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Brain distribution of c-*fos* expression as a result of prolonged rapid eye movement (REM) sleep period duration

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Abstract

Auditory stimulation (AS) or recovery from sleep deprivation (SD) has been shown to increase REM sleep periods in rats, cats and humans. This increment in REM has been credited to an amplified level of excitability in a widely distributed neuronal network throughout the brain. Fos-like immunostaining (FLI) has been useful in constructing maps of post-synaptic neuronal activity with single cell resolution, and has been proposed to be tightly related with progressing neuronal activation. This study utilized FLI as a marker to determine the number of neurons and structures which express c-*fos* in broadly distributed areas of the brain in animals with REM periods prolonged by either AS or SD. The results indicated that the brain stem and diencephalon present FLI increases in a variety of structures that possibly share various functional aspects of the REM sleep mechanism. These results are discussed in terms of the possibility that REM maintenance is related to an increase in the recruitment of REM-on neurons.

Keywords: REM sleep; Auditory stimulation; Brain stem; Diencephalon; c-Fos

1. Introduction

Different manipulations such as cholinergic microinjections in the pontine reticular formation (PRF) or selective sleep deprivation have been shown to increase REM sleep [4,5,50]. Recently, it has been reported that auditory and somesthetic stimulation, initiated at the beginning and continued throughout a REM sleep period produces a significant enhancement of each REM sleep period in rats, cats and human subjects [11,22,30,42]. Studies performed in cats have shown that this increment is not affected by cholinergic blockade and is unrelated to the increase in PGO spike density which occurs in conjunction with the REM increase [2]. However, kainic acid lesions of PRF cells, though not preventing the appearance of REM sleep, does prevent the duration increase induced by auditory stimulation (AS) [1].

On the other hand, the REM sleep increase induced by AS is accompanied by an increase in single unit activity frequency of PRF cells [12]. Since REM sleep increase by cholinergic stimulation or sleep deprivation also produces an increase in single unit activity of PRF or pedunculo pontine (PPT) cells, respectively [20,51], it has been suggested that REM sleep prolongation by different approaches is related to an increase in the excitation of certain neuronal groups, and that this may be causally related to the maintenance of longer REM sleep periods.

Recent evidence suggests that REM sleep and the polygraphic features of this sleep phase, are generated by a broadly distributed neuronal network throughout the brain stem, rather than by a particular structure [40,45,47,49]. Nonetheless, the specific sets of neurons within the brain stem that are responsible for REM sleep have not been clearly outlined. This problem is related to the limitations of methodologies previously employed, such as electrophysiology and classic anatomical techniques.

Several studies have shown that c-*fos* expression is related to neuronal activation under certain conditions. Moreover, Fos-like immunostaining (FLI) has been useful in constructing maps of post-synaptic neuronal activity with single cell resolution [29]. However, it is important to

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take into consideration the experimental designs and the appropriate controls in order to determine optimal baseline and experimental c-*fos* expression. Under the right conditions by using FLI it may be possible to obtain the anatomical correlates of neuronal activation which occur during a behavioral state such as REM sleep [10,37].

In this context, we have previously reported that REM sleep increases by AS and SD induce an increase in FLI in several brain stem structures [22]. Moreover, subsequent studies have demonstrated FLI increases in the dorsolateral pontine regions [44] as well as in the medial and lateral portions of the reticular formation when REM sleep is induced by cholinergic stimulation [52]. Neuronal early genes expression, has also been shown to occur in response to sleep deprivation [17,32,33].

Since it has not yet been determined how and which of brain structures participate in the regulation of REM sleep maintenance, in the present study we utilized Fos-like immunoreactivity as a marker to determine the number of neurons and structures which express c-*fos* throughout the brain, in animals having different durations of REM sleep obtained either by auditory stimulation (AS) or by sleep deprivation (SD).

2. Materials and methods

Twenty-four male Wistar rats (180–200 g) were implanted for conventional sleep recordings. Screw electrodes were placed in the parietal bone for recording the EEG and in the left hippocampus (4 mm posterior to Bregma and 3.8 mm lateral). Wire electrodes were additionally placed in the neck muscles for recording EMG. After a week recovery period, the animals were habituated for 3 days to the recording chamber which contained a plexiglass cage within a sound attenuated room and connected to a Grass Model 79 D polygraph. During this period the animals were also habituated to the inverted flower pot method for sleep deprivation.

