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REVIEW

Sleep as a biological problem: an overview of frontiers in sleep research

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Abstract Sleep is a physiological process not only for the rest of the body but also for several brain functions such as mood, memory, and consciousness. Nevertheless, the nature and functions of sleep remain largely unknown due to its extremely complicated nature and lack of optimized technology for the experiments. Here we review the recent progress in the biology of the mammalian sleep, which covers a wide range of research areas: the basic knowledge about sleep, the physiology of cerebral cortex in sleeping animals, the detailed morphological features of thalamocortical networks, the mechanisms underlying fluctuating activity of autonomic nervous systems during rapid eye movement sleep, the cutting-edge technology of tissue clearing for visualization of the whole brain, the ketogenesis-mediated homeostatic regulation of sleep, and the forward genetic approach for identification of novel genes involved in sleep. We hope this multifaceted review will be helpful for researchers who are interested in the biology of sleep.

Keywords Sleep · Thalamus · Amygdala · Tissue clearing · Ketogenesis · Forward genetics

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Introduction

From a historical perspective of sleep research

One feels tired and falls asleep every night and wakes feeling refreshed every morning. At first glance, the structure of sleep and wakefulness is pretty simple, as if a computer turns on and off by itself. As is often the case in biology, despite of its simple appearance, illuminating a comprehensive picture of sleep is not without complications. A vast amount of research indicates that all aspects of sleep are inextricably linked with the brain and that the brain does not work properly without sleep. Thus, sleep is described briefly and clearly as follows: sleep is of the brain, by the brain, and for the brain [1]. The considerations of sleep from its outward aspects have been documented since ancient times (e.g., the earliest hypothesis of sleep was proposed in the fifth century B.C.). On the other hand, neurobiological insights into sleep rested quietly until the two breakthroughs in the early 20th century, which are the invention of electroencephalography [2] and the localization of sleep centers in the brain [3].

There is no doubt that electroencephalography was the first and only methodology for disclosing the relationship between sleep and brain activity. Electroencephalography shattered the concept that the brain is silent during sleep, and nowadays is used for monitoring sleep states. Somewhat surprisingly, in the process of a study of 'telepathy', German neurologist Berger [2] discovered the electroencephalogram (EEG) in humans and showed that EEG exhibits low-amplitude fast activity during wakefulness. Within a few years after that, it was demonstrated that the large-amplitude oscillations dominate the EEG under some types of anesthesia [4] and during sleep [5, 6]. The large and slow EEG wave (1–4 Hz in frequency) is termed slow-wave or delta activity. Sleep accompanied by slow-wave activity is called slow-wave sleep (SWS) or non-rapid-eye-movement (NREM) sleep. The name of NREM sleep is an antonym of another sleep state, rapid eye movement (REM) sleep. REM sleep was discovered later [7], which is characterized by the low-voltage and rapid EEG as wakefulness, the lower level of electromyogram (EMG) relative to that of NREM sleep, and, as the name suggests, the high activity of electrooculogram (EOG). Based on its unique characteristics, REM sleep is also called paradoxical sleep (PS) or active sleep.

In parallel with the discovery of EEG, neuroanatomical research of sleep began with close observation of an infection by Austrian neurologist von Economo [8]. Encephalitis lethargica (also called von Economo disease) became epidemic for about 10 years from 1915. The disease causes inflammation in several brain regions and disturbance of sleep. von Economo proposed that, owing to the correlation between the site of inflammatory lesion and the sleep disorder, (1) an arousal system is localized in the brainstem and provides the waking signal to the forebrain, (2) a sleep center is present in the basal ganglia or the anterior hypothalamus, (3) the posterior hypothalamus is involved in narcolepsy, which is one of the sleep disorders and characterized by frequent transitions between sleep and wakefulness [3]. Later experimental studies have confirmed to some extent von Economo's hypotheses: (1) arousal state is regulated by the reticular activating system (RAS) including monoaminergic and cholinergic neurons in the brainstem [9]; (2) sleep-active GABAergic neurons reside in the ventrolateral preoptic nucleus (VLPO) of the anterior hypothalamus and innervate the arousal systems in the brainstem and hypothalamus [10, 11]; (3) narcolepsy is caused by a loss of neuropeptide orexin/hypocretin, produced by a neuron group in the lateral hypothalamic area (LHA) of posterior hypothalamus, or its receptor [12, 13].

For understanding sleep as a biological process, it is necessary but not sufficient to localize the elements required for regulation of sleep. Circuit-level models of the regulatory mechanisms for sleep have also been proposed and revised several times over the years (e.g., flip-flop switch model for sleep and wakefulness [14–16], brainstem circuits for REM sleep [17–19], and thalamocortical networks for neural oscillations during sleep [20–22]). Even at present, however, it is truly difficult to find clear answers as to basic and practical questions on sleep such as what is the status of the brain during sleep, how does the brain control sleep, and what are the benefits of sleep? In this review, consisting of six sections covering a wide range of research fields, we provide an overview of the current knowledge and methodologies in sleep biology.

Section 1: Cortical dynamics during sleep

What is sleep? This fundamental question must be answered before we can achieve an understanding of the mechanisms and functions of sleep, however, it has been virtually ignored in the biology of sleep. Sleep is generated by the brain. Therefore, a neurophysiological perspective is an essential consideration for biology of sleep [23]. Although sleep is a global phenomenon not only in the brain but also in the whole body, it is not so reliable to judge the sleeping state in many species of animals only by the behavioral aspects such as posture, body motion, and opening and closing of eyelids. As described above, the EEG reflects closely the state of sleep. In fact, EEG is generally used to score sleep stages in mammals including humans. In other words, the neurophysiological features of cerebral cortex are the keys for understanding the state of sleep itself. Unfortunately, however, it is almost impossible to estimate the electrophysiological behavior of individual cells from EEG date, because, in principal, this inverse problem is ill-posed [24].

Ideally, the state of sleep should be understood from a comprehensive picture of the neural activity all over the cortex in vivo with high spatial (subcellular–cellular) and temporal (millisecond–second) resolution. In reality, conventional techniques do not simultaneously meet the requirements [25]. It should be noted that functional imaging of the whole brain with a cellular resolution has been achieved with light-sheet microscopy in the larval zebrafish in vivo [26] and in the larval *Drosophila* ex vivo [27]. At this point, however, it is technically difficult to shed light on the mammalian brain in vivo with light sheets due to the larger size and the lower optical transparency compared to that of the larvae. For obtaining accurate knowledge of individual neural dynamics, currently available methods to measure a wide-range of cerebral activity are partly prospective but still need further improvement. Hence, in the mammalian brain, we can still learn a lot from the so-called 'bottom-up (from parts to the whole)' approaches with in vivo cellular physiological techniques which have continued since the era of classical single-unit studies.

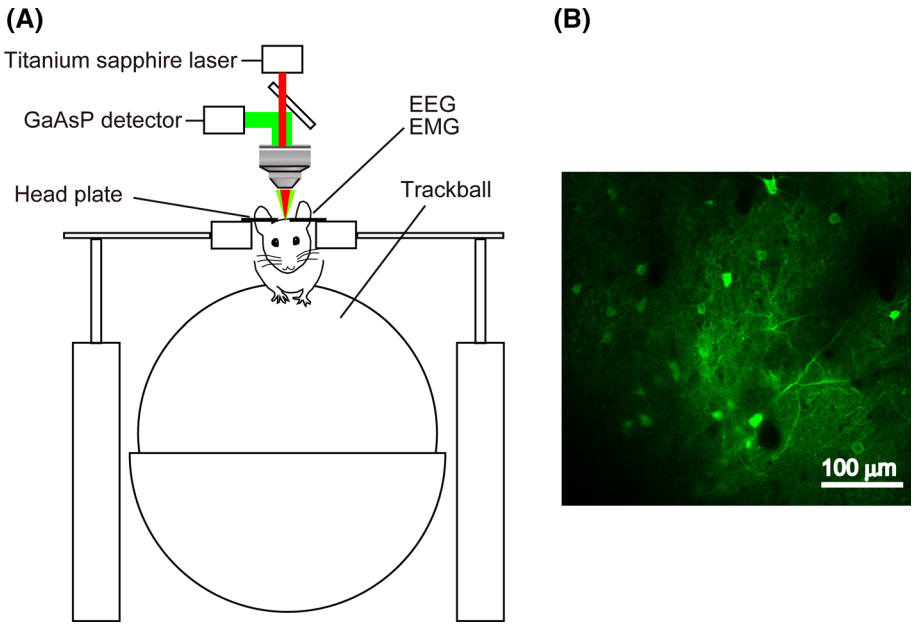

Fig. 1 The two-photon imaging of sleeping mice. **a** Schematic of a two-photon microscopy apparatus for naturally waking/sleeping mice. This experimental apparatus is based on the rig proposed by David Tank's laboratory [118]. Mice can fall asleep spontaneously even under the head-restrained condition owing to a floating trackball. For detection of sleep state, EEG/EMG signals are recorded simultaneously with two-photon imaging. **b** Two-photon image of GCaMP-expressing neurons (*green*) in the layer 2/3 of primary motor cortex

