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NEURAL ARCHITECTURE OF CIRCADIAN RHYTHMS: A SUBSTRATE TO SLEEP-WAKE STUDIES

Luciana Pinato, Eduardo Garcia Rodrigues, Luiz Fernando Takase and Maria Ines Nogueira

Laboratório de Neurociências, Departamento de Anatomia, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, Brazil

Corresponding author
M.I.Nogueira
Laboratory of Neurosciences, Department of Anatomy
Institute of Biomedical Sciences
Universidade de São Paulo,
05508-900, São Paulo – Brazil
Phone: +55-11 3091-7401
Fax: +55-11 3091-7366
e-mail: minog@usp.br

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ABSTRACT

The nervous system is a complex arrangement of neurons and glial cells, which architecture reflects the diverse structural and functional roles played by them, involving different active chemical substances. Understanding the sleep-wake cycle, is a task that illustrates this complexity. Narrowing this perspective, the present study explored the involvement of the rat's raphe nuclei in the sleep-awake cycle, using Fos expression as the tool adopted to appraise the neuronal activation in different stages of that cycle. The awake state was studied with animals in a cage with standard or enriched conditions. The results revealed that the raphe nuclei are more involved with the functions of the animal's activity period. The rostral raphe nuclei showed a significant number of Fos_IR cells in relaxed conditions than in the attentive ones. In the caudal raphe nuclei these two conditions were not different, but interestingly they presented a considerable number of cells in the sleep state. These results taken into account with literature data on 5-HT and the fact that the raphe nuclei are its principal source, favor the concept of serotonin being mainly related to the activity period of the sleep-wake cycle.

Key words: biological rhythms, raphe nuclei, sleep/wake cycle, serotonin, Fos protein

INTRODUCTION

The biological success of a species depends on its ability to establish harmonic relations internally and with its surrounding environment. The assembly of structures enabling synchronization of the various physiological systems in an organism, and its entrainment to environmental "zeitgebers" might be described as a neuronal net, which reflects the structural elements composing the nervous architecture of biological rhythms.

Briefly, this architecture comprises many neuronal clusters in the central nervous system (CNS); some of them receiving inputs through nerves arriving from sensorial peripheral receptors; some processing those coming information, and others to distribute them, as output, to other regions in the nervous system, somatic or visceral muscles or endocrine glands. All together these different neuronal groups promote physiological and behavioral adjustments.

In mammals the central synchronizers are the suprachiasmatic nuclei (SCN), also known as the master clock. This bilateral nucleus occupies the ventral hypothalamus, resting on the optic chiasm. Each one composed by more than 10,000 cells, in rats for instance, synchronically oscillating, generating and imposing rhythms around 24 h upon other structures, at both the CNS or other peripheral clocks in the body, like adrenal glands, lungs, kidneys and liver. The rhythms are expressed in physiological or behavioral

events, such as sleep/wake cycle, body temperature, feeding behavior or secretion of glucocorticoids, among others (Hastings et al., 2003).

The SCN have endogenous rhythm, they entrain the other oscillators independently of light (Abrahamson and Abrahamson 2001; Menaker 2003). However, direct projections from the retina upon the SCN the retinohypothalamic tract, are the pathway which entrains the animal to the day-night cycle, and so far entrains the other oscillators. Indirect retinal afferents to SCN from the medianus (MnR), dorsal raphe (DR), and intergeniculate leaflet (IGL) thalamic nuclei, also modulate circadian rhythms (Morin, 1992; Kalsbeehk et al., 1993; Costa et al., 1999).

Serotonergic fibers from the raphe nuclei to SCN represent their third major source of afferent fibers (Azmitia & Segal, 1978; Van De Kar & Lorens, 1979). These nuclei innervate also the IGL (Mantyh & Kemp, 1983; Morin et al., 1992). In these circuits 5-HT inhibits light effects on circadian rhythms (Rea et al., 1994; Shibata & Moore, 1993; Cavalcante et al., 2002).