Three experimental groups were used. The sleep-deprived group (SD) was maintained on the inverted flower pot for 48 h, while the other groups; control and auditory stimulation (AS) were sleep-deprived for only 3 h, before the recording session. At the onset of recording, only slow wave sleep (SWS) and waking were recorded for the first 30–60 min, thereafter only those animals which presented 3 REM sleep periods during the next 30–60 min were utilized in each of the 3 groups (control, AS and SD). The AS group received an auditory stimulus in the form of a beep (80 dB, 20 ms, 1 kHz) which was applied every 20 s throughout each of the three REM periods. Furthermore, the animals of the three groups were maintained in a stressfull situation in the habituation period and during the experimental day due to the flower pot method, but only the SD group presented a sleep rebound, which was determined in a previous study [36].

After the recording session, all the animals were left in a sound proof chamber and observed through a one-way mirror for 30 min in quiet waking, and then deeply anesthetized and perfused with PBS 0.1 M followed by 4% paraformaldehyde. The brains were removed and placed in 30% sucrose in 0.1 M phosphated buffer until they sank. Frozen sections (40 μm thick) were washed 3 times (10 min per wash) in 0.01 M PBS with 0.1% Triton X-100, and then incubated with 10% normal goat serum (Vector Labs.) for 20 min the slices were then incubated with pp55 c-*fos* polyclonal antibody, generously provided by Tom Curran of the Roche Institute for Molecular Biology, at a dilution of 1/2000 for 72 h at 4° C. After washing with PBS, the sections were processed for ABC immunohistochemistry as previously described [9]. The number of nuclei stained with pp 55 c-*fos* antibody per structure in the brain stem, were quantified with the aid of an Image Analyzer (MCID). The MCID enables one to define vari-

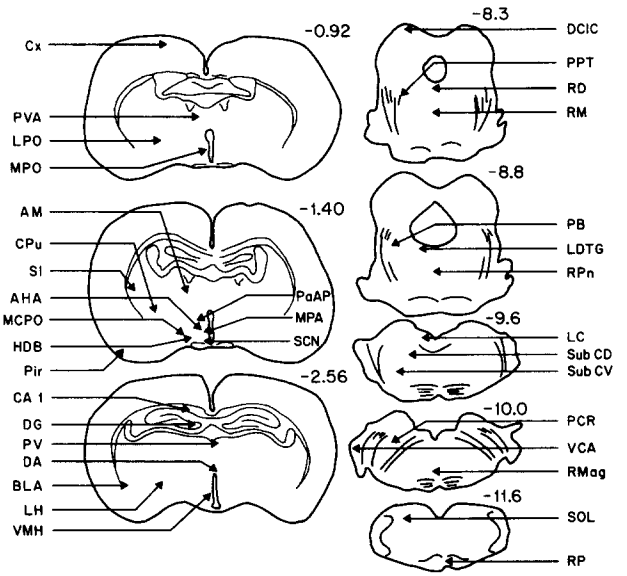

Fig. 1. Schematic drawings of the eight antero-posterior coronal sections with arrows pointing to the 36 forebrain, diencephalic and brain stem nuclei that were investigated in this study. Cx, cortex; PVA, paraventricular thalamic nucleus (anterior); LPO, lateral preoptic area; MPO, medial preoptic nucleus; AM, anteromedial thalamic nucleus; CPu, caudate putamen; SI, substantia innominata; PaAP, paraventricular hypothalamic nucleus (anterior); MPA, medial preoptic area; MCPO, magnocellular preoptic nucleus; HDB, horizontal diagonal band; SCN, suprachiasmatic nucleus; Pir, piriform cortex; CA1, CA1 field of Ammon's horn; DG, dentate gyrus; PV, paraventricular thalamic nucleus; DA, dorsal hypothalamic area; BLA, basolateral amygdaloid nucleus (anterior); LH, lateral hypothalamic area; VMH, ventromedial hypothalamic nucleus; DCIC, dorsal cortex of the inferior colliculus; PPT, pedunculo pontine tegmental nucleus; RD, raphe dorsalis; RM, raphe medialis; PB, parabrachial nucleus; LDTG, lateral dorsalis tegmental nucleus; RPn, raphe pontis; LC, locus coeruleus; SubCD, subcoeruleus (dorsal); SubCV, subcoeruleus (ventral); PCR, parvocellular nucleus; VCA, ventral cochlear nucleus (anterior); RMag, raphe magnus; SOL, solitary tract nucleus; RP, raphe pallidus.