The rhythmic activity in the cortex during SWS is a common feature across areas, species, and dimensions, which is being explored with the use of electrophysiological techniques such as unit, intracellular, and patch-clamp recordings in vivo. In intracellular studies, the sequential depolarized (UP) and hyperpolarized (DOWN) states appear in the membrane potentials of cortical neurons under anesthesia, which are correlated with the oscillations in the EEG [28–30]. The UP/DOWN states are observed also in naturally sleeping animals [31, 32]. In natural sleep, the transition of UP/DOWN states is synchronous not only between nearby neurons [33] but also between distant neurons (up to 12 mm apart) [34]. In extracellular studies, sleep provokes a change in firing behaviors of pyramidal tract neurons: a regular spiking during wakefulness and a rhythmic burst firing during SWS [35]. During SWS, multiple cortical neurons cease to fire in synchrony for a short period, and that the silent periods are associated with surface EEG and local field potentials (LFP) in rats [36, 37] and in humans [38]. The period of spontaneous discharge and the period of quiet in a group are referred to as ON and OFF period, respectively. However, it has not been proven directly whether ON/OFF periods in the extracellular studies correspond to UP/DOWN states in the intracellular studies. In addition, the origin and the mechanisms involved in the rhythmic cortical activity for SWS remain controversial [22, 39].

Two-photon microscopy, another standard technique for cellular physiology in vivo, recently began to be used in sleep research. Currently available two-photon microscopes cannot scan the whole brain in vivo, but can illuminate aspects of cortical dynamics different from electrophysiological insights. Ca^{2+} imaging of cortical neurons reveals that there is a synchrony in activity of cortical neurons in immature mice, but the synchronous activity is not correlated with the ratio of low-frequency (0.5–4.0 Hz) to high-frequency (20–60 Hz) EEG power [40]. Sleep is liked with learning and memory [41, 42] in which morphological changes in dendritic spines have an important role [43, 44]. The observation of dendritic spines in the cortex in vivo have shown that sleep contributes to turnover of dendritic spines in immature mice [45–47]. Furthermore, formation of spines after motor learning depends on SWS but not on REM sleep [47]. Interestingly, visualization of the influx of cerebrospinal fluid into the cortex indicates that clean-up of the interstitial space in the brain is enhanced under ketamine/xylazine anesthesia and declined when awakened from sleep [48]. In the two-photon experiments, however, there is still almost no direct information on the cortex during 'natural' sleep. To gain precise understanding of spatio-temporal dynamics of the cortex during natural sleep, we designed and constructed the two-photon imaging system for naturally sleeping animals, which allow to visualize physiology and morphology of cortical cells during wakefulness, SWS, and REM sleep (Fig. 1). Fluorescence imaging of the sleeping brain makes it possible to directly answer open questions in neurophysiology of sleep (e.g., how do synchronized neural activity during SWS travel within a microscopic field, what type of cortical neurons are activated or inactivated during sleep, does astrocytic activity respond to sleep, and is the motility of microglial fine processes influenced by sleep?).

Section 2: Novel classification of rat thalamic neurons on the basis of single-cell labeling studies

The thalamus not only acts as a relay between subcortical areas and the cerebral cortex but also appears to play an important role in regulating attention and arousal [15]. On the basis of the functions and input–output organizations, the thalamic nuclei have been traditionally divided into three groups, "specific nuclei", "association nuclei", and "nonspecific nuclei" [49]. Specific nuclei receive specific signals from subcortical regions and relay the information topographically to specific regions of the cerebral cortex. Association nuclei receive a few afferents from subcortical regions, but form a strong reciprocal connection with specific regions of the cerebral cortex, especially association areas. Nonspecific nuclei, consisting of the intralaminar and midline thalamic nuclei, project to wide regions of the cerebral cortex rather than to restricted areas as the specific and association nuclei do, and are thought to function in regulating the excitability of wide regions of the cerebral cortex, and in the arousal system.

Recently, a novel classification of thalamic neurons has been proposed: core-type neurons and matrix-type neurons [50, 51]. The core and matrix classification of the thalamic neurons was originally proposed mainly in the sensory thalamus of primate and carnivore. Core-type neurons are found principally in primary sensory thalamic nuclei, express parvalbumin, and send axon fibers to middle layers (mainly layer 4) of restricted cortical fields. In contrast, matrix-type neurons are distributed throughout the thalamic nuclei, and produce calbindin D28 k and project preferentially to the superficial layer of the widespread cortical areas. The most different point of the novel "core-matrix classification" from the "specific–nonspecific classification" is that diffusely projecting thalamic neurons (matrix-type neurons) are not restricted only in the intralaminar and midline thalamic nuclei (nonspecific nuclei), but distributed throughout the thalamus. In rodent brain, however, because no thalamic relay neurons produce parvalbumin, it is difficult to differentiate thalamic neurons only by their chemical or molecular characteristics. Instead, the morphological analysis of cortical axonal arborizations helped us classify the thalamic neurons to core- and matrix-type neurons.

By a single neuron labeling method with Sindbis viral vectors, Kaneko and colleagues analyzed axonal arborization of the rat thalamic neurons in the ventral anterior–ventral lateral nuclei (VA–VL), ventromedial nucleus (VM) [52, 53], posterior medial nucleus [54], and lateral posterior nucleus [55]. The present review focuses on the motor thalamic nuclei, the VA–VL and VM, and tried to apply the core and matrix classification to the results. The motor thalamic nuclei are known to receive inputs from the basal ganglia and cerebellum. The inhibitory afferents from the output nuclei of the basal ganglia principally enter the VM and inhibitory afferent-dominant zone (IZ) of the VA–VL [52, 56], the latter being located in the rostroventral portion of the VA–VL. On the other hand, the caudodorsal portion of the VA–VL receives glutamatergic excitatory afferents mainly from the deep cerebellar nuclei [56, 57], and has been named excitatory subcortical afferent-dominant zone (EZ). Interestingly, in the cerebral cortex, the largest difference in axonal arborizations between VM/IZ neurons (recipients of the basal ganglia input) and EZ neurons (recipient of cerebellar input) was found in their laminar preference. All five of the EZ neurons reconstructed in the single neuron labeling study projected more than 85 % of axon fibers to cortical layers 2–5 like core-type neurons, however, axonal arborizations of single EZ neurons were not restricted in single cortical areas [52]. Thus, there might be at least two subtypes in the core-type neurons: focal-core-type neurons, projecting to the cortex in an area-specific manner; and diffuse-core-type neurons, projecting to multiple areas.

In contrast, VM and IZ neurons sent their axon fibers predominantly to the superficial layer of widespread cortical areas [52, 53], indicating that these neurons are classified into the matrix-type. Thus, as Jones suggested, diffusely projecting thalamic neurons (matrix-type neurons) were not restricted in the intralaminar and midline thalamic nuclei (nonspecific nuclei). Further, it should be noted that the axonal arborizations of matrix-type neurons (IZ and VM neurons) showed a widespread distribution even at a single-neuron level, as compared with those of core-type neurons (EZ neurons). These results suggest that even when a small number of matrix-type neurons are activated, many pyramidal neurons in widespread cortical areas would be activated through the apical dendrites, and may thus be associated with general arousal or attentional mechanisms.

