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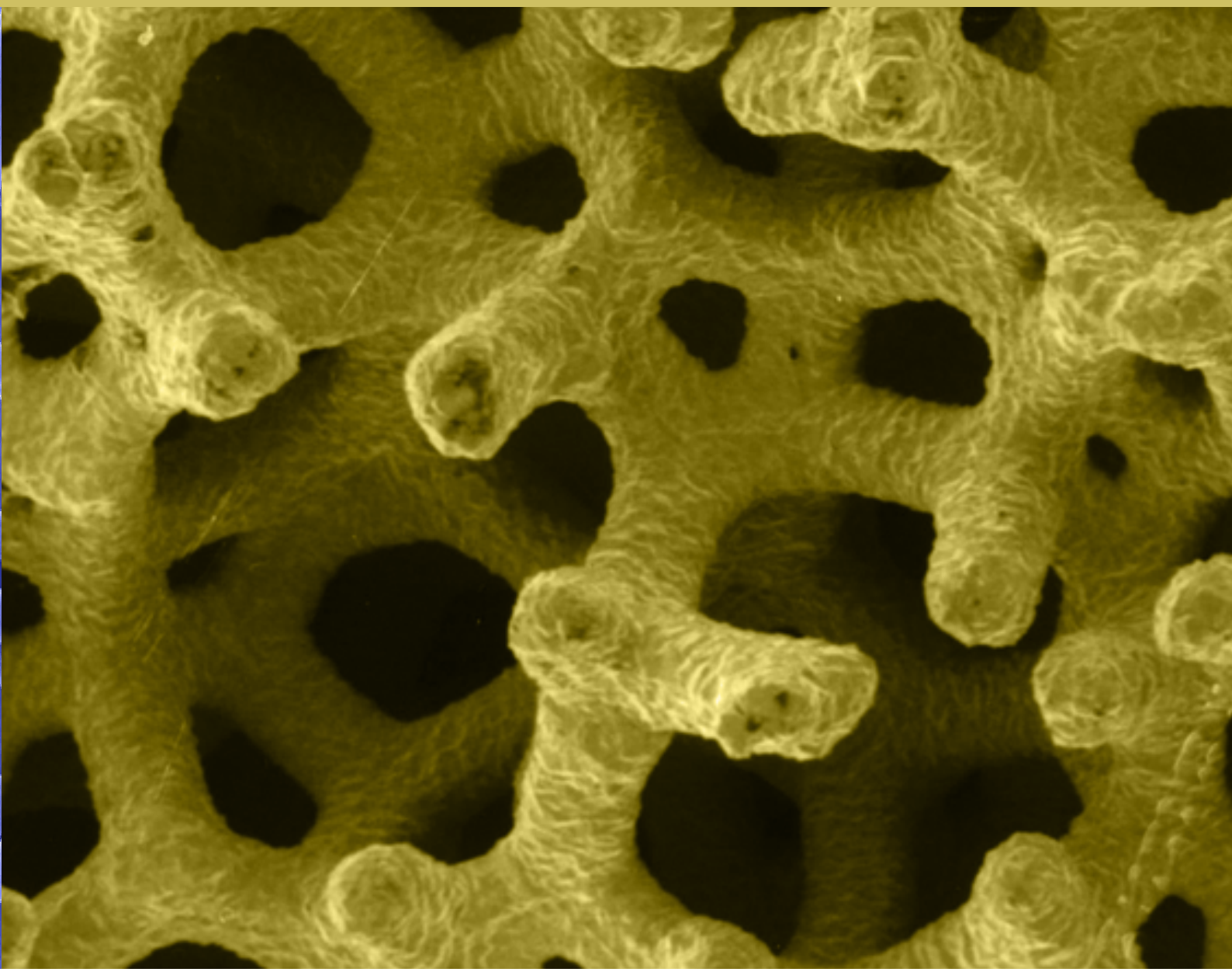

