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SLEEP ALTERATIONS IN MOUSE GENETIC MODELS OF HUMAN DISEASE

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SLEEP ALTERATIONS IN MOUSE GENETIC MODELS OF HUMAN DISEASE

DISSERTATION

A dissertation submitted in partial fulfillment of the Requirements for the degree of Doctor of Philosophy in the College of Arts and Sciences at the University of Kentucky

By
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ABSTRACT OF DISSERTATION

SLEEP ALTERATIONS IN MOUSE GENETIC MODELS OF HUMAN DISEASE

Sleep is a process essential for the well-being of an animal and in humans as much as one-third of our life is spent in sleep. Yet, the biological need for sleep still remains a conundrum. Our knowledge of the genes influencing sleep and the mechanisms regulating the process can be advanced with the utilization of genetic and genomic approaches which, in turn, may inform us about the functions of sleep as well. With this goal, I have investigated and examined sleep-wake phenotypes for a variety of transgenic and knock out animals.

For the first part of my research (Chapter 3), I examined mouse models of Alzheimer's disease, and a combined model of Alzheimer's disease (AD) and Diabetes. Sleep disturbances in case of AD are evident long before the onset of cognitive decline. I investigated sleep-wake alterations in 5XFAD, a double transgenic mouse model of AD which displays an early onset of AD pathology and cognitive impairments. We found that these mice have shorter bout lengths under baseline conditions. This was true for both sexes, however, the effect was more prominent in females. Additionally, females also had a shorter duration of sleep compared to control animals. These overall bout length reductions are indicative of increased sleep fragmentation similar to the ones seen in human AD patients.

Inadequate sleep is associated with increased risk for metabolic disorders such as diabetes besides neurodegenerative diseases such as AD. There is also growing evidence that type 2 diabetes mellitus (T2DM) poses an increased risk of AD. To understand how the two conditions interact, we studied a combined mouse model of AD and diabetes (db/AD) which was generated by crossing of db/db (diabetic obese mice) and APP-PS1 (knock-in AD mouse model). The resulting mice showed profound cerebrovascular as well as AD pathology. Both females and males, diabetic AD animals had longer sleep duration compared to non-diabetic AD animals. They also exhibited attenuated sleep-wake rhythms. Females were found to have shorter sleep bouts than males. In addition, significant two way interactions were found for the age and db/AD genotype. Our findings suggest that db genotype and not cerebrovascular pathologies affect sleep in our mouse model. For the last part of my research, we analyzed over 300 single gene knock out mouse lines generated on a C57BL6/NJ background, monitored at The Jackson Laboratory. With this unbiased approach where the knockouts were chosen at random, we identified 55 novel genes affecting various sleep traits, utilizing a variety of statistical approaches. Sex differences were found for a number of knockouts as well as controls. Control females were found to have shorter bout lengths and less sleep duration compared to male littermates.

Keywords: amyloid beta, sleep fragmentation, diabetes, phenotyping, piezoelectric system, knockout mice

Mansi Sethi
December 9, 2016

SLEEP ALTERATIONS IN MOUSE GENETIC MODELS OF HUMAN DISEASE

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December 9, 2016

To the inspirational women of my life,
my mom and my grandmothers

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"All progress takes place outside the comfort zone." - Michael John Bobak

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Chapter 1 Introduction

Sleep is a complex behavior regulated by circadian and homeostatic processes. The circadian process gives time context to most physiological processes including sleep by entraining internal rhythms to the daily photoperiod. On the other hand, the homeostatic process works like an hour glass and tracks sleep need. In the absence of sleep, sleep need accumulates and in the presence of sleep, it dissipates (Borbely 1982, Flores, Flores et al. 2007, Franken, Thomason et al. 2007, Maret, Dorsaz et al. 2007, O'Hara, Ding et al. 2007, Wisor, Striz et al. 2007, Andretic, Franken et al. 2008). Though the two processes develop independently of each other, their interaction determines the sleep and wake parameters of timing, duration, and quality (O'Hara, Jiang et al. 2017).

Many genes related to the circadian system have already been well characterized, however much remains unknown about the homeostatic process of sleep and its general functions (Flores, Flores et al. 2007, Franken, Thomason et al. 2007, Maret, Dorsaz et al. 2007, O'Hara, Ding et al. 2007, Wisor, Striz et al. 2007). Early evidence of genetic regulation of sleep comes from twin studies which showed that monozygotic twins have more similarity in their brain architecture and higher concordance for EEG spectrum traits than dizygotic twins (Stassen, Lykken et al. 1988, Ambrosius, Lietzenmaier et al. 2008, De Gennaro, Marzano et al. 2008). Further, it was found that for many EEG traits more than 80% of the phenotypic variance could be attributed to genetic factors (i.e. 80% heritability).

Sleep disturbances and disorders are common in today's society. An estimated 30 to 40% of adults show signs of insomnia and nearly 5 to 15% have problems with excessive daytime sleepiness (Bamne, Mansour et al. 2010). Inadequate sleep results in cognitive deficits which include increased reaction time, and impaired reasoning and decision making. Such deficits are a serious cause of concern in operational set-ups where they elevate risks of accidents and chances of making fatal errors (Killgore, Rupp et al. 2008), including medical errors (Ker, Edwards et al. 2010, Mongrain, Hernandez et al. 2010, Rillich, Schildberger et al. 2011, Bertram and Rook 2012, Callander, Bolton et al. 2012, Reichert and Gerhardt 2013). Apart from day to day risk, there is growing evidence that sleep disturbances are also a risk factor for disorders such as neurodegenerative diseases

including Alzheimer's and Parkinson's disease, hypertension, obesity, depression, diabetes, heart attack and the metabolic syndrome (Bamne, Mansour et al. 2010, Bertram, Rook et al. 2011, Pack and Pien 2011). Efforts aimed at improving sleep quality and treatment of sleep disorders are limited by our rudimentary knowledge of sleep functions and the mechanisms that regulate these functions. Identification of the genetic influences and elucidation of underlying mechanisms would therefore be critical in developing novel drugs targeting these regulators or other molecules that influence sleep.

One approach to broaden our understanding of sleep regulation is by use of genetic resources such as transgenic and mutant animals. Given that sleep studies in humans tend to be difficult and expensive, it calls for generating animal models for human diseases that recapitulates sleep alterations seen in human conditions. As mentioned earlier, sleep disturbances are often seen in many neurodegenerative diseases including AD. AD is the most common form of dementia and associated sleep perturbations such as increased daytime sleepiness, fragmented sleep is the single biggest cause for institutionalization of patients, as middle of the night wanderings put both AD sufferer and caregivers at increased risk (Pollak and Perlick 1991, Vitiello, Poceta et al. 1991).

AD pathology begins at least 10-15 years before clinical onset of the disease (Kang, Lim et al. 2009, Potvin, Lorrain et al. 2012, Sterniczuk, Theou et al. 2013, Hahn, Wang et al. 2014). Drug regiments successful in animal models have not offered any symptomatic relief or delay in progression in the case of human clinical trials, which calls for a different approach towards the treatment and mechanistic dissection of AD. It is proposed that therapeutic interventions may prove more successful if targeted at preclinical stages; before pathological changes become irreversible or too extensive to repair. This calls for potential biomarkers or behaviors that could identify the preclinical pathologies and provide alternative drug targets. Improving sleep and wake quality is an exciting potential target not only to improve life quality, but may even directly impact disease progression. Sleep alterations precede cognitive impairments and begin in parallel with accumulation of Aβ plaques (Anafi, Pellegrino et al. 2013, Aoyama and Nakaki 2013, Dyakonova and Krushinsky 2013, Fitzsimmons and Bertram 2013, Ju, McLeland et al. 2013, Malkki 2013, Möller-Levet, Archer et al. 2013, Simon, Greenaway et al. 2013, Spira, Gamaldo et al. 2013, Stevenson and Schildberger 2013, Vagelatos and Eslick 2013, Yoo, Mohawk et al.

2013, Ju, Lucey et al. 2014). Also, sleep is shown to have a role in clearance of toxic metabolites like Aβ resulting from neuronal activity (Xie, Kang et al. 2013). Recent developments suggest that a positive feedback loop might exist between sleep perturbations and AD pathology, such that each factor might amplify the other, driving the progression of disease. Therefore, for my first project, detailed in chapter two, I evaluated sleep-wake alterations in 5XFAD, a mouse model for AD (Oakley, Cole et al. 2006).

Insufficient sleep also has been linked to elevated risk of metabolic disorders such as obesity and diabetes (Knutson, Spiegel et al. 2007). Previous studies have shown that the leptin-resistant diabetic animals have diminished sleep wake rhythms (Laposky, Bradley et al. 2008). Also, there is increasing evidence that diabetes and obesity present a risk for AD development (Ott, Stolk et al. 1999, Kroner 2009). To study how the interaction of the two disorders effect sleep, we took advantage of the combined model of AD and diabetes generated by Dr. M. Paul Murphy's lab (Niedowicz, Reeves et al. 2014). The combination of the two diseases results in additional pathologies such as aneurysms and strokes in addition to the ones that are characteristic of the parental AD mouse line.

Similar to transgenic animals, and disease models described above, mutant mice such as knockouts also have the potential to add to our knowledge of the genes involved in sleep regulation. With this aim, I examined a large-scale knockout population phenotyped for their sleep-wake traits in addition to many other physiological aspects at the Jackson Laboratory (JAX) as part of the IMPC (International Mouse Phenotyping Consortium) project, as described next.

The number of protein coding genes for the mouse genome stands at around 25,000. With developments in genome sequencing, and the advancement in gene targeting technology, it became feasible to knockout every single gene of the mouse genome. This led to the establishment of the International Knockout Mouse Consortium (IKMC) in 2007 with the aim of functionally annotating the entire mammalian genome. The first phase involved creating ES (embryonic stem) cell lines for each targeted mouse gene (Abbott 2010, Bradley, Anastassiadis et al. 2012). By 2011, over one-third of the mouse genes were established in ES cell lines. At this point, the second phase of the project, KOMP2 under the umbrella of IMPC began; an effort to turn these ES cell into "knockout mice" and then

comprehensively phenotype over 5000 knockout mouse lines generated in this phase (Brown and Moore 2012). Our collaborator, The Jackson Laboratory, is one of the primary KOMP2 centers. Behavioral assays such as rotarod, holeboard exploration, and especially sleep, are unique to the JAX phenotyping pipeline. Such a large scale endeavor has many advantages over individual lab efforts, in part due to use of a standardized mouse background for the knockouts, standard protocols, and quality control.

Traditional sleep analysis makes use of EEG/ EMG (Electroencephalogram/ Electromyogram) recordings which have been considered the "gold standard". EEG/EMG techniques pose many limitations when it comes to recording rodents, especially on a large scale, as required for KOMP2. The system requires extensive surgery to fix the electrodes, recovery time, hooking up cables, and limited animal movement, in addition to labor intensive signal analysis (Flores, Flores et al. 2007). Alternative less invasive techniques like infrared beam breaking and wheel running have a lower correlation rate (70-80%) with sleep, depending upon the mouse strain under observation (Koteja, Garland et al. 1999). To overcome all these limitations, our lab designed the Piezoelectric system for non-invasive higher throughput monitoring of sleep in rodents.

The use of this system for phenotyping the sleep-wake traits has been central to my research projects. This non-invasive sleep-wake monitoring system consists of a Polyvinylidine Difluoride (PVDF) sensor pad placed on the cage floor which detects gross body movements of the animal to generate signals (Fig. 1). During sleep, rhythmic 3 Hz signals produced by breathing is the principal motion detected by the system in contrast to wakefulness which is characterized by high frequency and erratic movements which masks this breathing (Donohue, Medonza et al. 2008, Mang, Nicod et al. 2014). The system is capable of recording various sleep parameters including percent sleep, bout lengths, peak activity, activity onset and breath rate. Current efforts are directed toward adding more features and training the classifier based on correlation of Piezosystem recording with human observation and EEG. With these features, the system would be expanded to resolve sleep further into REM (Rapid Eye Movement) and NREM (Non-rapid eye movement), which would make the system more valuable for additional sleep assessments. In the future, the current data will be reanalyzed by these newer algorithms, but we can already determine sleep and wake with accuracy comparable to EEG/EMG.

To date, I have analyzed more than 6000 animals representing over 300 knockout lines, and more than 1800 control mice have been recorded. Around 20% of these knockout lines demonstrated altered sleep phenotypes, depending on the specific sleep traits assessed and the statistical approaches utilized. Some genes were found to specifically alter total sleep amounts or sleep fragmentation (sleep bout lengths) primarily in the light phase, others in the dark phase. Among controls and knockouts, males sleep slightly more than females in most but not all cases. The high hit rate seen in our study may also reflect that a high percentage of genes are expressed in the brain, and that many factors affect sleep. In addition, the same piezoelectric system also enabled us to look for altered breathing rhythms. Several genes were found to affect breathing variables as well.

In summary, in the following chapters of this dissertation I describe in detail my studies to fill the gaps in our knowledge of the genetic regulators of sleep by examining a variety of mouse models. We utilized novel screening technology to phenotype sleep-wake traits and identified novel candidate genes that regulate sleep and perhaps sleep functions. Also, we evaluated mouse models of AD and Diabetic conditions for their sleep alterations that may be relevant for human AD and Diabetic diseases, and serve as a tool for the development of drugs to improve sleep and potentially slow the progression of disease.

Chapter 2 Review-Large-scale sleep phenotyping projects in mice: application of high-throughput screens

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Abstract

Mouse models are an essential tool to dissect the genetic mechanisms that underlie complex behaviors such as sleep. A variety of genetic and genomic techniques are available to manipulate the mouse genome, and identify the genetic influences regulating sleep. Forward genetic techniques such as Quantitative trait loci (QTL) and mutagenesis screens are aimed at characterization of gene/s responsible for the phenotype in question. In contrast, reverse genetic methods may utilize knockout or other transgenic mice to ascertain the novel functions associated with a gene of interest. To fully exploit these available resources, and disentangle the pathways that regulate sleep, it is essential to implement robust phenotypic screens. In addition, for the detection of subtle phenotypes, analysis of a large number of animals is required. Conventional EEG/EMG is a popular sleep recording method, however, it is invasive and labor-intensive, and therefore not suitable for use in large-scale projects. There are many other alternative tools available such as video tracking, wheel running, light beam breaking, and piezoelectric systems. The choice for any of the particular alternative methods depend upon the specific needs of a research study. This paper reviews the large-scale projects as implemented in the field of sleep, and the advantages and shortcomings of the various phenotypic paraphernalia.

Introduction

Sleep is a complex behavior that results from multiple underlying neuronal pathways, and is regulated (in part) by gene networks. In the last few decades, there has been increasing realization that sleep is critical for the overall well-being of an animal. Sleep has been suggested to be restorative in nature and critical in memory consolidation (Nishida, Pearsall et al. 2009, Walker 2009). In addition, it also has a role in modulating energy balance and immunity. Furthermore, sleep has been shown to facilitate clearance of toxic metabolites, including beta amyloid, a neuronal by-product of the APP protein that is a major constituent of the plaques in Alzheimer's Disease (AD). Currently, more than 50 million Americans are affected by sleep disorders or disturbances. These sleep deficiencies, in turn, are associated with a variety of health problems. For instance, inadequate sleep, which is becoming increasingly common due to the demands of a modern lifestyle, has been implicated in a variety of diseases including neurodegenerative and metabolic disorders (Knutson, Spiegel et al. 2007, Rothman and Mattson 2012, Suzuki, Miyamoto et al. 2012). Sleep apnea is linked to pathological conditions such as obesity, diabetes, hypertension, stroke, renal failure, and myocardial infarction (Shamsuzzaman, Gersh et al. 2003). Some of these sleep problems arise as a result of a desynchronized biological clock, while others seem to be linked to disrupted physiological functions of sleep. Efforts aimed at improving sleep quality and treatment of sleep disorders are limited by our rudimentary knowledge of the functions of sleep and the mechanisms that regulate these functions. Identification of the genetic influences on sleep, and elucidation of the regulatory mechanisms at the molecular and biochemical level, would therefore be significant for the development of drugs targeting these regulators or other molecules that influence sleep and

wake. This review provides an overview of the large-scale genetic approaches undertaken to identify the sleep-related genes in mice, and also the scalable technology available to phenotype and characterize the sleep traits in these large mouse populations.

Mouse-an animal model for sleep studies

Human studies tend to be expensive and difficult to control in many cases. Given that there is 90% homology between the mouse and human genome (in the critical coding and gene regulatory regions), in addition to the strong conservation of most brain and physiological systems, the mouse has become the preferred animal model to emulate a wide variety of both normal and human pathological conditions. In areas relevant to sleep research, the mouse shares striking similarities to humans at the level of neural circuits and the neurotransmitters involved in sleep regulation, in addition to nearly 100% identity for critical circadian and sleep-related genes (at least among the few that are known). Furthermore, the availability of hundreds of inbred mice strains, and a wide-array of genetic tools allow for easy manipulation of the mouse genome. Therefore, use of mice in parallel with well-established genetic resources can provide insight into the genetic determinants of sleep physiology.

Forward Genetic Approaches

QTL- mapping genomic loci

A higher concordance of various sleep parameters and sleep architecture in mono- vs. di-zygotic twins provided early evidence for the genetic regulation of sleep (Stassen, Lykken et al. 1988, Ambrosius, Lietzenmaier et al. 2008, De Gennaro, Marzano et al. 2008). Genetic studies conducted by Valatx, on several inbred, recombinant inbreds (RI)

and hybrid mice further strengthened the notion that sleep is under genetic control (Valatx, Bugat et al. 1972, Valatx 1978, Kitahama and Valatx 1980). Forward genetic techniques, which involve screening for sleep-related phenotypes, followed by scanning the genome in pursuit of genes affecting the trait in question, is one mechanism to determine the genetic components of sleep regulation. Traditional QTL analysis is one such phenotype-driven approach to identify genetic variants associated with complex traits. It typically involves crossing of two inbred strains that differ for the trait of interest. The F1 progeny is inter-crossed, or back-crossed to one of the parents to generate an F2, which are then subjected to phenotypic assessment. In some cases, these crosses are followed by several generations of brother-sister matings to obtain homozygosity, and a set of defined Recombinant Inbred (RI) strains. Using the QTL approach, sleep-related genes such as *Homer 1a*, involved in recovery from glutamate-induced neuronal hyperactivity, *Acads* (acyl-coenzyme A dehydrogenase), which influences theta oscillations, and *Rarb* (Retinoic acid receptor beta), involved in long-term potentiation, were identified (Tafti, Petit et al. 2003, Drager 2006, Flores, Flores et al. 2007, Franken, Thomason et al. 2007, Maret, Dorsaz et al. 2007, O'Hara, Ding et al. 2007, Wisor, Striz et al. 2007). In a similar study, a QTL linked to phase advancement relative to dark onset, was mapped to chr 18 in "early runner" mice (Wisor, Striz et al. 2007). A comprehensive study by Winrow and colleagues, utilizing more than 250- BL6 (BL6 and BALB/c) mice for the analysis of 20 sleep-wake traits, identified 52 significant QTLs represented by 20 genomic loci (Winrow, Williams et al. 2009). In a follow up study, using pharmacological compounds, the investigators validated six of the candidate genes shortlisted previously- *Chrm3, Chrna4, Drd5, Htr1d, Glp1r* and *Cacnali,* for their role in regulating various aspects of sleep (Brunner, Gotter et al. 2011).

Furthermore, the same research group also narrowed down a QTL on chr 17, influencing REM sleep parameters, to the gene *Ntsr1* by microarray expression analysis of over 28,000 brain transcripts (Fitzpatrick, Winrow et al. 2012).

A disadvantage of a typical QTL analysis is that the F2 progeny are unique, and thus require genetic mapping each time to identify the recombination patterns. There is also only a single individual mouse representing each F2 genotype, limiting the reliability of each phenotypic measure. Furthermore, even with accurate phenotyping, such crosses present limited mapping resolution, where a QTL can span several hundred genes (Flores, Flores et al. 2007, Franken, Thomason et al. 2007, Maret, Dorsaz et al. 2007, O'Hara, Ding et al. 2007, Wisor, Striz et al. 2007). Additionally, conventional crosses suffer from the issue of low genetic diversity present in most of the common laboratory mice as a result of historical development of inbred strains. In contrast, RI strains have fixed recombination patterns, and multiple mice of each genotype can be tested. Since the genotype of each RI strain is fixed, they only need to be mapped once, and can then be used repeatedly by many investigators, in many different experiments, and can be replicated as often as desired in the future. Lastly, each RI strain represents multiple generations of recombinations, and thus has increased mapping power, although the limited number of RI lines has historically limited mapping resolution. To overcome these shortcomings of traditional QTL studies using RI lines, existing reference panels are being expanded as in the case of BXD strains, along with the development of new reference panels such as the Collaborative Cross (CC) using eight diverse parental strains (see below), and then using these same eight parental strains in Diversity Outbred (DO) mice (Peirce, Lu et al. 2004).

Collaborative cross for precision mapping

CC and DO mouse resources exhibit a marked increase in genetic diversity, as much as 4 times that of the classical inbred mice, and can facilitate high resolution mapping, sometimes to the level of a single gene (Bradley, Anastassiadis et al. 2012, Gkoutos, Schofield et al. 2012, Schofield, Hoehndorf et al. 2012). The CC reference panel utilizes random crossing of eight founder strains. The founder strains are comprised of five traditional inbred laboratory strains and 3 wild-derived strains, which capture around 90% of the genetic diversity found in the laboratory inbred mice strains (Bradley, Anastassiadis et al. 2012, Gkoutos, Schofield et al. 2012, Schofield, Hoehndorf et al. 2012, Threadgill and Churchill 2012). These mice are being inbred to achieve a goal of generating 1000 strains. CC mice provides another advantage- as with other RI strains, all lines are reproducible and, for each line, the genotyping is needed only once (Churchill, Gatti et al. 2012, Svenson, Gatti et al. 2012, Threadgill and Churchill 2012).

Diversity Outbred mouse- genetic panel with improved genetic diversity

The Diversity Outbred panel is a heterogeneous stock of highly diverse mice from the same eight parental strains. They were designed to maximize genetic diversity similar to what is found in human populations. The DO founder mice were obtained from the incipient CC mice. Unlike the CC panel, the DO mice are maintained through random outbreeding rather than inbreeding, leading to additional recombinations and minimizing the chance of allelic loss. Random mating and the outbred nature yields a unique genetic constitution for each mouse and therefore non-reproducibility of DO animals (Churchill, Gatti et al. 2012, Svenson, Gatti et al. 2012, Threadgill and Churchill 2012). Thus, each mouse is required to be genotyped for the QTL analysis- a disadvantage compared to CC

mice. Overall mapping results from DO can be independently validated in CC lines (Bradley, Anastassiadis et al. 2012, Churchill, Gatti et al. 2012, Gkoutos, Schofield et al. 2012, Schofield, Hoehndorf et al. 2012, Svenson, Gatti et al. 2012, Threadgill and Churchill 2012, Threadgill and Churchill 2012). Even though, CC and DO animals are yet to be utilized to their full potential, their successful application is already evident. One such noteworthy example of utilization of pre-CC (incipient CC mice) comes from a study conducted by Philip et al (Philip, Sokoloff et al. 2011). A QTL for peak activity after sleep deprivation, was mapped on chr 9, spanning 530 kb and harboring only 3 genes. In addition, another QTL, linked to sleep percentage during the dark phase, mapped to a genomic region on chr 7 representating a region of about39 genes.

ENU mutagenesis- random point mutations of genes

As the name suggests, ENU mutagenesis projects utilize ENU (N-ethyl-N-nitrosourea), a chemical mutagen that randomly induces base substitutions in mouse spermatogonia. Mutagenized mice are then screened for a phenotype that shows significant deviation from the population mean for the trait of interest (Gondo 2010). Using this approach, the circadian genes, *Clock* and *Rab3a* in mice, and *Per* and *Dbt* in flies, were successfully isolated (Takahashi, Pinto et al. 1994, Vitaterna, King et al. 1994, Flores, Flores et al. 2007, Franken, Thomason et al. 2007, Maret, Dorsaz et al. 2007, O'Hara, Ding et al. 2007, Wisor, Striz et al. 2007). These genes further helped in discerning several other clock genes, such as *Bmal1, Cry1,2, Per1,2,3,* which regulate circadian and homeostatic aspects of sleep. Such successes lead to the initiation of many large-scale ENU mutagenesis projects (Gondo, Fukumura et al. 2009), targeting the entire mouse genome, each one of them focused on a phenotype of specific interest. Vitaterna and colleagues in their

mutagenesis screen, concentrated on behavioral and nervous system phenotypes, and reported 46 confirmed circadian mutants as of 2005 (Goldowitz, Frankel et al. 2004, Vitaterna, Pinto et al. 2006). In follow up studies, *Ovtm* was identified as a mutation in *Fbxl3* gene*,* which was linked to a long circadian period (25.8 h) (Siepka, Yoo et al. 2007). In contrast, *Prtm,* a short circadian period (21.4 h) mutation, resulted from a loss-of-function mutation in the *Cry1* gene, and *Psttm,* another short period mutation (22.9 h) was traced to *Fbxl21* gene (Siepka, Yoo et al. 2007, Yoo, Mohawk et al. 2013).

A shortcoming of ENU mutagenesis is that it requires follow up with positional cloning for the identification of the causative gene, which is a difficult and time consuming process. However, with further advancement in genome sequencing, the mutations can now be identified in a cost and time efficient way. The process is still quite labor intensive in the case of mammals. Despite these difficulties and shortcomings, QTL studies and ENU mutagenesis are arguably our most powerful tools to find novel genetic pathways, as no a priori assumptions are made about the functions of any given gene. Given the pleiotropic nature of most genes, and our lack of knowledge regarding most of the 22,000 protein coding genes in mammals, these "blind" approaches are necessary.

KOMP2- a large-scale, gene-driven approach

Reverse genetics, in contrast to forward genetics, is a genotype-driven approach that utilizes knockouts and other genetically altered mice to find novel functions associated with a gene of interest. It has helped further characterize, the roles of neurotransmitters and canonical circadian genes, such as *Clock, Bmal* and *Npas* (O'Hara, Jiang et al. 2017). Individual lab efforts are most often biased towards a small number of genes of interest, due to limitations of resources and expertise. These individual efforts at producing single

gene knockouts are also marked by heterogeneity of genetic background. To overcome these drawbacks, a more systematic and comprehensive project commenced in 2007, with the establishment of the International Knockout Mouse Consortium (IKMC). This large-scale undertaking aims at generating single gene knockout ES cells for the entire mouse genome on the C57BL/6N background (Abbott 2010, Bradley, Anastassiadis et al. 2012). Knockout animals thus generated, are being comprehensively phenotyped for over 200 physiological parameters as part of the Knockout Mouse Phenotyping Program (KOMP2) (Brown and Moore 2012). Many of the behavioral assays, such as rotarod and sleep assessments are unique to the Jackson Laboratory (JAX), one of the KOMP2 centers. KOMP2 has been instrumental in uncovering new candidate sleep genes. In collaboration, we have established a role for more than 50 genes, not implicated in sleep regulation previously (see Chapter 5).

Recording systems for analysis of sleep

EEG/EMG limitations

Phenotyping sleep-wake traits is an essential component of both forward and reverse genetic approaches towards a better understanding of sleep. The different sleep-wake parameters that can be screened range from duration of sleep states (REM, NREM sleep), the number of such sleep bouts, and other variables such as sleep latency (time to fall asleep, which works better in humans that consolidate most sleep in one long sleep period at night, as opposed to the polyphasic sleep of mice). Sleep in all mammals is associated with distinct electrophysiological changes in brain activity. Traditionally, EEG/EMG techniques have been exploited for the measurement of these brain changes with high accuracy. However, for rodents which have smaller brains, it requires surgical

implantation of electrodes followed by recovery periods of many days, and labor intensive scoring. In addition, it entails tethering the animal to the EEG recording system that restricts the free movement of an animal. These drawbacks limit the application of EEG for high-throughput behavioral studies that require large numbers of mice for discerning phenotypic effects. Multiple, non-invasive, alternative systems have been developed in the past few decades for the assessment of sleep-wake related behaviors. There are several automated recording systems available, such as those based on monitoring of activity or video recordings which can assess behaviors related to sleep, and also some variations to make EEG/EMG easier, as discussed next.

Telemetry- alternative EEG/EMG method to record sleep

Telemetry provides another way of doing EEG/EMG, which does not require animals to be tethered for data acquisition. It allows recording animals under a variety of conditions such as group housing, as well as more enriched environments. Furthermore, it can measure various physiological parameters in addition to sleep, such as body temperature and activity of an animal. Similar to typical EEG/EMG recordings, it still requires surgical implantation of electrodes as well a transmitter. Typical radio transmitters tend to be relatively heavy and big for the size of the skull of a mouse (for instance, 3.9 g at a volume of 1.9 cc for DSI PhysioTel EA-F20 and ETA-F10; https://www.datasci.com/products/implantable-telemetry/mouse-(miniature)). However, the newer generation of these systems can be as light as 1.4 g, which is still extremely heavy and causes problems for typical inbred strains weighing 20-30g. There are other improvements being made to this system. Recently, a wireless telemetry system was developed that does not require anchor screws to affix the system to the skull, but just the

burr holes, and can be used in young pups as well (Zayachkivsky, Lehmkuhle et al. 2013). However, despite these improvements, these telemetry systems typically have much higher noise and poorer sleep scoring than traditional cabled EEG/EMG systems.

Minimally-invasive approach

A less-intensive surgery method based on locomotor activity, was developed by Storch et al (Storch, Hohne et al. 2004). This method requires implanting small magnets subcutaneously near neck muscles of mice. Mice activity is characterized by magnet movement relative to a sensor plate placed beneath the cage. The system can detect sleep/wake quantitatively with limited accuracy, in addition to activities such as grooming and rearing. However, it cannot discern quiet rest, or sub-stage sleep into REM and NREM. Additionally, it has a low resolution, and still requires surgery which makes the system difficult for higher throughput studies (e.g. more than 200 mice).

