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Fos expression in arousal and reward areas of the brain in grass rats following induced wakefulness

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Abstract

In the diurnal grass rat nocturnal voluntary wakefulness induces Fos expression in specific cellular populations of arousal and reward areas of the brain. Here, we evaluated whether involuntary wakefulness would result in similar patterns of Fos expression. We assessed this question using male grass rats that were sleep deprived for 6 h by gentle stimulation (SD group), starting 2 h before lights off (12:12 LD cycle). Then, we examined expression of Fos in cholinergic cells of the basal forebrain (BF), as well as in dopaminergic cells of the reward system, and compared these results to those obtained from an undisturbed control group. Different from previous results with grass rats that were voluntary awake, the BF of SD animals only showed a significant increase in Fos expression in non-cholinergic neurons of the medial septum (MS). These observations differ from reports for nocturnal rodents that are sleep deprived. Thus, our results show that voluntary and induced wakefulness have different effects on neural systems involved in wakefulness and reward, and that the effects of sleep deprivation are different across species. We also investigated whether other arousal promoting regions and circadian and stress related areas responded to sleep deprivation by changing the level of Fos expression. Among these areas, only the lateral hypothalamus (LH) and the ventro lateral preoptic area showed significant effects of sleep deprivation that dissipated after a 2 h period of sleep recovery, as it was also the case for the non-cholinergic MS. In addition, we found that Fos expression in the LH was robustly associated with Fos expression in other arousal and reward areas of the brain. This is consistent with the view that the arousal system of the LH modulates neural activity of other arousal regions of the brain, as described for nocturnal rodents.

Keywords

sleep deprivation; grass rat; diurnal; wakefulness; reward; circadian

1. Introduction

In modern human society, a significant proportion of the population is awake throughout the night due to, for example, job duties or social demands. This practice poses physiological

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challenges to the brain and body, since being a diurnal species, humans have evolved to be active during the day and to be resting during the night. The consequences of sleep deprivation range from cognitive impairments [reviewed in 1] to the development of a wide range of health problems, including late-onset diabetes, gastrointestinal and cardiovascular disease [reviewed in 2], prostate [3] and breast cancer [4, 5]. Whether and how these consequences are influenced by the conditions and motivational factors responsible for nocturnal activity remains to be elucidated.

Work with animal models has shed light on the etiology of some of the health problems associated with chronic sleep deprivation during the regular rest phase [6–9]. The interpretation of the findings, however, becomes problematic because the majority of these studies uses nocturnal rodents, and also, because the sleep deprivation during the rest phase is induced by the researcher, and not produced voluntarily by the animals. In the diurnal grass rat, *Arvicanthis niloticus*, access to a running wheel results in voluntary shifts in the temporal distribution of activity in some individuals (night-active, NA), but not in others [day-active, DA; 10]. This makes the grass rat a suitable model with which to study the physiological consequences of being voluntarily active during the natural rest phase of a diurnal species.

In a previous study, we found that in comparison to grass rats that have no wheel access [11], and therefore are likely to be sleeping at night [12], grass rats that are actively running during the night [euthanized at zeitgeber time (ZT) 16 (lights on at ZT 0, off at ZT 12)] show elevated Fos expression in areas of the brain related to reward and arousal, such as cholinergic (ACh) and non-cholinergic (nACh) regions of the basal forebrain (BF), and non-dopaminergic cells (as determined by lack of tyroxine hydroxylase; TH) of the supramammillary nucleus (SUM) and anterior ventral tegmental area (aVTA). Our observations of Fos expression in the BF of NA grass rats were not in agreement with those reported for nocturnal laboratory rats that were forced to stay awake during their rest phase [13, 14]. We reasoned that this discrepancy could be related to the fact that NA grass rats were voluntarily awake, rather than forced to be awake as it was the case in the experiments with laboratory rats [13, 14]. Further, the elevated expression of Fos seen in reward areas (i.e., SUM and aVTA) in grass rats with access to wheels suggests that enhanced voluntary exercise has rewarding properties, which are not likely shared with situations involving forced wakefulness.

