Poster Session

JST-CREST “Opt Bio” / WPI-IIIS Joint Symposium
Deciphering the Brain through “Opt Bio” Tools

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Poster Presentation	**13:50 – 14:30**

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1

Development of photocaged adenosine 2A receptor activator

Tsuyoshi Saitoh[1], Shuji Ioka[1], Mustafa Korkutata[1], Yohei Chitose[2], Manabu Abe[2], Michael Lazarus[1], Hiroshi Nagase[1]

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Adenosine (Ad) is an endogenous somnogen, which strongly promotes sleep through the activation of the adenosine 2A receptor (A2AR) in the nucleus accumbens (NAc).1 Although the increased accumulation of Ad during wakefulness leads to induce sleep, the detailed temporal and spatial mechanisms of adenosinergic control of the NAc have not been clarified. Though photocaged A2AR activator has been expected as a novel optical tool to control the single receptor activity by light irradiation, the chemical tools based on the known A2AR agonist have an issue for separation of the endogenous and exogenous effects due to the background activity. We recently discovered a novel positive allosteric modulator (PAM) YNT378.2 YNT378 enhanced the sensitivity of A2AR to endogenous Ad without showing agonist activity and induced slow-wave sleep in mice. These findings led us to develop the photocaged A2AR PAM (opto-YNT378) to facilitate the optopharmacology study of A2AR.
The firth generation opto-YNT378, carrying 6-nitroveratryl (Nv) group as a photolabile protecting group, had problematic properties such as water-insolubility, slow photoresponse, and short absorption maximum. To improve the photochemical and physicochemical properties of Nv group, we developed a novel photolabile protecting group A400 based on 3-aryl coumarine chromophore.3 The 2nd generation opto-YNT378 bearing A400 exhibited high water solubility, fast photoresponse, and an absorption maximum at 420 nm. Moreover, the time-dependent photoactivation of opto-YNT378 was observed in the cell-based assay.

2

Opto-chemical control of sleep in the nucleus accumbens using a photocaged adenosine A2A receptor allosteric modulator

Koustav Roy[1], Shuji Ioka[1], Mao Amezawa[1], Yoan Cherasse[1], Hiroshi Nagase[1], Masashi Yanagisawa[1], Kaspar Vogt[1], Manabu Abe[2], Tsuyoshi Saitoh[1], Michael Lazarus[1]

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Photopharmacology may offer the possibility of curing diseases and alleviating symptoms while preventing uncontrolled drug activity, i.e., the drug is active only at the times and places where it exerts its therapeutic effect. Although chemical photo switches have been used extensively in vitro, their use in vivo has been slow, largely because of the difficulties in applying these probes in mammalian models. We revealed a prominent role of indirect pathway neurons in the nucleus accumbens (NAc) in sleep/wake regulation and proposed that the NAc links motivation and sleep. This brain circuit may explain why we feel sleepy in the absence of motivating stimuli, i.e., when we are bored. Adenosine is a plausible candidate molecule for activating NAc indirect pathway neurons to induce slow-wave sleep (SWS) because caffeine, the most widely consumed psychostimulant in the world, produces its arousal effect in the NAc by blocking adenosine A2A receptors (A2AR) on indirect pathway neurons. However, the ability of adenosine in controlling NAc indirect pathway neurons for sleep induction remains to be elucidated. We recently reported the first positive allosteric A2AR modulator, named A2AR PAM, that evokes A2AR responses in the brain and developed a visible-light photoactivatable derivative of A2AR PAM (opto-A2AR PAM). Opto-A2AR PAM showed remarkable water solubility (>10 mM) and has an absorption maximum at 415 nm in aqueous solution. SWS was significantly increased for 5h in wild-type mice after intraperitoneal administration of opto-A2AR PAM and stimulation with violet light (405 nm) for 1 h after drug treatment, whereas no effect was observed in the absence of light exposure or in A2AR knockout mice. By using opto-A2AR PAM, we induced sleep for the first time in freely behaving mice by photopharmacologic allosteric A2AR modulation, suggesting that extracellular adenosine is involved in the regulation of sleep in the NAc.

3

Chemogenetic suppression of histamine receptor cells produces slow-waves in mice

Hikari Yashiki[1], Yo Oishi[1], Michael Lazarus[1]

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Sleep in mice is divided into 2 stages: non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep. Electroencephalogram (EEG) shows typical waves during these phases. The delta-range frequency (1–4 Hz) of EEG is dominant during NREM

sleep, while theta-range frequency (4–8 Hz) is dominant during REM sleep. Although these criteria are used generally, the mechanism to generate these typical waves is not clear.
Antihistamines induce sleepiness/sleep in humans. So, they have been used for sleep-promoting effect. Antihistamines work as inverse agonists of histamine H1 receptors (H1R, Gq-coupled receptors), causing pharmacological effects opposite to those of agonist. At present, the neuronal mechanisms of sleepiness/sleep-promoting effect of antihistamines are unknown and it is also unclear whether suppression of H1R-expressing cells affects on sleep/wake behaviors.
In this study, we examined the effect of neuronal suppression in H1R-expressing cells on sleep/wake-behaviors in mice by a chemogenetic method. After the chemogenetic suppression, the EEG with high amplitude and low frequency was observed for a few hours even during movement. These results suggest that suppression of H1R-expressing cells produces slow waves in mice.

4

Mechanisms of hippocampal adult-born neurons for memory consolidation during sleep

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Sleep plays critical roles in memory consolidation, yet the mechanisms are still unclear. Young adult-born neurons (ABNs) in the hippocampal dentate gyrus (DG) bestow unique plasticity to the memory circuit. We found that ABNs exhibit sparse activity during REM sleep, which is necessary for memory consolidation (Kumar and Koyanagi et al., Neuron, 2020). A prominent synchronous neural activity (i.e., theta rhythm) appears in the DG during REM sleep. Theta rhythm coordinates both synaptic plasticity and memory consolidation. To reveal the functions of the theta rhythm with ABNs during REM sleep, we examined hippocampal theta-phase specific ABN activity for memory consolidation by closed-loop optogenetic manipulation. This study provides insights into how the unique cellular plasticity in the adult brain operates synchronously with brain rhythm for memory consolidation during sleep.

5

Adult-born neuron activity in the establishment of fear generalization

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Overgeneralization of fear in safe contexts is considered a hallmark of post-traumatic stress disorder. Traditional behavior paradigm to elicit fear generalization requires more than ten days, which prevents the understanding of how fear generalization establishes in parallel with memory processing, including memory consolidation. It is known that adult-born neurons (ABNs) continuously generate in the hippocampus play key roles in contextual fear generalization. We have elucidated the dynamic activity change of ABNs in REM sleep and their necessity in memory consolidation. However, their activity patterns responsible for fear generalization are completely unknown. In this study, we first constructed a robust behavior paradigm to analyze the process of contextual fear generalization. To reveal the temporal and spatial activity of ABNs, we employ calcium imaging in freely moving mice. This study provides new insights into understanding the mechanisms of establishing contextual fear generalization.

6

Synchronous young and matured neuron activity for memory consolidation

Yuteng Wang[1], Pablo Vergara[1], Sakthivel Srinivasan[1], Jiahui Yu[1], Toshie Naoi[1], Yoan Cherasse[1], Takeshi Sakurai[1], Masanori Sakaguchi[1]

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Brain plasticity shifts from cellular to synaptic during development. However, cellular plasticity remains largely functional in the hippocampal dentate gyrus

(DG) throughout life; adult-born neurons (ABNs) are continuously generated in DG that play pivotal roles in memory. Indeed, we found that the sparse activity of ABNs during REM sleep is necessary for memory consolidation (Kumar et al., Neuron, 2020). It suggests synchronized ensemble activity between ABNs and the developmentally-born matured neurons for memory consolidation during sleep. However, the activity and its functional significance remain completely unknown. To tackle this issue, we have tested various methods utilizing the Calcium-imaging and miniaturized microscope we developed. Our study aims to pave a way to uncover how cellular plasticity plays distinct roles in concert with already existing circuits in the adult brain.

