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c-*fos* proto-oncogene changes in relation to REM sleep duration

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Auditory stimulation has been shown to increase REM sleep periods in cats and humans. This effect has been attributed to an elevation of the level of excitability in a variety of brain stem neuronal groups. Fos-like immunostaining (FLI) has been useful in constructing maps of post-synaptic neuronal activity with single cell resolution, and has been suggested to be tightly correlated with ongoing neuronal activity. This study used FLI to quantify neurons from structures expressing c-*fos* in brain stem areas in animals with normal REMs and compared them with those showing extended REM periods. The results basically indicated that brain stem areas which in other studies have been described as having REM-ON cells, showed an increase in FLI, while no FLI changes occurred in areas described as having REM-OFF cells. These results are discussed in terms of the possibility that REM maintenance is related to a widespread increase in brain stem excitability.

Different manipulations such as sleep deprivation[4] or chemical stimulation[3,34] have been shown to increase REM sleep. Recently it has been reported that auditory stimulation (AS) initiated at the beginning and continued throughout a REM sleep period produces a very significant enhancement of each REM sleep period in both cats[9] and human subjects[19,28]. Animal work has 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]. On the other hand, kainic acid lesions of pontine reticular formation (PRF) cells, though not preventing the appearance of REM sleep, does prevent the duration increase induced by AS[1]. Finally, the REM sleep increase induced by AS is accompanied by an increase in single unit activity frequency of PRF cells[8]. This latter observation suggests that prolongation of REM sleep by AS induces augmentation of excitability of certain neuronal groups, and that this may be causally related to the maintenance of longer REM sleep periods. Moreover, since recent evidence suggests that REM sleep is generated by a broadly distributed neuronal network throughout the brain stem, rather than by a particular structure[26,29,32,35], it seemed appropriate to study Fos-like immunostaining (FLI) which has been useful in constructing maps of post-synaptic neuronal activity with single cell resolution[18]. Moreover, it has been suggested that c-*fos* proto-oncogene expression is tightly correlated with ongoing neuronal activity, although there are some exceptions[6,18,23]. In the present study, therefore, we utilized FLI for quantifying the number of neurons and structures which express c-*fos* in the brain stem in animals presenting normal REM sleep periods and compared them with those showing extended REM sleep periods induced either by AS or sleep deprivation.

Twenty-three 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 one week recovery, the animals were habituated for three days to the inverted flower pot method for sleep deprivation[4] and to the recording system which consisted of a cage within a sound attenuated room, where the animal was then connected to a Grass Model 79 D polygraph.

Four experimental groups were used. A sleep-deprived group (SD) which was maintained on the inverted flower pot for 48 h and three other groups: control, auditory stimulation (AS), and slow wave sleep (SWS) which were sleep deprived for only 3 h, before the recording session. At the beginning of the recording session, only SWS and waking were recorded during 30–60 min, following which only those animals within the control, AS and SD groups which presentend 3 REM periods during the next 30–60 min were utilized. 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 the three REM periods. For the SWS group only

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TABLE I

Percent ± S.E.M. values of the different phases of the sleep–wake cycle for each group, and x̄ S.E.M. duration of individual REM periods

Groups		*Awake*	*Slow wave sleep*	*REM sleep*	*REM duration (min)*
Control	(n = 6)	38.9 ± 6	52.1 ± 7	9.01 ± 1	1.7 ± 0.3
Auditory stimulation	(n = 6)	27.6 ± 9*	61.2 ± 9*	12.98 ± 3*	2.92 ± 0.3**
Sleep deprivation	(n = 6)	38 ± 7	43.1 ± 8	19 ± 4*	3.14 ± 0.4**
Slow wave sleep	(n = 5)	43.3 ± 9	57 ± 10	0 ± 0	0 ± 0

*$P < 0.05$, **$P < 0.001$.

those animals presenting waking and slow wave sleep during the recording session were included.

After the recording session, all the animals were maintained awake in a sound attenuated room for 30 min, 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 p 55 c-*fos* antibody, generously provided by Tom Curran of Roche Institute for Molecular Biology, at a dilution of 1/2000 for 72 h. After washing with PBS, the sections were processed for ABC immunohistochemistry as previously described[7]. 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). Sections which corresponded from Bregma to six different antero-posterior levels –12.3, –11.6, –10.3, –9.3, –8.8 and –8.3[21] were analyzed. Within these sections the following structures were quantified: solitary tract nucleus (NTS), parvocellular nucleus (PCR), magnocellular nucleus (Mc), raphé pallidus (RP), raphé medialis (RM), raphé dorsalis (RD), locus coeruleus (LC), parabrachial nucleus (PB), pedunculo pontine tegmental nucleus (PPT), laterodorsalis tegmental nucleus (LDTG), subcoeruleus nucleus (SubC), and the pontine reticular formation (PRF). The data obtained from sleep recordings and FLI quantifications were evaluated by a one-way ANOVA and the Fisher test.