Non-invasive approaches

As described above, both EEG/EMG and telemetry involves surgical implants, which can affect the natural behavior of an animal. In addition, these methods are expensive as well, and thus not suitable for assessment of a large number of mice. To address these deficiencies, a number of other, non-invasive devices for sleep phenotyping have been developed. One such scoring system designed by Pack et al utilizes object recognition algorithms in combination with video-scoring and infrared beam breaks (Pack, Galante et al. 2007). The characterization of sleep and wake was based on the investigators' premise that an activity of more than 40s can be classified as sleep. This setup showed high correlation with EEG assessments. Fisher et al also utilized video tracking- based on locomotor analysis, which shows high correlation with EEG/EMG assessment of sleep-

wake, however, it cannot discern sleep architecture- REM and NREM sleep, similar to the system developed by Pack et al (Fisher, Godinho et al. 2012). There are other limitations associated with video-based devices such as adjustment of camera angle, and need for proper illumination at all times. There are other systems available as well, such as infrared beam breaking which is limited to detecting gross body movements. It cannot distinguish quiet rest from sleep, and also does not work well when the mouse does not change its location frequently.

Respiratory patterns have also been used as an alternative to EEG/EMG, as in the case of a system developed by Zeng and colleagues (Zeng, Mott et al. 2012). They utilized a Doppler radar system, and support vector machine, a supervised learning system, to classify different vigilance states. The system has high classification accuracy for wakefulness and NREM (91% and 85% respectively) but low accuracy for REM sleep (~70%).

PiezoSleep- a novel, non-invasive and high-throughput system, is based on periodic signals generated during respiration. It utilizes a motion sensor- piezoelectric film, placed at the bottom of the cage floor, which transforms the pressure variations into electrical signals. During sleep, the prominent movement is from breathing, in contrast to wakefulness that is characterized by more erratic signals is the basis of discerning sleep and wakefulness by this system (Flores, Flores et al. 2007, Donohue, Medonza et al. 2008, Mang, Nicod et al. 2014). The system demonstrates a classification accuracy of over 90% for sleep and wakefulness compared to traditional EEG/EMG (Donohue, Medonza et al. 2008, Mang, Nicod et al. 2014). Additionally, the system due to its highly sensitive nature can discriminate quiet wake from sleep, and also detect short arousals which almost all

other non-invasive systems fail to. This automated system is equipped to record a variety of sleep parameters- sleep percent across 24h, dark and light phase, and bout length across 24h, dark and light phase. The systems' additional hourly data assessment feature finds its use in time-sensitive studies such as those involving sleep deprivation, or effect of dosage of drugs as exemplified by my research project on 5XFAD, as described in chapter 3 (Sethi, Joshi et al. 2015). It has also been equally effective, as validated by IR camera and EEG/EMG in recording sleep for larger rodents such as Spiny mice.

Recently, the systems capabilities were enhanced further, and it integrated assessment of breath rate, a supplemental tool in determining aberrant sleep physiology in animals. The system was recently tested for its assessment of sub-stages of sleep, and was found to have a high classification rate for wake and NREM sleep but underestimated REM sleep (Yaghouby, Donohue et al. 2016). With further improvements in its algorithm, classification of REM sleep as well is expected to improve in near future. In its current form, the piezosystem has been significant in identifying more than 50 sleep-related genes, and several other genes affecting breath rate in mice, as part of KOMP2, an ongoing large-scale endeavor, within a short time span (Chapter 5). Thus, PiezoSleep, an important component of KOMP2, contributed massively to such unprecedented success in the field of sleep. A pre-CC study, also utilized this system and found reasonable success in finding QTLs affecting a variety of sleep parameters (Philip, Sokoloff et al. 2011).

Conclusion

No single technique of gene analysis is complete by itself and has limitations of its own. EEG/EMG technique is invasive, and can alter the natural behavior of an animal, and on other hand, most of the other, non-invasive alternatives fail to record sleep measures

such as NREM delta waves, a measure of sleep intensity. For high-throughput behavioral studies, the relatively inexpensive, non-invasive alternatives, such as PiezoSleep can serve well as a first pass. Later, the animals with phenotype of interest can be followed with EEG/EMG if needed, for more comprehensive sleep phenotyping. This 2-step process can reduce time and efforts by many fold. Further improvements in existing techniques as in case of PiezoSleep, and also ongoing development of new strategies will equip us better at the task of annotating genes without the need of EEG/EMG eventually. Large scale phenotype- or gene- driven approaches are invaluable tools to unravel novel sleep-related genes, and identify novel pathways depicting relationship among them by utilizing various bioinformatics tools. Furthermore, different approaches can complement each other and reveal further the association between protein domains of a gene and their functions. In summary, advancements in sleep phenotyping techniques in parallel with systematic phenotyping pipelines is critical in identifying novel candidate genes underlying sleep modulation.

Chapter 3 Increased fragmentation of sleep-wake cycles in the 5XFAD mouse model of Alzheimer's disease

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Abstract

Sleep perturbations including fragmented sleep with frequent night-time awakenings and daytime naps are common in patients with Alzheimer's disease (AD), and these daily disruptions are a major factor for institutionalization. The objective of this study was to investigate if sleep-wake patterns are altered in 5XFAD mice, a well-characterized double transgenic mouse model of AD which exhibits an early onset of robust AD pathology and memory deficits. These mice have five distinct human mutations in two genes, the amyloid precursor protein (APP) and Presenilin1 (PS1) engineered into two transgenes driven by a neuron specific promoter (Thy1), and thus develop severe amyloid deposition by 4 months of age. Age matched (4-6.5 months old) male and female 5XFAD mice were monitored and compared to wild-type littermate controls for multiple sleep traits using a non-invasive, high throughput, automated piezoelectric system which detects breathing and gross body movements to characterize sleep and wake. Sleep-wake patterns were recorded continuously under baseline conditions (undisturbed) for 3 days and after sleep deprivation of 4 hours, which in mice produces a significant sleep debt and challenge to sleep homeostasis. Under baseline conditions, 5XFAD mice exhibited shorter bout lengths (14% lower values for males and 26% for females) as compared to controls ($p<0.001$). In females, the 5XFAD mice also showed 12% less total sleep than WT ($p<0.01$). Bout length reductions were greater during the night (the active phase for mice) than during the day, which does not model the human condition of disrupted sleep at night (the inactive period). However, the overall decrease in bout length suggests increased fragmentation and disruption in sleep consolidation that may be relevant to human sleep.

The 5XFAD mice may serve as a useful model for testing therapeutic strategies to improve sleep consolidation in AD patients.

Key words: sleep, sleep homeostasis, amyloid beta, diurnal rhythm, sleep fragmentation

Introduction

Alzheimer's disease (AD), which is characterized by accumulation of extracellular amyloid beta (Aβ) plaques and intra-neuronal hyperphosphorylated neurofibrillary tau tangles in the brain, is the most common form of dementia (Glenner and Wong 1984). Aside from severe cognitive deficits, approximately 25 to 40% of AD patients also display profound circadian rhythm and sleep-wake disturbances, which may precede overt cognitive impairments (Carpenter, Strauss et al. 1996, Moran, Lynch et al. 2005, Snyder, Nong et al. 2005). These disturbances include fragmented sleep, frequent nighttime awakenings, and excessive daytime sleepiness (Prinz, Peskind et al. 1982, Bliwise 2004, Bliwise, Mercaldo et al. 2011). Altered sleep architecture in AD includes reduced rapid eye movement (REM) and slow wave (SWS) sleep in addition to increased latency to REM sleep (Prinz, Peskind et al. 1982, Bliwise, Tinklenberg et al. 1989, Perry, Walker et al. 1999, Stevenson and Schildberger 2013). Fragmented sleep, which is also common in many other pathological conditions including Parkinson's Disease, Diffuse Lewy Body Disease (DLBD), sleep apnea, and neuromuscular disorders, has wide spread consequences ranging from excessive daytime sleepiness to impaired memory consolidation (Kimoff 1996, Dauvilliers 2007, Deschenes and McCurry 2009, Rolls, Colas et al. 2011). Recent studies suggest that reduced slow wave sleep, which has been shown to have restorative functions, might be the contributing factor to this impaired memory consolidation (Nishida,

Pearsall et al. 2009, Walker 2009). However, there is still much debate regarding the contribution of different sleep stages in the consolidation of different type of memories, with some data supporting a role for all stages of NREM in declarative memory and a greater role for REM in non-declarative memory (Tucker, Hirota et al. 2006, Marshall and Born 2007, Nishida, Pearsall et al. 2009, Diekelmann and Born 2010).

In regard to circadian system dysfunction, Saitlin et al found that AD subjects have reduced locomotor activity and phase delays of approximately four hours in their activity rhythms and three hours for the core body temperature rhythm compared to healthy elderly subjects (Satlin, Volicer et al. 1995). Often, AD patients also display "sundowning"; a behavioral state characterized by increased aggressiveness, restlessness and anxiety seen towards the afternoon and evening hours (Vitiello, Bliwise et al. 1992). These changes in sleep and circadian rhythms, which correlate positively with the degree of progression of AD, not only affect the quality of life of patients and their care givers but also constitute one of the major factors for institutionalization (Pollak and Perlick 1991, Vitiello, Poceta et al. 1991).

Aggregation of amyloid beta (Aβ) in the brain has been implicated in sleep perturbations as well as in the pathogenesis of AD (Hardy and Higgins 1992, Hardy and Selkoe 2002). Various findings suggest that Aβ aggregation, as indicated by reduced cerebrospinal fluid (CSF) Aβ42 levels, begins as early as 15 years prior to the appearance of clinical symptoms (i.e. the preclinical stage) (Morris and Price 2001, Perrin, Fagan et al. 2009, Sperling, Aisen et al. 2011, Fitzsimmons and Bertram 2013). Even in asymptomatic individuals, Aβ is associated with neural dysfunction of the brain networks subserving memory formation (Sheline, Raichle et al. 2010). Among cognitively

unimpaired individuals, those with higher levels of Aβ accumulation had poorer sleep quality and shorter sleep duration compared to controls without Aβ plaques (Anafi, Pellegrino et al. 2013, Aoyama and Nakaki 2013, Dyakonova and Krushinsky 2013, Fitzsimmons and Bertram 2013, Ju, McLeland et al. 2013, Malkki 2013, Möller-Levet, Archer et al. 2013, Simon, Greenaway et al. 2013, Spira, Gamaldo et al. 2013, Stevenson and Schildberger 2013, Vagelatos and Eslick 2013, Yoo, Mohawk et al. 2013, Ju, Lucey et al. 2014).

Various studies performed in mouse models of AD also indicate an association between sleep perturbations and AD pathogenesis. Using microdialysis, Kang et al demonstrated that young Tg2576 mice (a model of AD), and wild type mice (C57BL6) have diurnal oscillations of brain interstitial fluid (ISF) Aβ, with higher levels during the active phase (night-time) (Kang, Lim et al. 2009). In aged APPswe/PS1δE9 mice with prominent Aβ plaques, sleep is disrupted as well as ISF Aβ diurnal rhythm is lost (Bertram, Rook et al. 2011, Roh, Huang et al. 2012). Further, sleep deprivation increases ISF Aβ, which decreases during sleep recovery (Kang, Lim et al. 2009, Bertram, Rook et al. 2011, Roh, Huang et al. 2012). Similar diurnal oscillations were found for CSF (cerebrospinal fluid) Aβ in healthy human subjects, with higher Aβ levels in day and reduced levels at night (Kang, Lim et al. 2009). These studies suggest that sleep loss accelerates the Aβ deposition and therefore sleep alterations may serve not only as an early marker of AD but also raise the possibility that improved sleep could slow progression of the disease. The extent to which the changes in sleep-wake patterns contribute to or are the result of AD progression is poorly understood. Because studies in AD patients are difficult and

expensive, an animal model displaying sleep alterations that mimic those found in AD is necessary.

Our present study aimed at investigating whether 5XFAD mice (MGI: 3693208), a well-characterized, double transgenic model of familial, early onset AD, show alterations in sleep-wake patterns. These mice have five distinct human mutations: three in the amyloid precursor protein (APP) namely Swedish, Florida and London mutations (K670N/M671L, I716V, V717I) and two in the Presenilin1 protein (PS1), i.e., mutations M146Ll and L286V engineered into two transgenes driven by a neuron specific promoter (Thy1). Each of these PS1 and APP mutations increase $A\beta_{42}$ production but when present together act additively to bring about an excessive $A\beta_{42}$ burden and hence early onset and aggressive AD pathology (Citron, Westaway et al. 1997, Eckman, Mehta et al. 1997, Citron, Eckman et al. 1998, Oakley, Cole et al. 2006, Ohno, Chang et al. 2006). These mice thus develop severe intraneuronal $A\beta_{42}$ at an early age of 1.5 months, amyloid deposition at 2 months, and loss of synapses around 9 months of age (Oakley, Cole et al. 2006, Ohno, Chang et al. 2006). As well as aggressive neuropathology, 5XFAD mice exhibit memory deficits as early as 4-6 months of age, in a range of behavioral assays such as Y maze, Morris water maze, contextual fear conditioning, auditory trace fear conditioning paradigm, and olfactory H maze (Oakley, Cole et al. 2006, Ohno, Chang et al. 2006, Ohno 2009, Devi, Alldred et al. 2010, Devi and Ohno 2010, Girard, Baranger et al. 2013). Since the 5XFAD mice exhibit well characterized, early onset AD-like neuropathological changes and cognitive impairments, the current study investigated whether these mice also exhibit sleep alterations similar to those reported in AD patients. Since AD affects men and women, we included both male and female 5XFAD mice in our study. More women are

known to have AD compared to men, possibly because of longer life expectancy in women (Hebert, Scherr et al. 2001) or due to hormonal alterations late in life (Morinaga, Ono et al. 2011, Barron and Pike 2012, O'Hagan, Wharton et al. 2012, Anaclet, Ferrari et al. 2014, Bhattacharya, Haertel et al. 2014, Civelek and Lusis 2014, Ju, Lucey et al. 2014, Lan, Zhao et al. 2014, Lim, Ellison et al. 2014, Lim, Gerstner et al. 2014, Mang, Nicod et al. 2014, Niedowicz, Reeves et al. 2014, Webster, Bachstetter et al. 2014). In this study, we analyzed the following sleep traits: sleep during the day and night, sleep bouts during the day and night under baseline conditions, and then examined sleep behavior again after 4 hour sleep deprivation in an effort to find if 5XFAD mice model some aspects of the sleep alterations reported in human AD patients.

Experimental procedures

Animal and housing conditions

This study utilized individually housed 5XFAD mice (males: N=10; females: N=7) and wild type mice (males: N=7; females: N=11) for baseline recording and sleep deprivation protocol was applied on 5XFAD (males: N=9; females: N=6) and wild type mice (males: N=6; females: N=11) of 4-6.5 months of age (lost data for few mice because of system failure), obtained from a breeding colony maintained at University of Kentucky. The original 5XFAD stock was provided by The Jackson Laboratories. Originally, 5XFAD were generated on B6/SJL background as previously described (Oakley, Cole et al. 2006, Ohno, Chang et al. 2006). This mouse model co-expresses three APP (Swedish: K670N/M671L, Florida: I716V and London mutation: V717I) and two PS1 human familial mutations (M146L, L286V) under the regulation of neuron-specific murine Thy1 promoter. These mice show intracellular Aβ accumulation at the age of 1.5 months. Plaque

deposition can be detected since 2 months of age; first appearing in deep layers of cortex and subiculum and eventually spreading to most of the cortex, subiculum and hippocampus. Apart from neuroinflammation, these mice also present neuronal loss, a characteristic often missing in most of the other transgenic mouse models of AD (Oakley, Cole et al. 2006, Ohno, Chang et al. 2006, Jawhar, Trawicka et al. 2012, O'Hagan, Wharton et al. 2012, Eimer and Vassar 2013, Girard, Baranger et al. 2013, Ju, McLeland et al. 2013, Lim, Yu et al. 2013, Malkki 2013, Sterniczuk, Theou et al. 2013, Vagelatos and Eslick 2013, Ju, Lucey et al. 2014). In 5XFAD mice, synaptic degeneration as evident from reduced expression of synaptic markers is seen commencing at the age of 4 months, the same age at which various hippocampal- and cortical- dependent memory impairments have been observed (Oakley, Cole et al. 2006, Ohno, Chang et al. 2006, Ohno 2009, Devi, Alldred et al. 2010, Devi and Ohno 2010, Girard, Baranger et al. 2013). The 5XFAD mice have also been reported to have lower body weight (~10%) than wild-type controls at 6-7 months of age (Jawhar, Trawicka et al. 2012, Bhattacharya, Haertel et al. 2014); whether this results from changes in food intake or metabolism has not been reported, to the best of the authors' knowledge.

In the current study, all mice were exposed to an alternating light (L): dark cycle (D), with lights on from 7 AM to 7 PM. Food (pellets) and water were provided ad libitum. All experimental procedures (described below) were approved by the Institutional Animal Care and Use Committee at the University of Kentucky and are consistent with the Institute of Laboratory Animal Resources Guide for Care and Use of Laboratory Animals, 8th edition.

Sleep recording with piezoelectric system

Sleep and wake states were determined using a piezoelectric system, as described previously (Flores, Flores et al. 2007, Donohue, Medonza et al. 2008). The system is comprised of plexiglass cages lined with piezoelectric films across the bottom that detect pressure variations. For all sleeping postures of the mouse, pressure variations from breathing are detected. Sleep states are characterized by quasi-periodic signals with low variations in amplitude, whereas wakefulness and rest states are characterized by irregular transient and high amplitude pressure variations corresponding to conscience body movements and weight shifting. Signal features sensitive to the differences between the sleep and wake states are extracted from the short-time pressure signal segments, and classification is automatically performed every 2 seconds. Data collected from the piezo system were binned over specified time periods (e.g. 5 minutes, 1 hour) using a rolling average of the percent sleep, as well as binned by length of individual bouts of sleep and the mean bout lengths were calculated. The sleep bouts were computed as the duration of contiguous sleep states. Sleep bouts were terminated by any arousal more than 2 seconds in duration. When counting all short arousals and short sleep bouts, average bouts in mice are typically less than 1 minute (Franken, Malafosse et al. 1999). The piezo system has been validated with EEG and human observations and demonstrates a classification accuracy of over 90% (Donohue, Medonza et al. 2008, Mang, Nicod et al. 2014).

Prior to sleep recording, the mice were acclimated in the plexiglass cages for 2-4 days. For the baseline measurements, mice were recorded for 3-5 days, during which time the mice were undisturbed except for monitoring once daily for food and water. The parameters that were analyzed under baseline conditions included total sleep time averaged over 24 h, average percentage of sleep across day (light phase), average percentage of sleep

across night (dark phase), average sleep bout length (across 24 hours, day and night), activity onset defined as the time relative to dark onset when the first sharp increase in percent wake states computed over a 2-hour sliding window occurs between 3 hours before and 3 hours after dark onset, on each day. This is typically the largest increase in this period, increasing from below 40% wake time to over 80% wake time. Diurnal wake ratios are related to the differential wake percentages during the light and dark phases, and are defined as the ratio of maximum wake-state percent in the dark phase to the minimum wake-state percent in the light phase, where percentages are computed over 3-hours intervals. Activity onset, as defined above, was also used a phase marker for the daily rhythm of sleep and activity.

For the second part of the study, the mice were sleep deprived for 4 hours beginning either at 8 or 9 AM. Sleep deprivation was accomplished by transferring the mice to novel cages. To keep the mice awake, nestlets (squares of cotton fibers that mice shred to build nests) and other novel objects were introduced to the cages, and cages were tapped gently when mice appeared ready to sleep. As the four hours progressed, more action was needed to keep mice awake. First, cage lids were gently removed and then replaced, providing additional air flow and olfactory and visual stimulation. If this failed to arouse the mice, then they were gently manipulated to induce movement. At the end of the sleep deprivation protocol, the mice were transferred back to their piezoelectric cages to continue monitoring sleep bout lengths and total amount of sleep.

Data analysis

The data were analyzed using SPSS statistics software version 20.0. Group data was analyzed by a general linear model of analysis of variance (ANOVA) for the baseline

studies. Initial assessment showed that males and females differed significantly from each other (not shown), consequently all the data were pooled and analyzed separately for the two sexes. Before conducting ANOVA, the data were tested for normal distribution and homogeneity of variance. For all of the sleep-wake parameters under consideration (listed above), P-value less than 0.05 were considered significant. Genotype was considered as an independent variable and the parameter under observation as the dependent variable. Hourly sleep percentage and bout length after 4 hour of sleep deprivation were analyzed with mixed ANOVA.

Post-mortem genotyping

After the sleep recording was complete, the mice were euthanized by CO_2 inhalation and decapitation. From dissected brains, cortex was preserved at -70° C for genotyping. Genotyping was conducted using conventional PCR or/and three-step serial extractions of Aβ with sequentially increasing denaturing conditions followed by quantification with a two-site (sandwich) ELISA as described previously (Kukar, Murphy et al. 2005, McGowan, Pickford et al. 2005, Beckett, Niedowicz et al. 2010, Bruce-Keller, Gupta et al. 2011). The primers used are listed here:

PCR Primer Sequence (5' to 3')

APP Forward: AGAGTACCAACTATGACTACG
APP Reverse: ATGCTGGATAACTGCCTTCTTATC
PS1 Forward: ATGACAGAGTTACCTGCACCGTTG
PS1 Reverse: CTGACTTAATGGTAGCCACGACCA

Results

Sleep under baseline (undisturbed) conditions

Sleep-wake patterns were monitored in 5XFAD mice in order to determine the effects of $A\beta_{42}$ overexpression. Statistical analyses (ANOVA) showed that 5XFAD male

mice did not differ from control littermates in the average total amount of sleep [i.e., sleep across 24h ($F_{(1,49)=}$ 1.11, P=0.298)]; daytime sleep ($F_{(1,49)}$=0.48, P=0.493), or nighttime sleep ($F_{(1,49)}$=0.63, P=0.431) (Figure 1 and Table 1)]. In the case of females, 5XFAD mice showed a significant reduction in average total amount of sleep ($F_{(1,52)}$=7.09, P=0.01) as well as nighttime sleep ($F_{(1,52)}$=7.54, P=0.008), compared to wild-type mice (Figure 1). Additionally, average total sleep bout length (across 24h) was reduced in 5XFAD mice of both the sexes; 14% in males and 26% in females (Figure 1). The decreased bout length in 5XFAD mice was observed during both the light phase and the dark phase (male mice: average bout length across 24h, $F_{(1,49)}$=12.12, P=0.001; daytime, $F_{(1,49)}$=7.97, P=0.007; nighttime, $F_{(1,49)}$=8.77, P=0.005) and for female mice: average bout length across 24h, $F_{(1,52)}$=24.18, P<0.001; daytime, $F_{(1,52)}$=10.48, P=0.002; nighttime, $F_{(1,52)}$=34.48, P<0.001).

Table 1 Effect of genotype on sleep wake traits under baseline conditions

Parameter	Wild type	5XFAD	Wild type	5XFAD
	Male	Male	Female	Female
Percent sleep day	56.5±0.8	55.7±0.8	55.1± 1.4	52.5± 0.8
Percent sleep night	27.1±1.5	25.4±1.4	23.2± 1.3	17.2± 1.8**
Percent sleep total (24h)	41.8±0.8	40.6±0.8	39.1± 1.1	34.8± 0.9*
Bout length day	60.4±2.0	54.0±1.3**	65.6± 2.1	54.0± 3.0**
Bout length night	46.1±2.5	37.0± 1.9**	33.7± 1.2	21.0± 1.9***
Bout length total (24h)	54.5±1.9	46.9±1.2**	50.4± 1.2	39.9± 2.0***
Activity onset	0.11±0.13	0.13±0.18	0.14±0.17	0.02±0.99

Values represent means ± SEM. *: P < 0.05; **P <0.01, ***P < 0.001. All comparisons are between WT and 5XFAD of the same sex.

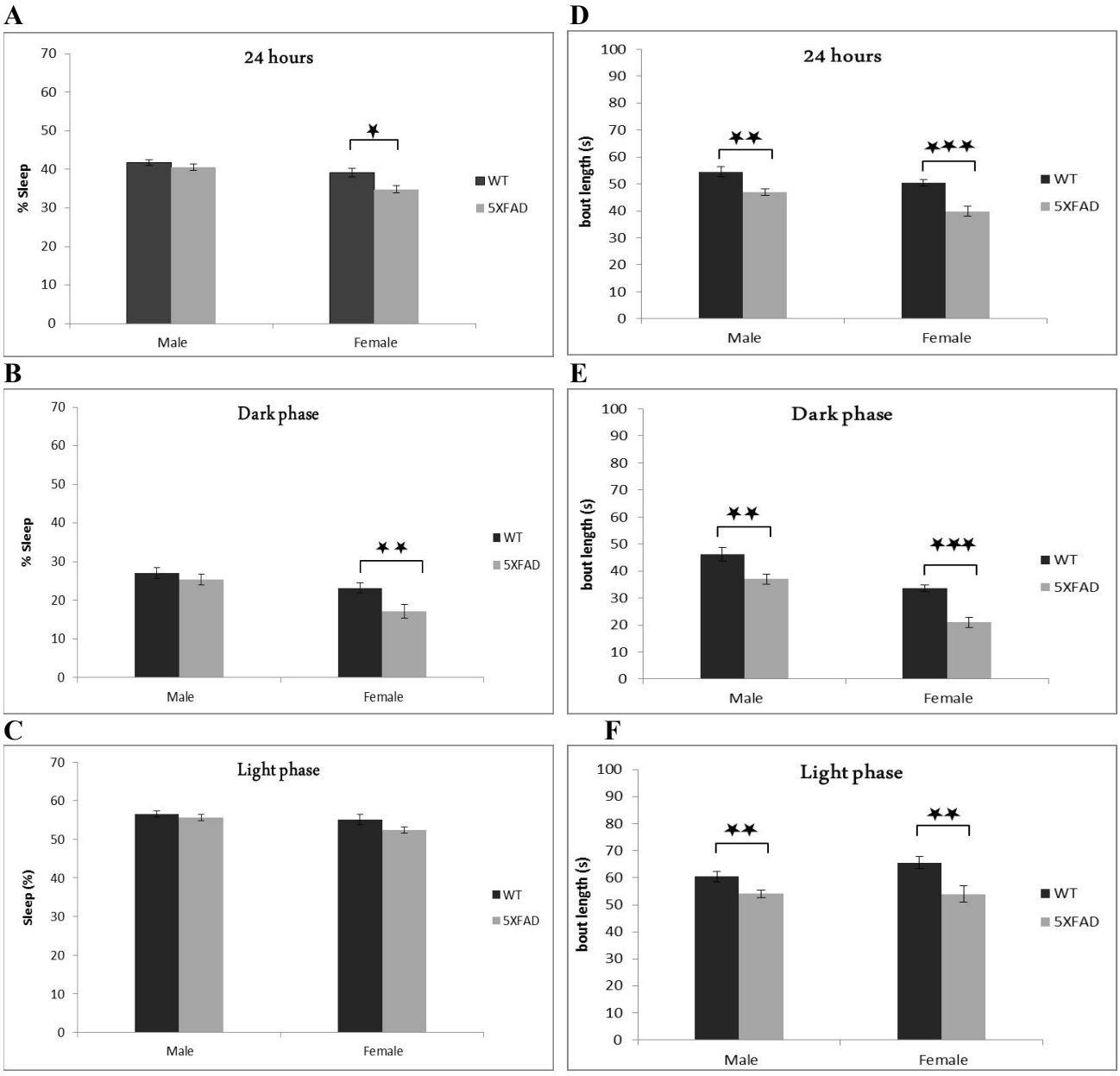

Figure 1 Sleep-wake patterns in 5XFAD and WT littermates under baseline conditions. Average percent sleep across 3 consecutive days analyzed over (A) 24 hours, (B) dark phase, and (C) light phase. Female but not male 5XFAD mice show reduction in sleep duration across 24 h and during the dark phase. (D to F) depicts average bout length in seconds (s) over (D) 24 h (E) dark phase, and (F) light phase. 5XFAD mice of both sexes had shorter average bout lengths across all phases in both the sexes. Values represent means ± SEM. *: $P < 0.05$; **$P < 0.01$, ***$P < 0.001$

There was no apparent genotypic difference in the sleep wake profile for both the sexes compared to their control littermates (Figure 2). Both WT and 5XFAD mice had activity onsets closely coinciding with dark onset, suggesting that there was no apparent change in phase of the daily sleep-wake rhythms. Similarly, there was no change in the peak activity or diurnal wake ratio. For males, the diurnal wake ratio (mean $\pm$ S.E.M.) was: WT, 2.67±0.21, and 5XFAD, 2.93±0.34. For females, the diurnal wake ratio (mean $\pm$ S.E.M.) was: WT, 4.46±0.33 and 5XFAD, 4.38±0.58. The higher ratio in females was due to less sleep during the dark period, as is typical for female mice.