7

CaliAli: A tool for inter-session alignment of 1-photon calcium imaging data allowing tracking neurons in the non-rigidly moving brain

Pablo Vergara[1], Sakthivel Srinivasan[1], Toshie Naoi[1], Yoan Cherasse[1], Takeshi Sakurai[1], Masanori Sakaguchi[1]

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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 each recording session. However, because the brain is a non-rigid structure, movements across sessions are often not expressed as a simple translation transformation. In this scenario, the spatial footprints of neurons are commonly not sufficient to align imaging sessions, especially when neurons overlap with each other, are sparsely distributed, or when their activities remap (i.e., neurons disappear in some recording sessions). To address this issue, we developed CaliAli (Calcium imaging Inter-session Alignment), a tool that utilizes blood vessels in addition to spatial cues of neurons to align video sessions automatically. Because CaliAli aligns video sessions before neural extractions, it is possible to extract weak calcium signals that would be otherwise undetected by independent analysis of each recording session. Moreover, CaliAli outperforms other tracking approaches in conditions of high neural overlap or remapping of neuron activities and can be applied to highly condensed populations such as the granule cells in the dentate gyrus and also sparsely distributed population such as the hippocampal adult-born neurons.

8

Transient recruitment of an adult-born neuron ensemble for fear memory consolidation in REM sleep

Sakthivel Srinivasan[1], Iyo Koyanagi[1], Yuteng Wang[1], Pablo Vergara[1], Jiahui Yu[1], Toshie Naoi[1], Kaspar E. Vogt[1], Yoan Chérasse[1], Takeshi Sakurai[1], Keita Lio[1], Noriki Kutsumrura[1], Hiroshi Nagase[1], Masanori Sakaguchi[1]

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Memory replay during sleep is suggested to contribute to memory consolidation during sleep. The dentate gyrus (DG) in the hippocampus encode contetual fear memory trace. Moreover, we showed that the activity of the adult-born DG neurons (ABNs) during REM sleep is necessary for memory consolidation (Kumar and Srinivasan et al., Neuron, 2020). Therefore, we examined whether re-activation of context eoncoding ABNs during REM sleep is necessary for memory consolidation. Our study reveals the coding mechanisms critical for fear memory consolidation during sleep.

9

Whole brain mapping and manipulation of activated neuronal populations by exhausted exercise

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Exhausted exercise induces a variety of physiological responses including increased core body temperature, glycogen reduction, hypoglycemia, described with so-called “Fatigue”. However, neural mechanism that integrates these physiological alterations has been largely unknown. To depict the neural populations which are active during exhausted exercise, we utilized TRAP (Targeted Recombination in Active Populations) system. We crossed TRAP2-mice with Rosa26-CAG-lsl-hM3Dq (hM4Di)-mCherry mice and used motor-driven treadmill for capturing and manipulating neurons activated in exhausted exercise in whole brain. We found that exhausted exercise induced increased expression of hM3Dq-mCherry in several brain regions including the POA, DMH, PVN, PH, Pons, PAG.

Chemogenetic activation of the trapped neurons by intraperitoneal injection of CNO (Clozapine-N-Oxide) showed reduction of body temperature and energy expenditure with immobility. Chemogenetic inactivation of these neurons showed no change in energy expenditure and body temperature. We found surface body temperature decreased during exhausted exercise on treadmill. These results suggest that neurons which are activated during exhausted exercise might regulate surface body temperature to maintain core body temperature during exhausted exercise.

10

Capturing and manipulating neurons activated by exhausted exercise

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Exhausted exercise induces a variety of physiological responses including increased core body temperature, glycogen reduction, hypoglycemia, described with so-called “Fatigue”. However, neural mechanism that integrates these phenomena is largely unknown. First, we utilized TRAP (Targeted Recombination in Active Populations) system by using TRAP2-iCre mice which express iCre under the c-fos promoter. We crossed TRAP2-mice with Rosa26-CAG-lsl-hM3Dq-mCherry and utilized motor-driven treadmill for capturing and manipulating neurons activated in exhausted exercise in whole brain. We found that exhausted exercise induces increased expression of hM3Dq-mCherry in several brain regions. Activation of these neurons by intraperitoneal injection of CNO (Clozapine-N-Oxide) showed reduction of body temperature and energy expenditure with increased immobility. To search the brain regions which controls these phenomena, utilized Cre-dependent AAV expressing hM3Dq-mCherry with TRAP2-mice. We injected AAV into the AVPe, DMH, PH regions to capture and manipulate neurons which are activated during exhausted exercise. Chemogenetic activation of AVPe neurons showed decreased body temperature and energy expenditure with immobility. On the other hand, activation of DMH and PH neurons showed increased energy expenditure and body temperature accompanied with tail vasodilation. Also, we found body temperature decreases during exhausted exercise on treadmill. These results suggest that neurons which are activated during exhausted exercise showed opposite effect on body temperature and energy expenditure regulation.

11

Rapid eye movement sleep is initiated by dopamine signaling in the basolateral amygdala in mice

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The sleep cycle alternates between REM (rapid eye movement) and NREM (non-rapid movement) sleep, which is a highly characteristic feature of sleep. However, the mechanisms by which this cycle is generated have not been well-characterized. We found that a periodic transient increase of dopamine (DA) level in the basolateral amygdala (BLA) during non-rapid eye movement (NREM) sleep terminates NREM sleep and initiates REM sleep. DA acts on dopamine receptor D2 (Drd2)-expressing neurons in the BLA to induce a transition from NREM to REM sleep. This mechanism also plays a role in cataplectic attack, which is a pathological intrusion of REM sleep into wakefulness in narcoleptics. These results show a critical role of DA signaling in the amygdala in REM sleep regulation and provide a neuronal basis of sleep cycle generation.

12

GABAergic neurons in the ventrolateral periaqueductal gray are implicated in cataplexy of narcoleptic mice

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The loss of orexinergic neurons cause narcolepsy, a sleep disorder characterized by chronic sleepiness and cataplexy. Cataplexy is a sudden muscle atonia during wakefulness, which is considered as the pathological intrusion of REM sleep into wakefulness. The midbrain region, especially the ventrolateral periaqueductal gray(vlPAG) is known to regulate REM sleep. However,

the role of vlPAG in cataplexy is poorly understood. We identified vlPAG and several brain regions as the upstream of glutamatergic neurons in sublaterodorsal tegmental nucleus(SLD) projecting to ventromedial medulla (VMM; GluSLD→VMM), which is known as the common pathway of cataplexy and REM atonia (Uchida et al., 2021). Based on the previous research, we injected Cre-dependent AAV expressing hM3Dq-mCherry into the vlPAG in vGAT-IRES-Cre mice. Chemogenetic activation of vlPAG GABAergic neurons decreased the amount of cataplexy in narcoleptic orexin-ataxin3 mice. These observations suggest that vlPAG GABAergic neurons suppressed cataplexy through SLD, and a lack of orexin signaling might induce the abnormal activity of vlPAG GABAergic neurons leading to muscle atonia.

