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c-Fos protein expression is increased in cholinergic neurons of the rodent basal forebrain during spontaneous and induced wakefulness

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Abstract

It has been proposed that cholinergic neurons of the basal forebrain (BF) may play a role in vigilance state control. Since not all vigilance states have been studied, we evaluated cholinergic neuronal activation levels across spontaneously occurring states of vigilance, as well as during sleep deprivation and recovery sleep following sleep deprivation. Sleep deprivation was performed for two hours at the beginning of the light (inactive) period, by means of gentle sensory stimulation. In the rodent BF, we used immunohistochemical detection of the c-Fos protein as a marker for activation combined with labeling for choline acetyl-transferase (ChAT) as a marker for cholinergic neurons. We found c-Fos activation in BF cholinergic neurons was highest in the group undergoing sleep deprivation (12.9% of cholinergic neurons), while the spontaneous wakefulness group showed a significant increase (9.2%), compared to labeling in the spontaneous sleep group (1.8%) and sleep deprivation recovery group (0.8%). A subpopulation of cholinergic neurons expressed c-Fos during spontaneous wakefulness, when possible confounds of the sleep deprivation procedure were minimized (e.g., stress and sensory stimulation). Double-labeling in the sleep deprivation treatment group was significantly elevated in select subnuclei of the BF (medial septum/vertical limb of the diagonal band, horizontal limb of the diagonal band, and the magnocellular preoptic nucleus), when compared to spontaneous wakefulness. These findings support and provide additional confirming data of previous reports that cholinergic neurons of BF play a role in vigilance state regulation by promoting wakefulness.

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Author contributions: JTM designed and conducted the experiments, analyzed data and wrote the paper; JWC, BJ, and SW conducted the experiments; JWC and BF analyzed data; CPW assisted with statistical analysis; RES and RWM designed research, analyzed data, and wrote the paper.

Keywords

sleep; wake; EEG; rat; stress; acetylcholine

INTRODUCTION

Extensive evidence indicates that basal forebrain (BF) cholinergic neurons are an important part of the neuronal circuits responsible for the neocortical activation associated with wakefulness and attention, whereas the role of BF GABAergic and glutamatergic neurons is less clear [for review, 28,29,42,49]. The cholinergic BF is comprised of the medial septum/diagonal band of Broca (MS/DBv) which has important hippocampal projections, while neocortical projections are prominent in the horizontal limb of the diagonal band of Broca (HDB), magnocellular preoptic nucleus (MCPO), and substantia innominata (SI). Furthermore, BF subnuclei project to subcortical thalamic nuclei which are also involved in vigilance state regulation [1,26,36,46,52], and particularly to the thalamic reticular nucleus in the rat [1].

Immunohistochemical detection of the protein c-Fos has been extensively used to indicate neuronal activation. Early studies documented an elevation of c-Fos protein labeling in select brain regions during wakefulness, compared to sleep [2,11,12,13,21,22,55]. Of particular interest, c-Fos protein expression was previously co-localized to approximately 10–12% of BF cholinergic neurons during sleep deprivation (SD) [24,43], and significantly less co-localization (approximately 0–2%) following sleep deprivation recovery (when sleep rebound occurred) or spontaneous sleep. However, c-fos genetic activation and c-Fos protein expression in the forebrain and hypothalamus is sensitive to stressors, sensory stimulation, and cognitive processing [9,10,14,33,40,41,63]. Thus, previous investigations studying c-Fos protein expression produced by sleep deprivation are potentially confounded by the stress and sensory stimulation of the sleep deprivation procedure. The present study added a condition in which the animal is spontaneously awake and not stressed in order to determine how the activity of BF neurons during sleep deprivation/forced wakefulness and recovery would compare to their activity during spontaneous wakefulness and sleep.

