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Sleep as a default state of cortical and subcortical networks

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Sleep has been conceptualized as 'activity-dependent', hence a response to prior waking experience, and proposed to be 'the price the brain pays for plasticity during wakefulness'. We here propose that at the level of neuronal networks, particularly those arising from isolated embryonic thalamocortical cells maintained in culture, it represents a default mode of functioning. We show that cell assemblies in *ex vivo* cultures express powerful sleep specific patterns of oscillatory activity, as well as metabolic and molecular signatures of the sleep state. We summarize recent evidences that support our hypothesis and discuss potential applications of developing *ex vivo* sleep models to answer open questions in the field.

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Introduction

The brain alternates between wake and sleep states. In mammals, and probably all vertebrate phyla, sleep further partitions in non-rapid eye movement (NREM) and rapid eye movement (REM) sleep [1,2]. While the functions of sleep are still a matter of debate and may include synapse consolidation [3], synaptic homeostasis [4], and clearance of the brain's metabolic waste [5], substantial knowledge exists about the neural circuitries that regulate arousal and sleep. The arousal system relies on monoaminergic and cholinergic cell populations located in the brainstem and basal forebrain, and histaminergic and hypocretinergic (orexin) neurons within the hypothalamus (for a recent review see Ref. [6]). The NREM sleep promoting system relies on the inhibitory neuromodulators, gamma-amino butyric acid (GABA) and galanin, which are produced by neurons of the ventrolateral preoptic and the median preoptic nuclei of the anterior hypothalamus, the parafacial zone of the brainstem, and the nucleus accumbens of the basal ganglia [7,8].

The bistability of wake and sleep states roots in mutually inhibitory circuitries between these two systems [6]. During NREM sleep, inhibitory pathways that innervate the arousal system project to monoaminergic, cholinergic and hypocretinergic neurons, and suppress their excitatory influence on the forebrain. During wakefulness activity of monoaminergic cells inhibits the sleep promoting nuclei, thus relieves both monoaminergic and hypocretinergic systems from inhibition [9]. Complex interactions between these mutually inhibitory circuitries mediate the spontaneous transitions in vigilance states, but the exact mechanisms whereby the brain rapidly and globally transitions from one state to another are not well known. The hypocretin system innervates and coordinates all the arousal-promoting nuclei [6], and is an essential component for state stability and proper regulation of sleep-wake transitions, sustaining long periods of wakefulness and NREM sleep, and suppressing REM sleep [10,11]. Therefore, an influential concept has been the 'flip-flop' switch model, whereby hypocretinergic neurons stably control the switching mechanisms between the mutually inhibitory pathways [12]. Here we discuss recent findings and propose that NREM sleep is a default state of cortical and thalamocortical circuitries.

Sleep as a default global brain state in *Caenorhabditis elegans*

The group of Zimmer was the first to model sleep neuronal dynamics in an invertebrate [13••]. When *C. elegans* lethargus animals are in a favorable environment, most brain neurons become silent and a global quiescent brain state, akin to sleep, arises spontaneously in the absence of external cues [13••]. Under arousing cues, this state however swiftly ceases, and dynamical brain activity is reactivated. The authors used computational methods to model the dynamics of the animals' wake and sleep states as trajectories between attractor states, where dynamics converge to stable points, and proposed that sleep is an emergent property of neuronal networks. Mammals arguably feature more complex brains and sleep-associated neuronal activities, but modeling stable vigilance states as attractors may one day be possible, and an *in vitro* 'sleep-in-a-dish' [14••] approach as we describe here may facilitate this conceptualization.

Sleep slow oscillation as the default state of cortical networks

Inhibitory pathways suppress activity of arousal nuclei during NREM sleep, thus terminating the release of wake-promoting neuromodulators. This switches the

cortex and subcortical regions (e.g. thalamus) to sleep and allows them to generate their inherent network oscillations in the absence of modulatory inputs [15]. Intrinsic interactions between excitatory and inhibitory neurons, which shape recurrent local cortical networks, depolarize and hyperpolarize membrane potentials periodically. These induce consistent alternations between synchronized burst firing and quiescence of neuronal populations, known as UP and DOWN states, respectively. The slow oscillation (<1 Hz) is the reflection of UP/DOWN alternations at the EEG or LFP level.

