博士論文（要約）

The discovery of PKCγ as a novel sleep-regulating element and analysis of its phosphorylation signaling.

（新規睡眠制御因子としてのPKCγの発見とリン酸化シグナリングの解析）

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Sleep-wake is a phenomenon that is widely conserved across species and plays a vital role in animal's survival. Sleep state is defined as several features such as (1) a prolonged period of immobility that results in a specific posture for sleep, (2) an increase in the arousal threshold to elicit a response from the animal during sleep, and (3) a homeostatic mechanism to keep sleep time per day constant, such as prolonged sleep duration after sleep deprivation [Campbell and Tobler 1984]. Wakefulness is characterized behaviorally by consciousness , voluntary motor activation, and high responsiveness to environmental stimuli [Alexangre et al., 2013]. Because either of the states need to be sustained for a certain period of time, stabilization of these states is fundamentally important. For example, certain continuous period of sleep is necessary to efficiently reduce sleepiness [Stepanski et al., 1984] and consolidate a memory [Djonlagic et al., 2012]. On the other hand, sustained wakefulness is an essential for eating, mating, and defending themselves from predators. The clinical findings also emphasize the importance of sleep-wake state stabilization. Patients with narcolepsy are characterized by the loss of such state stabilization; they show the inability to maintain wakefulness and the nocturnal awaking [Gelineau et al., 1880].

Multiple molecular mechanisms are known as sleep-wake regulators. In particular, several kinases, including PKA [Joiner, Crocker et al., 2006], ERK (p38, a member of mitogen activated protein kinase family) [Vanderheyden et al., 2013; Mikhail et al., 2017], CaMKII α / β [Tatsuki et al., 2016], SIK3 [Funato et al., 2016] and protein kinase C (PKC) [Li et al., 2015], are candidate molecules for important sleep and wake regulators. Among them, PKC family molecules have unique features that seems to be related to sleep and wake states. They are enriched in post-synaptic density during wake phase [Diering et al.,2017], and dynamically phosphorylated in anti-phase with a sleep-promoting kinase CamKII α [Bruning et al., 2019]. In this sense, PKC family might be involved in the wake state regulation. PKC consists of ten isoenzymes encoded by nine genes and is divided into three subfamilies based on co-factor dependency [Callender and Newton, 2017]. These PKC isoenzymes play a diverse role. For example, PKC γ is involved in eliminating climbing fiber-Purkinje cell synapse in the cerebellum [Kano et al., 1995]. PKC γ has also been implicated in neuropathic pain in the dorsal horn [Malmberg et al., 1997]. However, the functions of these PKC isozymes in sleep and wake state regulation has not been fully investigated.

To unravel the role of these PKC isozymes in sleep and wakefulness, I begun by analyzing sleep-wake phenotypes of bi-allelic KO mice for each of them, which were produced by triple-CRISPR technology [Sunagawa et al., 2013, Tatsuki et al., 2016]. In this study, I used the Snappy Sleep Stager (SSS) which enables the non-invasive phenotyping of sleep/wake states by measuring the respiration of mice [Sunagawa et al., 2016]. SSS can also quantify the sleep duration as Pws (= the transition probability from wake to sleep) and Psw

(= the transition probability from sleep to wake). Notably, I evaluated Pws and Psw as indicators for the stability of wake and sleep states, respectively.

Among the bi-allelic KO mice, Prkcg (the gene for PKCγ) knockout caused a significant increase in sleep duration (903.7 ± 37.8 min as mean ± SEM, n = 10), which was 179.0 min longer than that of wild-type (WT) mice ($p < 0.001$). This long sleep phenotype was attributed to increased Pws (i.e, increased frequency of state transitions from wake to sleep), implying that the wake state of Prkcg KO mice was destabilized. Next, I performed a series of whole-brain over-expression (OX) experiments of PKCγ mutants using a latest adeno-associated virus (AAV) serotype (AAV-PHP.eB [Deverman et al., 2016]). I found that when a gain-of-function mutant of PKCγ (PKCγ-A24E) was over-expressed in the mouse brain, significantly shorter sleep duration (611.4 ± 11.0 min as mean ± SEM, n = 6), which was 101.8 min shorter than that of PKCγ-WT-overexpressing mice ($p < 0.001$), was observed. Notably, PKCγ-A24E-overexpressing mice had significantly lower Pws (i.e, decreased frequency of state transitions from wake to sleep) than PKC-WT-overexpressing mice, indicating that the expression of PKCγ-A24E stabilized wakefulness. In addition, a kinase-dead mutant of PKCγ (PKCγ-A24E/K380R) totally canceled the longer wakefulness phenotype. Taken together, these results suggest that kinase activity of PKCγ is important for stabilizing wakefulness.

To investigate the effect of phosphorylation status of PKCγ on sleep/wake phenotype, I performed comprehensive phospho-mimetic mutants screening of PKCγ in vitro and in vivo. I developed the mobility shift assay system to evaluate the kinase activities of each phospho-mimetic mutants in vitro. On the other hand, I screened the change of sleep-wake phenotypes by AAV-based phospho-mimetic mutant OX in vivo. In in vitro experiments, I found that phospho-mimetic mutants on C2 domain (S192E, T208E, T212E, T214E, S251E, S349E and S352E) showed increased kinase activities compared to PKCγ-WT, suggesting that PKCγ's kinase activity might be regulated by the phosphorylation status on these sites. On the other hand, I found that mice overexpressing PKCγ-T39E and PKCγ-S664E in vivo had significantly shorter sleep times (612.1 ± 10.2 min/day and 612.8 ± 16.0 min/day, respectively) compared to the levels of PKCγ-WT mice. However, the kinase activity of PKCγ-T39E and PKCγ-S664E did not alter in the in vitro experiments. This discrepancy might be attributable to the problem that the artificial substrate used in the in vitro study is not physiological peptide, and to the difficulties of controlling intracellular localization and cell-type specific expression of PKCγ. Nevertheless, I finally concluded that the effect of PKCγ on the wake state can be regulated by its phosphorylation status via potential alteration of its kinase activity.

Finally, to investigate the downstream phosphorylation targets of PKCγ in the wake

state regulation, I performed a phospho-proteomics analysis to elucidate altered phosphorylated peptides in the brain of WT mice, Prkcg KO mice. I assumed that the phosphorylation on the PKCγ substrates would be down-regulated by KO of Prkcg gene. I found that long-term potentiation (LTP)-related proteins, the dopamine signaling and calcium signaling-related proteins, such as CACNA1A, CACNAB4, and CaMK2α were down-regulated in Prkcg KO mice. Therefore, the functional modification of LTP pathway and dopamine/calcium signaling by PKCγ may play an important role in stabilizing wakefulness. We also conducted a preliminary experiment to search for peptides up-regulated by PKCγ-A24E overexpression. Further validation is needed to conclude this result, but it has been suggested that PKCγ targets functional proteins in neurons.

Taken together, these results suggest that PKCγ may have a role in wake state stabilization through its phosphorylation status and downstream dopamine or calcium signaling. In this scenario, the phosphorylation on T39 and S664 may potentially modulate the kinase activity of PKCγ in vivo, although I have not yet acquired the direct evidence for the hypothesis in in vitro kinase assay. In conclusion, I revealed that the novel molecular mechanism for stabilizing wakefulness through PKCγ phosphorylation signaling in this thesis.