In our previous study, we also observed that in animals that were active during the night there were strong correlations between Fos expression in orexin cells (orexin A and B; OXA and OXB, respectively) of the lateral hypothalamus (LH) and Fos expression in ACh and nACh cells of two major nuclei in the BF- the medial septum (MS) and vertical diagonal band of Broca [VDB; 11]. We also identified putative appositions between OXA positive fibers and ACh and nACh cell bodies of the BF [11]. These observations are in agreement with findings in nocturnal species that suggest that the orexinergic system modulates neural activity of other arousal systems, including the ACh system of the BF [reviewed in 15].

In the present study, we examined the question of whether in grass rats experimenter-induced wakefulness during the night would elicit changes in Fos expression in arousal and reward areas that are similar to those seen in grass rats that voluntarily become active at night [11]. Specifically, we examined patterns of Fos expression in the BF, SUM, and aVTA after 6 h of induced wakefulness produced by gentle stimulation. We also included in our analyses other brain areas that are known to promote wakefulness or sleep, i.e., the ventro lateral preoptic area (VLPO), LH, tuberomammillary nuclei (TMM), raphe nuclei, locus coeruleus (LC), and nucleus incertus (NI), as well as areas of the brain involved in circadian control, i.e., the suprachiasmatic nucleus (SCN) and ventral subparaventricular zone (vSPZ).

Since experimenter-induced wakefulness may be stressful, we also measured Fos expression in areas related to the stress response, i.e., the parvocellular and magnocellular subnuclei of the rostral paraventricular nucleus of the hypothalamus (pPVN and mPVN, respectively). In addition, because in nocturnal rats a period of sleep recovery induces the return of Fos expression to baseline levels [13, 16, 17], we investigated how 2 h of sleep recovery, following the sleep deprivation episode, influences the expression of Fos in the brain regions affected by sleep deprivation. Finally, to explore in a diurnal species a potential modulatory role of the LH on neural activity of other wakefulness promoting areas, we used correlation analyses to study the relationships between Fos expression in the LH and Fos expression in other arousal areas.

2. Methods

2.1. Animals

Twenty-nine adult male grass rats bred in our laboratory were used in this study. All animals were housed individually in plexiglass cages (17 × 34 × 28 cm) for at least one month before the behavioral manipulation. The animals were kept on a 12:12 light-dark cycle with a red light (< 5 lux) on at all times and were provided with *ad libitum* access to water and food (Harlan Teklad 8640 rodent diet, Harlan Teklad Laboratory, Madison, WI). All experiments were performed in compliance with guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Michigan State University Institutional Animal Care and Use Committee.

2.2. Induced wakefulness

Fifteen grass rats were stimulated to stay awake for 6 h from ZT (lights on at ZT 0) 10 to ZT 16 (Fig. 1). Wakefulness was induced by gently touching the animals with a Q-tip when they showed signs of sleepiness, that is, when their eyes were closing as they were sitting or resting on their side. After the period of induced wakefulness, one group of grass rats (sleep deprived group, SD; n=7) was perfused immediately, and the other group was left undisturbed for 2 h (recovery group, R; n=8), and perfused at ZT 18 (Fig. 1). Two additional groups of undisturbed animals (n=7 per group) were perfused at ZT 16 and ZT 18 and were used as control groups for the SD and R groups (CSD and CR, respectively; Fig. 1). At the time of perfusion, intraperitoneal injections of sodium pentobarbital (Ovation Pharmaceutical, Deerfield, IL) were used to deeply anesthetize the animals. The anesthetized animals were fit with an aluminum foil hood over their heads to avoid exposure to light. Then, they were intracardially perfused with 0.01 M phosphate buffer saline (PBS), followed by 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) with 75 mM lysine (Sigma-Aldrich) and 10 mM sodium periodate (Sigma-Aldrich; PLP) in 0.1 M phosphate buffer (PB). Brains were post-fixed for approximately 4 h in PLP and then transferred to 20% sucrose solution in 0.1 M PB at 4°C. Brains were left in this solution until they sunk to the base of the vial. Then, coronal sections were cut on a freezing sliding microtome at 30 µm. Alternate sections were collected in three series in cryoprotectant solution at -20°C and stored under those conditions until further processing.

