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学位論文題目	A step *forward* towards demystifying sleep physiology: Dominant screening of sleep and wake behavior in mice （フォワード・ジェネティクスで同定した新規睡眠制御遺伝子解明）

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論文の要旨
Abstract of thesis

Background

Sleep-wake cycle is an intricate process controlled by complex cascade of effectors. The homeostatic propensity to sleep increases as “sleep need” accumulates during wakeful state, driving animals to commence in sleep behavior. However, the molecular and cellular mechanism that facilitates such switch is largely unknown. Forward genetic screening based on a heritable phenotype is useful for searching the causal genetic change without requiring a specific working hypothesis.

Purpose

The ENU-driven mutagenesis screen by the Yanagisawa’s group is the world’s first unbiased (non-candidate gene-based) attempt to causally identify regulatory genes in the sleep physiology in mice. The advantages of sleep research using mice over fruit fly include reliable staging of three vigilance states, wakefulness, NREMS and REMS through EEG/EMG-based somnography analysis. Furthermore, the high similarity of the genome and brain

structure between mouse and human predicts a conserved mechanism regulating sleep/wakefulness. The applicant et al expected that findings in mice can be directly applied to human sleep physiology and pathology, and sought to understand the pathway that governs the sleep regulation with attempts to identify key factors in the regulatory cascade.

Materials and Methods

First, C57BL/6J G0 male mice were treated with ENU at 100 mg/kg body weight at weekly interval for three weeks. At age of 25-30 weeks, sperms were collected from the G0 male mice for in vitro fertilization with C57BL/6N female eggs to produce F1 offspring. A comprehensive set of sleep parameters is measured in the F1 male progeny through EEG/EMG-based somnography analysis. Each F1 showing perturbed sleep behavior was selected as founder for mutant pedigree and proceeded for N2 offspring production by either natural mating or in vitro fertilization with C57BL/6N female. The established mutant pedigree is, then, subjected to heritability test and genetic identification by linkage analysis and whole exome sequencing.

Result

ENU induces ~3000 mutations randomly across the genome per gamete in ENU-treated G0 mice. Since their progeny of F1 and N2 still carries hundreds of random mutations that may have inherited from the founder mice, the causality between the mapped gene mutation and observed phenotype must be shown. Traditionally, counter strains genetically very distant from the mutagenized strain were preferred because polymorphic markers were more readily available. However, the availability of the complete mouse genome sequences with genome-wide list of genetic markers between C57BL/6J (B6J) and C57BL/6N (B6N), together with the next-generation sequencing, has made use of such distant counter strains and fine-grained linkage analyses unnecessary. To evaluate the similarity in sleep and wake behavior between B6J and B6N, two strains against C57BL/10J were examined. Consistent with the genetic proximity, B6J and B6N showed similar sleep/wakefulness compared to B10J. Thus, based on their near-identical sleep/wake behavior, C57BL/6J and C57BL/6N are chosen as mutagenized and counter strains, respectively. The reproducibility of the sleep/wake parameters were also examined by recording the same animal twice in 4+ weeks interval. Among the parameters tested, the total time spent in the wake and non-rapid eye movement (NREMS), as well as the REMS episode duration, show sufficient reproducibility with small coefficients of variance, indicating that these parameters are most suitable for quantitative phenotype-driven screening. This is consistent with our previous finding that *Sik3*^{Sleepy} mutant and *Nalcn*^{Dreamless} mutant pedigrees were isolated based on total wake/NREM sleep time and REM sleep episode duration, respectively. Whole-exome sequencing combined with coarse linkage analyses can immediately provide a list of candidate gene mutations associated with sleep abnormality. Our simulations calculate the achievable LOD score as a function of the

phenotype strength and the numbers of mice examined. With the advances in genome sequencing capabilities and the nuclease-mediated targeted mutagenesis system, the major obstacles of time and cost for genetic validation is greatly reduced. The system successfully identified *Sleepy* and *Dreamless* pedigrees with abnormal sleep/wake behavior as reported by Funato *et al* in *Nature* (2016). Here, a pedigree with a mild decrease in total wake time linked to a mutation in the *Cacna1a* gene is presented as an example.

Discussion

Our screening at ~10,000 scales succeeded in finding 10 heritable loci and identified 3 causal gene mutations linked to sleep abnormalities. While low occurrence of sleep phenodeviant was expected, possibly due to redundant regulatory mechanisms in sleep/wakefulness and low penetrance of the phenotype, our screening output seems to be comparable to other trials where a single biological trait is screened. The identified genes in the study usually encode proteins with large molecular weights, probably because the cumulative frequency of ENU-induced mutation is proportional to the gene size; some of known sleep regulatory genes, such as the very compact orexin and orexin receptor genes, have not been detected in this screening. Also, for unknown reasons, the majority of our sleep phenodeviants has been long-sleep mutants. Phenotype-driven screening at such large scale, indeed, is labor-intensive and costly. It is not recommended for an attempt to furthering the knowledge of largely defined mechanisms since there is a high chance of identifying already known genes, as it was the case of our obesity screening. Also, in a dominant screening, a highly focused screen is recommend rather than trying to examine multiple biological traits in a workflow; after all, the most expensive step in the screening is phenotyping, not the production of mutagenized mice. It is difficult to draw estimation on further identification of candidate genes and whether/when saturation can be reached in a dominant screen. Nevertheless, more causal gene mutations in sleep/wake regulation is expected to be found by enlarging the scale and continuing the search. The screening strategy is easily modifiable for studying other physiological phenomena in mice with careful selection of the screening criteria and the biology to be studied.

審査の要旨

Abstract of assessment result

【批評 Review】

Sleep-wake cycle is an intricate process controlled by complex cascade of effectors. However, the molecular and cellular mechanism that facilitates such switch is largely unknown. The applicant described the optimized streamline of dominant screening of randomly mutagenized mice in sleep and wake behavior, which had led to successful identification of multiple mutations responsible for strong sleep/wake phenotype. By using the forward genetic screening based on a heritable phenotype, the applicant successfully identified more candidate genes,

including Cacna1a showing a mild decrease in total wake time resulting from a heterozygous mutation. In conclusion, the applicant demonstrated that their screening system is modifiable for application in any behavioral analysis other than sleep/wakefulness.

【最終試験の結果 Result】

The final examination committee conducted a meeting as a final examination on July 2nd, 2019. The applicant provided an overview of dissertation, addressed questions and comments raised during Q&A session. All of the committee members reached a final decision that the applicant has passed the final examination.

【結論 Conclusion】

Therefore, the final examination committee approved that the applicant is qualified to be awarded a Doctor of Philosophy in Human Biology.