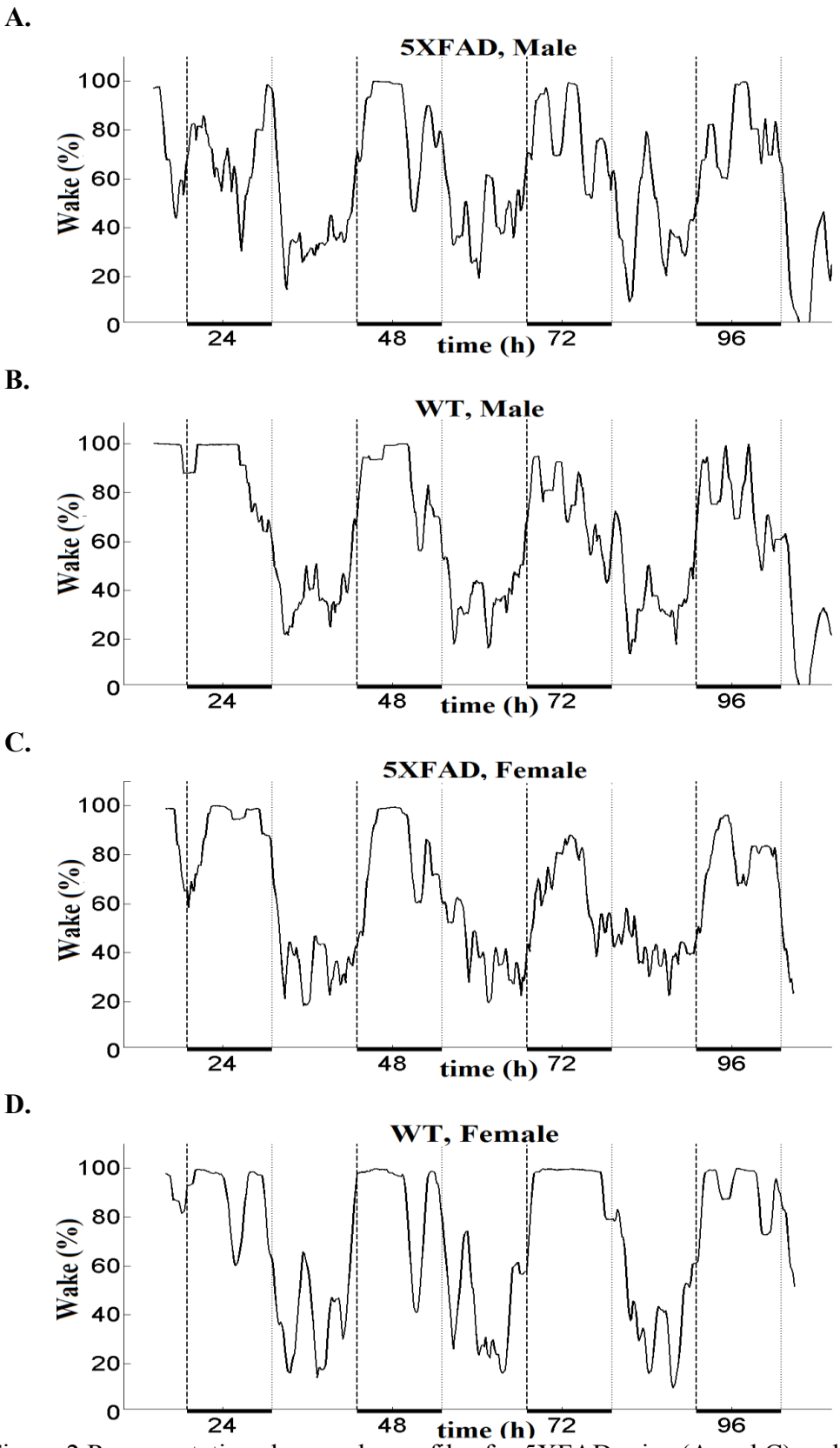

Figure 2 Representative sleep-wake profiles for 5XFAD mice (A and C) and control littermates (B and D). The percent wake plotted on the Y axis is represented as a sliding average over a 2 hour window. Hours of recording are plotted on the X-axis where 0 represents the midnight of day 1. Broken vertical lines demarcate the dark phase, which is also indicated by a heavy horizontal black line at the bottom.

Sleep after 4-h sleep deprivation

Percent of sleep and bout length (dependent variables) in the 6 hours immediately after sleep deprivation (4 hours) was compared between wild type and transgenic (5XFAD) groups using mixed ANOVA model with the genotype as the between-subjects variable and time (6 time points) as the repeated measure (or within subject variable). There was no interaction between genotype and time for bout length (males: $F_{(5,65)}$=0.49, P=0.782; females: $F_{(5,75)}$=0.580, P=0.715) and sleep duration (males: $F_{(5,65)}$=1.55, P=0.186; females: $F_{(5,75)}$=0.922, P=0.471) after sleep deprivation for either sex (Table 2 and Figure 3).

Table 2 Effect of genotype on sleep wake traits after sleep deprivation (SD)

Percent sleep post-SD						
	1st h	*2nd h*	*3rd h*	*4th h*	*5th h*	*6th h*
WT Male	24.4±6.2	65.9±7.3	46.9±8.5	30.0±7.5	41.4±10.3	10.4±8.2
5XFAD Male	24.9±5.1	49.1±5.9	49.8±7.0	39.9±6.1	35.3±8.4	23.7±6.7
WT Female	60.4±5.9	67.4±4.2	67.8±4.6	62.8±4.2	54.2±6.2	59.3±4.1
5XFAD Female	50.3±8.1	57.4±5.7	54.3±6.3	56.3±5.7	55.6±8.5	42.5±5.6
Bout length (seconds) post-SD						
	1st h	*2nd h*	*3rd h*	*4th h*	*5th h*	*6th h*
WT Male	22.9±4.5	55.9±6.7	51.6±10.3	37.5±7.0	48.8±23.3	22.1±10.5
5XFAD Male	20.5±3.7	36.7±5.5	43.5±8.4	34.9±5.7	50.6±19.0	25.9±8.6
WT Female	42.5±5.1	50.4±4.7	52.0±5.1	48.4±5.3	45.0±4.6	50.5±5.5
5XFAD Female	45.4±6.9	44.2±6.4	46.6±6.9	43.7±7.1	37.7±6.3	39.6±7.4

Values represent mean ± SEM

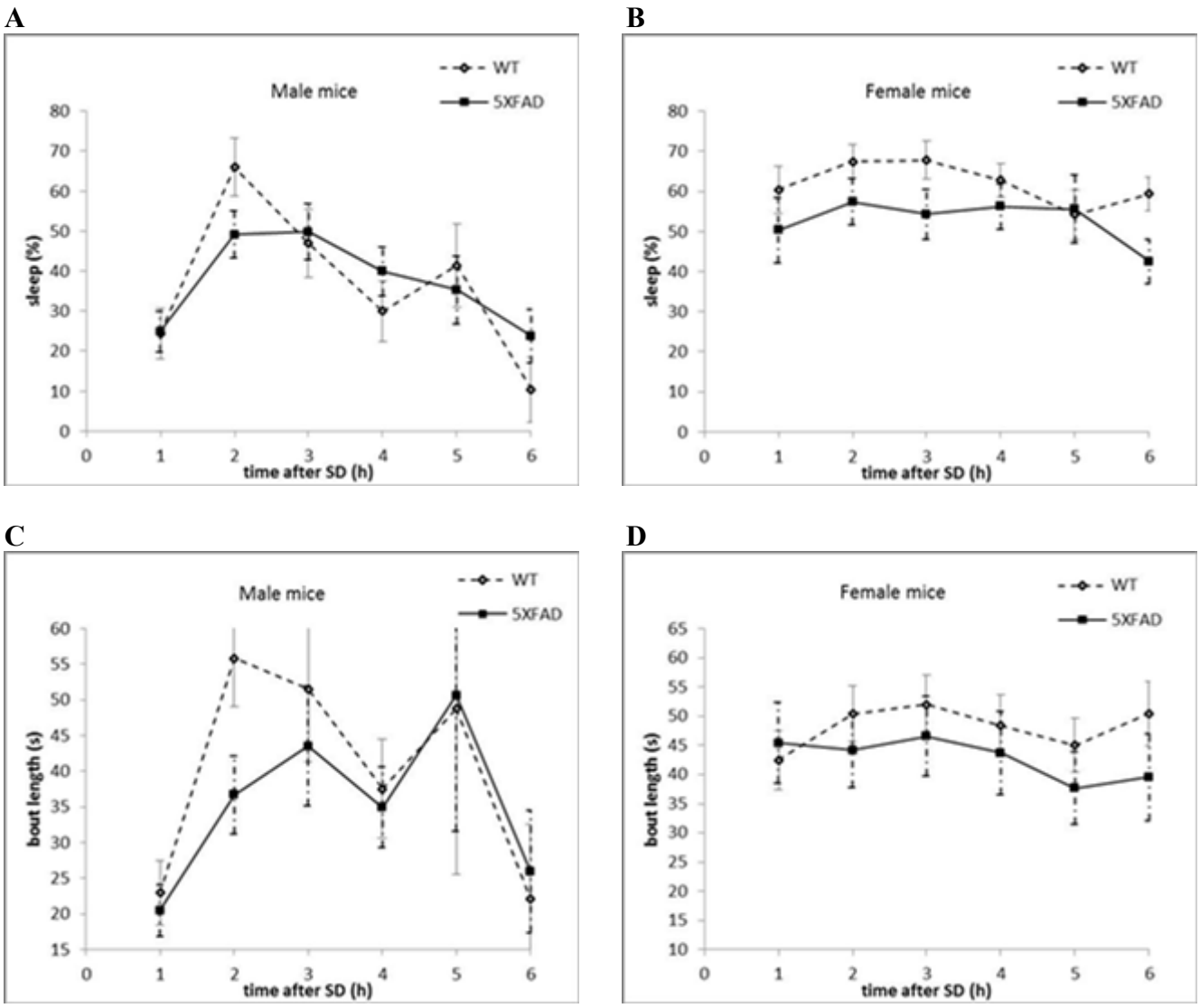

Figure 3 Comparison of the sleep-wake patterns of WT and 5XFAD mice after a sleep deprivation of 4 h. Average percent sleep in males (A), females (B), and average bout length in male (C) and female mice (D) was analyzed for 6 h of the recovery period. No genotype difference was found using Mixed ANOVA.

Discussion

Sleep has become a key avenue of research in the quest to find mechanisms underlying Alzheimer's disease and development of effective therapeutics. It plays a variety of roles crucial to maintaining optimal brain functions and has been found to be

closely linked to AD pathology. One source of evidence comes from a study where improved sleep lowered the risk of AD in people with at least one APOE Ɛ4 allele (Lim, Yu et al. 2013, Simon, Greenaway et al. 2013). This finding is consistent with other studies showing that AD patients frequently have poor quality of sleep, even before the onset of clear symptoms. Poor sleep may be one factor contributing to their compromised cognition since sleep plays a critical role in learning, memory, and other brain functions (Durmer and Dinges 2005, Killgore, Balkin et al. 2006, Killgore, Rupp et al. 2008, Ker, Edwards et al. 2010, Mongrain, Hernandez et al. 2010, Rillich, Schildberger et al. 2011, Bertram and Rook 2012, Callander, Bolton et al. 2012, Reichert and Gerhardt 2013). In a recent study, Lim and colleagues found that loss of neurons in the intermediate nucleus, a proposed homologue of the rodent ventrolateral preoptic nucleus (VLPO), is a potential contributing factor for the fragmented sleep seen in older individuals including Alzheimer's patients (Lim, Ellison et al. 2014).

This study aimed at identifying sleep-wake alterations in the AD mouse model-5XFAD, that recapitulates certain features of the human AD condition and may help in understanding the underlying mechanisms of this disease. Both male and females 5XFAD mice belonged to the age group, 4 to 6.5 months, which shows many of the pathological characteristics of AD including accumulation of intraneuronal Aβ, cerebral plaque deposition, gliosis, synaptic degeneration, neuronal loss, and memory deficits (Oakley, Cole et al. 2006, Ohno, Chang et al. 2006, Jawhar, Trawicka et al. 2012, O'Hagan, Wharton et al. 2012, Eimer and Vassar 2013, Girard, Baranger et al. 2013, Ju, McLeland et al. 2013, Lim, Yu et al. 2013, Malkki 2013, Sterniczuk, Theou et al. 2013, Vagelatos and Eslick 2013, Ju, Lucey et al. 2014). Early onset of the robust pathology seen in these mice

attributable to the incorporation of five additive mutations lead to increased total Aβ production which makes the 5XFAD mouse a useful experimental tool for investigating the effects of increased $A\beta_{42}$ levels which is thought to be one of the key factors involved in disease progression. Also, only a handful of previous studies of AD mouse models have included both sexes or investigated sleep fragmentation.

Our findings show that under baseline conditions, average length of sleep bouts was reduced in both male and female 5XFAD mice. In addition, female mice also had significant reduction in total sleep time averaged over 3 days and sleep occurring during the dark periods. However, male mice did not differ from control littermates in their sleep duration. In contrast to initial expectations, reductions in bout length were found to be greater during the night (the active phase in mice), which does not necessarily model the human condition of disrupted sleep at night (the usual inactive phase for humans). However, the overall decrease in bout lengths in the 5XFAD mice suggests increased fragmentation and disruption in sleep consolidation throughout the day. This finding is likely to be relevant to human sleep disturbances, since mice (unlike humans) usually exhibit considerable amounts of sleep during both the day and night. Assessment of the sleep-wake parameters for the 6 h immediately after sleep deprivation (for 4 h) indicated that genotype did not affect bout length or sleep percentage in either males or females, although there was a general trend of reduced bout length in both sexes.

In general, our findings of decreased sleep bout lengths in 5XFAD mice support and extend previous findings of differences in sleep physiology in other AD mouse models. Reduced NREM duration has been reported in $PLB1_{triple}$ knock in mice (hAPP/hTau/hPS1), whereas lower REM sleep (during light period) was observed in PDAPP (overexpresses

hβAPP) and Tg2576 mice (Huitron-Resendiz, Sanchez-Alavez et al. 2002, Zhang, Veasey et al. 2005, Platt, Drever et al. 2011). APPswe/PS1δE9 mice aged 9 months had reduced REM and NREM sleep stages across both light and dark phases (Bertram, Rook et al. 2011, Roh, Huang et al. 2012). However, some AD mouse models, such as APP/PS1 knock-in mice, do not exhibit obvious changes in sleep (Bassett, Gogakos et al. 2012, Duncan, Smith et al. 2012, Jiang, Franklin et al. 2012).

In the current study, we did not find any change in the phase of the rhythm in 5XFAD which replicates previous findings in APP/PS1 mice and other AD mouse models (Sterniczuk, Dyck et al. 2010, Bassett, Gogakos et al. 2012, Duncan, Smith et al. 2012, Jiang, Franklin et al. 2012). In this respect, the AD mice do not closely resemble the AD patients, which show large delays in the phase of their activity and temperature rhythms compared to those of normal elderly subjects. However, there were sex differences in the 5XFAD transgenic mice. The mechanisms causing the sex differences in sleep over 24 hours and sleep at night in the 5XFAD mice are unknown. It is possible that sex disparity in Aβ levels contributes to the sleep differences. Oakley et al in their studies on 5XFAD have reported that $A\beta_{42}$ levels were higher in young females compared to age-matched males (until at least 9 months of age). This may explain the differences found between the two sexes in our study. Further, this observation indicates that the extent of sleep disruptions may be linked to the levels of Aβ as proposed by previous studies. In addition, some studies indicate that hormonal alterations in the later part of life in women may pose a higher risk of AD for them as compared to men, although some studies indicate otherwise (Morinaga, Ono et al. 2011, Barron and Pike 2012, O'Hagan, Wharton et al. 2012, Anaclet, Ferrari et al. 2014, Bhattacharya, Haertel et al. 2014, Civelek and Lusis 2014, Ju, Lucey et

al. 2014, Lan, Zhao et al. 2014, Lim, Ellison et al. 2014, Lim, Gerstner et al. 2014, Mang, Nicod et al. 2014, Niedowicz, Reeves et al. 2014, Webster, Bachstetter et al. 2014). It is possible that there may be other underlying causes present which require further investigation. In the 5XFAD mice, Devi et al illustrated that stressful conditions resulted in higher Aβ42 levels and plaque burden in hippocampus of females but not in males (Devi and Ohno 2010). Sex differences were also seen in a study of Tg2576 mice (Wisor, Edgar et al. 2005, Wisor and Kilduff 2005). Post AD pathology (22 months old), females in addition to exhibiting sleep-wake alterations common to males also showed increased REMS. However, Tg2576 mice (15-17 months) did not show any significant effect of sex or sex X genotype interaction on theta to delta ratio in the EEG (Wisor, Edgar et al. 2005, Wisor and Kilduff 2005). In another study in 3XTg mice, males with AD pathology did not show genotypic differences in circadian phase shifts post AD pathology but females had a tendency towards large circadian phase shifts in response to light pulses presented in the early subjective night (Sterniczuk, Dyck et al. 2010).

While the present findings indicate that the 5XFAD mice exhibits some sleep alterations that are relevant to AD, there were also some limitations to this study. One limitation was that the algorithms currently used by the piezoelectric system do not distinguish REM sleep from NREM sleep, although algorithms under development may be able to do this in the future. Also, it should be kept in mind that this mouse model represents the advanced stage of AD with its early onset and extensive pathology.

5XFAD mice show amyloid pathology- an important characteristic of Alzheimer's disease - but fail to exhibit hyperphosphorylated tau. Those AD mouse models that do show tau pathology differ from human clinical presentation in important AD features like

neuronal loss and intraneuronal Aβ (Wirths and Bayer 2010, Barone and Menna-Barreto 2011, Bertram, Rook et al. 2011, Bliwise, Mercaldo et al. 2011, Bruce-Keller, Gupta et al. 2011, Brunner, Gotter et al. 2011, Holtzman, Morris et al. 2011, Jiang, Striz et al. 2011, Keane, Goodstadt et al. 2011, Li, Cheung et al. 2011, Lloyd 2011, Moreno-De-Luca, Helmers et al. 2011, Morinaga, Ono et al. 2011, Pack and Pien 2011, Pavlova and Sheikh 2011, Philip, Sokoloff et al. 2011, Platt, Drever et al. 2011, Raizen and Wu 2011, Rillich, Schildberger et al. 2011, Ringwald, Iyer et al. 2011, Rolls, Colas et al. 2011, Skarnes, Rosen et al. 2011, Sperling, Aisen et al. 2011, Barron and Pike 2012, Bertram and Rook 2012, Callander, Bolton et al. 2012, Jawhar, Trawicka et al. 2012, Sakurai 2012, Zeng, Mott et al. 2012, Dyakonova and Krushinsky 2013, Fitzsimmons and Bertram 2013, Reichert and Gerhardt 2013, Stevenson and Schildberger 2013). In spite of their various limitations, the 5XFAD mice and other AD mouse models exhibit sleep alterations that resemble some aspects of the sleep disruptions reported in AD patients. As described above, the 5XFAD mouse model is especially useful because it exhibits neuronal loss, similar to AD patients, and the early onset pathology in the 5XFAD mice allows them to be studied at younger ages than other AD mouse models. Therefore, this mouse model is useful for investigations of the role of sleep loss in the progression of AD.

Recent studies show that sleep impacts Aβ levels in the brain. Diurnal oscillations of Aβ levels in human cerebrospinal fluid (CSF) and in mouse hippocampal interstitial fluid (ISF) exhibit lowest values during the rest phase. Furthermore, sleep deprivation during the normal rest phase elevates these Aβ levels (Kang, Lim et al. 2009). The diurnal rhythms of Aβ levels become attenuated and eventually lost as Aβ deposition in the brain progresses (Bertram, Rook et al. 2011, Roh, Huang et al. 2012). These changes begin in

parallel to onset of sleep disruptions in mice (Bertram, Rook et al. 2011, Roh, Huang et al. 2012). Further, a recent study by Xie and colleagues demonstrated that sleep strongly increases clearance of Aβ, one of the metabolites generated by neuronal activity, which is greatest during wakefulness (Xie, Kang et al. 2013). These studies further support the concept that sleep disruption may be one of the causal factors involved in progression of the AD. A feedback loop might exist where Aβ accumulation might deteriorate sleep quality which could lead to further Aβ accumulation and increasing the susceptibility of the patients further to the pathophysiological changes associated with AD.

Conclusions

The 5XFAD mouse model of AD overexpresses amyloid β at an early stage and is therefore useful in studying the effect of Aβ on sleep. Our findings showed various sleep-wake alterations in both male and female 5XFAD mice under baseline conditions and also after sleep deprivation. The overall decrease in bout length suggests increased fragmentation and disruption in sleep consolidation that may be relevant to human sleep disturbances in AD and other neurological diseases. Because sleep disturbances precede overt AD symptoms by ten years or more, and experimental sleep disruption accelerates Aβ deposition, sleep enhancement may be a valuable therapeutic target for treatment of AD that can be investigated in 5XFAD mice.

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Chapter 4 Altered sleep-wake behavior in a novel murine model of type 2 diabetes and Alzheimer's disease

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Author contributions: Mansi Sethi and Ren Gurriero performed the experiments. Mansi Sethi designed the experiments, and analyzed the data with supervision from Dr. Arnold Stromberg; Mansi Sethi wrote the manuscript; Mansi Sethi, Marilyn Duncan and Bruce O'Hara interpreted the results, and edited the manuscript. Alex M. Helman and Teresa Macheda genotyped the animals. Michael P. Murphy generated the db/AD animals.

Running title: Sleep changes in combined mouse model of Alzheimer's Disease and diabetes

Abstract

Sleep has been suggested to play a variety of roles ranging from learning, memory consolidation, and clearance of toxic metabolites such as amyloid beta (Aβ), to regulation of energy metabolism. Current data suggest bidirectional roles for sleep and Alzheimer's Disease (AD), with poor sleep promoting AD, and AD increasing sleep disruption. Insufficient sleep is also correlated with an increased risk of obesity and type 2 diabetes mellitus (T2DM), which in turn are increasingly linked to AD. Previous studies have shown that leptin-resistant db/db mice, which have T2DM and obesity, have attenuated sleep-wake rhythms in addition to increased sleep duration and sleep fragmentation. AD mouse models often show fragmented and/or reduced sleep. To examine the interaction of T2DM and AD, a novel mouse model was created by combining the db/db mutations with a commonly used mouse model of AD (APP/PS1). Mice with these combined mutations display severe cerebrovascular pathology, without increased Aβ deposition (Neidowicz et al., 2014). Cognitive impairments in the db/AD mice were more profound than those in db/db or APP/PS1 mice (Neidowicz et al., 2014). Given the association of sleep disruptions in both of these disorders, we examined multiple sleep variables in db/AD, and the individual mouse models db/db and APP/PS1. In the db/AD mice, we found significant alterations in baseline sleep including age associated reduced sleep duration across 24h, and in the dark as well as the light phase. The most significant differences were found during the dark phase. We also found sex and age effects for many of the sleep parameters. These db/AD mice may serve as an important tool to study the mechanisms involved at the intersection of T2DM and AD, and to examine sleep interventions to slow the progression of disease.

Keywords: phenotyping, piezoelectric system, sleep, knockout mice, mutant mice

Introduction

Sleep is indispensable for the role it plays in learning, memory consolidation, energy conservation, and the overall health of an animal. Sleep is also important for optimal cognition over short time periods, and probably also over longer time periods of many years (Ellenbogen 2005, Killgore and Weber 2014). Sleep perturbations are seen in a wide array of diseases- neuromuscular disorders, neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease, and Diffuse Lewy Body disease, as well as metabolic disorders such as obesity and type 2 diabetes mellitus (T2DM) (Knutson, Spiegel et al. 2007, Rothman and Mattson 2012, Suzuki, Miyamoto et al. 2012). With the increased life expectancy of the human population, the prevalence of age-related ailments such as AD and T2DM have reached epidemic proportions; therefore, it is imperative to interrogate the underlying mechanisms by which sleep modulates these pathologies.

AD is one of the most common types of dementia; characterized by amyloid beta (Aβ) plaques and neurofibrillary tangles as the hallmarks (Barker, Luis et al. 2002). Approximately, 25-60% of AD patients are affected by profound circadian and sleep-wake aberrations which are the major reason for their institutionalization (Pollak, Perlick et al. 1990, Bianchetti, Scuratti et al. 1995, Moran, Lynch et al. 2005, Guarnieri, Adorni et al. 2012). Circadian dysfunction is presented as a reduction of locomotor activity, and a phase delay of as much as 4 hours in body temperature and activity rhythms (Satlin, Volicer et

al. 1995, Harper, Stopa et al. 2001, Harper, Volicer et al. 2005). The sleep alterations are manifested as a reduction in slow wave sleep (Loewenstein, Weingartner et al. 1982, Martin, Loewenstein et al. 1986, Vitiello, Prinz et al. 1990) and rapid eye movement (REM) sleep, increased latency to REM sleep, increased sleep fragmentation associated with reduced nighttime and increased daytime sleepiness (Holth, Patel et al. , Prinz, Peskind et al. 1982, Martin, Loewenstein et al. 1986, Vitiello, Prinz et al. 1990, Petit, Gagnon et al. 2004). These sleep impairments in turn are positively correlated to the degree of progression of AD and also believed to lead to memory deficits (Walker 2009).

Aggregation of Aβ in the brain has been implicated in sleep perturbations and also in AD pathogenesis. Aβ deposition has been reported to begin as early as 10-15 years before the onset of clinical symptoms, and sleep disruptions as well can be seen since preclinical stages of AD (Kang, Lim et al. 2009, Potvin, Lorrain et al. 2012, Sterniczuk, Theou et al. 2013, Hahn, Wang et al. 2014). In one study, cognitively normal individuals with increased Aβ deposition were found to have shorter sleep and poorer quality of sleep (Ju, McLeland et al. 2013, Malkki 2013, Spira, Gamaldo et al. 2013). Studies in animal models also corroborate these findings and demonstrate an association between sleep perturbations and AD pathogenesis. In the APPswe/PS1dE9 AD mouse model, normal 24-hour fluctuations of Aβ in the hippocampal interstitial fluid (ISF) dissipated in older animals with Aβ plaque deposition along with disruption of sleep-wake cycle (Roh, Huang et al. 2012). Recently, Xie and colleagues have shown that sleep is linked to as much as 60% increase in brain interstitial space, which in turn facilitates increased Aβ clearance (Xie, Kang et al. 2013). Thus, the existing data posits that there exists a bidirectional role

for sleep and AD, with poor sleep increasing Aβ deposition and reducing clearance, and increasing Aβ leading to more sleep disruption.

T2DM, a chronic metabolic disorder, is a major health concern that affects 8% of adults in America (Harris, Flegal et al. 1998). T2DM and associated obesity, in turn are risk factors for other diseases including cardiovascular diseases as well as metabolic syndrome (Kahn, Buse et al. 2005). Similar to bidirectional relationship between Aβ deposition and AD, it has been postulated that a feedback loop exists between sleep impairments and type 2 diabetes (Barone and Menna-Barreto 2011). Additionally, reduced sleep is another risk factor for diabetes (Laposky, Bradley et al. 2008). Multiple studies have shown that sleep restriction (or deprivation) leads to reduced insulin sensitivity and impaired glucose metabolism (Knutson, Spiegel et al. 2007, Donga and Romijn 2014). Conversely, in rodents, diet (type, its availability and timings) has been shown to affect sleep patterns (Mavanji, Billington et al. 2012). The db/db mice have attenuated sleep-wake rhythms in addition to increased sleep fragmentation and increased overall sleep duration, which further strengthens the idea that sleep and metabolism are linked inversely (Laposky, Shelton et al. 2006, Laposky, Bradley et al. 2008).

AD and T2DM, which are both age-associated diseases, share several similarities such as cognitive deficits (Stolk, Breteler et al. 1997, Saedi, Gheini et al. 2016, Zilliox, Chadrasekaran et al. 2016), degenerative changes, and sleep alterations (Kuusisto, Koivisto et al. 1997, Luchsinger, Tang et al. 2004, Peila, Rodriguez et al. 2004, Yaffe, Blackwell et al. 2004, Ronnemaa, Zethelius et al. 2008, Ronnemaa, Zethelius et al. 2009, Schrijvers, Witteman et al. 2010). Amyloid is conceived to be a main factor responsible for the observed cellular degeneration of brain and pancreas in AD and T2DM, respectively

(Abedini and Schmidt 2013). Since the first Rotterdam study, many other studies have shown that individuals with diabetes have a doubled risk of AD (Ott, Stolk et al. 1999, Kroner 2009). Janson et al in a study of Mayo Clinic Alzheimer Disease Patient Registry (ADPR) found that diabetic patients are more vulnerable to AD compared to non-diabetic individuals (Janson, Laedtke et al. 2004). Thus, it is proposed that there may be common pathophysiological mechanisms related to both the diseases.

The patients with AD and a prior history of T2DM have cerebrovascular anomalies, in addition to the typical AD-related neuropathology without showing additional Aβ burden. The role of cerebrovascular anomalies or vascular dementia, a frequent comorbidity with Alzheimer's disease, is poorly understood. An animal model recapitulating both disease aspects is critical to understand how the two conditions interact at the cellular and molecular level, and promote cerebrovascular abnormalities**.** Previous attempts to model key features of both- diabetes and AD in rodents includes utilization of streptozotocin, which induces type 1 rather than type 2 diabetes (Park 2011, Thibault, Anderson et al. 2013). Other approaches such as feeding of a high-fat or western diet had only short term effects, while combined models of APP and insulin-resistant, or APP and leptin-resistant mice showed only a limited spectrum of pathology (Killick, Scales et al. 2009, Studzinski, Li et al. 2009, Julien, Tremblay et al. 2010, Kohjima, Sun et al. 2010, Takeda, Sato et al. 2010).

Recently, Niedowicz and colleagues created a novel mouse model by crossing leptin resistant db/db mice with APP/PS1, a knock-in mouse model of AD (Neidowicz et al., 2014). The parental strains- db/db animals are obese since the age of 3-4 weeks, display elevated plasma insulin at an early age of 10 days and increase in blood sugar levels since

4 to 8 weeks. They have a shorter life span and tend to live upto 18-20 months of age (Coleman 1978). Aβ deposition in APP/PS1 animals can be observed since the age of 6 months (Anantharaman, Tangpong et al. 2006). Neuritic plaques do not appear until 9 months of age (Murphy, Beckett et al. 2007). Cognitive and memory deficits have been reported during old- age (Bruce-Keller, Gupta et al. 2011).

The db/AD mice resulting from the cross of the above 2 strains, displayed additional pathologies than either of the parental lines, in particular, severe cerebrovascular abnormalities including aneurysms and small strokes, without increased Aβ deposition (Neidowicz et al., 2014). Cognitive impairment on the Morris water maze task was markedly profound in these mice compared to db/db and APP/PS1 mice alone. These db/AD mice are obese and glucose intolerant beginning at an early age and have parenchymal amyloid plaques similar to parental lines (Neidowicz et al., 2014). Thus, the db/AD mouse model mimics several of the significant features of humans with T2DM and AD. Given the role of sleep in both of these disorders, in this study we aimed to evaluate multiple sleep variables in the combined mouse model (db/AD) and also, the individual mouse models db/db and APP-PS1 and WT mice for comparisons.

