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Sleep-active cells in the cerebral cortex and their role in slow-wave activity

Dmitry Gerashchenko[#], Jonathan P. Wisor[†], and Thomas S. Kilduff

Center for Neuroscience Biosciences Division, SRI International, Menlo Park, CA 94025 USA

Abstract

We recently identified neurons in the cerebral cortex that become activated during sleep episodes with high slow-wave activity (SWA). The distinctive properties of these neurons are the ability to produce nitric oxide and their long-range projections within the cortex. In this review, we discuss how these characteristics of sleep-active cells could be relevant to SWA production in the cortex. We also discuss possible models of the role of nNOS cells in SWA production.

Keywords

EEG; slow waves; electroencephalographic delta power; immediate early genes; neuronal nitric oxide synthase; GABAergic neurons; interneurons

Functional role of EEG slow waves

Synchronization of cortical EEG activity in the low frequency range results in production of EEG slow waves; i.e., activity in the 1–4.0 Hz (delta) range. SWA is considered a reliable indicator of sleep need that increases with time awake and decreases as sleep need is satiated during sleep¹. SWA is thought to mediate the restorative function of non-rapid eye movement (NREM) sleep^{2–4}. SWA has been linked to the induction of cortical plastic changes because it increases locally after a learning task and is positively correlated with post-sleep performance improvement⁵. Slow oscillations might help synaptic consolidation or produce synaptic downscaling to increase signal-to-noise ratios in relevant neural circuits³. Synaptic downscaling may occur because burst firing of pyramidal neurons during slow wave sleep (SWS) leads to LTD⁶. Another possible mechanism of the restorative function of SWA is replenishment of the glycogen that is depleted during wakefulness⁷. These mechanisms may explain why short naps can be efficient for improving performance or preventing decrements in performance⁸, since even brief naps are associated with an increase in cortical SWA of the EEG. SWS may have restorative properties not only for the brain, but also for peripheral organs. A selective reduction in SWS and SWA for only a few nights was shown to result in a clear adverse effect on glucose homeostasis and increased risk of type 2 diabetes in young healthy adults⁹. Taken together, these studies suggest that SWA generation is important for normal functioning of both the brain and peripheral organs.

Thalamocortical mechanisms of SWA production

The awake state is associated with a so-called “desynchronized” EEG, which consists of tonic and irregular firing of cortical neurons at relatively high frequencies (particularly in the

Corresponding Author: Dmitry Gerashchenko Tel. 857-203-6294 Fax: 857-203-5592 dmitry_gerashchenko@hms.harvard.edu.

[#]Current address: Harvard Medical School/VA Medical Center 1400 VFW Parkway West Roxbury, MA 02132

[†]Current address: Washington State University 320K Health Sciences Bldg. Spokane, WA 99210

30–50 Hz gamma band)¹⁰. Gamma-band oscillations during wakefulness produce activation geometry that is characterized by discrete clusters of brain activity. This complex pattern of brain activity has been suggested to be essential for cognition¹⁰. During NREM sleep, the activity of the EEG is dominated by large-amplitude delta oscillations (1–4 Hz). Low-frequency oscillations result in broad-band activation coherence (i.e., many cells fire together over wide areas) and the absence of cognition. This “synchronized” pattern of EEG activity occurs during sleep or anesthesia and is a product of oscillations between depolarized (“up”) and hyperpolarized (“down”) membrane potential states^{11,12}. Delta-frequency activities originate primarily from intrinsic thalamic oscillators, but these oscillators are synchronized by corticothalamic feedback¹³. Thus, delta waves depend on both activity of cortical origin and activity driven by the thalamus. Recent data suggest that temporal dissociations between the cortical and thalamic activity often occur. Thalamic and cortical activities may alternate periods of coupling and decoupling during sleep¹⁴, but a decoupling typically occurs during the transition from wakefulness to sleep in which thalamic deactivation most often precedes cortical deactivation by several minutes¹⁵. Deactivation of cortex before the thalamus could occur during anesthesia induction¹⁶. Since SWA can be generated in the surgically-isolated cortex¹⁷ or when the cortical and thalamic activities are decoupled, it is expected that the cortex should contain neurons that sustain SWA generation in the absence of thalamic input. Furthermore, since SWA involves both depolarizing and hyperpolarizing phases, inhibitory neurons are likely to be among the cortical neurons involved in SWA. Whereas thalamocortical mechanisms of SWA production have been studied in great detail, the intracortical mechanisms of SWA production have received little attention.

