Poster Session

The 11th Annual WPI-IIIS Symposium

～Deciphering the Mysteries of Instinctive Behaviors～

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Poster Presentation	**14:55 – 16:00**

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1

Synaptic plasticity in prefrontal cortex regulates sleep

Yusuke Iino[1], Takeshi Sawada[1,2], Kensuke Yoshida[1,2,3], Chika Shimizu[1], Motoki Juichi[1], Masashi Yanagisawa[1], Takeshi Sakurai[1], Haruo Kasai[2], Shoi Shi[1,2]

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The synaptic strength in the cerebral cortex has been shown to fluctuate according to the sleep-wake cycle. Several pieces of empirical evidence show that synapses are primarily potentiated during wakefulness and subsequently downregulated during sleep. However, the causal relationship between synaptic potentiation and sleep has remained elusive. In the present study, we developed novel molecular tools to induce structural long-term potentiation and depression (sLTP and sLTD). By utilizing a chemical inducer of dimerization, we confirmed that specific effector molecules could be accumulated in dendritic spines. Accumulation of molecule X or Y in dendritic spines resulted in sLTP or sLTD, respectively, in dissociated cortical neurons. By using the two photon imaging, we verified that this manipulation induced sLTP in cortical neurons in vivo. Using this tool, we found that sLTP in the excitatory neurons of the medial prefrontal cortex, but not in the inhibitory neurons, causally increased non-rapid eye movement (NREM) sleep in mice. Conversely, synaptic potentiation in excitatory neurons in the hippocampus decreased NREM sleep, suggesting a circuit-dependent regulation of NREM sleep by synaptic potentiation. This study provides evidence for a causal link between synaptic potentiation and the regulation of NREM sleep.

2

Both ablation and activation of POA cause sleep fragmentation

Ayako Imamura[1], Koichi Miyatake[2], Akiko Ihara[2], Sakiko Honjoh[2]

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Sleep and wakefulness are controlled by specific brain regions. These regions have been identified by classical elimination of brain regions or by acute manipulation of neural activity. However, acute manipulation of these regions often has only a temporary effect on the sleep-wake cycles and chronic inhibition of neural activity in these areas has little effect. These may have been an adaptation and compensation by other brain regions.
The preoptic area (POA) has long been recognized as the sleep center first proposed by von Economo. However, a recent optogenetic study found that photostimulation of POA^{GAL} neurons at 10 Hz failed to increase sleep and surprisingly increased wake. These results call into question whether POA neurons promote sleep.
In this study, we investigated the effects of brain region-specific cell ablation and chemogenetic activation in the POA on the sleep-wake cycle. In this ablation method, we injected taCasp3 AAV, which overexpresses procaspase-3 in a Cre-dependent manner and induces apoptosis, in the POA-specific to avoid the effects of passing axons and ablate the cells in the brain region.
The cell ablation results showed the fragmentation of the sleep-wake cycle and a decrease in sleep duration. This effect was sustained for more than four weeks, and no compensation by other brain regions was observed. Next, chemogenetic activation of POA by intraperitoneal injection of CNO (Clozapine-N-Oxide) surprisingly observed the fragmentation of the sleep-wake cycle. These results suggest that the POA may not be simply a sleep-promoting region, as previously reported.

3

Regulation of Sleep and Wakefulness by Neuronal Circuits linking Ventrolateral Preoptic Nucleus and Lateral Hypothalamic Area

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Uncovering of connectivity and functional relationships between the preoptic area (POA) and lateral hypothalamic area (LHA) is crucial for comprehensive mechanistic understanding of regulation of sleep and wakefulness. GABA- and galanin (GAL)-producing neurons in the ventrolateral preoptic nucleus (VLPO) of the POA are known to participate in promotion of sleep, while orexin (hypocretin)-producing neurons (orexin neurons) in the LHA play a vital role in maintenance of wakefulness. Using retrograde rabies virus-mediated tracing combined with *in situ* hybridization, we found that vesicular GABA transporter (*Vgat*)- and galanin (*Gal*)-expressing neurons in the VLPO make monosynaptic inputs to orexin neurons in the LHA.

Notably, over half (56.3±8%) of the VLPO inputs were *Vgat*- and *Gal*-double-positive. Next, we examined input neurons of the GABA- and GAL-producing neurons in the VLPO projecting to the LHA and determined that the POA and LHA contained the biggest numbers of inputs. Among other upstream brain areas were the nucleus accumbens, bed nucleus of stria terminalis, and wake-promoting regions, such as the tuberomammillary nucleus. Further, using multidimensional scaling and Spearman correlation analyses of input neurons, we suggested that the GABA- and GAL-producing VLPO neurons projecting to the LHA compose a common neuronal population. We next found that optogenetic stimulation of the $VLPO^{GABA}$→LHA pathway induced short bouts of wakefulness, rather than impacted sleep. This study reveals multilevel connectivity of the VLPO and LHA and reports a new function of the $VLPO^{GABA}$→LHA pathway in regulation of sleep and wakefulness.

