Sleep-related c-Fos protein expression in the preoptic hypothalamus: effects of ambient warming

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Gong, Hui, Ronald Szymusiak, Janice King, Teresa Steininger, and Dennis McGinty. Sleep-related c-Fos protein expression in the preoptic hypothalamus: effects of ambient warming. Am J Physiol Regulatory Integrative Comp Physiol 279: R2079–R2088, 2000.—Preoptic area (POA) neuronal activity promotes sleep, but the localization of critical sleep-active neurons is not completely known. Thermal stimulation of the POA also facilitates sleep. This study used the c-Fos protein immunostaining method to localize POA sleep-active neurons at 22°C and mildly elevated (31.5°C) ambient temperatures. At 22°C, after sleep, but not after waking, we found increased numbers of c-Fos immunoreactive neurons (IRNs) in both rostral and caudal parts of the median preoptic nucleus (MnPN) and in the ventrolateral preoptic area (VLPO). In animals sleeping at 31.5°C, significantly more Fos IRNs were found in the rostral MnPN compared with animals sleeping at 22°C. In VLPO, Fos IRN counts were no longer increased over waking levels after sleep at the elevated ambient temperature. Sleep-associated Fos IRNs were also found diffusely in the POA, but counts were lower than those made after waking. This study supports a hypothesis that the MnPN, as well as the VLPO, is part of the POA sleep-facilitating system and that the rostral MnPN may facilitate sleep, particularly at elevated ambient temperatures.

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METHODS

Surgery. All experiments were conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals. Experiments were performed on 27 adult male Sprague-Dawley rats weighing 280–320 g at the time of surgery. Rats were anesthetized with ketamine-xylazine (80 mg/kg and 10 mg/kg, respectively, ip) and surgically implanted for EEG and electromyogram (EMG) recording and hippocampal temperature recording. Briefly, stainless steel screw electrodes were threaded in the skull over frontal and parietal cortex for EEG recordings, and flexible insulated wires were inserted into neck muscle for EMG recording. A reentry tube for a thermocouple was stereotaxically placed in the posterior hippocampus (AP 6.0, L 4.5, H −5.5 from bregma). Electrodes were connected to a miniature connector, and the assembly was imbedded in dental acrylic.

Adaptation and recording procedures. After recovery from surgery, animals were maintained on a 12:12-h light-dark cycle (lights on 0800) with ad libitum access to food and water. Rats were adapted to the recording procedures, including connection to recording cables, for 2.5 h for 2–3 days before testing within a temperature-controlled chamber ($T_a = 22 \pm 0.1^\circ C$). Ten days after surgery, at 0830, rats were again connected by recording cables, and experiments were initiated. Data were obtained on a computerized acquisition system (Delta Software) for recording EEG, EMG, and brain temperature.

Recordings were initiated at 0900, and all rats were killed immediately at the end of a 2-h recording period at 1100. Groups of 6 rats each were studied under one of four conditions: 1) Control Sleep (CS) and $T_a = 22^\circ C$, 2) Control Wake (CW) and $T_a = 22^\circ C$, 3) Heat Sleep (HS) and $T_a = 31.5 \pm 0.1^\circ C$, and 4) Heat Wake (HW) and $T_a = 31.5^\circ C$. Electrophysiological data were monitored throughout recordings. CS and HS animals were undisturbed and allowed to sleep ad libitum. CW and HW animals were kept awake by 1-s-duration auditory stimuli or small remotely controlled cage movements that were applied when appearance of EEG slow-wave activity heralded sleep onset. An additional three rats were originally assigned to the CS group, but they slept <65% of the time. These rats were used to provide additional data points for correlations between sleep amounts and c-Fos immunostaining (see Fig. 9).

Immunohistochemistry. Animals were perfused transcardially with 0.1 M phosphate buffer (PB) solution followed by 300 ml of PB 4% paraformaldehyde (pH 7.0) and 100 ml each of 10% and 30% sucrose solutions. The brains were stored in 30% sucrose at 4°C until they sank. Coronal sections were cut at 40 μm on a freezing microtome. For immunohistochemistry, sections were incubated with a rabbit anti-c-Fos primary antiserum (AB-5) polyclonal 1:10,000 (Oncogene Science) for 48 h. Sections were subsequently incubated with a biotinylated goat anti-rabbit IgG (Vector Laboratories; 1:200) for 2 h and then reacted with avidin-biotin complex (Vector Elite Kit, 1:100) and developed with diaminobenzidine tetrahydrochloride, which produced a black reaction product in the cell nuclei. Omission of the primary antiserum resulted in an absence of specific cellular staining.