2.3. Immunocytochemistry (ICC)

Unless indicated otherwise, all ICC procedures were carried out at room temperature, and all incubations involved gentle agitation. In addition, sections were rinsed 3 times (5min/rinse) in 0.01 M PBS between all the steps of the ICC protocol, and all incubations included 0.3% Triton X-100 (TX; RPI, Elk Grove Village, IL; TX).

2.3.1. Basal forebrain—Free-floating sections containing the forebrain were rinsed (6 times, 10 min/rinse) in 0.01 M PBS and blocked for 30 min in 0.01 M PBS with 3%

hydrogen peroxide (J.T. Baker, Phillipsburg, NJ). Then, sections were rinsed in 0.01 M PBS (6 times, 10 min/rinse), blocked for 30 min using 5% normal donkey serum (NDS; Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS, and incubated overnight in a rabbit anti-Fos antibody (Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:20,000 in PBS and 3% NDS) at 4° C. The sections were then incubated for 1 h in a donkey anti-rabbit biotinylated antibody (Jackson; diluted 1:200 in PBS and 3% NDS) and then for 1 h in avidin-biotin peroxidase complex (AB complex, 0.9% each avidin and biotin solutions; Vector Laboratories, Burlingame, CA; in PBS). After 3 rinses (10 min/rinse) in Tris buffer (pH 7.2), the sections were preincubated in 0.025% diaminobenzidine (DAB; Sigma-Aldrich) enhanced with 2.5% nickel sulfate (Sigma-Aldrich) in Tris buffer for 1 min, and then 3% hydrogen peroxide was added for the reaction (0.66 μ l 3% hydrogen peroxide/ml buffer). After a 2.5 min reaction, the tissue was rinsed 3 times in PBS with 0.03% TX (10 min/rinse), followed by one rinse in PBS (10 min). Then, sections were blocked for 30 min in 5% normal horse serum (NHS; Vector) in PBS and incubated overnight in a goat anti-choline acetyltransferase (ChAT) antibody (Chemicon, Temecula, CA; diluted 1:2,000 in PBS and 3% NHS) at 4° C. Following primary incubation, the sections were incubated for 1 h in a horse anti-goat biotinylated antibody (Vector; diluted 1:200 in PBS and 3% NHS). Then, the tissue was incubated for 1 h with AB complex (0.9% each avidin and biotin solutions; Vector; in PBS). After 3 rinses (10 min/rinse) in Tris buffer, the sections were preincubated with 0.02% DAB in the same buffer for 1 min, and then 30% hydrogen peroxide (0.35 μ l 30% hydrogen peroxide/ml buffer) was added for the reaction. After a 1 min reaction, the tissue was rinsed in 0.01 M PBS (4 times, 5 min/rinse). Then, all sections were mounted onto gelatin-coated slides, dehydrated, and later coverslipped with dibutyl phthalate xylene (Sigma-Aldrich; DPX).

2.3.2. Reward system—Free-floating sections containing the caudal diencephalon and midbrain were processed for Fos and TH following ICC procedures similar to the ones described above, but with the exceptions noted below. The reaction for Fos staining lasted 3 min. For TH staining, the tissue was blocked with 5% NHS (Vector; in PBS) for 30 minutes and then incubated in a mouse anti-TH antibody (Immunostar, Hudson, WI; diluted 1:150,000 in PBS and 3% NHS) for 24 h at 4° C. The biotinylated secondary antibody incubation with horse anti-mouse (Vector; diluted 1:200 in PBS and 3% NHS), as well as the AB complex incubation (in PBS), were for 1 h. The pre-incubation step with the DAB solution was for 45 sec, and the reaction with hydrogen peroxide lasted 1.5 min.