In the present study, rats were divided into the following four groups that characterized treatment (non-manipulated or manipulated): spontaneous wakefulness (SW), spontaneous sleep (SS), sleep deprivation (SD, wakefulness produced by gentle sensory stimulation), and the recovery sleep (SDR) that occurred in a 2h period following 6h of sleep deprivation. SD and SDR treatments were performed for comparison to the two spontaneous treatment groups (SW and SS), as well as to replicate previous findings [24,43]. The SW time point was selected to capture when the animal was most awake, which, from our polysomnographic data, was at the beginning of the dark/active period. The SD time point was selected to capture when animals would have a minimal amount of sleep preceding SD, at the beginning of the light/inactive period. Following treatment, brain tissue was processed to determine the activity of cholinergic BF neurons by combining c-Fos immunohistochemistry with choline acetyltransferase (ChAT) double-labeling. Previous studies have described elevated c-Fos expression following focal brain and spinal cord injury, including regions far from localized lesions [4, 16,18,50,54]. Such techniques as EEG/EMG screw electrode placement, as well as non-specific effects due to surgical implantation or tethering techniques, may produce false c-Fos protein expression. Hence, for comparison to the histological data, sleep recordings were conducted in a separate group of rats exposed to SW, SS, SD, and SDR. In order to demonstrate possible site-specificity of the distribution of wake-active cholinergic neurons, the subnuclei of the BF were examined based on the regional delineation found in the rat atlas of Paxinos and Watson [47].

EXPERIMENTAL PROCEDURES

Adult male Sprague-Dawley rats (Charles River, Wilmington, MA, USA) weighing between 280 and 350 grams were housed under constant temperature and 12:12 light dark cycle with food and water available ad libitum. All animals were treated in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care's policy on care and use of laboratory animals. All experiments conformed to U.S. Veterans Administration, Harvard University, and U.S. National Institutes of Health guidelines on the ethical use of animals. All measures were taken to minimize the number of animals used and their suffering, and were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23).

Immunohistochemical detection of choline acetyl-transferase and the c-Fos protein

Rats were placed on 8AM/8PM light/dark schedule. Each rat was habituated for 10 minutes on experimental days 4, 5 and 6 to the sleep deprivation technique of gentle sensory stimulation, which involved auditory stimulation, such as tapping on the side of the cage; or tactile stimulation, such as gentle brushing of the rat's back with a piece of gauze or cotton ball. On day 7, rats were exposed to one of four treatment groups: 1) SW: when the rat was left undisturbed, sacrificed at 10 PM (n=6); 2) SS: when the rat was left undisturbed, sacrificed at 10AM (n=6); 3) SD: 2 h of SD, sacrificed at 10AM (n=6); and 4) SDR: 6 h of SD, followed by 2 h of undisturbed recovery, sacrificed at 4PM (n=6). Figure 1 depicts the timeline for each of these four treatment groups.

For sacrifice, these rats were anaesthetized with pentobarbital (150 mg/kg, intraperitoneal) and perfused transcardially with PBS, followed by 10% formalin (Sigma). The brain was removed and placed in 30 % sucrose. Sections containing BF were cut on a freezing microtome at 40 μ m and stored in PBS in the fridge (4 °C) for 1–2 days. Sections were first blocked in a normal donkey serum buffer for one hour at room temperature (RT, 22°C), and then placed overnight in goat anti-c-Fos antibody (Santa Cruz Biotechnology, SC-52-G, 1:2000) in the fridge. On the next day, tissue was washed and placed in donkey anti-goat IgG antibody (Chemicon, 1:400) for one hour at RT, followed by one hour incubation in Avidin-Biotin Complex (100 ml avidin reagent, 100 ml Biotin reagent, 5 ml of buffer solution)(Vector Laboratories) at RT. Tissue was washed and placed for 2 minutes in a solution of DAB (0.06%), Nickel-ammonium sulfate (0.01%), in the presence of hydrogen peroxide (0.02%) (black precipitate, DAB kit, Vector). Tissue was washed, and then placed overnight in the fridge incubated in rabbit anti-ChAT (Chemicon, AB143, 1:2000). The next day, tissue was rinsed, incubated in Avidin-Biotin Vector blocking kit for one hour, followed by one hour RT incubation in biotinylated donkey anti-rabbit IgG (Chemicon, 1:400). Tissue was then rinsed, followed with one hour incubation in Avidin-Biotin Complex (described above). Tissue was washed, and placed for three minutes in DAB (0.06%) in the presence of hydrogen peroxide (0.02%), but without nickel-ammonium sulfate (brown precipitate). Tissue was mounted from PBS onto chrome-alum gelatin-coated slides, dried, and coverslipped using Permount mounting medium.