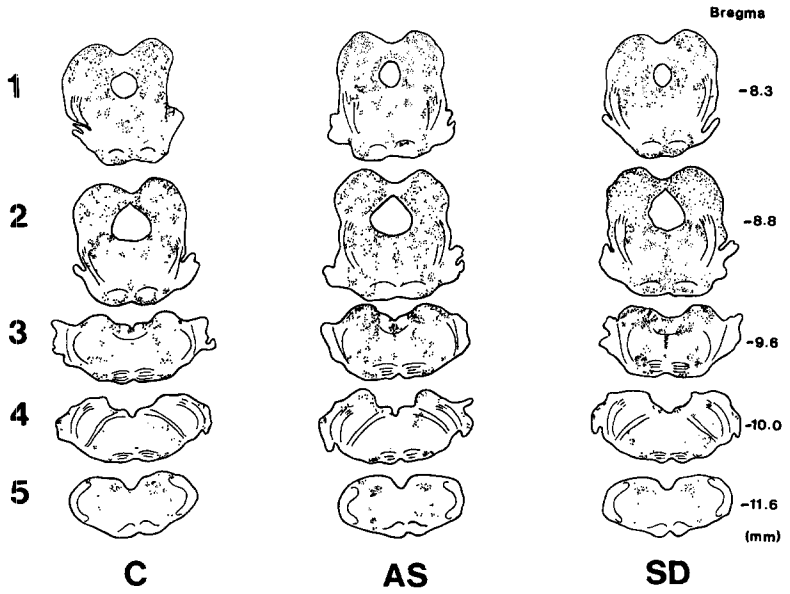

Fig. 2. Camera lucida drawings of brain stem coronal sections showing positive FLI neurons from the three experimental groups.

ous sample areas for each structure in question, and quantifies cell density accordingly. Sections which corresponded from Bregma to eight different antero-posterior levels −11.6, −10.04, −9.68, −8.8, −8.3, −2.56, −1.40, and −0.92 [34] were analyzed. Within these sections the following thirty-six structures were separated into two groups; brain stem structures and areas found in the diencephalon and forebrain. The brain stem structures were:

Table 1
Percent and mean period duration (±S.E.M.) of the different phases of the sleep-wake cycle for each group

Group	Wakefulness		Slow wave sleep		REM sleep	
	Percent %	Mean period duration (min)	Percent %	Mean period duration (min)	Percent %	Mean period duration (min)
Control	38.1 ± 2.8	2.9 ± 0.5	53.3 ± 3.0	4.4 ± 0.4	8.0 ± 1.5	1.8 ± 0.3
AS	26.1 ± 0.03 ●	3.2 ± 0.5 ●	63.7 ± 3.2 ●	4.6 ± 0.5	10.0 ± 1.0 ●	2.9 ± 0.3 ●
SD	36.5 ± 0.03	3.4 ± 0.5	42.3 ± 3.2 ●	4.4 ± 0.5	21.1 ± 1.0 ●	3.8 ± 0.3 ●

● $P < 0.05$.

