Chapter 17
Imaging Sleep and Wakefulness

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17.1 Introduction: Behavioral Definition of Sleep

Sleep is considered to be a unique and essential phenomenon for life in all animals (Siegel 2008; Cirelli and Tononi 2008; Campbell and Tobler 1984). In fact, animals whose sleep is reduced for long periods eventually die under certain conditions (Rechtschaffen and Bergmann 2002; Driver et al. 2013; Shaw et al. 2002). The development of novel techniques for neuroscience research has rapidly increased our knowledge about the functions and mechanisms of sleep. First of all, what is the state of sleep? To answer this question, we must first define sleep. That is, a strict definition of sleep is the answer in and of itself. There are two major difficulties in defining sleep. First, while sleep seems to be a relatively simple behavior, it is not just a state of rest or immobility. For example, squirrels sleep immediately after arousal from hibernation, indicating that sleep is a distinct state with distinct functions from the energy-conserving rest state of hibernation. To distinguish between sleep and "non-sleeping" rest, sleep has been defined according to the following behavioral criteria: behavioral quiescence, characteristic stationary posture, rapid state reversibility, and elevated threshold to arousing stimuli (Piéron 1913). These criteria distinguish sleep not only from "non-sleeping" rest, but also from coma and anesthesia. Second, the sleep/wake cycle is linked with, but can be dissociated from,

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circadian rhythm. The sleep/wake cycle is controlled not only by the circadian rhythm, but also by homeostatic mechanisms (Borbély 1982). Homeostatic regulation is an additional criterion for defining sleep to distinguish between sleeping and circadian behaviors. In particular, animals exhibit recovery sleep after extended wakefulness. Based on these criteria, sleep can be defined behaviorally in diverse animal species, including genetic animal models such as the roundworm *Caenorhabditis elegans* (Raizen et al. 2008; Hill et al. 2014), fruit fly *Drosophila melanogaster* (Shaw et al. 2000; Hendricks et al. 2000), and zebrafish *Danio rerio* (Zhdanova et al. 2001; Yokogawa et al. 2007; Prober et al. 2006). Interestingly, according to these definitions, the jellyfish *Cassiopea spp*, which has a diffuse nervous system, also exhibits sleep-like behavior (Nath et al. 2017). On the other hand, some animals cannot be definitely judged to have sleep because they do not show behaviorally typical compensatory rebound sleep after sleep deprivation or reduced responsivity to stimuli during sleep-like behavior (Cirelli and Tononi 2008). Thus, the behavioral definition of sleep is highly variable, and could overlook sleep in certain animal species and several features of sleep, particularly the neurophysiological properties of sleep, as described below.

17.2   Oscillations in Sleep

Understanding the brain is critical for understanding sleep. In the early twentieth century, sleep in humans was reported to be controlled by several brain areas (von Economo 1931), which had also been experimentally investigated in non-human mammals (Jouvet 1962; Nauta 1946; Moruzzi and Magoun 1949). The brain not only controls sleep, but is also largely affected by sleep. Indeed, electroencephalogram (EEG), reflecting wide-range extracellular electrical activity in the brain, is currently used to provide a stricter definition of sleep (unless otherwise mentioned, EEG in this chapter refers to cortical EEG). The discovery of EEG brought two large changes to the concept of sleep: (1) the sleeping brain is not silent; (2) sleep comprises two main stages, non-rapid eye movement (NREM) and rapid eye movement (REM) sleep (Fig. 17.1). The first report of human EEG by Berger showed that
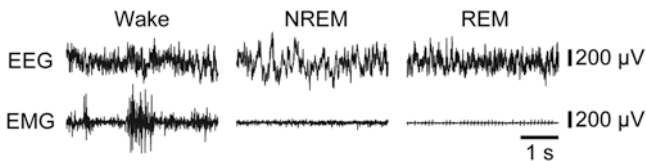

Fig. 17.1 EEG and EMG in a mouse during wakefulness (left), NREM (center), and REM sleep (right). EEG and EMG were recorded with screw electrodes inserted in the skull and stainless steel wires in the neck muscles, respectively. Note that EMG, especially of antigravity muscles, also reflects sleep/wake states: highest in wakefulness, lower in NREM, and lowest in REM sleep. (This figure is modified from Kanda et al. 2017)