4

GABAergic neurons in the BNST projecting to the DpMe induce immediate transition from NREM sleep to arousal

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Negative emotions like fear and anxiety can arise in threatened individuals, resulting in hyper arousal and increased blood flow, allowing them to escape and minimize risks. Growing evidence shows the effects of sleep on the regulation of emotional brain activity. Sleep disturbance is frequent in patients suffering from psychiatric stress disorders, such as depression, social anxiety disorder (SAD) or post-traumatic stress disorder (PTSD), who are unable to regulate their negative emotions. The bed nucleus of stria terminalis (BNST) is a crucial region of interest regarding human stress-related psychiatric disorders, including PTSD. Our group previously demonstrated that the optogenetic excitation of GABAergic neurons in the BNST ($GABA^{BNST}$) triggered an immediate transition from NREM sleep to wakefulness. Here, we describe the neuronal pathway responsible for this transition. We found dense innervation from $GABA^{BNST}$ neurons to the deep mesencephalic nucleus (DpMe), one of the arousal regulating centers, mainly populated by excitatory glutamatergic neurons ($GLUT^{DpMe}$). We demonstrate that optogenetic stimulation of $GLUT^{DpMe}$ neurons results in immediate transition from NREM sleep to wakefulness. Retrograde neuronal circuit tracing with a modified rabies virus vector demonstrates neuronal connectivity between the BNST and DpMe. Last, we hypothesize that some GABAergic interneurons in the DpMe ($GABA^{DpMe}$) might inhibit $GLUT^{DpMe}$ neurons and receive inhibitory projections from $GABA^{BNST}$ neurons. In conclusion, we could decipher the neuronal circuit responsible for the promotion of wakefulness between the BNST and the DpMe. Our data could provide precious insight to improve sleep disorders in people with stress-related sleep disorder.

5

Gastrin Releasing Peptide (GRP)-producing Neurons in the Hypothalamic Suprachiasmatic Nucleus (SCN) Mediates Photic Entrainment

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Most animals display fluctuations in physiological phenomena that are around 24 hours (circadian rhythms). In mammals, the hypothalamic suprachiasmatic nucleus (SCN) is the master clock that generates and regulates the circadian rhythms. SCN contains different subtypes of neurons that produce distinct neurotransmitters, such as vasoactive intestinal peptide (VIP), arginine vasopressin (AVP), and gastrin releasing peptide (GRP). Compared to the other subtypes, the GRP-producing neurons are scarce in numbers and the roles they play in circadian regulation is unclear. Therefore, we used newly generated transgenic mice, the *Grp-iCre* knock-in (KI) mice, and virus vectors coding target genes that are expressed Cre-dependently, to specifically manipulate the SCN GRP-producing neurons. When we deleted the SCN GRP-producing neurons by specifically expressing caspase, an enzyme that causes cell death, in the GRP-producing neurons, we discovered that the mice's behavioral rhythms were not significantly affected. But when we subjected these mice to jetlag, where the light onset was advanced for 6 hours, we discovered that the SCN GRP-producing neurons-deleted mice displayed significantly slower entrainment to the new light-dark cycle. We concluded that although SCN GRP-producing neurons may not be essential for circadian rhythms generation, they may play an important role in mediating photic

entrainment.

6

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24 hours QIH induction by OPN4-based optogenetics

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Optogenetics has been used for neuronal manipulation at a high temporal resolution to understand the neuronal mechanisms of many physiologies. Current tools have been reported to be effective for short-term manipulation (seconds to minutes), although it has been challenging for continuous stimulation of neurons on much longer time scale (hours to days) to induce long-term behavioral and physiological responses such as hibernation. In this study, we aimed to establish a method allowing stable 24-hour neuronal manipulation with high reproducibility by using OPN4 (melanopsin), which is a GPCR(Gq)-type photosensor with high-photosensitivity. We recently found that excitatory manipulations of *Qrfp*-expressing neurons in the preoptic area of the hypothalamus (Q neurons) induced a hibernation-like hypothermic/hypometabolic state (QIH) in mice. To control QIH with higher time resolution, we developed an optogenetic method using modified OPN4. The engineered-OPN4 stably and reproducibly induced QIH for at least 24 hours by illuminating low-power light (3 μW, 473 nm laser) with high temporal resolution. The optogenetic method would enable us to identify neural mechanisms underlying long-term dormancy states such as sleep, daily torpor and hibernation.

7

Male sexual behaviors are regulated by dopaminergic signaling in NAc in mice

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Male mice are known to exhibit sequential sexual behaviours such as sniffing, mounting, intromission and ejaculation. However, the neural mechanisms that regulate and coordinate these behaviours have remained elusive. Based on our observations of GRAB-DA dynamics in the nucleus accumbens (NAc), we found that there are several subregions in the NAc. Specifically, the ventral shell region (NAc-vs) shows elevated DA levels during intromission, while the medial shell region (NAc-ms) displays diminished DA levels during intromission. To identify the inputs to these subregions of the NAc, we employed two-colour cholera toxin B subunit (CTB)-mediated retrograde labeling to trace neuronal projections. We found that the NAc-vs receives input from the antero-lateral aspect of the ventral tegmental area (alVTA), while the NAc-ms receives innervation from the postero-medial aspect of the VTA (pmVTA). To identify the neuronal activity of the alVTA and pmVTA, we monitored the GCaMP dynamics of dopaminergic neurons in various midbrain regions. We observed that GCaMP fluorescence recorded from the alVTA were augmented during intromission, while those from the pmVTA were decreased during this phase. Thus, the neuronal activity of the VTA subregions projecting to the distinct subregions of the NAc corresponded to the DA release pattern. By utilizing optogenetic manipulation of the dopaminergic terminals in the NAc, we found that the behavioral expression pattern of sexual behavior in male mice was altered. These findings suggest that DA dynamics within the NAc are critical for regulating sexual behavior in male mice.