Section 3: Neural mechanisms for inducing fluctuations of autonomic nervous system during REM sleep

REM sleep (or PS) is characterized by EEG desynchronization and muscular atonia. In addition to these tonic events, several phasic events occur during REM sleep, including ponto-geniculo-occipital (PGO) waves, REMs, or fluctuations of the autonomic nervous system, which are expressed as abrupt changes of respiration, heart rate, or blood pressure, etc. PGO waves originate from the pons, conducted to lateral geniculate nucleus, then to occipital (visual) cortex, resulting in activation of the visual system during REM sleep. Since PGO waves occur in close relation with REMs, these events are considered to be involved

in forming visual images of dreaming [58, 59]. Comparing to PGO waves and REMs whose function and mechanisms have been investigated by many researchers [58–61], fluctuations of the autonomic nervous system during REM sleep have been paid less attention, although it is highly probable that the changes of autonomic nervous system during REM sleep reflect emotional changes during REM sleep or dreaming.

Emotional changes during waking are mediated by the amygdala, which is known to be a center of emotion and is involved in expression of emotion such as fear or aggression, and in forming emotional memory. During REM sleep, without external stimuli that cause emotional changes, some endogenous factors induce similar changes to those caused by the external stimuli during waking. Brain imaging studies in human and neural recording studies in animals have revealed that the amygdala becomes active during REM sleep [59, 62, 63]. So, the amygdala would have a role in emotional changes during REM sleep.

Generation of REM sleep is regulated by the cholinergic and glutamatergic neurons in the mesopontine tegmental area including laterodorsal/pedunculopontine tegmental nuclei (LDT/PPT) and the areas ventral to the LDT (named differently among researchers, such as nucleus pontis oralis; nRPo, peri-locus coeruleus α; periLCα, locus subcoeruleus; SLC or sublaterodorsal tegmental nucleus; subLDT) [19, 64]. A population of neurons in these areas exhibits specific firing during REM sleep, which are silent during waking, start to increase firing before the state shift from SWS to REM sleep, become maximally active during REM sleep (REM-on or PS-on neurons). Another population of neurons becomes active both during waking (W) and REM sleep (W/REM or W/PS active neurons) [65–67]. These neurons, by ascending projection to the thalamus, hypothalamus, basal forebrain, or directly to the cerebral cortex, activate cortical neurons to induce desynchronization of EEG, and by descending to the medulla, suppress muscular tonus, or induce muscular atonia during REM sleep [68]. In addition to these tonic firing neurons, there are still a variety of neurons in the LDT/PPT-subLDT area that discharge in a phasic manner during REM sleep. Some of these exhibit phasic firing synchronous with REM or PGO wave and are considered to be involved in the generation of these phasic events [69–71], while there are still many phasic firing neurons whose functions remain to be known.

Blood pressure fluctuation during REM sleep is a remarkable sign during REM sleep mediated by the autonomic nervous system [72]. To elucidate the mechanisms for regulating blood pressure during REM sleep, we focused on the amygdala and the cholinergic neurons in the LDT, crucial components for the generation of emotion and REM sleep. Single neuronal activity in the amygdala and LDT in relation with blood pressure fluctuation during REM sleep was investigated in unanesthetized, head-restrained rats.

Of 108 neurons recorded from the amygdala during sleep–waking cycles, 53 (about 50 %) displayed higher firing during REM sleep. Among them, 36 were most active during REM sleep (PS), and were called PS-active neurons, 12 became active during SWS and REM sleep (SWS/PS active neurons), and five were active during waking and REM sleep (W/PS active neurons). In contrast to PS-on neurons in the brainstem REM sleep-generating center, an increase in firing in these neurons during REM sleep started after the onset of REM sleep and the firing did not continue throughout the REM sleep period but appeared at some limited periods of REM sleep in phasic manner. A neuron in Fig. 2 exhibits phasic firing during REM sleep, which is preceding about 1 s to the increase in blood pressure. Of 50 neurons examined, 11 (22 %) showed firing correlated with and preceding blood pressure fluctuation during REM sleep, while another seven (14 %) showed correlation but the firing increase delayed to blood pressure changes.

Similar properties of neurons were obtained from the LDT. The cholinergic neurons in the LDT were discriminated form other types of neurons by the shape of action potentials (spikes) with longer duration of positive components and smaller amplitude, and longer duration of negative components [73]. Seven of 17 (41 %) LDT neurons discharged in close correlation with and preceding blood pressure fluctuation during REM sleep. Among them, six neurons were judged to be cholinergic from the spike shape. These results indicate that the amygdala and the cholinergic neurons in the LDT have a crucial role in driving blood pressure fluctuation during REM sleep.

The question that remains is: How is the blood pressure fluctuation during REM sleep regulated by these structures? It is required to clarify whether the emotional system is activated by the REM sleep-generating system or the REM sleep-generating system is driven by the emotional system during REM sleep. Preceding works have shown that after the brain transection at the mid-pontine level in cats, muscular atonia and REM, basic signs of REM sleep appear periodically, but blood pressure fluctuation disappeared, indicating that blood pressure fluctuation during REM sleep is driven by the forebrain structures rostral to the transection [74, 75]. Neuroanatomical studies have revealed that the LDT send ascending projections to various forebrain structures including amygdala [76]. These studies lead us to hypothesize that the ascending cholinergic neurons in the LDT activate amygdala neurons, which drive blood pressure fluctuation during REM sleep. It is still probable that the descending drive from the amygdala (which causes blood pressure increase

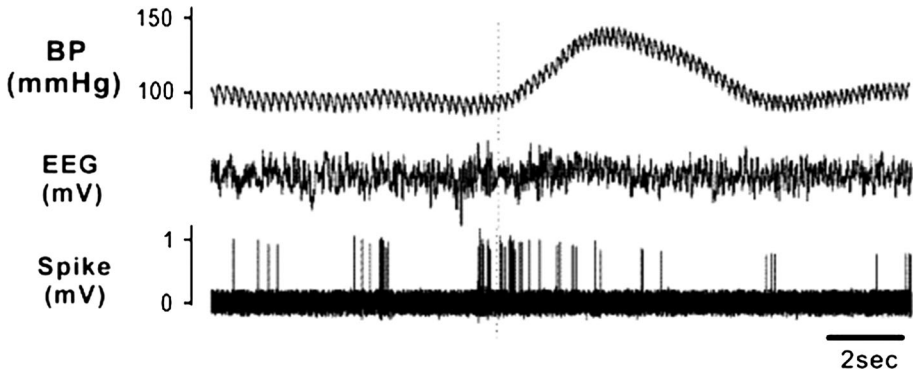

Fig. 2 Single-unit recording from neurons in the amygdala during REM sleep. PS-active neurons recorded from the amygdala, which shows phasic firing preceding blood pressure (BP) increase (*a vertical dashed line*) during REM sleep

responding to emotional events during waking) would also activate LDT during REM sleep. Further studies are required to elucidate the exact relations between the emotion system and the REM sleep-generating system during REM sleep.

Section 4: Whole-brain imaging to identify sleep-controlling circuits

The sleep–wake cycle is an organism-level biological phenomenon. The states are behaviorally and electrophysiologically defined, and its regulatory mechanism apparently implemented in the cellular circuit layer. However, experimental approach to such organism-level functions are still challenging due to the complexity of multi-cellular organisms. Systemic identification of cellular components and their connections are still needed to fully understand the features of sleep-controlling neural circuits. From such a viewpoint, recent technologies of three-dimensional (3D) imaging with tissue-clearing methods are providing a novel approach of identifying the sleep circuit. Now, researchers have been able to observe 3D cellular-to-subcellular structures of cleared tissues with two-photon or even single-photon microscopies [77–79] and a widefield fluorescence microscopy [80]. In addition, combination of efficient clearing methods with a light-sheet microscopy enables high-throughput imaging at the whole-brain scale [81–83]. These studies performed 3D imaging experiments related to circuit mapping such as axon tracing, 3D-immunohistochemical analysis, and rabies virus-based synaptic tracing [78–80, 84–89].

One of the author's groups also developed a high-throughput 3D imaging and analysis pipeline termed clear, unobstructed brain/body imaging cocktails and computational analysis (CUBIC) [90, 91]. CUBIC aims to provide a comprehensive cell detection and analysis throughout a whole brain or even a whole body. This purpose was achieved by three steps of CUBIC: (1) an efficient and reproducible tissue clearing, (2) a high-throughput whole-organ or whole-body imaging of the cleared tissue with a light-sheet microscopy, and (3) computational analyses of the acquired 3D images to extract biological information.