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NEW CONCEPTS IN THE NEUROPHYSIOLOGY OF SLEEP AND WAKEFULNESS

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ABSTRACT

The neural substrates of sleep and wakefulness form a highly distributed and, to some extent, redundant network, with hypocretin, monoaminergic and cholinergic systems largely promoting wakefulness and GABAergic systems in the preoptic area, hypothalamus and brainstem promoting sleep. The hypocretin/orexin system plays a special role in the promotion of wakefulness and suppression of REM sleep by providing excitatory input to the monoaminergic and cholinergic systems. Sleep is not a unitary state but involves a cyclic alternation between NREM and REM sleep; the pons is critical for generating the multiple components (ie, EEG synchronization, eye movements and muscle atonia) that characterize REM sleep. Recent findings have implicated the participation of hypothalamus, through MCH/GABA that provide a critical input to pontine generator of REM sleep. The timing of sleep and wakefulness is regulated by an interaction between the circadian pacemaker located in the hypothalamic SCN and a sleep homeostatic system whose anatomic location is yet to be definitively identified. Among various neurochemicals, extracellular AD and nNOS/NK1 accumulate in the BF as wakefulness is extended and inhibits cortically projecting cholinergic neurons, thereby influencing cortical activity. In the future, it seems reasonable to expect a spreading of these insights from basic to clinical grounds for a better understanding of the causes and mechanisms of sleep disorders and the generation of novel therapeutics in sleep medicine.

Keywords: REM, NREM, monoaminergic , cholinergic and GABAergic systems, Hypocretin, hypothalamus, circadian pacemaker, atonia. Original received: July 7, 2016; Final revision received: July 20, 2016; Accepted: July 26, 2016

Introduction

Although the mechanisms and function/s of sleep are still largely unresolved, a great advance has been made during the past decade on the understanding the mechanisms involved in the sleep-wake cycle. This new evidence was largely confined to research in animals, however it will still be very useful for medical translational approaches and to the "bench to bedside" paradigm in sleep disorders. In this Mini Review we will summarize recent evidence about arousal and sleep promoting neuronal pathways and the processes involved in the timing of sleep and wakefulness.

The reticular formation and the task of to be awake

During the mid-1930s the current concept relevant to sleep and wakefulness was generated by the pioneer research of Bremer [1] who demonstrated that lesions at low medullary levels did not modify the sleep-wake cycle ("encephale isolé") while a transection between pons and intercollicular midbrain, produced chronic sleepiness ("cerveau isolé"). Bremer's observations ruled out the initial concept that the sleep was a phenomenon merely passive produced by the inactivation of sensorial stimuli arriving at diencephalic/telencephalic structures (**Figure 1**).