Data analysis. Sleep-wake states were scored in 10-s epochs on the basis of the predominant state within the epoch. Wake was defined by low-voltage high-frequency activity combined with elevated neck muscle tone. NREM sleep was defined by higher-amplitude EEG with prominent activity in the 2- to 8-Hz range. Rapid eye movement (REM) sleep was defined by moderate-amplitude EEG with dominant theta frequency activity (6–8 Hz), combined with very low neck EMG tone except for occasional brief twitches. The percentages of each state were calculated for the total 2-h recording period and for the 2nd h. The mean brain temperature for the last hour of recording was also determined for each animal.

The EEG was filtered at 1.0 and 30 Hz, digitized at 256 Hz with the Pass Plus system (Delta Software, St. Louis, MO), and analyzed using the fast-Fourier transform (FFT) as follows. Overlapping 4-s Bartlett-tapered windows were analyzed in 0.5- to 1-Hz bins from 0.5 to 30 Hz and combined in 10-s samples. Power in the delta frequency range, 1–4 Hz, was then summed in 60-s blocks. To normalize values across animals, delta activity was expressed as a percentage of minimum power samples ($n = 4–6$) derived from active waking in the same record. EEG FFT analysis was completed on five animals in CS, HS, and CW groups, as these data were incomplete in one animal in each group.

The Neurolucida computer-aided plotting system (Microbrightfield) was utilized for plotting neurons exhibiting c-Fos-like immunoreactivity (Fos-IR). Individual section outlines were drawn at ×20 visualization, and Fos-IRNs were then mapped in the section outlines under ×200 visualization. Fos-IRN counts were calculated in constant rectangular grids (see Figs. 4–7 for depiction of the grids) in three areas found to have sleep-specific staining. 1) The rostral medial preoptic nucleus (MnPN) (30) grid was centered at the anterodorsal tip of the third ventricle rostral to the decussation of the anterior commissure in front of the bregma (~A: 0.1 mm), extending 600 μm laterally and 300 μm both dorsally and ventrolaterally. 2) The caudal MnPN grid was placed immediately above the third ventricle at the level of the decussation of the anterior commissure, extending 300 μm laterally and 600 μm dorsally just behind the bregma (~A: −0.26 mm). 3) The VLPO grid was placed such that the ventromedial corner abutted the lateral edge of the optic chiasm and extended 700 μm laterally and 300 μm dorsally into the lateral preoptic area at levels 160 μm or more caudal to the organum vasculosum of the lamina terminalis (OVLT), as described previously (37). If the base of the brain curved dorsally so that the grid covered areas outside of the brain, the position of this grid was adjusted slightly to cover brain tissue, with care taken to encompass the VLPO area. A treatment-blinded examiner marked clearly stained Fos-IRNs bilaterally in three consecutive sections containing the largest part of the nuclei. The resulting six counts were averaged for each animal. Care was taken that sections selected for cell counts were at exactly the same levels in each treatment group. Significance levels for group differences in cell counts in each site were compared using ANOVAs followed by Newman-Keuls tests for differences between treatment pairs (GB-STAT). Significance levels for regression analyses relating cell counts to sleep amounts in individual animals were determined by regression ANOVAs (GB-STAT).

RESULTS

Three sites exhibited pronounced and localized increases in the number of Fos IRNs (Fig. 1) in sleeping compared with waking animals at 22°C. Photomicrographs showing Fos IRNs at the levels of the rostral MnPN, caudal MnPN, and VLPO are shown in Figs. 2–4. The rostral MnPN was a midline cell group that widened to form a “cap” around the rostral end of the third ventricle just anterior to the decussation of the anterior commissure (Fig. 2, and see DISCUSSION for...
additional description of the MnPN). In caudal MnPN, Fos IRNs were found in the midline immediately above the third ventricle and included sites both dorsal and ventral to the decussation of the anterior commissure (Fig. 3). We have differentiated rostral and caudal MnPN because of differential effects of ambient warming on c-Fos expression in these two areas. The VLPO region containing Fos IRNs was lateral to the optic chiasm, extending caudally from behind the OVLT to ~150 μm rostral to the emergence of the supraoptic nucleus and extending dorsally from the base of the brain into the lateral POA without a distinct border (Fig. 4).