Table 2
Effect of AS and SD on the number ($\bar{x}$ ± S.E.M.) of Fos-like immunoreactive neurons in different brain stem structures

Brain structure	Control $n = 8$	Auditory stimulation $n = 8$	Sleep deprivation $n = 8$
Solitary tract nucleus	13.4 ± 2.5	27.3 ± 2.6 ●	27.3 ± 2.6 ●
Parvocellular nucleus	2.9 ± 1.0	8.2 ± 1.0 ●	8.8 ± 1.0 ●
Parabrachial nucleus	13.5 ± 3.0	40.9 ± 3.1 ●	38.7 ± 3.1 ●
Pedunculo pontine tegmental nucleus	18.1 ± 1.0	34.8 ± 4.0 ●	31.5 ± 1.0 ●
Lateral dorsalis tegmental nucleus	9.6 ± 1.2	13.7 ± 1.3 ●	14.9 ± 1.3 ●
Subcoeruleus (dorsal)	4.3 ± 1.1	9.2 ± 1.1 ●	9.2 ± 1.1 ●
Subcoeruleus (ventral)	1.4 ± 1.0	4.8 ± 1.0 ●	5.3 ± 1.0 ●
Raphe dorsalis	7.8 ± 1.2	9.1 ± 1.3	13.0 ± 1.3 ●
Locus coeruleus	7.0 ± 1.0	6.7 ± 1.0	6.4 ± 1.0
Raphe pallidus	1.8 ± 1.0	3.0 ± 1.0	2.6 ± 1.0
Raphe pontis	9.0 ± 1.0	11.1 ± 2.0	14.6 ± 1.0
Raphe medialis	6.1 ± 1.0	7.3 ± 2.0	12.0 ± 4.0
Raphe magnus	2.1 ± 1.0	2.0 ± 1.0	3.6 ± 1.0
Ventral cochlear nucleus (anterior)	5.2 ± 4.8	8.7 ± 6.4	2.7 ± 2.5
Dorsal cortex inferior colliculus	61.1 ± 9.0	72.1 ± 9.0	61.5 ± 12.0

● $P < 0.05$.

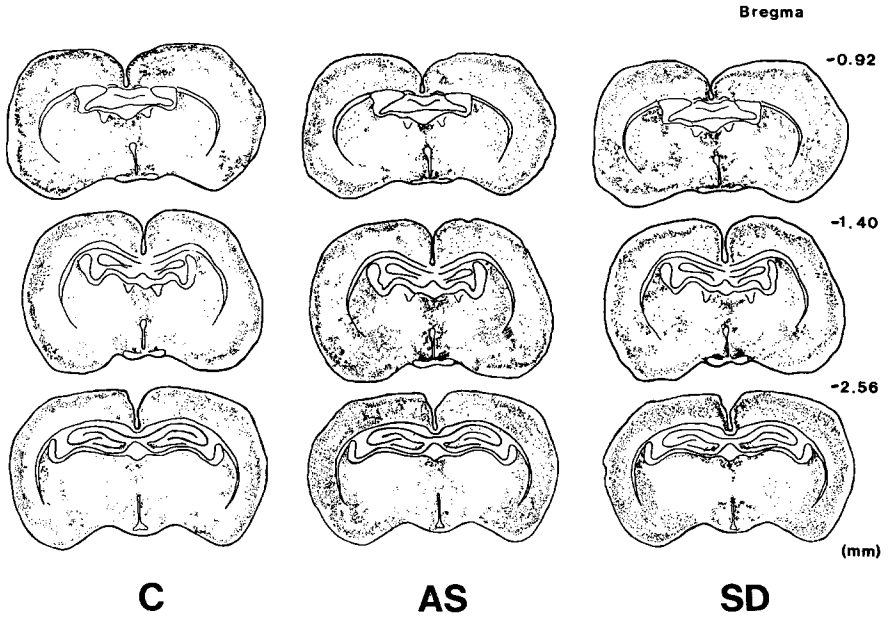

Fig. 3. Camera lucida drawings of diencephalic coronal sections showing positive FLI neurons from the three experimental groups.

SOL, PCR, PB, PPT, LDTG, LC, SubCD, SubCV, RP, RPn, RM, RD, RMg, VCA, DCIC; whereas the diencephalon and forebrain structures were: AHA, LH, AM, CA1, CPu, Cx, DG, HDB, DA, LPO, MCPO, MPA, MPO, PaAP, SCN, Pir, PV, BLA, PVA, SI, and the VMH. See Fig. 1 for a complete description of the abbreviations. The data obtained from sleep recordings and FLI quantifications were evaluated by a one-way ANOVA and the Fisher test.