The results showed that groups AS and SD had a significant increase in total REM sleep percent and mean REM sleep period duration ($P < 0.05$ and $P < 0.001$, respectively), in comparison to control and SWS groups (see Table I). The SWS group had no REM sleep at all, while the AS and SD groups presented an increment of approximately 60% in mean REM duration with respect to the control group. In addition, Table I shows a significant increase in slow wave sleep in the AS and SWS groups ($P < 0.05$), and a decrease in waking in the AS group ($P < 0.05$).

As for the FLI data it was observed that groups AS and SD which showed an increase in REM sleep also showed an increment in the FLI neurons in several brain stem structures. Analysis of variance showed that in the AS and SD groups there was a significant increase in FLI neurons in PPT ($P < 0.001$), PB ($P < 0.001$), SubC ($P < 0.01$) and NTS ($P < 0.01$) (see Table II and Fig. 1). On the other hand, there were structures such as LC, RD and RM that presented no changes in FLI between any of the groups. In addition, Table II shows that there was an increase in FLI in LDTG ($P < 0.05$) and RP (P

TABLE II

Effects of AS and SD on the number (x̄ ± S.E.M.) of Fos like-immunoreactive neurons in different brain stem structures

PCR, parvocellular nucleus; RP, raphé pallidus; NST, solitary tract nucleus; LC, locus coeruleus; PB, parabrachial nucleus; PPT, pedunculo pontine tegmental nucleus; RD, raphé dorsalis; SubC, subcoeruleus nucleus; LDTG, laterodorsalis tegmental nucleus; RM, raphé medialis; AS, auditory stimulation; SD, sleep deprivation; SWS, slow wave sleep.

		PCR	*RP*	*NST*	*LC*	*PB*	*PPT*	*RD*	*SubC*	*LDTG*	*RM*
Control	(n = 6)	7.3±2	4.7±4	19.8±4	13.6±4	19.0±8	19.6±6	8.1±3	5.9±1	9.1±1.5	8.6±3
AS	(n = 6)	9.5±2	4.8±1	28.4±3**	12.0±3	62.2±16***	41.2±5***	9.7±3	13.9±5**	11.7±3.6	5.6±4
SD	(n = 6)	12.4±4	8.95±3*	35.4±8**	13.6±5	61.7±12***	32.6±9***	12.2±1	12.7±4**	13.94±4*	10.3±3
SWS	(n = 5)	3.4±2*	6.0±2	16.7±3	11.3±3	20.6±3	14.0±2	10.7±3	5.6±1	9.0±2.01	9.0±2.5