8

Ventral tegmental area dopaminergic neurons mediate sleep reduction induced by ablation of the surrounding GABAergic neurons

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It is widely accepted that sleep is controlled by two processes, a homeostatic process and a circadian process. However, sleep is also modulated by external stimuli such as motivational stimuli. Dopaminergic neurons in the ventral tegmental area (VTA) not only play a central role in processing motivational stimuli,

but also regulate sleep. Recently, GABAergic neurons around the VTA were also revealed to regulate sleep. Chronic lesions of GABAergic neurons around the VTA (ventromedial midbrain/pons area: VMP) cause severe daily sleep loss, yet the mechanism was unclear. In this study, we examined the role of VTA dopaminergic neurons (VTA^{DA}) in the severe sleep loss caused by lesions of VMP GABAergic neurons (VMP^{GABA}). To this end, we ablated VTA^{DA} in mice with VMP^{GABA} lesions and measured their daily sleep. As a result, VTA^{DA} lesions attenuated the daily sleep loss in the light period in mice with VMP^{GABA} lesions. Furthermore, the analysis of sleep architecture suggested that the attenuation of the sleep loss occurred via the increased sleep episode number. In conclusion, this study revealed that VTA^{DA} mediate the severe daily sleep loss caused by chronic lesions of GABAergic neurons around the VTA.

9

Optochemical control of slow-wave sleep in the nucleus accumbens by a photoactivatable allosteric modulator of adenosine A2A receptors

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Optopharmacology, an emerging approach in pharmacology that involves the activation or deactivation of molecules with light for targeted drug delivery, may ease symptoms, cure diseases, and improve quality of life while preventing uncontrolled drug action. In-vivo use of optopharmacology to render neurons or glia photoresponsive without the need to rely on genetic engineering has been slow to develop mainly because of the inaccessibility of the brain to light irradiation and inadequate delivery of photoactivatable compounds through the blood-brain barrier (BBB). The nucleus accumbens (NAc) is a novel region for slow-wave sleep (SWS) regulation by integrating motivational stimuli. Adenosine is a possible candidate molecule for activating NAc indirect pathway neurons that express adenosine A_{2A} receptors (A_{2A}Rs) to trigger SWS but direct proof is lacking. Here, we developed a BBB-permeable, visible-light (λ > 400 nm) photoactivatable positive allosteric modulator of A_{2A}Rs (A_{2A}R PAM) and used it to optochemically induce SWS in the NAc of freely behaving mice. By contrast, optochemical activation of A_{2A}Rs has no effect on the ventrolateral preoptic area, a sleep center where A_{2A}Rs are thought to promote sleep. Our approach to A_{2A}R optopharmacology should help in the generation of photoactivatable compounds for virtually any druggable target.

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Development of photocaged drugs targeting adenosine 2A receptor

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Adenosine (Ad) is an endogenous sleep-inducing molecule that strongly promotes sleep through activation of adenosine 2A receptors (A_{2A}R) in nucleus accumbens (NAc). Increased accumulation of Ad during wakefulness induces sleep, but the spatiotemporal mechanism of adenosinergic regulation in NAc remains unclear. Photocaged drugs are known as photo-pharmacological tools to control the activity of target receptors by light irradiation, but photocaged drugs using A_{2A}R agonists have problems in differentiation from endogenous effects due to background activity. Our group recently found a positive allosteric modulator (PAM) YNT378 that enhances the sensitivity of A_{2A}R to endogenous Ad and reported that it induces slow-wave sleep in mice. In this study, we designed and synthesized novel photocaged A_{2A}R PAMs based on the structure of YNT378 to elucidate the spatiotemporal mechanism of A_{2A}R activity.
As the first generation photocaged YNT378 with a 6-nitroberatryl (Nv) photoremovable protecting group (PPG) showed poor water solubility, slow photoreaction rate, and short reaction wavelength, we designed a new water-soluble PPG, A400, based on 3-arylcoumarin, and synthesized A400-YNT378. A400-YNT378 showed a good water-solubility and rapid uncaging reaction at 420 nm. In this presentation, we will report the details of the photochemical and pharmacological properties of these photocaged drug.

11

Design and synthesis of novel κ opioid receptor agonists with bicyclo [2.2.2] octane skeleton

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κ opioid receptor (KOR) agonists have been expected as analgesics without serious side effects derived from the activation of μ opioid receptors (MOR) such as addiction, sleepiness, nausea, and vomiting. Nalfurafine, the first selective KOR agonist, succeeded in affording strong analgesic and antipruritic effects without addiction, while, during its clinical trials for postoperative pain, nalfurafine showed a severe sedative effect at the same dose as an antinociceptive effect. Therefore, the development of new KOR agonists without sedative effect has been required.
During the course of drug discovery research based on active conformational analysis of nalfurafine, we recently discovered that YNT-1612 with a bicyclo [2.2.2] octene scaffold, which enables the side chain orientation toward the active conformation of nalfurafine, exhibits potent antinociceptive activity without a sedative effect. In this study, to elucidate the mechanism of side-effect segregation, we conducted the structure-activity relationship (SAR) study between the orientation of amide side chains and analgesic/sedative effects.
Bicyclo [2.2.2] octane derivatives YNT-1623 and YNT-1624 having different amide side chain orientations were designed, synthesized, and evaluated for pharmacological activity. The results showed that both YNT-1623 and YNT-1624 exhibited strong analgesic effects, whereas YNT-1624 showed no sedative effects, while YNT-1623 was significantly more sedative. These results provide important information indicating that the orientation of the amide side chain contributes to the separation of sedative effects, which will help elucidate the mechanism of side-effect isolation in the future.