Photomicrographs were taken using an Olympus BHS System Microscope for conventional and Nomarski optics. Using a camera lucida attachment, the distribution of c-*fos*

Table 3
Effect of AS and SD on the number ($\bar{x} \pm$ S.E.M.) of Fos-like immunoreactive neurons in different diencephalic and forebrain structures

Brain structure	Control $n = 6$	Auditory stimulation $n = 6$	Sleep deprivation $n = 6$
Suprachiasmatic nucleus	19.6 ± 6.0	28.0 ± 3.2 ●	48.0 ± 6.1 ●
Lateral hypothalamic area	4.6 ± 4.2	11.0 ± 4.1 ●	16.5 ± 4.3 ●
Basolateral amygdaloid nucleus (anterior)	30.3 ± 7.2	62.0 ± 12.3 ●	34.6 ± 9.8 ●
Dorsal hypothalamic area	9.8 ± 3.2	28.0 ± 4.8 ●	16.8 ± 5.8
Anterior hypothalamic area (anterior)	5.0 ± 1.5	9.0 ± 2.3	10.6 ± 5.2
Anteromedial thalamic nucleus	7.2 ± 4.5	7.3 ± 5.1	4.5 ± 2.8
CA1 field of Ammon's horn	0.3 ± 0.2	0.6 ± 0.6	1.6 ± 1.0
Caudate putamen	18.0 ± 11.1	11.4 ± 5.1	12.2 ± 3.7
Cortex	45.6 ± 6.0	62.2 ± 9.4	56.3 ± 9.5
Dentate gyrus	9.8 ± 4.5	5.8 ± 2.0	9.5 ± 2.4
Horizontal diagonal band	6.0 ± 2.1	7.4 ± 1.0	6.7 ± 0.6
Lateral preoptic area	8.8 ± 2.0	13.3 ± 4.6	14.0 ± 2.8
Magnocellular preoptic nucleus	3.6 ± 1.7	1.2 ± 0.6	4.0 ± 1.3
Medial preoptic area	8.2 ± 2.3	12.4 ± 2.1	10.3 ± 1.3
Medial preoptic nucleus	16.5 ± 5.7	18.8 ± 5.3	13.3 ± 1.7
Paraventricular hypothalamic n. (ant.)	8.5 ± 3.0	12.5 ± 5.0	8.5 ± 2.6
Piriform cortex	17.5 ± 2.9	28.0 ± 9.9	23.5 ± 3.4
Paraventricular thalamic nucleus	36.3 ± 4.2	43.8 ± 9.9	34.5 ± 4.5
Paraventricular thalamic nucleus (anterior)	16.3 ± 3.8	21.3 ± 8.0	28.2 ± 2.8
Substantia innominata	4.8 ± 1.4	3.3 ± 0.8	4.6 ± 1.6
Ventromedial hypothalamic nucleus	15.5 ± 1.9	12.3 ± 4.3	17.6 ± 4.3

● $P < 0.05$.

expression was drawn at 10× for each tissue section of the eight antero-posterior levels for control, AS, and SD groups.

3. Results

3.1. Polygraphic recording

The results showed that groups AS and SD had a significant increase in total REM sleep percent and mean REM sleep period duration with respect to control group. The increment in REM sleep in the AS and SD groups was of approximately 60% with respect to control group.

On the other hand, the AS group presented an increase in SWS percent and a decrease in wakefulness in relation to the control group. However, the SD group and control group were not significantly different in mean period duration of SWS and wakefulness, while SWS of SD group showed a significant decrease with respect to control (Table 1).

