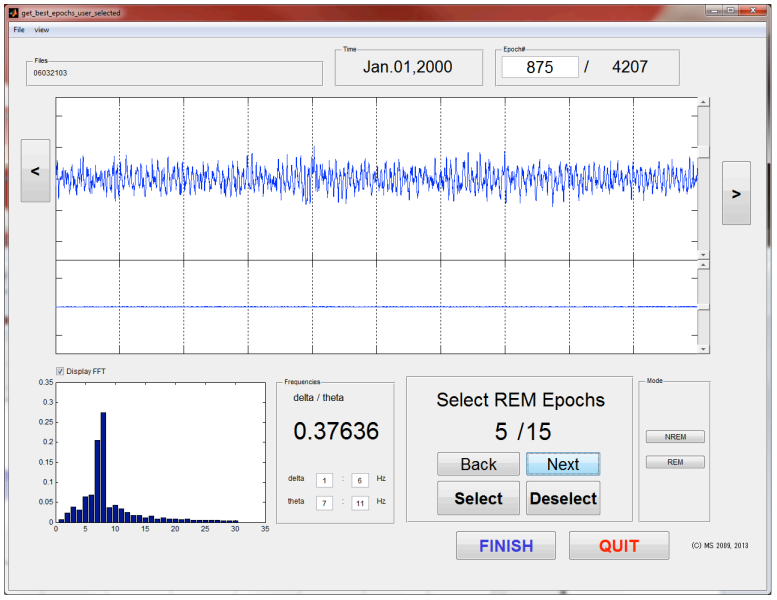


Figure 3-8 Abnormal EEG micro-architecture during REM sleep on *Gscl*-null mice. A typical REM EEG from a *Gscl*-null mice showing abnormal delta-power contaminations (A) as compared with a typical, "clean" REM epoch in a wildtype mice (B). See the frequency spectrum display in the lower left panel of the window. Although SleepyScore is unable to automatically detect EEG/EMG micro-architecture abnormalities, users have an ample opportunity to examine the potential micro-architecture ablations when they select reference epochs at the beginning of executing the software.

CHAPTER FOUR

FORWARD GENETICS OF SLEEP/WAKE IN MICE

Introduction

With the enabling technologies as described in previous chapters at hand, we have now been conducting a large-scale forward genetic screening of sleep/wake behaviors in mice. We specifically chose a dominant screening of ENU-mutagenized G1 or F1 mice, which has a significant potential in primarily detecting strong gain-of-function mutations with a relatively modest throughput of phenotypic assay. In this chapter, I will describe the approaches and our findings, with a highlight on two promising mutant lines recently identified.

Approaches

Mice

ENU-mutagenized C57BL/6 male mice were provided from the laboratory of Dr. Joseph Takahashi at UT Southwestern and the laboratory of Dr. Shigeharu Wakana at RIKEN BioResource Center (Tsukuba, Ibaraki, JAPAN). Mice were provided food and water ad lib, housing in regular cages pre- and post-recording periods, and switched to special recording cages with food and hydrated gels during EEG/EMG recordings. All mice were 12-16 weeks old at time of surgery and singly housed, kept always under conditions of controlled temperature and humidity, and maintained on a 12:12-hour light:dark cycle.

All animal experiments were performed under protocols approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center at Dallas and were in strict accordance with NIH guidelines.

For the primary screen, we have now been conducting two slightly different screens, at the G1 generation and F1 generation as shown in Figure 4-1, aiming for up to 10,000 mice. While G1 mice are generated by crossing ENU-injected (G0) C57BL/6J males with wildtype C57BL/6J females, F1 primary screening utilizes mice that are generated by directly crossing ENU-injected (G0) C57BL/6J males with wildtype C57BL/6N females, which skips one generation thus enabling us to shorten the experimental duration towards genetic mapping. As shown in Table 4-1, wildtype male mice with the C57BL/6J, C57BL/6N, and C57BL/10J backgrounds exhibit essentially identical sleep phenotype with regard to all major quantitative parameters of sleep. This suggests that the subtle differences of genetic background within the C57BL family of inbred strains are unlikely to affect sleep parameters in the primary screening either at the G1 or F1 (C57BL/6J x C57BL/6N) mice..This also. assures that interference from inter-strain QTL is unlikely with respect to sleep phenotype in the later stage of genetic mapping at the N2 and N3 generations.

Recording Methods

For individual mice, we are measuring the hourly total time and mean episode durations (reflecting sleep-state fragmentation) of wake, NREM, and REM states, as well as the EEG power spectra (in particular, delta power in NREM and theta power in REM). EEG delta power is the most reliable marker for the “depth” of NREM sleep (Franken et al., 2001;

Bjorness et al., 2009)and is closely monitored during the recovery NREM sleep after 4 hours of sleep deprivation. Each mouse is completely acclimated to the housing condition with controlled light/dark cycle and to the tethered recording setup for at two weeks before the actual recording. EEG/EMG is recorded for at least 3 days in a special cage. We also optimized our methods for automated sleep deprivation for genetic screening, using gyratory shakers (120 rpm, programmed to shake/stop for 1-10 seconds at random) (Sinton et al., 2009).

Identification of causal mutations

Once we find a phenotypically interesting G1 or F1 mouse, we cross him with wildtype C57BL/6N or C57BL/10J females in order to test the heritability of the phenotype. Since the G1 mice carry heterozygous (dominant) mutations, up to one half of the offspring is expected to exhibit the same phenotype, if the phenotype is indeed due to an induced mutation. Once the heritability of sleep phenotype is confirmed, we will map the causal mutation by classical linkage analysis, after generating ~100 N2 mice by backcrossing the mutant mouse with the same inbred counter strain we used for generating F1 mice. For genotyping single-nucleotide polymorphisms across the genome, we use a microfluidic PCR device (Fluidigm BioMarkTM HD System) that can simultaneously genotype up to 96 loci for up to 96 N2 mice in a single run. The relative paucity of ENU-induced mutations (estimated 30-40 coding mutations across the genome) ensures that we can identify the causal mutation once we find a heritable mutant trait. Simultaneously, we analyze the ENU induced mutations across the exome on the N2 mice by whole-exome sequencing with ~50X read coverage (McDermott

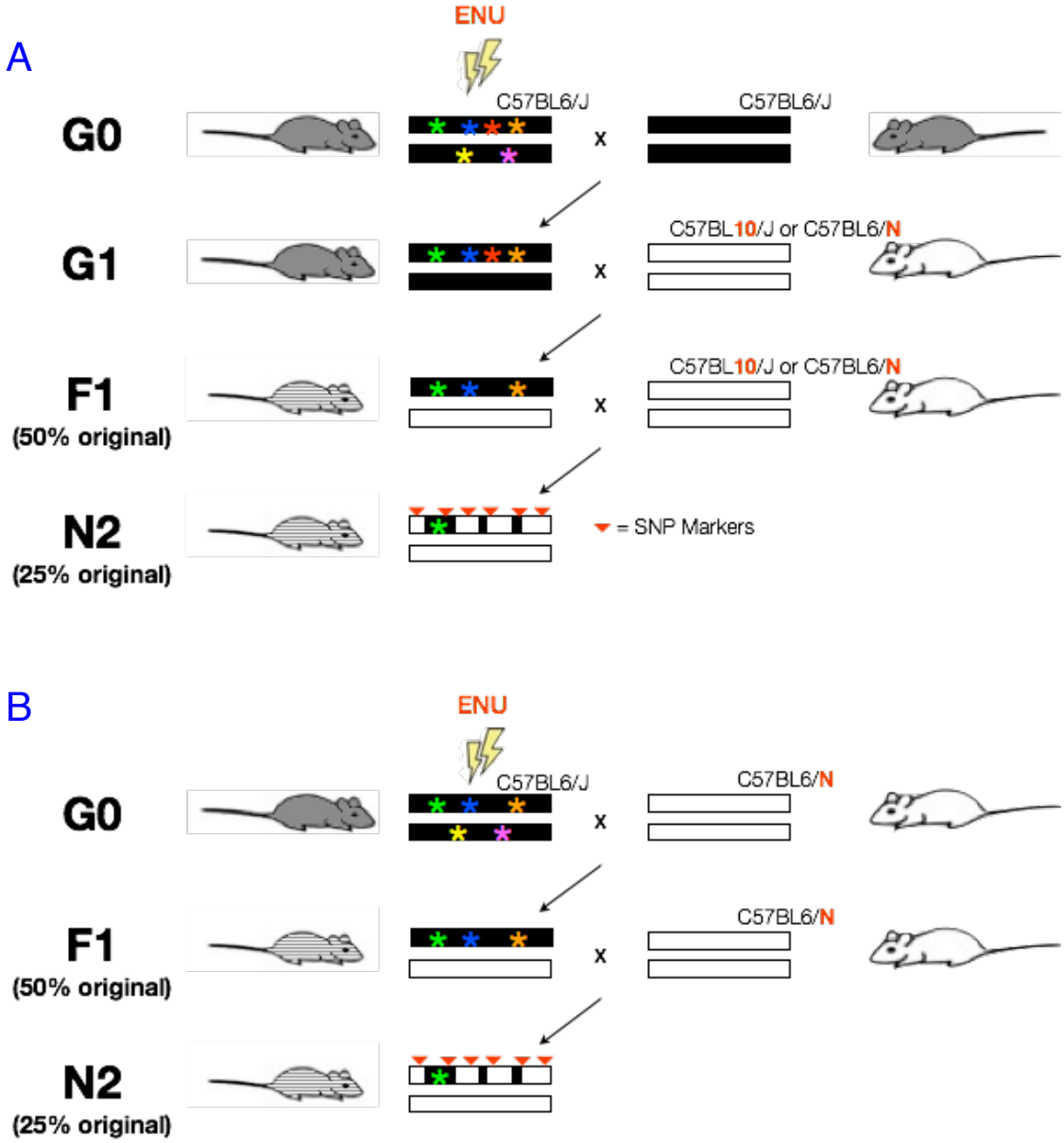


Figure 4-1 Breeding Diagram for Mapping Causal Mutations. (A) Primary screening at G1 generation with a pure C57BL/6J background. Once a phenodeviant (putative mutant) mouse from G1 generation is identified, two additional crosses with a different inbred strain are required to map the mutation that causes sleep abnormalities. (B) After we confirmed that all sleep parameters are identical between the C57BL/6J and C57BL/6N strains, we now skip one generation by crossing C57BL/6J G0 male mice directly with wildtype C57BL/6N female mice, and conduct the primary screen at F1 generation.