Methods and Design

Generation of combined- db/AD mice

As described previously, the db/AD mice were generated by crossing $APP^{\Delta NL/\Delta NL}$ /$PS1^{P264L/P264L}$ (APP/PS1) knock-in mice (Citron, Oltersdorf et al. 1992, Mullan, Crawford et al. 1992) with leptin receptor-deficient mice ($Lepr^{db/db}$) (Niedowicz, Reeves et al. 2014). Briefly, the original heterozygous $Lepr^{+/db}$ stock (homozygous db/db animals are infertile) on a C57BL/6J background was purchased from The Jackson Laboratories, and APP/PS1

mice on CD-1/129 background were obtained from a breeding colony maintained at University of Kentucky and derived from stock purchased from Cephalon. F1 mice, heterozygous for the 3 alleles were then crossed to generate offspring of the genotypes: wild type, and homozygous db mice that were in turn wild type and homozygous for APP and PS1. For our study, we utilized the genotypes as listed: $Lepr^{db/db}$ X $APP^{\Delta NL/\Delta NL}$ /PS1 $^{P264L/P264L}$ *(db/AD)*, $Lepr^{+/+}$ X $APP^{\Delta NL/\Delta NL}$ /PS1 $^{P264L/P264L}$ *(AD)*, $Lepr^{db/db}$ X $APP^{+/+}$ /PS1 $^{+/+}$ *(db)* and $Lepr^{+/+}$ X $APP^{+/+}$ /PS1 $^{+/+}$ *(WT)*.

Experimental procedures

Animal housing and sleep phenotyping

This study utilized individually housed control and mutant mice generated on B6 X CD1/129 background. We included both males and females that were 2 to 16 months old. The animals were provided with ad-libitum access to water and food. Prior to the sleep recordings, mice were group-housed and exposed to a 14:10 alternating light/dark (LD) cycle (lights on at 8 h). We recorded a total of 119 animals in several subgroups as listed in Table 1. The animals underwent sleep recording in 2 batches based on their availability at two different times. The first batch included 29 animals, and the rest of the animals were recorded in the second batch. The baseline sleep data were collected for 3 days from most animals but for only 2 days in 30 animals because of issues at the animal facility. During sleep recording, the mice were exposed to a 12:12 LD cycle (lights on at 4am for first batch and 8am for second batch). The PiezoSleep system used in the study is comprised of 8 quad cage units, which allowed assessment of up to 32 mice per experiment. Pine shavings were added as bedding to the cages, and cotton squares (nestlets), which could be shredded and used to build nests, were provided as environmental enrichment. All experimental

procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky and are consistent with the Institute of Laboratory Animal Resources Guide for Care and Use of Laboratory Animals, 8th edition.

Genotyping

Tail snips were collected before weaning for genotyping for APP, db and PS1. Genomic DNA was isolated and purified using the Promega Wizard Genomic DNA kit (Promega; Madison, WI). db genotyping was then carried out using a single nucleotide polymorphism Taqman genotyping kit purchased from Applied Biosystems Life Technologies. As for APP and PS1, genotyping was performed using Promega GoTaq Flexi DNA Polymerase (as described previously; (Anantharaman, Tangpong et al. 2006)).

Sleep recording with piezoelectric system

Sleep and wake states were determined using the PiezoSleep System as described elsewhere (Flores, Flores et al. 2007, Donohue, Medonza et al. 2008, Mang, Nicod et al. 2014). The system is comprised of plexiglass cages lined with piezoelectric films across the cage floor that detect pressure variations. For all sleeping postures, pressure variations from breathing are detected. Sleep states are characterized by quasi-periodic signals with low variations in amplitude, whereas wakefulness and rest states are characterized by irregular transient and high amplitude pressure variations corresponding to voluntary body movements and weight shifting.

Signal features sensitive to the differences between the sleep and wake states are extracted from 8-second pressure signal segments, and classification is automatically performed every 2 seconds using overlapping windows. Sleep-wake decisions in the 2-second intervals are binned over specified time periods (e.g., 5 minutes, 1 hour) for local

percent sleep/wake statistics. In addition, durations of consecutive sleep states are used to compute mean sleep bout lengths. For the current study, the PiezoSleep system eliminates commonly occurring short arousals (as seen during instances of stirring or twitching) during sleep bouts. These arousals were counted as wake instances in the previous chapter, resulting in shorter bout lengths.

The sleep bout is calculated as the amount of time from initial sleep state to the first 30-second interval when the mouse is awake for over 50% of the time. The sleep bout length computed with the 50% wake in the 30-second interval rule typically marks a clear transition into an extended wake period, where the mouse does not quickly transition back into the sleep state. The piezo system has been validated with simultaneous EEG/EMG and human scoring of mice demonstrating a classification accuracy of over 90% in mice (Donohue, Medonza et al. 2008, Mang, Nicod et al. 2014). Mice unlike humans, are nocturnal and polyphasic i.e. they have fragmented sleep (each episode termed as bout), distributed across 24 h, and more so during daytime. Thus their sleep is measured in terms of number and length of such sleep bouts. The sleep statistics analyzed under baseline (undisturbed) conditions for experiments in this report were: percent sleep over 24 h periods, percent sleep during light phase, percent sleep during dark phase, average sleep bout length (across 24 h, light phase, and dark phase periods).

Statistical Analysis

Females and males were broadly grouped into 2 age groups: young (2-8 months), and old (8.5-15 months), given the predicted life span of db/AD mice is 15-16 months (Niedowicz, Reeves et al. 2014). For baseline data, we conducted ANOVA (analysis of variance) to determine statistically significant differences for the sleep-wake parameters

between different genotypes; segregated into subgroups by sex and age. As mentioned earlier, the animals were recorded in 2 batches. Initial assessment showed that there was no difference between first and second batch of animals therefore, the data was merged for subsequent analysis.

To examine the association between sleep parameters and interaction effects (genotype X sex, genotype X age, age X sex), baseline data was examined using multiple regression model. Each of the six sleep-wake parameters was treated as the outcome of interest. Genotype (db/AD, AD, db, WT), sex (male and female) and age (young and old) were included as categorical predictors. Backward selection approach was utilized with a significance level of five percent for the models, starting with the main effects of all 3 covariates and all the possible 3 two-way interactions (described above). Individual predictor terms and their interaction terms were removed if the P value was less than 0.05. JMP statistics software version 12.0 was used for all modeling analysis and ANOVA. Plots were used to test the data for normal distribution and homogeneity of variance.

Results

In all, 119 animals- males and females, categorized as young and old were utilized. The number of animals per sub-group is listed in table 1. The means and standard error for each group is presented in table 2.

Females

Genotypic differences

Total sleep time (% sleep per 24h) was increased in old db females compared to age-matched all other 3 genotypes- db/AD ($p<0.0011$), wild type ($p=0.019$) and AD ($p=0.0224$). A similar genotype effect was seen concerning sleep during the dark phase, in

that db mice had increased sleep amounts compared to the other 3 groups of animals-db/AD (p=0.0017), AD (p=0.001) and WT (p=0.0017). The order follows: db>WT and AD>db/AD for sleep across 24h and dark phase. This indicates that genotypic differences in sleep duration mainly occur during the active or dark phase. The genotypic effect was less prominent in younger animals; db/AD had elevated sleep compared to controls across 24 h (p=0.0291) as well as during the dark phase (p=0.0041). db/AD and db had comparable sleep durations. There was no genotype difference in bout length durations for any of age groups (Figure 1 and Table 2).

Effect of age

Old db/AD mice slept less than the young animals across both light and dark phase. In contrast, bout length did not reflect an age difference. We did not have any females in the AD young group. For the remaining groups, we did not find any effect of age.

Males

Genotypic differences

Younger males displayed more prominent genotypic differences unlike females which showed marked differences in older age. Both db and db/AD mice have increased duration of sleep across 24 h, and dark phase compared to WT and AD animals. The total sleep duration is comparable between db mice and db/AD mice, and between WT mice and AD mice. Concerning sleep duration during the light phase, db/AD and db mice mice had similar sleep durations that were elevated compared to WT (p=0.0151 and 0.0129 respectively). Sleep durations tend to be equal for db/AD and db across light phase. Bout length across 24h was in the same mean range for all the groups. Mice of the db/AD genotype had shorter sleep bouts than WT mice during the dark phase, and AD mice had

increased bout lengths with respect to db animals for the light phase. AD mice did not differ from WT for bout lengths across 24 hours or during the dark or light phase. Concerning the older subjects, no db mice were available. For the other three groups, there were no differences in sleep amounts or the bout lengths.

Effect of age

The older males of the db/AD genotype slept less during the light phase than younger mice. Like db/AD females, younger male littermates slept longer than old animals during light phase. AD young males had less sleep duration compared to old mice during 24h and dark phase. Control males in general had increased bout lengths during night compared to females. db/AD young males during light phase displayed increased bout lengths than females of same age.

Multiple regression

The adjusted multiple regression models are shown in Table 3, with each sleep parameter as an outcome of interest, and genotype, age, sex or/and the interaction terms- ageX genotype and sex X genotype as predictor variables. The co-variates included in each of the model is depicted in the Table 3. The selection of the variables is based on backward elimination as described in the Methods section. For sleep across 24h, the $R_{adjusted}^2$ is 0.19 which means that the model explains 19% of variation seen in overall sleep. The diabetic animal group has significantly higher sleep percent ($p=0.0007$) than the control group after controlling for age and ageXgenotype interaction effect. Age and genotype interaction effect was significant for db/AD group compared to WT animals. For sleep across dark phase, the model explained 26% of the variation. After adjusting for other covariates in the model, diabetic animals were estimated to sleep 10% more during the dark phase compared

to controls ($p<0.0001$). In addition, AD animals slept approx. 6% less than the reference (control) group ($p=0.0045$). Similar to total sleep, age and genotypic interaction for db/AD animals were associated with sleep across the dark phase ($p=0.0002$) as well as across the light phase ($p=0.0034$).

Sex was included as covariate in the models for bout length. In case of bout length across 24h, AD animals had increased bout length (42 s/bout, $p=0.0236$) after controlling for other covariates in the model. Females compared to males had shorter sleep bout across 24 h (31.5 s; $p=0.0055$) as well as during the dark phase (28.63 s; $p=0.0077$). Additionally, sex Xage interactions were significant for the old animals ($p=0.0159$) for bout length during the dark phase. Older animals had shorter bout length during light phase (39.32s; $p=0.0359$) than the younger mice after adjusting for the other predictors in the model. Females also have reduced bout lengths for the light phase (39.48 s; $p=0.013$). AD animals have increase in light phase bout lengths by an estimated mean of 100s ($p=0.0004$) with respect to the controls.

Table 3 List of the sample size for each of the group divided based on genotype, sex and age

Animal count

Genotype-sex-age	Count
Db/AD -F-old	7
Db/AD -F-Young	17
Db/AD -M-old	4
Db/AD-M-Young	8
Db-F-old	3
Db -F-Young	8
Db -M-Young	11
AD -F-old	19
AD -M-old	7
AD -M-Young	3
WT -F-old	14
WT -F-Young	6
WT -M-old	7
WT -M-Young	4
Total	119

Table 4 . Effect of genotype on sleep wake traits under baseline conditions for males and females

Males			**Genotype**		
Parameter	*Age group*	*WT*	*DB*	*AD*	*AD/Db*
% Sleep 24 h	*Old*	47.6±2.3	N.D.	50.7±1.6	43.8±7.5
	Young	43.5±2.2	55.6±1.6	44.3±3	53.7±1.3
% Sleep Dark	*Old*	38.7±3	N.D.	37.2±2.5	35.8±3.8
	Young	31.4±2.5	45.3±1.8	25.3±3.7	41.3±2.5
% Sleep Light	*Old*	56.5±2.3	N.D.	64.2±2	51.9±11.4
	Young	55.7±3.5	65.9±2.4	63.3±2.4	66.1±1.5
Bout length 24h	*Old*	439.6±44.6	N.D.	528.2±41.1	465.2±119.7
	Young	501.2±52.3	466.7±27.1	542.2±31.8	462.7±21.5
Bout length Dark	*Old*	384.6±43.4	N.D.	429.9±42.6	412.1±97.3
	Young	438.4±57.2	372.8±23.1	313.4±41.2	330.2±27
Bout length Light	*Old*	496.7±53.4	N.D.	633.9±44	512±142.4
	Young	562.3±51.1	577.6±36.1	763.4±14.5	660.7±57.7

Females			**Genotype**		
Parameter	*Age group*	*WT*	*DB*	*AD*	*AD/Db*
% Sleep 24 h	*Old*	48.7±1.8	58.2±2.1	49.2±0.8	43.3±3.9
	Young	40.9±8.2	51.9±4	N.D.	53.5±1.7
% Sleep Dark	*Old*	33.9±2.9	52.9±2.1	33.4±1.5	32.4±4.2
	Young	26.4±6.2	42.7±4.4	N.D.	43.6±2.4
% Sleep Light	*Old*	63.5±1.9	63.6±2.4	65±1.4	54.3±4.4
	Young	55.4±11.3	61.1±3.8	N.D.	63.5±1.6
Bout length 24h (s)	*Old*	395±29.3	324.5±15.1	443.5±29	404.8±66.7
	Young	385.8±59.3	438.7±34.5	N.D.	419.6±24.7
Bout length Dark (s)	*Old*	272.6±16.8	275.6±22.3	324.4±21.6	310.3±72.5
	Young	280.3±29.5	369.1±34.5	N.D.	360.3±31.4
Bout length Light (s)	*Old*	476.5±34.1	386.1±4.6	571.1±52.8	485.9±65.8
	Young	445.9±104.8	511.7±38.1	N.D.	494.2±24.2

Values represent mean ± SEM. N.D. No data

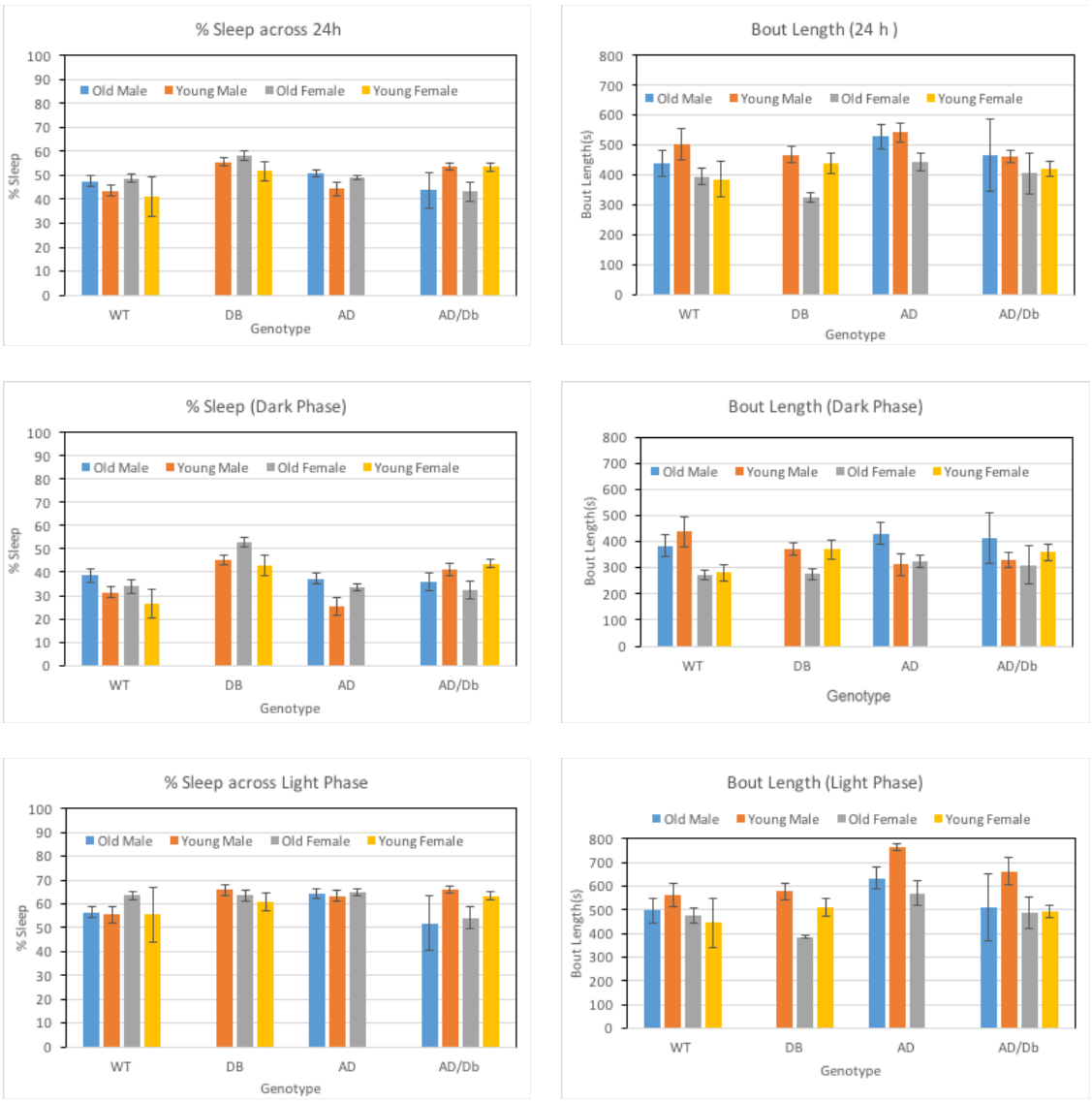

Figure 4 Sleep–wake patterns in AD, db, db/AD and WT littermates under baseline conditions. Average percent sleep analyzed over (A) 24 h, (B) dark phase, and (C) light phase. (D–F) depicts average bout length in seconds (s) over (D) 24 h (E) dark phase, and (F) light phase.

Table 5 Multiple linear regression results using sleep parameters as the outcome of interest. Estimate corresponds to the respective non-intercept parameter estimates in the final model.

Predictor	**Estimate**	**Standard error**	**P value**
1. Sleep % across 24 hours		**R_{adj}^2=0.19**	**p<0.0001**
Age: Young	Reference		
Old	0.62	0.96	0.5187
Genotype: WT	Reference		
db/AD	-0.76	1.38	0.5840
db	6.83	1.97	0.0007
AD	-1.91	1.77	0.28
AgeXGenotype: AgeXWT	Reference		
AgeXdb/AD	-5.66	1.38	<.0001
AgeXdb	1.49	1.97	0.45
AgeXAD	1.59	1.77	0.37
2. Sleep % across dark phase		**R_{adj}^2=0.26**	**p<0.0001**
Age: Young	Reference		
Old	1.49	1.10	0.1791
Genotype: WT	Reference		
db/AD	0.63	1.59	0.6921
db	10.92	2.26	<.0001
AD	-5.89	2.03	0.0045
AgeXGenotype: AgeXWT	Reference		
AgeXdb/AD	-6.11	1.59	0.0002
AgeXdb	2.83	2.26	0.2136
AgeXAD	1.20	2.03	0.5549
3. Sleep % across light phase		**R_{adj}^2=0.08**	**p=0.0225**
Age: Young	Reference		
Old	-0.25	1.20	0.8371
Genotype: WT	Reference		

db/AD	-2.15	1.74	0.2185
db	2.75	2.47	0.2688
AD	2.06	2.22	0.3543
AgeXGenotype: AgeXWT	Reference		
AgeXdb/AD	-5.20	1.74	0.0034
AgeXdb	0.15	2.47	0.9514
AgeXAD	1.99	2.22	0.3726

Predictor	**Estimate**	**Standard error**	**P value**
1. Bout length 24h		**R_{adj}^2=0.07**	**p=0.0135**
Genotype: WT	Reference		
db/AD	-7.37	17.51	0.6746
db	-12.10	20.79	0.5617
AD	42.56	18.55	0.0236
Sex: Male	Reference		
Female	-31.50	11.14	0.0055
2. Bout length Dark Phase		**R_{adj}^2=0.08**	**p=0.0162**
Age: Young	Reference		
Old	-2.77	12.49	0.82
Genotype: WT	Reference		
db/AD	-3.99	17.29	0.8178
db	5.62	21.41	0.7934
AD	21.80	19.54	0.2668
Sex: Male	Reference		
Female	-28.63	10.55	0.0077
SexXAge: SexXYoung	Reference		
SexXOld	-26.25	10.72	0.0159
3.Bout length Light phase		**R_{adj}^2=0.13**	**P=0.0012**
Age: Young	Reference		
Old	-39.32	18.51	0.0359
Genotype: WT	Reference		
db/AD	-14.93	25.63	0.5615

db	-52.83	31.74	0.0988
AD	106.01	28.96	0.0004
Sex: Male	Reference		
Female	-39.48	15.64	0.013
SexXAge: SexXYoung	Reference		
SexXOld	16.89	15.89	0.29

The percentage of total variation in any of the sleep parameter that can be explained by our model is indicated by value of Radj2 as listed in the table.

Discussion

Sleep is a multifaceted process with diverse indirect and/or direct functions in cognition, metabolism, learning and memory consolidation, and amyloid beta clearance (Tucker, Hirota et al. 2006, Knutson, Spiegel et al. 2007, Marshall and Born 2007, Diekelmann and Born 2010, Xie, Kang et al. 2013, Krueger, Frank et al. 2016). Dysfunctional sleep therefore has detrimental effects on health, and is linked to many pathological conditions including AD and T2DM (Grandner, Seixas et al. 2016). Previously, sleep aberrations were considered to be a byproduct of these pathologies but mounting evidence supports the proposition that a reciprocal relationship exists between sleep loss and AD, and sleep apnea and T2DM.

Apart from cognitive decline, T2DM can also lead to renal impairments, vision loss and cardiovascular disease (Association). It also increases the risk of dementia, specifically vascular dementia and AD (Ott, Stolk et al. 1999, Xu, Qiu et al. 2004, Biessels, Staekenborg et al. 2006, Luchsinger, Reitz et al. 2007, Reijmer, van den Berg et al. 2010, Vagelatos and Eslick 2013). Furthermore, in individuals with T2DM who also exhibit dementia, there is an increased incidence of cerebrovascular pathology (Ahtiluoto,

Polvikoski et al. 2010). In addition to the pathways regulating interaction of AD and T2DM, the cerebrovascular dysfunction co-occurring with other dementias or in isolation is poorly understood (Grinberg and Heinsen 2010). A mouse model was thus generated by crossing a db/db mouse with APP/PS1- a knock-in AD animal model. This novel db/AD mouse model can further our understanding of diabetes promoting neurodegenerative diseases, specifically AD and vascular dementia, and other secondary implications resulting from interaction between AD and db. The db/AD mouse recapitulates the key aspects of both AD and db parental lines. These mice are obese, insulin resistant and glucose intolerant, and have amyloid deposition similar to their parental lines. In addition, they also exhibit exacerbated cerebrovascular abnormalities such as aneurysms and strokes, without any additional burden of amyloid beta similar to the human disease condition. They have a significant decline in cognition- more than either of the AD or db parents, which might be due to the vascular abnormalities. In our current studies, we were interested in evaluating the effect of db/AD genotype on sleep phenotypes. We assessed several sleep-wake parameters in db/AD animals as well as their parental lines to understand holistically the effect of the interaction between db and AD on sleep, and the origin of these sleep differences. In addition to the main effect of genotypes, we also examined 2-way interaction amongst genotype, sex and age.

Multiple regression indicated that bout length is affected by sex and females tend to have shorter bouts compared to males. Shorter sleep duration during the dark phase was found in case of AD animals, after controlling for other variables. Increased bout lengths were found across 24h as well as light phase. In a previous study conducted on the parental APP/PS1 strain, our research group did not find any effect of genotype on sleep for the

male mice. In that study, there was an effect of age on percent sleep during day and bout length during either day or night, with older animals sleeping less and having shorter bouts of sleep (Duncan, Smith et al. 2012). In the current study, one-way ANOVA indicated that aging increases sleep in male AD mice, in contrast to the earlier study. This difference might be related to several factors, including differences in the age ranges, low sample size for younger group, or a genetic difference in the background strain (i.e., additional BL6 composition of the mice in the current study). Our findings are similar to the ones reported in 5XFAD AD mouse model. 5XFAD male and female mice, with early onset of AD pathology, exhibited increased sleep fragmentation (as indicated by longer sleep bouts) with prominent effects evident during the dark phase than light phase (Sethi, Joshi et al. 2015). In addition, females also had reduced overall sleep due to marked reduction in sleep in dark phase. Other AD models such as $PLB1_{triple}$ (hAPP/hTau/hPS1) and TgCRND8 mice were found to have reduced NREM sleep. In contrast, PDAPP (overexpresses hBAPP) and Tg2576 have less REM sleep during dark phase (Huitron-Resendiz, Sanchez-Alavez et al. 2002, Zhang, Veasey et al. 2005, Platt, Drever et al. 2011, Colby-Milley, Cavanagh et al. 2015). Another, related mouse- APP/PS1 with a different PS1 mutation (APPswe/PS1dE9), was reported to have reduced REM and NREM sleep (Roh, Huang et al. 2012).

Sleep and metabolism are mutually exclusive processes (Adamantidis and de Lecea 2008), with inadequate sleep resulting in reduced leptin and elevated ghrelin levels, in addition to an increase in BMI in a dose dependent way (Gupta, Mueller et al. 2002, Sekine, Yamagami et al. 2002, Taheri, Lin et al. 2004). In contrast, food deprivation in rats was linked to reduction in sleep duration, and refeeding to increase in its duration (Borbely

1977, Danguir and Nicolaidis 1979, Shemyakin and Kapas 2001, Minet-Ringuet, Le Ruyet et al. 2004). Sleep architecture is also modulated by the type of diet and the time of availability. As observed in mice, a high fat diet resulted in increased duration of NREM sleep, as well as number of episodes (Jenkins, Omori et al. 2006). The leptin deficient ob/ob mice and leptin insensitive db/db mice have altered sleep architecture reflected as fragmented sleep, and increased total NREM sleep. Furthermore, their sleep-wake rhythms are diminished, and NREM delta power and locomotor activity is reduced as well (Laposky, Shelton et al. 2006, Laposky, Bradley et al. 2008). Additionally, recovery sleep followed by sleep deprivation is also reduced in both db/db and ob/ob mice (Laposky, Shelton et al. 2006, Laposky, Bradley et al. 2008, Mavanji, Billington et al. 2012). Similar alterations in sleep-wake patterns have been reported in several other rodent models of obesity including obese zucker and spontaneously hypertensive rats, and diet-induced obese mice. Our results also depict similar sleep patterns for both db and db/AD animals for both the sexes. Marked differences were seen in particular during the dark phase where both db and db/AD animals slept longer than controls implicating the effect of db gene. Multiple regression reflected that age interacts with db/AD genotype for the sleep duration variables. Although we did not find any evidence of sleep fragmentation in these db/AD and db mice, increased sleep during the active phase of the mouse emulates the human condition. We found similar sleep phenotype for db/AD and db animals. However, db/AD has been reported to have severe cognitive impairments relative to db animals (Niedowicz, Reeves et al. 2014). Our findings thus indicate that the cerebrovascular pathology seen in db/AD mice, believed to be responsible for their cognitive deficits does not lead to additional sleep aberrations.

Since sleep modulates energy metabolism, and the reverse holds true as well, some common neural circuits can be involved in regulating both sleep and metabolism (Collet, van der Klaauw et al. 2016). Some of those neural pathways might be mediated through the hypothalamus, a brain region central to sleep regulation as well as energy homeostasis. A set of SCN neurons have been shown to project to the arcuate nucleus of the hypothalamus, which then forms reciprocal connections with orexigenic neurons expressing NPY/AGRP, and anorexigenic neurons expressing POMC and CART (Saeb-Parsy, Lombardelli et al. 2000, Yi, van der Vliet et al. 2006, Huang, Ramsey et al. 2011). These neuropeptides are modulated by leptin- a satiety hormone under circadian regulation which is released in increased amounts at night (Sinha, Ohannesian et al. 1996, Licinio, Mantzoros et al. 1997).

Leptin can be one of the upstream molecular components involved at the intersection of AD and diabetes. Leptin mediates its functions through JAK/STAT signaling pathway, activating kinases such as MAPK, AKT and mTOR (Marwarha and Ghribi 2012). These kinases are known to inhibit Glycogen synthase 3 Kinase (GSK3B) which then suppresses GSK3 dependent tau phosphorylation. Down-regulation of leptin signaling as seen in T2DM can therefore result in increased tau phosphorylation which can subsequently promote AD development (Chen, David et al. 2004). Niedowicz et al found that db/AD and db mice do in fact have upregulated tau phosphorylation along with increased PS1 expression. Unlike expected, these increased levels of PS1 were not accompanied by additional Aβ deposition, either in the brain or in vasculature (Neidowicz et al., 2014). Though, Aβ oligomers were found to be elevated in db and more so in AD/db mice. Hyperinsulinemic individuals have an increased tau phosphorylation, and are at a

higher risk of developing AD and cognitive decline (Luchsinger, Tang et al. 2004). Thus, another possible instigator of tau pathology can be insulin, the release of which is temporally regulated.

Leptin also acts at the level of hippocampus and modulates synaptic plasticity and is presumed to be a cognitive enhancer. A study utilizing data from AD Neuroimaging Initiative (ADNI) reported low plasma leptin in 70% of subjects with MCI. Leptin treatment in AD mice reduces AD pathology, i.e. tau phosphorylation and amyloid beta, which consequently also improved cognition (Freude, Plum et al. 2005). The mechanism listed above, explaining the interplay among sleep, AD and db is possibly only one aspect of the complex cascade of events. There may be several yet unappreciated signaling pathways involved; with other neuroendocrine and metabolic components playing a critical role as well. For instance, GSK3β, in addition to their involvement in the leptin pathway, also affects rhythm of clock genes in SCN (Avila, Leon-Espinosa et al. 2012, Besing, Paul et al. 2015). Reduction in leptin can result in upregulated GSK3β which might subsequently result in fragmented sleep, as found in transgenic mice overexpressing GSK3β (Ahnaou and Drinkenburg 2011). Orexin can be another possible component considering that is implicated in metabolism in addition to sleep. Inhibition of orexin system has been reported to not only improve sleep but also glucose metabolism in db/db animals (Tsuneki, Kon et al. 2016). Collectively, these studies suggest that diabetes associated dysfunctions promote AD pathology which can then disrupt sleep. Conversely, sleep impairments might be augmenting the AD and T2DM diseased states. Further interrogation of the underlying pathways can bridge the gap in our understanding of how sleep, AD and diabetes are inter-connected.