13

Visualizing input-output architecture of orexin neurons with double-color projection-selective retrograde tracing

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Orexin-producing neurons (orexin neurons), located in the lateral hypothalamic area (LHA), play a highly important role in maintaining wakefulness. Orexin neurons send widespread projections to nuclei containing monoaminergic neurons such as VTA, LC, TMN, and raphe nuclei, all of which contain monoaminergic neurons. We previously identified input neurons that make direct synaptic contacts to orexin neurons with modified rabies vector-based retrograde tracing. That study showed orexin neurons received input from various regions of the brain.

In this study, we used projection sites-specific rabies-mediated monosynaptic retrograde tracing to identify the neuronal inputs to the orexin neurons with projections to particular regions. In addition, we used newly generated orexin-iCre KI mice to analyze the input-output relationship of orexin neuronal circuits using the modified multi-color simple-cTRIO method. This new method allowed us to detect more than two different input-output pathways in the same brain.

This study revealed that orexin neurons projecting to each output region also send projections to all brain regions we previously examined. However, we found some biased input and output architectures. Orexin (LHA→LC), Orexin (LHA→VTA), and Orexin (LHA→DR) neurons have mostly overlapping but partly distinct distributions of input neurons.

14

Delineation of Neural Circuits of Galaninergic Neurons in the VLPO Implicated in Regulation of Sleep

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Sleep and wakefulness are regulated through dynamic reciprocal interactions between sleep- and arousal-inducing neuronal networks. In this intricated circuitry, orexin neurons located in the lateral hypothalamus (LH) play a pivotal role in consolidation of wakefulness, while GABAergic and galaninergic (GAL) neurons in the ventrolateral preoptic nucleus (VLPO) participate in initiation and maintenance of sleep. Previous studies showed that orexinergic neurons are innervated by GABAergic neurons in the VLPO. However, anatomical connection and functional interaction between orexin neurons and galaninergic VLPO neurons has not yet been examined. The aim of this study is to identify sleep-implicated neural circuits comprising of the galanin-producing neurons in the VLPO and orexinergic neurons. We conducted monosynaptic retrograde rabies-mediated tracing from orexin neurons using newly generated Orexin-iCre knockin mice and visualized galanin (Gal) and vesicular GABA transporter (Vgat) mRNAs in the VLPO inputs implementing FISH. We found that Gal-positive VLPO neurons, mainly co-expressing Vgat, make direct synaptic inputs to orexin neurons. Further, we conducted projection-specific rabies-mediated tracing from the GAL(VLPO→LH) neurons and identified that they receive monosynaptic inputs from various functionally diverse brain areas. We are currently performing optogenetic stimulation of the axons of galaninergic and GABAergic VLPO neurons in the LH to assess contribution of the GAL/GABA(VLPO→LH) pathways to regulation of sleep. These findings uncover connectivity of galaninergic VLPO neurons and may shed light on the role of their circuits in governance of sleep.

15

DpMe pathway induces arousal

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Sleep disorders are known as one of the core symptoms of depression, anxiety disorders and PTSD patients. We previously demonstrated that the optogenetic stimulation of GABAergic neurons in the Bed Nucleus of the Stria Terminalis (BNST) can trigger immediate transition from nonrapid eye movement (NREM) sleep to wakefulness in male mice. We confirmed dense innervation from GABA-BNST neurons to the deep mesencephalic nucleus (DpMe), one of the regions implicated in promoting arousal. We demonstrated that optogenetic stimulation of GLUT-DpMe neurons resulted in immediate transition from NREM sleep to wakefulness. We hypothesized that some GABA-DpMe interneurons might inhibit GLUT-DpMe neurons and receive inhibitory projections by GABA-BNST neurons. We also performed retrograde neuronal circuit tracing with a modified rabies virus to examine the neuronal connectivity between BNST and DpMe. Combined with in situ hybridization, we concluded that GABA-BNST neurons do not make direct synaptic connections to GLUT-DpMe neurons. However, we found that GABA-DpMe neurons receive direct synaptic input from GABAergic and glutamatergic neurons in the DpMe as well as GABA-BNST neurons. This research revealed the neuronal circuits involving GABA-BNST and GLUT-DpMe neurons that play an important role in quick switching from NREM sleep to wakefulness. We hope that this research can help decipher the etiology of some stress or fear related insomnia symptoms observed in some psychiatric disorders, like post-traumatic stress disorder (PTSD) and social anxiety disorder (SAD) in the future.

16

Gastrin-releasing Peptide-producing Neurons in the Suprachiasmatic Nucleus play an Essential Role in Regulating Circadian Rhythm

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Most organisms exhibit behavioral, physiological, and molecular fluctuations in cycles of around 24 hours, known as circadian rhythms, which are regulated by the circadian master clock, or the hypothalamic suprachiasmatic nucleus (SCN) in mammals. The SCN contains distinct subtypes of neurons expressing different neurotransmitters, such as arginine vasopressin (AVP), vasoactive intestinal peptide (VIP), and gastrin-releasing peptide (GRP). Studies assessing the role of each neurotransmitter have been conducted over the past few decades, but little is known about the roles of the different neurons that express each peptide. In this study, we focused on unraveling the role of gastrin-releasing peptide-producing neurons in regulating circadian rhythm, as little research has been done to identify the properties of these neurons. We used a newly generated mouse line, *Grp-iCre* knock-in (KI) mice and Cre-dependent adeno associate virus (AAV) to specifically manipulate SCN GRP-producing neurons. We discovered that when we inhibited the SCN GRP-producing neurons with tetanus toxin light chain (TeNTLC), mice display attenuated behavioral rhythmicity compared to control mice that express only green fluorescent protein (GFP). When we examined the clock gene expression rhythm within the SCN of mice with inhibited SCN GRP-producing neurons, we discovered that the oscillation amplitude of PER2 expression is drastically lower in these mice than in control mice. These results suggest that SCN GRP-producing neurons are crucial for sustaining SCN molecular rhythmicity and in turn, is important for regulating behavioral rhythm.

17

Induction of mouse hibernation-like state using high sensitive optogenetics

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Although a wide variety of optogenetic tools has been developed, a specific opsin that can be stimulated continuously for a long time duration (e.g., 24 hours) has not been reported yet. To create a new optogenetic tool for long term photostimulation, we modified a certain opsin (here called HPN1) and applied it for the hibernation-like hypometabolic state named "Q neurons-induced hypometabolic state (QIH)" in mice, which we recently established. Optical stimulation of HPN1 expressed in Q neurons induced hypothermia for a long period by illuminating quite low-power light with high spatiotemporal resolution. The HPN1-mediated QIH recapitulated some kinetics of physiological

changes observed in the natural hibernation. The extremely high-sensitive optogenetics will allow us for much more localized light stimulation than has been achieved so far. This could also enable proper optogenetic manipulation in experiments that would be affected by light or heat, such as research on circadian rhythms, thermoregulation.

18

Roles of monoamines and their regulatory systems in motivation and arousal

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The exact mechanisms by which monoamines (MA) such as dopamine, serotonin, histamine and noradrenaline regulate human behaviours and how their abnormalities result in several psychiatric disorders have been incompletely understood. Our project aims at producing a constructive understanding between monoamines and psychiatric disorders, with a particularly focus on the depression-related symptoms of motivation and arousal.
We generated genetically modified mice expressing Cre recombinase under the control of MA promoters with a conditional KO mouse for GTP cyclohydrolase 1 (GCH1), the gene indispensable for production of MA. We then evaluated psychiatric disorders in addition to sleep and circadian rhythm.
Our experiments on serotonin deficient mice showed a reduced wheel-running activity compared to control groups. The noradrenergic deficient mice exhibited unusual increase of electromyogram during NREM sleep. Mice deficient in dopamine showed a drastic increase of wakefulness during the dark phase compared to control mice, accompanied by a massive reduction of sleep and low anxiety. Finally, mice depleted in MA of neurons projecting to the prefrontal cortex exhibited an abnormal response to fear conditioning. We also analyzed effect of icv orexin on sleep/wakefulness behavior in MA-deficient mice.