Sleep-active neurons in the cerebral cortex and their association with SWA

The immediate early gene Fos has been widely used as a marker of neuronal activity¹⁸. In a recent study¹⁹, we examined Fos expression during sleep and wakefulness in the types of neurons most likely to play an important role in sleep and wakefulness. We identified a cell type in the cortex in which the expression of Fos is induced during recovery sleep (RS) after a period of sleep deprivation (SD). We also found that the expression of Fos in these neurons varied in parallel with SWA in three mammalian species and was significantly correlated with NREM sleep delta energy in mice.

In both rat and mouse cortex, we found that a subset of GABAergic interneurons which express neuronal nitric oxide synthase (nNOS) showed greatly elevated Fos expression during RS after 6 h of SD¹⁹. The number of Fos⁺/nNOS-immunoreactive neurons in the cortex was greatly increased during RS, a period characterized by reduced wakefulness, increased total sleep time and increased SWA. An increased proportion of Fos⁺/nNOS-immunoreactive neurons during RS was found in all areas of the cortex examined; very few double-labeled cells were observed during SD. In contrast to the cerebral cortex, a greater proportion of Fos⁺/nNOS neurons was not observed during RS in subcortical brain regions²⁰. These results indicate that cortical nNOS-immunoreactive neurons are uniquely activated during RS when SWA is high. To determine whether nNOS neurons are active during “normal” sleep, we measured Fos expression in nNOS cells in mice undergoing spontaneous bouts of sleep and wakefulness¹⁹. Although the amounts of both NREM and REM sleep were similar in mice during the 2.5 h before sacrifice at ZT2.5 and ZT8.5 (ZT0 defined as light onset), the proportion of Fos⁺/nNOS cells was significantly higher in mice killed at ZT2.5 when SWA was high than at ZT8.5 when SWA was low. Across the day, the number of Fos⁺/nNOS double-labelled cells was more strongly correlated ($R=0.61$, $P<0.005$) with NREM delta energy (the product of NREM sleep time multiplied by SWA) during the 2.5 h prior to the animal's sacrifice than with either NREM sleep time or SWA alone.

Assessment of different regression models indicated a nonlinear relationship between these parameters (Figure 1).

Similar observations were made in the brain of a third rodent species, the golden hamster (*Mesocricetus auratus*), during free running/constant dark conditions¹⁹. Although we did not record EEG in this experiment, the proportion of Fos⁺/nNOS-immunoreactive neurons was very high early in the inactive phase of the circadian cycle (Circadian Time 0300 or CT3), when high levels of NREM sleep and SWA have been described in this species²¹. The proportion of double-labelled neurons decreased across the inactive phase with the lowest proportion observed early in the active phase, in conjunction with the hamster's peak locomotor activity (CT12-CT15). This temporal profile of Fos⁺/nNOS-immunoreactive neurons can be expected to correlate with NREM delta energy since both NREM sleep and SWA are at their highest levels early during the inactive phase and SWA exponentially decays during subsequent sleep in the inactive phase in the hamster²¹. A significantly higher proportion of Fos⁺/nNOS-immunoreactive neurons was also observed in the hamster cortex during the latter half of the active phase (CT21 to CT0). Higher NREM delta energy might be expected at this time because longer sleep episodes occur late in the active phase.

Role of NO in the EEG synchronization

NO is a signaling molecule produced from L-arginine and oxygen by a family of nitric oxide synthases (NOS). Higher levels of NO production have been consistently found in the cortex during the lights off period in nocturnal animals²²⁻²⁵. The diurnal variations in NO level, nNOS expression, and activity are tightly correlated within the frontal cortex in rats²⁵. While the diurnal nature of NO production is evident in these studies, the basis for this variation is unknown. At present, the prevailing point of view is that NO production and nNOS activity are coupled to wakefulness. Our data (Figure 1) suggest that NO production and nNOS activity may be elevated during sleep with high SWA. It is possible that such coupling has not been previously found because NO release, nNOS expression, and nNOS activity during wakefulness have not been compared with levels during sleep associated with increased homeostatic drive. In a recent study, voltammetric measurements of NO were performed in the frontal cortex during wakefulness, NREM sleep, and REM sleep²⁶. This study failed to show increased NO production during sleep. However, NO was not measured during RS after SD or during sleep periods associated with increased homeostatic drive and high levels of SWA²⁶. Other studies have measured NO during REM SD and sleep recovery, but they did not simultaneously record EEG^{24,25}. One of these studies demonstrated that the correlated changes observed in baseline conditions between NO release, nNOS expression, and NO activity in the frontal cortex were disrupted during REM SD and subsequent recovery²⁵. The authors noted in the discussion that the precise mechanisms underlying such disruption remained to be investigated²⁵.