Isolated cortical slices [16,17], cortical slabs [18••], and matured cortical cultures [14••,19•,20] consistently generate UP and DOWN states (Figure 1). Corner was the first to propose the term 'slow wave sleep' to the neuronal activity of isolated cortical networks [21•]. An elegant *in vivo* study deafferented a small cortical region from the thalamus and showed that the deafferented region could only generate slow oscillations, while the rest of the cortex could oscillate between sleep and wakefulness [18••]. LFP recordings from cortical slices even revealed that the slow oscillation travels across in *in vitro* preparations [17], similar to what it does *in vivo* [22]. We have shown that matured cortical cultures (>10 days *in vitro*, Figure 2a) show a robust slow oscillation [14••], which was later also demonstrated by independent laboratories [19•,20]. Membrane potential of cortical neurons cultured in a dish, obtained using patch-clamp recording, is hyperpolarized when the network is silent and depolarized when it is active (Figure 2b) [23•], similar to *in vivo* cortical recordings during slow oscillations (Figure 2c) [24]. The incidence and duration of the UP/DOWN states occurring in the isolated cortical islands and *in vitro* preparations lie within defined boundaries, preventing them to produce oscillations faster than the slow oscillation.

In a large study using primary cultures of mouse cortical neurons, multielectrode array recordings, and transcriptome and metabolome analyses, our laboratory established a simple *in vitro* model of sleep-wake cycle that recapitulates the major correlates of sleep and wakefulness [14••]. Independent laboratories also confirmed the feasibility of developing *in vitro* sleep-wake models using primary cultures of cortical neurons [19•,20,23•]. Stimulation of these *in vitro* cortical cell assemblies using wake-promoting neuromodulators or electrical stimulation switched the default state activity (synchronized UP/DOWN) to desynchronized tonic firing [14••,19•,20,23•]. In all these stimulation paradigms however, the *in vitro* preparations invariably returned to the default slow oscillatory activity after 24−48 hour. Altogether these studies strongly suggest that the slow oscillation, similar to NREM sleep, is the default mode activity of cortical neuronal assemblies.

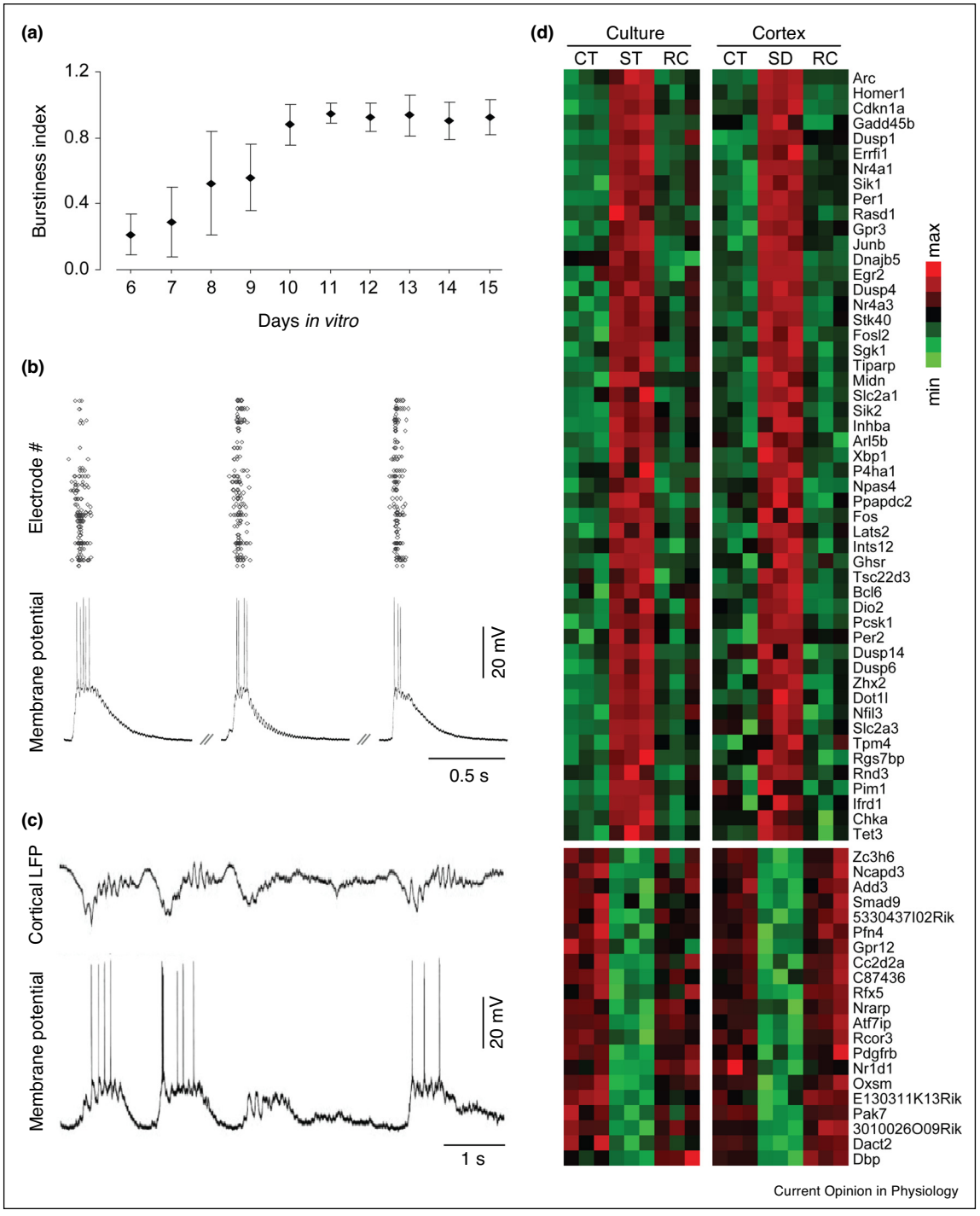
Cellular substrates of the cortical slow oscillation