Light microscopy was carried out using an Olympus BX51 microscope. Neurolucida (MicrobrightField, Williston, VT) rendition of tissue outlines and landmarks was performed at a low magnification (10 $\times$), and cell locations were mapped at higher magnification (20 $\times$). Cholinergic cells that were c-Fos positive (c-Fos/ChAT) were determined by the black punctate nucleus (c-Fos-positive) surrounded by brown (ChAT-positive) cytoplasm (e.g., figure 2). Individual cholinergic cells that were not labeled with c-Fos were also documented, detected by an absence of the black punctate nucleus, but evident brown cytoplasm indicating ChAT-positive. Cells identified under light microscopy to be in the BF were counted as either ChAT-positive, or c-Fos/ChAT double-labeled for the representative 4 sections per case. Estimated cell locations of representative cases were mapped onto coronal schematic sections [47] using

Adobe Illustrator 10. Subnuclei analysis was performed, employing the subdivision delineation of the widely-used Paxinos and Watson rat atlas [47]. Photomicrographs were captured using a Spot Camera (Microfire) mounted on the Olympus BX51 microscope.

BF cell counts between the four treatment groups were evaluated using parametric one-way ANOVA. In order to evaluate differences of double-labeling in BF subnuclei, two-way ANOVA was employed, where one independent variable was the type of treatment (SD vs. SW), and the other independent variable was the BF subnuclei of analysis (MS/DBv, HDB, SI, MCPO). Select pairwise comparisons of the data were performed using the independent t-test (with Bonferroni correction when appropriate). Statistical analysis utilized SPSS software (release 11.5), and differences were determined to be significant when $P < 0.05$.

Polysomnographic recording

Electroencephalograph (EEG) and electromyograph (EMG) surgery was performed. Animals were anesthetized by means of inhalation of isoflurane (2.5–3%). Two screw electrodes (Product#E363/20; Plastics One Inc., Roanoke, VA, USA) were fixed onto the skull above the temporal cortex (AP –2mm, ML $\pm$ 4mm) for recording EEG. EMG electrodes (Product#E363/76; Plastics One Inc.), which consisted of flexible stainless steel wires insulated with nylon, were placed in the nuchal muscles. A Grass model amplifier system polygraph (15LT Bipolar, Grass Technologies) with four amplifiers (Product#15A54 Quad AC Amplifiers, Grass Technologies) was used for all EEG and EMG data collection (GAMMA/SYS Acquisition software, Grass Technologies). EEG filter settings were set at 0.1 and 100Hz. Behavior was classified into 3 different vigilance states by means of EEG and EMG analysis: wakefulness (W), nonREM sleep (NREM), and rapid eye movement sleep (REM). Grass Rodent Sleep Stager (RSS) V4.2 (Grass-Telefactor) was used for off-line EEG and EMG analysis. Recordings were visually scored in 10s epochs.

Two weeks following EEG/EMG surgery, rats were connected for EEG/EMG recording (experimental day 1). This separate group of rats, not sacrificed for immunohistochemical analysis, was exposed to the same habituation procedures and treatment as that previously described for immunohistochemical detection of ChAT and the c-Fos protein ($n=6$ for each of the four treatment groups).

RESULTS

An average of 388 ChAT-Positive cells was identified and counted per tissue slice, and four slices per case were used for representation of BF. Therefore, approximately 37,000 cholinergic neurons were investigated in this study. Figure 2 is a photomicrographic depiction of c-Fos/ChAT double-labeled neurons in BF.

Figure 3 depicts schematic representation of cell labeling in BF for the four treatment groups. There appeared to be a relative increase of double-labeled c-Fos/ChAT neurons during SD (Figure 3A) and SW (Figure 3B) in these representative cases (Figure 3A), when the rat was probably most aroused, compared to SDR (Figure 3C) and SS (Figure 3D). This apparent trend was supported by the quantitative analysis presented next.

