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Invited review

Peeking into the sleeping brain: Using *in vivo* imaging in rodents to understand the relationship between sleep and cognition

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ABSTRACT

Sleep is well known to benefit cognitive function. In particular, sleep has been shown to enhance learning and memory in both humans and animals. While the underlying mechanisms are not fully understood, it has been suggested that brain activity during sleep modulates neuronal communication through synaptic plasticity. These insights were mostly gained using electrophysiology to monitor ongoing large scale and single cell activity. While these efforts were instrumental in the characterisation of important network and cellular activity during sleep, several aspects underlying cognition are beyond the reach of this technology. Neuronal circuit activity is dynamically regulated via the precise interaction of different neuronal and non-neuronal cell types and relies on subtle modifications of individual synapses. In contrast to established electrophysiological approaches, recent advances in imaging techniques, mainly applied in rodents, provide unprecedented access to these aspects of neuronal function *in vivo*.

In this review, we describe various techniques currently available for *in vivo* brain imaging, from single synapse to large scale network activity. We discuss the advantages and limitations of these approaches in the context of sleep research and describe which particular aspects related to cognition lend themselves to this kind of investigation. Finally, we review the few studies that used *in vivo* imaging in rodents to investigate the sleeping brain and discuss how the results have already significantly contributed to a better understanding on the complex relation between sleep and plasticity across development and adulthood.

1. Introduction

Experimental evidence in humans and animals suggests that sleep plays a central role in brain development, cognition and plasticity (Golbert et al., 2017; Puentes-Mestril and Aton, 2017; Tononi and Cirelli, 2014). Sleep is involved in physiological processes in the brain and periphery, such as temperature and hormone regulation, neuromodulation, brain activity, and gene expression. The interaction of these aspects is likely to be necessary for sleep to exert its beneficial effect on cognition. Therefore, it is of great importance to investigate the relationship between sleep and cognitive function in the intact brain (i.e. *in vivo*). Among the many *in vivo* approaches, electrophysiological recordings have led to some of the most influential insights into the physiology and function of sleep in humans and animals over the last 70 years. Large scale (e.g. electroencephalography [EEG], local field potential [LFP]) and microelectrode (e.g. intra and extracellular) recordings have been critical to reveal the complex dynamics of cellular and network activity during sleep, that varies not only between (Steriade et al., 2001) but also within sleep stages (Levenstein et al., 2017; Watson et al., 2016). Recording large scale activity using EEG or LFP revealed that sleep is dominated by brain oscillations represented by synchronized rhythmic activity in specific cellular ensembles (Buzsáki et al., 2013). Brain oscillations are thought to be central for brain maturation, information processing and memory (Muller et al., 2018; Watson and Buzsáki, 2015), and are integral to current theories of the cognitive function of sleep across the lifespan (Khazipov and Luhmann, 2006; Navarro-Lobato and Genzel, 2018; Tononi and Cirelli, 2014; Vyazovskiy, 2015; Vyazovskiy and Harris, 2013). Nevertheless, our understanding of cellular and network activity during sleep and its impact on off-line information processing and neuronal plasticity remains limited. Electrophysiology only provides incomplete information on the identity of the recorded neuron (i.e. *putative* inhibitory or excitatory) and its activity (i.e. somatic spiking). However, the activity of neuronal circuits and their modulation by experience are shaped by the interaction of many neuronal and non-neuronal cell types. In the cortex, the function and connectivity of excitatory neurons varies across layers

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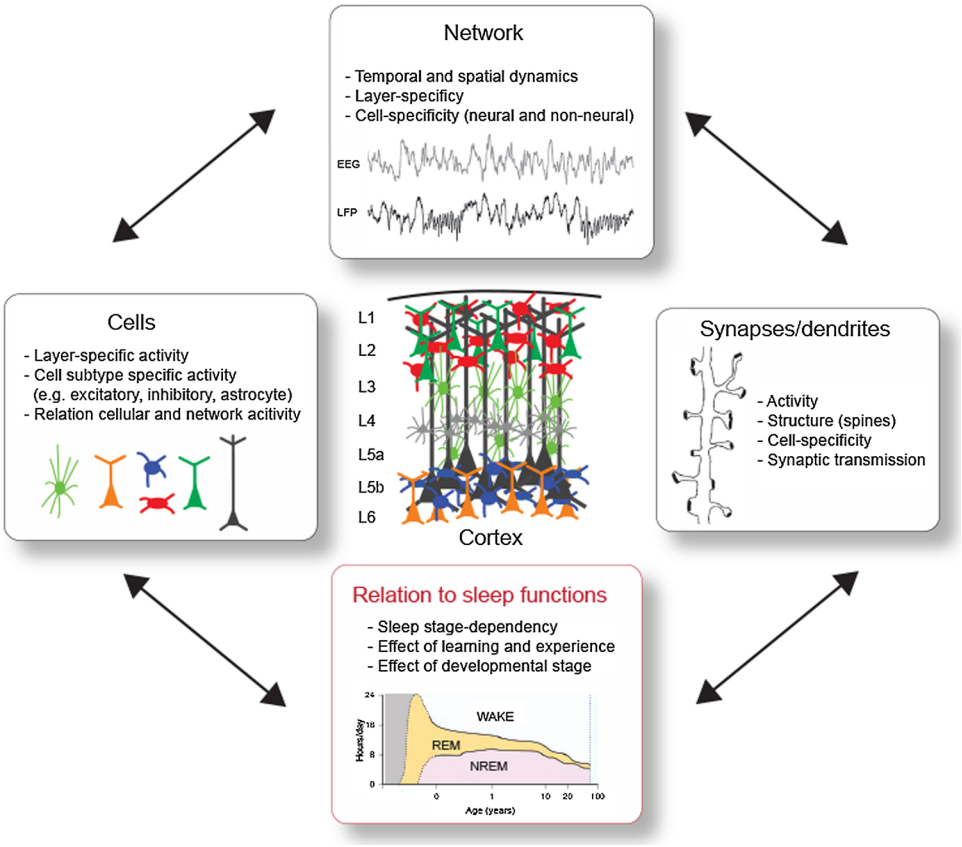

Fig. 1. Schematic representation of key aspects relevant to sleep research that cannot be addressed using standard electrophysiological approaches. *Middle:* The different layers of the cortex are populated by different types of neurons (inhibitory = red, blue and light green; excitatory = light and dark grey, orange, and dark green). Non-neural cells are not represented here, but populate the entire cortex. *Outside:* Electrophysiology does not provide access to defined neuronal subpopulations on a single cell *(left)* or network level *(top)* and does not allow the observation of subcellular structure and function *(right)*. However, these aspects of neuronal circuits are key when investigating sleep *(bottom)* (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

and subtypes (Zingg et al., 2014). Similarly, distinct inhibitory cell types within different cortical layers modulate excitatory tone in a laminar and brain state-dependent fashion *in vivo* (Muñoz et al., 2017; Tremblay et al., 2016). On a single cell level, electrophysiology is not ideal for resolving membrane potential changes arising in various neuronal compartments, such as dendrites which have been proposed to be of great importance for information processing and memory formation (Kastellakis et al., 2015). Lastly, accumulating evidence emphasizes the role of non-electrical activity, originating for example from glial cells, in shaping brain function (Araque and Navarrete, 2010). While it is of great importance to understand how sleep modulates the activity of these neural circuit components, electrophysiological approaches are not adequate for such investigations (Fig. 1).