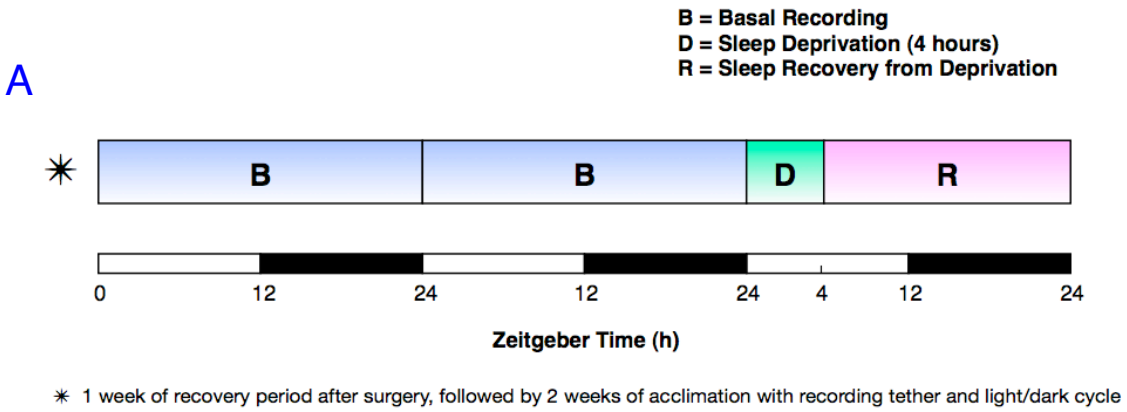
	Wake								
	C57BL/6J			C57BL/6N			C57BL/10J		
24 hr									
Total time (min)	746.5	± 39.4	(5.3%)	717.0	± 61.4	(8.6%)	747.6	± 27.7	(3.7%)
Episode duration (min)	11.9	± 3.0	(25.0%)	10.4	± 3.1	(30.0%)	3.6	± 0.9	(23.8%)
Light priod									
Total time (min)	227.0	± 20.2	(8.9%)	206.0	± 29.3	(14.2%)	291.3	± 35.7	(12.2%)
Episode duration (min)	5.3	± 0.9	(16.0%)	4.7	± 1.3	(26.8%)	2.4	± 0.3	(13.6%)
Dark period									
Total time (min)	519.5	± 38.5	(7.4%)	510.9	± 46.3	(9.1%)	456.3	± 31.1	(6.8%)
Episode duration (min)	27.3	± 14.9	(54.4%)	20.9	± 11.4	(54.5%)	5.7	± 2.6	(46.4%)

	NREM Sleep								
	C57BL/6J			C57BL/6N			C57BL/10J		
24 hr									
Total time (min)	622.5	± 41.8	(6.7%)	653.8	± 61.5	(9.4%)	621.4	± 27.4	(4.4%)
Episode duration (min)	6.4	± 0.6	(9.9%)	6.8	± 1.0	(14.0%)	3.0	± 0.7	(23.7%)
Light priod									
Total time (min)	439.1	± 16.5	(3.8%)	457.9	± 27.2	(5.9%)	378.7	± 33.0	(8.7%)
Episode duration (min)	6.5	± 0.7	(10.8%)	7.1	± 1.2	(16.5%)	3.1	± 0.7	(21.5%)
Dark period									
Total time (min)	183.4	± 37.7	(20.5%)	195.9	± 45.5	(23.3%)	242.7	± 24.5	(10.1%)
Episode duration (min)	6.4	± 0.9	(13.3%)	6.3	± 1.2	(19.7%)	2.9	± 1.0	(32.9%)

	REM Sleep								
	C57BL/6J			C57BL/6N			C57BL/10J		
24 hr									
Total time (min)	70.2	± 8.1	(11.5%)	68.6	± 7.6	(11.0%)	71.1	± 9.3	(13.1%)
Episode duration (min)	1.6	± 0.1	(7.1%)	1.7	± 0.2	(11.0%)	1.3	± 0.1	(10.2%)
REM latency (min)	7.8	± 0.9	(11.0%)	8.5	± 1.3	(15.6%)	3.8	± 0.8	(22.0%)
Inter-REM interval (min)	28.2	± 3.4	(12.2%)	30.7	± 4.9	(16.0%)	24.1	± 3.7	(15.2%)
Light priod									
Total time (min)	53.9	± 8.6	(15.9%)	55.9	± 6.9	(12.3%)	50.0	± 6.8	(13.6%)
Episode duration (min)	1.6	± 0.1	(8.5%)	1.8	± 0.2	(11.6%)	1.4	± 0.2	(12.2%)
REM latency (min)	7.6	± 0.9	(12.4%)	8.5	± 1.5	(17.6%)	3.9	± 0.9	(23.2%)
Inter-REM interval (min)	17.8	± 3.0	(16.8%)	19.2	± 3.0	(15.8%)	17.3	± 3.5	(20.3%)
Dark period									
Total time (min)	16.4	± 4.4	(26.9%)	12.7	± 4.3	(33.8%)	21.0	± 8.4	(40.1%)
Episode duration (min)	1.6	± 0.2	(12.2%)	1.3	± 0.3	(23.4%)	1.1	± 0.2	(19.0%)
REM latency (min)	8.5	± 1.4	(16.8%)	8.6	± 2.1	(24.3%)	3.6	± 1.0	(27.1%)
Inter-REM interval (min)	46.2	± 23.9	(51.7%)	48.8	± 24.3	(49.9%)	33.7	± 13.3	(39.4%)

Data represent the mean values ± SD (%RSD). Each strain consists of 15 mice.

Table 4-1 Quantitative sleep/wake characteristics are identical among three inbred strains of the C57BL subfamily. Every sleep parameter was extensively examined before proceeding to the actual mutagenesis screening. Parameters for which the %RSD values are constantly less than 20% across the strains are shown in red; these can be considered “tight” parameters, suitable for use in the primary genetic screening as described in the text.



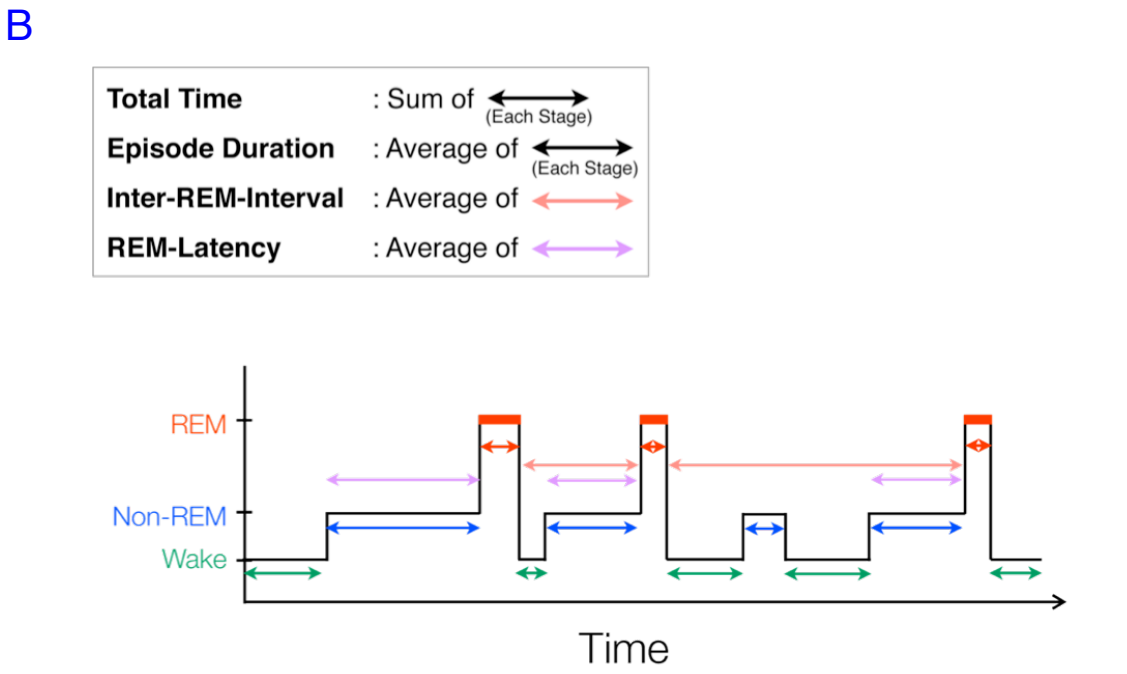


Figure 4-2 Experimental protocol for primary screening and some of the quantitative sleep parameters for phenotyping. (A) Before the actual recording session is started, mice are fully acclimated with the wired recording environment and light/dark cycles. At least 2 days of basal EEG/EMG are recorded first. On the following day, the mice are sleep-deprived using gyratory shakers (120 rpm, programmed to shake/stop for 1-10 seconds at random) for 4 hours at the beginning of light phase, followed by a recovery sleep recording until the end of the day. The housing cage is changed before sleep deprivation, so that normal mice will not sleep for another hour or so even after the end of the deprivation period. This allows screening for abnormal response to forced as well as spontaneously prolonged waking. (B) Once all the sleep stages are determined by the automated sleep-scoring system, the software calculates basic sleep parameters on an hourly basis.