Clearing reagents of CUBIC were developed based on a hydrophilic reagent Sca/e [77], and contain aminoalcohols instead of glycerol in the original recipe. The CUBIC clearing reagents can clear tissues efficiently and reproducibly by its ability of not only lipid removal and refractive index adjustment but also an active heme removal. Such efficient clearing ability as well as preservation of fluorescent protein signals enable 3D fluorescent imaging of whole organs or even a whole body by using light-sheet microscopy. Many applications of CUBIC in cell and cellular circuit studies were demonstrated. For example, 3D immunohistochemical analysis of the hypothalamic regions identified neurons with specific neuropeptides in the suprachiasmatic nucleus. Extractions of anatomical structures and their quantitative comparison in a pathological state were also performed. In a more complicated case, 3D images of whole-brain neural activities with or without light stimuli were collected by using Arc-dVenus transgenic animals [92]. These images were aligned to a 'standard brain' and then subtraction of these neural activity signals was calculated to directly compare two brains in the two experimental conditions. The informatics analysis clearly depicted the brain regions responsible for the light stimuli. Thus, CUBIC, together with other tissue clearing and imaging methods, have the potential to facilitate our understandings about sleep circuits in the brain.

Section 5: Regulation of sleep homeostasis and energy metabolism focusing on PPAR and ketone body

Multiple studies have demonstrated an association between sleep and energy metabolism. Numerous epidemiologic studies have provided evidence that short-duration or poor-quality sleep induces appetite dysregulation, increases

body mass index (BMI), and decreases glucose tolerance and insulin sensitivity, which increases the risk of type 2 diabetes [93–96]. However, the mechanisms of the relationship between sleep restriction and metabolic disorders have remained elusive. We recently demonstrated that peroxisome proliferator-activated receptors (PPARs) and ketone bodies (acetoacetate, AcAc and β-hydroxybutyrate, BHB), both of which are important for energy metabolism, also participate in sleep regulation.

PPARs are transcription factors and are members of the steroid hormone nuclear receptor superfamily. PPARs include three known isotypes, PPARα, PPARβ/δ, and PPARγ; these isotypes have many shared biological effects including effects on lipid and glucose metabolism [97–99]. The expression of PPARα is known to be directly controlled by the Clock gene [100, 101]. It was reported that mice treated with bezafibrate, an anti-hyperlipidemic PPAR ligand, showed phase-advanced circadian locomotor activity [102]. We therefore examined whether PPARs are involved in sleep regulation in the following experiments.

In mice provided bezafibrate-supplemented food for 2 weeks, the acrophase of wakefulness and NREM sleep rhythm was advanced by approximately 3 h in comparison with controls [103]. In bezafibrate-treated mice, slow-wave activity (SWA, power density of EEG delta band between 0.5 and 4.0 Hz) during NREM sleep, an indicator of sleep depth, was greater than that in controls over 24 h [103]. These bezafibrate-treated mice showed no significant rebound in SWA for NREM sleep after sleep deprivation [103]. Increased SWA during NREM sleep was also observed in mice with intracerebroventricular (ICV) injection of bezafibrate (unpublished data), suggesting that the enhancement of SWA observed in our study was due to central rather than peripheral effects. These results suggest that central PPARs action plays an important role in the regulation of circadian rhythms and sleep homeostasis.

In that study, bezafibrate treatment significantly affected plasma levels of ketone bodies. Ketone bodies are generated from the breakdown of fatty acids and become major fuels in most tissues under conditions of reduced glucose availability, such as starvation or consumption of a high-fat diet [104, 105]. We have found that mice treated with bezafibrate showed increased AcAc and decreased BHB in plasma, accompanied by increased SWA in NREM sleep [103]. In addition, mice treated with bezafibrate showed increased expression of genes encoding ketogenic enzymes such as 3-hydroxy-3-methylglutarate-CoA synthase 2 (Hmgcs2) and carnitine palmitoyltransferase 1a (Cpt1a) in the liver. PPARα activation leads to stimulation of ketogenesis, and both Hmgcs2 and Cpt1a are downstream genes of PPARα [104, 106]. However, it was not clear whether ketone body metabolism itself in the brain was responsible for the changes in sleep homeostasis. In order to address this issue, we next investigated the role of ketone bodies in the regulation of sleep homeostasis.

We found that sleep deprivation for 6 h induced a marked increase in plasma AcAc and BHB accompanied by enhanced SWA during subsequent NREM sleep [107]. In addition, sleep deprivation increased the expression of ketogenic genes (PPARα and Hmgcs2) and decreased ketolytic enzyme gene expression (succinyl-CoA-3-oxoacid CoA transferase, Scot, and acetoacetyl-CoA synthetase, Aacs) in the hypothalamus and cortex. In contrast, sleep deprivation did not affect the expression of these genes in the liver. A recent study reported that astrocytes in the brain can produce ketone bodies [108, 109], although the liver is known to be the major organ that supplies the brain with ketone bodies [110]. Our results suggest that sleep deprivation may activate ketogenesis mainly in the brain, rather than in the liver.

In addition, direct injection of AcAc into the lateral ventricle decreased the amount of REM sleep and increased SWA during NREM sleep, while BHB injection did not affect it [107]. Circulating AcAc can suppress the activity of vesicular glutamate transporters (VGLUTs) leading to decreased glutamate release and consequent suppression of excitatory neurotransmission [111]. Cortical projections of the glutamatergic system are known to be important for cortical activation and wakefulness [112]. We used in vivo microdialysis to investigate whether icv injection of AcAc would suppress glutamate release, and confirmed that ICV injection of AcAc did decrease glutamate release in the lateral ventricle, while injection of vehicle or BHB did not affect glutamate release [107]. These results suggest that AcAc would be more important for regulation of sleep homeostasis than BHB.

At the present time, we can not determine the origin of the change in peripheral plasma ketone bodies because of technical limitations in the measurement of ketone bodies in mouse brains. We are now planning to investigate the effects of central inhibition of ketogenesis on regulation of sleep homeostasis in a future study. In conclusion, sleep deprivation increased ketone bodies and ketogenesis-related genes accompanied by enhanced SWA during subsequent NREM sleep. ICV injection of ketone body (acetoacetate) enhanced SWA and suppressed glutamate release. These results suggest that sleep loss activates brain lipid metabolism and increases ketone body (AcAc), which results in suppression of neuronal activity, leading to deeper sleep. Our results provide additional evidence for an interaction of energy metabolism and sleep/wake regulation, and indicate that alteration of metabolic function affects sleep quality. In this regard, the development of a detailed nutritional approach to the treatment of sleep disorders may contribute to a novel therapeutic strategy.

Section 6: A forward genetic approach to identifying novel genes regulating sleep/wakefulness behavior

Advances in optogenetic and pharmacogenetic research enable us to directly examine whether the specific neural circuitry can regulate sleep/wakefulness states. However, an acute effect on sleep/wakefulness behavior induced by optogenetic and pharmacogenetic manipulation does not necessarily mean that the neural circuitry is responsible for the physiological sleep/wakefulness. Furthermore, prolonged manipulation to suppress NREM sleep or REM sleep eventually becomes less effective because there is a homeostatic drive to restore deprived NREM sleep or REM sleep. The homeostatic regulation of sleep/wakefulness and the molecular entity of "sleep need" remain unknown.

Genetic components shape sleep/wakefulness. Monozygotic twin pairs show higher similarity in spectrum profiles of EEG during NREM sleep compared with dizygotic twin pairs [113]. Each inbred mouse strain has specific sleep characteristics such as amount of waking time, NREM sleep duration [114], and the distribution of EEG spectral peak frequency [115]. Along these lines, there have been many studies examining sleep/wakefulness of gene-modified mice, which is called a reverse genetic approach [116]. Except for the serendipitous discovery of narcoleptic phenotypes of orexin-deficient mice [12], most studies of gene-modified mice turned out to confirm the results that had been predicted from pharmacological studies because many of these genes are selected based on their pharmacological effects on sleep. The normal wake duration of histidine decarboxylase-deficient mice, which lack histamine, a wake-promoting neurotransmitter, enhanced our awareness of a possible masking of sleep phenotype by tight homeostatic feedback [117]. Another substantial drawback of research using gene-modified mice, most of which is gene-deficient type modification, is that homozygous mutants are lethal or unhealthy and therefore cannot be examined or are not suitable for sleep analysis.