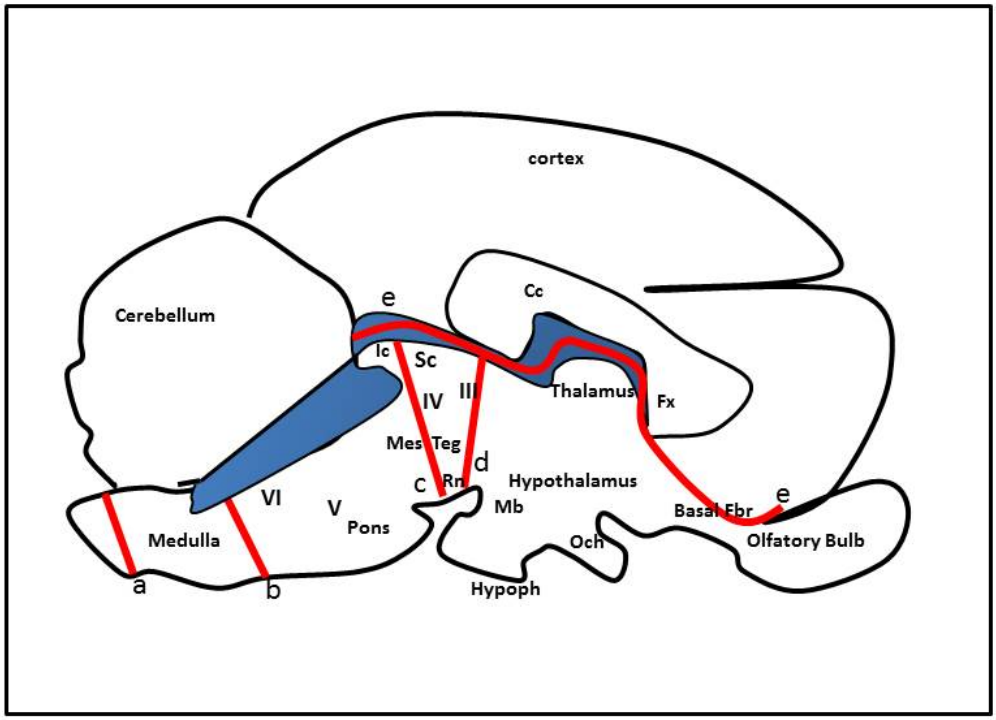

Figure 1. Representative drawing of a sagittal section of a cat's brain illustrating several transection levels (red lines). (a) Isolated encephalon (b) Medullary-pontine (c) Mesencephalic intercollicular (d) Mesencephalic precollicular (e) Telencephalon removed. Cc (corpus callosum); Fx (fornix); Hypoph (hypophysis); Ic (inferior colliculus); Lc (nucleus locus coeruleus); Mb (mammillary bodies); Och (optic chiasm); Pyr (pyramid); Rn (red nucleus); Sc(superior colliculus); III, IV, V, and VI cranial nerves (Modified from Villablanca J.R., J. Sleep Res 2004, 13:179-208[3]). In blue: Ventricular cavities.

Following these ideas, Moruzzi and Magoum [2] demonstrated at the 1940's that the electrical stimulation of the reticular formation of brainstem (pontine reticular formation-PRF) either activated the cortex or produced awakenings. Moruzzi and Magoum concluded that the forebrain was kept alert by the tonic activity of the reticular formation. Thus, during those years, the general framework of knowledge held that the reticular formation maintains wakefulness from an ascendant activation to the thalamus and cortex producing recordings of EEG activation or wakefulness. It was hypothesized that a passive inactivation of the reticular formation (FR) caused a reduction of sensorial input and consequently generated sleep. However, some observations did not fit with this "hypothetical reticular theory of sleep generation". For example Hess et al. [4] using thalamic stimulation produced sleep or wakefulness depending on the frequency of stimulation and Batini et al. [5] demonstrated that transections of pons, rostral to V cranial nerve, induced wakefulness. Both findings suggested that inputs from the lower pons or medulla inhibited a wakefulness center in the rostral pons or rostral sites (thalamus) to induce sleep. The data indicated that sleep was not merely a result of deactivation of arousal centers but an active state of the brain.

The "classic" ascending reticular activating system (ARAS) involved in arousal and EEG activation and consequently in wakefulness is composed by the cholinergic laterodorsal tegmental nucleus (LDT) and pedunculopontine tegmentum nuclei (PPT), the noradrenergic locus coeruleus (LC), serotonergic (5-HT) raphe nuclei, dopaminergic ventral tegmental area (VTA), substantia nigra (SN) and periaqueductal gray projections (vPAG), that stimulate the cortex directly and indirectly via the thalamus, hypothalamus and basal forebrain (BF). This system and others neuronal pools like glutamatergic and GABAergic midpontine neuronal pools, histaminergic and hipocretinergic neurons of the posterior hypothalamus and cholinergic neurons of the basal forebrain, constitute the general neural network for wakefulness. New evidence on this matter indicates that, a) the activating systems are not only limited to the RF but also include specific areas of posterior hypothalamus and basal forebrain (the histaminergic (HA) and orexinergic (ORX) neurons of the posterior-lateral hypothalamus (PLH) constitute the posterior hypothalamic wake promoting center), b) the activity of several FR neurotransmitters, neuromodulators on target neurons and their pathways produces arousal, thus challenging the concept of a unique mechanism of FR activation, c) the unexpected finding of the activation of BF GABAergic neurons that produce sustained wakefulness and EEG gamma activity [6] and d) the pontomesencephalic parabraquial nucleus (PB) and precoeruleus area (PC) to BF pathway that seem very important for EEG activation and wakefulness, since lesions of PB-PC, produced a coma-like state in the rat [7]. An important next step will be to determine how these areas interact and synchronize the network framework responsible of wakefulness (**Figures 2 A y 2 B)**.