To quantify these observations, Fos IRNs were labeled using the Neurolucida (Microbrightfield) computer-aided plotting system and then were counted within

Fig. 1. High-magnification photomicrograph of Fos-immunostained nuclei in the rostral median preoptic nucleus (MnPN). In this site, black-stained nuclei were seen only after sleep.

Fig. 2. Photomicrographs of representative brain sections showing Fos immunoreactive neurons (Fos IRNs) in rostral MnPN area under 4 different experimental conditions, Control Sleep (CS, A), Heat Sleep (HS, B), Control Wake (CW, C), and Heat Wake (HW, D). Note the increased numbers of immunolabeled neurons capping the third ventricle in the sleep conditions, particularly HS (B). Laterally, diffuse immunostaining was seen in both sleep and wake conditions.
a rectangular grid system (see METHODS). Examples of the application of these grids in typical sections are shown for rostral MnPN (Fig. 5), caudal MnPN (Fig. 6), and VLPO (Fig. 7). These data are summarized in Fig. 8.

We found 1) in the rostral MnPN, the number of Fos IRNs was significantly higher in the CS group compared with CW. Similarly, the number of Fos IRNs was significantly greater in the HS group compared with the HW group. In addition, the number of Fos IRNs was significantly greater in HS compared with CS (Fig. 8A).

2) In the caudal MnPN, the number of Fos IRNs was significantly higher during CS compared with CW. The number of Fos IRNs was greater in the HS group

Fig. 3. Photomicrographs of representative brain sections showing Fos IRNs in caudal MnPN area under the same 4 different experimental conditions described in Fig. 2, CS (A), HS (B), CW (C), and HW (D). Note increased no. of immunostained cells in the midline in the sleep condition. Laterally, more labeled cells were found in the wake conditions, although some cells were seen after sleep.

Fig. 4. Photomicrographs of representative brain sections showing Fos IRNs in the ventrolateral preoptic (VLPO) area under 4 different experimental conditions, A: CS; B: HS; C: CW, D: HW. Note the cluster of immunostained cells near the base of the brain in the CS condition (A). This cluster was absent in the other conditions. Dorsally in the preoptic area (POA), diffuse staining could be seen under all conditions.
compared with HW (Fig. 8B). The numbers of Fos IRNs in HS and CS groups were not significantly different.

3) In the VLPO area, the number of Fos IRNs was significantly higher during CS compared with CW. The number of Fos IRNs was also significantly higher during CS compared with HS (Fig. 8C). HS and HW or CW did not differ significantly.

Table 1 shows the sleep-wake parameters, EEG delta activity, and brain temperature data for each group. The awake groups had 3–5% sleep and the sleep

Fig. 5. Rostral MnPN. Line drawings of representative sections showing Fos IRNs and the grid area used for analysis under the 4 different experimental conditions: CS (A), HS (B), CW (C), and HW (D). Fos IRNs were identified by direct visualization (×20) with the Neurolucida counting system. See Fig. 2 and text for discussion of the distribution of Fos IRNs.

Fig. 6. Caudal MnPN. Line drawings of representative sections showing Fos IRNs in the grid area used for analysis under 4 different experimental conditions: CS (A), HS (B), CW (C), and HW (D). Fos IRNs were identified by direct visualization (×20) with the Neurolucida counting system. See Fig. 3 and text for discussion of the distribution of Fos IRNs.
groups 68–85% sleep. These differences were significant at both $T_{a}$s ($P < 0.05$). Sleep amounts did not differ significantly between HS and CS groups for either the 2-h treatment period or the 2nd h of treatment, although sleep time was lower in the HS group. Mean delta per minute across the 2-h treatment period, expressed as a percentage of minimum awake values, was slightly higher in the CS than in the HS group, but this difference did not approach significance ($P = 0.57$, 2-tailed $t$-test). The low but persistent levels of delta activity seen in the awake animal groups is characteristic of the FFT analysis method. Hippocampal temperature was elevated by 1.1°C in the HS compared with the CS group and by 0.7°C in HW group compared with the CW group, respectively. Brain temperatures at the elevated $T_{a}$ were within the typical normal circadian range of the rat (46).