Aiming to understand the involvement of the raphe nuclei on the sleep/wake cycle, experiments were conducted in rats sleeping or awake in standard or enriched conditions. The neuronal activity was approached by Fos expression, considered a physiological marker.

MATERIAL AND METHODS

Animals

Adult male *Rattus norvegicus*, Wistar strain, 3-4 months old, were used in this study. The animals were housed in the facilities of the Institute in a light dark cycle 12:12 h (lights on at 7:00 h) and constant temperature (23 ºC). All the experiments followed the International Guiding *for Biomedical Research Involving Animals* (Cobea, Colégio Brasileiro de Experimentação Animal, 1991) and were approved by the CEA – Committee of Ethics on Experimental Animals of the Institute of Biomedical Sciences of the Universidade de Sao Paulo.

Three experimental groups, n=5 each, were composed as follows: Sleep, awake standard (AWS) and awake enriched (AWE). The animals of the sleep group underwent surgery to implant electrodes for EEG recording. After 7 days of surgery recovery their EEG were monitored for 5 days, from 11 to 13 h, to establish their pattern of sleep. In the AWE group the animals were housed in cages that encouraged exploratory behavior, by daily-introduced new objects; while the AWS groups were housed in standard conditions.

Immunocitochemistry

According to the EEG registers, 2 h after the time in which the animals showed higher amount of sleep, they were deeply anesthetized with sodium pentobarbital. After

confirmation of the anesthetic effects, they were perfused through the left ventricle with 100 ml of saline 0.9% at room temperature, followed by 300 ml of cold 4% paraformaldehyde plus borax, 4 °C, pH 9.5. The brains were removed from the skull, post-fixed for 4 h in the same fixative plus 20% sucrose for cryoprotection. Then they were moved to flasks with PBS plus 20% sucrose overnight and cut into 40μm thick coronal sections in a sliding microtome with dry ice. Every fifth section was collected in separate series of wells containing anti-freeze solution (5:5:1) and stored at -20°C. One series of tissue from each animal was incubated with primary and secondary antibodies against Fos protein, revealed through ABC-DAB method (Hoffman *et al*., 1993).

Data analyses

The histological and quantitative analyses were carried under bright light field illumination on a Hund-Wetzlar 500 microscope, coupled with the VIA 170 Video image maker-measurements, Boeckler Instruments, Inc. Only the cells with clearly dark brown stained nucleus were considered. The boundaries of the raphe nuclei were established with an adjacent series Nissl's stained and references were according to the brain atlas (Paxinos & Watson, 1998).

The results expressed as median ± SE, represent the sum of all cells counted in the slices of one series for each raphe nucleus in each animal. For statistical comparisons between experimental groups ANOVA and Tuckey post-hoc test were used. A probability value $p \leq 0.05$ was considered as statistically significant.

RESULTS

The EEG registers revealed variable pattern of sleep in respect to the amount and quality of sleep presented by the 5 animals. The sleeping duration was not proportional to the recorded amount of paradoxical sleep, characterized by rapid eyes movements (REM). The higher and lower times the animal spent sleeping varied in a range of 96.2 % to 63.0 % of the recording time. The amount of REM sleep in these same animals was respectively 20.9 % and 11.6 % (table 1).

