Sleeping with the clock: pacemaker neurons enter the scene

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ABSTRACT

Theoretical contributions provided by Giuseppe Moruzzi in 1972 and Giovanni Berlucchi in 1970 are here revisited, highlighting an itinerary of knowledge on the relationships between sleep-wake alternation and biological clocks, and on the role of pacemaker neurons. With a modern insight, Moruzzi dealt with the role of homeostatic mechanisms and of “timing devices” in sleep and wake, and he referred to a theory formulated by Berlucchi. This theory, which has remained hidden in a book chapter, stemmed from a careful critical evaluation of previous experimental approaches and theories. With a remarkable intuition, Berlucchi proposed that the sleep-wake cycle is an endogenous biological rhythm, as other body rhythmic functions with which it interacts, and that this cycle is generated, as other rhythms, by a functional group of pacemaker neurons, endowed with endogenous rhythmic properties. Berlucchi viewed pacemaker neurons as hierarchically organized cells, entrained by the environment, controlled by intercellular, synaptic and nonsynaptic communication. All these hypotheses have been subsequently confirmed by discoveries that are here summarized. These issues are still at the forefront of research; many questions, however, are still open.

Key words
Sleep • Wakefulness • Homeostasis • Circadian rhythms • Pacemaker neurons • Oscillators • Giuseppe Moruzzi • Giovanni Berlucchi

Abbreviations
EEG, electroencephalogram • REM, rapid eye movement • SCN, suprachiasmatic nucleus

Revisiting concepts on sleep-wake timing

Forty years ago, Moruzzi (1972) presented in a seminal article an overview of knowledge accumulated by then on neural mechanisms underlying sleep, wakefulness and their alternation. We here wish to revisit, in the light of modern knowledge, sections of the last part of this article, as well as a review written by Berlucchi (1970) to which Moruzzi (1972) refers. These texts are especially inspiring nowadays for their foresight on the relationships of sleep-wake states with biological clocks, and the key role played by pacemaker neurons in these processes. Tracing the full itinerary of the knowledge gained in these fields of investigation in the last four decades is beyond the limits and scope of the present article; current knowledge will, therefore, be briefly dealt with, highlighting key concepts. This itinerary shows, however, that novel ideas may enter the scene on tiptoe and knowledge is then accelerated and bursts out.
Homeostatic mechanisms in sleep physiology

The section on “The Biological Clocks” of Moruzzi’s (1972) article is preceded by a short section in which, discussing an issue a physiologist had to take into account, Moruzzi analyzes the possibility that sleep could represent a homeostatic mechanism. The concept of homeostasis, introduced by Claude Bernard (1885) and Cannon (1929) referring to the capacity of an organism to maintain a stable internal environment, was a pervasive framework in physiology at the time of Moruzzi’s writing.

In Sherrington’s view, to which Moruzzi refers, sleep, which restores the effects of the “wear and tear” occurring during wakefulness, certainly contributes to the maintenance of a steady state in neurons and their complex wiring (Sherrington, 1946). Concerning the possibility that sleep contributes to a “local homeostasis” of interactive neurons, Moruzzi recalls: “It has been claimed that the internal environment may be modified by sleep deprivation” and he refers here to the studies which pioneered the “humoral regulation” of sleep (Legendre and Pieron, 1913; Pappenheimer et al., 1967). “It is puzzling that sleep-promoting substances may appear in the blood or in cerebral spinal fluid both as a consequence of prolonged wakefulness and of induced sleep” comments Moruzzi, adding that uncertainties in those experiments are also shown by studies on Siamese twins who do not exhibit the same sleep-wake cycles (Alekseeva, 1958). Presumably with an afterthought, Moruzzi also comments that “a physiologically meaningful result would be obtained only if it might be shown that a sleep inducing factor accumulates in the blood or in the cerebral spinal fluid during a normal period of wakefulness and disappears during natural sleep. Were such a result obtained it would have important theoretical implications”.

Moruzzi hence considers that “the regulation of local processes of recovery should be basically different from that involved in classical homeostasis” also because, if sleep is related to cognitive processes which occur during wakefulness, as previously demonstrated by Fejnberg and Evans (1969), and if sleep has the function to “reinstate or restore a condition of the brain that was present at the beginning of the previous period of wakefulness, such a condition could not be restored without abolishing the effects of learning”. Moreover, “regulation of sleep is a centralized process, while neurochemical needs are probably different in different parts of the cerebrum. The need of slow recovery is in fact likely to depend both on the intensity and on the type of activity during the previous periods of wakefulness. But recovery cannot occur at random, only when needed; it must be concentrated in a given period of time, called sleep. This, incidentally, is likely to be the functional meaning of a centralized control of sleep by the brainstem and the diencephalon”.