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The anatomical and functional understanding of neural dynamics of VTA dopaminergic neurons in female sexual behavior

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The neural mechanisms of female sexual motivation have been unclear. To understand the neural basis of female sexual motivation, we focused on dopamine which is recognized as a crucial neural substrate for sexual behavior in both male and female animals. Here, we examined the neural activities of VTA dopaminergic neurons in a female mouse during sexual behavior by using in vivo imaging system, fiber photometry. We discovered the dramatic surge of the neural activity of VTA dopaminergic neurons in the female mouse immediately after male mouse ejaculation. Currently, we are trying to reveal the function or biological meaning of this dramatic dopamine surge of the female mouse at the timing of male mouse ejaculation by applying neural circuit-specific manipulation methods involving histological, optogenetic, and chemogenetic strategies. We will introduce those data at the meeting.

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Dopamine dynamics in NAc-vs control male sexual behaviors in mice

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In male mice, sexual behaviors consist of a series of stereotypic steps, such as sniffing, mounting, intromission, and ejaculation. However, the neural mechanisms by which these behaviors are regulated and coordinated have remained unclear. By using GRAB-DA sensor to monitor dopamine (DA) signaling in the nucleus accumbens (NAc), we found that DA dynamics in the NAc ventral shell region (NAc-vs) corresponded nicely to sequential steps of male sexual behaviors: 1) DA release is increased at the initiation of mounting; 2) DA level fluctuates rhythmically during intromission; 3) DA level is decreased right before ejaculation. To characterize the functional importance of DA dynamics in NAc-vs, we optogenetically manipulated the NAc-vs projecting dopaminergic neurons in the ventral tegmental area. Interestingly, inhibiting the dopaminergic neurons at the onset of sniffing or mounting stopped sexual behaviors. Moreover, inhibition of the dopaminergic neurons

during intromission induced ejaculation, whereas activation during intromission prolonged ejaculation latency. These results suggest that DA dynamics in NAc-vs contribute critically to the regulation of sexual behaviors in male mice.

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Analyses of the effects of prior social stress on brain activity during social interaction in mice using a novel semi-automated c-Fos mapping program

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Depression is a major psychiatric disorder worldwide. Stressful social experience is a factor for depression. However, the precise mechanism that links social stress to depression remains unclear. Mice exhibit depression-like phenotypes following chronic stress. Social defeat stress (SDS) is one of the methods to apply chronic stress to mice. Here, we investigated how chronic SDS affects BALB/c mice. While BALB/c mice are reported to show higher stress susceptibility compared to the widely used C57BL/6 mice, there are few studies that applied SDS to BALB/c mice. We found that BALB/c mice showed depression-like states including reduced social interest after chronic SDS and the effects remained even after 2 weeks. Furthermore, we aimed to investigate the brain regions that show altered activity following social interaction in mice that underwent SDS by means of c-Fos mapping. To this end, we established a novel method to conduct whole-brain c-Fos mapping based on semi-automated image processing, and the screening revealed several brain regions that showed different c-Fos expression levels in stressed mice compared to the control mice. These results contribute to understanding how prior social stress affects the brain activity during social interaction and provide a novel method for efficient c-Fos mapping across various brain regions.

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Is increased REM sleep an adaptive response or an exacerbating factor in stress resilience?

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Our sleep consists of two stages: rapid-eye-movement (REM) sleep and non-REM (NREM) sleep. Currently, the function of REM sleep is largely unknown. Here, we focused on depression. Patients with depression almost inevitably exhibit sleep disorders, and in particular, abnormalities in REM sleep, most commonly increases in REM sleep amounts, are frequently observed. There are controversies as to whether increased REM sleep helps recovery from depression or rather contributes to worsening of the depression-related symptoms. As a first step to address this in mice, we aimed to analyze the effects of 10 days of social defeat stress on sleep in mice. Similar to patients with depression, stress exposure in mice increased REM sleep. Moreover, the increase in REM sleep that observed after acute stress exposure tended to attenuate after chronic stress exposure. Based on these results, we next investigated the effects of artificially increased REM sleep at specific timings by chemogenetic activation of the REM sleep-promoting neurons recently identified in our laboratory. Surprisingly, we found that repeated activation of this neurons has antidepressant effects on a decrease in social interests induced by chronic social defeat stress. Now we are trying to reveal how artificially increased REM sleep contributed to those behavioral phenotypes. Our future work will provide new insights to address the causal relations between stress resilience in mice and REM sleep.

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Characterization of the neuronal activity of a newly identified REM sleep-regulating neuron using glass pipette extracellular unit

recording combined with optogenetics

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Human beings spend about one-third lifetime in sleep. Mammalian sleep comprises non-rapid eye movement sleep (NREM) sleep and rapid eye movement (REM) sleep. Previous studies revealed that the brainstem plays a crucial role in the regulation of REM sleep. However, the mechanism of REM is still largely unknown. Glass pipette extracellular unit recording, a conventional electrophysiological technique, has been used to characterize neuronal activity in the sleep/wake cycle. This method is robust in terms of temporal resolution and can record even a small neuron. A new REM sleep-regulating subtype neuron has been identified in the dorsal pons and the activity of this neuron in the sleep/wake cycle should be characterized. However, it has been demanding to record a specific subtype neuron and a neuron that fires at low frequency in the method. To overcome this limitation, we combined the opto-tagging method with glass pipette extracellular unit recording and made a cell-type-specific unit recording. We found that some of the REM sleep-regulating neurons showed increased activity during REM sleep and these neurons may play important role in REM sleep regulation.

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

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During rapid eye movement (REM) sleep, the cerebral cortex becomes activated and produces vivid dreams. Yet, it normally does not lead to motor output owing to expression of muscle atonia. However, patients with REM sleep behavior disorder (RBD) exhibit impaired muscle atonia during REM sleep and frequently act out of their dreams. For example, they talk loudly or exhibit violent movements such as hitting and kicking. RBD patients often wake up following such movements, which can lead to reduced sleep quality.
A majority of RBD patients co-suffer from synucleinopathies including Parkinson's disease and dementia with Lewy bodies or eventually develop these diseases within 10-14 years. Thus, RBD is considered as a prodromal of synucleinopathies. Here, we aimed to understand the mechanisms underlying the link between RBD and synucleinopathies and establish an RBD mouse model for future development of effective treatment for RBD. A rare G51D α-synuclein mutation leads to production of toxic α-synuclein fibril and patients carrying this mutation show rapid progression of Parkinson's disease. We produced and injected G51D α-synuclein fibrils into the brainstem pontine tegmental area in mice, and observed effects on atonia during REM sleep, behavior and motor symptoms.

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Effects of chronic cell ablation in the preoptic area on sleep-wake cycles

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Sleep and wakefulness are controlled by specific brain regions. These regions have been identified by classic electric stimulation of non-specific cell types and acute neural activity manipulation. However, despite many experiments that show that acute manipulation of these regions has a temporary effect on the sleep-wake cycles, chronic inhibition of neural activity often has no effect.
This is thought to be due to adaptation and compensation by other brain regions. In this study, we focused on the preoptic area (POA), which has been

reported to promote sleep . With conventional methods such as electrolytic lesions, axons passing through the region are also affected, so functional inhibition is likely not brain region-specific. The effect of brain region-specific cell ablation in the POA on sleep-wake cycles is unclear. Then, we investigated the effect of brain region-specific cell ablation in the POA on the sleep-wake cycle. In this 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. As a result, the fragmentation of sleep-wake cycles was observed, the frequency of transitions from wakefulness to sleep increased, and the duration of sleep decreased. This effect was sustained for more than four weeks, and no compensation by other brain regions was observed. These results suggest that POA is necessary for consolidated sleep-wake cycles.