Figure 9 shows regression analyses based on individual animals between sleep amounts and c-Fos IRN counts in each site, including correlation coefficients and significance levels. These regressions combine the data within control $T_{a}$ and warm $T_{a}$ populations. In rostral MnPN, in both warm and control $T_{a}$s, we found significant correlations between sleep amounts and cell counts, but the relationship was particularly strong in the warm ambient population ($r^2 = 0.89, P < 0.0001$). In caudal MnPN, at both $T_{a}$s, there were also significant correlations between sleep amounts and cell counts, but the relationship was particularly strong in the warm ambient population ($r^2 = 0.89, P < 0.0001$). In caudal MnPN, at both $T_{a}$s, there were also significant correlations between sleep amounts and the number of Fos IRNs. In VLPO, a significant correlation was found at the control $T_{a}$ but not at the warm $T_{a}$. Correlation analyses were congruent with group data reported above. The regression analyses include three additional CS animals with lower sleep amounts (mean: 53.6% of recording time). These animals also had relatively low Fos IRN counts (Fig. 9). Mean cell counts in these three rats were in rostral MnPN, 21, in caudal MnPN, 12, and in VLPO, 4.3. These values were similar to those of the wake groups (Fig. 9). The analyses reported here are based on total sleep time. Separate analyses based on NREM or REM amounts yielded weaker correlations (data not shown).

In both CS and HS animals, in addition to the MnPN and VLPO sites, moderate numbers of Fos IRNs were also found diffusely in the medial and lateral POA, and bed nucleus of the stria terminalis (Figs. 5–7). Usually, the total numbers of Fos IRNs in these sites were reduced in sleeping compared with awake animals. Compared with CS and HS animals, CW and HW animals displayed greatly increased numbers of Fos IRNs in the lateral septal area and throughout neocortex areas. The finding of increased c-fos expression in most brain areas associated with spontaneous waking or with sleep deprivation confirms findings in previous studies (5, 8, 28, 32). The paraventricular thalamic nucleus exhibited moderate-to-strong staining in both wake and sleep groups.

**DISCUSSION**

Increased c-fos gene expression is a marker of neuronal activation in most brain sites (25). In the VLPO, a site previously shown to exhibit sleep-related c-fos expression (41), we found a high density of sleep-active neurons by use of electrophysiological methods (42).
Sleep-related Fos IRNs in the VLPO, rostral MnPN, and caudal MnPN. Under warm ambient temperature conditions, compared with baseline sleep, sleep-related Fos immunostaining was significantly increased in rostral MnPN but not in caudal MnPN. This was a rationale for differentiating these two areas. We found that some rats that spontaneously exhibited less sleep, without sleep deprivation procedures, also lacked increased Fos immunostaining in MnPN or VLPO (Fig. 9). This observation supports the interpretation that the absence of c-fos expression in the sleep-active sites in the CW and HW groups was associated with reduced sleep and was not due to the mild stimulation associated with sleep-deprivation procedures. The occurrence of relatively sustained sleep, exceeding 60–65% of the recording time, appears to be crucial for selective c-fos expression in these sites. There was variability in sleep-related c-fos expression in animals sleeping 60–65% of the time, indicating that additional factors, such as sleep depth, may also be important.

Sleep-related Fos immunostaining in the MnPN has not been reported previously. The MnPN is a midline structure that is comprised of a column of cells abutting the rostral edge of the third ventricle and surrounding the rostral, ventral, and dorsal surfaces of the decussation of the anterior commissure (30). The MnPN extends caudally to the organum vasculosum of the lamina terminalis. In our analysis, the MnPN region rostral to the anterior commissure was designated as the rostral MnPN, and the MnPN region at the level of the anterior commissure was designated as the caudal MnPN. Both rostral and caudal divisions of this nucleus exhibited increased numbers of Fos IRNs in both CS and HS groups, compared with awake controls. These results suggest that MnPN neurons become activated during sleep. Mean sleep-related Fos IRN densities without stereological correction in coronal sections through rostral and caudal MnPN and VLPO were 141, 276, and 127 cells/mm², respectively, in the CS condition, within the defined grids used in this study. In the HS condition, corresponding densities were 288, 331, and 37 cells/mm², respectively. The rostral-caudal extent of the MnPN was greater than that of the VLPO. In the absence of stereological correction, these numbers do not accurately show absolute cell counts, but they can be used for comparison of sites within animals. These density numbers suggest that the MnPN contains a relatively large sleep-related cell population, exceeding that of the VLPO.