In contrast, current *in vivo* imaging techniques, such as two-photon microscopy or microendoscopy, combined with cell-specific labelling of neural circuits provide a novel and promising avenue to address these questions. For this purpose, neurons are visualized by the expression of fluorescent indicators. Optically recording neuronal activity typically relies on the fact that electrical communication between brain cells is accompanied by rapid changes in membrane voltage and ion concentrations across the cytoplasmic membrane, including calcium (Ca^{2+}). Here, activity-dependent changes in intracellular Ca^{2+} using fluorescent indicators provide an indirect readout of neuronal activity. In a wide range of transgenic mouse lines the expression of fluorescent dyes and molecular tags can be limited to neuronal subtypes using site specific recombinase technology. To date, *in vivo* optical imaging has been successfully applied in anaesthetized or awake animals, while only few studies have used imaging to investigate the role of sleep in cognition.

Here we first highlight which aspects of awake circuit activity have been revealed by imaging that are prime candidates for a similar investigation in the sleeping brain. Next, we also compare the advantages and limitations of currently available *in vivo* imaging methods in rodents, specifically focusing on their potential to investigate the physiology underlying the function of sleep. We then review those studies that have so far applied imaging techniques to investigate sleep and highlight how they contributed to our understanding of the influence of sleep on cognition.

Due to their widespread use in sleep research and *in vivo* imaging studies, our review will focus on rodents. We refer the readers to reviews on sleep regulation and function in other model systems with characterized sleep behaviour, such as Drosophila and Zebrafish, that combine various *in vivo* experimental approaches, including imaging techniques (Chiu and Prober, 2013; Frank and Cantera, 2014; Tononi and Cirelli, 2014).

2. State-dependent information processing and plasticity in neuronal networks – insights from *in vivo* imaging studies in the awake brain

Processing and storage of information in neural circuits involves complex dynamics which can be studied at different levels, including neuronal ensembles, single cells and subcellular compartments. Awake imaging of neuronal ensembles (excitatory, inhibitory, astrocytes) has provided important insights into how information processing is modulated by behavioural states. Zooming in onto subcellular compartments, imaging of dendrites or single spines has significantly improved our understanding of how new experiences are stored on a cellular level. Such studies highlight how imaging could be leveraged to investigate neuronal activity during sleep which can be viewed as another type of brain state that is essential for information storage.

2.1. Brain-state modulation of information processing

Similar to sleep, wakefulness can be divided into substates, active wake (AW) and quiet (inactive) wake (QW), which differ in their degree

of cortical synchronization and underlying behaviour. During QW, neuronal membrane voltage (Vm) displays fluctuations similar to those during NREM sleep, marked by large amplitude oscillations at slow frequencies (< 5 Hz) that are highly correlated across neighbouring neurons and layers in the cortex (Poulet and Petersen, 2008; Zhao et al., 2016). AW, including behaviours such as locomotion or active whisking, shifts the cortex into a more desynchronized state accompanied by a reduction in Vm oscillatory frequency (Crochet and Petersen, 2006; Polack et al., 2013; Poulet and Petersen, 2008; Zhao et al., 2016). These changes in Vm oscillations across wakefulness are also reflected in the LFP (Okun et al., 2010; Poulet and Petersen, 2008). Interestingly, shifts in brain states are associated with altered processing of sensory information by cortical neurons as measured by intracellular recordings (Bennett et al., 2013; Niell and Stryker, 2010; Polack et al., 2013). In primary visual cortex (V1), this translates into an increased responsiveness of excitatory neurons to visual stimulation during active behaviour (Fu et al., 2014). Two-photon Ca^{2+} imaging of different cortical cell types showed that the increase in gain of V1 excitatory responses was dependent on behavioural state modulation of interneuron activity. More specifically, somatostatin (SST) interneurons were depressed during active behaviour while vasoactive intestinal peptide (VIP) interneurons remained excited regardless of sensory stimulation (Fu et al., 2014). Interestingly, Reimer et al. (2014) observed subtle fluctuations in Vm synchronization even within QW, where bouts of desynchronization were accompanied by enhanced visually evoked cortical responses even in the absence of behaviour. Similar to the findings of Fu et al. (2014), desynchronization was also associated with the activation of VIP and inhibition of SST interneurons. Sensory evoked responses in the cortex are not only influenced by waking states but show distinct changes across REM and NREM sleep in humans and animals (Colrain and Campbell, 2007; Rector et al., 2005; Wehrle et al., 2007). Therefore, it will be interesting to use functional imaging of neuronal subtypes to investigate how off-line processing of external and internal (i.e. self-generated) stimuli is shaped by sleep state-specific circuit activity.

At the circuit level, information processing within and between brain regions is thought to benefit from coordinate activation of ensembles of neurons which is promoted by large scale network oscillations (Buzsáki et al., 2013). A good example is spatial information coding in the hippocampus. Here electrophysiological recordings showed that individual hippocampal cells (i.e. place cells) are sequentially activated at specific spatial locations during exploration (e.g. AW) and coincides with theta oscillatory (4–10 Hz) activity. When the animal is inactive (i.e. QW, NREM sleep), these sequences of activation are “replayed” during fast ripple oscillations (150–250 Hz, Girardeau and Zugaro, 2011; Sadowski et al., 2011). Using chronic two-photon Ca^{2+} imaging of hippocampal cells combined with LFP recordings in running and inactive mice, Malvache et al. (2016) have, for the first time, optically detected replay sequences. In contrast to electrophysiology, imaging provided stable access and identification to more than a hundred cells over repeated imaging sessions. This revealed that replay sequences during wakefulness were represented by the functional binding of different anatomically preconfigured cell ensembles. While this study did not investigate sleep states, hippocampal replay activity occurs during both NREM and REM sleep and is thought to be important for off-line information processing and memory formation (Girardeau and Zugaro, 2011; Sadowski et al., 2011). It will thus be interesting to examine whether replay during sleep involves similar binding mechanisms of anatomically defined cell ensembles.

2.2. Synaptic plasticity

The ability of the brain to store information in response to experience is thought to rely on synaptic plasticity which involves the strengthening or weakening of synaptic connections (e.g. long-term potentiation [LTP] and depression [LTD]). Changes in synaptic strength between two neurons are often measured at the cellular output level using electrophysiology. Since electrodes are not suited to investigate the role of dendrites (but see Smith et al., 2013), spines and astrocytes in plasticity, novel imaging methods provide promising new avenues.