Table 1 – Percentage of time spent by the animals in the awake and sleep stages during the 120 min of EEG recording

Animal	Awake	SWS	REM
1	2.9	76.2	20.9

2	18.5	72.9	8.6
3	36.9	51.5	11.6
4	20.0	31.1	48.8
5	26.6	20.0	53.3

The Fos expression pattern in the MnR nucleus, in the 3 experimental groups studied illustrates the general result achieved in the raphe nuclei. The sleep group had a lower number of labeled cells in comparison to the AWS and AWE groups (Figure 1 A-D). This nucleus has direct projections to the SCN, its boundaries are observed in Nissl's staining. The superior limit is immediately below the superior cerebelar decussating peduncle, while its laterals portions are restricted between the tectospinal tract, and its inferior part rests above the pontine nuclei (Figure 1 A). The reticular formation characteristic disposition of cells scattered among fibers is illustrated in the MnR (Hay-Schmidt et al., 2003). The number of activated neurons in the AWS group was twice the one observed in the AWE group at the MnR. It is interesting to point that the labeled cells are scattered all over the nucleus in the AWE, but in the AWS they are more concentrated in the dorso-ventral midline (figures 1B, C and D).

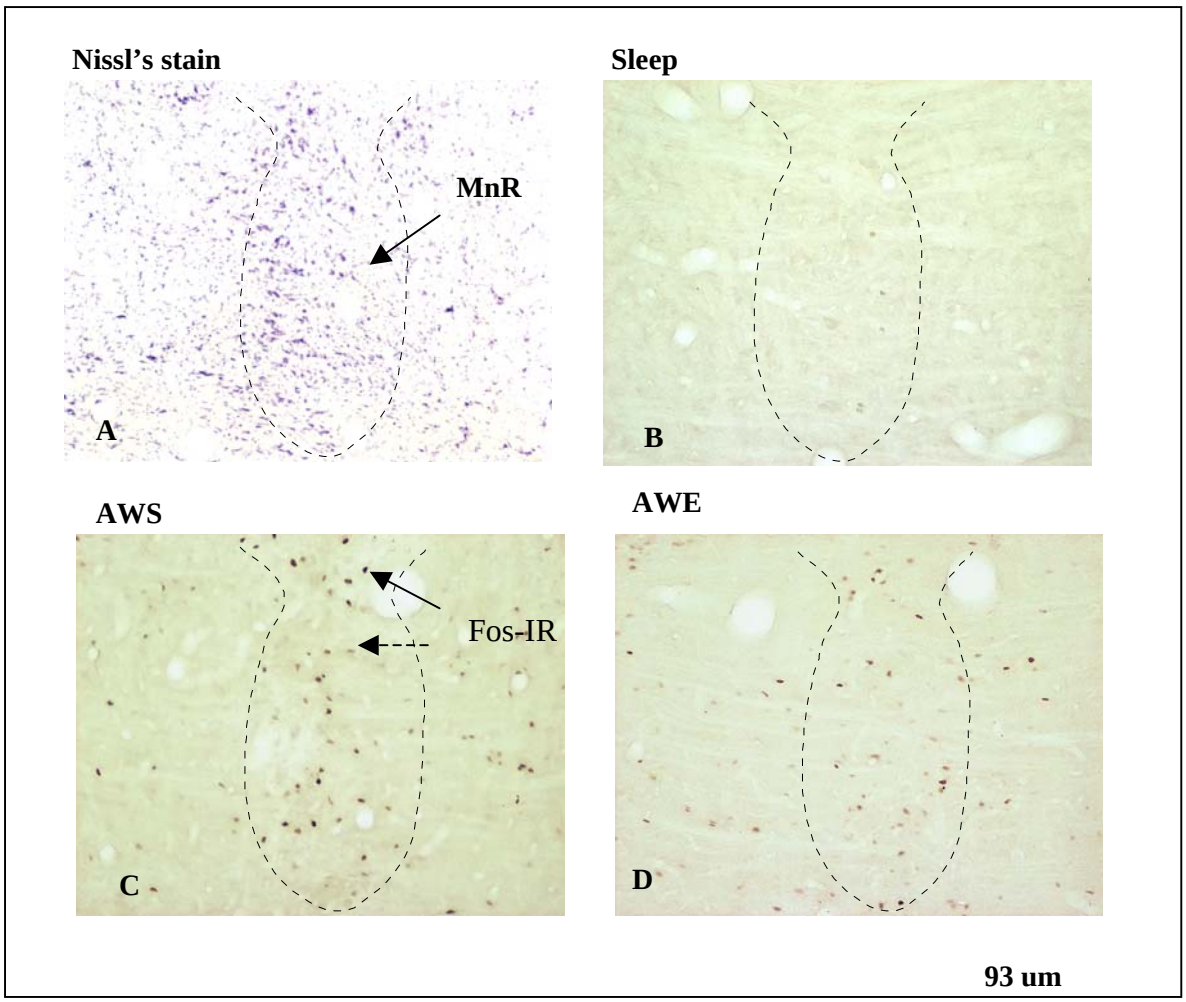
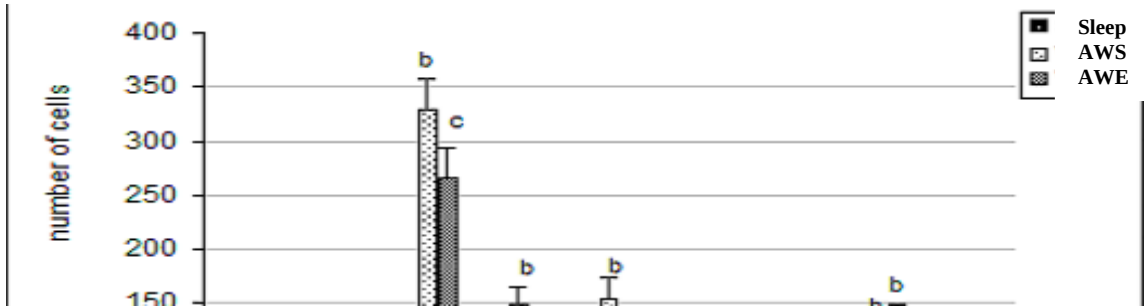