In contrast, forward genetic research is free from any assumptions and can begin from a clear sleep abnormality to find a gene or gene mutation responsible for sleep/wakefulness. The dominant screening method is based on dominant phenotypes, which appear in heterozygous mutants. The biggest drawback of forward genetic research in mammals is that it requires vast amounts of labor, time, and money. Because we employed EEG/EMG-based sleep/wakefulness staging, we need to develop a team composed of well-trained surgeons for implanting EEG/EMG electrodes, and we also require a streamlined system of EEG/EMG recording and staging.

Let us briefly consider the number of mutagenized mice we have to examine. The expected number ($E1$) of mutagenized mice showing sleep abnormality is proportional to the number of mice screened (N).

$$E1 = N \times P1$$

where $P1$ is the probability of mutagenized mice having a sleep phenotype. For qualitative data such as total wake time, we select mice showing sleep/wakefulness parameters that deviate from an average value by three times the standard deviation. When a parameter follows a normal distribution, the probability of deviation beyond three standard deviations is 0.0028. Thus, $P1$ should be higher than this value.

Because the majority of screened mice show sleep abnormalities by chance or due to the summation of weak effects derived from multiple gene mutations, most of their offspring do not show sleep abnormality similar to their father. The expected number of mouse pedigrees showing sleep abnormality ($E2$) is given by

$$E2 = E1 \times P2$$

where $P2$ is the probability of heritable sleep abnormality recognized in the offspring of a founder mouse.

We usually examine whether sleep abnormality is heritable using 15–20 male mice of N2; then, if the pedigree passes the heritability test, we obtain a total of 60–100 N2 male mice for linkage analysis. Linkage analysis gives us a LOD score, which indicates the likelihood of genetic loci linked to a certain phenotype. Importantly, the LOD score depends on the extent of the phenotype. As shown in Fig. 3, I performed simulations examining the relationships among LOD score, daily total wake time, and the number of N2 mice, showing that the LOD score is dependent on daily wake time and on the number of N2 mice. Higher LOD scores mean a stronger sleep phenotype if the number of N2 mice is constant. Another simulation also showed that the pedigree having a LOD score < 7–10, when based on the analysis of 80 N2 mice, is not suitable for further investigation because the sleep phenotype is so weak that it is difficult to obtain statistically significant differences when compared with wild-type littermates. However, it is well worth examining the homozygous mutants because homozygous mutants may have stronger and more robust sleep abnormality than heterozygous mutants. The expected number of mouse pedigrees showing high LOD score is given by

$$E3 = E2 \times P3$$

where $P3$ is the probability of N2 mice of heritable sleep-abnormal pedigrees showing a sufficiently high LOD score.

The number (Y) of mutagenized mice necessary to establish at least one pedigree showing a high LOD score with the probability X is given by

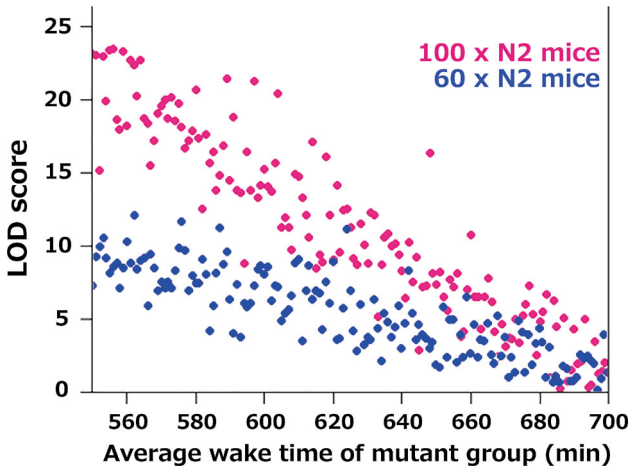

Fig. 3 LOD score depends on the strength of the sleep phenotype. Simulation of the LOD score when the average wake time of the mutant group (half of all N2 mice examined) of the pedigree varies using statistical software R. *Blue and magenta circles* indicate the simulated LOD score and daily wake time using 60 and 80 N2 mice, respectively. Half of the N2 mice have the wild-type allele, and their daily wake time is set as 740 min

$$Y = \frac{\log(1 - X)}{\log(1 - P1 \times P2 \times P3)}$$

If we assume the values as follows: $P1 = 0.005$, $P2 = 0.25$, $P3 = 0.3$, approximately 3000 mutagenized mice are necessary to obtain at least one pedigree showing robust sleep abnormality with the probability of 0.7. Thus, the number of animals is highly important in a forward genetics project.

We have set up an EEG/EMG recording system that can obtain EEG and EMG signals simultaneously from up to 80 mice. A team of well-trained research staff and students has been working on EEG/EMG electrode surgery, recording, and staging of sleep/wakefulness of up to 80 mutagenized mice per week (Fig. 4). Through this effort, we have established several pedigrees showing sleep abnormalities, including *Sleepy* and *Dreamless* mutant pedigrees. *Sleepy* mutant mice are characterized by shorter daily wake time, while *Dreamless* mutant mice show reduced time spent in REM sleep and short REM sleep episode duration. We are now working on to elucidate how the *Sleepy* and *Dreamless* genes regulate sleep and are continuing the screening of mutagenized animals to establish another sleep abnormal pedigree and identify another sleep regulatory gene.

Conclusions

In this review we have presented six differently focused pictures of what is going on in the current biology of sleep. Two-photon imaging in the sleeping brain is a promising method for discovering novel phenomena in the cortex during sleep. Single-cell tracing with Sindbis viral vectors discloses the exact axonal arborizations of thalamic neurons. Single-unit recording during sleep shows that the amygdala and the LDT are involved in the fluctuating blood pressure during REM sleep. Fast-evolving tissue-clearing technologies are promoting a systematic analysis of the brain without missing out on any details. The studies focusing on energy metabolism indicate a close connection between sleep homeostasis and ketogenesis via PPARα. The large-scale forward genetics in mice has already identified several novel genes involved in the regulation of sleep and wakefulness. Needless to emphasize, sleep is a global state in a living animal. The approaches shown in this review provide fruitful information on each aspect of sleep, but are still in the process to achieve a systematic understanding of sleep. Further exploring sleep requires seeing everything about sleep form so many sides. Thus, the progress of sleep biology is expected to be accelerated by development of methodologies, further multidisciplinary studies, and a theoretical integration of the experimental evidence on sleep. Finally, we hope that many inspired by this review will join in this quest to find out the truth about this mysterious research field.

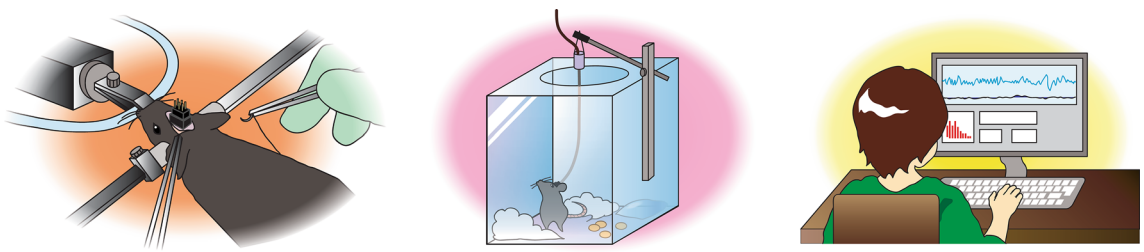

Fig. 4 EEG/EMG-based screening of mutagenized mice. (*Left*) It takes 20–40 min to implant an EEG/EMG electrode in a mouse under isoflurane anesthesia. (*Middle*) After full recovery from the surgery, the mouse is tethered with a thin and flexible cable to transmit EEG/EMG signals. The tethered mouse can move freely in a cage. (*Right*) Each epoch of the recorded EEG/EMG is visually scored using a staging-assist software