2.4. Cell counts

In order to quantify Fos and ChAT expression, we selected three sections through the MS and VDB and five sections that included the horizontal diagonal band of Broca (HDB). For the MS and VDB, sections corresponded approximately to plates 15 through 17 of the rat brain atlas by Paxinos and Watson [18], whereas for the HDB sections corresponded to plates 15 through 20. For every section selected, cells expressing Fos, ChAT, and Fos +ChAT were counted using a 600 μ m (width) by 300 μ m (height) sampling box placed at the center of the studied areas, as described previously in Castillo-Ruiz et al. [11]. To quantify Fos and TH expression, we selected three sections through the VTA. These sections corresponded to levels 1 to 3 described previously [11] which in here we referred to as aVTA, medial VTA (mVTA), and posterior (pVTA), respectively. More caudal regions of the VTA were not analyzed due to the scarce cell labeling seen in those areas. For every section selected, cells expressing Fos, TH, and Fos+TH were counted using 160 μ m $\times$ 160 μ m sampling box placed lateral to the medial border of the VTA, in an area rich in TH staining, following the criteria outlined in Castillo-Ruiz et al. [11].

Sampling boxes were used to count Fos in one section through each of the following areas: 190 μm $\times$ 190 μm in the rostral VLPO; 215 μm (width) $\times$ 160 μm (height), medial vSPZ; 100 μm (width) $\times$ 200 μm (height), rostral mPVN and pPVN; 1200 μm (width) $\times$ 700 μm (height), medial LH (one hemisphere chosen at random was counted for each animal); 120 μm $\times$ 120 μm , the dorsal tuberomammillary (DTM); 150 μm $\times$ 150 μm , the ventral tuberomammillary (VTM); 160 μm $\times$ 160, the SUM; 150 μm (width) $\times$ 650 μm (height), the lateral dorsal raphe (IDR); 150 μm (width) $\times$ 700 μm (height), the median raphe (MR); and 200 μm $\times$ 200 μm , the NI. The rat brain atlas by Paxinos and Watson was used to identify the study areas [18]. Additionally, we quantified Fos in one section of the medial SCN. The borders of this nucleus were determined from Nissl-stained sections through the anterior hypothalamus of a representative animal. In addition, we counted Fos and/or TH positive cells in 2 sections containing the LC and overall Fos expression in 3 sections containing the medial dorsal raphe (mDR). For the LC we used a 400 μm (width) $\times$ 700 μm (height) sampling box. For the mDR, the 3 sections analyzed corresponded to levels 3 through 5 of the mDR following the nomenclature of Janusonis et al. [19]. Levels 1 and 2 of the mDR were not analyzed since Fos staining was rare in that region, and because these levels in the grass rat do not show rhythmic Fos expression (A. Castillo-Ruiz, unpublished observations). The sampling boxes used for the analysis of the mDR had the following dimensions: 150 μm (width) $\times$ 650 μm (height) for levels 3 and 4; and 160 μm $\times$ 160 μm for level 5. Figure 2 shows the placement of sampling boxes in areas for which such placement has not been previously shown in published work from our group [11, 12, 20–23].

For areas located distal to the midline, cells were counted bilaterally and cell numbers were averaged per section. If more than one section was used for counts, cell numbers were averaged across sections, with the exception of the VTA and mDR. For these two areas, every section was treated as a different level given that previous studies, including one from our group, suggest that the VTA and DR display rostro-caudal gradients of neural activation [11, 24, 25]. After the cell counts were completed, the number of cells expressing Fos was divided by the area occupied by the box for the IDR, as well as for levels 3 and 4 of the mDR. These adjustments were necessary because for some sections part of the box fell outside the study area. The results for these areas are expressed as number of cells/mm². For all other study areas the results are expressed as number of cells within the sampling region. All counts were done at 25x, except for the counting of labeled cells in the LH, which was done at 10x, under a light microscope equipped with a drawing tube (Laborlux 8, Leitz Wetzlar GBH, Wetzlar, Germany). An investigator unaware of the source of the tissue collected all the anatomical data.