Taking into account our observations that activity of cortical nNOS cells correlates with delta energy, all previously published results can be explained by the hypothesis that NO level, nNOS expression, and activity are increased during sleep. NO production has a clear diurnal pattern²²⁻²⁵, but it is secondary to the homeostatic drive that increases after long bouts of wakefulness. Substantial increases in cortical NO release that last for a few hours after the end of REM SD have been shown in two studies (see Figure 4 in²⁵ and Figure 3B in²⁴). These previously unexplained increases in NO release support our model because they occur during recovery after SD, when homeostatic drive and SWA are high. We hypothesize that NO release is higher during the night in nocturnal animals, especially during the latter part of the night^{24,25}, because NO production increases during nighttime sleep when homeostatic drive is high. However, we recognize the need to be cautious regarding whether the changes described above are due to nNOS activity in the sleep-active population of

cortical neurons. There are three isoforms of NOS: nNOS (also known as NOS-1), inducible NOS (NOS-2) and endothelial NOS (NOS-3).

Although nNOS is presumed to be the source of NO released in the cerebral cortex, other sources cannot be ruled out. Even presuming that nNOS makes the greatest contribution to cortical NO levels of the three isoforms, it is not certain that the relevant pool of nNOS is located in the sleep-active neuronal population since the neuronal isoform is also present in terminals of basal forebrain cholinergic cells that project to the cerebral cortex²⁷. Therefore, changes in cortical NO levels may also depend on activity of other neurons in addition to the sleep-active population.

NO appears to play a role in regulation of regional blood flow²⁸⁻³⁰, but it also modulates neural activity more directly. NO may play a key role in short-term dynamic variations of the strength of synapses on cortical pyramidal neurons³¹. NO is also implicated in long-term synaptic plasticity as a retrograde messenger in several regions of the brain, including the cortex³². NO may also affect neuronal activity by modulation of gap junction permeability^{33,34}.

Rather than being released by exocytosis from synaptic vesicles and acting on membrane-bound receptor proteins³⁵, NO rapidly diffuses through membranes in target neurons, where it can be stabilized through reaction with protein carriers³⁶. Because NO readily crosses membranes, diffusing to act nearly simultaneously on a large number of cells throughout a volume of tissue (potentially as much as several hundred microns from the site of release³⁷), it is especially well-suited for large-scale modulation of brain activity^{38,39}. Several studies have documented effects of NO on the electrical activity of neuronal networks. In invertebrates, NO modulates the frequency of network oscillations in both sensory and motor systems⁴⁰. NO-related modulation of the rhythmic activity of neuronal ensembles have also been observed in mammals⁴¹.

NOS knockout studies

If NO is critically involved in the generation of SWA, a blunted SWA response to SD in nNOS KO mice would be expected. Unfortunately, the response of nNOS KO mice to SD has not been evaluated to this point. Without this key experiment, it is difficult to assess the extent to which the SWA generating system is functional in nNOS KO mice, although the existing evidence suggests a defect in this system. First, when compared to the control strain, hourly amounts of SWA are consistently higher across the 24 h period in nNOS KO mice⁴². Increased SWA most likely indicates that nNOS KO mice have high homeostatic pressure under baseline conditions, presumably due to a failure to discharge sleep need even when sleep occurs. A similar explanation has been proposed for the differences in SWA in humans, in which short sleepers are thought to live under a higher “NREM sleep pressure” than long sleepers⁴³. Second, systemic administration of tumor necrosis factor alpha (TNF α) increased NREM sleep and SWA during NREM sleep in control and iNOS KO mice, but failed to increase these parameters in nNOS KO mice⁴⁴. TNF α is a key cytokine that regulates NREM sleep responses. Systemic injections of TNF α in animals enhance both NREM sleep amounts and SWA during NREM sleep^{45,46}, whereas inhibition of TNF α has the opposite effect on NREM sleep and SWA⁴⁶, attenuates sleep rebound after SD⁴⁷, and prevents NREM sleep increases after mild increases in ambient temperature⁴⁸. The failure of TNF α to increase NREM sleep and SWA in nNOS KO mice suggests that nNOS is required for mediation of TNF α sleep responses.