Moruzzi concludes that “the fixity of the internal environment is not the aim of sleep or at least not its major task”. Despite Moruzzi’s cautious attitude, or even perplexity, on the role of homeostatic mechanisms in sleep, the questions he raised will subsequently be reconsidered in a perspective that remarkably suits his formulation. Ten years later, the influential “two process model” of sleep regulation (Borbély, 1982; Daan et al., 1984) proposed that sleep is generated by the interaction of homeostatic and circadian mechanisms. Thus, Moruzzi’s “need of recovery depending both on the intensity and on the type of activity during the previous periods of wakefulness” found a concrete physiological correlate in the electroencephalogram (EEG) slow-wave activity as a marker of homeostatic sleep pressure.

The “two process model”, widely validated in humans and several other species (e.g. Tobler and Borbély, 1986; Dijk and Czeisler, 1995; Deboer and Tobler, 2003), introduced two additional concepts Moruzzi had somehow anticipated. The first concept that “in addition to its global aspect, sleep may have also a local, use-dependent facet” (Borbély, 2001) has received firm support (e.g. Vyazovskiy and Tobler, 2008; Krueger et al., 2008; Nir et al., 2011), and was anticipated by Moruzzi’s statement on “different needs in different parts of cerebrum”.

The second concept posits “that sleep propensity rises during waking and declines during sleep” (Borbély and Achermann, 2000). The biological mechanisms underlying sleep propensity still need to be clarified, but numerous molecules are nowadays potentially implicated in this function. Among these, the role of adenosine in the local, use-dependent accumulation of sleep propensity has been established (review in Porkka-Heiskanen and Kalinchuk, 2011). This reflects Moruzzi’s expectation “of a
physiologically meaningful result”, even though his expectation of a substance which accumulates during sleep and disappears during wakefulness has actually been met by a substance, adenosine, which accumulates during wakefulness. It is noteworthy that through the elegant door of the “two process model” of sleep regulation homeostatic mechanisms had entered sleep physiology together with circadian rhythms.

**Biological clocks ticking for sleep**

In the section focused on biological clocks, Moruzzi (1972) discussed the possibility that internal clocks could play a role in the sleep-wake cycle. The context from which his considerations emerge is of special interest and historical relevance.

In the mid-1960s, investigators engaged in the study on circadian rhythms (and among them Franz Halberg, the father of American chronobiology, who coined the term “circadian” from the Latin *circa dies*, that he translated as “about one day”) had proposed to consider chronobiology as a discipline with a dignity on its own. This is certainly peculiar when one considers that the rampant dogma of a constant internal environment was prevailing at that time, and that chronobiology was presented even in physiology textbooks as the opposite of the steady state (see, for example, Giese, 1973).

A few years earlier, Halberg and coworkers (1954) and Aschoff (1954) had simultaneously developed the idea that cyclic variations in the environment, including the light-dark cycle, could influence the expression of circadian rhythms, and had coined for such environmental factors the terms “synchronizer” and “Zeitgeber” (“time giver” in German) in their respective publications. However, it was also found that the manipulation of circadian rhythms by environmental factors does not modify significantly the period (24 ± 2 h) of such rhythms in humans (Aschoff, 1960), pointing to endogenous mechanisms.

Kleitman was actually the first to conduct an experiment in humans in the absence of periodic environmental time cues in the Mammoth Cave in Kentucky (USA) in 1938 (Kleitman, 1987; Cajochen et al., 2006). Other pioneering experiments were performed by Aschoff and Wever (1962) and Mills (1964) on human subjects kept for long periods of time in isolation in a deep bunker or underground cave, and instructed to adopt regular habits (of sleeping, eating, etc.), and whose sleep-wake cycle exceeded 24 h though persisted in a range of 24.7-25.8 h. Moruzzi (1972) defined biological clocks as “timing devices which regulate important functions, frequently instinctive in nature, such as drinking and feeding activities. In some animals, such as the rat, sleep also appears to be entirely under their influence”. He also summarized the findings of Richter (1967), who had demonstrated that these functions are regulated by an inborn clock which is not dysregulated by alterations of the light cycles or conditions of starvation or dehydration. Richter (1967) had also pointed out in the rat one-way relationship between the clock and sleep-regulating centers, since the clock seemed to regulate sleep centers while sleep deprivation did not seem to modify the clock. On this basis, Moruzzi (1972) wrote: “Thus the clock is not controlled, via humoral or neural feed-backs, by the structures which are in need of sleep recovery”.