To analyze the effect of sleep deprivation and sleep recovery on Fos expression in each of the brain regions, we compared each treatment group to their control separately (i.e., SD to CSD and R to CR). We, however, did not compare the two treatment groups to each other because of the confounding effect of ZT. For these group comparisons, the data rarely met the assumptions of parametric statistical methods, even after transformations, therefore, all comparisons were done with non-parametric Mann-Whitney U tests. To explore further differences between groups, effect sizes (Cohen's d) were calculated when comparisons approached, but missed significance. In addition, we used Pearson's r correlation tests to determine if Fos expression in the LH was associated with Fos expression in other brain regions. We accomplished this by pooling the data of all groups for each region. The data from this large sample was amenable to parametric statistical analysis. For all comparisons differences were considered significant when p was less than 0.05 (two-tailed tests). The software used for the statistical analyses was SPSS 17 (SPSS Inc., Chicago, IL, USA).

3. Results

Significant differences in Fos expression among treatments were seen only in some of the study areas, particularly those related to the modulation of wakefulness and sleep. Figure 3 shows, for each group, the patterns of Fos expression in the areas where significant effects and non-significant trends were seen, and Table 1 shows Fos expression for all other areas pooled across treatment groups. Table 1 also shows the p values associated with the treatment's comparisons that did not approach statistical significance.

3.1. Patterns of Fos expression in wakefulness and sleep promoting areas

The cholinergic BF—The total number of ACh cells in the MS, VDB, and HDB did not differ significantly for any comparison (all p values > 0.12; data not shown). Also, the numbers of ACh cells that contained Fos in these areas were not significantly affected by the treatments (all p values > 0.23; Table 1). In the MS, however, there was a significant difference in the expression of Fos in nACh cells between the SD and CSD groups ($U = 7.50$, $p = 0.03$; Fig. 3A and Fig. 4), with more Fos seen in the SD group. A non-significant trend for the same effect in nACh cells of these two treatment groups was observed in the VDB ($U = 10.00$, $p = 0.07$, $d = 1.24$; Fig. 3B). All other comparisons in terms of Fos expression in the BF failed to reach statistical significance (all p values > 0.15; Fig. 3A and 3B and Table 1).

TMM—In the VTM, there was a trend for more Fos to be expressed in R compared to CR animals ($U = 10.50$, $p = 0.07$, $d = 1.13$; Fig. 3C). All other comparisons between SD and CSD in the VTM and DTM, as well as between R and CR in the DTM did not reach statistical significance (all p values > 0.23; Fig. 3C and Table 1).

LH—For the LH more cells expressed Fos in the SD group than in the CSD group ($U = 3.00$, $p = 0.04$; Fig. 3D). In addition, there was a non-significant trend for higher Fos expression in the R group than in the CR animals ($U = 12.00$, $p = 0.07$, $d = 1.23$; Fig. 3D).

LC—In this region, none of the comparisons found significant effects in the total number of TH cells (all p values > 0.73; data not shown) or in the number of nTH cells expressing Fos (all p values > 0.08; Table 1). There was, however, a non-significant trend for higher Fos expression in TH cells of SD in comparison to CSD animals ($U = 2.00$, $p = 0.06$, $d = 1.44$; Fig. 3E). In contrast, the comparison between expression of Fos in TH cells of the R group and the CR group did not approach statistical significance ($U = 5.00$, $p > 0.08$; Fig. 3E).