Center Sequencing Core in UT Southwestern Medical Center). Combining the linkage analysis result, which has a resolution of chromosomal region about ~25Mbp, and the next-generation deep sequencing data, we are able to pinpoint the single mutation that causes the sleep phenotype.

Results of G1/F1 screening

Examining ~7,000 G1/F1 mice through the primary screen, we have so far found 39 initial phenodeviant G1/F1 mice. Although many of these mice represented non-heritable deviations, several promising pedigrees were identified. Now, we have already mapped 4 causal mutations from 4 different pedigrees/phenotypes out of 11 pedigrees with confirmed heritability.

In the next two sections, the two pedigrees for which the causal mutations were already identified will be described in some details.

Example phenodeviant 1 - Markedly long NREM sleep time / short wake time

This mouse shows significantly increased total sleep time in the basal recordings (Figure 4-3A and 4-3B). Although mice are nocturnal species, his wake time does not increase during the dark period, and he spends most of the day in sleep. The total time of wakefulness during the basal period is markedly diminished as compared to the entire G1 cohort, with values more than 3.8 standard deviation below from the population mean (Figure 4-3C). Importantly, the mouse is otherwise healthy and his other behaviors including sleep homeostasis and reductions of delta power during recovery phase after forced sleep

deprivation are overtly normal on visual inspections. This mouse has been bred with wildtype C57BL/6N females in order to examine phenotype heritability. We observed clear double peaks in both of F1 and N2 generations, confirming the heritability (Figure 4-3D and 4-4).

After we took tail DNAs from all the N2 mice (n=110 with 55 putative m/+ and 55 putative +/+), we performed QTL analysis using R/qtl software (Broman et al., 2003; Smith et al., 2009). Genotyping was based on a panel of SNPs between C57BL/6J and C57BL/6N, which was recently assertained and confirmed in RIKEN BioResource Center in collaboration with Vivek Kumar (Kumar et al., 2013) in Takahashi laboratory at UT Southwestern (Figure 4-5). The result of the QTL analysis (with 24-hour wake time as quantitative trait) was impressive since a single peak with LOD score of >35 was detected on chromosome 9 (Figure 4-6). While there are more than 400 genes within this QTL region, we utilized a next-generation sequencing, particularly whole-exome sequencing, to narrow them down to a single nucleotide level. Indeed, the sequencing identified only one ENU-induced mutation within the region, and we named the mutated allele as *Sleepy*, after we confirmed the mutation by conventional direct sequencing on multiple affected mice, which predicted the hypersomnia phenotype with a 100% accuracy (Figure 4-7).

It is important to note that we would be unable to isolate the *Sleepy* mutant if we had used home cage locomotor activity as a surrogate readout for sleep/wake behavior (Tang et al., 2002) in the large-scale screening (Figure 4-8). Even though the retrospective statistical analysis shows a significant reduction of the dark-phase and 24-hour home cage activity in the *Sleepy* mutants, the reduction is within 1 standard deviation from the mean value of G1 population, which could not have been detected in the primary screen. The surrogate

parameter would not clearly separate two populations of mutant and normal mice at F1/N2 generations, either.

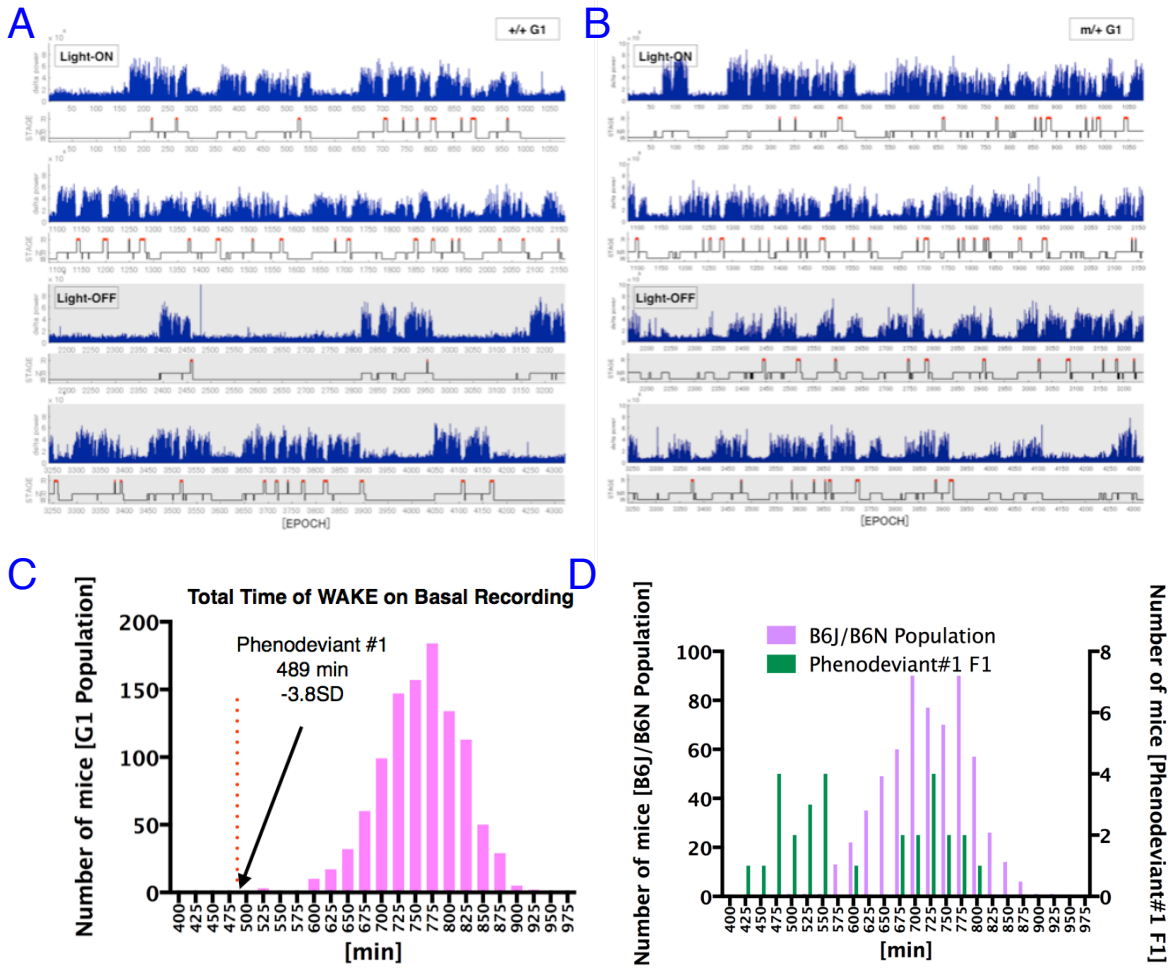


Figure 4-3 Sleep/wake characterization of phenodeviant #1. (A and B) Representative 24-hr basal hypnograms for a wild-type mouse (A) compared with Phenodeviant #1 (B). W represents a period of Wakefulness; NR, NREM sleep; R, REM sleep. (C and D) Histograms showing the distributions of 24-hour total time of wakefulness for basal recording in the G1 primary screen (C) and in the F1 generation (D).

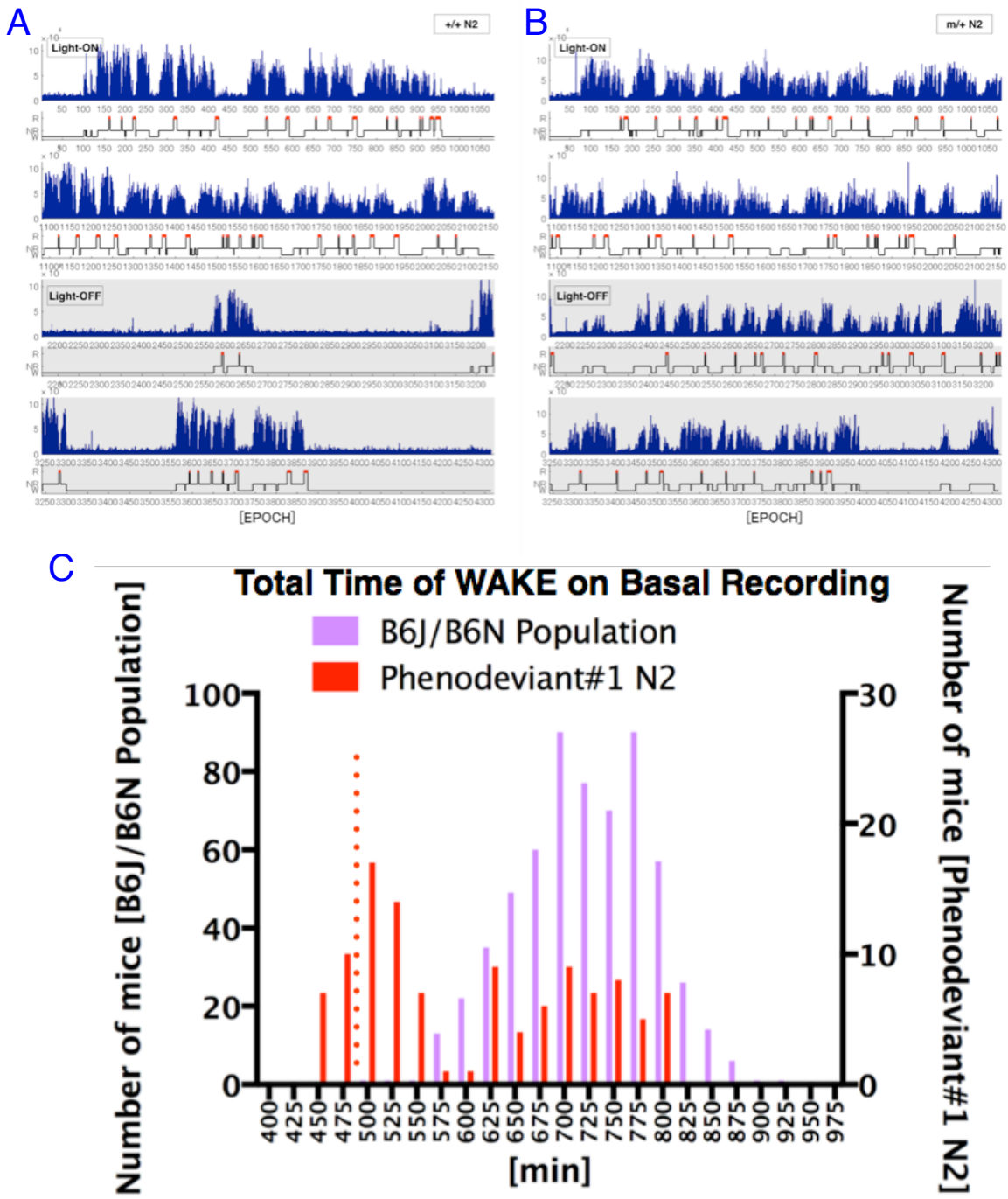


Figure 4-4 Hypersomnia phenotype is transmitted to N2 generation of phenodeviant #1. (A and B) Representative 24-hour basal hypnograms for N2 siblings with (B) and without (A) the phenotype. Clear double peaks for 24-hour wake time in the N2 generation are observed (C).