2.2.1. Dendrites and dendritic spines

Accumulating evidence has shown that storage of information in the brain relies on intrinsic regenerative events within dendrites that lead to compartmentalised Ca^{2+} changes and subsequent, localized dendritic plasticity (Williams et al., 2007). Functional imaging using two-photon microscopy is well suited to visualize Ca^{2+} in dendrites. This approach showed that Ca^{2+} activity of CA1 dendrites is more predictive of the spatial precision and long-term stability of place cells than somatic activity in the hippocampus (Sheffield and Dombeck, 2014), supporting an important role of dendritic activity in spatial coding and memory. Furthermore, Ca^{2+} imaging of dendrites combined with motor learning demonstrated that different tasks engage activity in separate dendritic branches and that this compartmentalization is regulated by a subset of dendrite targeting interneurons (Cichon and Gan, 2015). At the structural level, the dynamic shape of spines (i.e. site of synaptic communication on dendrites) and their clustering have been hypothesized to serve as morphological substrate of information storage (Kastellakis et al., 2015). Longitudinal two-photon imaging of spine morphology combined with behavioural tasks revealed that spinogenesis and pruning (Hofer et al., 2009; Xu et al., 2009; Yang et al., 2009), spine turn-over rate and spine clustering (Frank et al., 2018) are all important features of experience-dependent plasticity and memory formation. In a very recent study, using functional and structural imaging combined with cell-specific optogenetic manipulation *in vivo*, El-Boustani et al. (2018) showed that experience-dependent plasticity in the visual cortex is accompanied by the strengthening of potentiated spines and the simultaneous weakening of neighbouring spines. Together these studies support the notion that previously encoded experience translates into complex functional and bidirectional anatomical changes within dendrites. Given that sleep is often proposed to favour unidirectional changes in synaptic strength (i.e. weakening: Tononi and Cirelli, 2014; strenghening: Rasch and Born, 2013), the application of compartment-specific functional and structural imaging now allows a more detailed analysis of dendritic information processing, not only during behaviour but also during subsequent sleep.

2.2.2. Astrocytes

Beyond their well-characterized role in brain homeostasis (Verkhratsky and Nedergaard, 2018), recent evidence support an important role for astrocytes in synaptic plasticity. Due to their close contact with synapses, astrocytes can dynamically regulate synaptic transmission by responding to neurotransmitters and the release of gliotransmitters (Araque and Navarrete, 2010; Theodosis et al., 2008). The complex mechanisms by which astrocytes modulate synaptic plasticity involve the modification of synaptic structure (Bernardinelli et al., 2014; Theodosis et al., 2008), homeostatic plasticity (De Pittà et al., 2016; Min et al., 2012; Singh and Abraham, 2017) and LTP/LTD (Min et al., 2012). More recently, *in vivo* manipulation of astrocyte function has been shown to directly modulate memory performance via the modification of synaptic plasticity (Adamsky et al., 2018; Robin et al., 2018), underlining the view that their function is behaviourally relevant.

The main way by which astrocytes respond and process information is intracellular Ca^{2+} modulation (Min et al., 2012), making astrocytes a prime target for Ca^{2+} imaging (Losi et al., 2017). Here, *in vivo* two-photon imaging confirmed previous *in vitro* results showing that Ca^{2+} signalling in astrocytes is highly compartmentalised and occurs predominantly within their tiny peripheral processes (Rusakov, 2015; Zheng et al., 2015). In contrast to the slow dynamics of transients within the soma, *in vivo* sensory-evoked Ca^{2+} responses in astrocytic processes are much faster (i.e. hundreds of milliseconds, Lind et al.,

2017; Winship et al., 2007) and therefore more relevant to the time course of synaptic transmission and plasticity events (e.g. spike timing-dependent plasticity). Two-photon imaging also showed that Ca^{2+} activity in astrocytes is modified by neuromodulators such as acethylcholine (Takata et al., 2011) or norepinephrine (Monai et al., 2016) which are both important for plasticity and memory. These observations are significant for two main reasons. First, Ca^{2+} imaging of astrocytes can reveal rapid and local changes in Ca^{2+} dynamics that may be important for the regulation of synaptic plasticity across brain states. Second, basal neuromodulator tone varies greatly across brain states and can be further modified during sleep in a use-dependent manner (e.g. norepinephrine release during NREM sleep, Eschenko and Sara, 2008). Neuromodulatory tone may therefore contribute to sleep-stage dependent plasticity via modulating astrocyte activity. In the context of sleep research, molecular studies have provided accumulating evidence implicating astrocytes in sleep regulation. Genetic manipulations have shown that astrocytes and gliotransmission can influence sleep architecture as well as NREM and REM sleep homeostasis and oscillations (Fellin et al., 2012, 2009; Foley et al., 2017; Frank, 2013; Gerstner et al., 2017). Sleep and sleep deficits, in turn, affect astrocytic gene expression and physical connectivity at synapses (Bellesi et al., 2015). Thus, tailoring novel imaging approaches to the distinct morphology and physiology of astrocytes is now necessary to better understand their role in information processing and plasticity during sleep.

in vivo imaging has provided unprecedented insight into the structure and function of neuronal and non-neuronal cells and their subcellular compartments that are involved in state-dependent information processing and plasticity in the awake brain. This approach is particularly powerful because it allows the simultaneously recording activity of (1) genetically defined components of (2) neuronal populations, single cells or subcellular compartments as well as (3) to monitor morphological changes. Using optical techniques to observe neuronal circuits across wakefulness and sleep will undoubtedly aid our understanding of cell-type specific contributions to off-line processing of waking experience (Fig. 1).

3. *In vivo* imaging of the rodent brain: advantages and limitations in the context of sleep researchin vivo*In vivo* imaging of the rodent brain: advantages and limitations in the context of sleep research

The development of novel optical tools to investigate the function of neuronal circuits *in vivo* has accelerated at an unprecedented speed over the last few years. While these techniques are suited to a plethora of different experimental strategies, there are several limitations that have to be carefully considered in their application in sleep research.

3.1. Visualizing neurons and their activity: fluorescent dyes

When recording neuronal activity across brain states, it is important to monitor both active as well as silent neurons over an extended period of time to accurately estimate population dynamics. Electrophysiology, in particular extracellular recordings, has been widely used to record spike trains of single cells in freely behaving animals. Two notable limitations of this approach are the indirect classification of recorded cells into excitatory and inhibitory neurons based on spike shape and the likelihood to oversample more active neurons, sometimes resulting in an overestimation of firing rates (Table 1, Harris et al., 2016). Imaging can alleviate these limitations when combined with the appropriate genetically engineered fluorescent dyes. Using transgenic mouse lines or viral vectors driven by specific promoters, expression can be limited to specific cortical layers, defined cell types (e.g. interneuron subtypes, astrocytes) or cells with specific anatomical connectivity (i.e. circuits) via axonal or trans-synaptic labelling (Osakada et al., 2011). Moreover, imaging can be less biased towards oversampling of active cells, when appropriate algorithms for detecting silent cells or cells with low baseline fluorescence are applied (Harris et al., 2016). In the future, the use of dual colour dyes to visualize morphology and function simultaneously will further improve detection of imaged cells (Rose et al., 2016). Available indicators enable monitoring of a variety of neuronal events, such as synaptic transmission (Liang et al., 2015), membrane voltage (Knöpfel et al., 2015) and intracellular Ca^{2+} concentration (Broussard et al., 2014). Genetically encoded Ca^{2+} indicators (GECIs), in particular the GCAMP family, provide a surrogate readout of electrical activity and are currently the most widely used (Tian and Looger, 2008). In contrast to electrophysiology, Ca^{2+} imaging only provides indirect access to neuronal spiking by reporting changes in Ca^{2+} concentrations in association with action potentials. The fluorescent change elicited upon binding Ca^{2+} ions is shaped by the specific characteristics of the indicator, including brightness and response kinetics. While information about action potentials is possible to obtain with high resolution imaging (Chen et al., 2013), the kinetics of most GECIs are still too slow (i.e. a decay time of hundreds of milliseconds) to reveal the precise spiking activity (Table1, Lin and Schnitzer, 2016) or track most oscillatory events found during sleep. Currently, typical Ca^{2+} transients captured with one- or two-photon imaging report durations often greater than 2 s, which is to slow to reflect oscillations even in the slow frequency bands (e.g. Delta: 1–4 Hz). In general, the analysis of the fluorescent GECI signal requires elaborate algorithmic processing for motion correction, image segmentation, the removal of neuropil contamination and spike deconvolution. Depending on the precise experimental question, recording spike rate is not always indispensable. Instead, changes in normalized fluorescence or peak amplitude of Ca^{2+} transients can yield meaningful comparisons with other physiological measures (e.g. EEG/LFP power, average firing rate) or across behavioural states. For example, population Ca^{2+} imaging of genetically defined neuronal subtypes provides insights into the cell-specific recruitment to network activity, independent of individual spike rates.