In Moruzzi’s view the pieces of the puzzle had found, therefore, a relatively fitting arrangement: the biological clock can influence sleep centers but, importantly, “biological clock and sleep center are separated structures” (a statement which might have been reassuring). In addition, sleep centers can be self-sufficient when endangered or perturbed, as in the case of sleep recovery, with no need of an intervention of the clock. Last but not least, sleep centers were sound evidence, while the clock, at the time of Moruzzi’s writing, was still an enigma.

With the simultaneous intuitions that frequently characterize scientific progress, in the same year in which Moruzzi published his review article the enigma was solved: the biological clock acquired the identity of a small cell group located in the hypothalamus, destined to celebrity. Key discoveries in this field appeared, in fact, in 1972 and, specifically, the first demonstration that the ablation of the hypothalamic suprachiasmatic nucleus (SCN) causes loss of circadian, endocrine (Moore and Eichler, 1972) as well as behavioral (Stephan and Zucker, 1972) rhythms. Shortly thereafter, Ibuka and Kawamura (1975) demonstrated that SCN ablation causes also a derangement of the temporal organization of rapid eye movement (REM) sleep and non-REM sleep.
during 24 h, without modifying the total amount of daily sleep. The first clock gene, denominated *per* (from “period”), had just been identified from three mutant strains of *Drosophila melanogaster* (Konopka and Benzer, 1971). Further molecular knowledge required many years and bursted in 1997, when circadian clock genes were identified in mammals (Antoch et al., 1997; King et al., 1997; Tei et al., 1997).

It is instead much less known that two years before Moruzzi’s (1972) paper, a young disciple of his had formulated a theory on the role of the biological clock in the circadian alternation of sleep and wakefulness (Berlucchi, 1970). Moruzzi (1972) stated that Berlucchi “has put forward the hypothesis that the cerebral activities underlying the alternation between sleep and wakefulness depend on an endogenous mechanism, related to the activity of pacemaker neurons which constitute the biological clock”. According to Berlucchi, however, the same neurons might be also be influenced by neural or humoral feed-backs. His conception is therefore different from that of Richter (1967), according to whom the biological clock and sleep center are separated structures, with a one-way relationship and without any feed-back control upon the timing device. Rat behavior is well explained by Richter’s theory, while what we know concerning cat physiology would fit better Berlucchi’s hypothesis”. Given that Moruzzi’s seminal studies on sleep and wake had notably been performed in the cat, the latter statement implies a benevolent view of “Berlucchi’s hypothesis”.

The section on biological clocks is then concluded by Moruzzi (1972) as follows: “In our opinion, the hypothesis that internal clocks, with their endogenous rhythm, control the sleep-waking cycle and possibly the alternation between the two stages of sleep deserves great attention. The differences between species are of course likely to be important, some clocks being completely independent of any other control, while others are submitted to several kinds of neural or hormonal influences. In either case, accumulation or depletion of local chemical factors might tilt the balance in the reciprocal control of antagonistically organized structures related to sleep and wakefulness”. This latter theme was then developed by Jouvet’s (1972) companion paper.

**Sleep-wake circadian alternation and pacemaker neurons**

Berlucchi’s theory had been published in German in a chapter of a volume on sleep and dreams (Berlucchi, 1970). Not sufficiently visible in this form, this article seems to have remained hidden and has not been cited in the numerous review papers published on this topic since then. Read nowadays in its original unpublished English version (kindly provided by Berlucchi), this text has a surprisingly modern vision. At a time when the problem of how we sleep and stay awake was highly debated, but the cause of the regular alternation of sleep and wakefulness had attracted little attention (Lavie, 2001), Berlucchi (1970) formulated hypotheses on the basis of a critical evaluation of previous experimental approaches and theories.

From experiments based on lesions and ablations at different levels of the neuraxis performed by Nauta, Bremer, Jouvet, Hobson, Moruzzi, Batini, Berlucchi himself and others, Berlucchi concluded that “the method of encephalic lesion makes it possible to localize neural centers involved in the integration of discrete behavioral or electrical events of sleep-waking cycle, but by no means allows to define the anatomical localization or even the existence of special separate centers for sleep and waking. The relationship between any given encephalic lesion and the resulting disturbances in sleep-waking pattern may be in most cases an indirect one, with the consequence that the value of the lesion for the localization of a center for sleep (or waking) is very limited or null”. However, importantly, spontaneous alternation between the two EEG activities (synchronized or desynchronized patterns) “is not observable in the prosencephalon disconnected from the diencephalon”. Therefore, the nervous mechanism for changes in general activity (the pacemaker) had to be located in the diencephalon (hypothalamus) and brain stem tegmentum. The localization in the hypothalamus was also supported by von Economo’s (1929) prediction of a sleep center located between the diencephalon and midbrain, based on his studies on encephalitis lethargica, that Berlucchi summarized in his paper.