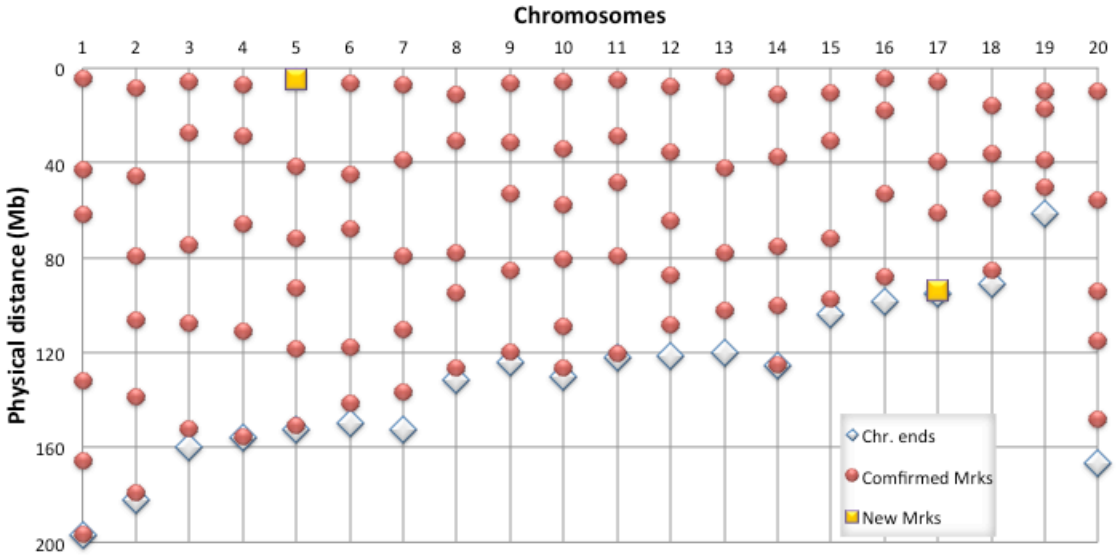


Figure 4-5 Panel of SNP markers between C57BL/6J and C57BL/6N strains. We utilized these markers to narrow the QTL region down to the subchromosomal level. Markers were originally constructed by Vivek Kumar in the Takahashi lab at UT Southwestern, and supplemented by Wakana lab at RIKEN Tsukuba.

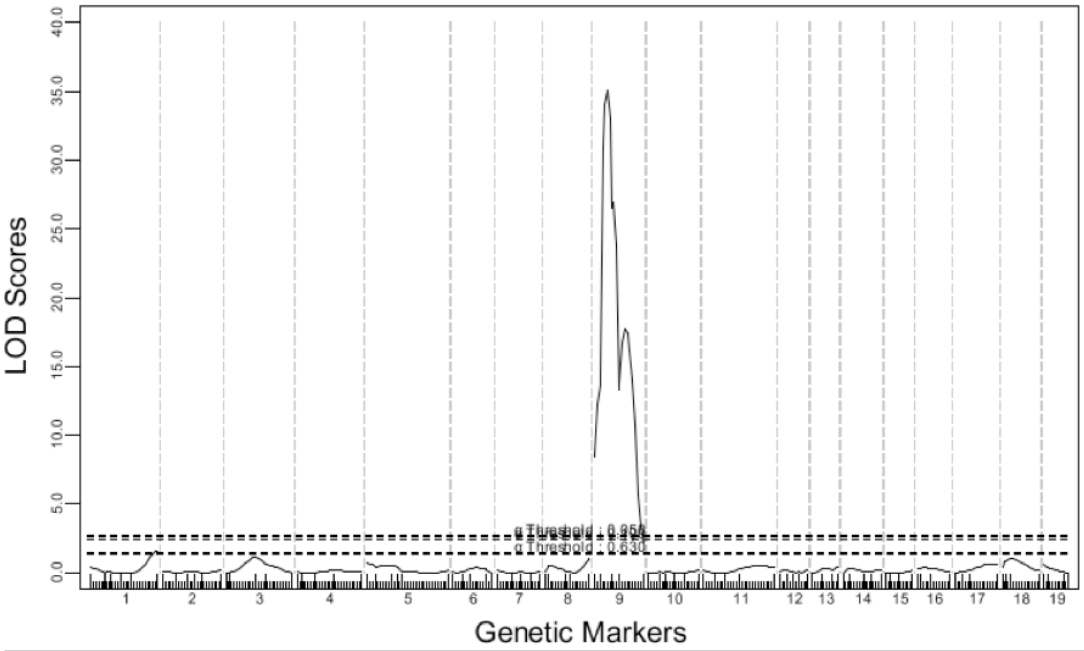


Figure 4-6 Linkage analysis for phenodeviant #1. The QTL analysis with C57BL/6J x C57BL/6N N2 mice successfully revealed single peak on chromosome 9 with a peak LOD score of more than 35. (putative m/+: n = 55, putative +/+: n = 55)

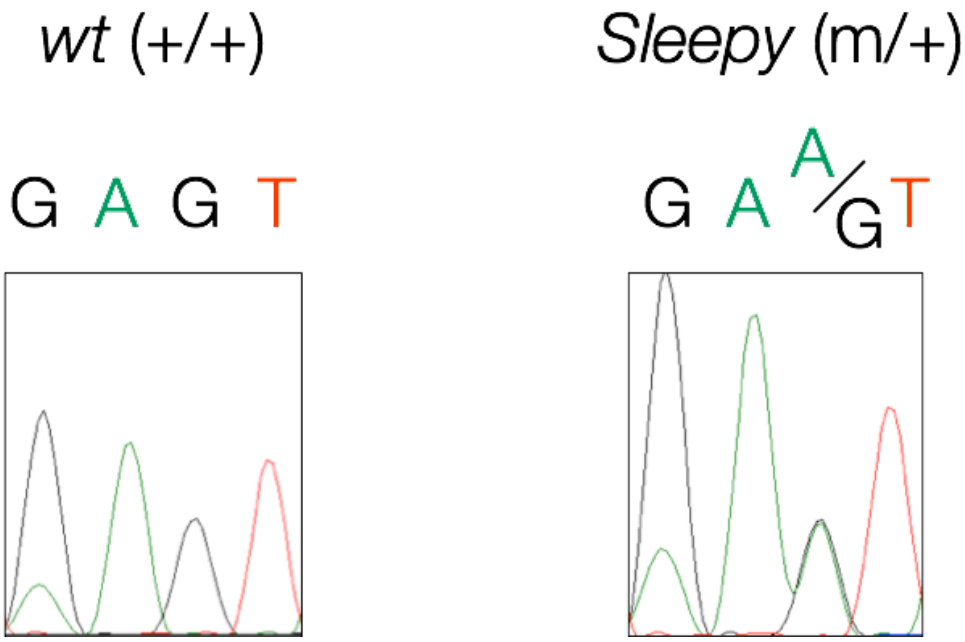


Figure 4-7 Direct sequencing on mutated region of phenodeviant #1. Whole-exome sequencing revealed that the mutant mice harbor a G to A transversion in the candidate locus named *Sleepy*.