Cortical UP states mostly result from the activity of cortical microcircuits between pyramidal neurons and parvalbumin-positive (PV^+) interneurons [25]. Whether the UP state is the resilient state of the cortex that is interrupted by the quiescent DOWN state is however object of debate. Most *in vivo* studies have pursued this hypothesis and investigated mechanisms that trigger and terminate the DOWN state [26–28]. The initiation and termination of DOWN states mainly involve inhibition by cortical GABAergic interneurons. Cortical somatostatin-positive (SOM^+) interneurons were shown to display minimum calcium changes during the UP state, but show a calcium increase that immediately precedes transition to the DOWN state [27,28]. Optogenetic activation of cortical SOM^+ interneurons during the UP state triggers the DOWN state, while their chemogenetic inhibition reduces the incidence of DOWN states [27]. Together these *in vivo* studies suggest that activity of cortical SOM^+ interneurons disrupts the ongoing UP state and initiates the DOWN state.

The alternate view is that the UP state interrupts the resilient DOWN state. In support of this view, the incidence of UP states increases over time by maturation of *in vitro* cortical networks (Figure 2a) [14••]. A role of astrocytes in triggering and maintaining the cortical UP state has been hypothesized, , as astrocytic calcium activity is seen to change preceding the UP state and to be coupled to neuronal activity during the UP state *in vivo* [29]. The exact mechanisms that trigger the UP state are however not clear and demand further investigations. Although the neocortex intrinsically develops the UP and DOWN firing patterns, thalamic afferents can modulate their initiation and duration (as reflected in alterations in LFP frequency), as discussed in sebsequent sections.

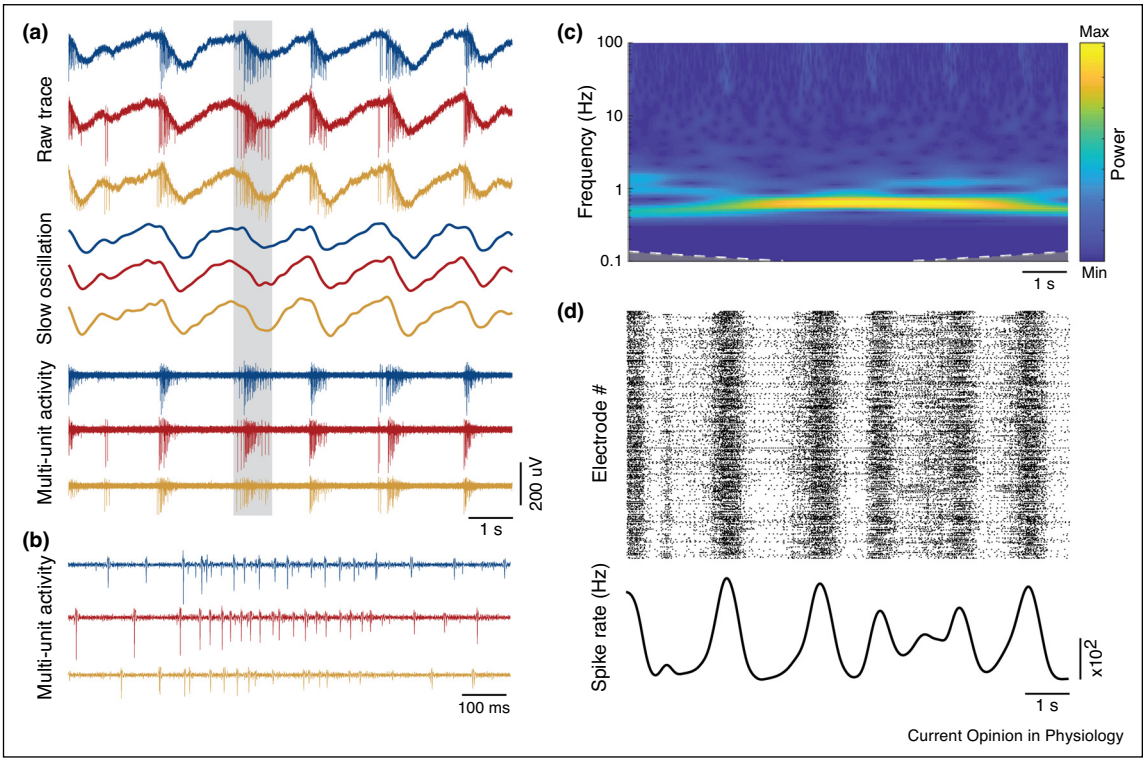
Sleep homeostasis is an intrinsic property of cortical networks