In summary, our study indicate that AD mice tend to sleep less during night compared to control mice. In contrast, db genotype (db/AD and db) resulted in increased overall duration of sleep culminating from increased sleep duration at night. The db/AD mice are obese, insulin resistant and glucose intolerant, and have amyloid deposition similar to their parental lines. Furthermore, they also display additional cerebrovascular abnormalities, and reduction in Aβ compared to AD mice, as have been reported in case of diabetic humans with AD-neuropathology. These mice thus emulate key aspects of human pathological condition and can well serve as model to interrogate how the interaction of db and AD leads to cerebrovascular abnormalities, and reduction in AD pathology.

Chapter 5 Noninvasive sleep monitoring in large-scale screening of knock-out mice reveals novel sleep-related genes

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Abstract

Sleep is a critical process that is well-conserved across mammalian species, and perhaps most animals, yet its functions and underlying mechanisms remain poorly understood. Identification of genes and pathways that can influence sleep may shed new light on these functions. Genomic screens of sleep enable the detection of previously unsuspected molecular processes of sleep. In this study, we report on a large scale phenotyping of sleep-wake parameters for a population of single-gene knockout mice at The Jackson Laboratory, a primary production and phenotyping center for the Knockout Mouse Program (KOMP2). Sleep-wake parameters were measured using a high throughput, non-invasive piezoelectric. Knockout mice generated on a C57BL6/N (B6N) background were monitored for sleep and wake parameters for five days under baseline conditions. Thus far, we have recorded sleep in over 6000 mice representing over 300 single gene knockout lines, and more than 1800 B6N control mice (females and males). Our study also integrated assessment of breath rates as a supplemental tool in determining aberrant physiology in these knockout lines. Significant sleep-wake differences in both light and dark phases were found for a number of knockout lines compared to controls. We have identified more than 60 genes influencing various sleep traits which have not previously been implicated in sleep. Additionally, sex differences were found for B6N mice and many of the knockout mouse strains. Control females exhibited shorter bout lengths and less total sleep compared to males as reported by other studies. Further studies investigating these genes, their correlation with other phenotypes and interaction with other known sleep related genes can provide insight into the pathways regulating sleep and its associated functions.

Keywords: phenotyping, piezoelectric system, sleep, knockout mice, mutant mice

Introduction

Sleep is a complex behavior common to all birds and mammals, and probably most or all other vertebrates and invertebrates with a nervous system. Regulated by a multitude of neuronal processes and indirectly by gene networks, it is a process vital for an organism's well-being. Sleep has been suggested to have a role in functions such as learning, memory consolidation, energy restoration, synaptic optimization and recently it has also been implicated in the clearance of metabolites, including amyloid beta (Aβ) (Tononi and Cirelli 2006, Tucker, Hirota et al. 2006, Marshall and Born 2007, Nishida, Pearsall et al. 2009, Diekelmann and Born 2010, Xie, Kang et al. 2013, Krueger, Frank et al. 2016).

Genetic manipulations have advanced our knowledge about some aspects of sleep, including influences on the sleep EEG, sleep disorders, brain areas regulating sleep processes, and molecular pathways underlying sleep and its regulation. However, relatively few gene mutations or gene knockouts in mice have been examined for effects on sleep, and there are still many unresolved questions regarding the biological need for sleep, functions of sleep, and the genetic and physiological basis of sleep homeostasis that could be addressed with insights from model organisms (Rechtschaffen 1998, Cirelli 2009, Vassalli and Dijk 2009).

There have been numerous efforts to address these questions utilizing a variety of animal models including mice. These efforts range from individual labs studying specific knockout mice, to large-scale QTL (quantitative trait loci) and genome-wide projects

including phenotype-driven ENU (N-ethyl-N-nitrosourea) mutant screens involving many labs, and gene-driven knockout mouse phenotyping programs (Gondo, Fukumura et al. 2009).

Circadian clock genes such as *Clock* and *Rab3a* in mice, and *Per,* and *Dbt* in flies, which influence both circadian timing and sleep homeostasis have been identified using ENU/EMS (N-ethyl-N-nitrosourea/Ethyl methanesulfonate) mutagenesis techniques (Vitaterna, King et al. 1994, Kloss, Price et al. 1998, Kapfhamer, Valladares et al. 2002, O'Hara, Ding et al. 2007, Cirelli 2009). Discovery of these clock genes led to identification of many others (*Bmal1/Cyc, Cry1,2, Per1,2,3,* etc.) that also underlie circadian and homeostatic aspects of sleep. However, many of these mutations produce only subtle phenotypes, which are difficult to detect, and, in addition are also affected by the genetic background of the mouse (Nadeau 2001). Approaches using traditional mouse strains, genetic crosses, and QTL strategies have also identified a modest number of genes that influence sleep including *Homer1a*, *Acads* (acyl-coenzyme A dehydrogenase), and *Rarb* (Retinoic acid receptor beta) (Tafti, Petit et al. 2003, Drager 2006, Maret, Dorsaz et al. 2007). A disadvantage of traditional QTL approaches is that they are subject to limited mapping resolution, and identifying the causal gene(s) is often difficult or not undertaken (Tabor, Risch et al. 2002, Churchill, Gatti et al. 2012), although this situation is improving with recent advances in mapping populations and related approaches (Jiang, Scarpa et al. 2015).

A major bottleneck in large scale genetic studies of sleep is the difficulty, expense, and time demands of traditional EEG/EMG studies. While knockout studies of selected

target genes such as neurotransmitter receptors have found modest effects on at least one sleep parameter, relatively few genes have been examined (O'Hara, Jiang et al. 2017). Using a higher throughput, non-invasive approach allows for much larger numbers of mice to be examined (Flores, Flores et al. 2007, Pack, Galante et al. 2007, Donohue, Medonza et al. 2008, Philip, Sokoloff et al. 2011, Mang, Nicod et al. 2014). Our approach utilizes a sensitive piezoelectric film across the mouse cage floor, and is especially well suited to characterization of large-scale resources such as the International Knockout Mouse Consortium (IKMC) (Ringwald, Iyer et al. 2011). IKMC aimed to generate mutant embryonic stem cells (ES) for every coding gene in the mouse genome on the B6N background. As live mice are made from the ES cell lines, these single-gene knockouts undergo a core set of broad-based phenotyping screens at the KOMP2 centers and as part of the International Mouse Phenotyping Consortium (IMPC) (Abbott 2010, Bradley, Anastassiadis et al. 2012, Brown and Moore 2012). At The Jackson Laboratory KOMP2 Center (JAX-KOMP2) sleep is part of this pipeline, and the results thus far are described in this report.

Methods and Design

Generation of KO mice

IKMC mouse mutants generated on a C57BL/6NJ mouse background have either null alleles, which have an entire locus removed or "knockout-first" alleles, which permits generation of conditional alleles by utilization of site-specific recombinase as described previously (Skarnes, Rosen et al. 2011, Schofield, Hoehndorf et al. 2012). Strain C57BL/6 is a well-characterized inbred strain which also serves as a reference strain for the mouse genome, making it an ideal choice for this effort, although differences between the

C57BL/6J and C57BL/6NJ substrains exist (Keane, Goodstadt et al. 2011, Simon, Greenaway et al. 2013, Mekada, Hirose et al. 2015)**.** Mice generated by the JAX-KOMP2 effort are non-conditional (Tm1b) null alleles. Homozygous mutants were screened for sleep wake phenotype in this study.

As part of the JAX-KOMP2 phenotyping pipeline, each mouse is comprehensively phenotyped for over 200 measurements, from age 4-18 weeks, for a range of morphological, physiological and behavioral traits including many disease relevant parameters pertaining to neurobehavior, metabolism, immune, cardiovascular, sensory, and musculo-skeletal systems, followed by terminal collection of blood and histopathology (Morgan, Simon et al. 2012) (Fig 1). Additional tests such as light/dark and hole-board exploration tests, rotarod, and sleep, are unique to the JAX-KOMP2 pipeline. Sleep is evaluated using a PiezoSleep System (*Signal Solutions, LLC, Lexington, KY*), a non-invasive, high throughput sleep-wake monitoring system (details provided in following sections). The primary traits analyzed are total sleep duration averaged across 24 h, across the 12 h light phase and 12 h dark phase, and average sleep bout lengths (across 24 h, dark, and light phase), and breath rate during all sleep periods.

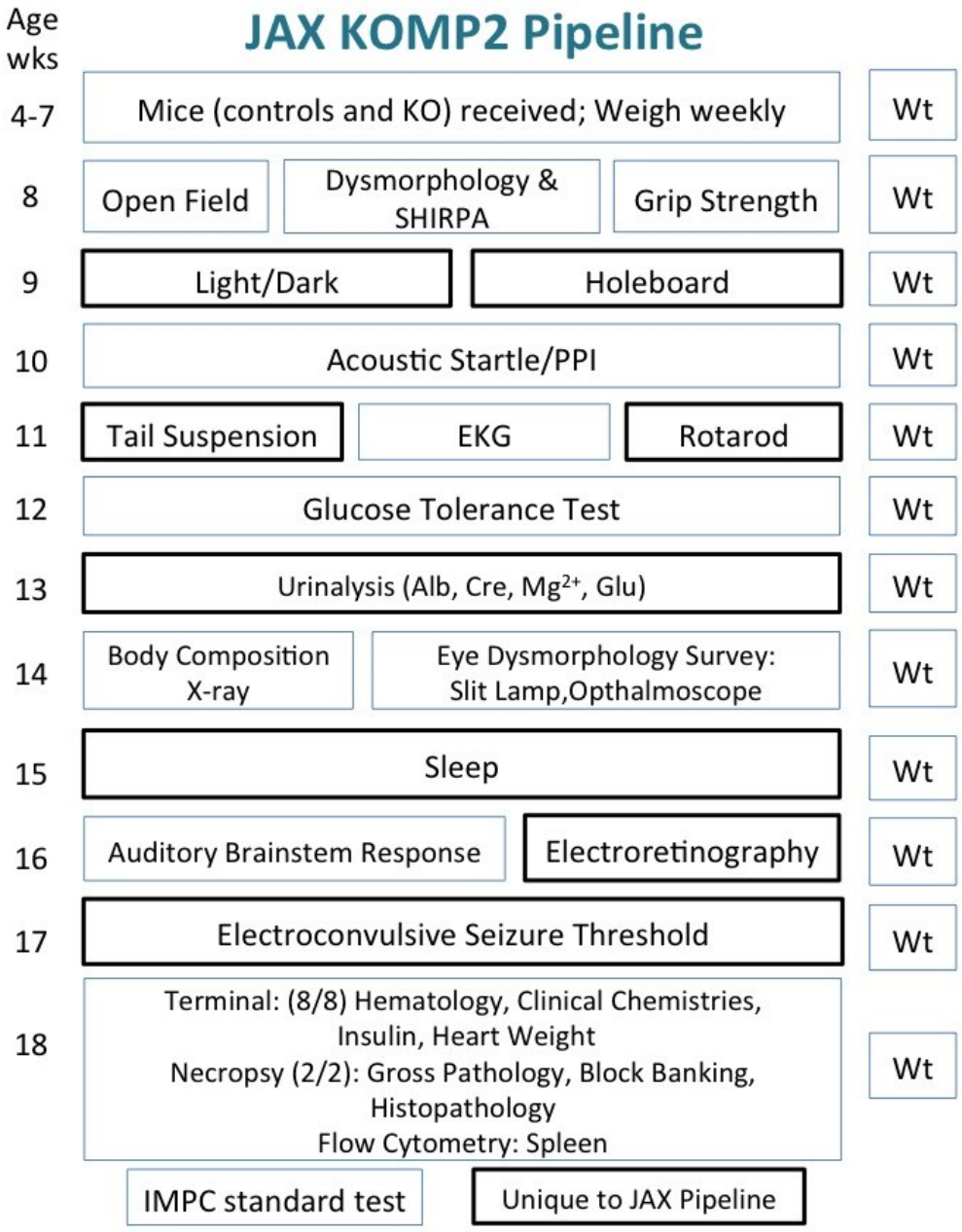


Figure 5 Phenotyping pipeline at The Jackson Laboratory

Sleep recording with piezoelectric system

Sleep and wake states were determined using the PiezoSleep System (Flores, Flores et al. 2007, Donohue, Medonza et al. 2008, Mang, Nicod et al. 2014). The system is comprised of plexiglass cages lined with piezoelectric films across the cage floor that detect pressure variations. For all sleeping postures, pressure variations from breathing are detected. Sleep states are characterized by quasi-periodic signals with low variations in amplitude, whereas wakefulness and rest states are characterized by irregular transient and high amplitude pressure variations corresponding to voluntary body movements and weight shifting.

Signal features sensitive to the differences between the sleep and wake states are extracted from 8-second pressure signal segments, and classification is automatically performed every 2 seconds using overlapping windows. Sleep-wake decisions in the 2-second intervals are binned over specified time periods (e.g., 5 minutes, 1 hour) for local percent sleep/wake statistics. In addition, durations of consecutive sleep states are used to compute mean sleep bout lengths. To eliminate the impact of short and ambiguous arousals on the bout length statistic, a bout length count is initiated when a 30-second interval contains greater than 50% sleep and terminates when a 30-second interval has less than 50% sleep. The sleep bout length computed with the 50% wake in the 30-second interval rule typically marks a clear transition into an extended wake period, where the mouse does not quickly transition back into the sleep state. The piezo system has been validated with simultaneous EEG and human scoring of mice demonstrating a classification accuracy of over 90% in mice (Donohue, Medonza et al. 2008, Mang, Nicod et al. 2014). The sleep statistics analyzed under baseline (undisturbed) conditions for experiments summarized in this report were: percent sleep over 24 h periods, percent sleep during light phase, percent

sleep during dark phase, average sleep bout length (across 24 h, light phase, and dark phase periods), and mean breath rate during sleep.

Animal housing and Phenotyping

This study utilizes control and KO mice generated on B6N background. From wean age, mice were housed at 3-5 mice per pen in pressurized, individually ventilated cages using pine shavings as bedding, with free access to acidified water and food (LabDiets 5K52, LabDiet, Scott Distributing, Hudson, NH). The housing facility was maintained on a 12:12 light/dark cycle starting at 0600. At 15 weeks of age, mice were removed from their home cages and placed into individual cages of the piezo system. The system used in the JAX-KOMP2 pipeline is comprised of 16 4-cage units, allowing assessment of up to 64 mice per 5-day experiment. Light cycle and food and water access were as that of standard housing conditions. A minimum amount of pine shavings was provided to each cage to allow sufficient detection of pressure signal. In each testing week, 10 control B6N animals (five females and five males) and 3-19 animals per KO line were tested. Females and males of all KO strains were analyzed, with a pipeline throughput goal of testing eight animals of each sex for all screens. Data presented in this report includes 1884 B6N mice (Females= 960, Males= 924) and 4467 KO mice (2243 females and 2224 males) from 318 KO strains.

Analysis

A data confidence metric that ranges from 0 through 1 assesses the signal quality and/or outlying signal behavior. Any of the sleep recordings with a data confidence value below the threshold of 0.6 was removed from the analysis. The control mice were also screened for outliers based on extreme high/low values for their sleep/wake parameters.

Control mice with Mahalanobis outlier distance (MD) above the upper control limit of 3.75 were excluded from the analysis. The final dataset used for analysis contained 1884 Controls (960 Females; 924 Males), and 4467 KO (2243 Females; 2247 Males). One-way analysis of variance (ANOVA) was performed for each sleep variable and breath rate, and posteriori multiple comparisons were done using Dunnett's test to identify genotypes showing significant difference/s with respect to the control group. Similar analysis was done to identify genotypes with sex-specific effects. 239 of the knockout cohorts containing at least 3 females and 3 males each were included in this analysis. For all of the sleep-wake parameters under consideration (listed above) measured, a P-value of less than 0.05 was considered significant. Family-wise error rate was controlled by Dunnett's test. Genotype was considered as an independent variable and the parameter under observation as the dependent variable. Initially, weight was accounted for as a co-variate. There was no effect of weight; therefore, it was excluded from the subsequent analysis. Also, multivariate outliers were identified using Mahalanobis distance (MD) in our data, as described elsewhere (Mitchell and Krzanowski 1985). In brief, it calculates multidimensional distance of each of the observations from the centroid mean vector of all measured variable scores, (Bassett, Gogakos et al. 2012).

Principal components analysis (PCA) was performed on six of the sleep variables under consideration: sleep duration in the light and dark phases and across 24 hours, bout length across 24 h and bout length in the dark and light phase, and breath rate. PCA is used to reduce the number of variables into principal components (PC) that account for most of the variance of the original variables (as reported in terms of eigenvalue) and also to get insight into the patterns in the data.

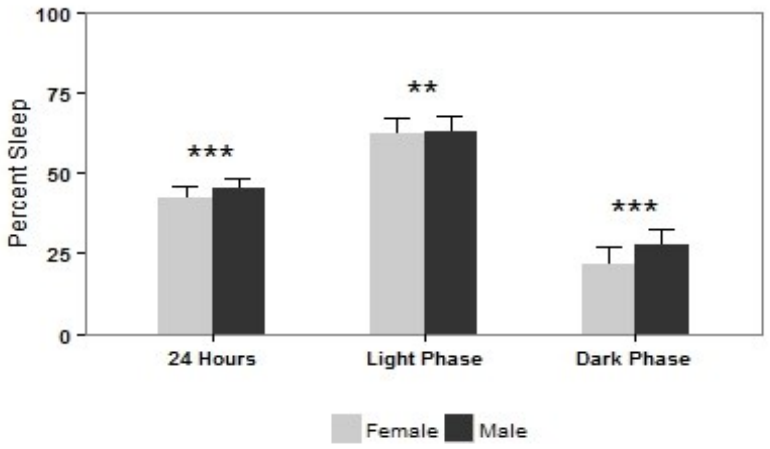
We investigated relationships between results from the targeted knockout genes and some of the known circadian and sleep-influencing genes, by implementing a network analysis using GeneMANIA plugin (University of Toronto) with Cytoscape (version3)(Warde-Farley, Donaldson et al. 2010).

Results

B6N mice

Mean values for sleep traits obtained from piezo system for B6N mice were established as a reference range, obtained separately for each sex. We found that female B6N have a significant reduction in total sleep time, as well as reduced sleep in both the light and dark periods (Fig 2A) compared to B6N males, though it is less pronounced during the light phase ($p < 0.05$) as compared to dark phase ($p<0.05$). The mean percent sleep across 24 h was 41.72 ± 0.11% for females and 45.09 ± 0.11% for males. During the dark phase, mean sleep duration was 21.49 ± 0.16% in females and 27.23 ± 0.17% in males, and in the light phase, for females sleep duration was 61.93 ± 0.15 % and 62.95 ± 0.14% in males. Similar to sleep duration patterns, females also show shorter bout lengths measured across 24 h (Females: 369.72 ± 1.98 s; Males: 444.56 ± 2.5 s), and during both the dark (Females: 199.48 ± 1.39 s; Males: 276.19 ± 2.16 s) and light phases (Females: 537.22 ± 2.92 s; Males: 618.97 ± 3.48 s) (Fig 2B). Overall, B6N female mice have reduced sleep duration and shorter bout length than their male counterparts.

A.



B.

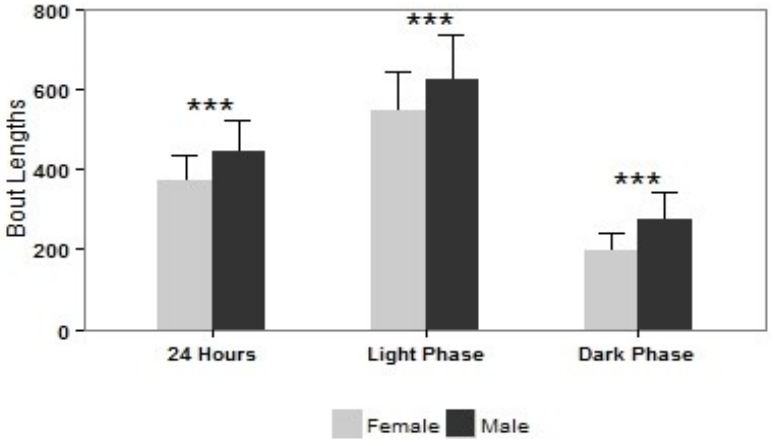
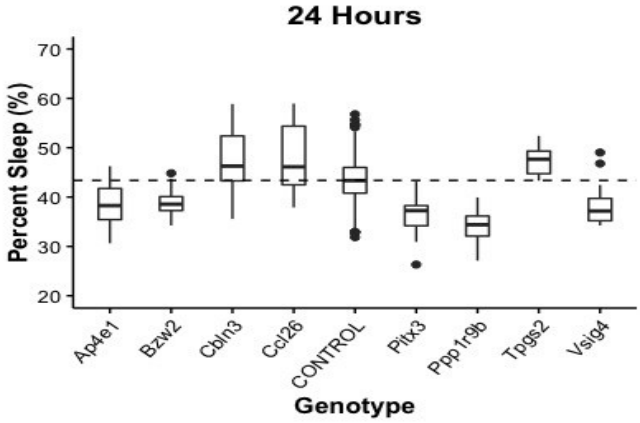


Figure 6 Sleep–wake patterns in control mice under baseline conditions. Average percent sleep across three consecutive days analyzed over (A) 24 h, dark phase, and light phase. Female mice show reduction in sleep duration across 24 h and during the light and dark phase. (B) depicts average bout length in seconds (s) over 24 h, dark phase, and light phase. Females had shorter average bout lengths across all phases. Values represent mean ± SD as obtained by Dunnett's test. **P< 0.01, ***P < 0.001.

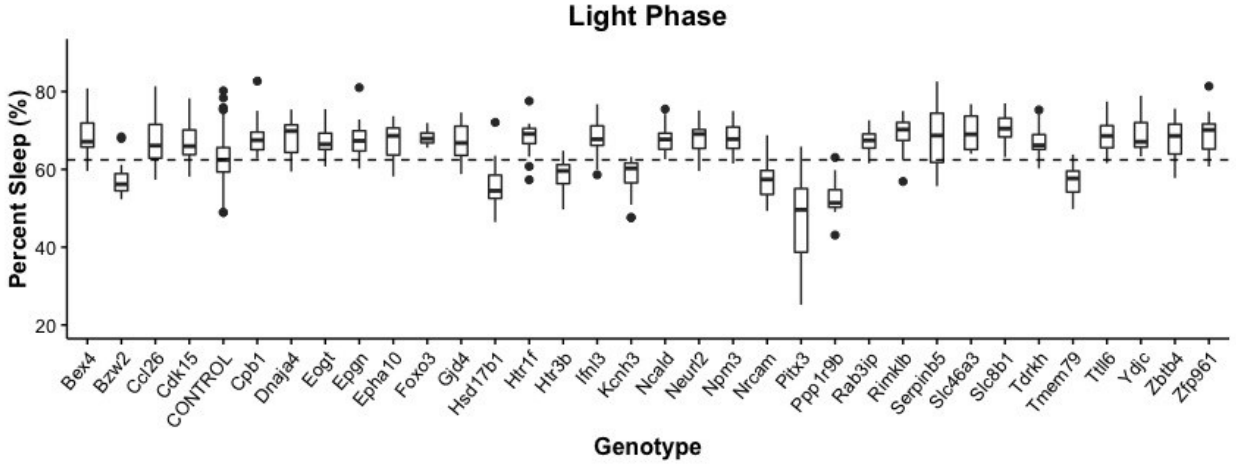
Knockout mice

This report presents the analysis of piezo system sleep recordings from 318 KO strains (Figure 4). Of these, 55 KO strains showed significance at $p<0.01$ in Dunnett's post hoc analysis for one or more of the sleep variables recorded. Across the 24-hour period, reduction in sleep percent (total sleep) was observed in *Ap4e1, Bzw2, Pitx3, Ppp1r9b,* and *Vsig4* KO strains, while increased total sleep was seen in *Ccl26, Cbln3* and *Tpgs2* compared to control mice. During light phase, the reduced sleep percent was recorded in *Kcnh3, Pitx3, Ppp1r9b, Hsd17b1, Htr3b, Nrcam, Tmem79* and *Bzw2,* and longer sleep duration in *Bex4, Ccl26, Cdk15, Cpb1, Dnaja4, Eogt, Epgn, Epha10, Foxo3, Gjd4, Htr1f, Ifnl3, Ncald, Neurl2, Npm3, Rab3ip, Rimklb, Serpinb5, Slc46a3, Slc8b1, Tdrkh, Ttll6, Ydjc, Zbtb4, Zfp961* and *Zbtb4* KO mice. During dark phase, sleep durations was reduced for *Mylip*, *Ppp1r9b, Rimklb,* and *Vsig4* knockouts, and increased for *Macrod2, Cbln3, Myh1,* and *Postn*. Additionally, as compared to controls, mean bout length was significantly reduced across 24h in *Ap4e1, Pitx3, Ppp1r9b, Hsd17b1, Myh1* and *Rnf10*, and increased in *Tmem136* mutant mice. In light phase, bout lengths were significantly shorter in *Ap4e1, Hsd17b1, Myh1, Nrcam, Ptpru, Pitx3* and *Ppp1r9b*, and longer in *Adck2*, *Arrb2, Ermp1*, *Htr1d, Ipp, Nfatc4, Slc8b1, Tmem136, Tmem79* and *Zfp961* relative to control mice. During dark phase, significantly shorter mean bout lengths were seen in *Ap4e1, Bex4, Mylip, Nefh, Nes, Ppp1r9b, Rab27b, Rimklb, Rnf10, Rnf25, Stx16, Tmem151b, Tmod2, and Zzef1,* and longer bout lengths were found in case of *Ghrhr* (Fig 3 and Table 1).

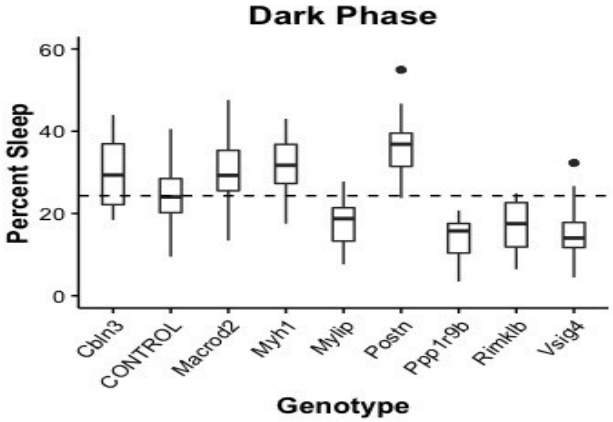
A.


24 Hours
Percent Sleep (%)
70
60
50
40
30
20
Ap4e1
Bzw2
Cbln3
Ccl26
CONTROL
Pitx3
Ppp1r9b
Tpgs2
Vsig4
Genotype

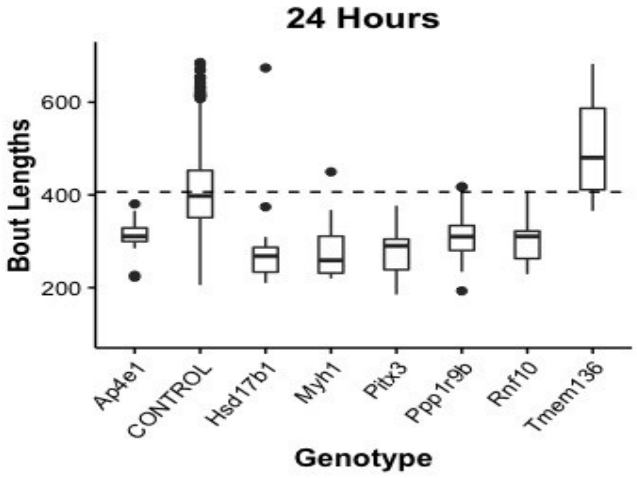
B.


Light Phase
Percent Sleep (%)
80
60
40
20
Bex4
Bzw2
Ccl26
Cdk15
CONTROL
Cpb1
Dnaja4
Eogt
Epgn
Epha10
Foxo3
Gjd4
Hsd17b1
Htr1f
Htr3b
Ifnl3
Kcnh3
Ncald
Neurl2
Npm3
Nrcam
Pitx3
Ppp1r9b
Rab3ip
Rimklb
Serpinb5
Slc46a3
Slc8b1
Tdrkh
Tmem79
Ttll6
Ydjc
Zbtb4
Zfp961
Genotype

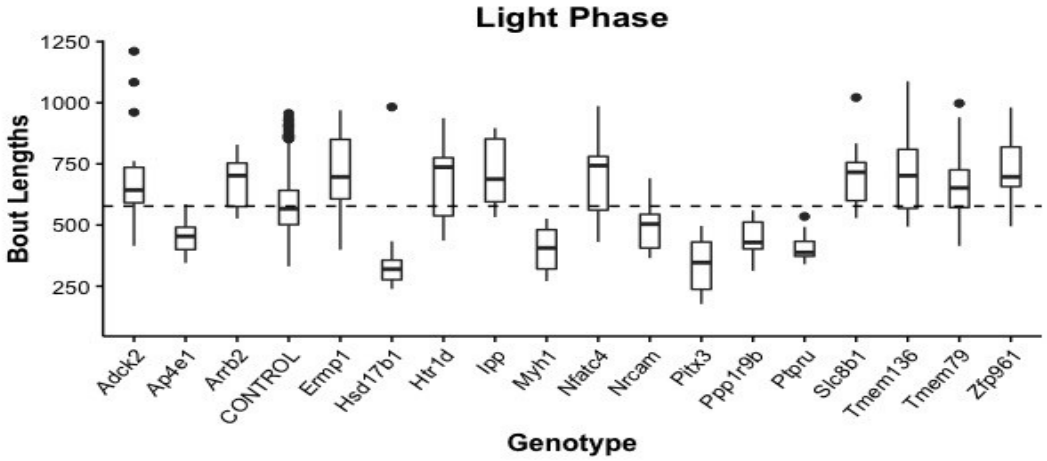
C.