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

1. Hobson JA (2005) Sleep is of the brain, by the brain and for the brain. Nature 437:1254–1256. doi:10.1038/nature04283
2. Berger H (1929) Über das Elektrenkephalogramm des Menschen. Arch Psychiatr Nervenkr 87:527–570
3. Von Economo CF (1930) Sleep as a problem of localization. J Nerv Ment Dis 71:1–5
4. Adrian ED, Matthews BH (1934) The interpretation of potential waves in the cortex. J Physiol 81:440–471
5. Loomis AL, Harvey EN, Hobart G (1935) Potential rhythms of the cerebral cortex during sleep. Science 81:597–598. doi:10.1126/science.81.2111.597
6. Loomis AL, Harvey EN, Hobart G (1935) Further observations on the potential rhythms of the cerebral cortex during sleep. Science 82:198–200. doi:10.1126/science.82.2122.198
7. Aserinsky E, Kleitman N (1953) Regularly occurring periods of eye motility, and concomitant phenomena, during sleep. Science 118:273–274. doi:10.1126/science.118.3062.273
8. Von Economo CF (1931) Encephalitis lethargica: its sequelae and treatment. Oxford University Press, New York
9. Moruzzi G, Magoun HW (1949) Brain stem reticular formation and activation of the EEG. Electroencephalogr Clin Neurophysiol 1:455–473
10. Sherin JE, Shiromani PJ, McCarley RW, Saper CB (1996) Activation of ventrolateral preoptic neurons during sleep. Science 271:216–219. doi:10.1126/science.271.5246.216
11. Sherin JE, Elmquist JK, Torrealba F, Saper CB (1998) Innervation of histaminergic tuberomammillary neurons by GABAergic and galaninergic neurons in the ventrolateral preoptic nucleus of the rat. J Neurosci 18:4705–4721
12. Chemelli RM, Willie JT, Sinton CM et al (1999) Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. Cell 98:437–451. doi:10.1016/S0092-8674(00)81973-X
13. Lin L, Faraco J, Li R et al (1999) The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. Cell 98:365–376. doi:10.1016/S0092-8674(00)81965-0
14. Saper CB, Scammell TE, Lu J (2005) Hypothalamic regulation of sleep and circadian rhythms. Nature 437:1257–1263. doi:10.1038/nature04284
15. Saper CB, Fuller PM, Pedersen NP et al (2010) Sleep state switching. Neuron 68:1023–1042. doi:10.1016/j.neuron.2010.11.032
16. Saper CB, Chou TC, Scammell TE (2001) The sleep switch: hypothalamic control of sleep and wakefulness. Trends Neurosci 24:726–731. doi:10.1016/S0166-2236(00)02002-6
17. Luppi P-H, Clément O, Fort P (2013) Paradoxical (REM) sleep genesis by the brainstem is under hypothalamic control. Curr Opin Neurobiol 23:786–792. doi:10.1016/j.conb.2013.02.006
18. Luppi P-H, Gervasoni D, Verret L et al (2007) Paradoxical (REM) sleep genesis: the switch from an aminergic–cholinergic to a GABAergic–glutamatergic hypothesis. J Physiol Paris 100:271–283. doi:10.1016/j.jphysparis.2007.05.006
19. Luppi PH, Clement O, Sapin E et al (2012) Brainstem mechanisms of paradoxical (REM) sleep generation. Pflugers Arch Eur J Physiol 463:43–52. doi:10.1007/s00424-011-1054-y
20. Steriade M, McCormick DA, Sejnowski TJ (1993) Thalamocortical oscillations in the sleeping and aroused brain. Science 262:679–685
21. Crunelli V, Hughes SW (2010) The slow (<1 Hz) rhythm of non-REM sleep: a dialogue between three cardinal oscillators. Nat Neurosci 13:9–17. doi:10.1038/nn.2445
22. Crunelli V, David F, Lőrincz ML, Hughes SW (2015) The thalamocortical network as a single slow wave-generating unit. Curr Opin Neurobiol 31:72–80. doi:10.1016/j.conb.2014.09.001
23. Jouvet M (1967) Neurophysiology of the states of sleep. Physiol Rev 47:117–177
24. Grech R, Cassar T, Muscat J et al (2008) Review on solving the inverse problem in EEG source analysis. J Neuroeng Rehabil 5:25. doi:10.1186/1743-0003-5-25
25. Sejnowski TJ, Churchland PS, Movshon JA (2014) Putting big data to good use in neuroscience. Nat Neurosci 17:1440–1441. doi:10.1038/nn.3839
26. Ahrens MB, Orger MB, Robson DN et al (2013) Whole-brain functional imaging at cellular resolution using light-sheet microscopy. Nat Methods 10:413–420. doi:10.1038/nmeth.2434
27. Lemon WC, Pulver SR, Höckendorf B et al (2015) Whole-central nervous system functional imaging in larval Drosophila. Nat Commun 6:7924. doi:10.1038/ncomms8924
28. Steriade M, Nuñez A, Amzica F (1993) A novel slow (<1 Hz) oscillation of neocortical neurons in vivo: depolarizing and hyperpolarizing components. J Neurosci 13:3252–3265
29. Steriade M, Nuñez A, Amzica F (1993) Intracellular analysis of relations between the slow (<1 Hz) neocortical oscillation and other sleep rhythms of the electroencephalogram. J Neurosci 13:3266–3283
30. Metherate R, Cox CL, Ashe JH (1992) Cellular bases of neocortical activation: modulation of neural oscillations by the nucleus basalis and endogenous acetylcholine. J Neurosci 12:4701–4711
31. Timofeev I, Grenier F, Steriade M (2001) Disfacilitation and active inhibition in the neocortex during the natural sleep–wake cycle: an intracellular study. Proc Natl Acad Sci 98:1924–1929. doi:10.1073/pnas.98.4.1924
32. Steriade M, Timofeev I, Grenier F (2001) Natural waking and sleep states: a view from inside neocortical neurons. J Neurophysiol 85:1969–1985. doi:10.1016/j.neuroimage.2009.03.074