The sleep homeostatic process regulates vigilance states to compensate for sleep loss after a prolonged period of wakefulness, or reduced sleeping time, by increasing sleep depth and/or duration in the next sleeping episodes [30]. The actual substrates of the homeostatic process are unknown, but several molecules such as adenosine, cytokines and Homer1a seem to be involved [31,32]. Our laboratory identified Homer1a, a protein regulating intracellular calcium, as a key marker of sleep homeostasis, suggesting that basic cellular mechanisms are at work in the compensatory processes mediating sleep homeostasis [32]. The major neurophysiological marker of the homeostatic process is an increased power in the EEG delta band, including slow wave activity, in both rodents and humans [33]. Since the slow oscillation is the intrinsic firing mode of the neocortex, and is thought to be instrumental in shaping delta oscillations



Comparison between sleep properties in culture and *in vivo*.
(a) The incidence of burst, or the UP state, increases in cultures during maturation. The graph shows the burstiness index of primary cultures of mouse cortical neurons at different ages *in vitro*. **(b)** Simultaneous intracellular and extracellular multielectrode array recordings of rat cortical neurons in culture indicate that the membrane potential of a cortical neuron is hyperpolatized when the network is silent and depolarized when it is active. **(c)** Intracellular recording of a cortical pyramidal cell and simultaneous LFP recording, in the vicinity of the neuron in somatosensory cortex of an anesthetized cat, show robust slow oscillation, very similar to cultured neurons in B. **(d)** *In vitro* models of the sleep-wake cycle show similar changes in gene expression in cortical cultures stimulated with wake-promoting neuromodulators, as in the neocortex of sleep-deprived living mice. The heatmaps represent gene expression levels of identified sleep-responsive genes within cortical neurons (CT, Control; ST, stimulated; SD, sleep-deprived; RC, recovery). Figure (a) and (d) are taken from Ref. [14••], (b) is adapted from Ref. [23•] with the authors' permission, and (c) is from Ref. [24] (Copyright 1995 Society for Neuroscience).

[34], it is likely that cortical networks can express properties of sleep homeostasis. We investigated this assumption using mouse cortical neuronal cultures and induced a desynchronized wake-like state using a cocktail of wake-enhancing neuromodulators. We found similar changes in gene expression in these stimulated cultures as in the neocortex of living mice enforced to stay awake (Figure 2d) [14••].



The sleep slow oscillation is the default state of isolated cortical networks.
(a) Representative extracellular recordings from primary cortical culture grown on a high-density microelectrode array (MaxWell Biosystems). Raw traces show recorded signals at 20 kHz sampling rate from 3 electrodes without preprocessing. Slow oscillation signals were obtained by applying a lowpass filter (<4 Hz), and multi-unit activity was extracted using a high-pass filter (>300 Hz). **(b)** Multi-unit activity of the highlighted period in A. **(c)** Time-frequency representation of recorded signal from the culture, the yellow trace in A, indicating a robust slow oscillation (<1 Hz) activity. **(d)** Raster plot and average spiking activity of the cortical culture.