EEG exhibits low-amplitude fast activity during wakefulness and large-amplitude alpha waves when the eyes are closed (Berger 1929). Adrian and Matthews later reported that slow waves are observed in EEG of deeply anesthetized animals (Adrian and Matthews 1934). Slow waves are also observed in EEG during sleep (Loomis et al. 1935a, b). Slow-wave activity (SWA, also known as delta wave activity, 1–4 Hz) is considered a hallmark of NREM sleep. Because of this feature, NREM sleep is also called slow-wave sleep. SWA is increased during recovery NREM sleep after sleep deprivation, suggesting that SWA reflects the homeostatic regulation of sleep (Borbély 1982; Tobler and Borbély 1986). The mechanism that underlies the generation of SWA, however, remains unknown. Aserinsky and Kleitman discovered another stage of sleep, REM sleep (Aserinsky and Kleitman 1953). Unlike in NREM sleep, EEG activity in REM sleep exhibits low-voltage desynchronized waves. As the name suggests, in REM sleep, electrooculogram shows high activity, whereas activity of electromyogram (EMG), reflecting the tone of antigravity muscles, shows lower activity during REM sleep than during NREM sleep (Fig. 17.1). Based on its unique characteristics, REM sleep is also called paradoxical sleep (PS) or active sleep. As described above, EEG activity closely reflects the state of sleep, including its homeostatic regulation. In addition, oscillatory activity in EEG during sleep occurs in mammals, as well as in amphibians, reptiles, and birds (Campbell and Tobler 1984). An EEG-based definition of sleep is more accurate but less applicable than the behavioral definition due to differences in nervous systems. EEG is usually recorded from the cerebral cortex, but most animal species have no nerve structure corresponding to the cerebral cortex of mammals. Importantly, however, although not recorded from mammalian cerebral cortex-like structures, electrophysiological oscillatory activity emerges in the sleeping central brain of the fly (Yap et al. 2017) and crayfish *Procambarus clarkii* (Mendoza-Angeles et al. 2007; Ramón et al. 2004*)*, implying that neuronal oscillations during sleep is a common feature across animal species, including invertebrates.

17.3   Electrophysiological Insights into the Sleeping Brain

Oscillations associated with sleep are also observed in other electrophysiological events in the cortex, such as local electric potentials (LFP), extracellular multi- and single-unit activity, and membrane potentials. Cortical neurons exhibit changes in their firing patterns that correlate with the state of sleep/wakefulness: a tonic pattern during wakefulness, a burst-and-pause pattern during NREM sleep, and a rhythmic bursting pattern during REM sleep (Evarts 1964; Hubel 1959). In addition, short-term and synchronous cessation of firing occurs in cortical neurons during NREM sleep, which correlates with slow waves in EEG and LFP (Vyazovskiy et al. 2009, 2011; Destexhe et al. 1999; Nir et al. 2011). The active and silent periods of the cortical neuronal population in NREM sleep are referred to as ON and OFF periods, respectively (Vyazovskiy et al. 2009). Slow waves are detected not only in LFP, but also in the membrane potential and EEG during NREM sleep (Steriade et al. 1993a;

Achermann and Borbély 1997). Surprisingly, slow waves in LFP appear during both NREM sleep and REM sleep in the middle layer of the primary cortices (Funk et al. 2016). Intracellular recordings in the cortex reveal that oscillatory activity, which consists of periodic depolarizing (UP) and hyperpolarizing (DOWN) states, appears in both EEG and the membrane potential under anesthesia and during NREM sleep (Steriade et al. 1993a, b, 2001; Timofeev et al. 2001; Metherate et al. 1992). UP/DOWN oscillations in membrane potentials disappear during wakefulness and REM sleep (Steriade et al. 2001; Timofeev et al. 2001).