Raphe nuclei and NI—In the IDR there was a non-significant trend for more Fos in the R group than in the CR group ($U = 10.50$, $p = 0.07$, $d = 1.05$; Fig. 3F). For all other comparisons in the raphe and NI no significant differences were found among treatment groups (all p values > 0.17; Table 1).

VLPO—In this area there was a significant difference between the SD and the CSD groups, with more cells expressing Fos in the latter group ($U = 0.00$, $p < 0.01$; Fig. 3G). There was, however, no difference between R and CR ($U = 17.00$, $p = 0.23$; Fig. 3G).

3.2. Patterns of Fos expression in reward systems

SUM and VTA—None of the comparisons found significant effects in the total number of TH cells in the VTA and SUM (all p values > 0.23; data not shown) or in the numbers of TH cells expressing Fos in the VTA (all p values > 0.46; Table 1) for any comparison. In the SUM, data on TH cells expressing Fos were not analyzed because double-labeled cells were very rare across all groups. In addition, no significant differences were seen in Fos

expression in nTH cells of the SUM and VTA for any comparison (all p values > 0.17 ; Table 1).

3.3. Patterns of Fos expression in circadian controlling areas

SCN and vSPZ—There were no significant differences among treatment groups in these areas (all p values > 0.41 ; Table 1).

3.4. Patterns of Fos expression in stress related areas

PVN—In the mPVN, all comparisons revealed no significant effects among treatments on Fos expression (all p values > 0.57 ; Table 1). In contrast, in the pPVN there was a non-significant trend for higher Fos expression in SD than in CSD animals ($U = 8.00$, $p = 0.07$, $d = 1.65$; Fig. 3H). In addition, in this region there was no difference between the R and CR groups ($U = 14.50$, $p = 0.23$; Fig. 3H).

3.5. Fos expression in the LH in relation to other brain regions

In the MS and HDB, we found that Fos expression in both nACh and ACh cells was positively correlated with Fos expression in LH cells; whereas in the VDB, Fos expression in only the nACh cell group was positively correlated with Fos expression in the LH (Table 2). In the LC, we detected a significant positive correlation between Fos expression in TH cells and Fos expression in LH cells, and a non-significant trend for a positive correlation on that measure between nTH cells and LH cells (Table 2). In the NI, however, we did not find evidence for a correlation between Fos expression in this nucleus and Fos expression in LH cells. For all other wakefulness promoting areas analyzed, overall patterns of Fos expression were positively correlated with Fos expression in the LH (Table 2). In addition, we found positive correlations between Fos expression in LH cells and Fos expression in the pPVN, mPVN, SUM, and aVTA (Table 2). In contrast, patterns of Fos expression in the SCN, vSPZ, VLPO, mVTA, and pVTA were not correlated with Fos expression in LH cells (Table 2).

4. Discussion

Fos expression has been used widely as a tool to assess neuronal activation induced by behavioral state, including sleep and wakefulness [reviewed in 26]. The presence of Fos in a cell, however, does not necessarily imply neuronal firing [reviewed in 26]. Nevertheless, at least in areas that control vigilance states, increased neuronal firing patterns [reviewed in 15] appear to be associated with increased Fos expression during the active phase of nocturnal rodents [27, 28]. Additionally, Fos expression has been used as a marker of neural activation in brain areas that mediate reward [29] and stress responses [30, 31], as well as a marker of circadian phase in the SCN and vSPZ [32]. Thus, due to the apparent reliability of Fos expression in identifying neuronal activity in our areas of interest, in this study we used it as a proxy for neural activation in the grass rat brain.

4.1. Voluntary vs. induced wakefulness: effects on arousal and reward areas of the brain

Previous reports from this lab demonstrated that in comparison to grass rats that show preference for wheel-running during the day, grass rats that voluntarily run during the night show specific patterns of Fos expression in arousal and reward areas of the brain [11, 20]. In this study, we sought to follow up those observations by asking whether grass rats that are forced to stay awake during the night show similar patterns of Fos expression. Overall, we found that induced and voluntary wakefulness have differential effects on those areas.