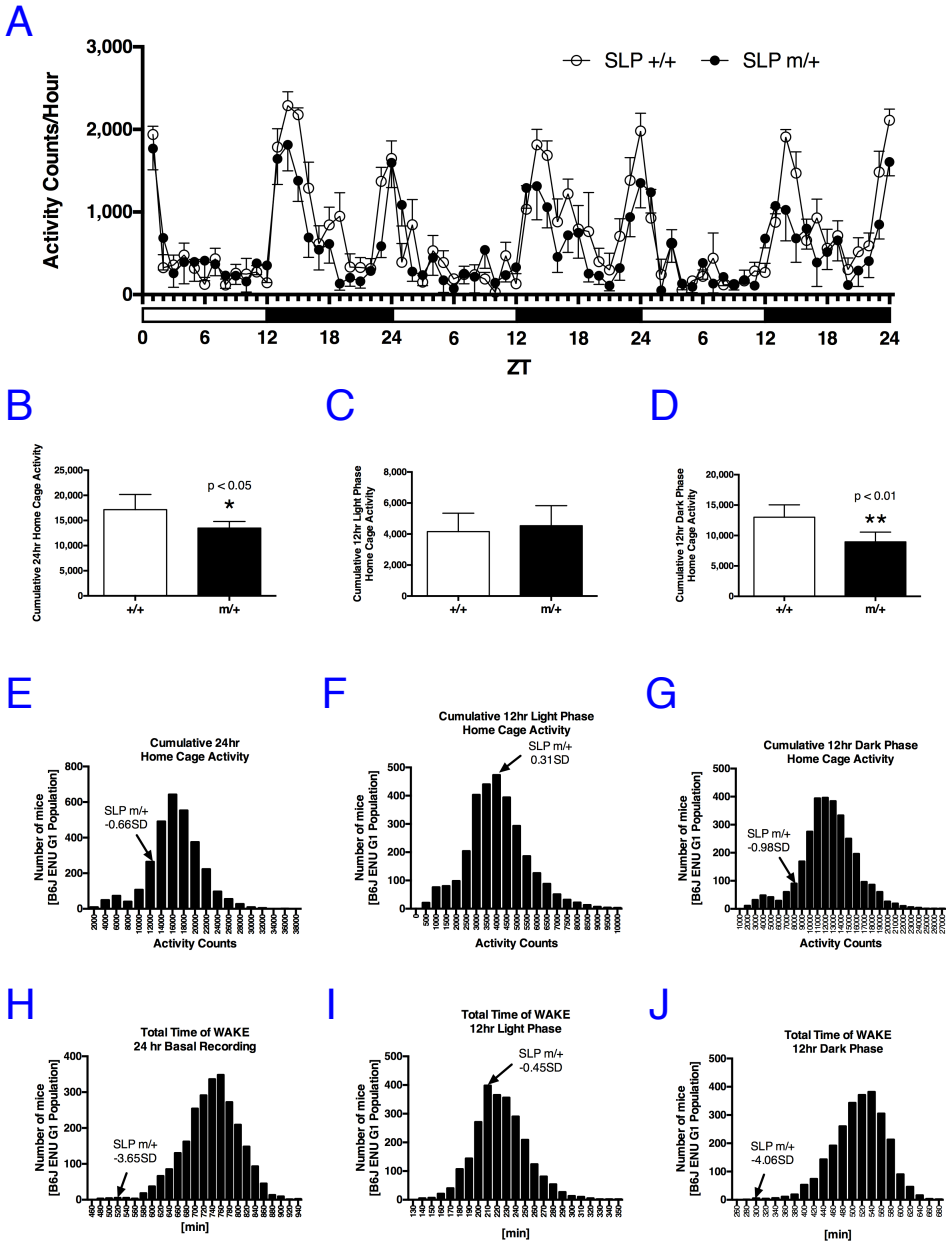


Figure 4-8 Sleep abnormalities in the *Sleepy* mutant could not have been detected using home cage locomotor activity as surrogate readout in large-scale screening. (A-D) Home cage activity of *Sleepy* (SLP) mutants and normal mice (n = 5 each) over 3 days of recording (A), and averaged total activity counts during 24-hour day (B), 12-hour light phase (C), and 12-hour dark phase (D). (E-J) Although statistical analysis of multiple mutant mice reveals reduced total home cage activities, these differences are within 1 standard deviation (SD) below the G1 population mean (E and G), which we would have been unable to pick up during the large-scale screen. In contrast, the EEG/EMG-based wake time in the *Sleepy* founder is ~4 x SD deviated from the population mean (H and J).

Example phenodeviant 2 - Short, highly fragmented REM sleep

This mouse shows a markedly reduced mean episode duration of REM sleep throughout the recording period (Figure 4-9). Other sleep parameters, such as total time of wakefulness and NREM sleep, are within normal ranges, except for a reduction of total REM sleep time during basal recording (Figure 4-9C and 4-9D). No overt abnormalities on sleep homeostasis or delta power reductions are detected by visual inspection during recovery period from forced sleep deprivation.

To test the heritability of this phenotype, we crossed this mouse with wildtype C57BL/6N females to produce F1 offspring. As shown in Figure 4-10, about half of the offspring from this phenodeviant G1 exhibit the same phenotype as G1, suggesting that this sleep behavior is indeed due to a Mendelian dominant mutation.

We then performed QTL analysis after generating 56 N2 males (23 putative m/+ and 33 putative +/+) from the F1 mouse which had the shortest mean episode duration of REM sleep (Figure 4-11). The linkage analysis revealed a clear, significant peak in a ~25 Mb interval on chromosome 14 (Figure 4-12), and from whole-exome sequencing, we identified a single ENU-induced missense mutation within that region. Also, the mutation was verified by conventional direct sequencing as in Figure 4-12B, and we named the allele as *Dreamless*.

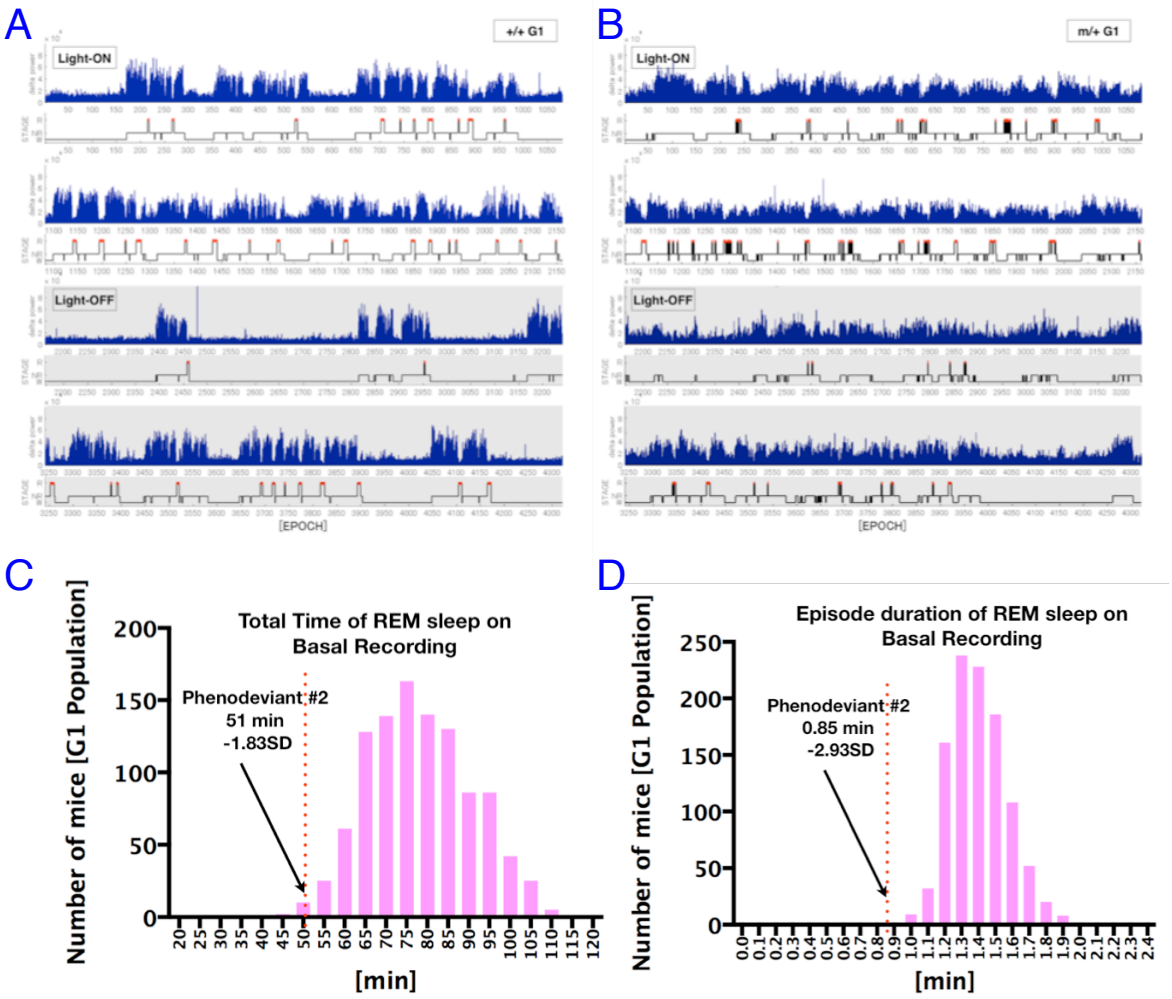


Figure 4-9 REM sleep abnormality of Phenodeviant #2. (A and B) Representative 24-hour basal hypnograms for a phenotypically normal mouse (A) compared with Phenodeviant #2 (B). Wrepresents a period of Wakefulness; NR, NREM sleep; R, REM sleep. Phenodeviant #2 shows highly fragmented REM sleep episodes throughout the EEG/EMG recording session. (C and D) Statistical analysis of total REM sleep time reveals a reduced total REM sleep time with 1.83 x standard deviation below the G1 population mean (C), whereas mean episode duration of REM sleep provides a stronger index of phenotype (D).