Previous reports have reported elevated ChAT activity associated with wakefulness [23,27]. Therefore, the number of cholinergic neurons (labeled with the antibody for ChAT) of the four slices per case was totaled, and reported in Table 1. There was no significant difference between the count of ChAT-labeled neurons of these four treatment groups (One-way ANOVA, $n=6$ per treatment group, $p=0.46$).

Table 1 and Figure 4A portray a significant difference of the number of ChAT cells that also expressed the c-Fos protein (c-Fos/ChAT) between the four conditions (one way ANOVA, $n=6$ per treatment group, $F(3, 20)=75.44$, $p<0.001$). Post-hoc pair-wise comparisons demonstrated significant differences between all groups, except SS vs. SDR (SD vs. SW, $P<0.01$; SD vs. SS, $P<0.001$; SD vs. SDR, $P<0.001$; SW vs. SS, $P<0.001$; SW vs. SDR, $P<0.001$). c-Fos/ChAT double-labeling in the SD treatment group was significantly elevated when compared to SW, and SW labeling was significantly elevated when compared to both SS and SDR.

As depicted in Table 1 and Figure 4B, a significant difference of the amount of wake in the last hour preceding sacrifice was demonstrated between the four conditions in a separate group of rats, not immunohistochemically analyzed (one way ANOVA, $n=6$ per treatment group, $F(3, 20)=98.84$, $p<0.001$). Post-hoc pair-wise comparisons demonstrated significant differences between all groups, except SS vs. SDR (SD vs. SW, $P=0.001$; SD vs. SS, $P<0.001$; SD vs. SDR, $P<0.001$; SW vs. SS, $P<0.001$; SW vs. SDR, $P<0.001$).

Table 2 depicts the percentages of c-Fos/ChAT double-labeled cells for each BF subnuclei (MS/DBv, HDB, MCPO, and SI) for the four treatment groups. The percentages of c-Fos/ChAT double-labeled cells were elevated following SD treatment, compared to SW, for MS/DBv, HDB and MCPO, but not for SI (Figure 4C). A significant difference was described, comparing the SD vs. SW treatment groups ($n=6$ per treatment group, $F(1, 40)=20.72$, $P<0.001$). Post-hoc pair-wise analysis revealed significant subnuclei differences, comparing double-labeling between the SD vs. SW treatment groups, for MS/DBv, HDB, and MCPO ($P<0.05$), but not SI. Also, differences were found to be significant between the four subnuclei ($F(3, 40)=9.10$, $P<0.001$). Post-hoc pair-wise comparisons demonstrated significant differences, comparing double-labeling between the four subnuclei (MS/DBv vs. HDB, $P<0.05$; MS/DBv vs. SI, $P<0.005$; HDB vs. MCPO, $P>0.05$; MCPO vs. SI, $P<0.005$), but not between MS/DBv vs. MCPO, or HDB vs. SI. The interaction of these two independent variables was also significant ($F(3, 40)=3.38$, $P<0.05$). The percentage of double-labeled cells was highest in the HDB and SI, followed by the MCPO and MS/DBv.

DISCUSSION

The pattern of c-Fos activation in cholinergic neurons of BF was as follows: $SD>SW>SS\approx SDR$. c-Fos/ChAT double labeling was highest following SD, similar to previous investigations. The elevation of double-labeling in the SD condition, compared to the SW condition, may be due to the gentle handling procedure of SD, which may introduce stress. c-Fos/ChAT double-labeling in BF was significantly elevated in the SW condition, when compared to SS and SDR. Double-labeling in the SW condition indicated that neuronal activation was due to wakefulness, for stress was minimized. The percentage of double-labeled cells was elevated following SD treatment, compared to SW, for the MS/DBv, HDB and MCPO subregions of BF, but not for the SI.

