Sleep as a default state of cortical and subcortical networks
Mojtaba Bandarabadi, Anne Vassalll and Mehdi Tafti

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.

Address
Department of Biomedical Sciences, Faculty of Biology and Medicine, University of Lausanne, 1005 Lausanne, Switzerland

Corresponding author: Tafti, Mehdi (mehdi.tafti@unil.ch)

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 animal [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 [19**]. LFP recordings from cortical slices even revealed that the slow oscillation travels across 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 somato-statin-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 subsequent 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.
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**].
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 \(I_T\) 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 affenter signals from diverse arousal-promoting cell groups [44]. Among the non-specific midline thalamic nuclei, the paraventricular nucleus (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 calcretinin 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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**References and recommended reading**

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


This paper is the first work that developed a model of sleep-wake cycle using cultured cortical networks and showed the feasibility of such *in vitro* modelling using thorough transcriptome and metabolome analyses.


This elegant in *vivo* study demonstrated that cortical networks deafferented from thalamic inputs express the slow sleep oscillation during all vigilance states, while the rest of the cortex oscillates between sleep and wakefulness.
Physiology of sleep


21. Corner MA: Spontaneous neuronal burst discharges as dependent and independent variables in the maturation of cerebral cortex tissue cultured in vitro: a review of activity-dependent studies in live model systems for the development of intrinsically generated biologic slow-wave sleep patterns. Brain Res Rev 2008, 59:221-244. This review proposed, for the first time, the concept of ‘slow wave sleep’ for the default neuronal activity of isolated cortical networks.


23. Kaufman M, Reinartz S, Ziv NE: Adaptation to prolonged neuromodulation in cortical cultures: an invariable return to network synchrony. BMC Biol 2014, 12:83. This paper used closed-loop continuous stimulation of rat cortical cultures with norepinephrine or cholinergic agonist to desynchronize the activity of the network, showing that synchronized UP/DOWN states invariably re-emerge after ceasing the neuromodulation.