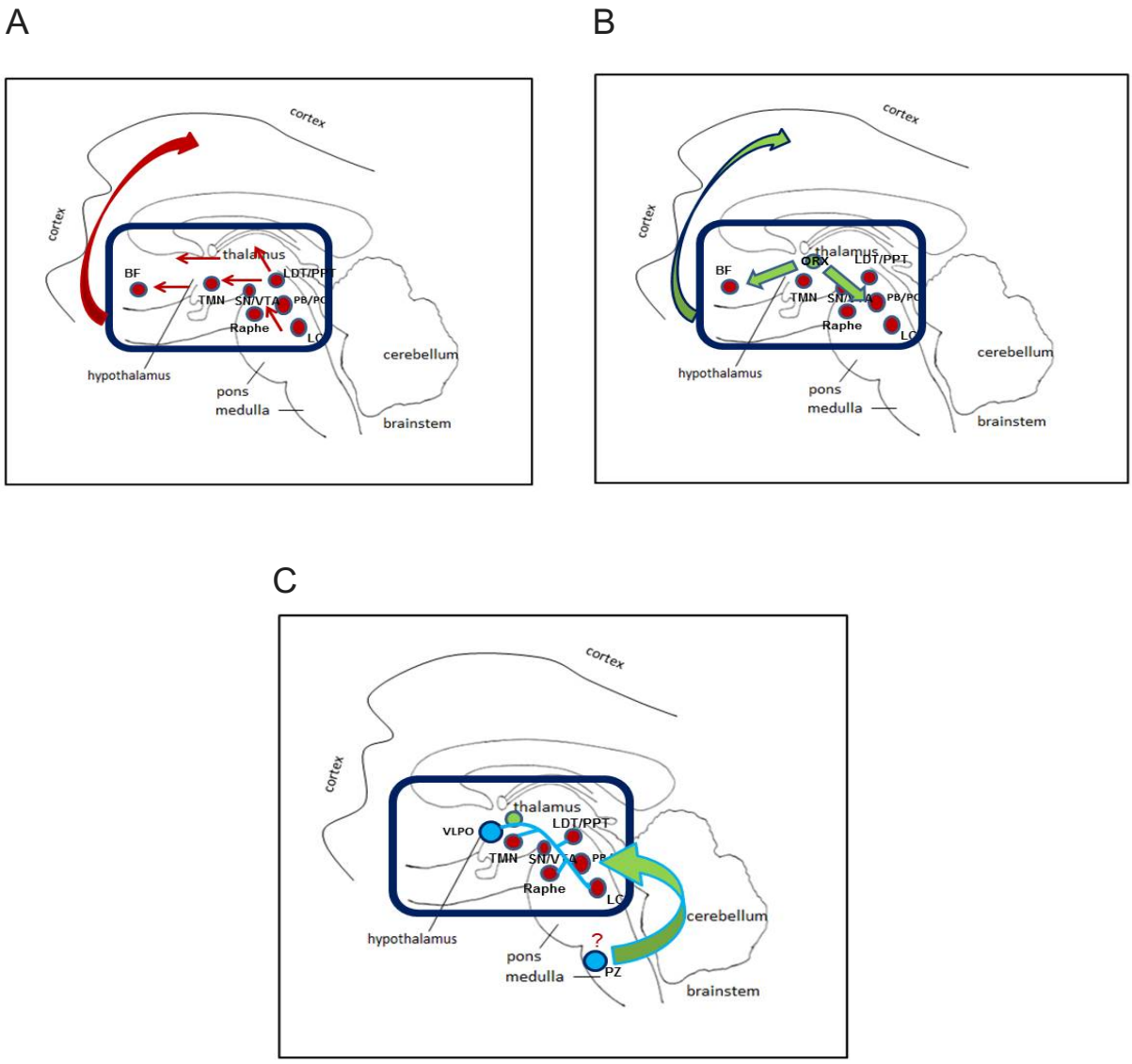

Figure 2. A. Schematic representation of neuronal pools promoting arousal to the forebrain. **B.** Sagital drawing of the orexin system (ORX) in the lateral hypothalamus innervating all the arousal system and consequently reinforcing mechanisms for wakefulness. **C.** Schematic drawing of sleep promoting pathway from VLPO/MnPO which is active during NoREM, and therefore, inhibits the activity of arousals centers of brainstem, hypothalamus and cortex. Medullary Pz area, inhibiting PB/PC area, would represent the counterpart for a dual control, rostro/caudal or caudo/rostral, for sleep generation or maintenance. BF: basal forebrain , TMN: tuberomammilary nucleus , SN/VTA: substancia nigra/ventral-tegmental area, LDT/PPT: laterodorsal and pedunculopontine tegmental nuclei, PB/PC: parabraquial/prelocuscoeruleus areas, LC: locus coeruleus. See text for further details.

