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学位論文題目	Elucidation of the involvement of REM sleep in cerebral blood flow regulation and its molecular mechanism （レム睡眠による脳血流制御への関与およびその分子機構の解明）

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論文の内容の要旨
Abstract of thesis

Mammalian sleep comprises of two distinct stages, rapid eye movement (REM) sleep and non-REM (NREM) sleep. Although various changes during NREM sleep are considered to be regenerative function of sleep, much less is known about the physiological changes during REM sleep. Cerebral blood flow (CBF) is critical for maintenance of energy-dependent processes and clearance of metabolic byproducts generated by neuronal activity, and is regulated independently from peripheral circulation. Previous studies have reported CBF dynamics across wake/sleep cycles, however, the results remain ambiguous due to different techniques used and different blood vessels measured. Therefore, in this doctoral dissertation, the author describes a new method using two-photon microscopy imaging to directly measure the capillary CBF during REM and NREM sleep in mouse. She found that capillary CBF was largely elevated during REM sleep. She further investigated the molecule and signaling pathway underlying these changes and found that adenosine 2A receptor-mediated signaling was crucial for capillary CBF upsurge during REM sleep.

（目的 Purpose）

The function of REM sleep has not been fully elucidated. The author aims to investigate the role of REM sleep by focusing on the brain circulatory system. The author seeks to develop a new method that allows her to directly

measure the blood flow of capillaries, where the actual substance exchange between blood and neurons/glia takes place. Using this new technique, the author aims to provide a conclusion on CBF changes during sleep and its implication in the maintenance of health.

（対象と方法　Materials and Methods）

The author used adult C57BL/6J and adenosine 2A receptor knockout ($A_{2A}R$ KO) mice in this study. Chronic cranial windows were made and placed in a stereotaxic frame. The skull over the parietal cortex was thinned circularly by a dental drill and removed, then a 4 mm-diameter glad coverslip was placed on the dura and sealed with cyanoacrylate glue. Two EEG electrodes were implanted epidurally over the cortex and cerebellum and EMG electrodes were placed bilaterally into the nuchal muscles and fixed with soldered. Finally, a rectangular aluminum head plate was placed on the skull and attached with dental cement. Two photon microscopy imaging was performed using upright two-photon microscope (Axio Examiner Z1 and LSSM 780 NLO, Zeiss) to directly measure the movement of individual red blood cells within capillaries, which was marked by FITC-Dextran. For each capillary, repetitive single-line scans were conducted along 20-50 mm range for 500 times with 1.3 milliseconds intervals per line to record the velocity and flow of red blood cell (RBC) in XT line scan. Both REM and NREM sleep were determined by EEG and EMG, and XT scans were taken in each sleep/wake state typically 5 to 10 times. XT line scan images were analyzed by MATLAB-based algorithm. For disturbance of REM sleep, the flowerpot method was used. For analysis of $A_{2A}R$ KO, NaHCO3 and $A_{2A}R$ PAM-1 (adenosine 2A receptor positive allosteric modulator) were used.

（結果　Results）

The author was able to target capillaries in the cortex layer I and simultaneously monitored sleep stages by EEG and EMG. The author found that capillary CBF upsurged during REM sleep, but showed no significant difference between wakefulness and NREM sleep. Capillary CBF slowly rose after entering REM sleep and slowly declined after waking up from REM sleep. The author also observed that there was larger elevation in capillary RBC flow during the rebound REM sleep after REM sleep disturbance. Since capillary CBF is elevated during REM sleep, the author predicted that adenosine might be involved in this change because 1) adenosine is known to be released by neurons and glia and acts as a vasodilator via $A_{2A}R$, and 2) adenosine is a target of caffeine in sleep inhibition. The author first examined the sleep architecture in $A_{2A}R$ KO mice and showed that they tended to have shorter REM sleep duration. Although there were no significant differences in total time spent in wakefulness, NREM sleep, and REM sleep compared to wild-type mice, time spent in REM sleep was significantly increased during the light phase in $A_{2A}R$ KO. The author next tested the effect of loss of $A_{2A}R$ on capillary CBF upsurge using $A_{2A}R$ KO. Capillary CBF response to NaHCO3 was similar between WT and $A_{2A}R$ KO, indicating that vascular responses were comparable between the two. The author finally tested whether the CBF during REM sleep is affected. Considering that $A_{2A}R$ KO exhibit shorter REM sleep duration, the author carefully picked REM sleep episodes of similar durations between WT and $A_{2A}R$ KO mice. As a result, the author showed that $A_{2A}R$ KO exhibited impaired upsurge of CBF during REM sleep.

（考察　Discussion）

The author showed that capillary CBF was drastically increased during REM sleep in all three cortical areas analyzed. This finding differs from the previous observation made by $H^2\ ^{15}O$-PET performed in humans, which showed the

highest CBF in wakefulness in many cortical regions, whereas the data is consistent with that of ultrasound imaging performed in rats. These differences are likely due to the data processing and normalization procedures, as well as the type of blood vessels observed. The advantage of the author's method is that it is subjected to minimal normalization procedures, and it detects blood flow from capillaries, where the actual substance exchange occurs. The author also demonstrated that the $A_{2A}R$ is crucial for the dramatic elevation of capillary CBF during REM sleep. In contrast, it is dispensable for maintaining capillary CBF during active wakefulness or NREM sleep.

The advantage of the author's approach is that it could detect changes in both capillary RBC velocity and flow. RBC flow is determined by RBC velocity and density, and the current results indicate REM sleep pressure enhanced RBC flow but not its velocity. However, it is not clear what the biological function of RBC velocity is. Also, it is not clear what is the significance of CBF elevation during REM sleep. CBF elevation may be important for a sufficient exchange of substances between the brain and the blood.

審査の結果の要旨
Abstract of assessment result

（批評　General Comments）

The applicant successfully established the method to directly analyze capillary RBC velocity and flow in the brain cortex of unanesthetized mice during the sleep/wake cycle by using two-photon microscopy. The applicant was able to show that capillary CBF was significantly elevated during REM sleep, while the CBF was comparable between wakefulness and non-REM sleep. The applicant also identified that $A_{2A}R$ was critical for CBF regulation during REM sleep but not required for CBF regulation in other sleep/wake stages. These observations may suggest that REM sleep acts as a drainage force for preventing capillary occlusion or involving meningeal lymphatics. The applicant's research provided new insight into sleep biology and a useful tool to investigate the role of REM sleep in CBF regulation.

（最終試験の結果　Assessment）

The final examination committee conducted a meeting as a final examination on May 29, 2020. 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）

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