12

Analysis of orexinergic function in chronic pain-like states

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Orexins neurons modulate pain sensitivity. However, it is unclear how orexin neurons process afferent pain information and produce analgesic effects in the central nervous system. In this study, to reveal this mechanism, we analyzed the neural pathways of pain regulation by orexin neurons using mice with chronic inflammatory pain induced by complete Freund's adjuvant (CFA).
We first demonstrated that CFA markedly increases c-fos-positive activated orexin neurons in the lateral hypothalamus of mice. To evaluate the effects of activated orexin neurons on pain, we activated orexin neurons chemogenetically and CFA-induced hypersensitivity was suppressed. These effects were recovered by intracerebroventricular administration of orexin 1 receptor (OX1R) antagonist. Next, to reveal the downstream of orexinergic neurons regulating pain, we focused on noradrenergic (NA) neurons in the locus coeruleus (LC), which dominantly express OX1R and play an important role in pain regulation. A retrograde tracer injected into the LC was transported to c-fos-positive orexin neurons. The analgesic effects by chemogenetic activation of orexin neurons were significantly suppressed by injection of NA receptor antagonist into the spinal cord, which is innervated by NA neurons in the LC. We also found that a novel selective OX1R agonist, originally developed in house, induced analgesic effects. These results suggest that orexin neurons are activated by afferent pain information and then suppress their transmission at the spinal cord through the activation of OX1R on NA neurons in the LC, and selective OX1R agonist may be a new type of analgesics.

13

Orexin receptor antagonist modulates sleeping energy metabolism without overt effects on sleep architecture

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Suvorexant, approved orexin receptor antagonist, targets the orexin-mediated wake-promoting system and offers an alternative mechanistic approach to treating insomnia. There is a crosstalk between regulators of energy metabolism and control of sleep / wakefulness. A molecule, such as orexin, acts on regulation of energy metabolism and sleep. Sleeping energy metabolism is related to sleep architecture, and orexin receptor antagonist may affect energy metabolism as well as sleep. Effect of suvorexant on sleeping energy metabolism in human is not known, while stimulating effects of orexin system on energy expenditure was reported in animal studies. However, effect of suvorexant on oxidized substrate is inconsistent among the animal studies.
In this placebo-controlled, double-blind, crossover intervention study, metabolic rate of 14 healthy young male without sleep problems was measured using whole-room indirect calorimetry, during which polysomnographic recording of sleep was made. The participants consumed suvorexant (20 mg) or placebo 10 minute before bedtime.
Fat oxidation was increased but protein catabolism was suppressed without changes in energy expenditure by suvorexant during sleep. Interestingly, sleep architecture was not notably affected by suvorexant. The only difference in sleep architecture between 2 conditions was observed in NREM stage 1. The present study suggested that the effect of suvorexant on energy metabolism was not mediated by major changes in sleep architecture.

14

Does different-intensity exercise affect the rest-activity rhythms among older adults?

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Sleep disturbances and poor rest-activity rhythms might reduce the quality of life for older adults. Whether exercise can affect circadian rhythms remain unknown. This randomized controlled trial investigated the effects of a home-based aerobic exercise intervention for 12 weeks in the evening on 54 older adults with lower sleep quality (PSQI $\geqq$ 6 points). Participants were randomly allocated to a low-intensity exercise group (60-70 steps per minute), a moderate-intensity exercise group (110-120 steps per minute), or a control group. Each intensity exercise group was performed for 30-min daily at home in the evening (18:00 to bedtime). Outcomes included the nonparametric approach actigraphy data of 24/7 (inter-daily stability, IS; intra-daily variability, IV; relative amplitude, RA; nocturnal activity, L5; daily activity, M10; L5 and M10 start times; the midpoint of L5 and M10 start times).
There were no significant differences in the IS, IV, RA, L5, and M10. The moderate-intensity exercise group showed a delay in L5 start time (23.8, 24,3, and 24.4 hours; P for trends = 0.002), and the midpoint of L5 and M10 start times (4.1, 4.5, and 4.6 hours; P for trends = 0.004) during the intervention. However, the low-intensity exercise group and control group did not change during the intervention.
Moderate-intensity exercise in the evening leads to delay rest-activity rhythms among older adults with lower sleep quality. Thus, it is necessary to consider the timing and intensity of exercise when suggesting exercise therapy (i.e., combination with CBT-I) to older adults with sleep disorders.

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Automatic Sleep Analysis for Individuals with Disordered Sleep Patterns

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Sleep stage scoring is the basis of sleep testing. Scored stages based on biological signals and other data are classified into five stages and used for sleep diagnosis. This scoring process used to be performed manually by trained technicians, but due to the rapid development of information technology in recent years, automated scoring methods using machine learning has been

studied actively. However, most of these methods have targeted only to healthy people, and there has not been sufficient progress for patients with sleep disorders. This is one reason why automatic scoring method has not been widely used in actual clinical practice. In this study, we develop a sleep stage scoring method for people who have sleep problems. In our experiment, we trained a classification model for sleep stage scoring with a dataset that contains biological signals taken from both healthy people and patients with sleep-disordered breathing, one of the most common sleep disorders. We found that the model's accuracy drastically dropped for the duration where apnea and hypopnea events occur. Then, we developed separate models dedicated to apnea/hypopnea duration and normal duration and applied them in a hybrid way. The results suggest that it is effective to apply the hybrid model to improve overall accuracy. We are planning to take a similar approach to sleep analysis for various sleep patterns in the future.