To be asleep (NoREM) or to be awake: lessons from the neuropathology of Encephalitis Lethargica

In the early 20th century the prevalent thought about sleep generation was that it was a passive phenomenon, a consequence of a reduction or inactivity of the arousal systems. After World War I, encephalitis lethargica (EL), a worldwide influenza related epidemic disease, led the outstanding Rumanian neuropathologist, Constantin von Economo to identify in patients with

EL three types of lesions associated with different effects on sleep and waking [8]. Type 1: Lesions from the posterior hypothalamus variably extending to mesencephalic RF were associated to somnolence or coma; Type 2: Lesions of anterior hypothalamus (ventrolateral preoptic area-VLPO- and median preoptic area MNPO-) and nearby areas of basal forebrain, were associated with insomnia and Type 3: Lesions of posterior-lateral hypothalamus, commonly in Type 1 survivors of somnolence/coma syndrome, were associated with narcolepsy. From these observations, von Economo concluded in his seminal paper about localization in sleep, that the posterior hypothalamus contains promoters of wakefulness whereas the anterior hypothalamus-adjacent BF contains promoters for sleep. These findings were confirmed by the research of Nauta [9] and other groups demonstrating that lesions of anterior hypothalamus or preoptic/BF (substancia innominata and horizontal limb of the diagonal band of Broca), reduced sleep and conversely their electrical stimulation produce sleep onset.

As mentioned above, the HA and ORX neurons of the posterior-lateral hypothalamus (PLH) constitute the posterior hypothalamic wake promoting center. HA of the tuberomammillary nucleus (TMN) are the only source of HA in the brain and these cells project extensively innervation to forebrain and brainstem. TMN firing rate has a decreasing pattern from a continuum from wakefulness to NREM sleep and REM sleep and plays a fundamental role in the generation of wakefulness. Administration of HA/H1 agonists/H3 antagonist increased wakefulness reducing NREM and REM sleep [10]. The hypocretin neurons are found only in the PLH and projects widely to brain and spinal cord. Neuropeptides hypocretin 1 and 2 (or orexin A and B) have been reported as excitatory through the receptor OX1 and OX2 and promote waking by activating forebrain and brainstem wake active cells groups. The most important evidence of the fundamental role of orexins in the regulation of wakefulness and sleep, was the demonstration that a loss of orexin signaling occurs in narcolepsy with cataplexy [11, 12].

The VLPO and MNPO are considered to have a key role for to promote sleep. The neurons in these nuclei contain GABA and the neuropeptide galanin and they innervate all the arousal-promoting regions, including the LDT/PPT, LC, DR, TMN, and also the orexin neurons. Thus, the VLPO and MNPO are hypothesized to promote sleep by coordinating the inhibition of arousal regions during NREM and REM sleep [13, 14] (**Figure 2 C**). Recently the identification of a slow wave GABAergic promoting center in the rostral medullary brainstem, at the parafacial zone(PZ), that inhibit PB glutamatergic neurons producing NREM sleep, adds a new pathway of to future investigations [15, 17].

REM sleep and its circuitry

After the discovering of REM sleep in humans by Aserinsky and Kleitman [18] in the mid-1950s, impressive amount of research demonstrated that the pons plays a key role in the generation of REM sleep. However the specific circuitries involved in promotion and regulation of this state remains in debate. In those years, brainstem monoaminergic cells groups showing a decreased firing pattern from a continuum of states of wakefulness to NREM and to REM sleep, were called “REM-off cells”; Those showing the opposite, with increased or maximal firing rate, were called “REM- on cells”. The seminal work of Hobson and McCarley in the mid-1970s proposed that the NREM/REM cycles arise from the reciprocal interaction, between both, REM off (monoaminergic) and REM on cells