For specific application in sleep research, the ideal indicators would be able to record the precise timing of subthreshold voltage changes of many hundreds of neurons with subcellular resolution, for which membrane voltage indicators seem well suited. With a temporal resolution in the millisecond timescale (Table 1, Lin and Schnitzer, 2016), several genetically encoded voltage indicators (GEVIs) have been successfully applied in live brains to detect population and, more rarely, even single cell voltage dynamics (Akemann et al., 2013; Cao et al., 2013). Unfortunately, a variety of severe limitations still prevent their widespread use. For many GEVIs, their kinetics are still insufficient to differentiate subthreshold events and action potentials. In addition brightness, response amplitude, and excessive photoinstability currently also limit their applicability (Platisa and Pieribone, 2018).

Genetically encoded fluorescent indicators can be imaged repeatedly over extended periods (i.e. weeks to months, Table 1). This makes them particularly appealing for sleep research which requires continuous recording of neuronal activity across sleep stages, as well as repeated recordings before and after behavioural manipulations across days. When imaging long continuous sessions, special care must be taken to avoid photobleaching and photodamage resulting from excessive illumination. This is particularly true for GEVIs which require a fast frame rate and greater illumination. This limits the imaging time with GEVIs to 15 to 30 min per day (St-Pierre et al., 2014). In comparison, GECIs can be imaged with virtually no photobleaching for at least 30 min (Tian et al., 2012). If longer durations are required, care should be taken to limit bleaching and appropriately correct for it during image analysis (Resendez et al., 2016). For this purpose traditional electrophysiological recordings are advantageous as they can be performed continuously over days (Table 1). When imaging repeatedly over several weeks, the level of indicator expression has to be monitored closely. Long term expression has been associated with cytotoxicity, altered intrinsic excitability and cell death (Resendez et al., 2016; Yang et al., 2018). Such side effects have been associated with abnormal accumulation of indicator in the nucleus and instable Ca^{2+}

Table 1
Main characteristics of *in vivo* electrophysiological and imaging techniques.

	Electrophysiology			***In vivo* imaging**		
	EEG/LFP	**Extracellular**	**Intracellular**	**Wide-field**	**One photon (fiberoptic)**	**Two/three photon**
Cell-type specificity	No	Ambivalent, only excitatory vs. inhibitory		Yes - based on type of indicator (e.g. synthetic, viral, mouse lines)		
Cellular resolution	No	Yes	Yes	No	Yes, when combined with a lens	Yes
Cellular compartments	No	No	Very challenging	No	Difficult	Yes
Sampling area/cells	Millimetres/~10 to thousands of neurons	~150 µm/~10	1-4 neurons at a time	Millimetres/tens to thousands of cells		~ up to 1 mm^2/ up to 1000 neurons
Temporal resolution	ms to sec (100-500 Hz sampling)	< 1 ms, 20-30KHz sampling	< 1 ms, 10-20KHz sampling	Based on type of indicator dynamics (e.g. < 10 ms [GEVI] to > 100 ms [GECI])		> 100 ms [GECI]
Sampling bias	N/A	Bias (active neurons)	Unbias	Bias (active neurons) + neuropil contamination	Less biased when combined with a lens (image segmentation)	Less biased (image segmentation)
Type of information	Electrical signal of a population of neurons	Local cell spiking	Subthreshold and action potentials	Population or single cell (miniscope) activity based on the type of indicator (e.g.Ca^{2+} [GECI], membrane voltage [GEVI])		Single cell activity (Ca^{2+}) or spine structure
Depth	Surface	Any	Mostly supragranular	Surface	Any	Up to 600 µm deep
Behaviour	Freely moving		Head-fixed	Head-fixed	Freely moving	Head-fixed
Recording time	Weeks to months/continuous per day		20 min	Weeks to months. Time restricting limiting factors: (1) Photobleaching, (2) head-fixation, (3) overexpression, (4) stability of indicator		
Stability of recordings	Stable	Drift	Very unstable	Stable	Drift	
Brain damage	Superficial	Mechanical by insertion		Superficial /photodamage	Mechanical by insertion /photodamage	Superficial /photodamage
Species	Difficult to impossible in invertebrates (e.g. flies, worm)			Small animals	Any	Mostly small animals and invertebrates
Price	Affordable					Expensive
Possible combinations	With any method		Challenging	With any method		
Brain state identification	Requires additional EMG or video recording			Requires additional EEG/LFP/EMG		

dynamics. While sparse labelling using low virus titres or double-crossed transgenic mouse lines have been recommended to avoid such phenomena, many double-crossed lines systemically expressing Ca^{2+} indicators suffer from epilepsy (Steinmetz et al., 2017). This drawback is particularly important in sleep research where oscillations, such as spindles, have been linked to epileptic episodes during sleep (Leresche et al., 2012), thus potentially biasing physiological measures. To diminish these concerns, viral expression should be limited in time, and side-effects such as photodamage, altered physiology and signal instability should be carefully assessed.

In summary, the main advantage of available fluorescent reporters is the access to genetically defined cell types. We also believe that the development of dual-colour dyes cannot only aid more balanced analysis of imaging data but also permit simultaneous visualization of neuronal structure and function (e.g. mRuby-GCaMP, Rose et al., 2016) which is particularly promising for the sleep field. Studying plasticity, such hybrid approaches have the unique advantage to provide access to both morphological and physiological substrates of memory formation. In the meantime, the above limitations have to be taken into consideration when designing and interpreting imaging experiments.

3.2. Imaging brain cells and activity: techniques

There are a variety of optical techniques to visualize the above fluorescent dyes. Similar to electrophysiology, the appropriate method should be chosen depending on the number of neurons to be recorded, their location, the physiological process under investigation and the behavioural paradigm. Of specific note for sleep research are the different scales at which imaging can operate. While population imaging of hundreds to thousands of cells can reveal experience-dependent changes in large neuronal networks, the resolution of multi-photon imaging is sufficient to monitor spine morphology, a key aspect of synaptic plasticity.