Thus c-fos expression seems to be a reliable method for detecting sleep-related neuronal activation in the POA. In the present study, we applied quantitative methods to c-Fos protein immunostaining to further localize sleep-related POA neuronal populations. We found sleep-related Fos IRNs in the VLPO, rostral MnPN, and caudal MnPN. Under warm ambient temperature conditions, compared with baseline sleep, sleep-related Fos immunostaining was significantly increased in rostral MnPN but not in caudal MnPN. This was a rationale for differentiating these two areas. We found that some rats that spontaneously exhibited less sleep, without sleep deprivation procedures, also lacked increased Fos immunostaining in MnPN or VLPO (Fig. 9). This observation supports the interpretation that the absence of c-fos expression in the sleep-active sites in the CW and HW groups was associated with reduced sleep and was not due to the mild stimulation associated with sleep-deprivation procedures. The occurrence of relatively sustained sleep, exceeding 60–65% of the recording time, appears to be crucial for selective c-fos expression in these sites. There was variability in sleep-related c-fos expression in animals sleeping 60–65% of the time, indicating that additional factors, such as sleep depth, may also be important.

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Table 1. Summary of sleep-wake state percentages, EEG delta activity, and brain temperature (°C)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total Wake</th>
<th>Total NREM</th>
<th>Total REM</th>
<th>Total Sleep</th>
<th>2nd Hour Total Sleep</th>
<th>EEG Delta Activity</th>
<th>Temperature, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS</td>
<td>14.86 ± 2.26</td>
<td>69.35 ± 1.73</td>
<td>15.79 ± 0.96</td>
<td>85.14 ± 2.07</td>
<td>76.39 ± 7.10</td>
<td>536.2 ± 56.1*</td>
<td>36.00 ± 0.13</td>
</tr>
<tr>
<td>CW</td>
<td>96.48 ± 2.45</td>
<td>3.52 ± 1.98</td>
<td>0</td>
<td>3.52 ± 1.98</td>
<td>1.94 ± 1.98</td>
<td>197.3 ± 23.2*</td>
<td>36.20 ± 0.16</td>
</tr>
<tr>
<td>HS</td>
<td>31.55 ± 5.19</td>
<td>55.77 ± 4.82</td>
<td>12.68 ± 1.65</td>
<td>68.45 ± 5.13</td>
<td>73.98 ± 5.13</td>
<td>489.2 ± 113.7*</td>
<td>37.10 ± 0.70</td>
</tr>
<tr>
<td>HW</td>
<td>94.90 ± 0.72</td>
<td>5.10 ± 0.79</td>
<td>0</td>
<td>5.10 ± 0.79</td>
<td>6.39 ± 1.14</td>
<td>229.2 ± 29.8</td>
<td>36.90 ± 0.20</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 in all groups except *n = 5. REM and NREM, rapid eye movement and non-rapid eye movement, respectively; EEG, electroencephalogram; CS, central sleep; CW, control wake; HS, heat sleep; HW, heat wake.
The functional role of the MnPN in sleep has not been studied previously. Sleep-associated processes such as lowered blood pressure, increased osmotic pressure, or episodic vasopressin secretion could stimulate c-fos expression in MnPN during sleep. Each of these processes is thought to be modulated by MnPN (18, 20). However, changes in vasopressin secretion, blood pressure, or osmotic pressure during sleep are small (29). Heat exposure may directly increase MnPN c-fos expression (38) or indirectly increase osmotic stimulation, but it would be necessary to explain why these stimuli would act during sleep but not during waking. Possibly, sleeping animals delayed drinking in response to osmotic stimuli, permitting development of a stronger osmotic signal. Further studies are required to determine whether neuronal activation in MnPN during sleep is simply a correlate of sleep or is an element of a sleep-promoting network.

The MnPN could be part of a diffuse hypothalamic sleep-promoting network that also includes the VLPO and other preoptic sites. There is evidence that both descending and ascending pathways from the POA may convey the hypnogenic output. Descending GABAergic and non-GABAergic projections from POA, including the VLPO, reach posterior hypothalamus and midbrain targets, including the major histaminergic, serotonergic, and noradrenergic cell groups that were shown to mediate arousal processes (19, 31, 40, 49). MnPN efferents also project to the serotonergic dorsal raphe nucleus and noradrenergic locus ceruleus (50), where they could regulate arousal processes. Pathways from POA to the basal forebrain (BF) cholinergic field (10) can mediate regulation of cortical projections from BF. A preliminary report also showed that some MnPN efferents also project to VLPO (7). Thus a hypnogenic output from the MnPN could be conveyed through VLPO. However, in the present study, in the HS condition, in which rostral MnPN was most activated, VLPO did not show Fos immunostaining. This suggests that a component of the output from the MnPN utilizes other pathways.