33. Chauvette S, Volgushev M, Timofeev I (2010) Origin of active states in local neocortical networks during slow sleep oscillation. Cereb Cortex 20:2660–2674. doi:10.1093/cercor/bhq009
34. Volgushev M, Chauvette S, Mukovski M, Timofeev I (2006) Precise long-range synchronization of activity and silence in neocortical neurons during slow-wave oscillations. J Neurosci 26:5665–5672. doi:10.1523/JNEUROSCI.0279-06.2006
35. Evarts EV (1964) Temporal patterns of discharge of pyramidal tract neurons during sleep and waking in the monkey. J Neurophysiol 27:152–171
36. Vyazovskiy VV, Olcese U, Hanlon EC et al (2011) Local sleep in awake rats. Nature 472:443–447. doi:10.1038/nature10009
37. Vyazovskiy VV, Olcese U, Lazimy YM et al (2009) Cortical firing and sleep homeostasis. Neuron 63:865–878. doi:10.1016/j.neuron.2009.08.024
38. Nir Y, Staba RJ, Andrillon T et al (2011) Regional slow waves and spindles in human sleep. Neuron 70:153–169. doi:10.1016/j.neuron.2011.02.043
39. McCormick DA, McGinley MJ, Salkoff DB (2015) Brain state dependent activity in the cortex and thalamus. Curr Opin Neurobiol 31:133–140. doi:10.1016/j.conb.2014.10.003
40. Gonçalves JT, Anstey JE, Golshani P, Portera-Cailliau C (2013) Circuit level defects in the developing neocortex of Fragile X mice. Nat Neurosci 16:903–909. doi:10.1038/nn.3415
41. Diekelmann S, Born J (2010) The memory function of sleep. Nat Rev Neurosci 11:114–126. doi:10.1038/nrn2762
42. Stickgold R (2005) Sleep-dependent memory consolidation. Nature 437:1272–1278. doi:10.1038/nature04286
43. Lamprecht R, LeDoux J (2004) Structural plasticity and memory. Nat Rev Neurosci 5:45–54. doi:10.1038/nrn1301
44. Bailey CH, Kandel ER (1993) Structural changes accompanying memory storage. Annu Rev Physiol 55:397–426. doi:10.1146/annurev.ph.55.030193.002145
45. Yang G, Gan W-B (2012) Sleep contributes to dendritic spine formation and elimination in the developing mouse somatosensory cortex. Dev Neurobiol 72:1391–1398. doi:10.1002/dneu.20996
46. Maret S, Faraguna U, Nelson AB et al (2011) Sleep and waking modulate spine turnover in the adolescent mouse cortex. Nat Neurosci 14:1418–1420. doi:10.1038/nn.2934
47. Yang G, Lai CSW, Cichon J et al (2014) Sleep promotes branch-specific formation of dendritic spines after learning. Science 344:1173–1178. doi:10.1126/science.1249098
48. Xie L, Kang H, Xu Q et al (2013) Sleep drives metabolite clearance from the adult brain. Science 342:373–377. doi:10.1126/science.1241224
49. Groenewegen HJ, Witter MP (2004) Thalamus. In: The rat nervous system. Elsevier, Amsterdam, pp 407–453
50. Jones EG (1998) Viewpoint: the core and matrix of thalamic organization. Neuroscience 85:331–345. doi:10.1016/S0306-4522(97)00581-2
51. Jones EG (2001) The thalamic matrix and thalamocortical synchrony. Trends Neurosci 24:595–601. doi:10.1016/S0166-2236(00)01922-6
52. Kuramoto E, Furuta T, Nakamura KC et al (2009) Two types of thalamocortical projections from the motor thalamic nuclei of the rat: a single neuron-tracing study using viral vectors. Cereb Cortex 19:2065–2077. doi:10.1093/cercor/bhn231
53. Kuramoto E, Ohno S, Furuta T et al (2015) Ventral medial nucleus neurons send thalamocortical afferents more widely and more preferentially to layer 1 than neurons of the ventral anterior–ventral lateral nuclear complex in the rat. Cereb Cortex 25:221–235. doi:10.1093/cercor/bht216
54. Ohno S, Kuramoto E, Furuta T et al (2012) A morphological analysis of thalamocortical axon fibers of rat posterior thalamic nuclei: a single neuron tracing study with viral vectors. Cereb Cortex 22:2840–2857. doi:10.1093/cercor/bhr356
55. Nakamura H, Hioki H, Furuta T, Kaneko T (2015) Different cortical projections from three subdivisions of the rat lateral posterior thalamic nucleus: a single-neuron tracing study with viral vectors. Eur J Neurosci 41:1294–1310. doi:10.1111/ejn.12882
56. Kuramoto E, Fujiyama F, Nakamura KC et al (2011) Complementary distribution of glutamatergic cerebellar and GABAergic basal ganglia afferents to the rat motor thalamic nuclei. Eur J Neurosci 33:95–109. doi:10.1111/j.1460-9568.2010.07481.x
57. Schwarz C, Schmitz Y (1997) Projection from the cerebellar lateral nucleus to precerebellar nuclei in the mossy fiber pathway is glutamatergic: a study combining anterograde tracing with immunogold labeling in the rat. J Comp Neurol 381:320–334. doi:10.1002/(SICI)1096-9861(19970512)381:3<320:AID-CNE5>3.0.CO;2-4
58. Roffwarg HP, Dement WC, Muzio JN, Fisher C (1962) Dream imagery: relationship to rapid eye movements of sleep. Arch Gen Psychiatry 7:235. doi:10.1001/archpsyc.1962.01720040001001
59. Miyauchi S, Misaki M, Kan S et al (2009) Human brain activity time-locked to rapid eye movements during REM sleep. Exp Brain Res 192:657–667. doi:10.1007/s00221-008-1579-2
60. Conduit R, Crewther SG, Coleman G (2004) Spontaneous eyelid movements (ELMS) during sleep are related to dream recall on awakening. J Sleep Res 13:137–144. doi:10.1111/j.1365-2869.2004.00397.x
61. Datta S (2011) Pontine-wave generator: a key player in REM sleep-dependent memory consolidation. In: Mallick BN, Pandi-Perumal SR, McCarley RW, Morrison AR (eds) Rapid eye movement sleep. Cambridge University Press, Cambridge, pp 140–150
62. Maquet P, Péters J, Aerts J et al (1996) Functional neuroanatomy of human rapid-eye-movement sleep and dreaming. Nature 383:163–166. doi:10.1038/383163a0
63. Sanford LD, Ross RJ (2011) Amygdalar regulation of REM sleep. In: Mallick BN, Pandi-Perumal SR, McCarley RW, Morrison AR (eds) Rapid eye movement sleep. Cambridge University Press, Cambridge, pp 110–120
64. Sakai K, Crochet S, Onoe H (2001) Pontine structures and mechanisms involved in the generation of paradoxical (REM) sleep. Arch Ital Biol 139:93–107
65. El Mansari M, Sakai K, Jouvet M (1989) Unitary characteristics of presumptive cholinergic tegmental neurons during the sleep–waking cycle in freely moving cats. Exp Brain Res 76:519–529. doi:10.1007/BF00248908
66. Steriade M, Datta S, Paré D et al (1990) Neuronal activities in brain-stem cholinergic nuclei related to tonic activation processes in thalamocortical systems. J Neurosci 10:2541–2559
67. Kayama Y, Ohta M, Jodo E (1992) Firing of "possibly" cholinergic neurons in the rat laterodorsal tegmental nucleus during sleep and wakefulness. Brain Res 569:210–220. doi:10.1016/0006-8993(92)90632-J
68. Sakai K, Koyama Y (1996) Are there cholinergic and non-cholinergic paradoxical sleep-on neurones in the pons? NeuroReport 7:2449–2453. doi:10.1097/00001756-199611040-00009
69. Saito H, Sakai K, Jouvet M (1977) Discharge patterns of the nucleus parabrachialis lateralis neurons of the cat during sleep and waking. Brain Res 134:59–72. doi:10.1016/0006-8993(77)90925-8
70. Sakai K, El Mansari M, Jouvet M (1990) Inhibition by carbachol microinjections of presumptive cholinergic PGO-on neurons in freely moving cats. Brain Res 527:213–223. doi:10.1016/0006-8993(90)91140-C