3.2. Fos-like immunoreactivity

FLI was observed in practically all the quantified structures of the brain stem, diencephalon and forebrain. The Fos-like immunostaining was restricted to neuron nuclei (Fig. 4). Moreover, the Curran pp55 Fos antibody that we used presents a clear nuclear immunostaining, except in the nucleolus, confirming previous findings [29]. It is important to mention that the antibody that we used in this study detects Fos and several lower molecular weight Fos family proteins (FF), where there is some basal expression in several brain structures of unstressed awake adult animals [27,29,46]. Cell counts were performed in 15 brain stem and 21 diencephalon structures. The total number of FLI neurons in all the analyzed structures was 3478 for the control group, 4985 for the AS group and 4620 for the SD group. The increment in global FLI in the AS and the SD groups with respect to control group can be clearly visualized in the camera lucida drawings of Figs. 2 and 3. However, since some structures did not show differences between groups, we described the Fos expression at length in separated sections.

3.3. Brain stem

Analysis of variance indicates that in AS and SD groups there were significant increases in FLI neurons in SOL ($P < 0.05$), PCR ($P < 0.05$), PB ($P < 0.05$), PPT ($P < 0.05$), LDTG ($P < 0.05$), SubCD ($P < 0.05$) (Table 2). In RD, a significant change was presented only in the SD group ($P < 0.05$). On the other hand, structures such as LC, RP, RPN, RM, RMag, VCA and DCIC presented no changes in FLI between any of the groups.

The increase in Fos expression in AS and SD groups can be seen in the Fig. 2. Although the control group presents a moderate FLI thoughout the brain stem, it is evident that AS and SD groups showed a marked increase in FLI neurons, specifically in dorsolateral and dorsomedial portions of sections 1, 2, 3 of Fig. 2, where the PPT,