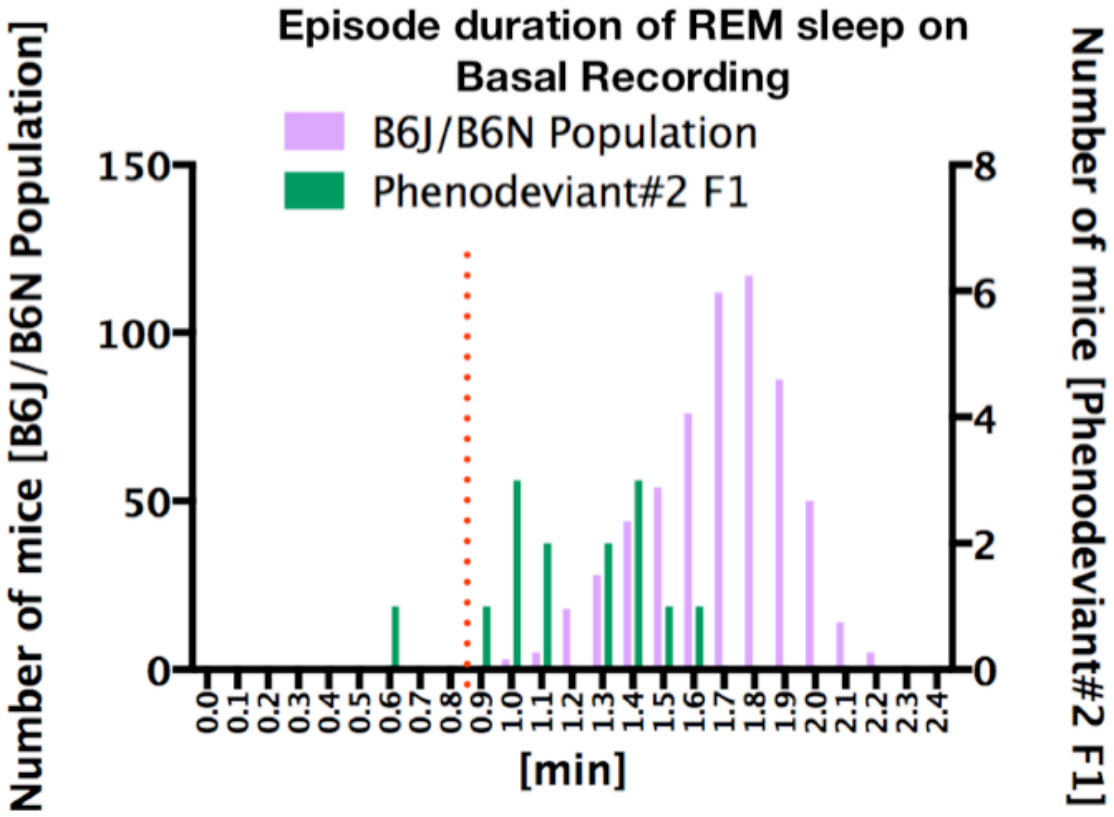


Figure 4-10 Histogram showing the distributions of mean episode duration of REM sleep on F1 mice of phenodeviant #2. The double peaks on F1 distribution are observed. The F1 mouse with shortest episode duration of REM sleep, which exhibited a duration even shorter than the G1 founder phenodeviant, was used for generating N2 mice.

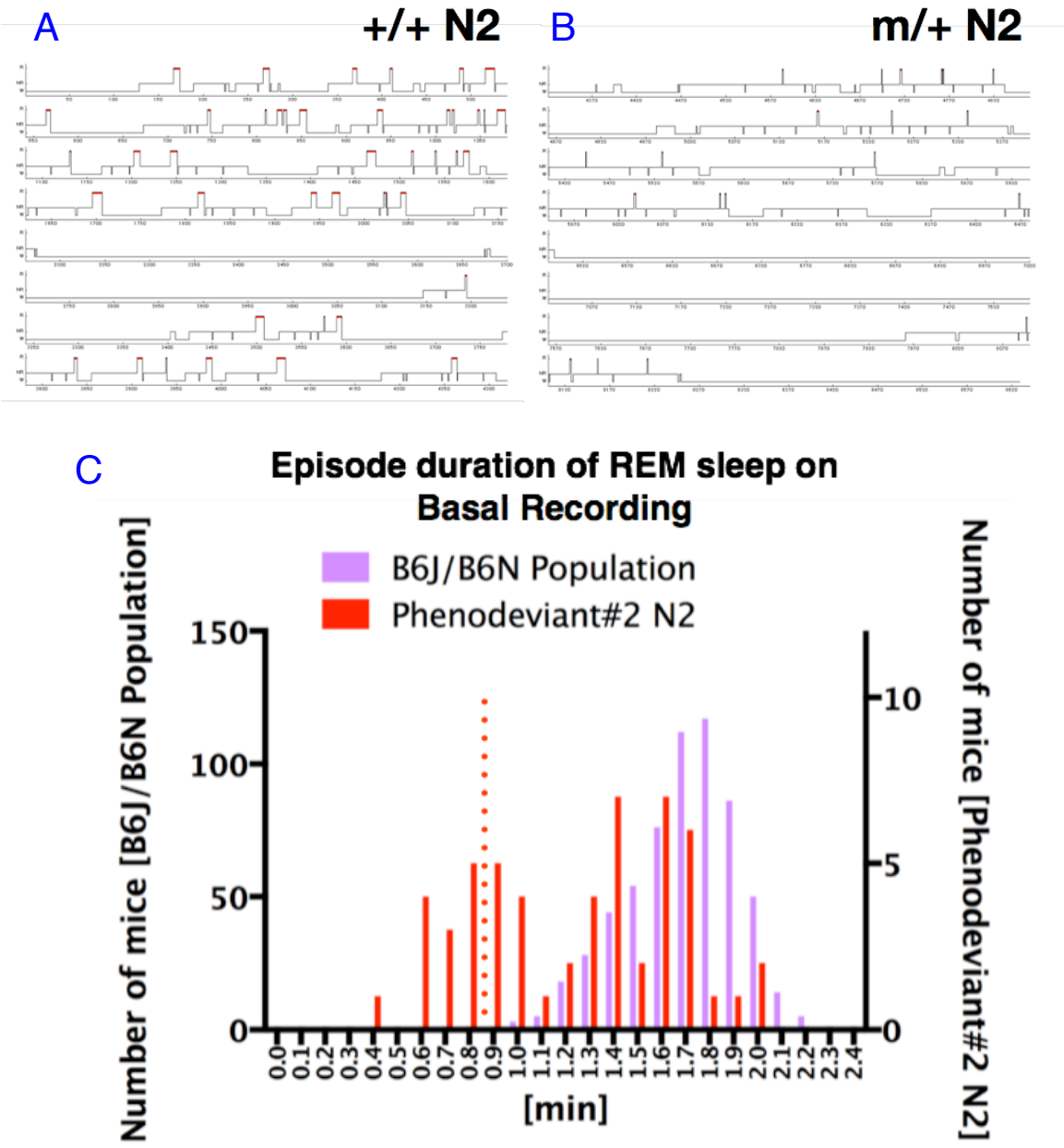


Figure 4-11 The REM sleep abnormality of Phenodeviant #2 is heritable at the N2 generation. (A and B) Representative 24-hour basal hypnograms for siblings of N2 mice with (B) and without (A) the short, fragmented REM phenotype. W represents a period of Wakefulness; NR, REM sleep; R, REM sleep.

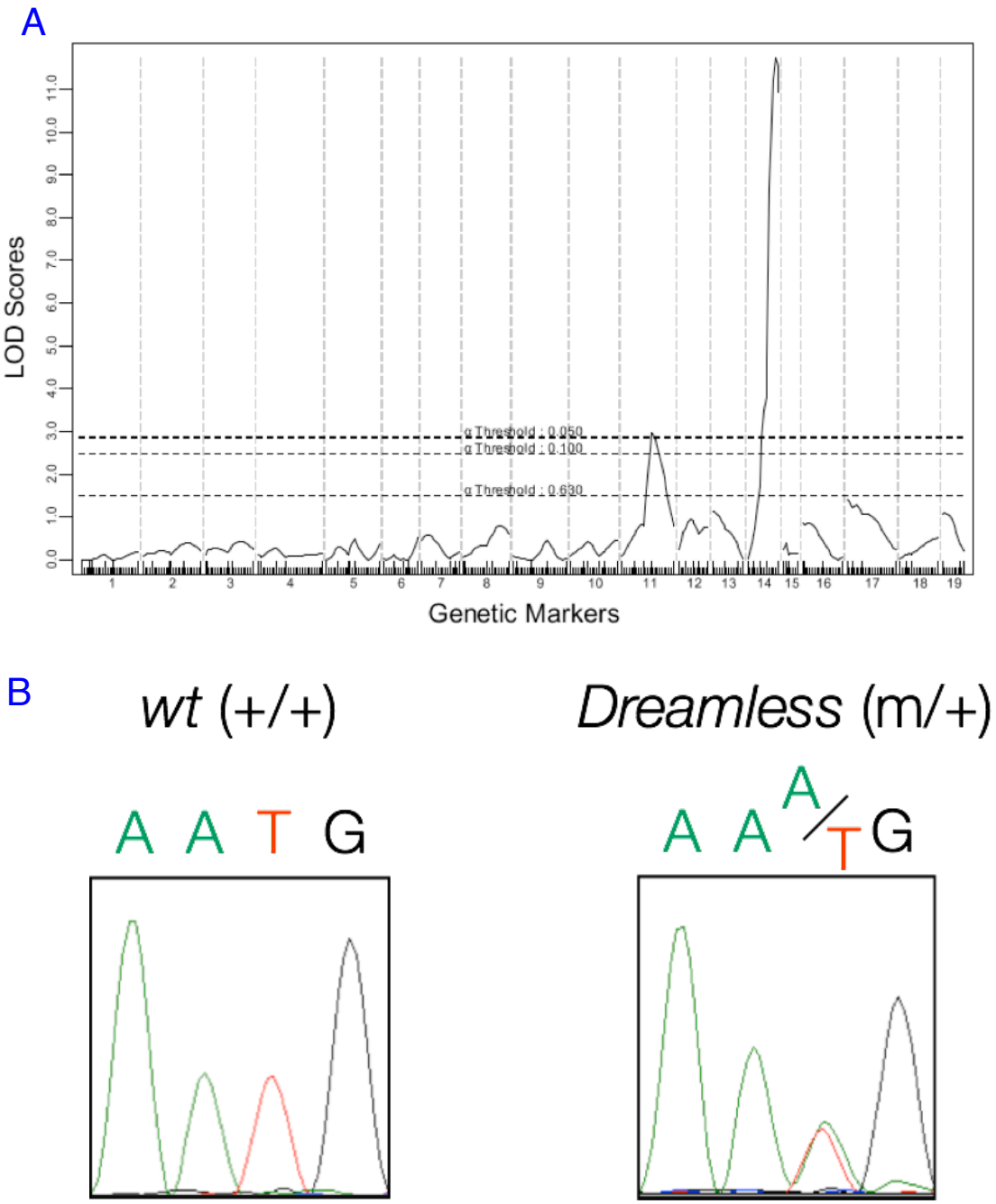


Figure 4-12 Mapping of Phenodeviant #2. Linkage analysis revealed a QTL peak on chromosome 14 with a LOD score of 11 (A). (putative m/+: n = 23, putative +/+: n = 33) Whole-exome sequencing revealed a T to A transversion on the candidate locus, named *Dreamless* (B).