Dark Phase
Percent Sleep
60
40
20
0
Cbln3
CONTROL
Macrod2
Myh1
Mylip
Postn
Ppp1r9b
Rimklb
Vsig4
Genotype

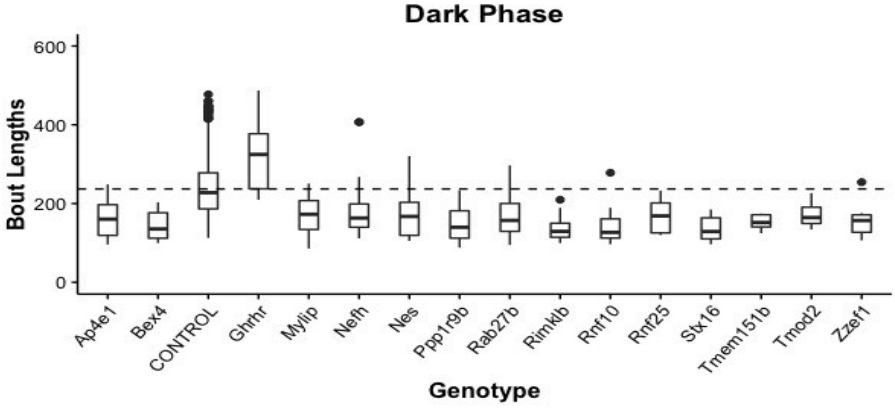
D.


24 Hours
Bout Lengths
600
400
200
Ap4e1
CONTROL
Hsd17b1
Myh1
Pitx3
Ppp1r9b
Rnf10
Tmem136
Genotype

E.


Light Phase
Bout Lengths
1250
1000
750
500
250
Adck2
Ap4e1
Arrb2
CONTROL
Emp1
Hsd17b1
Htr1d
Ipp
Myh1
Nfatc4
Nrcam
Pitx3
Ppp1r9b
Ptpru
Slc8b1
Tmem136
Tmem79
Zfp961
Genotype

F.


Dark Phase
Bout Lengths
600
400
200
0
Ap4e1
Bex4
CONTROL
Ghrhr
Mylip
Nefh
Nes
Ppp1r9b
Rab27b
Rimklb
Rnf10
Rnf25
Stx16
Tmem151b
Tmod2
Zzef1
Genotype

G.

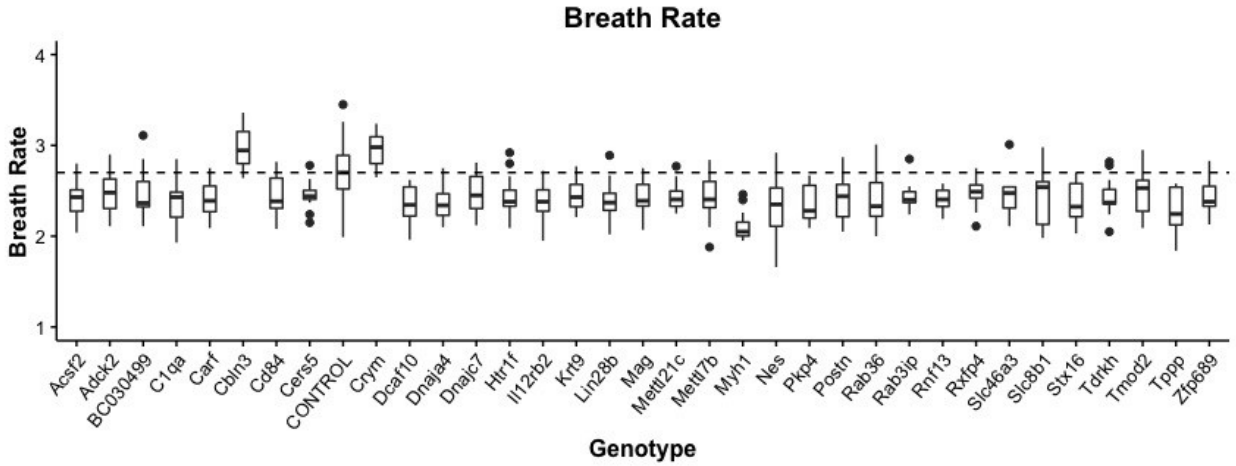
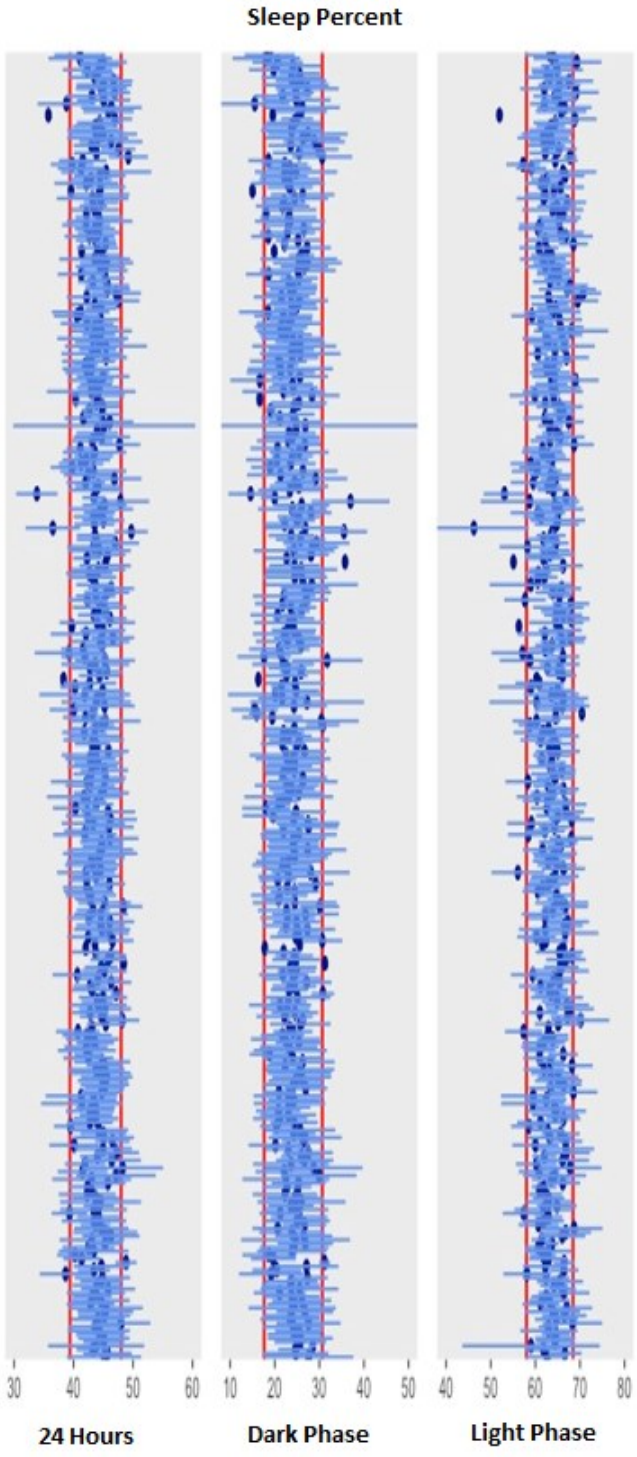


Figure 7 Represents mutant mice that differed significantly from control mice in percent sleep across 24 h (A), in light phase (B) and in dark phase (C); and bout length (D, E, F) across respective phases. (G) KO strains that differed significantly compared to control mice in their breathing rates.

A.


Sleep Percent
30
40
50
60
24 Hours
10
20
30
40
50
Dark Phase
40
50
60
70
80
Light Phase

B.

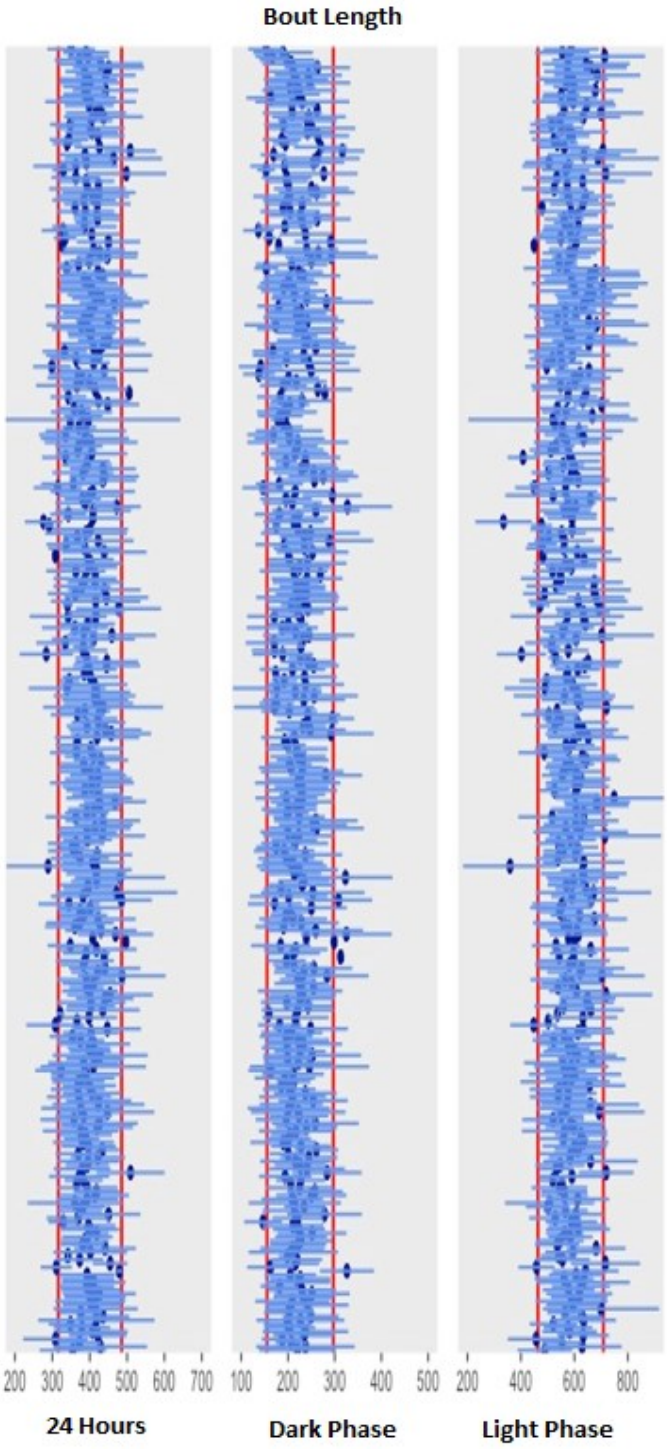


Figure 8 Represents complete dataset for all 318 KO strains arranged alphabetically for (A) sleep percent (24h, dark phase, light phase), and (B) bout length (24h, dark phase, light phase) measured in seconds as depicted on y axis. Each bar represents mean (dot) ±

Similar analysis was performed for 239 KO strains, each of which had at least 3 females and 3 males to evaluate sex-specific differences in sleep parameters measured. The results for *Pitx3* are similar to that observed in the overall data. In both males and females, as compared to controls, *Pitx3* has significantly lower sleep percent and bout length only during the light phase. *Ppp1r9b*, though significantly reduced for overall sleep percent in both males and females, was not found to be significant in both males and females for bout lengths during the light phase. *Myh1* had reduced sleep percent and bout lengths during light phase in males and higher sleep percent in females. *Hsd17b1* had reduced sleep percent in males and reduced bout lengths in both males and females only during the light phase. Increased sleep percent was seen in both males and females in *Postn* during dark phase, and *Slc8b1* during light phase. *Cbln3* and *Ccl26* had increased sleep percent in females with *Ccl26* affecting sleep only during the light phase and *Cbln3* affecting both light and dark phases. Significantly higher sleep percent during light phase were seen in females belonging to *Cpb1, Serpinb5, Slc46a3*, and *Ydjc*, while reduced values were seen in *Col18a1*. *Adck2* and *Ptpru* have significantly lower sleep percent during light phase while it is increased in *Ipp, Nrn1l*, and *Tmem136*. *Zbtb4* had increased values for sleep percent and bout lengths during the light phase in females, whereas *Vsig4* and *Ghrhr* had significantly reduced values during the dark phase. Among the genotypes specific to males, *Dnaja4* and *Epha10* have significantly higher values for sleep percent, while it is reduced in Prom2 during the light phase. Bout lengths are significantly reduced specifically during dark phase in *Ap4e1, Rimklb, Rnf10, Stx16* and *Zzef1*. In *Zfp961* males, sleep percent and bout length are both increased during the light phase in males while they are lower during the dark phase in *Tmem151b* and *Tmod2* males.

Table 6 Sex differences in genotypes significant for (A) sleep percent and (B) bout lengths with direction of change. ↑ represents values higher than Control animals, and ↓ represent values lower than Controls.

A.

	Daily Sleep Percent		Light Phase Percent		Dark Phase Percent	
Genotype	Male	Female	Male	Female	Male	Female
Ap4e1	--	0.009 ↓	--	--	--	--
Cbln3	--	<0.001 ↑	--	0.031 ↑	--	.003 ↑
Ccl26	--	<0.001 ↑	--	0.001 ↑	--	--
Col18a1	--	0.049 ↓	--	0.006 ↓	--	--
Cpb1	--	--	--	0.006 ↑	--	--
Dnaja4	--	--	0.002 ↑	--	--	--
Epha10	--	--	0.009 ↑	--	--	--
Ghrhr	--	--	--	--	--	.003 ↑
Hsd17b1	--	--	<0.001 ↓	--	--	--
Kcnh3	--	--	--	0.008 ↓	--	--
Macrod2	--	--	--	--	0.031 ↑	.004 ↑
Mettl7b	<0.001 ↓	--	--	--	<0.001 ↓	--
Myh1	--	--	0.038 ↓	--	--	<0.001 ↑
Nrcam	--	--	--	0.003 ↓	--	--
Pitx3	<0.001 ↓	0.005 ↓	<0.001 ↓	<0.001 ↓	--	--
Postn	--	--	--	--	<0.001 ↑	.002 ↑
Ppp1r9b	<0.001 ↓	<0.001 ↓	<0.001 ↓	<0.001 ↓	<0.001 ↓	<0.001 ↓
Prom2	--	--	0.002 ↓	--	--	--
Ptpn5	0.032 ↓	--	--	--	0.006 ↓	--
Serpinb5	--	--	--	<0.001 ↑	--	--
Slc46a3	--	--	--	0.003 ↑	--	--
Slc8b1	--	--	0.008 ↑	<0.001 ↑	--	--
Tmod2	--	--	--	--	0.005 ↓	--
Vsig4	--	--	--	--	--	.001 ↓
Ydjc	--	0.011 ↑	--	<0.001 ↑	--	--
Zbtb4	--	--	--	0.009 ↑	--	--
Zfp961	--	--	0.005 ↑	--	--	--

B.

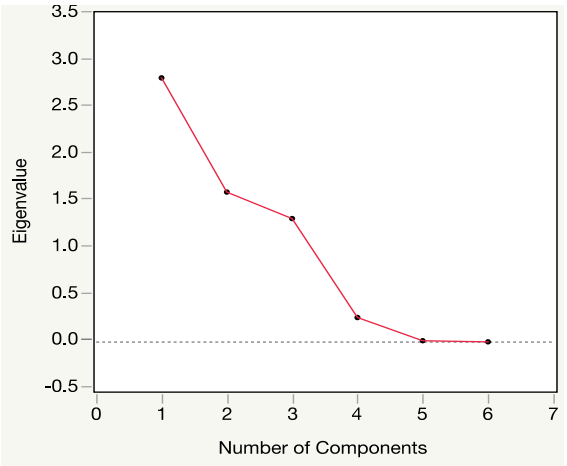
	Bout Lengths Mean		Bout Length Light Phase		Bout Length Dark Phase	
Genotype	Male	Female	Male	Female	Male	Female
Adck2	--	--	--	0.002 ↓	--	--
Ap4e1	0.001 ↓	--	--	--	0.003 ↓	--
Ghrhr	--	<0.001 ↑	--	--	--	<0.001 ↑
Hsd17b1	0.001 ↓	0.002 ↓	<0.001 ↓	<0.001 ↓	--	--
Ipp	--	--	--	<0.001 ↑	--	--
Kcnh3	--	--	--	--	--	0.001 ↑
Mettl7b	--	--	0.002 ↓	--	--	--
Myh1	0.003 ↓	0.034 ↓	0.002 ↓	--	--	--
Nat1	--	--	<0.001 ↑	--	--	--
Nefh	--	--	--	--	--	0.008 ↓
Nrn1l	--	--	--	0.005 ↑	--	--
Pitx3	<0.001 ↓	0.001 ↓	<0.001 ↓	<0.001 ↓	--	--
Ppp1r9b	0.001 ↓	--	--	--	<0.001 ↓	0.001 ↓
Ptpru	--	--	--	0.005 ↓	--	--
Rimklb	0.047 ↓	--	--	--	<0.001 ↓	--
Rnf10	<0.001 ↓	--	--	--	<0.001 ↓	--
Slc8b1	--	--	--	0.029 ↑	--	--
Stx16	--	--	--	--	<0.001 ↓	--
Tmem136	--	0.003 ↑	--	0.038 ↑	--	--
Tmem151b	--	--	--	--	0.001 ↓	--
Tmod2	--	--	--	--	0.002 ↓	--
Vsig4	--	--	--	--	--	0.048 ↓
Zbtb4	--	--	--	<0.001 ↑	--	--
Zfp961	--	--	0.033 ↑	--	0.023 ↓	--
Zzef1	--	--	--	--	0.001 ↓	--

Prinicpal Component and Outlier Analysis

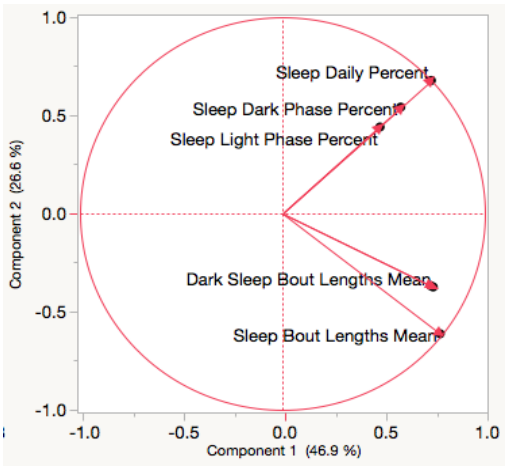
We performed additional analyses to identify sleep related genes based on multiple measures using Principal Component Analysis (PCA) . We included standardized values for sleep percent and bout lengths for 24 hours, light phase and dark phase in our analysis. For our dataset, as can be seen from the scree plot, the first three principal components had eigenvalues greater than 1 (a conventional threshold rule of thumb) and explained for more

than 95% variability in the data, with PC1 accounting for 46.92% of the variability (Fig. 5). Correlation between the principal component and the original variables is described in terms of loadings. There is a point of inflection in the eigenvalues after 4 components. However, fourth factor has eigenvalue less than 1, indicating that 3 components may best represent the data. Biplot reveals the relationship between different sleep variables in first two principal components.

The first PC1 had high loading values for each variable (mean loading = 0.68; Figure 5C). ANOVA with multiple comparison through Dunnett's post hoc test of PC1 resulted in 9 genotypes that showed significance. These genotypes are *Pitx3, Ppp1r9b, Ap4e1, Hsd17b1, Rnf10, Ghrhr, Fam186b, and Tnfsf18*. Interestingly, the PC2 had positive loadings for sleep percent and negative loadings for bout lengths, and *Tpgs2, Tmem79, Cbln3, Myh1, Ppp1r9b, Parp8, Rnf25, Arrb2, Macrod2, Dcaf10, and Ccl26* were significant. Similarly, PC3 had positive loadings for light phase and negative loadings for dark phase. In addition to others, the genes significant for PC3 include *Pitx3, Rimklb, Zfp961, Postn, Hsd17b1, Slc8b1, Serpinb5, Nrcam, Cpb1, Macrod2, Ipp, Dnaja4, and Mylip*. Based on the results from ANOVA of individual variables and PC3, we propose these genes as candidate genes that affect sleep in a specific circadian phase.



A. scree plot



B: Biplot

Loading Matrix

	Prin1	Prin2	Prin3	Prin4	Prin5	Prin6
Sleep Bout Lengths Mean	0.77928	-0.61304	0.04154	0.09501	-0.07840	0.00000
Light Sleep Bout Lengths Mean	0.73327	-0.36842	0.45257	-0.34714	0.03541	-0.00000
Dark Sleep Bout Lengths Mean	0.74518	-0.37461	-0.50148	0.22108	0.06341	-0.00000
Sleep Daily Percent	0.73559	0.67732	-0.00028	0.00840	-0.00815	-0.00042
Sleep Light Phase Percent	0.48215	0.44026	0.72222	0.22800	0.01080	0.00026
Sleep Dark Phase Percent	0.58471	0.54134	-0.57900	-0.17161	-0.01939	0.00032

C. *Loading matrix for PCs*

Figure 9 Scree plot depicting relationship between eigenvalue and number of components.

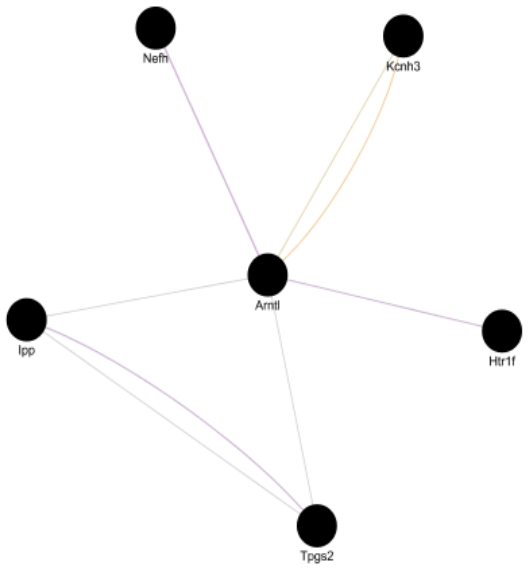
Outlier Analysis

As an additional outlier detection strategy, Mahalanobis distances (MD) for multivariate outliers were calculated for the sleep percent and bout length variable means of all KO strains and candidate genes were identified with upper control threshold of 3.75. Based on outlier analysis, the top candidate genes for sleep are *Pitx3, Ppp1r9b, Ap4e1, Akr1d1, Postn, Myh1, Tnfsf18, Ptpru, Mdk, Vsig4, Tmem79, Serpinb5, Ipp, Bex4, Gpr156, Stat5b, Hsd17b1, Plekha3, Esrra, Gpr19, Stx16, Slc1a1, Actrt3, Rabac1, Tpgs2, Arrb2,* and *Ghrhr.*

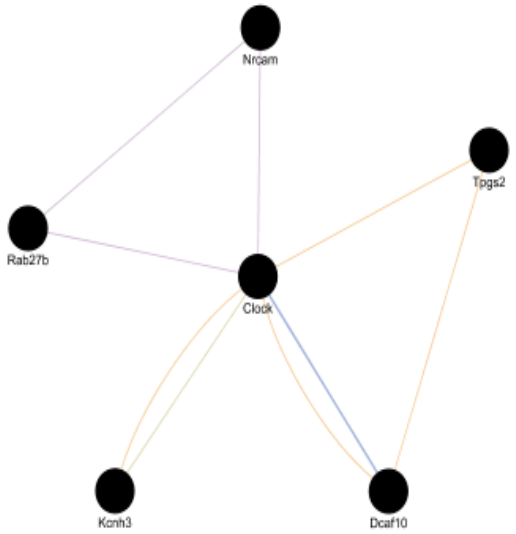
Gene Network Analysis

To further evaluate genes with significant sleep phenotypes discovered in our study, and to compare these to genes known to affect sleep, we used the GeneMANIA tool (Warde-Farley, Donaldson et al. 2010), implemented in Cytoscape software (version 3). The GeneMANIA application determines relationships between the query genes and other related genes based on their genetic and physical associations, shared protein domains, co-expression, co-localization and their participation in the shared pathways. Predictive gene networks were created as depicted in Figure 6, using candidate genes from our analysis with respect to the key genes previously implicated- *Arntl, Clock, Per1,* and *Per2* in regulation of sleep behavior.

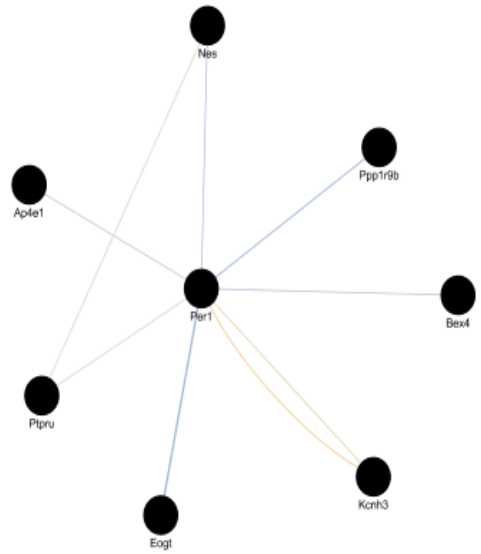
Arntl


Nefh
Kcnh3
Arntl
Ipp
Htr1f
Tpgs2

Clock


Nrcam
Tpgs2
Rab27b
Clock
Kcnh3
Dcaf10

Per1



Per2

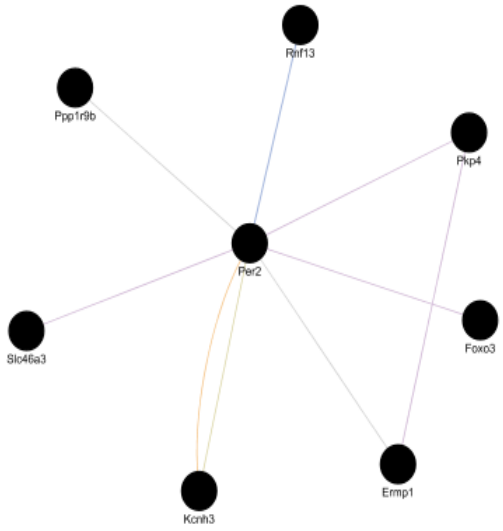


Figure 10 Gene networks as predicted by GeneMANIA based on genetic and physical associations, shared protein domains, co-expression, co-localization and their participation in the shared pathways for the candidate genes of interest with respect to the genes that are known to affect sleep- *Arntl, Clock, Per1* and *Per2*.

Breath Rate

Breath rate significantly differed from controls for 34 of the strains ($p < 0.05$) (Fig 3G and Table 1C). A few of these mice displayed a sleep phenotype in addition to varied breath rate and include knockouts for genes: *Myh1, Nes, Cbln3, Htr1f, Slc8b1, Tdrkh, Rab3ip, Slc46a3, Adck2, Postn, Tmod2, Cbln3, Nes, Dnaja4,* and *Stx16*. Sex differences were found for breath rate as well.

Discussion

We have reported here the results from a large-scale phenotyping study that systematically assesses sleep in knockout mice. With sleep wake recordings from more than 6000 mice, consisting of more than 1800 controls and nearly 300 gene knockouts, the KOMP2 pipeline at The Jackson Laboratory has generated a wealth of information-rich gene centric data that is unprecedented in sleep research. Through our analysis, we have been able to identify sleep-related genes that have been previously unknown to influence sleep. An experimental design using both sexes, and piezo monitoring system that consistently reports multiple sleep variables, has allowed for an in-depth analysis to identify genes that affect sleep in one or both sexes and during specific times of day. We found a considerably high hit rate with nearly 20% of gene knockouts tested differing significantly from controls for at least one sleep phenotype. This is perhaps not surprising given the dramatic changes in brain physiology and function that occur with sleep and wake transitions, and further suggests a potential to uncover many as yet unappreciated pathways affecting sleep. Although the purpose and functions of sleep are still unclear, we know that sleep is an essential physiological process and that even modest reductions in sleep have

substantial effects on health and cognitive functions (Shaw and Franken 2003, Durmer and Dinges 2005, Cohen, Doyle et al. 2009, Cespedes, Bhupathiraju et al. 2016).

The key findings from data in this report are best exemplified by results obtained from *Pitx3, Ppp1r9b Slc1a1,* and *Ap4e1* knockout mice that have a significant effect across many sleep traits. *Pitx3* is the Paired Like Homeodomain Transcription Factor 3 and its deletion was associated with reduced sleep and bout length across 24h and light phase in our study. Although little is known about the role of Pitx3 and sleep, Derwinska et al have reported that a hemizygous deletion on chromosome 10 involving Pitx3 resulted in sleep disturbances beginning early childhood in a Caucasian boy (Derwinska, Mierzewska et al. 2012). In addition, Pitx3 is well known for its role in regulating lens development and is therefore associated with ocular abnormalities as seen in a range of animals including xenopus, zebrafish, humans as well as mice where its deficiency is reflected as a form of aphakia (Flint, Valdar et al. 2005, Harper, Volicer et al. 2005, Hirai, Pang et al. 2005, Khosrowshahian, Wolanski et al. 2005, Shi and Chiang 2005, Shi, Bosenko et al. 2005, Laposky, Shelton et al. 2006, Popov, Kaminskaya et al. 2009, Huang and He 2010, Bertram, Rook et al. 2011, Bertram and Rook 2012, Callander, Bolton et al. 2012, Mavanji, Billington et al. 2012, Dyakonova and Krushinsky 2013, Reichert and Gerhardt 2013). In the KOMP2 pipeline, in addition to sleep, *Pitx3* is associated with a multitude of phenotypes including vision/eye, neurological/behavior, growth/size, homeostasis/metabolism, cardiovascular and skeleton. As expected, the *Pitx3* KO mice have anopthalmia or absence of eyes. In mice, Pitx3 is also thought to be essential in development of dopaminergic neurons in Substantia Nigra (SN). Besides SN, Pitx3 is also expressed in Ventral Tegmental area (VTA) Reviewed by (Peirce, Lu et al. 2004, Maret,

Dorsaz et al. 2007, Chandran, Shahana et al. 2009, Cirelli 2009, Cohen, Doyle et al. 2009, Deschenes and McCurry 2009, Gondo, Fukumura et al. 2009, Kang, Lim et al. 2009, Koethe, Schreiber et al. 2009, Kroner 2009, Li, Dani et al. 2009, Mekada, Abe et al. 2009, Millstein, Zhang et al. 2009, Miura, Matsuda et al. 2009, Miyake, Takahashi et al. 2009, Moy, Nonneman et al. 2009, Nishida, Pearsall et al. 2009, Ohno 2009, Panda, Patra et al. 2009, Perrin, Fagan et al. 2009, Popov, Kaminskaya et al. 2009, Regal, Amigo et al. 2009, Sorensen, Vermeulen et al. 2009, Vassalli and Dijk 2009, Walker 2009, Winrow, Williams et al. 2009, Wisor, Jiang et al. 2009, Devi and Ohno 2010, Sheline, Raichle et al. 2010, Wang, Liu et al. 2010, Rillich, Schildberger et al. 2011, Bertram and Rook 2012, Callander, Bolton et al. 2012, Dyakonova and Krushinsky 2013, Fitzsimmons and Bertram 2013, Reichert and Gerhardt 2013, Stevenson and Schildberger 2013). Not only these regions are associated with reward, addiction and movement, they also play an important role in sleep and alertness Reviewed by (Nishino 2013).