Several studies have described significant activation of cholinergic BF neurons during SD treatment, documenting increases in c-Fos protein expression [23,43]. The finding herein that approximately 13% of cholinergic cells were found to express c-Fos protein during SD was very similar to that of these previous studies. For example, Modirrousta et al. [43] reported approximately 11.7% c-Fos/ChAT double-labeling in BF following 3 hours of SD (produced by gentle sensory stimulation). Also similar to previous findings [23,43], sparse double-labeling was evident in the SS and SDR treatments in the present study. However, the first paper investigating c-Fos labeling in BF ChAT cells reported no difference in ChAT-cFos double labeled neurons between the SD and SDR [3]. These authors believe that this discrepancy may be due to the limited quantitative analysis that was performed in relatively

few sections. This bears on the discussion below describing the relatively small percentage of ChAT cells that express the c-Fos protein during SD (~13%) or spontaneous wakefulness (~9%). However, Basheer et al. did report an increase in BF c-Fos protein levels using western blots, as well as an increase in its DNA binding function in SD group when compared to the SDR group clearly suggesting an SD-induced increase of c-Fos in BF [3].

Numerous previous studies have documented an increase of c-Fos expression in the BF and neighboring preoptic regions following stress [6,7,9,14] and/or sensory stimulation [33]. In one study, c-Fos expression (without cholinergic co-localization) was analyzed following different SD procedures in the cat [34]. SD by means of gentle sensory stimulation produced a slight increase of c-Fos expression in such regions as SI and the nucleus of the diagonal band of Broca, and a rather large increase in neighboring preoptic regions. In this study [34], the more stressful water tank technique for SD, though, produced a substantial increase of c-Fos in all BF subnuclei analyzed. The relatively high percentage of c-Fos/ChAT expression in BF during the SW condition (approximately 9%), which is significantly elevated compared to SS and SDR counts, leads us to conclude that BF cholinergic neurons appear to be wake-active. Still, it remains possible that the increase of sensory stimulation that may occur in the SW condition may also contribute to c-Fos protein expression, although this study attempted to minimize the possible confounds of stress and sensory stimulation.

Select subnuclei of the BF (MS/DBv, HDB, and MCPO) exhibited elevated amounts of c-Fos/ChAT double-labeling in the SD treatment group, when compared to SW. The delineation of BF subnuclei was adapted from the widely-used rat brain atlas of Paxinos and Watson [47], so that these findings may be easily understood and replicated. Lu and colleagues described c-Fos activation in SI cholinergic neurons of spontaneously waking rats, but not in other BF subnuclei [37]. However, quantification and supporting photomicrographic evidence was not provided in this report, making it difficult to precisely compare this work to the present findings. Here, substantial c-Fos activation was described in areas outside of SI during SW; i.e., Figure 2, where c-Fos/ChAT co-localization in the horizontal limb of the diagonal band is photographically depicted.

The functional significance of these subnuclei differences is unclear, but may reflect neuroanatomical differences. The MS/DBv includes a distinct population of cholinergic neurons that project to the hippocampus, which play a part in the generation of the EEG theta wave [for review, 51,57,58]. Cholinergic neurons of HDB, MCPO, and SI, though, project to neocortical regions [25,48,59–62]. Furthermore, differences in neuromodulation by afferent systems may also be reflected in these subnuclei count differences. Also, the possibility remains that stress and sensory stimulation due to our SD method of gentle handling may be responsible for the increase of double-labeling, compared to SW, and these three subnuclei may be more sensitive to such manipulations. Also, the amount of wake in the SD group (99.8% of total time) was significantly elevated when compared to that in the SW group (70.8% of total time), which alone may be responsible for the significant increase in c-Fos/ChAT double-labeling in these subnuclei in the SD treatment group. Interestingly, double-labeling was not significantly increased in SI, when comparing SW and SD.