A second experimental approach, initiated by Hess, had been based on the induction of sleep or wake by electrical stimulation of different brain regions.
This was discussed by Berlucchi as follows: "Any electrical stimulation delivered to the brain makes all neurons in the area of the electrode discharge simultaneously, thereby just disrupting the cerebral organization present at that particular moment, rather than creating a new one". Therefore Berlucchi shared the opinion of Jouvet (1972), who, after reviewing the long list of brain regions found to be "hypnogenic" concluded that "almost the whole encephalon has hypnogenic capacities". Berlucchi considered anyhow unlikely that sleep and wake are the product of different structures. Even the arousal effect of the stimulation of the ascending reticular formation (Moruzzi and Magoun, 1949) was reinterpreted by Berlucchi as a pattern of physiological activity that could "trigger the shift toward waking in the pacemaker, which then promotes and supports the organization of the waking brain".

The experimental approach of single neuron recording in unanesthetized freely moving animals during the sleep-wake cycle, introduced in the 1960s and used for the investigation of many cortical and subcortical regions, was commented by Berlucchi as follows: "there is no doubt that the activity of single neurons varies in relation to the sleep-waking cycle under several respects. There are changes in amount of activity, but it is clear that sleep does not involve any paramount reduction of discharge relative to waking. The major differences between sleep and waking concern undoubtedly the temporal patterns of discharge and the relationship between the activities of different neurons. It is out of discussion that the primum movens of the sleep-waking cycle must be a primary change in neuronal activity".

Four main theories were debated at that time. The first, formulated by Kleitman (1929) and Bremer (1937) on the basis of their experimental data, proposed that sleep is due to lack of sensory input, and hence a passive state of deafferentation leading to cortical inactivity. An unlikely hypothesis, according to Berlucchi, given that sleep is a state of "relative sensory isolation" with no evidence of centrifugal mechanisms leading to "sensory deafferentation". Furthermore, sensory input is not the only source of excitation for the brain, an equally important source of intracerebral activation being represented by mental activity.

Another theory, Moruzzi’s "reticular hypothesis of sleep and waking", notably attributed to the brain stem reticular formation a unique function in providing upper brain centers with the background of excitation necessary for wakeful behavior, so that the transition between sleep and wake and vice versa was ascribed to fluctuations of ascending reticular activation. Berlucchi acknowledged that this was the first theory which did not consider sleep as a passive state. However, demonstrations of an active hypnogenic role of reticular formation centers (Batini et al., 1959; Jouvet, 1962), obtained also by multiple-unit recordings (Goodman and Mann, 1967), indicated that "the validity of the concept of ascending reticular activation can be upheld only if sleep, besides waking and arousal, is included among the processes activated". Furthermore, midbrain transection experiments performed in those years led Berlucchi to conclude that "the alternation between behavioral and EEG patterns of sleep and waking may take place in absence of the ascending reticulo-system, hence one must postulate that this alternation is controlled by other mechanisms rostral to the midbrain and evidence (presented above) suggests that these mechanisms are located in the hypothalamus. If the concept of the functional unit of the brain stem reticular formation is to be retained, it must be stressed that the reticular formation is an important, but by no means indispensable, system for the mediation of both waking and sleep".

The third theory was represented by Kleitman’s (1963) evolutionary theory of sleep and waking, as reconsidered by Kleitman himself from its first formulation (Kleitman, 1929). This theory stated that the newborn has a "wakefulness of necessity" dictated by vegetative stimuli, whereas the infant "learns" how to concentrate sleep during the night and wake ("wakefulness of choice") during the day. Sleep-wake alternation was attributed by Kleitman to activity/inactivity cycles of the brain stem reticular formation, while the eventual existence of hypnogenic centers rostrally or caudally to the reticular formation would have been irrelevant. Berlucchi, though reinforcing that "the concept of a common pacemaker controlling both sleep and waking is more economical and consistent with the experimental facts" shared with the evolutionary model the view that an autonomous rhythmic activity could be modulated by environmental inputs.