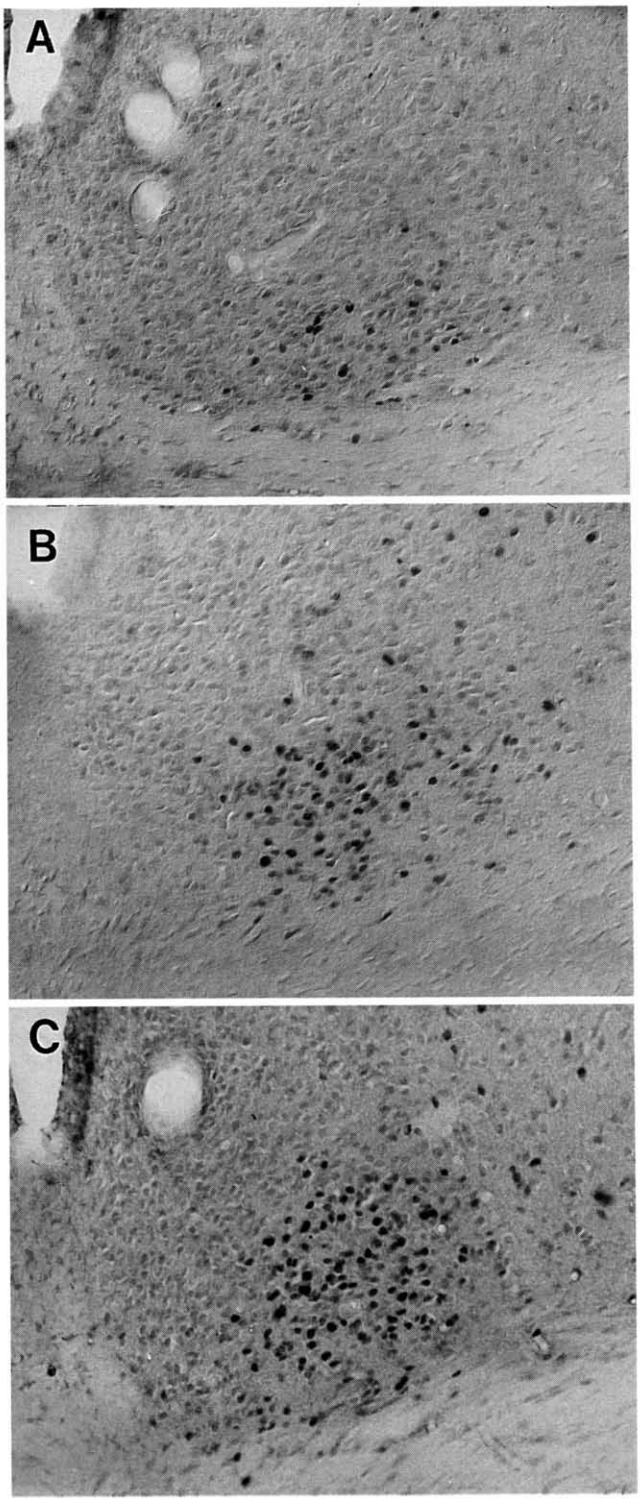

Fig. 4. Photomicrographs showing Fos-like immunoreactivity of the suprachiasmatic nucleus during the (A) control condition, (B) with auditory stimulation, (C) recovery from sleep deprivation (×20).

LDTG, SubC and PB are located. Finally, the pontine reticular formation (PRF) the magnocellular nucleus of the bulb (Mc) and the the inferior olive nuclei (IO) did not express Fos or FF proteins.

3.4. Diencephalon and forebrain

In Table 3, analysis of variance shows a significant increase in FLI for groups AS and SD in structures such as SCH ($P < 0.05$), LH ($P < 0.05$), BLA ($P < 0.05$) and for DA, a significant change was only found for group AS ($P < 0.05$). All other structures quantified in the diencephalon and forebrain presented no significant differences between any of the groups. Fig. 4 demonstrates the increase in FLI in the AS and SD groups in the suprachiasmatic nucleus. Moreover, in Fig. 3 it is evident that several structures such as the Cx, DG, PV, MPA and others present significant Fos expression in all the groups. However, only the SCN, BLA and LH nuclei present an increase in FLI in AS and SD groups with respect to the control.

4. Discussion

The results of this study show that there are changes in FLI in a variety of brain stem and diencephalon structures. These changes can be divided in those structures that showed an increase of FLI in AS and SD groups such as PPT, LDTG, PB, SubCD, SubCV, SOL, SCN, BLA, and LH, and those which showed no changes in AS and SD groups such as LC, RMag, RM, RP, RPn, VCA, DCIC, AHA, AM, CA1, CPu, Cx, DG, HDG, LPO, MCPO, MPA, MPO, Pa, Pir, PV, PVA, SI, and the VMH. In addition, there was a structure (the RD) which showed increased Fos expression only following recovery from 48 h of sleep deprivation, and another one (the DA) which showed a FLI increase only in the AS group. Finally, interestingly the giant cells of the PRF, did not show Fos and FF protein expression which in fact coincides with the observations of Yamuy et al. [52] in the model in which REM sleep is increased by carbachol stimulation.

4.1. Brain stem

The brain stem nuclei which showed an increase FLI in relation to the REM increment by AS or SD are the same that we previously reported [22]. These structures interestingly have been shown to be related to REM regulation in various experimental approximations: (1) they contain REM-on cells [13,15,38,39,45]; (2) the LDTG, PPT, and PB increase their glucose uptake in relation to REM sleep [19]; (3) carbachol microinjections in these structures induce an increase in REM sleep [3]. In the SOL no one has recorded during REM sleep. However, electrical stimulation of the vagus, an important afferent of the SOL, and the serotonergic denervation of SOL, produces significant increments in REM sleep [31,35]. It is interesting that carbachol induced REM sleep is also related with an increase in FLI in the PPT, LDTG, PB [44,52], demonstrating that the recruitment of active neurons in these structures is tightly related to REM sleep mechanisms.

Interestingly, the monoaminergic REM-off structures such as LC, RMag, RM, RPn, and RP [21,41,48], showed no changes in FLI in the different groups tested, which are the same as we previously reported [22]. Since it is not possible to observe a decrease in FLI expression as a result of a decrease in neuronal activity, it is conceivable that the absence of changes in Fos and FF in these REM-off structures reflect a decrease in neural activity. However, the RD, a serotonergic structure which contains REM-off neurons, showed in the present study an increment in Fos expression only in the SD group. Recently, Yamuy et al. [52] reported an increase in FLI not only in RD but also in the Raphe Magnus, Raphe obscurus and the Raphe centralis superior following carbachol-induced REM. These findings are difficult to explain although it is possible that the increased Fos and FF expression in raphe after carbachol microinjection or sleep deprivation is not located in serotoninergic neurons [52].