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Toward label-free molecular imaging of whole brain and brain cells using ultra-broadband multiplex CARS microspectroscopy

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Mapping the distribution of chemical bonds throughout brain tissue is inevitable for understanding brain functions. We applied label-free ultra-broadband multiplex CARS (MCARS) spectroscopic imaging system to construct the whole brain chemical molecular map. Our experimental setup enabled us to acquire the MCARS spectrum with the spectral coverage of approximately 3000 cm^-1 from the macro (tissue level) and micro (cellular level) scales. As a result, the MCARS images of the whole brain slice at CH_2 stretching and CH_2 scissoring bands were assigned as lipids, which mainly visualize the white matter. Next, we visualized the hippocampus and cortex on micro-scale. The ratio of the vibrational band due to CH_3 stretching to that of OH stretching indicates that the water content of these neurons is more abundant than other surrounding regions. In the cortex, localized lipid-rich areas were also observed. This probably corresponds to the distribution of lipid-rich glial cells such as astrocytes and oligodendrocytes, which are one of the most abundant cells in the brain. These results were suggesting that MCARS spectroscopic images can be used to distinguish cell types in the brain. In conclusion, the whole brain label-free molecular imaging with single-cell resolution will allow a comprehensive analysis of molecules in the brain and will lead to a better understanding of brain function and structure.

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The role of hypothalamic supraoptic nucleus in maintaining wakefulness.

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This project arisen from accidental finding that photostimulation of supraoptic (SO) nuclei resulted in prolonged wake state. Based on previous in-situ hybridization data we assumed that SO nucleus is involved in cannabinoid convulsive effects and therefore investigated the effect of optogenetic activation of SO nucleus. Bilateral photostimulation of SO nuclei did not induce electrographic seizures, however, we discovered that prolonged photostimulation (4 hours) resulted in increased wakefulness (time in wake) with complete absence of sleep (NREM and REM) during stimulation, which was highly significant and resulted in sleep rebound accompanied by increased NREM sleep amount and delta NREM amount immediately after the end of stimulation. We have also examined the effect of 8 hrs photostimulation, which resulted in normal wake behavior absence of sleep for the first 4 hours and significant decrease of sleep for the rest of photostimualtion time. This suggests that SO nucleus is strongly involved in inducing and possibly maintaining arousal state. To our knowledge this data has not been reported by anyone yet. We are planning to deeply investigate how activation of SO nuclei produces arousal and what mechanism underlie this phenomenon and to anatomically dissect inputs and outputs of such arousal effect.

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Recovering EEG Generators from Their Collective Stochastic Interference

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Mainstream descriptions of EEG rely on its analysis in the frequency domain and basically explain the observed EEG oscillations as emerging from neuronal synchronization processes. Traditionally, these oscillations are categorized into spectral bands whose correlates with different brain states are well established. Recently, however, some alternative views of EEG emphasize its arrhythmic nature, focusing on global properties of the spectrum as, for example, the observed 1/f spectrum. Following this line of thought, in the present study we modeled EEG as a stochastic process emerging from the superposition of arrhythmic pulses described by arbitrary functions. Even though this kind of superposition generates colored Gaussian noise, from our simulations we discovered that it is still possible to statistically recover the shape of the underlying pulse. Applying these concepts to actual EEG, unique patterns characterizing NREM sleep, REM sleep, quiet wake, and active wake can be identified. Remarkably, using the presented methodology, all these behavioral states can be determined directly from EEG, not requiring EMG. In addition, although it is well-known atropine induces EEG delta waves that can be confused with NREM sleep EEG, our method shows a distinctive pattern for the atropine effect, very different from those patterns associated with NREM sleep or any other physiologic state.

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Dihydropyridine calcium blockers do not interefere with slow wave sleep

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Slow wave or non-rapid eye movement (NREM) sleep is tightly homeostatically regulated and essential for survival. Slow waves are observed in the electroencephalogram (EEG) as oscillations in the delta (0.5-4 Hz) range. Slow wave activity is to date the best indicator for homeostatic sleep regulation; it is increased after prolonged waking and slowly reduced during NREM sleep. The precise mechanisms underlying sleep homeostasis and the generation of slow waves are unknown. Activity-dependent neuronal calcium influx has been hypothesized to play an important role in generating slow oscillations and might be involved downstream signaling that mediates sleep function. Dihydropyridine blockers of L-type voltage gated calcium channels (VGCCs) are in wide clinical use to treat hypertension and other cardiovascular disorders and are readily blood-brain-barrier (BBB) penetrant. We therefore wanted to investigate their potential effect on slow wave generation and homeostatic NREM sleep regulation. In-vivo two-photon imaging of cortical neurons showed larger spontaneous calcium transients in slow wave sleep compared to waking. Application of the dihydropyridine calcium blocker nicardipine significantly reduced cortical calcium transients, without affecting slow wave generation. Nicardipine also did not affect the slow wave activity over 24h following application. Time spent in slow wave sleep and episode duration were also not affected. We conclude that despite evidence that neuronal calcium influx may be involved in NREM sleep function, blocking calcium entry through L-type VGCCs does not interfere with slow wave generation or regulation.

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The effects of olfactory stimulation during REM sleep on dream emotionality: a study focusing on individual difference in olfactory perception

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Odors are useful stimuli in sleep research because its rarely cause arousal. In addition, odor has another unique characteristic that there are large individual differences of perception.
In the present study, we examined how the effects of presentation of olfactory stimuli during REM sleep on dreams differed according to individual differences in odor perception.
We focused on the preference at study 1, and the familiarity at study 2, the effects of odor were stronger

when the subjects were preferring and familiar to the odor, and their dream became more negative.
However, no such effect was found when the subjective intensity was focused on at study 3.
These results, preference and familiarity related and subjective intensity did not relate to the effect that make dream more negative, were confirmed at the pooled analysis of study 2 and 3.
In our presentation, we will present these results and why preferred and familiar odor induced negative dream and subjectively intense odor did not.

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Recovery from systemic-inflammation-induced altered sleep is potentiated by sevoflurane preconditioning

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Background: Despite extensive evidence on the organ protective effects of sevoflurane, its effect on disturbed sleep remains unclear. We hypothesized that sevoflurane preconditioning positively impacts disturbed sleep caused by systemic inflammation.Methods: A mouse model of lipopolysaccharide (LPS)-induced systemic inflammation was employed to investigate the effects of sevoflurane on sleep recovery. We evaluated symptoms recovery through electroencephalography/electromyography (EEG/EMG) and histological studies. The mice were exposed to 2% sevoflurane before and after peritoneal injection of LPS. The EEG/EMG were recorded for 24 h after the procedure. Brain tissue was harvested after the sevoflurane/LPS procedure and was immunostained using individual antibodies against choline acetyltransferase (ChAT) and Fos. We quantitatively analyzed the ChAT-positive and ChAT/Fos double-positive cells in the pedunculopontine tegmental nucleus and laterodorsal tegmental nucleus (PPTg/LDTg). Results: Mice preconditioned with sevoflurane showed a significant recovery in rapid eye movement (REM) sleep following the LPS challenge. They also demonstrated shorter REM latency, indicating an early recovery from LPS-altered sleep. We observed more ChAT/Fos double-positive cells in the PPTg/LDTg in the sevoflurane preconditioning plus LPS group than in the LPS-only group.Conclusions: Sevoflurane preconditioning promotes recovery from altered sleep induced by systemic inflammation. Activation of PPTg/LDTg is considered a mechanism underlying sleep reintegration. The recovery phenomenon shows potential for clinical application in cases of sleep disturbances induced by systemic inflammation..