(cholinergic) in the medial pons [19]. After refinements, this model recognizes also the glutamatergic and GABAergic participation as REM on/off cells [20]. Particularly, the neurons from the dorsolateral pons (LDT/PPT) are considered crucial for its generation. Microinjections of cholinergic agonists result in REM sleep state, starting at rostral LDT/PPT and projecting to subcoeruleus or sublaterodorsal tegmental nucleus (SLD) and nucleus pontis oralis [21, 22]. During REM sleep this activation, in the dorsolateral pons, is enough to produce EEG desynchronization and theta activity, ponto-geniculate-occipital (PGO) waves, rapid eye movements and atonia. A flip-flop model for REM-off and REM-on neurons was proposed by Lu [27]. This model includes a brainstem flip-flop switch with mutually inhibitory REM-off/REM-on areas in the tegmentum. The REM-off neurons are characterized by the overlap of inputs of orexin/external VLPO and melanin concentrating hormone (MCH) neurons. The vlPAG and LPT nd REM-on pools contain GABAergic neurons reciprocally inhibiting the SLD and PC. In spite of the PPT-LDT, REM-on neurons may inhibit LPT REM-off neurons but they are not mutually inhibited by them and thus they are not part of the REM flip-flop switch. The same unidirectional relationship occurs with the serotoninergic dorsal raphe and noradrenergic locus coeruleus (DRN-LC) that activate REM-off neurons but are not inhibited by the SLD REM-on neurons. The glutamatergic REM-on neurons project rostral, to the BF and regulate the EEG components of REM sleep, and caudal, to the medulla and spinal cord muscle atonia system (**Figure 3**). Recent findings have implicated the participation of hypothalamus, through GABAergic neurons containing the neuropeptide MCH as a co-transmitter to provide a critical input to the pontine generator of REM sleep [23, 24], while others groups showed that caudally at the ventral medulla (vM), REM sleep can be switched "on/off" by a population of glutamatergic cells [25, 26] (**Figure 3 and 4**). These findings suggest a "dual command", rostral-hypothalamic and dorsal-medullary for REM sleep.

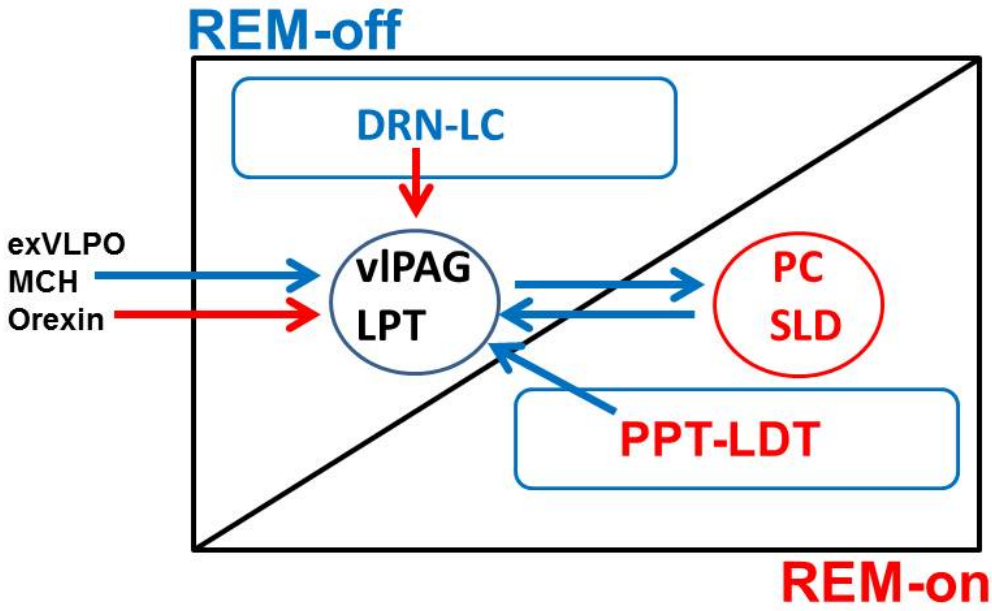

Figure 3. A Model for interactions between the REM-On and REM-Off System. Blue arrows denote inhibitory connections and red arrows excitatory connections. DRN-LC (dorsal raphe nucleus-locus coeruleus), vlPAG-LPT (ventrolateral periaqueductal gray matter and lateropontine tegmental areas), PC-SLD (peri-locuscoeruleus and sublaterodorsal tegmental areas), PPT-LDT (pontopedunculo tegmental-laterodorsal tegmental area).