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Assessing Wakefulness Intensity as a counterpart to Sleep Depth During the Sleep-Wake Cycle

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NREM sleep is associated with low frequency activity, which is conventionally assessed using EEG delta power, whereas wakefulness is associated with higher frequency activity, which is diffusely distributed over the EEG beta and gamma bands. EEG spectral power is organized according to a 1/f-like law, implying that the power of the frequencies identifying wakefulness is negligible in comparison to the power of the delta band. As a result, while EEG spectral analysis provides clear, easy-to-measure information that can be directly associated with the dynamics of sleep homeostasis, there is no equivalent for wakefulness. However, novel methods for extracting the arrhythmic components of EEG provide robust features specific to wakefulness for the first time. Using this information, a variable indicating the intensity of wakefulness can be defined, which serves as a counterpart to delta power-measured sleep depth. The dynamics of the wakefulness-intensity variable during the sleep-wake cycle in WT mice and the mouse sleep mutants Sleepy and Dreamless are investigated in this preliminary report. This variable anticorrelates with delta power and is highly correlated with the likelihood of being awake strongly supporting its interpretation as the opposite of sleep depth. Sleepy and Dreamless have subnormal and supranormal levels of the wakefulness-intensity variable respectively.

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Binaural beats at slow frequencies less than 1 Hz shorten the latency of slow-wave sleep

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Binaural beats are an auditory phenomenon in which listeners hear a single tone equal to the frequency difference between two tones transmitted to two ears separately and simultaneously. Previous studies suggested binaural beats can entrain neural oscillations and induce related behavioral states. A previous study showed listening to a 1-Hz binaural beat during a nap increased the duration of slow-wave sleep. However, the effect of binaural beats in the range of slow oscillations ($<$ 1-Hz) on sleep is poorly understood. We hypothesized the 0.25-Hz beats could entrain neural oscillations and enhance slow-wave sleep by shortening the latency or increasing the duration. Twelve healthy participants (6 women, 25.3±2.6 years) were included in this study, approved by the research ethics committee. The experiment included four 90-minute afternoon nap sessions consisting of a sham condition (without acoustic stimulation) and three binaural beats conditions (0-Hz, 0.25-Hz, or 1-Hz) with a 250-Hz carrier tone. Participants were not informed of their conditions. The acoustic stimuli were delivered through earphones and continued for a 90-minute nap. Our results showed both the N2- and N3- latencies of the 0.25-Hz binaural beats condition were shorter than those in the sham condition. This study shows the potential capability of 0.25-Hz binaural beats for sleep induction in generally healthy populations.

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Targeted memory reactivation during REM sleep selectively enhances brain responses to unpleasant images

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Reactivation of emotional memories during REM sleep has been hypothesized to lead to memory consolidation and a decrease in emotional charge. However, previous studies relying on subjective measures or remaining under the influence of circadian rhythms have provided conflicting evidence for this hypothesis. To address this issue, the present study used the targeted memory reactivation (TMR) procedure to reactivate specific memories during sleep that are associated with a sensory stimulus during wakefulness. Additionally, this study used the late positive potential (LPP), a component of event-related brain potentials, time-locked to the presentation of affective images. Sixteen healthy adults participated in the study. EEGs were recorded while viewing unpleasant, neutral, or pleasant images (old images) with an odor stimulus. During subsequent REM sleep, the same odor was presented in the TMR condition, while an odorless stimulus was presented in the control condition. Upon awakening, they performed the same task as before sleep adding new images to test memory for the images. They also rated their mood before and after sleep. The results showed that TMR increased the LPP amplitude after sleep for unpleasant old images, but there were no changes in the LPP for unpleasant new images as well as neutral or pleasant images. In addition, there was a positive correlation between LPP amplitude for unpleasant old images and memory performance. Furthermore, negative mood was reduced after sleep in the TMR condition. These results suggest that TMR during REM sleep selectively enhances attention to previously encountered unpleasant stimuli while ameliorating negative affect.

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Exploration of a novel mechanism of sleep regulation that depends on feeding condition

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Our sleep comprises two states: rapid eye movement (REM) sleep and non-REM (NREM) sleep. The physiological function of REM sleep is poorly understood. Recent human studies showed that low amount of REM sleep is associated with a high risk of dementia (Pase et al., 2017) and increased mortality (Leary et al., 2020). Moreover, in mice, the cerebral blood flow increases during REM sleep (Tsai et al., 2021), suggesting that nutrition supply and the clearance of waste products occur actively during REM sleep. Although the causal relationships are unclear, these studies imply that REM sleep is important for maintenance of our health. However, currently it is difficult to reliably increase the REM sleep amount in humans. Here, we unexpectedly found that a certain type of feed can increase the REM sleep amount in mice. Switching to a normal feed reversed the REM sleep amount to normal levels. We also found that this feed condition-dependent change in sleep can be enhanced by the perturbation of certain neuronal circuits. Now, we are trying to elucidate the underlying mechanisms. This study contributes to understanding a novel regulatory system of sleep. Moreover, it might provide clues for developing methods to increase REM sleep amount in humans.