Figure 1 – Pattern of Fos-IR neurons in the MnR nucleus in the sleep and awaken stages of male rats. A- Nissl's staining, B- Animal sleeping, C - Animal in Awake Standard condition, and D- Animal in Awake enriched condition.

The involvement of the various raphe nuclei in the sleeping and awake stages, appraised by the number of Fos immunoreactive (Fos –IR) cells they presented, is clearly related in most of them to the activity period of that cycle, statistically significant to $p \leq 0.05$ (Figure 2). In the caudal raphe nucleis (RMg, RPa, and ROb the mount of Fos-IR in the sleep stage represents more than 30% of the cells involved in the AWS condition, suggesting their involvement in the regulatory functions subjacent to that stage. The DR, in the AWS presented 20% more cells than the AWE group, meanwhile the MnR, PMnR and PnR nuclei of the AWS group showed around 50% more cells than the AWE group.



Similar pattern of Fos-IR labeling was qualitatively observed analyzing the following structures comprising the neural architecture of sleep/wake circadian rhythms: the central amigdala (CeM), lateral parabraquial (LPB) medianus pre-optic and locus coeruleus nuclei. They had more Fos –IR in the awake than in the sleep state confirming former data on sleep studies, although the SCN presented considerable amount of Fos-IR cells in both stages of the sleep/wake cycle.

DISCUSSION

The clear and pronounced involvement of the raphe nuclei with the period of the animal's activities, and their evident different recruitment in the functions related to the sleep/wake cycle, are the main contributions of this research.

The results are in agreement with the electrophysiological responses of the serotonergic neurons, which increases from sleep to awareness (Heym et al., 1982b) Although interrelated sleep and awaken states are alternating and mutually exclusive behaviors. The relaxed posture, increased threshold to sensory stimuli and synchronized EEG are characteristic of sleep unlikely awareness. However, REM sleep in spite of representing deep sleep and showing absence of muscles tonus, presents besides rapid ocular movements, cortical desynchronization and autonomic alterations similar to the awake state, hence named paradoxical sleep (Timo-Iaria et al., 1970).