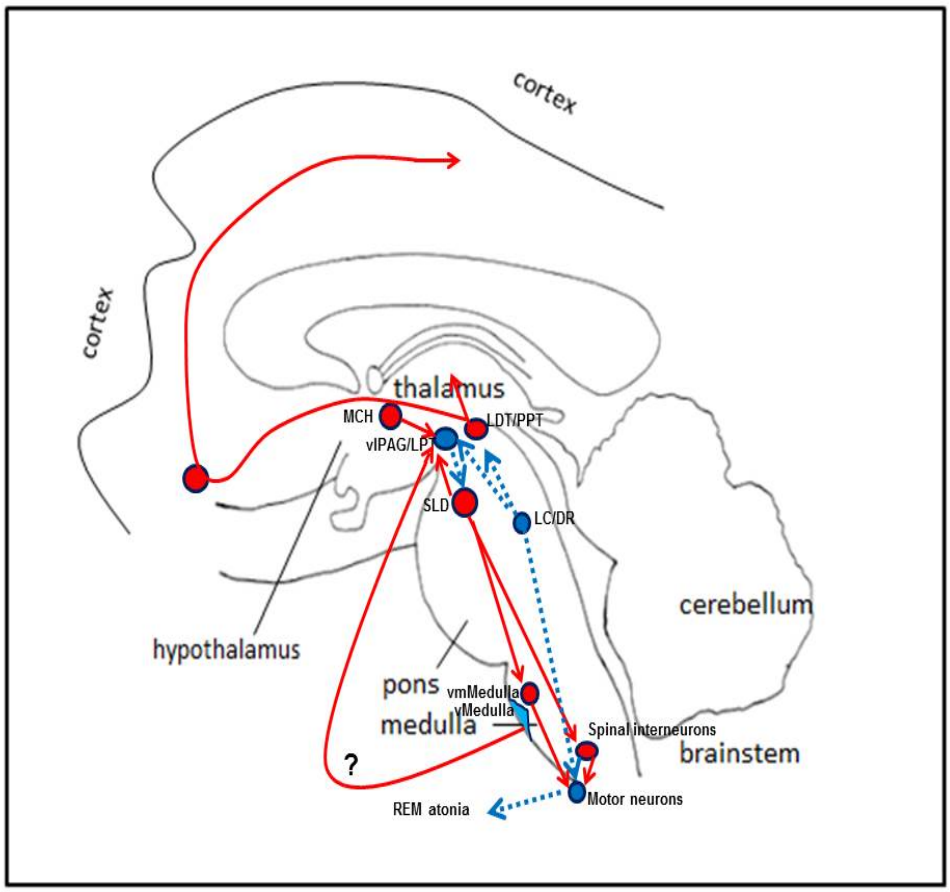

Figure 4. Schematic drawing illustrating the hypothetical circuitry involved in REM sleep regulation. A flip-flop mutual inhibition between SLD REM-on neurons and vlPAG/LPT REM-off neurons are proposed to regulate transitions into and out of REM sleep. During REM sleep, SLD neurons activate GABA/glycine neurons in the ventromedial medulla and spinal cord that inhibit motor neurons. The vlPAG/LPT inhibits the SLD, but during REM sleep, the vlPAG/LPT may be inhibited by neurons making melanin concentrating hormone (MCH) and other neurotransmitters. Solid lines denotes pathways active during REM sleep; dashed lines pathways inactive during REM sleep.

TRANSITIONS FROM WAKEFULNESS-SLEEP AND NREM/REM SLEEP: THE FLIP-FLOP SWITCH MODEL.

The wake-sleep switch

As mentioned above, several arousal-promoting projections arising from the brainstem (cholinergic, monoaminergic and glutamatergic) activates the hypothalamus, BF and cortex producing wakefulness (**Figure 2 A**). ORX neurons have a dual arousal-promoting mechanism, i.e. by reinforcing activity of the arousal centers and directly exciting cortex and BF [27, 29] (**Figure 2 B**). The main sleep-promoting pathways from the VLPO and MnPO inhibit the components of the ascending arousal pathways from the hypothalamus and the brainstem (**Figure 2 C**). However, the ascending arousal systems are also capable of inhibiting the VLPO. This mutually inhibitory relationship of the arousal and sleep-promoting pathways

produces the conditions activation/deactivation of the flip-flop switch discussed above. Also, wake–active TMN and sleep-active VLPO are reciprocally connected and contributed to the flip-flop switch mechanisms.