One well-established approach for capturing the population level is wide-field microscopy (Ma et al., 2016). Here, a conventional camera is used to track hemodynamics or bulk fluorescent activity of large areas of exposed cortex. Although this approach makes single cell resolution problematic, it is attractive for imaging population activity at a high frame rate in head-fixed animals (Ma et al., 2016). For bulk fluorescence imaging in freely moving animals, fibreoptics (i.e. photometry) offers an attractive alternative. Here, the same optical fibre is used to excite fluorophores and collect emitted light which is then transmitted to a camera (Adelsberger et al., 2005) or photodiode (Seibt et al., 2017). Due to its small diameter (< 400 µm), the fibre can be chronically implanted into the brain providing access to deep brain areas which is of particular interest for sleep and memory research, where areas such as the hippocampus or amygdala are especially interesting. Moreover, the development of multi-fibre implants has made photometry a powerful and affordable tool to record from multiple remote brain areas simultaneously (Calipari et al., 2017; Guo et al., 2015; Kim et al., 2016; Lu et al., 2018). When implementing population imaging using these methods, several limitations have to be taken into account, beyond those associated with functional indicators (see Section 3.1). Namely, the slow kinetics of many available dyes prevent the exploitation of the potentially high frame rate of population imaging. Fluorophore expression should also be limited to the cell-type and structure of interest to avoid background contamination. Furthermore, due to its low spatial resolution, activity of the recorded population cannot be directly inferred as the signal mainly reflects activity synchronization (Adelsberger et al., 2005). Finally, and similarly to electrophysiology, these approaches are biased towards active cells since estimating the fraction of active versus silent cells is not possible. To

Table 2
Summary of the studies using *in vivo* imaging to investigate sleep in rodents. Abbreviations: VSD = voltage sensitive dye, P = postnatal day; W = weeks of age; SS = somatosensory cortex; M = motor cortex.

Imaging approach	Species (age)	cortical area/cells	Main findings	References
Functional imaging				
Population activity				
Ca^{2+} (fiberoptic)	Mouse (P3-4)	Parietal/mixed cells	Large cortical Ca^{2+} waves during rest phases - reflect Early Network Oscillations (ENOs)	Adelsberger et al., 2005
VSD (wide field)	Rat (P4)	Barrel/mixed cell	Increased membrane voltage activity within 500 ms following twitching activity during active sleep	Tiriac et al., 2012
Ca^{2+} (wide field)	Mouse (> 8 W)	SS/excitatory neurons	Population activity of excitatory neurons in upper and lower layers = Wake > NREM > REM	Niethard et al., 2016
Ca^{2+} (fiberoptic)	Rat (5-6 W)	SS/dendrites of L5 neurons	Populations of dendrites increase activity during - and correlate with - spindle-rich oscillations (IS)	Seibt et al., 2017
		SS/mixed L2/3 cells	Population activity of L2/3 neurons (excitatory + inhibitory) = AW > QW = NREM = IS = REM	
CSF tracers (two-photon)	Mouse (adult)	Parietal/glymphatic system	Sleep helps clearance of toxic waste and enables metabolic homeostasis	Xie et al., 2013
Single cell activity				
Ca^{2+} (two-photon)	Mouse (> 6 W)	SS/dendritic shaft (L5 neurons)	Ca^{2+} = AW < QW < NREM < IS = REM Ca^{2+} synchronisation correlates with spindle-rich oscillations	Seibt et al., 2017
		SS/L5 (mixed neurons)	Ca^{2+} activity = Wake < NREM < REM	
Ca^{2+} (two-photon)	Mouse (adult)	M/dendritic tuft (L5 neurons)	Increased Ca^{2+} activity in dendrites during REM after motor learning is functionally related to selective pruning and stabilization of newly formed spines	Li et al., 2017
Ca^{2+} (two-photon)	Mouse (> 8 W)	SS/inhibitory neurons subtypes + excitatory neurons (L2/3)	PV interneurons = Wake = REM > NREM SST interneurons = Wake > NREM > REM Other neurons (mainly excitatory) = Wake > NREM > REM	Niethard et al., 2016
Structural imaging				
Development				
Two-photon	Mouse (3 W)	SS/dendritic tuft (L5 neurons)	Spine formation sleep = wake Spine elimination sleep > wake	Yang and Gan, 2012
Two-photon	Mouse (4 W)	SS/dendritic tuft (L5 neurons)	Spine formation sleep < wake Spine elimination sleep > wake	Maret et al., 2011
Two-photon	Mouse (3 W)	M/ dendritic tuft (L5 neurons)	Newly formed spines are selectively prunes and stabilized during REM sleep	Li et al., 2017
Adulthood				
Two-photon	Mouse (13 W)	SS/dendritic tuft (L5 neurons)	Spine formation and elimination sleep = wake	Maret et al., 2011
Two-photon	Mouse (adult)	M/dendritic tuft (L5 neurons)	Branch specific spine formation after learning is stabilised during sleep	Yang et al., 2014
Two-photon	Mouse (adult)	M/dendritic tuft (L5 neurons)	New spines induce by learning are selectively prunes and stabilized during REM sleep	Li et al., 2017

rescue single cell resolution, an optical lens (i.e. Gradient-index (GRIN)) can be implanted that can alter the focal distance to be in close proximity to cell bodies near the fiber and allows for wide field fluorescence to be recorded at its small, two-dimensional tip (Resendez et al., 2016). GRIN lenses are readily combined with small, head-mounted miniature microscopes that include a miniature camera for signal collection attached to the cranial implant. With a weight of ~2 g such miniscopes provide an often affordable way to monitor activity of single neurons in freely behaving animals (Table 1, Ziv et al., 2013). While they are mostly used in a one-photon configuration with low spatial resolution, increasing efforts are invested into the development of head-mounted two-photon microscopes (Zong et al., 2017), rendering it an even more promising technique for the future.

For high resolution functional imaging, two-photon resonant scanning microscopy has become the most widely used technique. It minimizes light scattering, reduces photobleaching and provides a high axial resolution sufficient to resolve single spines (~2-3 µm). These characteristics allow recordings as deep as 500 µm with a field of view (FOV) between 200µm^2 and 1 mm^2 (Table 1). Imaging depth can be further extended to 1700 µm by applying adaptive optics (Booth, 2014) or three photon excitation (Ouzounov et al., 2017) which also reduces light scattering. Similar to the fiberoptic approach described above, more distant brain areas can be reached with two-photon microscopy using chronically implanted microprisms which have recently been used to image insular cortex and developmental spine dynamics in medial prefrontal cortex (Livneh et al., 2017; Pattwell et al., 2016). More invasive strategies rely on the removal of brain tissue covering the hippocampus (Sheffield and Dombeck, 2014; Velasco and Levene, 2014) which also strongly interferes with long range connectivity and may thus bias results. Recently, significant improvements in depth penetration without interfering with brain tissue have been provided by the combination of high-peak power output lasers together with red shifted fluorophores (Dana et al., 2016; Kawakami et al., 2015). Besides imaging depth, the FOV size can be extended using mesoscale two-photon microscopes that allow Ca^{2+} imaging across millimetre wide areas with cellular resolution (Sofroniew et al., 2016). Apart from increasing the size and depth that can be visualized, great effort has gone into imaging the morphology of neurons. Standard two-photon microscopy has provided great insight into the complex dynamics of axonal and dendritic plasticity over almost two decades (Holtmaat and Svoboda, 2009). Today, advances in super resolution imaging are able to reveal nanoscopic features *in vivo,* such as actin distribution and single spine shape changes with a resolution of < 100 nm in volumes of brain tissue (Urban et al., 2018). Overall, the above advances promise to alleviate many of the methodological limitations of two-photon microscopy. However, the feasibility of such new developments is oftentimes limited given their high cost or the time and expertise that is necessary for implementation and appropriate interpretation of results. In the context of sleep research, it has to be emphasized that both standard two-photon imaging and wide-field imaging require head-