Former research established serotonin as the most important neurotransmitter related to sleep, after demonstration that severe insomnia due to lesion or inhibition of the raphe nuclei was reverted by its administration (Jouvet et al., 1966). This theory was replaced by the complex theory of hypnogenic peptides (Boberly & Tobler 1989), after

observing the depressed electrophysiological behavior of 5-HT neurons in sleep and absence of alterations in its levels at thalamic or cortical areas (Cespuglio et al., 1983). Later studies, however, restored 5-HT role in sleep (FONOFF et al., 1999), taking in account its role in the homeostatic regulation of the sleep-wake cycle (Boberly et al., 1989). According to this theory, the 5-HT, mainly from the DR nucleus, informs the intensity and duration of awareness through projections to the pre-optic lateral area (POAL) (Mcginty & Sterman, 1968; Sallanon et al., 1989). This circuit encompasses gabaergic fibers from APOL, which once activated by 5-HT, inhibits histaminergic neurons, responsible for awareness, in the posterior hypothalamus (Kitahama et al., 1989). In addition, 5-HT afferent fibers to the SCN modulate VIP synthesis, the hypnogenic agent at POAL (Riou et al., 1981).

The brainstem monoaminergic groups (serotonin and noradrenalin) have prominent action in awareness, while acetylcholine predominates during sleep. These three systems have intense, reciprocal and complementary connections at prosencephalic areas (Hobson, 1999). Similar profile is present in other systems related to this cycle. Orexin, like serotonin although produced in restricted areas of the brain have widely spreaded connections, but they act in opposite stages of the sleep/wake cycle (Peyron et al., 1998). Orexin projections are mostly located in areas related to REM sleep, very close to the ones histamine also projects, suggesting cooperation in this state of the cycle, besides influencing feeding behavior (Erickson et al., 2001). So does MCH, melanin-concentrating hormone, which has also been considered a hypnogenic factor (Verret et al., 2003).

Therefore modulation of the sleep/wake cycle results from interactions of various neuroactive substances at different sites of the neuronal net related to that rhythm (Portas et al., 1998).

The Fos-IR methodology do not reveal all interactions in a circuit nor the identity of the cell's neurotransmitter but it has been validated in many studies (Gozal et al., 2001; Szymusiak et al., 2001) as a reliable physiological marker. In this research it clearly showed the relation of raphe nuclei with the awaken stage. As raphe nuclei are the main source of brain's 5-HT, it is possible to conclude that serotonin is also related to this state as previously reported. Studies evaluating 5-HT content in the various raphe nuclei showed that it starts increasing even before lights are off, when it peaks in nocturnal animals, reinforcing 5-HT role with animal's activity period (Pinato et al., in press).

The pattern of labeling in the raphe nuclei favors their involvement with autonomic and regulatory functions of the awaken state (Timo-Iaria, 1970) as demonstrated in previous studies using Fos expression and different stimulus (Takase et al., 2001; 2004). The Fos-IR cells observed in the caudal raphe nuclei; RPa, RMg and ROb were not different in both experimental conditions of the awaken state. However, these

nuclei unlike the others presented a considerable number of cells labeled in the sleep state. This finding in addition to the DR, MnR-PMnR and PnR activation significantly different in the awaken relaxed and attentive states challenge research on the interactions among these nuclei and the nervous system during these diverse situations.

In summary, the raphe nuclei are more involved with the functions of the activity period. The rostral raphe nuclei showed significant number of cells Fos-IR in relaxed conditions than in the attentive ones. In the caudal ones these two conditions were not different, but interestingly these nuclei presented considerable number of cells in the sleep state. These results taken into account with literature data, favor the serotonin concept for its main role in the activity period, although double labeling, Fos-IR + 5-HT, was not performed. Therefore, the study of the raphe nuclei's involvement in the sleep/wake cycle reflects complex organization and interactions that require multiple approaches to be elucidated.

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