The NREM-REM sleep switch

Two neuronal pools of mutually inhibitory neurons in the upper pons form a switch for controlling transitions between NREM and REM sleep. GABAergic neurons in the vlPAG and the adjacent LPT fire during NREM states to inhibit entry into REM sleep. During REM sleep, they are inhibited by a population of GABAergic neurons in the sublaterodorsal region that fire during REM sleep. This mutually inhibitory relationship produces a REM-NREM flip-flop switch, promoting rapid and complete transitions between the two states. The core REM switch is also modulated by other neurotransmitter systems. Noradrenergic neurons in the LC and serotoninergic neurons in the DR inhibit REM sleep by actions on both sides of the flip-flop switch (exciting REM-off and inhibiting REM-on neurons) and during REM sleep they are silent. Cholinergic neurons promote REM sleep by having opposite actions on the same two neuronal populations. The orexin neurons inhibit entry into REM sleep by exciting neurons in the REM-off population (and by presynaptic effects that excite monoaminergic terminals), whereas the VLPO neurons promote the entry into REM sleep by inhibiting this same target. During REM sleep, a separate population of glutamatergic neurons in the SLD activates a series of inhibitory interneurons in the medulla and spinal cord, which inhibit motor neurons, thus producing the atonia of REM sleep. Withdrawal of tonic excitatory input from the REM-off regions may also contribute to the loss of muscle tone. At the same time, ascending projections from glutamatergic neurons in the PB and PC activate forebrain pathways that drive EEG desynchronization and hippocampal theta rhythms, thus producing the characteristic EEG signs of REM sleep. Further research using optogenic tools showed that REM episodes (duration and frequency) can be increased by photostimulation of MCH projections in TMN and median septum. Thus, hypothetically, MCH neurons, constitute another input to consider in the REM-NREM flip-flop model [23, 27, 28], **Figures 3 and 4**. Also the finding of vM GABAergic control of REM sleep, suggests an extended hypothalamic/midbrain/brainstem, perhaps redundant, controlling REM sleep [26].

Pressing flip-flop switches: Role of homeostatic and circadian loads

Sleep is a process homeostatically controlled and sleep deprivation in humans and animals are a proof of that. If an individual is sleep deprived for a period of time there is a subsequent increase in the amount of sleep for an adequate compensation. The best candidate in the search of the hypnogenic substance that mediates the homeostatic drive appears to be the increase of a nucleoside astrocyte-derived, adenosine (Ado), acting on the sleep-active GABAergic neurons of MnPO and VLPO [30]. Also a cortical neuronal population expressing neuronal nitric oxide synthase (nNOS) has recently emerged as a candidate for involvement in homeostatic physiological sleep response [31, 32].

The homeostatic drive has been incorporated in the 2-process model of sleep regulation, the process S and the process C. The process S, (homeostatic sleep related), builds up during wakefulness and when a threshold is reached, sleep appears only if process C is an appropriate

circadian phase [33]. The input of the circadian system constitutes a fundamental influence on sleep state switching. In mammals, daily rhythms are driven by the suprachiasmatic nucleus (SCN) through sub-paraventricular zone dense projections to dorsomedial nucleus of the hypothalamus (DMH). Lesions of DMH reduce the total amount of wakefulness suggesting that the circadian system promotes wakefulness through inhibition of the VLPO and excitation of lateral hypothalamic neurons[13]. Melatonin is produced by the pineal gland during the night in both, diurnal and nocturnal species. Specific receptors for melatonin are found in the cortex, SCN, and hypothalamic regions involved in thermoregulation. This has led to the idea that melatonin is an internal sleep 'facilitator' in humans. Melatonin can influence sleep-promoting and sleep/wake rhythm-regulating actions through the specific activation of MT_1 and MT_2 receptors, the two major melatonin receptor subtypes found in mammals. Both receptors are highly concentrated in the SCN [17, 34].

In addition, our adaptation to situations on the daily life requires alterations of a specific physiological response. Changes in the wake-sleep cycle could be observed as a consequence of stressors, seasonal changes, migrations or lack of food which in turn produces a need for adaptation to these new situations, which was called by McEwen the allostatic load [35]. Excellent examples of this situation occurred in insomnia animals exposed to behavioral stress in which an unexpected simultaneous activation of arousal and sleep promoting centers suggests a simultaneous activation of homeostatic and circadian drives and additional activation of noradrenergic(LC)/histaminergic(TMN) systems driven by the allostatic load [36]. Another example occurred when humans sleeping are exposed to a novel environment, during the first night in a sleep laboratory. The subjects keep one hemisphere partially more vigilant than the other hemisphere, during sleep. This is equivalent to keep an eye-on of wakefulness, a phenomenon which may play a protective role similar to that played in marine mammals and birds [37].

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