Ppp1r9b is the Protein Phosphatase 1 Regulatory Subunit 9B (or Neurabin II or Spinophilin), and as the name indicates is a regulatory subunit of protein phosphatase (PP1). It is a protein highly enriched in dendritic spines (Feng, Yan et al. 2000). Sleep is reported to promote formation of dendritic spines for memory consolidation (Yang, Lai et al. 2014). In addition, PP1 regulates AMPA channels that are believed to play a role in synaptic plasticity, and learning and memory Reviewed in (Prince and Abel 2013). Further, *Ppp1r9b* is one of the substrates of *GSK3B* (Glycogen Synthase Kinase 3 Beta) which is a crucial circadian clock regulator (Kaasik, Kivimae et al. 2013). Casein Kinase I enzymes have been shown to play a critical role in regulating clock genes such as *Per2*, and *Ppp1r9b* may dephosphorylate some of these same sites and work in opposition (Fukuyama 2003

and Padiath 2005). There is increasing evidence that clock genes not only influence circadian aspects of sleep and wake, but are fundamentally tied to sleep homeostasis as well, which appears to be altered in the *Ppp1r9b* knockout mice (Flores, Flores et al. 2007, Franken, Thomason et al. 2007, Maret, Dorsaz et al. 2007, O'Hara, Ding et al. 2007, Curie, Mongrain et al. 2013, Franken 2013, Mang and Franken 2013).

Ap4e1 codes for the Epsilon subunit 1 of Adaptor Protein (AP) 4 complex that is involved in vesicle trafficking. *Ap4e1* has been associated with Cerebral Palsy, and mutations in humans have been known to cause intellectual disabilities, of which abnormal sleep behavior is one of the symptoms (Moreno-De-Luca, Helmers et al. 2011). Like *Pitx3*, *Ap4e1* is associated with multiple additional aberrant phenotypes observed in the KOMP2 pipeline, and is also found to be within the QTL Cplaq15 (Circadian Period of Locomotor Activity 15) (Hofstetter, Trofatter et al. 2003).

Another KO that showed significant changes in sleep-wake traits is *Kcnh3 (Kv12.2)* a subunit of potassium channels that regulate neuronal excitability. *Kcnh3* KO mice have shorter sleep duration in the light phase. A similar but less pronounced reduction was also seen in *Kcnh3* heterozygous mice (data not included). Its overexpression has been associated with deficits in learning, and its ablation with enhanced cognitive functions (spatial and working memory), hippocampal hyperexcitability and spontaneous seizures (Miyake, Takahashi et al. 2009, Zhang, Bertaso et al. 2010). Many other Kv channels are known to modulate sleep-wake. A well-known example is Shaker in drosophila (Cirelli, Bushey et al. 2005). Flies mutant for the *Shaker* gene have reduced sleep and are short sleepers. In *Kcnc1, Kcnc3, Kcnc1/3 and Kv1.2* KO mice less NREM sleep has been observed, with a similar magnitude to sleep phenotypes observed for Kcnh3 reported here

Reviewed in (Rechtschaffen 1998, Cirelli 2009). Hence, our study identifies and adds another novel potassium channel associated gene that affects sleep. Lack of certain K channels may reduce the resting membrane potential of neurons, leading to increased firing, and reduced sleep. While this would occur in both inhibitory and excitatory circuits, the net effect is presumably increased excitation. Recently, Ding et al has shown that reduction seen in brain extracellular potassium ion levels is associated with sleep and anesthetized mice supporting this hypothesis (Holth, Patel et al. , Cespedes, Bhupathiraju et al. 2016, Ding, O'Donnell et al. 2016, Yaghouby, Donohue et al. 2016).

Slc1a1 (EAAC1 or EAAT3), a glutamate transporter is found mainly in neurons. Lack of this transporter may increase glutamate transmission, and similar to the K channel story, may lead to greater excitatory responses and more difficulty falling asleep (Kanai, Bhide et al. 1995, Aoyama and Nakaki 2013). *Slc1a1* knockout mice have both lower sleep amounts and shorter bout lengths.

Nrcam KO mice have reduced sleep and bout length during light phase. Nrcam (Neuronal cell adhesion molecule), a neuronal cell adhesion molecule, serves many functions such as axonal guidance, clustering, and maintenance of sodium channels essential for action potential propagation at nodes of Ranvier (Sakurai 2012, Amor, Feinberg et al. 2014). It also regulates density of dendritic spines (Demyanenko, Mohan et al. 2014). *Nrcam* has been shown to be associated with autism in humans as Reviewed in (Sakurai 2012). Cognitive deficits were also found in Nrcam null mutant mice (Reviewed in (Moy, Nonneman et al. 2009).

Cbln3 KO mice had increased duration of sleep across 24 hours and dark phase. Cbln3 belongs to precerebellin family and studies conducted by Miura and colleagues show

that it co-localizes at purkinjee cell synapse along with *Cbln1*, another cerebellum specific protein, important for synaptic plasticity (Hirai, Pang et al. 2005, Miura, Matsuda et al. 2009).

With a multitude of phenotypes and rich data in the KOMP2 pipeline, cross trait analysis is the logical extension of this study. As an initial step, we conducted cross trait analysis between sleep and neurobehavioral phenotypes performed as part of the JAX KOMP2 pipeline. In the 38 KO strains we identified with abnormal sleep, we found significant associations with abnormal behavioral responses to light and hyperactivity. Specifically, *Chn1, Rnf10, Rimklb, Ap4e1,* and *Macrod2*. *Pitx3, Foxo3, Ccl26,* and *Ghrhr* were associated with abnormal behavioral response to light, and *Mylip, Nxn* and *Zbtb4* to hyperactivity. *Ppp1r9b* had abnormal responses to new environments, whereas *Mylip* and *Pitx3* showed increased exploratory activity in new environments. Startle reflex was increased in *Pitx3, Ajap1,* and *Nxn*, while it was decreased in *Foxo3*. As seen with the gene networks, several of these genes have a circadian footprint and may contribute to these coincident phenotypes.

Sex differences were seen for many of the KO lines as well as in B6N controls throughout our study. Previous studies in mice and humans also report similar sex differences in sleep and circadian rhythms, although with relatively small sample sizes. Sex differences in sleep have also been observed in BL6J mice for baseline sleep parameters such as REM and NREM sleep, with lower values observed in female mice (Koehl, Battle et al. 2006, Paul, Dugovic et al. 2006). Sex differences observed in human studies have generally been small, but include higher EEG power density in women (Carrier, Land et al. 2001). EEG profiles in primary insomnia patients also show sex

differences (Buysse, Germain et al. 2008). Sex hormones are thought be one of the contributing factors for these differences (Collop, Adkins et al. 2004, Krishnan and Collop 2006, Pavlova and Sheikh 2011).

In addition to small but significant sex differences in our control mice, several genotypes had highly significant sleep trait differences for a specific sex. KO strains in which sex differences were found might be helpful in identifying causes for the intriguing sexual dimorphism seen in sleep behavior, such as those in which female mice sleep less than males. In our data, the majority of significant findings are sex specific, suggesting at least some biochemical differences in female vs. male sleep regulation.

Our study demonstrates the utility of rapid-non-invasive sleep phenotyping in high throughput mouse screens. This initial set of approximately 300 genes was not selected to have sleep phenotypes and yet a high percentage were found to have altered sleep phenotypes, and of a magnitude as large as any that have been selected specifically for sleep studies over the past 25 years (Reviewed by (Cirelli 2009). This supports the utility of an unbiased selection and phenotyping for mouse knockouts, especially given that a majority of genes are not well understood. Unlike most individual studies of KO mice that examine genes predicted to influence a trait of interest, the IMPC/KOMP2 is a comprehensive, unbiased approach, having examined more than 2000 genes to date, and thus holds potential for detecting and identifying unexpected and pleiotropic effects of the knocked out genes (Brown and Moore 2012).With fewer than 300 KOs analyzed to date, we demonstrate the potential of this large scale effort to find novel sleep phenotypes for a significant percentage of coding genes.

Some of the potential sleep regulating genes identified in our study can be broadly categorized into transcription factors (*Pitx3, Foxo3*), immune system related (*Ccl26*), membrane transporters (*Slc46a3, Slc8b1)* ion channels (*Kcnh3*), neurotransmitters receptors (*Htr3b, Htr1f, Htr1d*), signal transduction (*Ppp1r9b, Rab27b, Ap4e1*), and metabolism (*Ghrhr*). A wide variety of genes belonging to these functional categories have been previously implicated in influencing sleep parameters. Furthermore, these categories are likely not exhaustive. Several of these genes have brain-associated functions such as myelin formation, neuronal differentiation, synaptic transmission, neuronal signal transduction and yet for many others such as *Adck2* and *Zfp961,* there is little information available in the literature. None of the target genes, with the exception of *Ghrhr and Pitx3*, have been implicated in sleep regulation before to the best of our knowledge. Continued broad phenotyping for sleep in this resource is likely to open many new avenues for sleep research.

There are some limitations to this study. The current analysis software does not distinguish REM sleep from NREM sleep, however, an algorithm to distinguish these sleep states from the piezoelectric recordings is in development and will be utilized in future studies once it has had sufficient validation. This algorithm and others will also be used to re-analyze data from the present study. Although we were able to detect many novel genes, the extensive filtering of sleep signals used in our study most likely excluded genes affecting sleep in subtle ways. Genes involved in sleep that have extensive redundancy or compensatory mechanisms in place to mask the effect of a gene ablation would also be missed. Finally, the knockout method is in general limited by the fact that a gene is ablated in all tissues, so KO of essential genes that may in fact be involved in sleep result in a non-

viable animal, preventing sleep phenotyping. However, utilization of the conditional allele obtained from these KOs in the future may overcome this limitation. It is also unclear to what extent any of the sleep alterations from gene ablation are due to direct or indirect effects of the gene in question. These questions may be partially addressed by examining the multiple phenotypes for each knockout, and can be pursued in other ways for the most interesting cases. There is no reason to believe that there are any genes whose sole functions are related to sleep, as even the so-called core circadian clock genes are pleiotropic.

Because the piezoelectric system monitors breath rate as the primary movement during sleep, it may also be useful to detect sleep apneas. There is limited overlap between genotypes significant for sleep wake alterations, and those for breathing rate. The results thus far suggest it is not a variable directly related to sleep percent and bout length, and so it was excluded from PCA and MD analysis. The non-invasive nature of the piezo set up allows assessment of breathing without the stress of most other alternative methods for assessing breathing.

Several genes were found to affect breath rate. Majority of these genes affected breath rate only as is the case with *Tppp* (p25 alpha/p24) mice which had shorter breath rate. A study conducted by Lehotzky et al suggests that *Tppp*, a tubulin-binding protein plays an important role in oligodendrocyte differentiation (Lehotzky, Lau et al. 2010). In addition, it also functions as a glycogen synthase kinase 3 inhibitor (Martin, Vazquez et al. 2002, Reichert and Gerhardt 2013). However, some of the genes that affect breath rate were also implicated in influencing various aspects of sleep phenotype. For instance, *Nes* mice have reduced breath rate compared to control mice as well as shorter bout length during dark phase. Nestin is an intermediate filament protein expressed predominantly in

the initial developmental stages of stem cells of the CNS as well as skeletal muscle (Frederiksen and McKay 1988, Lendahl, Zimmerman et al. 1990, Sejersen and Lendahl 1993).

We don't know yet if breath rate regulating mechanisms are in any way associated with other brain functions or sleep-related pathways, but the ability to assess breathing should provide additional variables of interest to the larger efforts to understand the multiple roles of all protein coding genes.

Conclusions

This study utilizes data generated from the KOMP2 project at The Jackson Laboratory, a large-scale project intended to phenotype knockout mice in alignment with the IMPC. This study reports on 6000 mice representing 300 different gene knockouts, along with over 1800 BL6NJ control mice all assessed for sleep and wake as a unique part of the JAX phenotyping pipeline. Our findings showed altered sleep and wake in many of the knockout lines compared to the controls.

Many of these strains also exhibit sex differences in sleep traits. In all, we identified a large number of genes in which target deletion resulted in high to modest effects on one or more of the observed sleep-wake traits, as well as breath rate. Follow up of these potential sleep regulating and breath rate modulating genes will likely suggest new signaling pathways underlying these two processes.

Acknowledgements

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We dedicate this paper to Martin Striz, who died unexpectedly in August 2014. He was an outstanding and dedicated student, teacher and scientist, and a kind and wonderful friend, who always gave generously of his time and expertise. He helped provide critical training and assistance to several individuals in this current work as well. He is missed daily, and will continue to be missed by all who knew him.

Supplementary Data

Table 7 Genotypes with significant differences compared to controls for (A) sleep percent and (B) bout lengths and (C) breath rate.

	Sleep % 24h	Dark Sleep%	Light Sleep%	Bout Length 24h	Dark Bout Length	Light Bout Length	Breath Rate
Genotype	**p-Value**	**p-Value**	**p-Value**	**p-Value**	**p-Value**	**p-Value**	**p-Value**
1700016K19Rik	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
4921509C19Rik	0.99962	1.00000	0.11773	1.00000	1.00000	1.00000	1.00000
A1cf	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Abca7	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Abcg2	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Acap1	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Acsf2	1.00000	1.00000	1.00000	0.86965	0.99985	1.00000	0.00016↓
Acsm2	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Actrt3	0.99971	1.00000	0.98181	1.00000	1.00000	1.00000	1.00000
Adad2	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Adck2	1.00000	1.00000	0.17446	1.00000	1.00000	0.00806↑	0.03159↑
Adck5	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Adgrb2	1.00000	1.00000	0.33340	1.00000	1.00000	1.00000	1.00000
Adora2b	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Ahrr	1.00000	1.00000	1.00000	1.00000	1.00000	0.99997	1.00000
AI464131	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Ajap1	1.00000	1.00000	0.32801	1.00000	0.44121	1.00000	0.10606
Akap11	0.99984	1.00000	0.76679	1.00000	1.00000	1.00000	1.00000
Akip1	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000

Akr1b8	1.00000	1.00000	0.95400	1.00000	1.00000	1.00000	1.00000
Akr1d1	1.00000	1.00000	1.00000	1.00000	0.99940	1.00000	1.00000
Ap4e1	0.00076↓	0.21807	0.05671	0.00048↓	0.00221↓	0.00760↓	0.99971
Arf2	1.00000	1.00000	0.93698	1.00000	1.00000	0.04677	0.99992
Arhgef10	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Arrb1	1.00000	0.67450	1.00000	1.00000	1.00000	1.00000	0.68630
Arrb2	0.32676	0.05610	1.00000	1.00000	1.00000	0.04312↑	1.00000
Arrdc1	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.99964
Arsk	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Asb10	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Bbox1	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
BC030499	0.79457	1.00000	0.54030	1.00000	1.00000	1.00000	0.04413↓
BC100451	1.00000	1.00000	0.37081	1.00000	1.00000	1.00000	0.97249
Bex4	1.00000	1.00000	0.02559↑	0.77634	0.02136↓	1.00000	0.74879
Bhlhe40	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.99953
Bmp2k	1.00000	1.00000	1.00000	0.99997	0.99442	1.00000	1.00000
Btg2	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Bzw2	0.01586↓	1.00000	0.00405↓	1.00000	1.00000	0.86152	0.17542
C1qa	1.00000	1.00000	1.00000	0.99993	1.00000	0.99804	0.00003↓
C1qb	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.15766
C1qtnf5	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.25933
C3	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.99842
C9	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Car12	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Carf	0.99883	1.00000	0.43063	1.00000	0.97268	1.00000	0.00060↓
Cast	1.00000	1.00000	1.00000	0.79872	1.00000	0.85807	1.00000
Cbln3	0.00048↑	0.00081↑	0.99998	1.00000	1.00000	1.00000	<0.00001↑
Ccdc120	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Ccl26	0.00161↑	0.88741	0.00451↑	1.00000	1.00000	0.83682	1.00000
Cd33	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Cd84	0.84255	1.00000	0.70273	1.00000	1.00000	1.00000	0.02760↓
Cdh4	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Cdk15	0.05326	1.00000	0.04206↑	1.00000	1.00000	1.00000	0.52529
Cdk19	1.00000	1.00000	0.98743	1.00000	1.00000	1.00000	1.00000
Ceacam16	0.84604	0.99926	1.00000	1.00000	1.00000	1.00000	1.00000
Cers5	0.73257	1.00000	0.18859	1.00000	1.00000	1.00000	0.03310↓
Ces4a	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.09808
Cfb	1.00000	1.00000	0.92660	1.00000	1.00000	1.00000	0.00702↓
Chek2	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000

Chn1	0.13375	0.99862	0.53975	0.99981	0.99972	1.00000	1.00000
Cited4	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Cldn13	1.00000	1.00000	0.65678	1.00000	1.00000	0.10247	0.98646
Cldn19	1.00000	1.00000	1.00000	1.00000	0.99999	1.00000	1.00000
Clvs1	1.00000	0.99994	1.00000	1.00000	1.00000	0.86035	0.77300
Cml2	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Col18a1	0.99999	1.00000	0.88021	1.00000	1.00000	1.00000	1.00000
CONTROL	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Cp	1.00000	1.00000	0.99663	1.00000	1.00000	1.00000	1.00000
Cpb1	1.00000	1.00000	0.00126↑	1.00000	0.88755	0.99035	0.92633
Crym	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.02616↑
Cyb5d1	1.00000	1.00000	0.58110	1.00000	0.99866	1.00000	1.00000
Cyb5d2	1.00000	1.00000	0.99854	1.00000	1.00000	1.00000	0.99938
Dcaf10	1.00000	1.00000	1.00000	0.15692	0.05769	1.00000	0.00008↓
Dennd2d	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.87328
Dixdc1	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Dnaja4	1.00000	1.00000	0.00039↑	1.00000	0.99990	0.52167	0.00003↓
Dnajb3	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Dnajb7	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.95353
Dnajc14	1.00000	0.41574	0.69828	1.00000	0.99999	1.00000	0.99975
Dnajc28	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Dnajc5g	1.00000	1.00000	1.00000	1.00000	1.00000	0.99957	0.85968
Dnajc7	1.00000	1.00000	1.00000	0.96069	0.99463	1.00000	0.03437↓
Dnase1l2	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Dntt	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Dpf1	1.00000	1.00000	0.99997	0.99392	1.00000	0.99969	1.00000
Elk1	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.99999
Eogt	1.00000	1.00000	0.01830↑	1.00000	1.00000	0.70973	0.99682
Epb4	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Epgn	1.00000	1.00000	0.00406↑	1.00000	0.99960	0.99923	0.29782
Epha10	1.00000	1.00000	0.02732↑	1.00000	1.00000	0.98108	0.89822
Ermp1	1.00000	1.00000	1.00000	1.00000	1.00000	0.01685↑	1.00000
Espnl	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.99999
Esrra	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
F2rl1	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Fam161a	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.20642
Fam186b	0.98672	1.00000	0.99980	0.21741	0.99658	0.05921	1.00000
Fam217b	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Far2	0.85965	1.00000	0.95150	1.00000	1.00000	1.00000	0.99488
Fastkd5	1.00000	1.00000	0.98823	1.00000	1.00000	1.00000	1.00000

Fdxacb1	1.00000	1.00000	0.96367	1.00000	0.99998	1.00000	0.16071
Foxi2	0.99915	1.00000	0.09700	1.00000	1.00000	1.00000	0.06559
Foxo3	0.99170	1.00000	0.00847↑	1.00000	1.00000	0.98206	1.00000
Foxred2	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Ghrhr	0.73473	0.05054	1.00000	0.74584	0.00048↑	1.00000	1.00000
Ghsr	1.00000	1.00000	0.99846	1.00000	1.00000	1.00000	1.00000
Gimap6	0.96312	1.00000	0.10616	0.99961	0.86516	1.00000	0.97581
Gimap8	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Gipc3	1.00000	1.00000	0.12197	1.00000	1.00000	0.15981	0.99964
Gjd4	0.97336	1.00000	0.02652↑	1.00000	0.99824	1.00000	0.80596
Glycam1	1.00000	1.00000	1.00000	0.99956	0.88946	1.00000	1.00000
Gpnmb	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Gpr142	1.00000	1.00000	1.00000	0.99561	0.54208	1.00000	1.00000
Gpr156	0.98860	1.00000	1.00000	1.00000	0.99999	1.00000	1.00000
Gpr183	1.00000	1.00000	0.92334	1.00000	1.00000	1.00000	1.00000
Gpr19	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
H1fx	1.00000	0.99983	0.99999	1.00000	0.34013	0.99422	1.00000
Hc	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Hdac10	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Hemgn	1.00000	0.99991	1.00000	0.95509	0.10681	1.00000	1.00000
Heyl	1.00000	0.99891	1.00000	1.00000	1.00000	1.00000	1.00000
Hfe2	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Hsd17b1	1.00000	0.99632	0.00003↓	<0.00001↓	1.00000	<0.00001↓	1.00000
Hsd17b11	1.00000	1.00000	0.51096	1.00000	1.00000	1.00000	0.45232
Hsf2	1.00000	1.00000	1.00000	1.00000	0.99900	1.00000	0.99991
Hsf4	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Hspb1	1.00000	1.00000	1.00000	0.99991	0.96012	1.00000	0.99969
Hspb2	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Hspb3	1.00000	0.99942	1.00000	0.98860	1.00000	1.00000	1.00000
Htr1a	1.00000	1.00000	0.79472	1.00000	1.00000	1.00000	1.00000
Htr1d	1.00000	1.00000	0.99929	1.00000	1.00000	0.00075↑	0.97581
Htr1f	0.91148	1.00000	0.00074↑	1.00000	0.99990	1.00000	0.00245↓
Htr3b	1.00000	1.00000	0.03519↓	1.00000	1.00000	1.00000	1.00000
Htr7	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Hyal3	0.99996	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Ifnk	1.00000	1.00000	0.99981	1.00000	1.00000	1.00000	1.00000
Ifnl3	0.96795	1.00000	0.00229↑	1.00000	0.99997	1.00000	0.13360
Igsf11	1.00000	0.64460	0.99978	1.00000	1.00000	1.00000	0.31266
Il12rb2	1.00000	1.00000	0.63810	1.00000	1.00000	0.67855	0.00558↓
Il24	0.99990	0.60789	1.00000	0.99999	1.00000	0.76901	1.00000

Ipp	1.00000	1.00000	0.40140	1.00000	1.00000	0.00010↑	0.47273
Iqcj	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Iqgap2	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Irf8	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Jam2	1.00000	1.00000	0.33528	1.00000	1.00000	1.00000	0.05637
Jmjd8	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.99969
Kcnh3	1.00000	1.00000	0.04012↓	1.00000	0.74510	1.00000	1.00000
Klk14	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Krt77	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Krt9	1.00000	1.00000	0.74664	1.00000	1.00000	1.00000	0.01956↓
Lcn2	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Lima1	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Limch1	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Lin28b	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.00001↓
Lipn	0.99993	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Lman1l	1.00000	1.00000	1.00000	1.00000	1.00000	0.98382	0.99551
Loxl1	1.00000	1.00000	1.00000	0.99995	0.81536	1.00000	0.80418
Lpar6	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Lrch1	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Lrrc15	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Ltbp2	1.00000	0.97685	1.00000	1.00000	0.99999	1.00000	1.00000
Macrod2	0.94355	0.00004↑	0.98001	0.96051	1.00000	0.19091	1.00000
Mag	1.00000	0.46726	1.00000	1.00000	1.00000	1.00000	0.00078↓
Mdk	1.00000	0.17186	1.00000	1.00000	0.99983	1.00000	1.00000
Mdp1	1.00000	1.00000	0.50462	1.00000	1.00000	1.00000	1.00000
Mepce	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.81046
Mettl21c	1.00000	1.00000	0.77394	1.00000	1.00000	1.00000	0.04942↓
Mettl7b	1.00000	0.91667	1.00000	0.29576	0.10787	0.59416	0.00082↓
Mfsd10	1.00000	1.00000	0.99993	1.00000	1.00000	1.00000	0.94635
Mmp8	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Moxd1	1.00000	1.00000	0.47566	1.00000	1.00000	1.00000	0.99096
Mpdz	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Mrgpre	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Myh1	1.00000	0.01319↑	0.91948	0.00007↓	0.98694	0.00005↓	<0.00001↓
Mylip	1.00000	0.00669↓	0.41814	1.00000	0.03605↓	1.00000	1.00000
Myo3b	1.00000	1.00000	1.00000	0.29230	0.07871	1.00000	1.00000
Myo7b	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.97413
Nat1	1.00000	1.00000	0.96747	0.99997	1.00000	0.10895	0.99927
Ncald	0.44568	1.00000	0.00167↑	1.00000	1.00000	1.00000	0.05868
Nefh	1.00000	1.00000	1.00000	0.62263	0.04344↓	1.00000	0.92851

Nek11	1.00000	1.00000	0.99636	1.00000	1.00000	1.00000	1.00000
Nes	1.00000	1.00000	1.00000	0.44716	0.03251↓	1.00000	<0.00001↓
Neurl2	0.96514	1.00000	0.00251↑	1.00000	1.00000	1.00000	0.94709
Nfatc4	1.00000	0.99960	0.46211	0.23840	1.00000	0.03742↑	1.00000
Nmrk2	1.00000	1.00000	0.99911	1.00000	1.00000	1.00000	0.17522
Npm3	1.00000	1.00000	0.00402↑	1.00000	1.00000	0.23074	0.46484
Nrcam	1.00000	0.99856	0.00003↓	0.64614	1.00000	0.01323↓	1.00000
Nrn1l	1.00000	1.00000	0.98253	1.00000	1.00000	0.29187	0.88305
Nrsn1	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Nsun7	0.99945	1.00000	0.99840	1.00000	1.00000	0.91420	0.99999
Nt5c	1.00000	1.00000	0.99866	1.00000	1.00000	1.00000	1.00000
Oard1	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Ocstamp	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.81566
Ogn	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.94635
Osm	1.00000	1.00000	0.48178	1.00000	1.00000	1.00000	0.96424
Pacsin2	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Paqr7	1.00000	0.99998	1.00000	1.00000	1.00000	1.00000	1.00000
Pard6a	1.00000	1.00000	1.00000	0.90411	0.99999	0.91909	1.00000
Parp16	1.00000	0.97431	0.18518	1.00000	0.72654	1.00000	1.00000
Parp8	0.28412	0.57899	1.00000	1.00000	1.00000	0.99620	0.99809
Pcdh12	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Pcsk4	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Pitx3	<0.00001↓	1.00000	<0.00001↓	<0.00001↓	1.00000	<0.00001↓	0.83024
Pkn3	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Pkp4	1.00000	1.00000	1.00000	0.80459	0.64098	1.00000	0.00003↓
Pla2g2d	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Plekha3	1.00000	1.00000	1.00000	0.99998	0.13563	1.00000	1.00000
Plk5	1.00000	1.00000	0.99997	1.00000	1.00000	1.00000	1.00000
Pnmt	1.00000	1.00000	1.00000	1.00000	0.99685	0.83489	0.95626
Postn	0.08822	<0.00001↑	0.96233	1.00000	0.79094	1.00000	0.04203↓
Ppp1r26	1.00000	1.0000	0.99999	1.00000	1.00000	1.00000	1.00000
Ppp1r9b	<0.00001↓	<0.00001↓	<0.00001↓	0.00010↓	<0.00001↓	0.00019↓	0.65755
Prkab1	1.00000	1.00000	1.00000	0.99904	0.52823	1.00000	1.00000
Prokr1	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.38500
Prom2	1.00000	1.00000	0.35239	0.99999	0.62360	1.00000	1.00000
Prss40	0.99517	0.99992	1.00000	1.00000	1.00000	1.00000	1.00000
Prss56	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.99997
Ptpn20	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Ptpn5	0.20353	0.30580	1.00000	1.00000	0.99755	1.00000	1.00000