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Validation experiment using in-home sleep electroencephalography and its clinical application

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To construct a system that can evaluate sleep as accurately as polymsomnografy (PSG), the gold standard for sleep evaluation, we developed an in-home EEG. We conducted a series of experiments to validate the accuracy of the device.The first study included 50 healthy subjects and verified the validity of sleep evaluation for in-home EEG using simultaneous measurements of in-home EEG, PSG, and Fitbit Charge 3, an accelerometer with HRV analysis, in the subjects. The mean ± standard deviation (SD) of the accuracy and kappa coefficient of the in-home EEG, in which the scoring result of the PSG for the correct answer, was 86.8% ± 3.8% and 0.80 ± 0.05, respectively. The lowest accuracy and kappa coefficient were 77.4% and 0.65, respectively. The accuracy in stage N1 was 59.5%, which was low, but good accuracy was obtained in the other sleep stages. The mean ± SD of the accuracy and kappa coefficient of the Fitbit was 69.5 ± 8.3% and 0.50 ± 0.14, respectively, and the accuracy and kappa coefficient of the Fitbit sleep stage scoring was significantly lower ($P < 0.05$) than those of the in-home EEG. Another empirical study of sleep assessment including 100 patients with sleep disorders of breathing (SDB) using in-home EEG, has just begun. A high proportion of patients have been diagnosed as obstructive sleep apnea syndrome (OSAS), and simultaneous measurement of their sleep using in-home EEG and PSG showed that the accuracy of in-home EEG depends largely on their severity of the disease. In patients with 5-15 AHI (apnea and hypopnea index), the accuracy was as high as that of healthy subjects, but in

patients with over 30 AHI, who are considered as having severe OSAS, the accuracy was below 70%.

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Automatic sleep stage and arousal assessment of sleep recordings from a portable IoT EEG device

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Since its inception, EEG recording and scoring has become a standard procedure for sleep research and sleep diagnosis. EEG has traditionally been recorded in a laboratory setting, as part of the Polysomnogram (PSG), but recently it is possible to record it in other spaces thanks to the advent of portable EEG devices. EEG is scored following the R&K or AASM guidelines, an activity that requires the visual inspection of each sleep epoch in a whole sleep recording, a very time consuming process that is prone to error. With the interest of speeding up this process, different machine learning models have been proposed, but they are limited to specific tasks, such as stage scoring or arousal scoring but not both; therefore, limiting the possibility to evaluate other sleep variables. For this reason, I am proposing a model called SAAS U-Net, for the simultaneous assessment of sleep stages, arousal events and other sleep variables using the recordings of a portable IoT EEG device, with the purpose of improving the early diagnosis of sleep disorders, benefitting the users of this device and at the same time, contributing to the promotion of a better quality of sleep.

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Metabolomic and pharmacologic analyses of brain substances associated with sleep pressure in mice

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Sleep need is accumulated during wakefulness and dissipated during sleep. Sleep deprivation (SD) has been used as a method to investigate the molecular changes under high sleep need. However, SD induces changes not only reflecting increased sleep need but also inevitable stresses and prolonged wake state itself. The Sleepy mutant mice exhibit constitutively high sleep need despite sleeping longer, and have been useful as a model of high sleep need. Here we conducted a cross-comparison of brain metabolomic profiles between SD versus ad lib slept mice, as well as Sleepy mutant versus littermate wild-type mice. 203 metabolite were quantified in total, of which 43 metabolites showed significant changes in SD, whereas 3 did in Sleepy mutant mice. The large difference in the number of differential metabolites highlighted limitations of SD as methodology. The cross-comparison revealed that a decrease in betaine and an increase in imidazole dipeptides (IDs) are associated with high sleep need in both models. Furthermore, we found that central injection of IDs increased subsequent NREM sleep time, suggesting the possibility that IDs may participate in the regulation of sleep in mice.

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The novel intracellular signaling pathways for the regulation of homeostatic sleep need

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Sleep is an ubiquitously conserved behavior, and homeostatic sleep need is the fundamental element for sleep regulation. However, the molecular/cellular basis

of sleep need remains unknown. Our recent study suggested that changes of sleep need can be translated into cumulative phosphorylation of certain brain proteins, and SIK3 kinase has an essential role in this regulatory mechanisms. In this study, to reveal the molecular/cellular basis of sleep need, we aim to identify upstream and downstream components of SIK3. As a possible upstream regulator of SIK3, we focused on LKB1 kinase. We generated postnatal neuron-specific LKB1 knockout (nKO) mice, and LKB1 nKO mice showed weaker sleep pressure. Furthermore, this phenotype is recovered by constitutively active mutation of SIK3. These results suggest that LKB1 regulates sleep/wake behavior by acting upstream of SIK3.

To identify the downstream signaling pathway of SIK3, we conducted in vitro substrate screening of SIK3 by kinase-oriented substrate screening (KIOSS) method (Nishioka et al., 2015) and the pathway analysis. These results suggest that Rho-GTPase signaling pathway may function downstream of SIK3 in sleep/wake regulation.

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Natural history study of sleep disturbances in CDKL5 deficiency disorder mice

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CDKL5 deficiency disorder (CDD) is an X-linked severe neurodevelopmental disorder caused by pathogenic mutations in the Cyclin-dependent kinase-like 5 (*CDKL5*) gene. Patients with CDD display a variety of clinic symptoms including early-onset seizures, intellectual disability, and autism spectrum disorder. The majority (>86%) of patients have sleep problems such as difficulty falling asleep, sleep fragmentation, frequent night awakenings, night screaming and excessive daytime sleepiness. Sleep disturbances impair neural plasticity and exacerbate illness state in patients with autism and epilepsy. However, little is known about the sleep phenotypes in *Cdkl5* mutant mice. To this end, we characterized baseline sleep and recovery sleep after sleep deprivation in young and older *Cdkl5* knock-out (KO) male mice and their wildtype littermates using electroencephalography (EEG) and electromyography (EMG) recording. At both baseline and following sleep deprivation, young and older *Cdkl5* KO mice exhibited significantly increased sleep latency, shorter sleep episode duration and frequent transitions between sleep and wakefulness compared with their littermate controls, which resemble sleep disturbances observed in human CDD patients. The results suggest that *Cdkl5* KO mouse model may be a valuable genetic model for studying sleep disruptions in CDD patients.

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OX2R-selective orexin agonism is sufficient to suppress narcoleptic symptoms, cataplexy and wake fragmentation, without inducing drug-seeking behavior in mouse models

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Acquired loss of orexin neurons causes a chronic sleep disorder narcolepsy-cataplexy. Narcoleptic symptoms are characterized by cataplexy and sleep/wake fragmentation. Orexin receptor agonist is expected as a mechanistic treatment of narcolepsy. However, it has been unclear whether the activation of only OX2R, or both OX1R and OX2R, is required to replace the endogenous orexin functions in the brain. To examine whether the selective activation of OX2R is sufficient to ameliorate the cataplexy and sleep/wake fragmentation, we compared the therapeutic efficacy by peptidic orexin in narcolepric model mice. Here we concluded that OX2R selective orexin agonism is sufficient to ameliorate both cataplexy and wake fragmentation without reward or reinforcing effects. These findings provide a proof-of-concept for a safer mechanistic treatment of narcolepsy-cataplexy through OX2R-selective agonism.