Role of long-range projections in EEG synchronization

Recent data indicate that slow waves originate locally in the cortex and then travel throughout different cortical regions. High-density EEG recordings demonstrated that slow waves are associated with large currents in several distinct cortical areas that are highly interconnected and overlap with many parts of the default network⁴⁹. SWA has been also analyzed using voltage-sensitive dyes (VSD), which report changes in membrane potential over large regions of the cortex and correspond closely with cortical activity measured by surface EEG electrodes⁵⁰. In a recent study, VSD signals in both hemispheres were shown to strongly correlate in both time and space in wild-type mice anesthetized with urethane, but not in anesthetized acallosal I/LnJ mice⁵⁰. This result suggests that neuronal connections through the corpus callosum are needed for the synchronization between cortical hemispheres. Communication between cortical areas depends largely on glutamatergic neurons because these neurons constitute the majority of long-distance connections with either other pyramidal neurons (80%) or inhibitory interneurons (20%)⁵¹. Until recently, the general view was that glutamatergic activation of local-circuit GABAergic neurons was required to produce inhibitory effects in the course of interactions between distant cortical areas. Several studies have now demonstrated that inhibitory effects can also be produced monosynaptically by a subgroup of GABAergic neurons that project over long distances in the cortex. These neurons may provide the morphological basis for direct intra-⁵²⁻⁵⁵ and interhemispheric inhibition⁵⁶⁻⁵⁸. The presence of nNOS in the majority (71–94%) of these GABAergic long-range projecting cortical neurons has been demonstrated in mice⁵⁹, cats⁶⁰, and monkeys⁶¹. Thus, nNOS neurons can play a role in SWA synchronization between hemispheres, but additional studies are needed to determine the type of neurons that are responsible for the interhemispheric SWA synchronization.

Possible models on the role of nNOS cells in SWA production

During the transition from wakefulness to sleep, thalamic deactivation most often precedes that of the cortex by several minutes¹⁵. Slow oscillations originate at various locations within the cortex (typically, in deep layers)^{62,63} and then travel across the brain⁴⁹. These findings suggest that the slow wave triggering occurs in the cortex, and not in the thalamus. However, what triggers slow waves is not known. Although the switch from silence to activity of pyramidal cells seems to be mediated by spontaneous synaptic events⁶³, the frequency of these events could be affected by external inputs. Such inputs may arise from within the cortex or be transmitted from subcortical regions, such as the basal forebrain^{64,65}.

After extended periods of wakefulness, high levels of sleep-promoting substances such as adenosine accumulate in the cortex and basal forebrain^{65,66}. These substances could initiate EEG synchronization through mechanisms that are presently unknown but may involve cortical nNOS neurons. If cortical nNOS neurons become activated by sleep-promoting substances while their activity is no longer under inhibition from wake-active brain regions (see details of our proposed model in⁶⁷), nNOS cells could facilitate activity of the corticothalamic loop that is responsible for SWA generation. According to this hypothesis, nNOS neurons would not be directly involved in the generation of SWA, but would instead play a permissive role in SWA and in maintaining SWA at a high level. As the concentration of sleep-active substances decreases during sleep, activity of nNOS cells and their facilitation of SWA would decline.

A specific role for nNOS cells in SWA is yet to be experimentally addressed. At present, we cannot conclude that nNOS neurons are an essential part of the SWA generating system. In this case, loss of nNOS neurons in the cortex should result in a large reduction or disappearance of SWA. We think that this alternative is very unlikely, since previous

publications have identified other cells that are sufficient for the generation of SWA⁶⁸. It is also possible that the activity of nNOS cells is driven by SWA, and that nNOS cells do not play a causative role in SWA production or initiation. We think that this is also unlikely because it would not explain why nNOS neurons are highly active during sleep episodes with high SWA. Testing these hypotheses will require procedures that allow selective activation, inhibition or ablation of the sleep-active neurons in the cortex.

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Figure 1.

Fos expression in nNOS-immunoreactive neurons of mouse cortex during spontaneous sleep and wakefulness. Note the similarity in profiles of % of Fos in nNOS cells (upper panel) and NREM delta energy (middle panel). The proportion of Fos⁺/nNOS double-labeled cells was highly correlated with NREM delta energy (lower panel). * $P < 0.05$. Reproduced from¹⁹; copyright 2008 National Academy of Sciences, U.S.A.