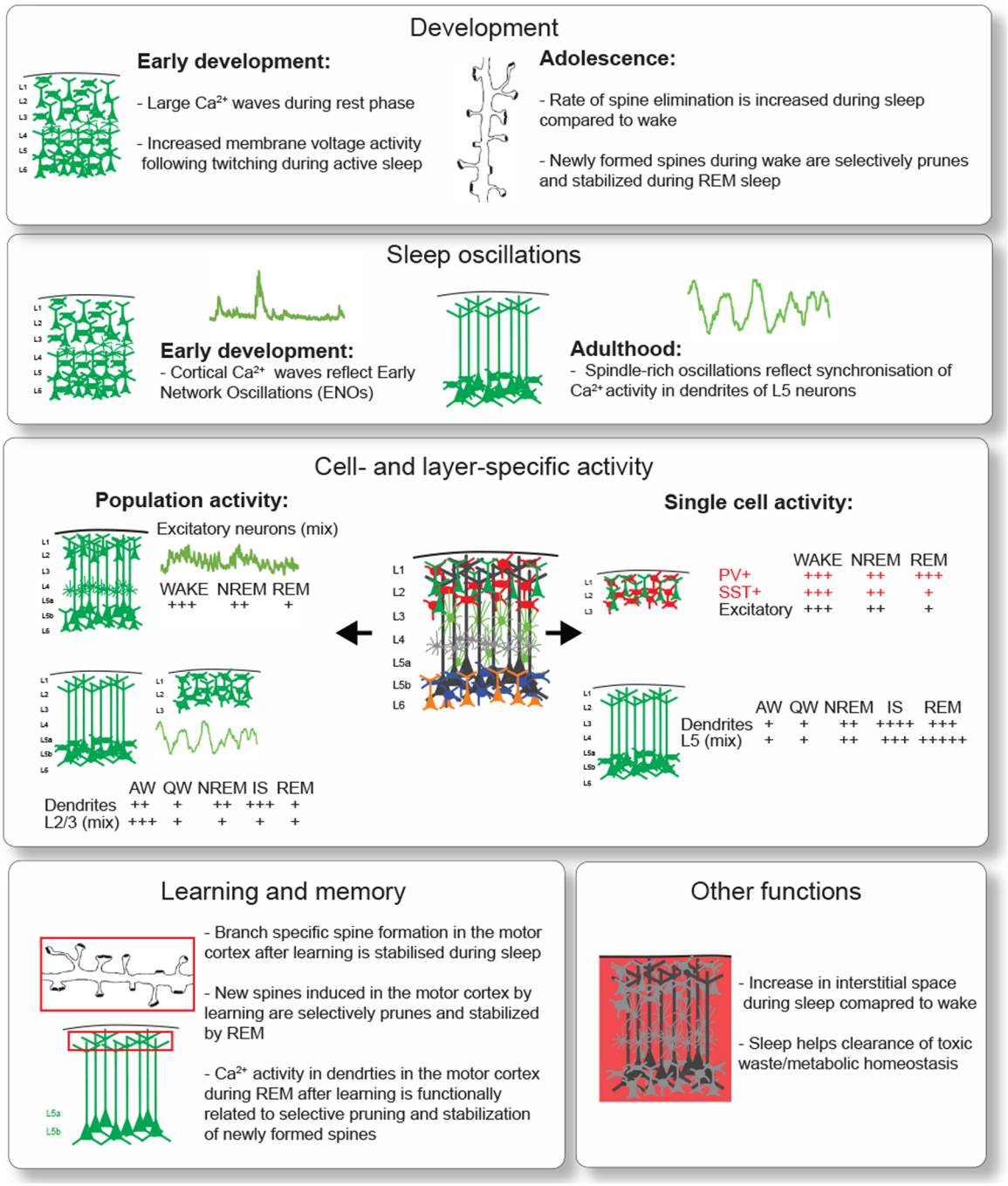

Fig. 2. Summary of the main findings using *in vivo* imaging applied to sleep separated into functional categories. Details for each study are found in the text and Table 2.

fixation. Immobilizing the animal can increase stress levels, a phenomenon known to influence spine dynamics via increased corticosterone levels (Liston and Gan, 2011). Head-fixation also results in more fragmented sleep which in turn can bias physiological and behavioural measures. If head-fixation cannot be avoided, care should be taken to closely monitor behavioural state (EEG/LFP), optimize head-fixation to mimic the natural sleep position (Yüzgeç et al., 2018) and compare observations to freely moving conditions.

4. Imaging the sleeping brain

Despite the important information it can provide, studies using *in vivo* imaging to investigate the function of sleep are remarkably sparse. A few research groups have started to implement these methods in recent years with specific focus on development, learning and memory, and state-dependent regulation of neuronal activity. Keeping in mind the technical limitations discussed above, we next discuss the main findings of these studies which are summarized in Table 2 and Fig. 2.

4.1. Functional imaging

4.1.1. Population activity

Brain oscillations, revealed in the EEG and LFP, represent a predominant neuronal signal during sleep. Neuronal oscillations are also among the first manifestations of brain activity during early development, when sleep takes up a large share of time and plasticity is at its