A contrasting result of the present study in relation to the studies that induced REM sleep by pontine carbachol microstimulation is that we did not find FLI in the PRF and the Mc, whereas others find an increase in FLI in these structures related to the carbachol induced REM-like state [44,52].

Several authors have shown that PRF and Mc play a regulatory role in REM sleep [14,39]. Since there are certain neurons that cannot express Fos under certain conditions [28], it is conceivable that the methodology used here does not induce FLI in PRF and Mc in relation to REM sleep and the discrepancy with the carbachol studies may be due to the fact that under the latter conditions REM periods are of much longer duration than those seen in this study.

The auditory related regions, such as the VCA, DCID and inferior olive, did not show changes in FLI in either of the different groups tested. These results though unexpected, may suggest that the auditory stimulus although intense (90 dB), is much too brief (20 ms) to be able to induce Fos and FF protein expression.

4.2. Diencephalon

The diencephalic structures which presented increased FLI expression by way of AS or SD suggest that some structures involved in REM regulation are outside of the brain stem. In this context, studies involving the BLA have demonstrated that electrical stimulation of this structure produces an increment in REM sleep and PGO activity [7] and microinjections of carbachol in the BLA induce significant increases in REM sleep for several days.

On the other hand, the increase in Fos expression in the LH and SCN when REM sleep was augmented, holds some interesting possibilities for future studies. The LH has been classically associated with wakefulness, since electrolytic or excitotoxic lesions of this structure induce persistent somnolence in cats [43]. Moreover, muscimol microinjections in this structure also induced a marked decrease in wakefulness and somnolence [18]. The increase in FLI neurons in the LH in the AS and SD groups is as difficult to understand as the RD increment. However, it is possible that the increment in Fos expression in these wakefulness related centers is involved with the autoinhibition of such structures during the increment of REM sleep produced by the different manipulations.

The SCN of the anterior hypothalamus is known as a critical structure for the expression of circadian rhythmicity in a variety of physiological and behavioral functions [24]. In the SD group the increment in FLI in the SCN may reflect alterations in the circadian cycle due to the 48 h of sleep deprivation which disturbed the circadian and homeostatic propensity towards sleep as demonstrated in Borbely's two-process model of sleep regulation [6]. The fact that c-*fos* was also expressed in SCN with AS is a rather interesting result. It is known that visual stimuli or brief exposure to light is capable of eliciting circadian entrainment as well as increased Fos expression [16]. However, this is understandable considering there is a direct pathway between the retina and the SCN [25]. Another external cue that has shown circadian phase entrainment is the use of food-anticipatory activity models [23]. However, our use of AS is not an anticipatory response; the animals never had prior knowledge or exposure to the stimulus until the moment of recording and therefore, were not habituated to the AS. It is a conceivable possibility that by producing these increases in REM sleep a phase shift could have occurred in the animals. Since this was an unexpected finding, we have no formal explanation for this result.

On the other hand, since we consider the other diencephalic and forebrain nuclei which showed no changes in FLI to be structures not related to REM sleep regulation [26], we propose that these structures act as controls. This implies that the changes in FLI in some structures of the brain in relation to REM duration, are not due to unspecific Fos and FF expression in certain nuclei, but rather work in relation with the dynamic mechanism of REM sleep regulation.

It is important to mention that there is an elevated amount of Fos and FF expression in the control group. These results may be dependent on the stressfull situation [8,10] of the methodology used here, although all animals in the present study were habituated for 3 days to the environmental condition, before beginning the experiments. However, the elevated Fos and FF expression in the control group may reflect the normal basal expression of these proteins in unstressed awake adult animals [29].

In sum, it can be suggested that when REM sleep is augmented, FLI expression increases in a widely distributed set of structures in the brain stem and diencephalon which have at least in part, been linked to some functional aspects of REM sleep regulation. This study further suggests that the maintenance of REM sleep may depend not only on an increment in the activity of REM-on cells [12], but also an increase in the recruitment of such cells, together with a decrease in the number of REM-off cells.

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