Labeling of the c-Fos protein may be employed in order to detect neuronal activation, where sacrifice of the animal reflects neuronal subpopulation activation within the preceding hour [44,31,56]. The percentage of c-Fos/ChAT double-labeling described here is somewhat low (i.e., 12.9% double-labeling for the SD treatment); that is, the vast majority of cholinergic neurons did not express the c-Fos protein following SD. Interestingly, Deurveilher et al. [15] recently reported that systemic caffeine injection in the rat, which increased wake activity, resulted in approximately 8–9% c-Fos/ChAT double-labeling in SI (30 mg/kg dosage of caffeine). It could be proposed that not all BF cholinergic cells express c-fos genetic activation/

c-Fos protein expression, although previous studies have reported that co-localization of the c-Fos protein in BF cholinergic neurons can be quite high following pharmacological manipulation; e.g., up to 68% of cholinergic neurons of HDB expressed the c-Fos protein following intracerebroventricular injection of nerve growth factor [20,45]. In conclusion, the large proportion of ChAT+ neurons (~ 80%) that are cFos negative during SD or SW indicates the importance of systematic quantification of the double labeled cells in order to detect meaningful differences between SD and SDR groups.

Although the use of the c-Fos protein antibody as an indicator of neuronal activation is quite useful, it has been clearly documented that not all neurons discharging action potentials express the c-Fos protein, and that expression of the c-Fos protein does not always imply action potential discharge [17,32]. The c-fos genetic activation/c-Fos protein expression is known to reflect phenotypic alteration such as genetic regulation of transcription, but does not always correlate directly with neuronal depolarization and discharge [32]. Although the documentation of c-Fos and other immediate early gene protein expression is useful, the electrophysiological recording of neuronal discharge remains the technique of choice to directly measure neuronal activity/depolarizations.

It has been proposed that cholinergic BF neurons are wake-active, for a cessation of cholinergic BF neuronal activity has been postulated to allow the occurrence of NREM sleep [19,35,38, 39,53; for review, 28,29,42,49]. Juxtacellular labeling, identifying cholinergic neurons, supports the postulate of their being wake-active [35]. Lesions of BF which spared fibers of passage [5], local inactivation of BF [8], and lesions specific to cholinergic neurons of BF [30] reduced the time spent in wakefulness.

In conclusion, cholinergic BF neurons were more active when the rats were spontaneously awake. Since stress and other potential confounds were minimized in the spontaneous wakefulness condition, this observation is consistent with a BF role in promoting cortical activation in the SW condition, in addition to the SD condition which has been previously reported. These findings further support the proposal that cholinergic neurons of BF are part of the brain circuitry responsible for the high frequency desynchronous cortical activity indicative of arousal.

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ABBREVIATIONS

AHA, anterior hypothalamic area
 ANOVA, analysis of variance
 BF, basal forebrain
 BST, bed nucleus of stria terminalis
 ChAT, choline acetyl-transferase
 CPu, caudate putamen
 DAB, diaminobenzidine
 EEG, electroencephalograph
 EMG, electromyograph
 GABA, gamma-aminobutyric acid
 HDB, horizontal limb of the diagonal band
 IgG, immunoglobulin

LGP, lateral globus pallidus
 LH, lateral hypothalamic area
 LPO, lateral preoptic area
 MCPO, magnocellular preoptic nucleus
 MnPO, median preoptic nucleus
 MPA, medial preoptic area
 MPO, medial preoptic nucleus
 MS/DBv, medial septum/vertical limb of the diagonal band
 NREM, nonREM sleep
 SS, spontaneous (non-manipulated) sleep
 SW, spontaneous (non-manipulated) wake
 PBS, phosphate buffered saline
 REM, rapid eye movement sleep
 RT, room temperature
 SD, sleep deprivation
 SDR, recovery following sleep deprivation
 SI, substantia innominata
 VLPO, ventrolateral preoptic nucleus
 VP, ventral pallidum
 W, wake

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Figure 1.

Graphic depiction of the timeline of the four treatment groups: 1) spontaneous wake (SW): when the rat was left undisturbed, sacrificed at 10 PM; 2) spontaneous sleep (SS): when the rat was left undisturbed, sacrificed at 10AM; 3) sleep deprivation (SD): 2 h of SD, sacrificed at 10AM; and 4) sleep deprivation recovery (SDR): 6 h of SD (8AM–2PM), followed by 2 h of undisturbed recovery (2–4PM), sacrificed at 4PM. Rats were placed on a 8AM/8PM light/dark cycle. Time of sacrifice for spontaneous wake and sleep treatment groups denoted by the “X”.

Figure 2.