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

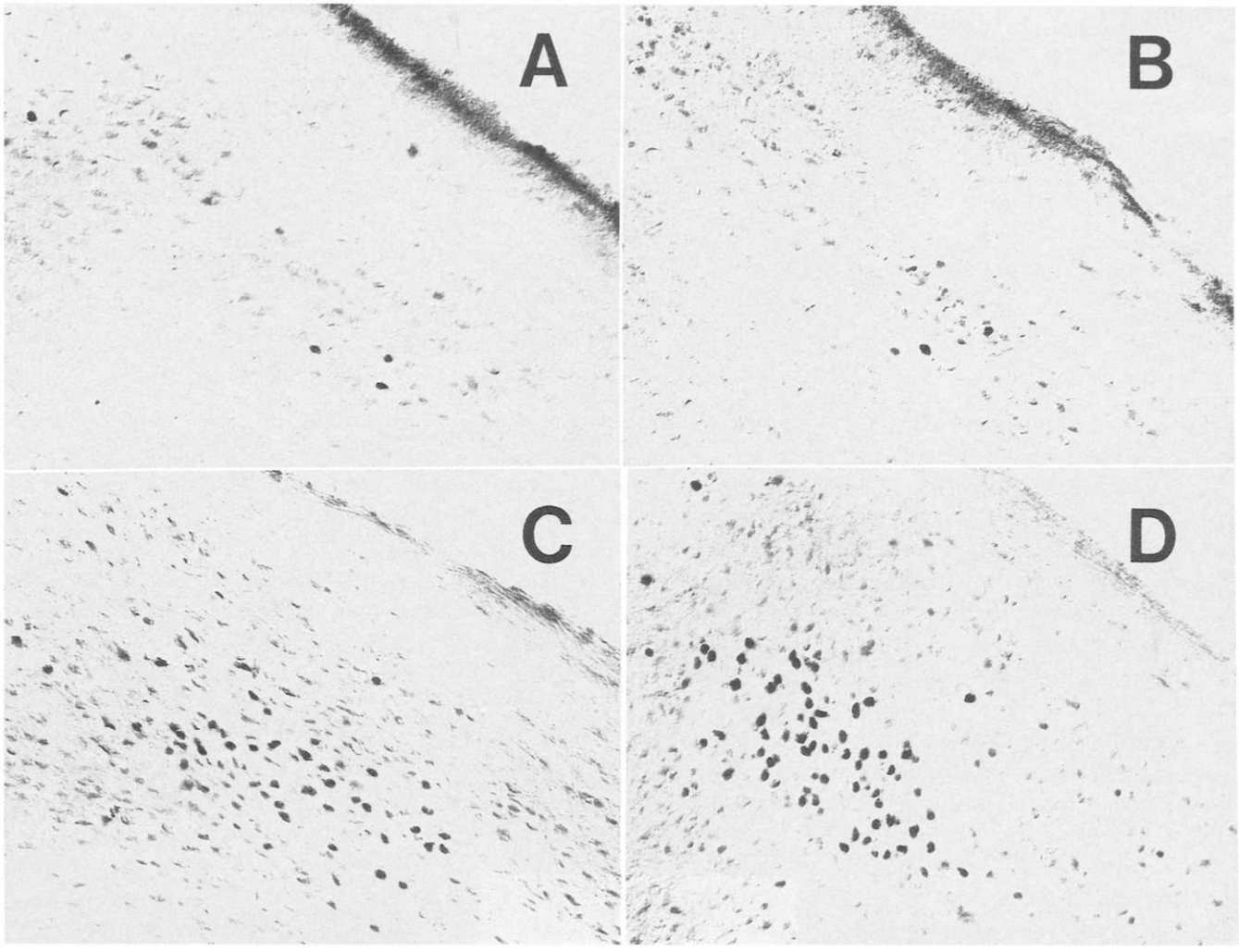

Fig. 1. Photomicrographs showing the Fos-like immunoreactivity of the parabrachial nucleus, in the different experimental groups. Note that panels D (auditory stimulation group) and C (sleep deprivation group) present a notable increase in immunoreactive cells, in relation to panels A (control) and B (slow wave sleep group).

< 0.05) only in the SD group. The PCR was the only structure where FLI was slightly lower ($P < 0.05$) in the SWS group as compared to all other groups. Finally, the PRF and Mc did not express Fos protein.

The results of this study show first of all that auditory stimulation also produces increases in REM sleep period duration in the rat. Therefore, since auditory stimulation influences REM duration in rats, cats[9] and humans[19,28], it suggests that this phenomenon is not an isolated event restricted to a particular species.

The results further show that there are changes in FLI in a variety of brain stem structures. These changes can be divided into those structures which increased FLI, such as PPT, PB, SubC, NST, and those which showed no changes such as LC, RD and RM. In addition, there were structures such as RP and LDTG which increased only following recovery from 48 h of sleep deprivation. Moreover, PCR showed a diminished expression of FLI in the rats belonging to the SWS group, whose only difference with the other groups was that they had no REM sleep periods. Finally, interestingly PRF and Mc did not show Fos protein expression.

In relation to the nuclei which showed increases in FLI it is interesting to note that they all have been shown to contain REM-ON cells[10,24,26,30]. There is one exception here, the NST, where no one has recorded from these cells during REM sleep. However, one interesting observation is that NST cells are part of the relay cells of vagal afferents, where Puizzillout et al.[22] have shown that stimulation of these afferents induce prolonged REM periods.

On the other hand, those nuclei which have been reported to contain REM-OFF cells such as the LC, RD and RM[12,15,31] showed no changes in FLI. It should be noted that it is not possible to observe a decrease in FLI expression as a result of a decrease in neuronal activ-

ity[17]. It is therefore conceivable that the absence of change in FLI reflects a decrease in neuronal activity. A notable exception were the RP cells, which despite having REM-OFF cells[27] presented a very small but significant increase in FLI during recovery from 48 h of REM deprivation. This difference, however, may be artifactual in view of the very low levels of c-*fos* expression in all groups. In relation to the LDTG cells which have REM-ON cells[30] they also showed a small increase in FLI but in the SD group only. We have no current explanation for the RP and LDTG results. However, the LDTG and coincidentally PPT are also structures shown to have an increase in glucose uptake in relation to REM sleep[14]. It should further be noted that FLI and glucose uptake are tightly correlated, although there are several exceptions[23].

The PCR was the only structure in the entire study in which the number of FLI neurons was found to be less in a particular group. This occurred in the SWS group. It is possible that PCR which have REM-ON cells[20,26] have a specific c-*fos* expression in relation to REM sleep, and since the SWS group has no REM at all, it expressed less c-*fos*.

Finally, extensive work has shown that PRF and Mc play a regulatory role in REM sleep generation[5,11,26,35], however, the present results show that these structures are unable to express c-*fos*. There are certain situations[13,16] in which FLI fails to map adequately functional pathways. We suppose that the methodology used here, does not induce FLI in PRF and Mc in relation to REM sleep. In sum, it can be suggested that FLI increases signifantly when REM sleep is augmented and that this increase appears to be correlated with those areas where most of the REM-ON cells have been reported to exist[20,24,26,30]. Moreover, in some of these nuclei, microinjection of carbachol induces significant increases in REM sleep[26], while their destruction produces either a decrease in REM sleep or at least induces important modifications of their polisomnografic characteristics[25,33].

This study further suggests that the maintenance of REM sleep may depend not only on an increment in the activity of REM-ON cells, but also on an increase in the recruitment of active neurons. It is thus conceivable that REM duration is the result of an elevated tonus of excitability over a widespread group of nuclei.

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