The rostral MnPN exhibited greater Fos immunostaining in animals sleeping in a warm ambient temperature compared with control sleep animals. During waking, the same ambient warming did not induce MnPN c-fos expression. Elevated MnPN Fos immunostaining was reported previously after 2 h of heat stress accompanied by a sharp rise in core temperature (38) and in association with fever after intravenous administration of PGE2 (48). Our results suggest that much milder heat exposure, when accompanied by sleep, also induces increased MnPN c-fos expression. In cats, POA WSNs become both more active and more thermostensitive during NREM sleep (1–3). Thus sleep may amplify the sensitivity of WSNs to ambient temperature, increasing the stimulus for c-fos expression. To our knowledge, the thermosensitivity of MnPN neurons has not been studied systematically, but an in vitro mapping study that included a small sample of MnPN neurons found a moderate density of WSNs in this site (11). Thermal activation of the MnPN may be
mediated by induction of PGE$_2$, which induces MnPN c-fos activation (48), probably acting on prostaglandin EP3 receptors localized in this area (26).

Thermoregulation and sleep regulation are integrated. In several mammalian species, activation of POA WSNs by local warming induces and sustains sleep and enhances EEG slow-wave activity within sleep (see introductory remarks). These WSNs also exhibit increased discharge during spontaneous NREM sleep. Because activation of WSNs by local warming is sufficient to induce NREM sleep, and this activation also occurs before spontaneous NREM sleep, we have hypothesized that the activation of these neurons generates the hypnogenic output from the POA (1, 3). We have shown that local POA warming can suppress discharge of putative wake-promoting neurons in the posterior hypothalamus (16), the cholinergic BF (4), and the dorsal raphe nucleus (DRN), including putative serotonergic neurons (13). Projections from the MnPN to the DRN (50) may mediate this effect of POA warming. On the basis of our findings that sleep-related c-fos expression is prominent in the MnPN and that it may be enhanced by ambient warming, we hypothesize that the MnPN participates in the integration of thermoregulatory and sleep regulatory functions. It is notable that, in the present study, mild ambient warming did not increase concurrent sleep in conjunction with increased rostral MnPN c-fos expression. However, we can hypothesize that increased c-fos expression in this site could play a role in the augmentation of subsequent sleep that has been found after ambient warming (24).

Our results confirm and extend previous reports. Sherin et al. (41) demonstrated that the number of c-Fos IRNs in VLPO was positively correlated with the amount of time spent asleep in the hour before animals were killed and was prominent when sleep time exceeded $\sim 60-65\%$ of recording time. We also found sleep-related c-fos expression in VLPO at normal ambient temperatures in animals sleeping $>60-65\%$ of the time. However, as confirmed by both mean counts and a correlation analysis, in the HS group, in VLPO, c-Fos IRNs were not increased compared with HW or CW groups, although three HS animals slept $>75\%$ of the time (Fig. 9). There were no significant differences between HS and CS groups in total sleep, 2$^{nd}$ h sleep, or EEG delta activity. This evidence suggests that VLPO c-fos expression during sleep is reduced at elevated T$_a$. Confirmation of our findings would suggest that the hypnogenic activation of the POA has distinct components depending on the stimuli modulating hypnogenic drive. Pointing to evidence that sleep may be facilitated by several distinct sleep factors or presleep conditioning factors, some investigators have suggested that there are multiple hypnogenic processes (12, 17). Our data provide limited support for this concept. However, we cannot exclude the possibility that the elevated T$_a$ altered the quality or depth of sleep in the HS group. Although the differences were not significant, sleep amounts and EEG delta power were slightly lower in the HS compared with the CS condition. As noted above, c-fos expression during sleep appears to occur when sleep amounts exceed a critical threshold. c-fos expression in VLPO may be particularly dependent on this threshold sleep amount or sleep quality.

In summary, Fos immunostaining showed that segregated sleep-active neuronal groups are localized in VLPO and two divisions of the MnPN. In the rostral MnPN, the number of sleep-induced Fos IRNs was increased strongly by ambient warming. Additional sleep-related Fos immunostaining was found diffusely in the POA, but these cell populations could not be anatomically separated from waking-related Fos-labeled populations in the same sites. Except in the MnPN, we could not identify segregated groups of WSNs, possibly because they constitute only $\sim 20\%$ of the population (6). If POA cells with specific wake-related or sleep-related activation, or having warm responsiveness, use different neurotransmitters or have other distinctive phenotypes, including expression of different immediate early genes, they might be differentiated with double-labeling or other labeling techniques.

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