Neuronal activity of isolated cortical networks recorded using microelectrode arrays also demonstrated homeostatic response. Krueger's group induced a wake-like state in cortical cultures using electrical stimulation and assessed the dynamics of network activity following stimulation. They found that both the power of the slow oscillation and network synchronization significantly increased in the recovery period following stimulation [19•]. Our laboratory also showed that primary cortical cultures demonstrate a homeostatic response after stimulation with a cocktail of waking neuromodulators, and found that recovery was associated with a significant and dose-dependent increase in UP state incidence and duration, as well as in network synchrony [35]. Another study found that after continuous stimulation of cortical cultures with norepinephrine, or the cholinergic agonist carbachol, cultures required a 9 hour recovery period in absence of any waking neuromodulator before the network could be tonically stimulated again [23•]. Altogether these data suggest that sustained tonic firing of cortical neurons activates homeostatic processes that appear to act as powerful attractors to bring the neuronal activity back to a default set-point, which is the slow oscillation. These studies confirm that cortical networks can intrinsically establish compensatory mechanisms, and the cultures used provide a powerful *in vitro* model to investigate the cellular and molecular substrates that underlie the homeostatic process of sleep regulation.

Thalamic contribution to the default state oscillation

Cortical networks can generate the slow oscillation (<1 Hz) in isolation, but faster oscillations of NREM sleep, that the delta band (1–4 Hz) and spindles (10–15 Hz), require the interaction of the cortex with other brain regions. The thalamus projects densely to the cortex and locally modulates its oscillatory activities. Thalamic inputs can influence the incidence and duration of the cortical UP and DOWN state, reflected in the frequency of the slow waves [36,37]. Blocking thalamic afferents reduces the peak frequency of the delta band to the slow oscillation frequency and abolishes spindles during NREM sleep [37]. Spontaneous firing of centromedial thalamus (CMT) neurons is phase-advanced to the global cortical UP state, and their optogenetic burst activation mimics cortical UP states and increases the synchronization of slow waves [36]. Neuronal firing of the ventral posteromedial thalamus can excite cortical PV^+ interneurons and thereby prolongs the DOWN state [38]. Furthermore, thalamic reticular nucleus (TRN) and thalamocortical (TC) relay cells modulate local cortical slow

waves [39,40], and generation of NREM sleep spindles relies on an interplay between the TRN, TC projections and cortico-thalamic (CT) feedback [41]. How much of the thalamic modulation of cortical networks can be emulated in *ex vivo* systems, notably in relation to the generation of spindles and K-complexes, using mixed thalamocortical cocultures, or dual-chamber systems with communicating milieu, or axons, is a much anticipated and exciting question.

Intrinsic properties of TC neurons create state-dependent thalamocortical resonant loops

Studies by Steriade *et al.* on thalamic and cortical neurons contributed founding principles in our understanding of the cellular mechanisms shaping vigilance states [42]. They showed that TC neurons are endowed with electrical properties that allow them to act as global brain states' arbiters. In wake, high neuromodulatory tone and the ensuing neuromodulatory receptor signaling cause depolarization of thalamic neurons, whereupon they fire tonically and generate gamma oscillations [43]. During sleep's low neuromodulatory tone, TC cells enter a well-defined cyclical activation pattern: in response to sustained membrane hyperpolarization, TC neurons de-inactivate a Ca^{2+} conductance and activate an I_h conductance, leading to Na^+ entry, depolarization and the opening of low-threshold T-type Ca^{2+} channels, and resulting Ca^{2+} influx. This Ca^{2+} current becomes regenerative as it activates Ca^{2+}-dependent spikes that in turn activate high-frequency bursts of Na^+ spikes. These patterns will be maintained for as long as the cells remain hyperpolarized. As the cortex also generates slow oscillations, these low frequency rhythms get sustained by the recurrence of TC and CT axons, creating a robust resonant system. In Llinas and Steriade's own words: 'it is no wonder then, that the most invariant activities in the nervous system are those that generate slow-wave sleep' [43].

Thalamus as a relay in cortical arousal

The non-specific intralaminar and midline thalamic nuclei receive afferent signals from diverse arousal-promoting cell groups [44]. Among the non-specific midline thalamic nuclei, the paraventricular thalamus (PVT) receives diverse inputs from the brainstem and hypocretin system, and projects to forebrain regions including the nucleus accumbens [45]. Optogenetic activation of a class of PVT interneurons that express calretinin was shown to promote wakefulness [45,46]. Interestingly, these cells represent the major target of hypocretin neurons in the thalamus. In addition, optogenetic activation of PVT glutamatergic neurons induced sleep-to-wake transitions, while their inhibition reduced wakefulness [47]. Tonic optogenetic activation of excitatory neurons of the CMT, another non-specific midline nucleus, caused rapid arousal from NREM sleep, whereas their burst activation deepened NREM sleep [36]. The ventromedial thalamic (VM) nucleus receives strong afferents from the basal ganglia and targets layer I of most cortical regions [48]. Optogenetic activation of calbindin1-positive matrix cells of VM induced transitions from NREM sleep to wakefulness, but not from REM sleep, while their chemogenetic inhibition reduced wake duration [49].