The fourth theory, Jouvet’s chemical theory of biogenic amines, had for Berlucchi the merit to com-
bine sound neurophysiological and neuropharmacological data. Previous chemical theories had indeed failed to provide explanations on the mechanisms of functioning of hypothetical arousing and sleep-inducing factors, an explanation provided instead by Jouvet’s theory, which, however, had the limit to ascribe complex functions such as wake and sleep stages to strictly localized neural centers. What really mattered for Berlucchi was if and how those experimental approaches and theories provided answers to the key questions on i) the mechanisms generating the periodic variations of neuronal activity which underlie sleep and wake alternation, ii) the location of the responsible pacemaker neurons, and iii) the functional factors which could influence them. Berlucchi thus turned the page formulating “a theory which makes no claims for originality” but presents an intuitive and coherent amalgam of previous data. The theory was based on the key concepts briefly presented below.

The sleep-wake cycle is a circadian rhythm

“The alternation of sleep and waking is expression of a physiological periodicity, integrated in the basic circadian rest-activity cycle of the organism and interlocked with several other about-the-day bodily rhythms, such as the periodic changes in endocrine activity, temperature regulations and other metabolic functions. All these physiological rhythms are considered to be the result of the entrainment of the activity of inherent timing mechanisms (biological clocks) to cyclic changes in the environment”. With this statement Berlucchi (1970) sent to the community of sleep physiologists the crucial message that the sleep-wake cycle is not a special rhythm, but one of many bodily rhythms with which sleep and wake also interact, and involves not only the brain but also the other body functions.

Full recognition of the sleep-wake cycle as an endogenous biological rhythm has been slow to come (Lavie, 2001), but forty years later the sleep-wake cycle is referred to as “the most dramatic overt manifestation of the circadian timing system” (Rosenwasser, 2009).

The enigmatic clock that in the 1970s has found its identity in the hypothalamic SCN has become today a clockwork network, entrained by cyclic changes in the environment, capable to generate and coordinate the circadian rhythms of the entire organism, obviously including the sleep-wake cycle (Fig. 1), as Berlucchi had foreseen.

In mammals, the most robust environmental cues are represented by the light-dark cycle and the time of feeding. The first, conveyed to the SCN by the axons of photosensitive melanopsin-containing retinal ganglion cells, is the main synchronizer of the SCN. Other neural (Abe et al., 2002) and non-neural (Yamazaki et al., 2000; Buijs and Kalsbeek, 2001) clocks are subordinated to this master clock (see also section “Pacemaker neurons are distributed in a kingdom”). The output ultimately drives daily rhythms in behavior and physiology (reviews in Mistlberger, 2005; Moore, 2007; Dibner et al., 2010; Herzog and Taghert, 2010). On the other hand, the feeding time entrains peripheral clocks with great efficacy whilst it exerts a relatively weak effect on the SCN clockwork (Stephan, 2002; review in Green et al., 2008) (Fig. 1). In the complex regulation of these processes, some outputs exert on input pathways a feedback which is likely to stabilize the entire circadian system (Herzog and Taghert, 2010).

There is now increasing evidence that also the sleep homeostat feeds back into the circadian process and vice versa (e.g. Dijk and Archer, 2010; Yasenkov and Deboer, 2011), thus linking homeostatic and circadian processes.

The sleep-wake cycle is generated by pacemaker neurons

“Central to the present theory is the hypothesis that the alternation between the cerebral activities underlying wakefulness and those subtending sleep depends primarily on an endogenous mechanism. The substrate of this mechanism is envisaged as a functional group of neurons, endowed with the inherent capacity of changing periodically their own spontaneously emitted activity and/or their own responsiveness to various synaptic inputs... Since these neurons are open to synaptic control, their inherently programmed schedule for action may be influenced by environmental factors, or by activities in other regions of the nervous system, often through feed-back mechanism... Neurons having intrinsic properties such as those here attributed to the hypothetical sleep-waking neural pacemakers are known to exist in invertebrates (Strumwasser, 1965; Kandel and Spencer, 1967)” (Berlucchi, 1970).
Inspired by the model proposed by Strumwasser of an intracellular mechanism responsible for the production of rhythmic activities in isolated neurons of the abdominal ganglion of *Aplysia* (Fig. 2A), Berlucchi had the lucid foresight that clock neurons could represent a key for understanding cellular and molecular mechanisms underlying sleep and wake. This will receive confirmation in the 1990s by the astonishing finding that single SCN neurons maintained in culture spontaneously exhibit a circadian rhythm in their firing rate (Welsh et al., 1995; Herzog et al., 1997) (Fig. 2B), and are therefore truly ticking devices from the electrophysiological point of view.