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Analysis of the mechanism of REM sleep with focus on Parkinson's disease

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During rapid eye movement (REM) sleep, the cerebral cortex is activated and produces vivid dreams. Yet, it usually does not lead to motor output owing to the expression of muscle atonia mechanisms. However, patients with REM sleep behavior disorder (RBD) exhibit impaired muscle atonia and frequently act out of

their dreams during REM sleep. Patients often wake up following such movements, which can lead to reduced sleep quality and excessive sleepiness or fatigue during the daytime. RBD is considered a prodrome of synucleinopathies; most RBD patients eventually develop synucleinopathies including Parkinson's disease and dementia with Lewy bodies within 10-14 years. In synucleinopathies, α-synuclein accumulates and causes nerve cell death, which leads to motor symptoms such as tremors, bradykinesia, and rigidity as well as non-motor symptoms including cognitive impairments and depression.
Here, we aimed to understand the mechanisms underlying the link between RBD and Parkinson's disease and establish an RBD mouse model for the future development of effective treatment for RBD. A rare G51D α-synuclein mutation is observed in familial Parkinson's disease. We produced and injected G51D α-synuclein fibrils into the brainstem pontine tegmental area in mice and examined the effects on muscle atonia during REM sleep, behavior, and motor symptoms across several months. Consequently, mice injected with α-synuclein fibrils exhibited impaired muscle atonia during REM sleep. Moreover, these mice exhibited motor impairment and propagation of phosphorylated α-synuclein to other brain areas. We expect this study will provide important insight into how RBD develops at the early stages of Parkinson's disease.

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Transient recruitment of adult-born neurons for fear memory consolidation in REM sleep

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Memory replay during rapid-eye-movement (REM) sleep is suggested to contribute to memory consolidation (Skaggs & McNaughton, Science). The dentate gyrus (DG) in the hippocampus hosts contextual fear memory trace (Liu et al., Nature) and adult-neurogenesis (Akers et al, Stem cells). Recently, we provided evidence that the activity of ABNs during REM sleep is necessary for contextual fear memory consolidation (Kumar et al., Neuron). However, the mechanism is not clear. Therefore, we hypothesize that re-activation of the ABNs, which represent the context to be associated with fear, during REM sleep is necessary for fear memory consolidation (Koyanagi et al., Neur Regen Res). In this study, we silenced the ABN activities representing the context during retrieval which did not impair the memory. However, silencing the ABNs during REM sleep impaired the memory. We also found a distinct dynamics of the ABN activity during REM sleep. Taken altogether, we propose that contextual fear memory consolidation relies on the transient recruitment of ABNs representing a specific context.

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CaliAli: neuronal tracing utilizing vasculature in calcium imaging

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Endoscopic calcium imaging allows monitoring the activity of neurons across days and weeks. Current algorithms to track neurons across sessions rely on the alignment of spatial footprints of neurons independently extracted from individual recording sessions. However, when neurons are intermittently detectable across sessions and misalignments are non-rigid, spatial footprints are commonly insufficient to correct misalignments and reliably track neurons. These issues are common in experiments involving unstable neural representation, such as global remapping or drifts of neural activities occurring through extensive periods. To address these issues, we developed CaliAli (Calcium imaging Inter-session Alignment): A tool that utilizes brain vasculature and spatial cues of neurons to track neurons across sessions automatically. Brain vasculature is used as a reference structure to track intermittently detectable neurons and to correct

misalignments. CaliAli uses an alignment-before-extraction approach, enabling the detection of weak calcium signals that would be otherwise undetected by analyzing isolated sessions. CaliAli outperforms state-of-the-art algorithms in conditions of high neuron overlap, sparsity, intermittent neuron detectability, and varying signal-to-noise ratios. This evidence suggests that CaliAli is a reliable tool for tracking neural activities.

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Sex differences in sleep/wakefulness from infancy to adulthood in a hypersomnia model, Sleepy mutant mouse

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It is known that there is a sex difference in sleep-wake behavior in adult mice. However, due to technical difficulties in recording of electroencephalogram and electromyogram (EEG/EMG)-based sleep-wake behavior in infant mice, sex differences in the developmental stages from infancy have not been investigated. Therefore, we established a method to record EEG/EMG every 4 days from postnatal day 21 immediately after weaning to postnatal day 57. Utilizing this methodology, we examined sex differences in the development of the hypersomnia phenotype observed in *Sik3_sleepy* mutant mice (*sleepy*). Furthermore, when EEG/EMG measurements were performed on *sleepy* and their wild-type littermates at 10–13 weeks and 30–33 weeks of age, females matured in sleep-wake behavior about 1 week earlier than that of male mice. We also found that prolong non-REM sleep time, observed in *sleepy* mutant mice, is more prominent in females than in males.

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Sleep disturbances in mice with a CDKL5 kinase-dead missense mutation, a novel mouse model of neurodevelopmental disorder

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Pathogenic variants in the Cyclin-dependent kinase like 5 (CDKL5) gene cause CDKL5 deficiency disorder (CDD), a severe neurodevelopmental disorder characterized by refractory early-onset epilepsy, intellectual disability, motor defects, cortical visual impairments, and autistic features. The majority (>86%) of patients have sleep problems such as difficulty falling asleep, sleep fragmentation, frequent awakening from sleep at night, night screaming and laughing, and excessive daytime sleepiness, which greatly impact the quality of life of patients and their families. However, sleep study using electroencephalography (EEG) and electromyography (EMG) recording in mouse models with CDD is limited. We have generated a novel mouse model of CDD, the *Cdkl5* kinase-dead K42R knock-in (*Cdkl5* K42R KI) mouse, which carries a mutation mimicking a human CDD lost-of-function missense mutation. In this study we have for the first time comprehensively analyzed sleep disturbances in both female and male *Cdkl5* K42R KI mice using EEG/EMG recording. The results show that *Cdkl5* K42R KI mice
recapitulate sleep disturbances observed in human CDD patients. Our study provides insights into better understanding of sleep disturbances in CDD and will facilitate identifying novel therapeutic strategies to treat sleep problems in CDD and neurodevelopmental disorder patients.