Discussion

An individual grows increasingly sleepy after a long period of wakefulness, and taking a nap is the only way to dissipate such sleepiness. The fundamental neural mechanism for this powerful feedback control, called homeostatic sleep regulation, remains a mystery. The amount of sleep per 24-h day is kept relatively constant for each species partly because of this feedback mechanism, but it also remains mysterious as to how the "set point" for sleep/wake amounts is determined and maintained. Forward genetic analysis can be a powerful approach for such a biological problem with no obvious exploratory entry point. However, as far as I know, forward genetic analysis of sleep based on true polysomnographic (EEG/EMG-based) phenotyping has never been attempted in mammals simply because it has been deemed too labor-intensive.

Here, I describe a forward genetic screen of "true" sleep phenotype in ENU-mutagenized mice, which we initiated three years ago. Indeed, our EEG/EMG-based screen identified a variety of mutants; such as a pedigree with markedly long NREM sleep time and a pedigree that exhibits short and fragmented REM sleep. ENU mutagenesis in inbred mice with a well-characterized genetic background ensured the determination of causal mutations for the identified sleep phenotypes, demonstrated in successful mapping of two causal mutations mentioned in this chapter. The use of two extremely closely related inbred strains, C57BL/6J and C57BL/6N with no detectable sleep/wake differences, ensured no interference from inter-strain QTLs, while providing enough inter-strain SNP markers at least for relatively crude linkage analysis. We found that, when combined with next-generation,

whole-exome deep sequencing, the relatively crude QTL analysis with ~100 SNP markers and 50-100 N2 mice was indeed sufficient to identify the single, ENU-induced causal mutation (Fairfield et al., 2011).

In order to claim a candidate mutation from a mapped QTL as a causal gene for the associated phenotype, the following types of evidence are required (Glazier et al., 2002; Abiola et al., 2003; Flint et al., 2005).

- Two or more different mutant alleles within the same gene
- Mutation resulting in an obviously drastic change of the encoded protein, such as frame shift, missense mutation at a highly conserved residue, and splicing mutation
- Mutation resulting in altered gene function or expression levels demonstrable in experiments in vivo or in vitro
- Duplication of phenotype by transgenic expression of the mutant allele or by reproducing the mutant allele by genetic knock-ins or genome editing strategies
- Genetic interaction of the mutant allele with null (or hypomorph) allele
- Demonstration of a mutant allele in another species

Currently, genetic verification of identified mutations is underway utilizing the zinc finger nuclease (ZFN) (Miller et al., 2007; Wood et al., 2011) and CRISPR/Cas (Cho et al., 2013; Cong et al., 2013) technologies, inducing the same mutations and/or functionally equivalent mutations on the mouse genome, which can be either homozygous or heterozygous already at the transgenic founder generation. In addition to the genetic verification, we are also performing in vitro functional studies of the mutated genes in transfected cells, which can be used to test the effects of the mutant alleles on relevant cellular phenotypes.

Outlook and Future Studies

Through this large-scale sleep screening in mice, we found that a heterozygous single-nucleotide change can induce a significant change in sleep behavior, implying the existence of nonredundant and so-far undiscovered molecular mechanisms of sleep regulation.

More studies must be performed to understand the role of the identified genes in vitro and in vivo in order to clarify the detailed biochemical/physiological mechanisms through which these genes govern sleep/wake regulations. In particular, we are analyzing the biochemical consequences of these mutations in vitro using transfected cell lines. We have been analyzing allelic interactions of the identified ENU-induced mutations with a number of targeted mutant alleles including knockouts, by measuring sleep/wake behavior in mice carrying different allele combinations. We have also been elucidating how and where in the brain these genetic changes eventually lead to the organismal-level sleep phenotype, utilizing viral- and transgene-mediated expression of mutant alleles.

With respect to furthering the genetic screen, a modifier screen, such as an enhancer or suppressor screen starting with the identified mutants, may be useful in order to discover additional genes working in the same genetic pathway. Even more straight forward, the continuation of the ongoing primary mutagenesis screen in order to identify additional mutant pedigrees will be also highly valuable, since accumulation of mutations in different genes that result in similar phenotypes (e.g., an increased sleep time) will eventually start to point to a biological pathway that dictates the phenotype. Since the fundamental molecular

mechanism of sleep/wake behavior remains mysterious, the results from these unbiased screens will continue to be highly valuable. We will eventually be able to connect the genetic "dots" with each other by finding missing pieces of the biologic puzzle, in order to reveal the entire picture.

We began our studies with the aim of gaining a fundamental understanding of sleep. Our forward genetic screening system in sleep/wake behavior in mice indeed has proven to be a powerful tool to identify candidate sleep-regulating genes. Since the mutant traits we found are very severe even in heterozygotes, we speculate that the mutated genes are at or near the very core of the molecular pathway regulating sleep in mice. Eventually, finding such sleep-regulating genes and pathways in mammals will make an enormous impact on the entire field of neuroscience.

BIBLIOGRAPHY

Abiola O et al. (2003) The nature and identification of quantitative trait loci: a community's view. Nat Rev Genet 4:911–916.

Allada R, Siegel JM (2008) Unearthing the phylogenetic roots of sleep. Curr Biol 18:R670–R679.

Allebrandt KV et al. (2013) A K(ATP) channel gene effect on sleep duration: from genome-wide association studies to function in Drosophila. Mol Psychiatry 18:122–132.

Aserinsky E, Kleitman N (1953) Regularly occurring periods of eye motility, and concomitant phenomena, during sleep. Science 118:273–274.

Aston-Jones G, Bloom FE (1981) Activity of norepinephrine-containing locus coeruleus neurons in behaving rats anticipates fluctuations in the sleep-waking cycle. J Neurosci 1:876–886.

Berthomier C, Drouot X, Herman-Stoïca M, Berthomier P, Prado J, Bokar-Thire D, Benoit O, Mattout J, d'Ortho M-P (2007) Automatic analysis of single-channel sleep EEG: validation in healthy individuals. Sleep 30:1587–1595.

Bjorness TE, Kelly CL, Gao T, Poffenberger V, Greene RW (2009) Control and function of the homeostatic sleep response by adenosine A1 receptors. J Neurosci 29:1267–1276.

Borbély AA (1982) A two process model of sleep regulation. Hum Neurobiol 1:195–204.

Borbély AA, Achermann P (1999) Sleep homeostasis and models of sleep regulation. J Biol Rhythms 14:557–568.

Brankack J, Kukushka VI, Vyssotski AL, Draguhn A (2010) EEG gamma frequency and sleep-wake scoring in mice: comparing two types of supervised classifiers. Brain Res 1322:59–71.

Broman KW, Wu H, Sen S, Churchill GA (2003) R/qtl: QTL mapping in experimental crosses. Bioinformatics 19:889–890.

Chamberlin NL, Arrigoni E, Chou TC, Scammell TE, Greene RW, Saper CB (2003) Effects of adenosine on gabaergic synaptic inputs to identified ventrolateral preoptic neurons. Neuroscience 119:913–918.

Chemelli RM, Willie JT, Sinton CM, Elmquist JK, Scammell T, Lee C, Richardson JA, Williams SC, Xiong Y, Kisanuki Y, Fitch TE, Nakazato M, Hammer RE, Saper CB, Yanagisawa M (1999) Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. Cell 98:437–451.

Cho SW, Kim S, Kim JM, Kim J-S (2013) Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. Nat Biotechnol 31:230–232.

Chong Y, Fryar CD, Gu Q (2013) Prescription Sleep Aid Use Among Adults: United States. NCHS Data Brief.

Cirelli C, Bushey D, Hill S, Huber R, Kreber R, Ganetzky B, Tononi G (2005) Reduced sleep in Drosophila Shaker mutants. Nature 434:1087–1092.

Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F (2013) Multiplex genome engineering using CRISPR/Cas systems. Science 339:819–823.

Datta S, Maclean RR (2007) Neurobiological mechanisms for the regulation of mammalian sleep-wake behavior: reinterpretation of historical evidence and inclusion of contemporary cellular and molecular evidence. Neurosci Biobehav Rev 31:775–824.

Doman J, Detka C, Hoffman T, Kesicki D, Monahan JP, Buysse DJ, Reynolds CF, Coble PA, Matzzie J, Kupfer DJ (1995) Automating the sleep laboratory: implementation and validation of digital recording and analysis. Int J Biomed Comput 38:277–290.

Flint J, Valdar W, Shifman S, Mott R (2005) Strategies for mapping and cloning quantitative trait genes in rodents. Nat Rev Genet 6:271–286.

Fornal C, Auerbach S, Jacobs BL (1985) Activity of serotonin-containing neurons in nucleus raphe magnus in freely moving cats. Exp Neurol 88:590–608.

Frankel WN, Yang Y, Mahaffey CL, Beyer BJ, O'Brien TP (2009) Szt2, a novel gene for seizure threshold in mice. Genes Brain Behav 8:568–576.

Franken P, Chollet D, Tafti M (2001) The homeostatic regulation of sleep need is under genetic control. J Neurosci 21:2610–2621.

Franken P, Malafosse A, Tafti M (1998) Genetic variation in EEG activity during sleep in inbred mice. Am J Physiol 275:R1127–R1137.

Funato H, Sato M, Sinton CM, Gautron L, Williams SC, Skach A, Elmquist JK, Skoultchi AI, Yanagisawa M (2010) Loss of Goosecoid-like and DiGeorge syndrome

critical region 14 in interpeduncular nucleus results in altered regulation of rapid eye movement sleep. Proc Natl Acad Sci USA 107:18155–18160.