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Pre-narcoleptics are more prone to suvorexant-induced cataplexy

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Orexins/hypocretins are key neuropeptides responsible for the regulation of the central arousal and reward circuits. Suvorexant, a dual orexin receptor antagonist, induces sleep in mice, dogs, and humans. Suvorexant induces sleep by reducing the orexin synthesis. We hypothesize that individuals with low brain orexin levels (pre-narcoleptics), maybe at a higher risk of cataplexy by suvorexant. Heterozygous orexin knockout (Ox-KO(-/+)) mice could mimic a perfect pre-narcoleptic condition. As expected, a biochemical analysis showed that Ox-KO(-/+) mice had a significantly low level of orexin peptide as compared to wild-type mice. The behavioral analysis showed that suvorexant administration in Ox-KO(-/+) mice induce narcolepsy as evident by the appearance of sleep onset REM (SOREM) sleep and cataplexy (direct transition from wake to REM sleep) in a dose-dependent manner. Sleep/wake did not show major changes. Our data suggest that Ox-KO(-/+) mice have insufficiently low brain orexin to show behavioral narcolepsy. However, upon suvorexant administration brain orexin may further decrease, which along with the inhibition of the orexin receptors, results in the appearance of narcoleptic symptoms in Ox-KO(-/+) mice.

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Orexin receptor antagonists ameliorate the symptoms of REM sleep behavior disorder in a novel mouse model and in human patients

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Rapid eye movement (REM) sleep behavior disorder (RBD), a parasomnia characterized by the loss of REM atonia, is associated with a high probability of later developing α-synuclein diseases. Novel transgenic models of RBD are useful for promoting the discovery of new RBD therapeutics. Here we generated transgenic mouse lines lacking the glycine receptor alpha1 subunit in cholinergic neurons (ChGlyR-KO) or in a subset of somatomotor neurons (MnGlyR-KO). Both ChGlyR-KO and MnGlyR-KO mice displayed excessive body and limb movements during REM sleep, including jerking, kicking, punching, and chewing behaviors that resemble human RBD. This phenotype was ameliorated by the administration of clonazepam, a benzodiazepine often used clinically to treat RBD symptoms in human, indicating that the ChGlyR-KO mouse is a good animal model for studying RBD. We also found the dual orexin receptor antagonist DORA22 was effective for treating the RBD phenotype of ChGlyR-KO mice. Further, in human RBD patients, the clinically available orexin antagonist suvorexant significantly reduced the loss of REM atonia. Our observations indicate that orexin signal blockade could be a potential treatment for RBD symptoms.

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Learning and memory deficit in adult dreamless mice

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Earlier REM sleep deprivation studies in rodents showed severe consequences of loss of REM sleep on learning and memory. However, finding of those studies is likely to affect by stressful sleep deprivation procedures that are difficult to bypass. To address this issue, we used Dreamless mice, which have inherently reduced REM sleep (Funato et al., 2016). Further, we used genetically encoded tools for neural circuit dissection to relevant cellular populations at learning and memory stages in dreamless mice. We crossed dreamless mice with TRAP2 (Targeted Recombination

in Active Population) and Ai27 (tdTomato) to label activated (TRAPed) cells at memory encoding, consolidation, and retrieval stages. Adult dreamless triple transgenic mice (3X: Drl -TRAP2-Ai27) and their littermates double transgenic (2X: TRAP2-Ai27) underwent a classical contextual fear conditioning paradigm. Next day when mice were placed in same context, dreamless 3X mice showed a significantly low (half) freezing percentage compared to littermates 2X mice. Histological examination of dreamless brain obtained at learning stage showed an overall low number of TRAPed cells in hippocampus, particularly in Dentate Gyrus region, indicating low hippocampal activation. Hippocampal activation is required for successful memory formation and storage, which is probably affected due to low REM sleep amount in dreamless mice.

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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 performed a large-scale screening of ENU-mutagenized mice in order to identify genes regulating sleep/wake behavior, and established the Dreamless mutant pedigree exhibiting ~50% reduction in 24-h REMS amount. We identified a single nucleotide substitution 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 a systemic Cre-expressing line Actb-iCre phenocopied the Dreamless mice on electroencephalogram and electromyogram (EEG/EMG) analyses. In Nalcn-flox mice, we confirmed a neuronal subtype-specific deletion of Nalcn mRNA in adult brain tissues. Recently we observed that NALCN has distinct roles in forebrain and pons-medulla regions for REM sleep regulation, with Foxg1-IRES-Cre or En1-Cre lines. Now we are analyzing the detailed sleep phenotype of these two Nalcn genetically-modified mice with sleep stage scoring.

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Sleepy mouse as a model of idiopathic hypersomnia

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Idiopathic hypersomnia (IH) is a neurologic disorder characterized by excessive daytime sleepiness, long night sleep, non-refreshing naps, and difficulty of awakening after naps or after night sleep, which presents as “sleep drunkenness.” Daytime sleep latency is shortened in IH patients, as typically assessed by multiple sleep latency test (MSLT). The pathophysiology of IH is still unknown and there is no approved drug for this disorder. The *Sleepy* mutant mouse, a mouse pedigree with a splicing mutation in the *Sik3* protein kinase gene, has longer sleep time and increased sleep need compared with wild-type mice. Therefore, we aimed to validate the *Sleepy* mouse as a mouse model for IH. We used a mouse version of MSLT and investigated sleep drunkenness in *Sleepy* mice. In addition, we aimed to evaluate the effectiveness of

orexin agonists in treating the sleepiness symptom of the *Sleepy* mouse. *Sleepy* mice showed normal sleep latency compared with wild-type mice in the light phase and shorter sleep latency for the first 3 trials of the MSLT in the dark phase. They also showed reduced decay of NREM delta density during wakefulness. Intracerebroventricular injection of orexin-A promoted wakefulness for 2 h and 3 h after injection in *Sleepy* mice and wild-type mice, respectively. Moreover, *Sleepy* mice but not wild-type mice showed a sleep rebound after the orexin-A-induced wakefulness. Intraperitoneal injection of the small-molecule orexin agonist YNT-185 promoted wakefulness for 2 h in *Sleepy* mice. These results indicate the validity of the *Sleepy* mutant mouse as a model for idiopathic hypersomnia, and the effectiveness of orexin agonists in treating sleepiness due to causes other than orexin deficiency, which makes them potential drugs to improve sleepiness in sleep disorders other than narcolepsy.

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Electrophysiological analysis of ion channel mutations in mice with REM sleep abnormalities

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We will perform a functional analysis of a novel mouse family with REM sleep abnormalities that was discovered in a previous study by random mutagenesis and EEG analysis.
The gene responsible for the sleep abnormalities in this mouse family has been identified as an ion channel, but how the mutation changed the biophysical properties of the ion channel as a result of the mutation is still unknown. The purpose of this study is to characterize the biophysical properties of this mutant ion channel using the patch clamp method.

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Sleep/wakefulness and body weight growth from infancy to adulthood in a hypersomnia model, Sleepy mutant mouse

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For technical reasons, few studies have been conducted to examine sleep/wakefulness during infancy using electroencephalogram and electromyogram (EEG/EMG). Therefore, the significance of sleep during infancy remains enigmatic and there are many unresolved issues regarding sleep during postnatal development, such as the onset of sleep abnormalities observed in adult mice. Here, we developed a method to record EEG/EMG from P21 immediately after weaning to P57 every 4 days. Using this system, we examined when SIK3 Sleepy mutant mice (SLP) start to exhibit the long sleep time observed in adulthood. In addition, EEG/EMG recordings were performed on these SLP mutant mice and their wild-type littermates at 10-13 weeks and 30-33 weeks of age. At P21, when sleep/wake behavior was immature, there was no significant difference in NREMS time between SLP mutant and wild-type littermates. Subsequently, the SLP mice showed a constant increase in NREM sleep time from P25, while the wild type showed a slight decrease. Furthermore, weekly weight measurements from 4 to 30 weeks of age confirmed that SLP mice begin to become obese after the onset of hypersomnia.