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NALCN in the forebrain and pons-medulla regions have distinct roles in REM sleep regulation

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Although REM sleep (REMS) is ubiquitous in mammals, the molecular/neural mechanism of REMS regulation remains unknown. We previously established the *Dreamless* mutant pedigree exhibiting abnormal EEG power spectra and ~50% reduction in REMS episode duration (Funato et al., *Nature* 2016). We identified an SNP specific to *Dreamless* mutant mice within the exon 9 of the *Nalcn* gene. The mutation leads to a single amino acid substitution (N315K) of the NALCN protein, a voltage-independent, non-selective leak cation channel. Introducing the same point mutation in wild-type mice through genome editing confirmed that the mutation was responsible for REMS abnormality, suggesting an important role of NALCN in REMS regulation. To elucidate the responsible brain regions and neuronal subtypes through which NALCN regulates REMS, we generated flox and FLEx (flip-excision) knock-in mice bearing Cre-dependent loss-of-function and gain-of-function *Nalcn* alleles, respectively. In *Nalcn*-FLEx mice, we confirmed that the mice crossed with systemic Cre-expressing lines phenocopied the *Dreamless* mice on EEG/EMG analyses. In *Nalcn*-flox mice, we confirmed a neuronal subtype-specific deletion of *Nalcn* mRNA in adult brain tissues. With *Foxg1*-IRES-Cre or *Emx1*-Cre lines, we observed that both gain-of- and loss-of-function mutants showed theta/delta dissonance during REMS. With *En1*-Cre, we observed fragmentation of REMS. It is suggested that NALCN in forebrain and pons-medulla regions has distinct roles for REM sleep regulation, and NALCN in excitatory cortical- and hippocampal neuron plays a key role for the maintenance of theta/delta oscillation during REMS.

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Sleep/wake behavior of mice lacking PKA-phosphorylation sites of SIK3

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SIK3 was identified as an important sleep regulator through a screening of randomly mutagenized mice. Moreover, mice lacking serine 551, the PKA phosphorylation site of SIK3, showed increased NREM sleep time and sleep need. These results suggest that the existence of PKA recognition sites, such as S551, is crucial for the normal sleep/wake regulation and maintenance of daily sleep need.
In addition to S551, there are two more PKA recognition sites, threonine 469 and serine 674. To examine whether the phosphorylation of T469 and S674 of SIK3 is also required for proper sleep/wake behavior, we generated mutant mice in which SIK3 T469 and SIK3 S674 were substituted by alanine.
Similar to *Sik3 S551A* mice, *Sik3 T469A* mice also show increased NREM sleep time and NREM sleep delta power, an index for sleep need. In contrast, *Sik3 S674A* mice show no change in NREM sleep time and NREM sleep delta power. Both SIK3 T469A and SIK3 S551A proteins showed reduced immunoreactivities for anti-phospho-S551 substrate and anti-phospho-T469 substrate. It can be assumed that phosphorylation of S551 and T469 may interact each other and synchronize. Moreover, SIK3 T469A and SIK3 S551A proteins have reduced binding to 14-3-3. Attenuation of the binding between SIK3 and 14-3-3 is consistent with a longer sleep phenotype.
This study indicated that the lacking PKA recognition site of SIK3, especially T469 and S551, is a gain of function and increased NREM sleep time and NREM sleep delta density. Also, SIK3 T469A and SIK3 S551A reduced the binding with 14-3-3 and it is thought to have a significant effect on sleep/wake behavior.

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Upstream and downstream pathways of SIK3 in the regulation of sleep and wakefulness

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Sleep behavior is conserved from vertebrates to invertebrates, and is regulated by the accumulation and dissipation of homeostatic sleep need. Despite its importance, the molecular and cellular basis of homeostatic sleep need remains unclear. We have recently revealed that salt-inducible kinase 3 (SIK3) in the brain plays a key role in this process (Funato et al., Nature, 2016, Kim et al., Nature, 2022). In this study, we aim to further explore the molecular and cellular basis of homeostatic sleep need by identifying upstream and downstream components of SIK3.

As a potential upstream regulator of SIK3, we focused on liver kinase B1 (LKB1). We generated postnatal neuron-specific LKB1 knockout (nKO) mice, and found that LKB1 nKO resulted in a significant decrease in EEG delta power during non-rapid eye movement sleep (NREMS), which is an indicator of sleep need. Furthermore, when we induced a constitutively active mutation of Sik3 (T221E) in LKB1 nKO mice, we observed a rescue of weaker NREMS EEG delta power. These results suggest that LKB1 regulates sleep/wake behavior by acting upstream of SIK3.