These recent studies indicate the important role of thalamic nuclei in the control of vigilance states and in inducing both electrocortical and behavioral arousal. Even if thalamic cell groups do not release wake-promoting neuromodulators, they contribute an ensemble of powerful and essential arousal-promoting circuits. Arousal induced in these studies results from activation of thalamic nodes in the dorsal arousal pathway through which the arousal system modulates the vigilance states. Ren *et al.* elegantly demonstrated this point by showing that hypocretin neurons excite PVT neurons to induce cortical arousal through a PVT-nucleus accumbens pathway [47]. Thus, it is crucial to separate contribution of a node in a multi-nodal pathway from the source of the pathway, and to investigate upstream of manipulated nodes in future studies on the role of brain regions in promoting arousal or sleep.

Dynamic manipulation of default state oscillations

Sleep oscillations are dynamically modulated within cortical, thalamic, and hippocampal networks [50–52], suggesting that external inputs influence their default state activities. With the advance of cell-type specific recording techniques in freely behaving rodents, several studies showed that arousal-promoting nuclei are not completely silent during sleep [53–57], thus release of waking neuromodulators is not completely abolished. Among them, serotonergic neurons of dorsal raphe [53], dopaminergic neurons of ventral tegmental area [54,55], and noradrenergic neurons of locus coeruleus [56,57] show sparse activity during NREM sleep, which may contribute to dynamic manipulation of default state oscillations and possibly sleep functions. Lack of hypocretin neurons significantly decreases activity in the slow delta range (0.75–2.25 Hz), but not the higher delta band (2.5−4 Hz), supporting a role for the hypocretinergic system in regulation of NREM oscillations [11]. Considering that sleep generating pathways induce sleep through inhibition of arousal-promoting pathways, these sparse activities of arousal-promoting nuclei may reflect the level of their inhibition by sleep generating pathways, and/or they may represent active players in the processes that herald, and indeed trigger, sleep-to-wake transitions. Future studies should investigate the relationship between dynamics of local sleep oscillations recorded from thalamocortical networks with cell-type

specific activity of both arousal promoting and sleep generating sites.

Conclusion

Sleep is a complex and dynamic process reflected in specific spatiotemporal patterns of neuronal oscillations. A major signature of NREM sleep, the slow oscillation, can however be closely reproduced by isolated cortical neuronal assemblies. Studies *in vivo* showed that thalamic inputs further modulate the frequency and localization of cortical slow waves, and generation of NREM spindles relies on closed-loop thalamocortical interactions. This order of thalamic-dependent regulation still awaits *in vitro* demonstration. Additionally, the mechanisms and pathways involved in generation of REM sleep and the transitions to and out of this state remain to be harnessed *in vitro*, and fully integrated to those of NREM and wake states.

While it is well established that both quantity and quality of sleep depend on prior wakefulness, we here highlighted the default origin of the slow oscillation. These two aspects of sleep, its neural activity-dependence and default nature, need not however be independent or mutually exclusive. While neocortical networks have the capacity to produce the default slow oscillation no matter what the previous experience of the network was, the slow oscillation still can be further modified by preceding activity, especially if activity resulted in lasting changes in the network connectivity.

Sleep is traditionally defined as a behavioral state, entailing macroscopic attributes such as a species-specific posture or behavioral quiescence, which cannot be emulated *in vitro*. But if sleep, unlike physical rest, is primarily a process 'from the brain and for the brain', evolved to sustain complex neuronal network activity, it may be approximated by modeling sleep-related patterns of brain activity. Recent research indeed provided forceful evidence in support of sleep as an at least partially self-sufficient cortical process, and suggests that *in vitro* sleep models provide powerful tools to study the cellular and molecular mechanisms that regulate the sleep-wake cycle. Bridging the gap between network activity and global behavior, two domains that may reveal surprising bidirectional relationships, will bring us closer to understand why we sleep.

Conflict of interest statement

Nothing declared.

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- •• of outstanding interest

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