17.4 Imaging Techniques Show Novel Aspects of Sleep

Electrophysiology reveals various aspects of the state of sleep. The dynamics of extracellular ionic composition during sleep and wakefulness were recently revealed with ion-sensitive microelectrodes: extracellular Ca^{2+}, Mg^{2+}, and H^+ increase, while K^+ decreases, when falling asleep, and the opposite occurs upon awakening (Ding et al. 2016). These changes not only correlate with, but also cause, sleep/wake states. Infusion of high Ca^{2+}, Mg^{2+}, and H^+, and low K^+ during wakefulness induces NREM sleep, while infusion of low Ca^{2+}, Mg^{2+}, and H^+, and high K^+ during NREM sleep induces wakefulness (Ding et al. 2016). The development of innovative imaging techniques has greatly contributed to elucidating the physiological aspects of sleep. This chapter mainly deals with fluorescence imaging. The most notable progress in fluorescence imaging techniques is the development of genetically-encoded calcium indicators (Lin and Schnitzer 2016), which allow for visualization of neural activity in microstructures up to the whole brain with diverse microscopy techniques. Although anesthesia is often used during imaging experiments, anesthesia is similar to, but different, from sleep. Thus, experiments with unanesthetized animals are preferred for sleep research (Fig. 17.2). One advantage of fluorescence imaging, especially in vivo two-photon microscopy, is that it provides images with high spatial resolution of microstructures, such as, in sleep studies, the morphology and Ca^{2+} signals of the dendritic spines. Morphologic changes in the dendritic spines are thought to be a cellular basis of learning and memory (Lamprecht and LeDoux 2004; Kasai et al. 2010). Dendrite spines are tiny bumps on dendrites whose size correlates with the efficiency of synaptic transmission. In addition, stimulating dendrites induces long-term potentiation of synaptic transmission and increases the size of dendritic spines. Although sleep contributes to memory consolidation (Stickgold 2005; Walker and Stickgold 2004), it has been unclear whether spine turnover occurs during sleep after learning. Two-photon imaging of the rodent motor cortex revealed that, after motor learning, NREM and REM sleep induce Ca^{2+} signal–dependent formation and elimination of dendritic spines, respectively (Li et al. 2017; Cichon and Gan 2015). When no specific task, such as motor learning, is performed, however, a net loss of spines is observed during NREM sleep (Yang and Gan 2012; Maret et al. 2011), supporting the synaptic homeostasis hypothesis: synaptic weight is potentiated to enhance learning in the awake state, and synaptic
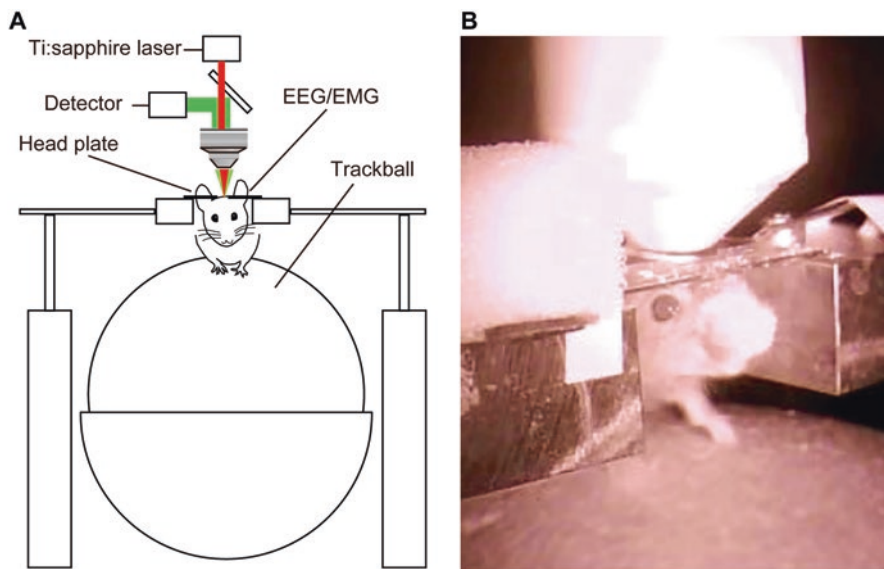
Fig. 17.2 Set-up for two-photon imaging from mouse brain during sleep and wakefulness. (**a**) Schematic showing arrangement of the head-restrained mouse, trackball, ball trackers, EEG/EMG electrodes, and custom-built two-photon laser-scanning microscope. (**b**) An infrared camera image depicting an awake mouse in the setup shown in (**a**). This setup allows for two-photon imaging in unanesthetized mice