Such finding was rapidly followed by an increasingly detailed dissection of the molecular mechanisms underlying the clock function. It was thus demonstrated that the rhythmic circadian firing of SCN neurons depends from the cyclic expression of a large family of clock genes and their interlocking transcription-translation negative feedback loops which result in cascades of gene expression within 24 h (reviews in Lowrey and Takahashi, 2004; Colwell, 2011) (Fig. 2C). Such molecular clockwork operates also in other mammalian cells, and more in general in eukaryotic cells. Recent findings have also deciphered molecular mechanisms of the negative feedback in the core transcriptional loop of the circadian clock of eukaryotic cells (Duong et al., 2011). Furthermore, selective deletion of some of these clock genes has been shown to exert profound effects on sleep in terms of circadian rhythmicity, sleep duration and structure, and EEG delta power (review in Franken and Dijk, 2009).

The Strumwasser’s model of clock neuron adopted by Berlucchi (Fig. 2A) thus foresees two key aspects destined to receive confirmation: i) the production of proteins via RNA migrating from the cell nucleus to the cytoplasm (future clock proteins and intracellular mediators, including ATP and cAMP) which also influences ion channels and therefore membrane excitability; ii) synaptic contacts (both excitatory and inhibitory) subserving intercellular communication.
Fig. 2. - Clock neurons over time. A: Pacemaker neuron isolated from the abdominal ganglion of *Aplysia* in the figure of Strumwasser (1967) reproduced by Berlucchi (1970). The original legend emphasizes that molecules of RNA enter the cytoplasm from the nucleus and determine the production of proteins (P) which act on “membrane permeability and ion pumps” resulting in excitation or inhibition. The dotted lines represent the communication between the subsynaptic membrane and the nucleus (N), which, as stated in the original legend, could be responsible for an external control of the endogenous rhythm. B: First recording of impulse frequency of an individual neuron of the suprachiasmatic nucleus performed from explants in the mouse (modified from Herzog et al., 1997). C: Model of the molecular mechanisms as currently viewed at the basis of rhythm generation in the circadian clock: RNA and proteins (as depicted in A four decades ago) undergo a transcription-translation feedback loop which oscillates with a 24 h period in neural and non-neural clock cells. The dimer Clock-Bmal1 activates transcription of the *per* (*period*) gene, and hence Per protein synthesis in the cytoplasm. Per accumulation in the cytoplasm and its association with partner proteins as Cryptochromes (Cry) determines Per-Cry translocation in the nucleus and repression of their own transcription via inhibition of the Clock-Bmal1 dimer activity. The gradual decrease of Per protein concentration triggers the derepression of Clock-Bmal1 activity and thus the initiation of a new 24 h cycle. The clock cell can receive environmental input, interact with other cells through synaptic (which include coupling through gap junctions) and nonsynaptic mechanisms, and send an output directly through the same clock proteins or indirectly through the rhythmic expression of transcription factors. Modified and adapted from Herzog (2007) and Mendoza and Challet (2009).
At the end of the 1990s it was reported that different types of sodium, potassium and calcium channels (modulated by both ATP and the cAMP system) are involved in the action potentials responsible for the electrical activity of SCN neurons, and that the membrane potential is more depolarized during the subjective day than during the subjective night, with a rhythm in phase with that of the neuronal firing rate (review in Schaap et al., 2003). However, the mechanisms of changes in the cell membrane driven by the molecular clockwork still need to be fully unraveled. These mechanisms “may involve rhythmic transcription and/or translation of ion channels, rhythmic regulation of ion channel trafficking and distribution as well as rhythmic regulation of the channel’s open state by phosphorylation” (Colwell, 2011).

In the 1990s it was also determined that communication between SCN neurons is primarily mediated by GABA (Moore and Speh, 1993) which, though typically inhibitory, can exert in the SCN an excitatory effect (Choi et al., 2008). A key role in interneuronal communication within the SCN has also been established for the neuropeptides vasoactive intestinal polipeptide, neurotensin, gastrin-releasing peptide (review in Welsh et al., 2010) and cholecystokinin (Hannibal et al., 2010).

Besides synaptic transmission, various types of non-synaptic communication have been recently identified in the SCN: ephaptic coupling, represented by feedback of extracellular field onto the electric potential across the neuronal membrane, independent of synapses; neuronal gap junctions (Fig. 2C), and glia-neuronal interactions (review in Schaap et al., 2003; see also section “Intercellular signaling regulates pacemaker neurons”).

Pacemaker neurons are distributed in a kingdom

Since, according to Berlucchi (1970) pacemaker neurons distributed in brain circuits can influence the entire brain activity, these neurons should have a precise organization: “It is possible that the hypothetical functional group of pacemaker neurons controlling the sleep-waking cycle is hierarchically parcellated into subunits, one particular assemblage of cells pacing the rhythmic activity of the others, as is the case, for example, with the specific excitation-conduction system of the heart. However, it seems more reasonable to anticipate that each pacemaker neuron in the group has its own endogenous rhythm” (Berlucchi, 1970).