71. Steriade M, Paré D, Datta S et al (1990) Different cellular types in mesopontine cholinergic nuclei related to ponto-geniculo-occipital waves. J Neurosci 10:2560–2579
72. Verrier RL, Harper RM, Hobson JA (2005) Cardiovascular physiology: central and autonomic regulation. In: Principles and practice of sleep medicine. Elsevier, Amsterdam, pp 192–202
73. Koyama Y, Honda T, Kusakabe M et al (1998) In vivo electrophysiological distinction of histochemically-identified cholinergic neurons using extracellular recording and labelling in rat laterodorsal tegmental nucleus. Neuroscience 83:1105–1112. doi:10.1016/S0306-4522(97)00439-9
74. Jouvet M (1972) The role of monoamines and acetylcholine-containing neurons in the regulation of the sleep–waking cycle. Ergeb Physiol 64:166–307
75. Kanamori N (1995) Effects of decerebration on blood pressure during paradoxical sleep in cats. Brain Res Bull 37:545–549. doi:10.1016/0361-9230(95)00030-I
76. Satoh K, Fibiger HC (1986) Cholinergic neurons of the laterodorsal tegmental nucleus: efferent and afferent connections. J Comp Neurol 253:277–302. doi:10.1002/cne.902530302
77. Hama H, Kurokawa H, Kawano H et al (2011) Scale: a chemical approach for fluorescence imaging and reconstruction of transparent mouse brain. Nat Neurosci 14:1481–1488. doi:10.1038/nn.2928
78. Ke M-T, Fujimoto S, Imai T (2013) SeeDB: a simple and morphology-preserving optical clearing agent for neuronal circuit reconstruction. Nat Neurosci 16:1154–1161. doi:10.1038/nn.3447
79. Chung K, Wallace J, Kim S-Y et al (2013) Structural and molecular interrogation of intact biological systems. Nature 497:332–337. doi:10.1038/nature12107
80. Kuwajima T, Sitko AA, Bhansali P et al (2013) ClearT: a detergent- and solvent-free clearing method for neuronal and non-neuronal tissue. Development 140:1364–1368. doi:10.1242/dev.091844
81. Dodt H-U, Leischner U, Schierloh A et al (2007) Ultramicroscopy: three-dimensional visualization of neuronal networks in the whole mouse brain. Nat Methods 4:331–336. doi:10.1038/nmeth1036
82. Ertürk A, Becker K, Jährling N et al (2012) Three-dimensional imaging of solvent-cleared organs using 3DISCO. Nat Protoc 7:1983–1995. doi:10.1038/nprot.2012.119
83. Tomer R, Ye L, Hsueh B, Deisseroth K (2014) Advanced CLARITY for rapid and high-resolution imaging of intact tissues. Nat Protoc 9:1682–1697. doi:10.1038/nprot.2014.123
84. Belle M, Godefroy D, Dominici C et al (2014) A simple method for 3D analysis of immunolabeled axonal tracts in a transparent nervous system. Cell Rep 9:1191–1201. doi:10.1016/j.celrep.2014.10.037
85. Ertürk A, Mauch CP, Hellal F et al (2011) Three-dimensional imaging of the unsectioned adult spinal cord to assess axon regeneration and glial responses after injury. Nat Med 18:166–171. doi:10.1038/nm.2600
86. Renier N, Wu Z, Simon DJJ et al (2014) iDISCO: a simple, rapid method to immunolabel large tissue samples for volume imaging. Cell 159:896–910. doi:10.1016/j.cell.2014.10.010
87. Niedworok CJ, Schwarz I, Ledderose J et al (2012) Charting monosynaptic connectivity maps by two-color light-sheet fluorescence microscopy. Cell Rep 2:1375–1386. doi:10.1016/j.celrep.2012.10.008
88. Menegas W, Bergan JF, Ogawa SK et al (2015) Dopamine neurons projecting to the posterior striatum form an anatomically distinct subclass. eLife. doi:10.7554/eLife.10032
89. Lerner TN, Shilyansky C, Davidson TJ et al (2015) Intact-brain analyses reveal distinct information carried by SNc dopamine subcircuits. Cell 162:635–647. doi:10.1016/j.cell.2015.07.014
90. Susaki EA, Tainaka K, Perrin D et al (2014) Whole-brain imaging with single-cell resolution using chemical cocktails and computational analysis. Cell 157:726–739. doi:10.1016/j.cell.2014.03.042
91. Tainaka K, Kubota SI, Suyama TQ et al (2014) Whole-body imaging with single-cell resolution by tissue decolorization. Cell 159:911–924. doi:10.1016/j.cell.2014.10.034
92. Eguchi M, Yamaguchi S (2009) In vivo and in vitro visualization of gene expression dynamics over extensive areas of the brain. Neuroimage 44:1274–1283. doi:10.1016/j.neuroimage.2008.10.046
93. Buxton OM, Pavlova M, Reid EW et al (2010) Sleep restriction for 1 week reduces insulin sensitivity in healthy men. Diabetes 59:2126–2133. doi:10.2337/db09-0699
94. Knutson KL, Spiegel K, Penev P, Van Cauter E (2007) The metabolic consequences of sleep deprivation. Sleep Med Rev 11:163–178. doi:10.1016/j.smrv.2007.01.002
95. Laposky AD, Bass J, Kohsaka A, Turek FW (2008) Sleep and circadian rhythms: key components in the regulation of energy metabolism. FEBS Lett 582:142–151. doi:10.1016/j.febslet.2007.06.079
96. Tasali E, Leproult R, Ehrmann DA, Van Cauter E (2008) Slow-wave sleep and the risk of type 2 diabetes in humans. Proc Natl Acad Sci USA 105:1044–1049. doi:10.1073/pnas.0706446105
97. Ahmed W, Ziouzenkova O, Brown J et al (2007) PPARs and their metabolic modulation: new mechanisms for transcriptional regulation? J Intern Med 262:184–198
98. Bordet R, Ouk T, Petrault O et al (2006) PPAR: a new pharmacological target for neuroprotection in stroke and neurodegenerative diseases. Biochem Soc Trans 34:1341–1346. doi:10.1042/BST0341341
99. Lee C-H, Olson P, Evans RM (2003) Minireview: lipid metabolism, metabolic diseases, and peroxisome proliferator-activated receptors. Endocrinology 144:2201–2207. doi:10.1210/en.2003-0288
100. Chen L, Yang G (2014) PPARs integrate the mammalian clock and energy metabolism. PPAR Res. doi:10.1155/2014/653017
101. Oishi K, Shirai H, Ishida N (2005) CLOCK is involved in the circadian transactivation of peroxisome-proliferator-activated receptor alpha (PPARalpha) in mice. Biochem J 386:575–581. doi:10.1042/BJ20041150
102. Shirai H, Oishi K, Kudo T et al (2007) PPARalpha is a potential therapeutic target of drugs to treat circadian rhythm sleep disorders. Biochem Biophys Res Commun 357:679–682. doi:10.1016/j.bbrc.2007.04.002
103. Chikahisa S, Tominaga K, Kawai T et al (2008) Bezafibrate, a peroxisome proliferator-activated receptors agonist, decreases body temperature and enhances electroencephalogram delta-oscillation during sleep in mice. Endocrinology 149:5262–5271. doi:10.1210/en.2008-0285
104. Fukao T, Lopaschuk GD, Mitchell GA (2004) Pathways and control of ketone body metabolism: on the fringe of lipid biochemistry. Prostaglandins Leukot Essent Fat Acids 70:243–251. doi:10.1016/j.plefa.2003.11.001
105. Robinson AM, Williamson DH (1980) Physiological roles of ketone bodies as substrates and signals in mammalian tissues. Physiol Rev 60:143–187
106. Cullingford TE (2004) The ketogenic diet; fatty acids, fatty acid-activated receptors and neurological disorders. Prostaglandins Leukot Essent Fat Acids 70:253–264. doi:10.1016/j.plefa.2003.09.008
107. Chikahisa S, Shimizu N, Shiuchi T, Séi H (2014) Ketone body metabolism and sleep homeostasis in mice. Neuropharmacology 79:399–404. doi:10.1016/j.neuropharm.2013.12.009
108. Auestad N, Korsak RA, Morrow JW, Edmond J (1991) Fatty acid oxidation and ketogenesis by astrocytes in primary culture.

J Neurochem 56:1376–1386. doi:10.1111/j.1471-4159.1991.tb11435.x
109. Blázquez C, Sánchez C, Velasco G, Guzmán M (1998) Role of carnitine palmitoyltransferase I in the control of ketogenesis in primary cultures of rat astrocytes. J Neurochem 71:1597–1606
110. McGarry JD, Foster DW (1980) Regulation of hepatic fatty acid oxidation and ketone body production. Annu Rev Biochem 49:395–420. doi:10.1146/annurev.bi.49.070180.002143
111. Jahn R (2010) VGLUTs-potential targets for the treatment of seizures? Neuron 68:6–8. doi:10.1016/j.neuron.2010.09.037
112. Brown RE, Basheer R, McKenna JT et al (2012) Control of sleep and wakefulness. Physiol Rev 92:1087–1187. doi:10.1152/physrev.00032.2011
113. De Gennaro L, Marzano C, Fratello F et al (2008) The electroencephalographic fingerprint of sleep is genetically determined: a twin study. Ann Neurol 64:455–460. doi:10.1002/ana.21434
114. Franken P, Malafosse A, Tafti M (1999) Genetic determinants of sleep regulation in inbred mice. Sleep 22:155–169
115. Franken P, Malafosse A, Tafti M (1998) Genetic variation in EEG activity during sleep in inbred mice. Am J Physiol 275:R1127–R1137
116. Cirelli C (2009) The genetic and molecular regulation of sleep: from fruit flies to humans. Nat Rev Neurosci 10:549–560. doi:10.1038/nrn2683
117. Parmentier R, Ohtsu H, Djebbara-Hannas Z et al (2002) Anatomical, physiological, and pharmacological characteristics of histidine decarboxylase knock-out mice: evidence for the role of brain histamine in behavioral and sleep–wake control. J Neurosci 22:7695–7711
118. Dombeck DA, Khabbaz AN, Collman F et al (2007) Imaging large-scale neural activity with cellular resolution in awake, mobile mice. Neuron 56:43–57. doi:10.1016/j.neuron.2007.08.003