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SIK3 in different hypothalamic areas mediates whole-body energy balance

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In addition to its role in determining sleep need, Salt-inducible kinase 3 (Sik3) is likely to regulate energy homeostasis since mice mutant for exon 13 of the Sik3 gene (Sleepy) display an obese phenotype. The main objective of this project is to identify the role of SIK3 in the central nervous system in the regulation of energy balance. To achieve this, neuron-specific mutant mice lines for the Sleepy gene were generated, as well as viral genetic approaches in Sik3-ex13 floxed mice were used to identify the brain regions involved in SIK3 actions. Our data showed that Vglut2-specific Sleepy mice also show an obese phenotype, suggesting dependency of

glutamatergic signaling. SIK3 gain of function, by AAV injection in Sik3-ex13 floxed mice, in the ventromedial nucleus of the hypothalamus induced feeding-independent increase in body weight, associated with altered glucose homeostasis. On the other hand, SIK3 in the paraventricular nucleus of the hypothalamus caused hyperphagia and reduced energy expenditure leading to an obese phenotype. Overall, these data suggest a role of SIK3 in the regulation of energy metabolism in a nucleus-specific manner.

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Where does Sleepy mutation of SIK3 cause sleep phenotypes?

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Recently, we found a gain-of-function Sleepy (Slp) mutation in the Sik3 gene, which produces the mutant SIK3(SLP) protein, increases sleep amount and non-REM sleep (NREMS) EEG delta density, an index of sleep need. However, it remains to be elucidated where SIK3(SLP) increase sleep amount and NREMS EEG delta density, since SIK3 is expressed in various tissues. Here, we investigated whether SIK3(SLP) in mature neurons is sufficient for the sleep phenotypes of Sleepy mutant mice with Synapsin1CreERT2; Sik3Sleepy-flox mice. Tamoxifen administrated Synapsin1CreERT2; Sik3Sleepy-flox mice exhibited increased NREMS time and NREMS EEG delta density. It suggested that SIK3 plays roles regulating sleep amounts and sleep need in mature neurons.

Furthermore, we explored neural populations which increase NREMS time upon SIK3(SLP) expression with AAV vectors. We found that SIK3(SLP) expression in medial parts of hypothalamus increased NREMS, but not NREMS EEG delta density. It implies that neural populations that increase NREMS and NREMS EEG delta power in Sleepy mutant mice are not identical.

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Sik3 regulates sleep need via glutamatergic neurons in cerebral cortex

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Sleep is regulated by sleep need in a homeostatic manner. We recently identified Sik3 as a gene regulating sleep/wakefulness through forward-genetics approach. The pedigree, Sleepy, which has a splicing mutation Sik3(Slp) in Sik3 resulting in the skipping of exon 13, exhibits increased sleep need and prolonged sleep time. On the other hand, the SIK3 kinase activity is tightly regulated by phosphorylation of T221 in the kinase domain T-loop. Interestingly, the phosphorylation of T221 is increased in wild-type mice after sleep deprivation, indicating SIK3 kinase activity increases in mice with a higher sleep need. However, how SIK3 kinase activity is involved in sleep regulation remains unknown.

Here, we report the functional analysis of SIK3 kinase activity for sleep/wake regulation using mice expressing a non-phosphorylatable T221A or phosphomimetic T221E mutant of SIK3(WT) or SIK3(SLP), respectively. Our EEG-EMG-based sleep analysis of those mutant mice and biochemical assays of those mutant SIK3 proteins indicate that SIK3 kinase activity regulates sleep need and is required to increase sleep amount following another functional alternation such as protein-protein interaction.

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Molecular mechanisms for SIK3(Sleepy)-mediated sleep/wake regulation

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Sleep/wake behavior of mice lacking PKA phosphorylation site in SIK3

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We previously identified a kinase, SIK3, as an important sleep regulator through screening of randomly mutagenized mice. Mice that express mutant SIK3 lacking the 52 amino acids encoded by exon 13 showed a decrease in wake time and an increase in NREM sleep time. SIK3 is an AMPK-family protein kinase containing a well-conserved protein kinase A (PKA)-phosphorylation site, serine 551. The skipping of exon 13 results in a deletion of 52 amino acids including S551. Also, Sik3 S551A knock-in mice showed reduced total wake time and increased sleep need. These results suggest that the existence of S551, a PKA recognition site, is crucial for the normal sleep/wake regulation and maintenance of daily sleep need.

In SIK3, there are three PKA recognition site, threonine 469, serine 551 and serine 674. To examine whether the phosphorylation of T469 and S674 of SIK3 is required for proper sleep/wake behavior, we generated mutant mice in which SIK3 T469 and SIK3 S674 were substituted by alanine through the CRISPR/Cas9 method. Sik3 T469A mice showed increased NREM sleep time and NREM sleep delta power, an index for sleep need. Sik3 S674A mice showed no changes in NREM sleep time and NREM sleep delta power. These findings indicate the PKA recognition sites of SIK3, especially T469 and S551 are required for regulation of sleep/wake behavior.

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Loss of canonical *Hdac4* signaling leads to dysregulated NREMS

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Sleep is observed universally across various species from nematodes to mammals and the properly maintained sleep is directly linked to the general well-being of the organism. Much advances has been made in the identification of the distinct groups of neuronal population that are involved in the transitions between sleep and wake states. These networks of neurons can drive vigilance stage switches and changes in neuronal activity related to sleep and wakefulness. Despite the growing interest and findings in the maintenance of sleep/wakefulness, the intracellular regulatory mechanism of the response to sleep need changes remains largely unknown.

Our EEG/EMG-based forward genetics approach in mouse sleep/wake behavior has successfully identified several sleep regulatory genes. We previously reported a long-sleep pedigree carrying a gain-of-function mutant allele, *Sleepy*, in the *Sik3* gene and showed SIK3 as a central regulator of sleep homeostasis. *Sik3-Sleepy* mice showed marked increase in daily total non-REM sleep (NREMS) sleep, as well as inherent increase in slow-wave activity during NREMS. Our effort in elucidating the key component of sleep/wake modulation has continued with discoveries of other sleep regulating genes. *Sleepy2* is one of such mutant

pedigrees with a loss-of-function of HDAC4. The heterozygous mutant mice showed increase in both NREMS time and slow-wave activity during NREMS.
Importantly, HDAC4 is known as a primary target of SIK3, where the phosphorylation by SIK3 of a conserved serine residue is essential in the nuclear-to-cytoplasmic transfer of HDAC4. Indeed, mice with a phosphodeficient mutation in this residue of HDAC4 showed an opposite phenotype: these mice showed increase in daily wake time and significant decrease in the slow-wave activity during NREMS. Taken together, sleep/wake maintenance and proper response to sleep need changes require canonical signaling of SIK3-HDAC4.
For further understanding of specific neuronal network where the SIK3 and HDAC4 may function, we employed a neuronal type-specific manipulation engineered by Cre-loxP recombination. From the comparison of various *Cre* reporter lines with *Hdac4*^{flox} or *Sik3-Sleepy*^{flox} mice, we discovered the NREMS time and slow-wave activity during NREMS are modulated by distinct neuronal types. Our results show that SIK3-HDAC4 mediated regulation of the amount and quality of sleep are controlled by discrete neuronal network in type- and region-specific manner.