height. This observation supports the prevalent hypothesis that sleep oscillations are fundamental to both brain maturation and plasticity across lifespan. During development, sleep is divided into very short bouts (< 50 s) of quiet (i.e. no movements) and active (i.e. rich in muscles twitches) sleep. During that time, the sleeping cortex is dominated by sparse activity constituting silent periods which are interrupted by "bursts" of network activity consisting mainly of spindle-burst (5–25 Hz) oscillations in rodents (Khazipov and Luhmann, 2006). The EEG signature of NREM sleep (i.e. Delta wave) first appears around postnatal (P) day 11 and an adult-like sleep pattern is detectable at around 3 weeks of age (Blumberg et al., 2014). While electrophysiology has greatly contributed to the characterization of cortical sleep oscillations in the adult and immature brain, the underlying physiology and their function remain largely unknown. One of the first studies that used imaging to investigate neuronal population activity during sleep was performed in neonatal mice, at an age when cortical layers had just developed (P3-4, Adelsberger et al., 2005). Fiberoptic imaging of the synthetic Ca^{2+} dye OGB1-AM in the immature cortex revealed large spontaneous Ca^{2+} waves reflecting synchronous neuronal activity occurring specifically during resting periods. While astrocytic Ca^{2+} fluctuations were excluded as potential contributors, the nonspecific labelling with OGB1-AM precludes any definitive statement about the nature of the underlying neuronal population. The dynamics of these synchronous waves correspond to the Early Network Oscillations (ENOs), first identified *in vitro* in the immature cortex (Garaschuk et al., 2000) and hippocampus (Garaschuk et al., 1998) which also show many similarities with the dynamics of spindle-bursts (Khazipov and Luhmann, 2006). Electrophysiology has shown that spindle-burst oscillations, which often occur spontaneously, can be triggered by early self-generated sensory stimuli (e.g. muscular twitches, retinal waves). This observation originally supported an important role for such oscillations in the development of the sensorimotor system (Blumberg, 2010). Here, imaging population voltage activity in the somatosensory cortex in rat pups (P4) showed that, during active sleep, ~85% of twitches were followed by an increased membrane voltage within 500 ms. This observation confirms that sleep twitches, previously linked to spindle-bursts, directly impact cortical activity (Tiriac et al., 2012). Since ENOs and spindle-bursts have been hypothesized to underlie activity-dependent wiring of synaptic connections during early postnatal development, these imaging studies support an important role for sleep in early cortical circuit maturation.

Imaging of population activity has also been performed in adult rodents, mainly to explore sleep-stage dependent Ca^{2+} activity. We recently combined EEG recordings with fiberoptic imaging to examine how the population activity of dendrites of L5 neurons in the rat somatosensory cortex is modulated by brain states. Using OGB1-AM and GCaMP6s as Ca^{2+} indicators, we found that dendritic populations exhibit the largest Ca^{2+} transients during NREM sleep, especially during NREM transitional state (intermediate state, IS). There were very few Ca^{2+} events during REM sleep and QW. Similar to the study by Adelsberger et al. (2005), we confirmed using two-photon imaging of single dendrites (see below, 4.1.2) that these large scale Ca^{2+} waves represented mainly synchronization of Ca^{2+} activity in individual dendrites. While NREM sleep is dominated by synchronized activity, the dynamics of these transients were specifically correlated with spindle-rich oscillations (Seibt et al., 2017). Our results therefore support the hypothesis that spindle-like oscillations in the cortex involve large-scale synchronized Ca^{2+} activity. While the source of Ca^{2+} waves in the developing cortex has not been identified, our study suggests that in adults these Ca^{2+} waves may be specific to L5 neurons since the Ca^{2+} activity of populations of L2/3 neurons did not show a similar trend (Seibt et al., 2017). Recently, Niethard et al. (2016) also investigated the state-dependent regulation of cortical Ca^{2+} activity of excitatory neurons using CaMKII-GCaMP6f transgenic mice and wide field imaging. The authors show that population activity across different cortical areas gradually decreased throughout wakefulness and NREM sleep, reaching its low during REM sleep. This marked decrease in activity during REM sleep was similar between supragranual and infragranual layers. Both our study and Niethard et al. (2016) found that cortical population activity is reduced during REM sleep compared to wakefulness. However, our results differ with regards to NREM sleep where we observed an increase in the population activity of dendrites during spindle-rich episodes and not a decrease from wakefulness which may be due to differences in the source of activity (L5 dendrites vs. all excitatory neurons). We further found that population Ca^{2+} activity of L2/3 neurons and dendrites exhibits a more complex pattern across different states of wakefulness and sleep: both markedly decreased during QW compared to AW. Moreover, dendrites displayed a gradual increase in activity during individual NREM and REM sleep episodes (Seibt et al., 2017). These observations are in line with electrophysiological data showing activity dynamics across NREM and REM sleep episodes (Levenstein et al., 2017; Watson et al., 2016) and call for a closer assessment of activity fluctuations *within* sleep stages, a time course that is often ignored in current sleep studies.

Taken together, functional imaging of population activity revealed large Ca^{2+} waves during development and adulthood that are linked to sleep oscillations known to be involved in brain maturation and plasticity (e.g. ENOs/spindle-bursts, spindles). Here, imaging has provided important insights into the physiology of cortical spindle-like oscillations that had so far not been characterized using electrophysiology (Andrillon et al., 2011; Peyrache et al., 2011; Seibt et al., 2017). Comparing the results from Niethard et al. (2016) and our own (Seibt et al., 2017) emphasizes that different sources of the Ca^{2+} signal (dendrites vs. somata) may result in very different observations. Therefore, it is important to apply genetic techniques to avoid unspecific dye loading and ensure the precise identification of the Ca^{2+} signal origin.

4.1.2. Single-cell and dendritic activity

High resolution imaging provides a unique opportunity to infer activity from individual cells as well as subcellular compartments across brain states. In addition to recording dendritic activity at the population level, we also performed two-photon Ca^{2+} imaging of single dendrites across wake and sleep in adult mice. We were able to show that activity of apical shaft dendrites of L5 neurons increased from wake to sleep, with highest activity during spindle-rich and REM sleep (Seibt et al., 2017). Interestingly, our results at the population and single dendrite level did not show the same trend for REM sleep (i.e. decrease vs. increase, respectively). This is a good example of how network synchronisation, which is low during REM sleep, influences observations made with different techniques. High, but desynchronized activity of single cells (or dendrites) will not appear as such when recording population dynamics but only when observing individual dendrites. We further demonstrated this by showing that the high correlation of population activity with spindle oscillations is a manifestation of increased synchronization of dendrites during spindle rich episodes (Seibt et al., 2017). An important role for REM sleep in the regulation of dendritic activity was further demonstrated by Li et al. (2017), who combined two-photon imaging in motor cortex with a behavioural learning paradigm in adult mice. Here, the authors showed that learning increased Ca^{2+} activity in tuft dendrites of L5 neurons specifically during REM, but not NREM sleep (Li et al., 2017). Combined, these two studies point at an important role for REM sleep in the modulation of activity of individual dendrites.

Furthermore, two-photon imaging has provided information on single cell activity in the cortex during sleep. Niethard et al. (2016) also explored brain-state dependent modulation of Ca^{2+} activity of individual L2/3 cortical neurons. Here, the authors combined global GCaMP6 transfection with cre-dependent TdTomato expression in parvalbumin (PV) or SST interneurons. Cells that were negative for TdTomato were considered mainly excitatory. Similar to their wide field imaging, single cell activity in L2/3 overall decreased from wake

to NREM to REM sleep. During REM sleep, SST interneurons activity was also depressed while PV interneuron activity was enhanced to a level similar to wakefulness. These observations point to a shift in the excitation/inhibition balance to predominantly PV-dependent inhibition, silencing pyramidal neurons and SST interneurons during REM sleep. Since SST interneurons are known to target dendritic activity (Tremblay et al., 2016), a decrease in SST inhibitory tone during REM sleep could therefore also explain the increased activity seen in single dendrites during that stage (Li et al., 2017; Seibt et al., 2017).