Downstream components of SIK3 have not been well investigated. To identify downstream signaling pathways of SIK3, we employed substrate screening and revealed that the RhoA-GTPase signaling pathway is strongly related to phosphoproteins identified in the screening. Furthermore, when expressing a constitutively active form of RhoA in the whole brain, NREMS EEG delta power during NREMS was decreased, suggesting that RhoA is also involved in sleep/wake regulation. Our studies provide novel insight into sleep/wake regulation.

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Molecular mechanisms for sleep/wake regulation by SIK3 kinase signaling

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Sleep amount within a day is tightly regulated by the homeostatic sleep need accumulating during wakefulness and dissipating during sleep. Although several studies suggests that the required sleep amount is under genetic control, the intracellular signaling pathway regulating sleep/wakefulness remains unknown. We recently identified Salt-inducible kinase 3 (Sik3) as a sleep promoting gene. The gain-of-function mutation (Slp) induces increased sleep need and amount. On the other hand, sleep deprivation specifically enhances the phosphorylation level at T221 in SIK3. Phosphorylation level at T221 in the SIK3 kinase domain modulates the kinase activity, suggesting that SIK3 kinase activity regulates sleep need.

Here, to examine the role of SIK3 kinase activity in sleep/wake regulation, we performed EEG/EMG-based sleep/wake analysis of mice carrying a T221A or T221E mutation in Sik3. Both Sik3T221A and Sik3T221E mice did not show any significant differences in sleep time. While Sik3T221A mice exhibited marked reduction of delta density during NREM sleep, which is a marker for sleep need, Sik3T221E mice also showed a tendency to decrease the delta density, which is consistent with SIK3(T221E) have a constitutive but less kinase activity. Moreover, we investigated the role of kinase activity of SLP mutant SIK3 in sleep/wake regulation. Sik3T221A-Slp mice exhibited the abolishment of hypersomnia phenotype of Sik3Slp mice. In contrast, Sik3T221E-Slp mice showed increased sleep time compared with Sik3T221E mice. These results suggest SIK3 kinase activity regulates sleep need and is crucial for the induction of increased sleep amount.

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SIK3-HDAC4 in the suprachiasmatic nucleus regulates the timing of arousal at the dark onset and circadian period in mice

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Sleep is a fundamental behavior conserved from vertebrates to invertebrates. Sleep/wakefulness is under the control of “two processes”: homeostatic sleep need and circadian rhythm. *Salt-inducible kinase 3* (*Sik3*) and histone deacetylase (HDAC) 4, a SIK3 substrate, are implicated in the sleep need signaling in glutamatergic neurons. Here, we demonstrate that the SIK3-HDAC4 pathway also regulates circadian behaviors, such as the strong arousal phase-locked to the beginning of the dark phase in laboratory mice, acting in the suprachiasmatic nucleus (SCN). SIK3 deficiency in the gamma-aminobutyric acid (GABA)-ergic neurons or neuromedin S (NMS)-producing neurons delayed the arousal peak phase and lengthened the behavioral circadian cycle under both 12-h light: 12-h dark condition (LD) and constant dark condition (DD) without changing daily sleep amounts. In contrast, the induction of a gain-of-function mutant allele of *Sik3* in GABAergic neurons exhibited advanced activity onset and a shorter circadian period. Loss of SIK3 in arginine vasopressin (AVP)-producing neurons lengthened the circadian cycle, but the arousal peak phase was similar to that in control mice. Heterozygous deficiency of HDAC4 shortened the circadian cycle, whereas mice with HDAC4 S245A mutation, which is resistant to phosphorylation by SIK3, delayed the arousal peak phase. Phase-delayed core clock gene expressions were detected in the liver of mice lacking SIK3 in GABAergic neurons. These results suggest that the SIK3-HDAC4 pathway regulates the circadian period length and the timing of arousal through NMS-positive neurons in the SCN, independent of the regulation of sleep amounts and depth.

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SIK3 regulates sleep amount in hypothalamic nuclei

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Sleep amount is regulated in a homeostatic manner. Although a number of sleep/wake switching neural circuits have been identified, the mechanisms by which these neural circuits contribute to homeostatic regulation of sleep remain to be elucidated. We previously found that a gain-of-function *Sleepy* (*Slp*) mutation in the *salt-inducible kinase 3* (*Sik3*) gene, which produces the mutant SIK3(SLP) protein, increases non-REM sleep (NREMS) and NREMS EEG delta density, a marker for the level of sleep need. We expect that SIK3 takes part in cellular signaling conveying seep need. However, it remains to be elucidated which cells and brain regions are responsible for the increased NREMS in *Sleepy* mutant mice. In this study, we aim to determine the cell type and neuronal groups that enhance quality and quantity of NREMS through SIK3(SLP) expression. First, we showed that *Sik3(Slp)* in mature neurons are sufficient to increase NREMS amount and NREMS EEG delta density with *synapsin1*^{CreERT2};*Sik3*$^{Slp\text{-}flox/+}$ mice which express *Sik3(Slp)* in neurons upon tamoxifen injections. Next, we explored neuronal groups through which SIK3(SLP) expression increases NREMS, by AAV-mediated expression of SIK3(SLP) in the hypothalamus. We found that SIK3(SLP) expression in the medial part of the hypothalamus increased NREMS. This result implies that SIK3(SLP) expression in the specific brain regions directly or indirectly modulates the activity of neurons executing the switch of sleep/wake states, which results in increased NREMS amounts. This will be an important step to connecting the intracellular signal representing sleep need with the circuit-level mechanism for sleep/wake switch.