Ptpru	1.00000	1.00000	0.98088	0.59129	1.00000	0.00014↓	1.00000
Pycr1	1.00000	0.99758	1.00000	1.00000	1.00000	1.00000	0.86458
R3hcc1l	1.00000	1.00000	1.00000	1.00000	1.00000	0.99968	1.00000
Rab11fip5	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Rab20	1.00000	0.99989	1.00000	1.00000	1.00000	1.00000	1.00000
Rab24	0.60031	1.00000	0.06027	1.00000	1.00000	1.00000	1.00000
Rab27b	1.00000	1.00000	1.00000	0.17053	0.01980↓	1.00000	0.87499
Rab36	1.00000	1.00000	0.87943	1.00000	0.99999	1.00000	0.00022↓
Rab39	1.00000	1.00000	1.00000	1.00000	0.99948	1.00000	0.99999
Rab3ip	0.99674	1.00000	0.03877↑	1.00000	0.77375	0.81519	0.01999↓
Rab43	1.00000	1.00000	1.00000	1.00000	0.99995	1.00000	0.99952
Rab5a	1.00000	1.00000	0.98602	0.99949	0.99517	1.00000	0.11026
Rabac1	1.00000	1.00000	1.00000	1.00000	1.00000	0.99996	1.00000
Rad21l	1.00000	1.00000	0.99001	1.00000	1.00000	1.00000	1.00000
Rap2b	1.00000	1.00000	1.00000	0.96079	0.95092	1.00000	1.00000
Resp18	1.00000	0.96304	0.99997	1.00000	1.00000	1.00000	1.00000
Rexo4	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Rhbdl2	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.98985
Rilpl2	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Rimklb	1.00000	0.00795↓	0.00055↑	1.00000	0.00015↓	1.00000	0.47273
Rin3	1.00000	1.00000	1.00000	1.00000	1.00000	0.98209	1.00000
Rnf10	1.00000	1.00000	1.00000	0.00013↓	0.00002↓	0.89154	1.00000
Rnf112	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Rnf13	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.03397↓
Rnf133	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Rnf25	1.00000	1.00000	0.09629	0.22376	0.04616↓	1.00000	1.00000
Rps6kl1	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Rrad	1.00000	1.00000	1.00000	1.00000	1.00000	0.96571	1.00000
Rxfp4	1.00000	1.00000	0.25707	0.99998	1.00000	0.87516	0.04697↓
Scg2	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Serpina1f	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Serpina7	1.00000	1.00000	0.99997	1.00000	1.00000	1.00000	1.00000
Serpinb5	1.00000	1.00000	0.00345↑	1.00000	0.14922	0.18500	0.87780
Setd6	1.00000	1.00000	0.95386	1.00000	1.00000	0.95874	1.00000
Sfxn4	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Sgta	1.00000	1.00000	0.99372	1.00000	1.00000	1.00000	1.00000
Sh3tc2	0.88140	1.00000	0.88515	1.00000	1.00000	1.00000	1.00000
Slc1a1	0.99998	0.07564	1.00000	1.00000	0.25652	1.00000	0.69838
Slc24a5	1.00000	1.00000	1.00000	0.69724	0.92114	0.99989	1.00000
Slc25a35	1.00000	1.00000	1.00000	1.00000	1.00000	0.99960	1.00000

Slc46a3	0.05127	1.00000	0.00001↑	1.00000	1.00000	0.60701	0.02093↓
Slc8a3	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Slc8b1	0.27322	1.00000	<0.00000↑	1.00000	0.98869	0.01925↑	0.00631↓
Smoc2	0.99999	1.00000	0.05730	1.00000	1.00000	0.95387	1.00000
Snx15	1.00000	1.00000	0.12902	1.00000	1.00000	1.00000	0.71906
Sorbs2	1.00000	1.00000	1.00000	1.00000	1.00000	0.93435	1.00000
Sox18	1.00000	1.00000	0.99831	1.00000	1.00000	0.14447	0.67412
Sp5	1.00000	1.00000	1.00000	1.00000	0.98822	1.00000	1.00000
Spag4	1.00000	1.00000	0.99997	1.00000	1.00000	1.00000	1.00000
Spp1	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Sprr1b	1.00000	1.00000	1.00000	1.00000	0.98382	1.00000	1.00000
Sprr3	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Sptssb	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Stag3	1.00000	1.00000	1.00000	0.99997	0.37120	1.00000	1.00000
Stat5b	1.00000	1.00000	0.99969	1.00000	1.00000	1.00000	1.00000
Stk16	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Stx16	1.00000	0.70597	0.67266	0.57495	0.00105↓	1.00000	0.00345↓
Stx19	1.00000	1.00000	0.99782	1.00000	0.99977	1.00000	0.05429
Syce1	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Syce1l	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Sycp3	1.00000	1.00000	0.99999	1.00000	1.00000	1.00000	1.00000
Syn3	1.00000	1.00000	0.99413	1.00000	1.00000	1.00000	0.11460
Tbc1d4	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Tbx22	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.99999
Tdrkh	1.00000	1.00000	0.04083↑	1.00000	1.00000	0.99994	0.00669↓
Tex101	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Tex29	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Thsd1	1.00000	1.00000	0.94754	1.00000	0.99941	1.00000	0.20642
Timp3	1.00000	1.00000	1.00000	1.00000	0.99943	1.00000	1.00000
Tmem136	1.00000	1.00000	1.00000	0.00254↑	0.99852	0.00042↑	1.00000
Tmem151b	1.00000	1.00000	0.74676	0.61905	0.03149↓	1.00000	0.99973
Tmem181	1.00000	1.00000	0.98210	1.00000	1.00000	1.00000	1.00000
Tmem181a	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Tmem79	0.12915	1.00000	0.00405↓	0.80889	1.00000	0.03757↑	1.00000
Tmod2	1.00000	0.13353	1.00000	1.00000	0.03712↓	1.00000	0.04314↓
Tnfsf18	0.38505	0.99958	0.97423	0.82131	0.94422	0.98041	0.71783
Tpcn1	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Tpgs2	0.03009↑	0.21296	0.99866	0.37887	0.99354	1.00000	0.95626
Tppp	1.00000	1.00000	1.00000	0.56355	0.62933	1.00000	<0.00001↓
Tprn	0.86892	0.80537	1.00000	1.00000	1.00000	1.00000	1.00000

Trip13	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Try4	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Tspan18	1.00000	1.00000	1.00000	0.91584	0.96123	1.00000	1.00000
Tssk5	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Ttll10	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Ttll6	0.57716	1.00000	0.00085↑	1.00000	1.00000	0.08389	0.99824
Vcpkmt	0.99999	1.00000	0.98888	1.00000	1.00000	1.00000	1.00000
Vegfb	0.69083	1.00000	0.16113	1.00000	1.00000	1.00000	1.00000
Vsig4	0.03580↓	0.00043↓	1.00000	1.00000	0.06173	1.00000	0.88800
Vsig8	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Wee2	1.00000	1.00000	1.00000	1.00000	1.00000	0.15328	0.99866
Ydjc	0.34380	1.00000	0.00005↑	1.00000	1.00000	1.00000	1.00000
Ypel1	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Zbtb32	1.00000	1.00000	0.98617	1.00000	1.00000	1.00000	1.00000
Zbtb4	0.41262	1.00000	0.00975↑	1.00000	1.00000	0.08996	1.00000
Zdhhc11	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
Zfp14	1.00000	1.00000	1.00000	0.99996	1.00000	0.70127	1.00000
Zfp219	1.00000	0.71832	1.00000	1.00000	1.00000	0.99909	1.00000
Zfp689	1.00000	1.00000	1.00000	1.00000	0.99982	1.00000	0.00579↓
Zfp961	1.00000	0.99999	0.00006↑	1.00000	0.05686	0.00726↑	0.91956
Zfyve26	1.00000	0.06976	1.00000	1.00000	0.93841	1.00000	1.0000
Zzef1	0.99999	0.33032	1.00000	0.96146	0.00138↓	1.00000	0.15004

Chapter 6 Discussion and conclusions

As much as one-third of human life is spent in sleep, a behavioral state characterized by reduced consciousness and increased arousal threshold. Sleep is well conserved across mammals and birds, and at least some form of sleep appears common in almost all animals. Sleep is thus believed to serve some important physiological function. It has been described as a process "of the brain, by the brain and for the brain" (Hobson 2005). However, getting sufficient sleep, which is a major health concern, has far-reaching health consequences beyond the more obvious deterioration of cognitive performance. Insufficient sleep has been implicated in many health conditions including metabolic disorders, such as obesity and diabetes, cardiovascular diseases, hypertension, depression, and neurodegenerative diseases such as Alzheimer's disease (AD) (Palagini, Bruno et al. 2013, Van Someren, Cirelli et al. 2015).

Reports on higher concordance of brain architecture and sleep phenotypes in monozygotic vs dizygotic twins provided some of the early evidence that sleep is under genetic control (Stassen, Lykken et al. 1988, Ambrosius, Lietzenmaier et al. 2008, De Gennaro, Marzano et al. 2008). In 1999, in a major discovery, a mutation in hypocretin receptor-2 was found to underlie canine narcolepsy (Lin, Faraco et al. 1999). Many other genes since then have been found to affect sleep; in a review, Cirelli summarizes more than 80 genes that have been reported to alter sleep parameters (Cirelli 2009). There have been many advancements in the field of sleep, however, we still lack understanding about the fundamental functions of sleep and the need for sleep. This in turn limits our efforts at improving sleep quality, and developing more effective drugs for sleep disorders. The

knowledge of genetic regulators thus may not only provide an insight into the general functions and genetic basis of sleep, but also might suggest novel drug targets to alleviate symptoms associated with sleep disorders. To this end, one approach to identify genetic components of sleep regulation is by exploiting existing genetic tools and utilizing rodent mouse models of human disease conditions. Another possible method to probe the genetic basis of sleep is through the use of knockout animals to discover novel sleep-related genes. For the first part of my project, I examined an AD mouse model, and a combined mouse model of AD and db (diabetes) for alterations in their sleep-wake patterns (Chapters 3 and 4). For the second part of my thesis, I analyzed data from a large-scale knock out project to identify novel sleep-related genes (Chapter 5).

An accurate measure of sleep and sleep-related traits is critical for both forward and reverse genetic approaches towards a better understanding of sleep. The different sleep-wake parameters that can be screened range from sleep duration (REM, NREM sleep), the number of such sleep bouts, and distribution of sleep across dark and light phases. To detect subtle effects of a gene mutation or an allelic variant in the phenotypic screens, a large population of mice is required. However, traditional EEG/EMG techniques used for sleep-wake assessment has limitations to its scalability for large-scale studies. Not only it is time and labor intensive, but also it is an invasive technique which can confound behavioral studies, in particular sleep. Furthermore, it requires animals to be tethered to the EEG system for data acquisition, which restricts the animal movement. To overcome these deficiencies, our lab developed the PiezoSleep system- a high throughput, non-invasive system suitable for sleep studies for large numbers of animals. It shows a classification accuracy of over 90% compared to human observation and EEG/EMG recordings

(Donohue, Medonza et al. 2008, Mang, Nicod et al. 2014). PiezoSleep comprises a highly-sensitive piezoelectric sensor pad placed on the cage floor. The sensor pad detects the pressure variations to classify sleep and wake states. During sleep, the prominent movement detected is breathing of an animal compared to wakefulness, which is characterized by erratic movements. The system can as well discriminate quiet rest from sleep and brief arousals, the feature that is missing in many other non-invasive alternative devices. PiezoSleep thus finds its use as an essential tool in all of my research projects. However, the system cannot as yet sub-stage sleep into REM and NREM sleep. A detailed description of the system can be found in Chapter 2.

AD, an age-related, progressive neurodegenerative disease, has become a health epidemic with more people living beyond their 60s. Sleep alterations are a common occurrence, in addition to circadian impairments in many of the AD patients. Epidemiological studies indicate that sleep disturbances are one of the primary causes of institutionalization of AD patients (Pollak, Perlick et al. 1990, Bianchetti, Scuratti et al. 1995, Moran, Lynch et al. 2005, Guarnieri, Adorni et al. 2012). Sleep disruptions are manifested in the form of reduced slow wave sleep (SWS) andREM sleep, in addition to increased nighttime awakenings, and excessive daytime sleepiness (Holth, Patel et al. , Prinz, Peskind et al. 1982, Martin, Loewenstein et al. 1986, Vitiello, Prinz et al. 1990, Petit, Gagnon et al. 2004). These sleep alterations in turn are believed to be linked to cognitive impairments seen in AD patients (Nishida, Pearsall et al. 2009, Walker 2009). Findings from human and mouse studies suggest that AD pathology begins as early as 15 years prior to the appearance of clinical symptoms, in parallel to sleep aberrations (Morris and Price 2001, Sperling, Aisen et al. 2011, Fitzsimmons and Bertram 2013). Improvement in sleep

has been found to lower the risk of AD in people with at least one APOE ε4 allele (Lim, Yu et al. 2013, Simon, Greenaway et al. 2013). Recently, in a seminal work, Xie el al have shown that sleep facilitates clearance of toxic metabolites, including Aβ, a neuronal by-product of the APP protein that is a major constituent of the plaques in Alzheimer's Disease (AD) (Xie, Kang et al. 2013). Taken together, these studies suggest that a bidirectional relationship exists between AD and sleep, with poor sleep enhancing the predisposition to amyloid beta (Aβ) deposition and reduced clearance, and increasing Aβ causing sleep disruption. Thus, sleep may be critical as both a therapeutic intervention for AD, and also a key symptom that needs relief. This information should support further development of therapeutics that can alleviate sleep aberrations in AD. Since human studies tend to be expensive, take many years, and are difficult to control, animal models are an important alternative. Given that a wide variety of genetic resources are well-established for mouse, and there is 90% homology between the mouse and human genome in the critical coding and gene regulatory regions, the mouse has become a preferred animal model to emulate a wide variety of both normal and human pathological conditions, including AD.

In chapter 3, I investigated whether 5XFAD, a well-characterized AD mouse model, exhibits sleep-wake alterations similar to those reported in human AD patients. These animals show early onset of AD neuropathology as a result of additive effects of 5 distinct human mutations: 3 in the amyloid precursor protein (APP) namely Swedish, Florida and London mutations, and 2 in the presenilin protein (PS1) i.e. M146L and L286V (Oakley, Cole et al. 2006). These mice thus develop severe intraneuronal $A\beta_{42}$ at an early age beginning at 1.5 months, amyloid deposition at 2 months, synaptic loss at 9 months of

age. Memory deficits as tested on behavioral assays such as Y maze, Morris water maze, can be seen at an age of 4 to 6 months onwards (Oakley, Cole et al. 2006). Considering that men as well as women are affected by AD, I included both male and female 5XFAD mice (4-6.5 months old) in my study. Sleep parameters that were recorded with the Piezo system include sleep across 24 h, dark and light phases, and bout length across 24h, dark and light phase. After baseline recording of 3-5 days, mice were sleep deprived for 4 hours.

Under baseline conditions, both male and female 5XFAD mice had shorter bout lengths compared to controls. Additionally, females also had reduced overall sleep due to marked reduction seen in sleep during the dark phase. However, males did not differ from controls in their sleep durations. The bout length reductions for 5XFAD mice (both males and females) were more prominent in dark phase (active phase of mice) than the light phase, which does not necessarily match the human condition. However, the overall decrease still reflects increased sleep fragmentation, which is akin to sleep disruptions seen in human AD condition. There was no effect of genotype for bout length and sleep duration in 6 h following sleep deprivation (Sethi, Joshi et al. 2015). Thus, in general, our findings are similar to previous reports of altered sleep-wake patterns in other AD animals. Reduced sleep including REM and NREM sleep, and/ or increased wake time has been reported in many AD mice- APP/PS1, PDAPP, TgCRND8, Tg2576 (Huitron-Resendiz, Sanchez-Alavez et al. 2002, Zhang, Veasey et al. 2005, Platt, Drever et al. 2011, Roh, Huang et al. 2012, Colby-Milley, Cavanagh et al. 2015). In summary, 5XFAD exhibits sleep alterations that resemble some key aspects of sleep disruptions reported in AD patients.

Sex differences found in our study might stem from difference in Aβ levels for the 2 sexes. Oakley et al have reported that $A\beta_{42}$ levels tend to be higher in young 5XFAD

females compared to age-matched males (Oakley, Cole et al. 2006). There may be other factors contributing to this disparity between the 2 sexes. An understanding of what drives these different levels of Aβ in the two sexes may provide further insight to human AD sleep alterations as well.

Type 2 Diabetes (T2DM), a chronic metabolic disorder, affects more than 8% of the American population. Obesity is known to increase the risk for T2DM. In addition, inadequate sleep has been linked to elevated risk of T2DM, independently of obesity. It is proposed that sleep restriction might alter energy metabolism, by upregulating appetite and reducing the energy expenditure (Knutson, Spiegel et al. 2007). These events in turn can lead to weight gain, and contribute to insulin resistance, which again is a risk factor for developing T2DM. Animal studies further corroborate the inverse relationship between sleep and metabolism. Leptin-deficient ob/ob, and leptin-resistant db/db mice and obese zucker rats have attenuated sleep-wake rhythms in addition to increased sleep fragmentation, and increased overall sleep duration (Mavanji, Billington et al. 2012). Conversely, type, timings and availability of diet, has been shown to affect sleep in rodents (Jenkins, Omori et al. 2006).

Furthermore, there is growing evidence that diabetes and obesity present an increased risk of AD development. The Rotterdam study, and several more recent studies, have shown that diabetic individuals have double the risk of AD prevalence (Ott, Stolk et al. 1999, Kroner 2009). In a clinical study, Janson et al found that AD patients are more prone to develop T2DM than non-Alzheimer controls (Janson, Laedtke et al. 2004). The individuals with AD, and a prior history of T2DM also exhibit cerebrovascular

abnormalities, in addition to the typical AD-related neuropathology without showing any additional Aβ burden. Effects of cerebrovascular anomalies or vascular dementia, a frequent comorbidity with Alzheimer's disease, are poorly understood. An animal model recapitulating- both AD and T2DM, is therefore critical to further our understanding about how the two conditions interact, and promote cerebrovascular abnormalities.

With the aim to evaluate how the interaction of AD and T2DM affect sleep, I took advantage of the combined mouse model of AD and T2DM (db/AD) created by Niedowicz et al. The db/AD mouse was generated by crossing leptin-resistant db/db with APP/PS1, a knock-in mouse model of AD (Neidowicz et al., 2014). db/AD mice exhibit additional cerebrovascular pathologies (aneurysms and strokes), not present in either of the parental lines. These animals are obese, insulin resistant and glucose tolerant beginning early age. Plaque deposition is reduced in db/AD animals compared to AD mice. Aβ levels do not differ between db/AD animals relative to AD mice across different age groups. However, $A\beta_{42}$ levels are reduced significantly in db/AD animals than AD mice, at 6 and 12 months of age (Neidowicz et al., 2014).

For my study, as detailed in chapter 4, I assessed sleep duration and bout length in db/AD mouse, as well as individual parental strains- db/db and APP/PS1, for comparisons. We included mice of both the sexes and different age groups (young and old), and examined the interaction effects between different predictor variables. Our findings are similar to previous reports on AD animals. In our study, AD animals slept less during dark phase, and also had longer bout lengths during the light phase and across 24h, after controlling for all other covariates. It is thus possible that AD animals tend to compensate for shorter sleep duration seen during dark phase, with longer sleep bouts during the light

phase (an increase in average bout lengths by approx. 100 s). db animals were found to have longer overall duration of sleep, attributable to prominent differences seen during the dark phase. This is similar to previous reports in case of leptin-deficient ob/ob and leptin-resistant db/db mice, and Obese zucker rats.

For db/AD mice (both males and females) similar to db animals, an increase in sleep duration across 24 h and dark phase compared to controls was found. Our findings thus indicate that the cerebrovascular pathology seen in db/AD mice, believed to be responsible for their cognitive deficits does not lead to additional sleep aberrations. Also, Niedowicz et al in their studies reported that db/AD animals have less plaque deposition and $A\beta_{42}$ levels than AD animals (Neidowicz et al., 2014). This might explain a more prominent effect of db genotype on sleep measures than AD in case of db/AD mice.

We found significant age and db/AD genotype interactions for sleep across 24h, light as well as dark phase, which means that as db/AD mice grow older; they tend to sleep less. Reduction in sleep duration tends to be more salient during the dark phase. These findings indicate that with an increase in age for db/AD animals, they sleep less than when they are young, and thus the sleep differences dissipate between db/AD and WT animals at later age.

Sex effects were seen in case of bout lengths, as females tend to have shorter bouts compared to males and these differences were more prominent in dark than the light phase. In regard to age difference, older subjects were found to have shorter bouts during the light phase. Interaction between sex and age was found to be significant for the older animals for bout length during the dark phase implying that there is reduction in bout length as the age progresses for any of the given genotype.

Leptin can be one of the upstream molecular components involved at the intersection of AD and diabetes. It is known to activate MAPK, AKT and mTOR kinases, which are known to inhibit Glycogen synthase 3 kinase (GSK3B) and thus GSK3B-dependent tau phosphorylation. Down-regulation of leptin signaling as seen in T2DM can therefore result in increased tau phosphorylation which can subsequently promote AD development (Shiromani, Xu et al. 2004, Bertram, Rook et al. 2011). Collectively, these studies suggest that diabetes associated dysfunctions promote AD pathology which can then disrupt sleep. Conversely, sleep impairments might be augmenting the AD and T2DM diseased states. Further interrogation of the underlying pathways can bridge the gap in our understanding of how sleep, AD and diabetes are inter-connected. In particular, it will be interesting to trace the molecular mechanisms that lead to reduced plaque deposition and $A\beta_{42}$ levels in db/AD animals, which might be significant in developing novel drug targets aimed at ameliorating AD pathology.

There are many mechanisms to determine genetic regulators of sleep. Each of the available techniques has its own strengths and limitations, and together have advanced our understanding about sleep. For my research, I mainly took advantage of reverse genetics techniques. In my earlier chapters, I utilized a variety of mouse models recapitulating human diseased conditions and assessed if they are similar in their sleep-wake phenotype to human conditions. For my fifth chapter, I report identification of novel sleep-related genes by screening sleep-wake parameters for a large-scale population of single gene knockout mice being recorded as part of Knockout mouse phenotyping program (KOMP2) (Abbott 2010, Bradley, Anastassiadis et al. 2012, Brown and Moore 2012). Sleep-wake traits were analyzed by PiezoSleep system. Knockout mice were generated on C57BL6/N

background, a common laboratory inbred strain. The mice are being recorded at the Jackson Laboratory (Jax), one of the KOMP2 centers. As part of the Jax phenotyping pipeline, each of the mouse is comprehensively screened for over 200 measurements, ranging from morphological, physiological to behavioral traits. Additional behavioral assays such as rotarod, holeboard exploration tests, and sleep, are unique to Jax pipeline.

These knockout mice were recorded for 5 baseline days, under 12:12 LD conditions at the age of 15 weeks. To date, I have analyzed data for more than 6000 animals, representing over 300 knockout lines, and over 1800 control animals. I have identified 55 novel sleep-related genes by utilizing multiple statistical approaches including ANOVA, Principal component analysis and Multivariate analysis (Mahalanobis distance). Significant sleep-wake differences were found for a number of knockout strains relative to controls, in both light and dark phase. Some of the shortlisted candidate sleep genes are Adck2, Bzw2, Cbln3, Cpb1, Htr1d, Pitx3, Ppp1r9b, Rab27b, Rab36, Rimklb, Rnf10 and Tmem136. For many of the genes not much is known as is the case for Tmem136 and Rimklb while others have been associated with a wide variety of functions such as neuronal differentiation, modulating dendritic morphology, protein-protein interactions, and signal transduction. But none of these genes have been implicated in sleep regulation before, other than *Htr1d* and *Pitx3*.

Additionally, sex differences were found for controls and many of the knockouts. Females slept less than males in most of these cases. Amongst other behavioral assays in the pipeline, sleep has been found to be the most consistent, which is not surprising given that sleep is a tightly regulated process and plays a critical role in overall health of an

animal. In addition to sleep traits, we also recorded breath rate for the knockout and control animals and found several genes affecting breathing variables as well.

This project demonstrates the potential of an unbiased approach such as KOMP2 in underscoring the role of novel genes, not implicated in sleep regulation earlier. The wealth of data generated by KOMP2 is unprecedented in sleep research. This analysis may not be the final step in functional annotation of sleep-related genes, but one crucial step towards our understanding of sleep regulation. Further follow up from our lab and other researchers would hopefully yield success in our quest to answer the questions of why do we need sleep and how are the genes implicated from these genetic studies responsible for its regulation and functions.

For the follow up, next logical extension of this project will be to elucidate the mechanisms through which our novel, candidate sleep genes regulate sleep. In particular, knockouts for the genes- *Ap4e1, Ppp1r9b* and *Cbln3,* which affect multiple sleep-wake traits can be followed primarily for the gene expression studies. Based on their RNA seq analysis results, consistently upregulated or downregulated genes (especially in brain) common to all the knockouts, can be studied further. Pathways of genes shortlisted based on their common occurrence can then be investigated, and knockout mice be generated for the gene/s thought to be upstream or downstream of the pathway of the deleted gene. Generation of knockouts is a time consuming process; an alternative could include the use of morpholinos, targeting the gene. Rescue experiments may be conducted for the suggested target downstream gene/s by introduction of its wild type transgenes in the knockout mouse. Pharmacological agonists/ antagonists could also be tried if appropriate. Complete/ partial rescue of the phenotypes and restoration of expression levels of the

affected genes, similar to the control, would implicate the gene as a downstream target and would lead us to hypothesize the pathway by which aforesaid genes might play a role in sleep regulation. Other assays/ approaches will be helpful in framing the hypothesis. In-situ hybridization (ISH) can locate the site of expression of the affected gene/s in the brain. Expression in some specific region of the brain might correlate with the observed phenotypes or might give further clues to the other possible functions of the gene. An alternative to ISH will be the use of public databases (such as Allen's Brain Atlas or BioGPS). Behavioral assays (such as learning and cognitive tasks) could be performed for the KO mice before and after transgene introduction to find if the behavioral phenotypes (if there were any), could be rescued too.

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Publications

- **Sethi M.**, Joshi S. S., Striz M., Cole N., Ryan J., Lhamon M.E., Agarwal A., Stacey J. Sukoff Rizzo S.J., Denegre J.M., Braun R.E., Fardo D.W., Donohue K.D., Chesler E.J., Svenson K.L., O'Hara B.F., Analysis of sleep traits in knockout mice from the large-scale KOMP2 population using a non-invasive, high-throughput piezoelectric system. ***BMC Bioinformatics*** **2015**, 16(Suppl 15):P15. (Poster Abstract)
- **Sethi M**., Joshi S. S., Webb R. L., Beckett T. L., Donohue K. D., Murphy M. P., O'Hara B. F., Duncan M. J., Increased fragmentation of sleep-wake cycles in the 5XFAD mouse model of Alzheimer's disease. ***Neuroscience*** **2015,** 290:80-89.
- **Sethi M.**, Joshi S. S., Striz M., Cole N., Ryan J., Lhamon M.E., Agarwal A., Stacey J. Sukoff Rizzo S.J., Denegre J.M., Braun R.E., Fardo D.W., Kumar V., Donohue K.D., Chesler E.J., Svenson K.L., O'Hara B.F., Non-invasive sleep-wake monitoring in large-scale screening of knockout mice reveals novel sleep-related genes *(in preparation)*
- **Sethi M.**, Guerriero L., Wang C., Helman A. M., Macheda T., Murphy M.P., Stromberg A.J., Duncan M.J., O'Hara B.F, Altered sleep-wake behavior in a novel murine model of type 2 diabetes and Alzheimer's disease *(in preparation)*

Select Presentations

- **Sethi M.**, Guerriero L., Wang C., Bernat R., Helman A. M., Macheda T., Agarwal A., Murphy M.P., Stromberg A.J., Marilyn J. Duncan, O'Hara B.F. Altered sleep-wake behavior in a novel murine model of type 2 diabetes and Alzheimer's disease *Annual Meetings/ Neuroscience*, **Poster presentation**, San Diego, California, USA **2016.**
- O'Hara B.F., **Sethi M.**, Striz M., Joshi S.S., Cole N., Ryan J., Ashley J., Lhamon M.E., Agarwal A., Sukoff Rizzo S.J., Denegre J.M., Braun R.E., Donohue K.D., Chesler E.J., Svenson K.L., Noninvasive sleep monitoring in large scale screening of mouse knockouts (KOMP2) produces high hit rate with implications for sleep and behavioral studies *Annual Meetings/ Neuroscience*, **Poster presentation**, San Diego, California, USA **2016.**
- **Sethi M.**, Striz M., Joshi S.S., Cole N., Ryan J., Ashley J., Lhamon M.E., Agarwal A., Sukoff Rizzo S.J., Denegre J.M., Braun R.E., Donohue K.D., Chesler E.J., Svenson K.L., O'Hara B.F., Noninvasive sleep monitoring in large scale screening of mouse knockouts (KOMP2) produces high hit rate with implications for sleep and behavioral studies, *30th Annual Sleep meeting*, **Oral and Poster presentation**, Denver, Colorado, USA **2016.**
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- Joshi S.S., **Sethi M.**, Striz M., Cole N., Ryan J., Ashley J., Lhamon M.E., Agarwal A., Sukoff Rizzo S.J., Denegre J.M., Braun R.E., Donohue K.D., Chesler E.J., Svenson K.L., O'Hara B.F., Improved Sleep related Gene Ontologies through analysis of KOMP2 sleep phenotyping data and gene expression studies, *Annual Meetings/ Neuroscience*, **Poster presentation**, Chicago, Illinois, USA **2015.**
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- **Sethi M.**, Striz M., Joshi S., Cole N., Ryan J., Ashley J., Denegre J.M., Donohue K.D., Chesler E.J., Svenson K.L., O'Hara B.F., Analysis of sleep and wake in the KOMP2 mouse population using an improved non-invasive, automated sleep

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- **Sethi M.**, Striz M., Joshi S., Webb R. L., Beckett T. L., Canter H., Brigham J., Donohue K. D., Murphy M. P., O'Hara B. F., Duncan M. J., Sleep alterations in 5XFAD mice, a model of Alzheimer's disease. *Annual Meetings/ Neuroscience*, **Poster presentation**, San Diego, California, USA **2013.**
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- Striz M., **Sethi M**., Zhang T., Joshi S., Gooch R., Yaghoubi F., Donohue K.D., O'Hara B.F, A Non-Invasive System for High-Throughput Monitoring of Mouse Sleep and Other Behaviors. *9th Kentucky Innovation and Entrepreneurship Conference*, **Poster presentation**, Lexington, KY, USA **2013.**

Teaching Experience

2010 - 2016	Teaching Assistant, University of Kentucky, Department of Biology Bio 152 Principles of Biology, Bio 302 Introduction to Neuroscience Bio 542 Histology (Lab) Bio 304 Principles of Genetics (Lab) Bio 155 Lab for Intro Biology I Bio 151 Principles of Biology Lab I Bio 153 Principles of Biology Lab II

Professional and Community Service

2015	*Human brain exhibit at Interactive Science Night*, Stonewall Elementary School, Lexington, KY *Poster Judge,* Showcase of Undergraduate Scholars, University of Kentucky
2014 - Present	*Team Leader*, Young Investigator Meeting, Boston, MA
2013	*Poster Judge for Biology Undergraduates,* Blue Grass Technical College, Lexington, KY *Science Fair Judge,* Ashland Elementary School, Lexington, KY