Photomicrographs of neurons in BF that are cholinergic (brown cytoplasm, expressing the synthesizing enzyme choline-acetyl transferase (ChAT)) and express the c-Fos protein (black nucleic stain). Examples include c-Fos/ChAT double-labeled cells in the horizontal limb of the diagonal band (Panels A and C) and the substantia innominata (Panels B and D) during sleep deprivation (A and B) and spontaneous wake (C and D). Arrowheads indicate examples of double-labeled cells.

Figure 3.

Representative coronal section schematics depicting neurons of BF that are cholinergic (black dots, ChAT-Positive). ChAT-Positive neurons that also express the c-Fos protein (c-Fos/ChAT) are denoted by red triangles. The four coronal schematic plates represent antero-posterior levels of 0.20 mm, -0.30 mm, -0.80 mm, and -1.30 mm from bregma. A relative increase of c-Fos/ChAT double-labeled neurons during sleep deprivation (Figure 3A) and the spontaneous wake conditions (Figure 3B) was observed, when the rat was most aroused, compared to few double-labeled cells during sleep rebound following sleep deprivation (Figure 3C) and the spontaneous sleep (Figure 3D) conditions. Abbreviations: AHA, anterior hypothalamic area; BST, bed nucleus of stria terminalis; CPU, caudate putamen; HDB, horizontal limb of the diagonal band; LGP, lateral globus pallidus; LH, lateral hypothalamic area; LPO, lateral preoptic area; MCPO, magnocellular preoptic nucleus; MnPO, median preoptic nucleus; MPA, medial preoptic area; MPO, medial preoptic nucleus; MS/DBV, medial septum/vertical limb of the diagonal band; SI, substantia innominata; VLPO, ventrolateral preoptic nucleus; VP, ventral pallidum.

Figure 4.

Graphic depictions of: (A) the percentages of BF cholinergic (ChAT-positive) neurons that express the c-Fos protein (indicating activation), represented as % c-Fos/ChAT double-labeling (n=6 per treatment group, as described in Figure 1) (B) the percentage of total time awake (% wake) in the hour preceding sacrifice times; and (C) the percentages of c-Fos/Chat double-labeled cells following SD and SW treatments for the medial septum/vertical limb of the diagonal band (MS/DBv), horizontal limb of the diagonal band (HDB), the magnocellular preoptic nucleus (MCPO), and the substantia innominata (SI). The percent of double-labeled cells was highest in the HDB following SD. The percent of double-labeled cells is calculated over the population of ChAT neurons of the individual subnucleus. The data plotted are means

±SEM. Abbreviations: sleep deprivation (SD), spontaneous wake (SW), spontaneous sleep (SS), and sleep deprivation recovery (SDR).

Table 1

The total number of cholinergic neurons (labeled with the antibody for ChAT), percentage of ChAT neurons also labeled with c-Fos, and the percentage of time awake in the last hour preceding sacrifice for the four treatment groups.

Treatment group	Total ChAT neurons	% ChAT/c-Fos	% Time awake (Hr before sacrifice)
Sleep Deprivation (SD)	1501±192	12.9±1.3%	99.8±0.2%
Spontaneous Wake (SW)	1343±124	9.2±1.8%	70.8±1.1%
Spontaneous Sleep (SS)	1630±216	1.8±0.4%	14.7±3.9%
Sleep Deprivation Recovery (SDR)	1269±135	0.8±0.3%	15.9±5.8%

Table 2

Percentages of c-Fos/ChAT double-labeled cells for each BF subnuclei (MS/DBv, HDB, MCPO, and SI) for the four treatment groups.

Treatment Group	MS/DBv	HDB	MCPO	SI
Sleep Deprivation (SD)	10.32±1.15	18.72±1.37	10.10±0.87	13.71±1.35
Spontaneous Wake (SW)	5.91±1.13	8.95±1.07	5.53±0.96	13.23±1.76
Spontaneous Sleep (SS)	1.13±0.61	2.33±0.34	2.14±0.57	1.87±0.81
Sleep Deprivation Recovery (SDR)	0.55±0.43	1.35±0.33	0.88±0.48	0.16±0.11