Traditionally, sleep is viewed as a time where cortical neurons fire action potentials at a similar, if not higher (i.e. REM sleep), rate compared to wakefulness (Steriade et al., 2001). However, the above evidence demonstrates that this does not apply to all cortical neurons. Imaging experiments suggests that activity in the cortex displays much more complex dynamics when recorded from individual cortical layers, cell types or neuronal compartments. Single cell resolution, which can be achieved by combining photometry with GRIN lenses, will also help to discriminate different Ca^{2+} sources within intricate networks. In this context, Yang Dan's laboratory performed functional microendoscope imaging to investigate the sleep regulation circuitry. Using cell type specific labelling in the hypothalamus (Chen et al., 2018), brainstem (Cox et al., 2016) and midbrain (Weber et al., 2018), they successfully identified discrete inhibitory and excitatory populations within these small regions contributing to REM-NREM sleep transitions. Such strategies combined with LFP/EEG to investigating the role of sleep stages in cognition should therefore help to resolve cell type specific contributions to network activity. Although at very early stages, these studies highlight some crucial advantages of optical approaches and demonstrate how their application will help to create a comprehensive model of sleep-dependent regulation of brain activity.

4.1.3. Metabolic imaging

Sleep has long been thought to participate in the metabolic regulation of the brain to, colloquially speaking, "clear the mind". Using two-photon imaging, Xie et al. (2013) were able to provide the first molecular evidence supporting this idea. Equivalent to the body's lymphatic system, the brain is innervated by a microscopic network of fluid-filled pipes carrying cerebrospinal fluid (CSF) to dispose metabolic waste. Visualizing the movement of distinct fluorescent CSF tracers (e.g. Texas red or FITC labeled dextran, tetramethylammonium) injected into this glymphatic system, the authors showed that during sleep the flow of CSF was significantly increased compared to wakefulness, owing to an enlargement of the interstitial space. This rise in CSF flow also cleared radiolabelled amyloid beta, a key molecule in Alzheimer's disease, much more effectively and prevented its damaging accumulation. This discovery of a sleep-dependent metabolic waste-clearing mechanism demonstrates how two-photon imaging can be applied to study sleep function beyond neuronal activity.

4.2. Structural imaging

In the context of sleep research, two-photon time-lapse imaging of neuronal morphology has been game changing as it offers the unique opportunity to visualize structural plasticity *in vivo* at the level of individual spines. Below, we review a number of studies that have leveraged this approach, using a transgenic mouse line that expresses the yellow fluorescent protein YFP in L5 pyramidal neurons to track individual spines of L5 apical tuft dendrites across wake and sleep.

Repeated imaging of spine morphology during sleep was first performed in juvenile mice, when spine turn-over is high, providing an interesting time window to examine structural plasticity modifications during sleep. Two studies from separate laboratories found that spine formation was promoted during both wake and sleep but that spine pruning was enhanced during sleep (Maret et al., 2011; Yang and Gan, 2012). Since spine formation and pruning during adolescence are experience-dependent (Zuo et al., 2005), these results provide additional evidence that sleep is important for synapse-specific refinement of brain circuits during development. Structural imaging was also applied in the context of learning and memory. Based on two-photon imaging of motor cortex, Yang et al. (2014) showed that several hours of sleep following motor learning was critical for the maintenance of newly formed spines. The formation of learning-induced spines was task and branch specific and was accompanied by neuronal reactivation during sleep (Yang et al., 2014). These findings suggest a compartmentalization of sleep-dependent structural plasticity. The same group also investigated the role of sleep stages, in particular REM sleep, in this process. Although overall the number of spines were not affected, identified new spines formed during learning were specifically strengthened and pruned during REM sleep (Li et al., 2017; Yang et al., 2014). The authors also revealed that the pruning and strengthening of spines during REM sleep depends on the increase of Ca^{2+} in dendrites (Li et al., 2017). These observations demonstrate that sleep affects synaptic structure in a highly differential manner, strengthening a few behaviourally relevant synaptic contacts while weakening others. However, the mechanisms underlying the selection of spines undergoing strengthening or pruning during sleep remain to be investigated in more detail and will benefit from approaches such as optical manipulation of spine morphology (e.g. photoactivable spine probe, Hayashi-Takagi et al., 2015).

Overall, structural imaging revealed that sleep can promote both spine formation and pruning across development and adulthood. The combination with a behavioural learning paradigm further showed that structural plasticity was experience-dependent and supported long-term memory storage. The idea that sleep is involved in the selection and redistribution of synaptic weights in response to experience is important and fits with the assumption that during sleep there is synaptic reorganization rather than overall synaptic strengthening or weakening. It is thus key to pursue this line of research to further understand the complex role of sleep in synaptic plasticity.

5. Conclusions and future perspectives

In Fig. 2 we present a summary of the main findings that imaging during sleep has provided so far, grouped into functional categories. Although not abundant, optical investigation of the sleeping brain has already provided important new information on the underlying physiology and the role of sleep in plasticity. Previously, the importance of NREM sleep for cognition and memory was particularly emphasized. More recently, studies that investigated dendritic physiology and morphology as well as network activity during development have shifted that focus towards REM sleep. This is an important step forward as REM sleep is often neglected, given its short duration in rodents. Similar biases are exhibited by studies using molecular techniques where screenings are often compared between sleep and wake. However, when sleep stages are considered, results indicate a significant influence of REM sleep on gene expression (Golbert et al., 2017). Functional imaging during development and adulthood also revealed that Ca^{2+} activity synchronization in the cortex is closely associated with specific sleep rhythms, such as spindle-like oscillations. There is hope that large scale functional imaging using fast voltage sensitive dyes will greatly contribute to a better understanding of sleep oscillations. This approach has already proven successful in this field mainly in anaesthetized animals (Muller et al., 2018). Ca^{2+} imaging in anaesthetized animals also points at an important role for astrocytes in generation of, for example, slow oscillations (Poskanzer and Yuste, 2016; Szabó et al., 2017), while molecular studies provide additional evidence that astrocytes contribute to sleep regulation (Fellin et al., 2012, 2009; Foley et al., 2017; Frank, 2013; Gerstner et al., 2017). Undoubtedly, imaging of non-electrical circuit components during natural sleep will complement these findings in the future.

To conclude, imaging provides a plethora of new approaches to investigate neural circuits during sleep. However, there are several

limitations associated with the indirect, optical measurement of neuronal activity that have to be carefully evaluated when designing experiments. Hence, we believe that many promising avenues will emerge when combining optical techniques with other, complementary methods. Electrophysiology will most often be needed to monitor brain states. Furthermore, combining extracellular recordings with imaging provides exciting new opportunities to gain more insight into the relationship between cellular and network activity. Moreover, targeted electrophysiological recordings using opto-tagging provides access to spiking activity of identified cell-types (Lima et al., 2009; Muñoz et al., 2017). Finally, the possibility to combine optogenetic and chemogenetic methods with imaging, both in head-fixed (Rajasethupathy et al., 2015) and freely moving (Kim et al., 2016) conditions, will be critical to ultimately uncover the causal relationship between activity, function and behaviour in sleep research.

Conflict of interest

The authors declare no conflict of interests.

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