Forty years later, the concepts that clock neurons are hierarchically organized and each neuron has its own rhythm have become core knowledge on circadian rhythms in general, and on the sleep-wake rhythm in particular, as witnessed by the SCN itself. Certainly an example of nervous function localization, the master clock is composed by pacemaker neurons each endowed with its own rhythm, and thus by single-cell circadian oscillators (Welsh et al., 1995), heterogeneous concerning the rhythmic expression of clock genes, peptidergic phenotype and response to environmental cues (review in Aton and Herzog, 2005). However, SCN neurons, in slices as well as in vivo, are synchronized via neuropeptide coupling/synaptic signaling and/or gap junctions (Fig. 2C). Given that SCN neurons differ also in pacemaking ability, they should be hierarchically organized (under the leadership of neurons with more robust pacemaking ability) to give origin to an output represented by a single peak of activity with a period of about 24 h (Aton and Herzog, 2005; Welsh et al., 2010).

However, the view that the output of a dominant biological clock (the SCN) drives circadian rhythms in physiology and behavior has been challenged by the demonstration of clock-like activities in many, if not all, neural and non-neural tissues (Guilding and Piggins, 2007) (Fig. 1), and the hierarchical organization of all these clocks is currently debated. The current view maintains the SCN as main circadian oscillator (Fig. 1), dominant on other brain oscillators (Abe et al., 2002) also because it processes photic information. Peripheral oscillators, though recipient of neural and humoral signals from the SCN, self-sustained and cell-autonomous, depend instead from different environmental cues (review in Dibner et al., 2010; Abraham et al., 2010).

The question raised by Berlucchi (1970) on the hierarchical parcellation into subunits of the “functional group of pacemaker neurons controlling the sleep-waking cycle” is, therefore, still open (see, for example, Mistlberger et al., 2005; Moore, 2007; Franken and Dijk, 2009; Lee et al., 2009; Rosenwasser, 2009).

Sleep and wake shift is due to activity shift of populations of pacemaker neurons

“A spontaneous shift from sleep to waking and the reverse is supposed to occur when, statistically, a
sufficient number of pacemaker neurons change their spontaneous activity and/or are coupled with one functional circuit and uncoupled from another, thus influencing the general cerebral organization. Hence, a shift in pattern of activity of an individual pacemaker neuron is not necessarily accompanied by a shift between sleep and waking, or between waking and sleep, if the majority of the other pacemaker neurons do not undergo a similar change in activity” stated Berlucchi (1970).

In support to the view that a shift in the activity of individual neurons may not be accompanied by a sleep-wake shift, a recent study by Tononi, Cirelli and their coworkers (Vyazovsky et al., 2011) has shown in freely behaving rats that, after sleep deprivation, cortical neurons can go briefly offline as in sleep while the animal is awake and performing a task, though such performance is impaired. These tired neurons which “take a nap” (Welberg, 2011) provide evidence that sleep can occur as a local condition at the neuronal level with no global shifting of state-dependent behavior, which would require instead the coordinated action of a “sufficient number” (according to Berlucchi’s definition) of neuronal populations.

Local phenomena during sleep have also been documented in humans with simultaneous scalp EEG and intracerebral EEG recordings, which have shown regional slow waves and underlying active and inactive neuronal states as well as local sleep spindles (Nir et al., 2011).

**Intercellular signaling regulates pacemaker neurons**

Once established, according to Berlucchi (1970), that sleep-wake alternation is caused by a rhythmic variation in the mode of activity of a sufficiently high number of pacemaker neurons, the sleep-wake shift should depend from the electrophysiological and biochemical brain organization. Therefore, “the synaptic bombardment to which the pacemaker neurons are submitted can interfere with the internally set rhythm of activity, so that a drift from the sleep organization to the waking organization or vice versa may be retarded or accelerated”.

Both intrinsic and extrinsic mechanisms should be involved in the regulation of pacemaker neurons, as foreseen by Berlucchi. Intrinsic mechanisms occur as in Strumwasser’s model (Fig. 2A): “the properties of the neuronal membrane are changed from the inside of the cell by a complex biochemical substrate endowed with memory and plasticity, and the assumption made in the present theory is that the alternation of sleep and waking depends primarily on similar intraneuronal mechanism”.