downscaling occurs to save space and energy in sleep (Tononi and Cirelli 2006). Downscaling of synapses in sleep is driven by group I metabotropic glutamate receptors and Homer1a at postsynaptic densities (Diering et al. 2017). In the hippocampus, sharp-wave ripple oscillations trigger the downregulation of synaptic weights during NREM sleep (Norimoto et al. 2018). Another advantage of fluorescence imaging is the ability to visualize neural activity with cellular resolution in a wide-field, and in some cases the whole-brain. Whole-brain Ca^{2+} imaging during sleep and wakefulness in C. elegans revealed that sleep is a global quiescent brain state (Nichols et al. 2017). Ca^{2+} imaging in the mouse cortex revealed that neural activity is suppressed during NREM sleep as compared with wakefulness, and further suppressed during REM sleep (Niethard et al. 2016). Ca^{2+} imaging studies suggest that GABAergic neurons could contribute to suppress neural activity during sleep (Nichols et al. 2017; Niethard et al. 2016). Fluorescence imaging is also effective for studies of glial cells, especially astrocytes. The role of astrocytes in the regulation of sleep/wake states is still largely controversial, because there is no evidence that astrocytic Ca^{2+} dynamics correlate with sleep/wake cycles. Definitive conclusions await further in vivo imaging studies. Interestingly, astrocytes control interstitial fluid flow in the brain for clearance of the extracellular environment (Iliff et al. 2012), which increases during sleep and decreases during wakefulness (Xie et al. 2013). Thus, astrocytes might contribute to sleep function rather than sleep regulation.

17.5 Future Directions

Sleep has been energetically investigated using various techniques (Kanda et al. 2016). Electrophysiology can acquire accurate electric signals with high temporal resolution and signal-to-noise ratio, and has recently revealed new properties of sleep (Siclari et al. 2017; Watson et al. 2016). Imaging techniques combined with genetic engineering enables neuron-type–specific recordings (Fig. 17.3) that show activity patterns of specific neurons during sleep and wakefulness (Cox et al. 2016; Weber et al. 2018; Chen et al. 2018). Another advantage of imaging techniques is spatial analysis (Fig. 17.3). By combining mathematical analysis and imaging techniques, it is possible to capture the network structure of the brain spatially. In addition, imaging techniques can be used to measure the dynamics of neuromodulators in the brain, helping to elucidate brain states (Kanda et al. 2017; Wang et al. 2018). In mammals, sleep is well-defined by EEG. An electrophysiology-based definition of sleep is missing for animals other than mammals, however, whose sleep is defined by behaviors. If imaging techniques can be used to extract characteristic patterns of neuronal activity in sleeping animals, sleep can be defined by parameters that can be applied more universally than EEG. Further development of various imaging techniques will contribute to enhance our understanding of sleep.
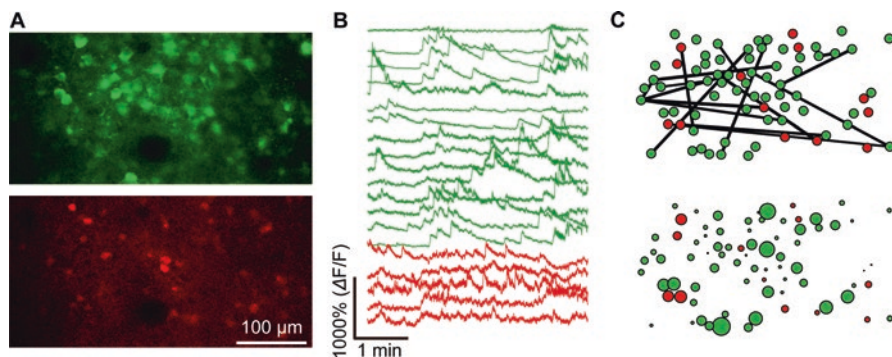

Fig. 17.3 In vivo two-photon Ca^{2+} imaging of cortical neurons. (**a**) A genetically encoded Ca^{2+} indicator, GCaMP6s (upper), and a fluorescent protein tandem dimer, Tomato (tdTomato) (lower), in the primary motor cortex layer 2/3. GCaMP6s was expressed in the primary motor cortex using an adeno-associated virus (AAV) vector. To identify GABAergic neurons, Vgat-tdTomato mice (Vgat-IRES-Cre; Rosa26-CAG-LSL-tdTomato-WPRE) were used. (**b**) Representative fluorescence signal ($\Delta F/F$) traces from somata in (**a**). (**c**) Spatiotemporal analysis of Ca^{2+} signals in the field of (**a**). (Upper) Circle size represents the fluorescence intensity. (Lower) Green and red denote pyramidal neurons and interneurons, respectively. Black straight lines show the connectivity between neurons

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