Furuse T et al. (2010) Phenotypic characterization of a new Grin1 mutant mouse generated by ENU mutagenesis. Eur J Neurosci 31:1281–1291.

Glazier AM, Nadeau JH, Aitman TJ (2002) Finding genes that underlie complex traits. Science 298:2345–2349.

He Y, Jones CR, Fujiki N, Xu Y, Guo B, Holder JL, Rossner MJ, Nishino S, Fu Y-H (2009) The transcriptional repressor DEC2 regulates sleep length in mammals. Science 325:866–870.

Hoebe K, Beutler B (2008) Forward genetic analysis of TLR-signaling pathways: an evaluation. Adv Drug Deliv Rev 60:824–829.

John J, Wu M-F, Boehmer LN, Siegel JM (2004) Cataplexy-active neurons in the hypothalamus: implications for the role of histamine in sleep and waking behavior. Neuron 42:619–634.

Ko EM, Estabrooke IV, McCarthy M, Scammell TE (2003) Wake-related activity of tuberomammillary neurons in rats. Brain Res 992:220–226.

Koh K, Joiner WJ, Wu MN, Yue Z, Smith CJ, Sehgal A (2008) Identification of SLEEPLESS, a sleep-promoting factor. Science 321:372–376.

Kohtoh S, Taguchi Y, Matsumoto N (2008) Algorithm for sleep scoring in experimental animals based on fast Fourier transform power spectrum analysis of the electroencephalogram. Sleep and Biological ….

Kumar V, Kim K, Joseph C, Kourrich S, Yoo S-H, Huang HC, Vitaterna MH, de Villena FP-M, Churchill G, Bonci A, Takahashi JS (2013) C57BL/6N mutation in Cytoplasmic FMRP interacting protein 2 regulates cocaine response. Science 342:1508–1512.

Kumar V, Kim K, Joseph C, Thomas LC, Hong H, Takahashi JS (2011) Second-generation high-throughput forward genetic screen in mice to isolate subtle behavioral mutants. Proc Natl Acad Sci USA 108 Suppl 3:15557–15564.

Kume K, Kume S, Park SK, Hirsh J, Jackson FR (2005) Dopamine is a regulator of arousal in the fruit fly. J Neurosci 25:7377–7384.

Lee MG, Hassani OK, Alonso A, Jones BE (2005) Cholinergic basal forebrain neurons burst with theta during waking and paradoxical sleep. J Neurosci 25:4365–4369.

Lloyd DJ, Hall FW, Tarantino LM, Gekakis N (2005) Diabetes insipidus in mice with a mutation in aquaporin-2. PLoS Genet 1:e20.

Mackiewicz M, Paigen B, Naidoo N, Pack AI (2008) Analysis of the QTL for sleep homeostasis in mice: Homer1a is a likely candidate. Physiol Genomics 33:91–99.

Maret S, Dorsaz S, Gurcel L, Pradervand S, Petit B, Pfister C, Hagenbuchle O, O'Hara BF, Franken P, Tafti M (2007) Homer1a is a core brain molecular correlate of sleep loss. Proc Natl Acad Sci USA 104:20090–20095.

Masuya H et al. (2004) Development and implementation of a database system to manage a large-scale mouse ENU-mutagenesis program. Mamm Genome 15:404–411.

Miller JC, Holmes MC, Wang J, Guschin DY, Lee Y-L, Rupniewski I, Beausejour CM, Waite AJ, Wang NS, Kim KA, Gregory PD, Pabo CO, Rebar EJ (2007) An improved zinc-finger nuclease architecture for highly specific genome editing. Nat Biotechnol 25:778–785.

Moruzzi G, Magoun HW (1949) Brain stem reticular formation and activation of the EEG. Electroencephalogr Clin Neurophysiol 1:455–473.

Naidoo N, Ferber M, Galante RJ, McShane B, Hu JH, Zimmerman J, Maislin G, Cater J, Wyner A, Worley P, Pack AI (2012) Role of Homer proteins in the maintenance of sleep-wake states. PLoS ONE 7:e35174.

NHLBI (National Heart, Lung, and Blood Institute). National Sleep Disorders Research Plan, 2003. Bethesda, MD: National Institutes of Health; 2003.

Peyron C, Tighe DK, van den Pol AN, de Lecea L, Heller HC, Sutcliffe JG, Kilduff TS (1998) Neurons containing hypocretin (orexin) project to multiple neuronal systems. J Neurosci 18:9996–10015.

Sakurai T et al. (1998) Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell 92:573–585.

Sakurai T, Nagata R, Yamanaka A, Kawamura H, Tsujino N, Muraki Y, Kageyama H, Kunita S, Takahashi S, Goto K, Koyama Y, Shioda S, Yanagisawa M (2005) Input of orexin/hypocretin neurons revealed by a genetically encoded tracer in mice. Neuron 46:297–308.

Sandberg JK, Ljunggren H-G (2005) Development and function of CD1d-restricted NKT cells: influence of sphingolipids, SAP and sex. Trends Immunol 26:347–349.

Saper CB, Scammell TE, Lu J (2005) Hypothalamic regulation of sleep and circadian rhythms. Nature 437:1257–1263.

Sharkey LM, Cheng X, Drews V, Buchner DA, Jones JM, Justice MJ, Waxman SG, Dib-Hajj SD, Meisler MH (2009) The ataxia3 mutation in the N-terminal cytoplasmic domain of sodium channel Na(v)1.6 disrupts intracellular trafficking. J Neurosci 29:2733–2741.

Sherin JE, Elmquist JK, Torrealba F, Saper CB (1998) Innervation of histaminergic tuberomammillary neurons by GABAergic and galaninergic neurons in the ventrolateral preoptic nucleus of the rat. J Neurosci 18:4705–4721.

Siepka SM, Yoo S-H, Park J, Song W, Kumar V, Hu Y, Lee C, Takahashi JS (2007) Circadian mutant Overtime reveals F-box protein FBXL3 regulation of cryptochrome and period gene expression. Cell 129:1011–1023.

Sinton CM, Kovakkattu D, Friese RS (2009) Validation of a novel method to interrupt sleep in the mouse. J Neurosci Methods 184:71–78.

Smith R, Sheppard K, DiPetrillo K, Churchill G (2009) Quantitative trait locus analysis using J/qtl. Methods Mol Biol 573:175–188.

Starzl TE, Taylor CW, Magoun HW (1951) Ascending conduction in reticular activating system, with special reference to the diencephalon. J Neurophysiol 14:461–477.

Steininger TL, Alam MN, Gong H, Szymusiak R, McGinty D (1999) Sleep-waking discharge of neurons in the posterior lateral hypothalamus of the albino rat. Brain Res 840:138–147.

Sunagawa GA, Séi H, Shimba S, Urade Y, Ueda HR (2013) FASTER: an unsupervised fully automated sleep staging method for mice. Genes Cells 18:502–518.

Tabeta K, Hoebe K, Janssen EM, Du X, Georgel P, Crozat K, Mudd S, Mann N, Sovath S, Goode J, Shamel L, Herskovits AA, Portnoy DA, Cooke M, Tarantino LM, Wiltshire T, Steinberg BE, Grinstein S, Beutler B (2006) The Unc93b1 mutation 3d disrupts exogenous antigen presentation and signaling via Toll-like receptors 3, 7 and 9. Nat Immunol 7:156–164.

Tafti M, Chollet D, Valatx JL, Franken P (1999) Quantitative trait loci approach to the genetics of sleep in recombinant inbred mice. J Sleep Res 8 Suppl 1:37–43.

Tafti M, Franken P, Kitahama K, Malafosse A, Jouvet M, Valatx JL (1997) Localization of candidate genomic regions influencing paradoxical sleep in mice. Neuroreport 8:3755–3758.

Takahashi JS, Pinto LH, Vitaterna MH (1994) Forward and reverse genetic approaches to behavior in the mouse. Science 264:1724–1733.

Takahashi JS, Shimomura K, Kumar V (2008) Searching for genes underlying behavior: lessons from circadian rhythms. Science 322:909–912.

Tang X, Orchard SM, Sanford LD (2002) Home cage activity and behavioral performance in inbred and hybrid mice. Behav Brain Res 136:555–569.

Theodoratos A, Whittle B, Enders A, Tscharke DC, Roots CM, Goodnow CC, Fahrer AM (2010) Mouse strains with point mutations in TAP1 and TAP2. Immunol Cell Biol 88:72–78.

Veasey SC, Valladares O, Fenik P, Kapfhamer D, Sanford L, Benington J, Bucan M (2000) An automated system for recording and analysis of sleep in mice. Sleep 23:1025–1040.

Vitaterna MH, King DP, Chang AM, Kornhauser JM, Lowrey PL, McDonald JD, Dove WF, Pinto LH, Turek FW, Takahashi JS (1994) Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. Science 264:719–725.

Wilkes JJ, Lloyd DJ, Gekakis N (2009) Loss-of-function mutation in myostatin reduces tumor necrosis factor alpha production and protects liver against obesity-induced insulin resistance. Diabetes 58:1133–1143.

Wood AJ, Lo T-W, Zeitler B, Pickle CS, Ralston EJ, Lee AH, Amora R, Miller JC, Leung E, Meng X, Zhang L, Rebar EJ, Gregory PD, Urnov FD, Meyer BJ (2011) Targeted genome editing across species using ZFNs and TALENs. Science 333:307.

Wu C, Wais M, Zahid T, Wan Q, Zhang L (2009) An improved screw-free method for electrode implantation and intracranial electroencephalographic recordings in mice. Behav Res Methods 41:736–741.

Wu MN, Joiner WJ, Dean T, Yue Z, Smith CJ, Chen D, Hoshi T, Sehgal A, Koh K (2010) SLEEPLESS, a Ly-6/neurotoxin family member, regulates the levels, localization and activity of Shaker. Nat Neurosci 13:69–75.