As for extrinsic mechanisms, Berlucchi assumed “that the function of some neurons in the central nervous system may be merely ‘neurocrinic’, i.e., these neurons would not be engaged in functional ‘impulse’ networks, but would simply provide the other neurons with metabolic materials (not necessarily synaptic mediators) necessary for their activity. For example, it may be supposed that the hypothetical sleep-waking pacemaker neurons are unable to elaborate the substances necessary for the functioning of their internal timing mechanism, and need therefore the collaborations of other neurons for this purpose”.

The hypothesis of cells “not engaged in functional ‘impulse’ networks”, but providing “metabolic materials” necessary for neuronal activity, seems to have set the stage for astrocytes rather than for “collaborating neurons”. We now know that astrocytes, indispensable energy-supplying devices through metabolic coupling with neurons, show marked variations in the expression of genes involved in glial glycogen metabolism during the sleep-wake cycle (Magistretti, 2006). Moreover, cultured astrocytes display circadian rhythms in extracellular ATP accumulation (Womac et al., 2009), and such circadian ATP release depends from the expression of circadian clock genes (Marpegan et al., 2011). ATP and/or its dephosphorylated products (ADP, AMP and adenosine) could therefore represent circadian signals elaborated by astrocytes. Recent findings obtained in *Drosophila* also indicate that calcium-dependent astrocyte-to-neuron signaling is needed for the expression of the circadian rhythm of locomotor activity (Ng et al., 2011). We are perhaps getting very close to the “neurocrinic” function Berlucchi had postulated.

**A waking body and a sleeping body**

Berlucchi (1970) concluded that “all this is utterly conjectural, but it is clear that a better insight into the mechanism of sleep and waking will be gained by trying to assess the way in which interneuronal
communication in terms of electrical impulses and in terms of humoral exchanges contribute to the general organization of the central nervous system”. By formulating a hypothesis he defined “conjectural” but with an amazingly broad view, Berlucchi had intuitions that one by one have received confirmations and have, in turn, opened new questions.

“A waking body is the end result of the work of a waking brain, as well as a sleeping body is the end result of the work of a sleeping brain” was Berlucchi’s straightforward introductory “statement of the problem”. Despite theories, models, key discoveries and dramatic progress in knowledge, we are still confronting with this problem.

Addendum by Giovanni Berlucchi

In response to the request of the Guest Editor of this issue, Cesira Batini, to provide a comment on the present article, Berlucchi has replied with the following note.

“The authors mention a review on the neurology of sleep and wakefulness which I wrote many years ago as an adieu to the research field in which I had worked for some time under the masterly teaching of Moruzzi. After unearthing my review because it was cited in Moruzzi’s (1972) monumental analysis of the sleep-waking cycle in Ergebnisse der Physiologie, the authors have arrived at the conclusion that I was the first to postulate the possible importance of pacemaker neurons for the genesis of both sleep and waking. They provide an extensive description of my review in a modern context, and I can only thank them for their overgenerous evaluation of my work. And yet I must stand by my original statement that when I was drafting my paper I was not proposing a particularly new theory. At that time I was under the influence of Hebb’s (1949) and Kleitman’s (1963) views of sleep as an endogenous cerebral organization alternative to wakefulness, along with Moruzzi’s (1963) powerful theoretical and experimental arguments in favor of an active production of sleep by brainstem mechanisms. During my stay in 1964-65 as a post-doc at the California Institute of Technology, I had learned of the discovery by Strumwasser of circadian activities of single neurons in the parieto-visceral ganglion of Aplysia. This led me to suggest that similar pacemaker neurons, endowed with a capability for autonomous activity, but open to exogenous synaptic control from sensory organs and other nervous centers, might exist in mammals, and that a network containing such pacemaker neurons could engender and maintain both sleep and waking by imposing on the whole cerebral organization the patterns of activity resulting in the appropriate behavioural outputs. On the basis of the then available results of lesion and stimulation experiments, this network could be localized in the hypothalamus and brainstem.

My review, published in German in 1970 in a book edited by my former collaborator Walter Baust, who translated my English version, was totally neglected in the neuroscientific literature, in spite of Moruzzi’s citation, so that it would be absurd of me to claim that it had an even minimal impact on the subsequent theoretical and practical developments in the relations between pacemaker neurons, circadian rhythms and the sleep-wake cycle. These developments are described by the authors in their informative survey of the currently accepted views and current controversies about biological clocks in the central nervous system and the control of circadian and other cyclic bodily functions. Some of Moruzzi’s ideas about sleep as an instinct, or sleep as a local recovery process selectively required by neuronal systems recently undergoing synaptic plasticity, or consciousness as a graded phenomenon linked to different levels of reticular activations seem worthy of reconsideration in the light of modern work cited in the present article”.

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