In the BF, we found that both induced and voluntary wakefulness elicited increased Fos expression in nACh cells of the MS. A similar trend with a large effect size was observed in

nACh cells of the VDB. In addition, increased Fos expression in response to induced and voluntary wakefulness during the night was observed in neurons of the LH. Together our results suggest that cellular populations in the MS, VDB, and LH are responsive to wakefulness *per se*, regardless of the procedure that elicits it.

In contrast, the HDB, VTA, and SUM did not show increased Fos expression following induced wakefulness, as observed when grass rats are voluntarily awake during the night [11]. These results suggest that induction of Fos in those areas in grass rats that are voluntarily running during the night may be related to the rewarding effects of wheel running [33]. In fact, one of our predictions for this study was that in comparison to grass rats that are voluntarily awake, grass rats forced to stay awake would not show differential patterns of Fos expression in reward areas of the brain. The lack of elevated Fos expression in the SUM and VTA seen here supports that prediction, which may also apply to the lack of an effect in the HDB, since reward areas of the brain project to the HDB [34]. Alternatively, the differential results obtained in our previous and present studies may stem from features of the experimental conditions other than induced vs. voluntary wakefulness. Those features include the length of exposure to the procedure (i.e., a month vs. a night) and the degree of physical activity exerted by the animals (i.e., wheel running vs. no wheel access). Thus, future work needs to evaluate whether the effect we previously observed in the HDB, SUM, and VTA of grass rats with access to wheels is in fact related to reward rather than to enhanced physical activity, and whether the effect is seen after a single night of wheel running.

A surprising finding of this study was that in contrast to reports on nocturnal rodents, in grass rats ACh cells of BF are unresponsive to induced wakefulness. For example, in lab rats, 2–6 h of induced wakefulness during the resting phase, following procedures similar to the one used in here, elicits high Fos expression in ACh cells of the BF [13, 14, 17]. The discrepancies between our results and the findings in nocturnal rodents could be related to species differences, or are perhaps related to chronotype (i.e., being diurnal vs. being nocturnal).

4.2. Induced wakefulness in brain areas related to wakefulness, sleep, circadian rhythms, and stress

4.2.1. Patterns of Fos expression in brain areas related to wakefulness and sleep—In this study, we also examined whether induced wakefulness has effects on Fos expression in areas that promote wakefulness that were not included in our voluntary wakefulness study [11]. These areas were the TMM, mDR, MR, LC, and NI. Of all these areas, only TH cells of the LC appeared to show increased Fos expression following induced wakefulness as shown by the non-significant trend with a relatively large effect size. These results are in agreement with the hypothesis that the LC is involved in modulating attention to salient stimuli in the environment [35]. Our results, then, could reflect an increase in the grass rats' alertness in response to the experimenters' presence during the procedure.

The fact that the TMM, IDR, mDR, MR, and NI did not show changes in Fos following induced wakefulness while other areas did, is interesting because it suggests that some wakefulness-promoting areas, such as the MS, VDB, and LH, may be more sensitive than others to the effects sleep deprivation. In addition, the results in the NI are of particular interest because this nucleus projects directly to areas of the brain examined in this study that showed a change in Fos expression following induced wakefulness, i.e., the MS and VDB [36]. Then, taken together our findings suggest that sleep deprivation may induce dissociations in the functioning of wakefulness-promoting areas. Because these areas are involved in cognitive processes [37], our results could in part explain the impairments in

cognition observed in individuals that stay awake during the normal resting phase of the species [reviewed in 1].

In this study, we also analyzed a sleep-promoting region, i.e., the VLPO [reviewed in 15]. Our prediction was that sleep deprivation should suppress Fos expression in the VLPO. Consistent with this prediction, we found that sleep deprived animals had lower levels of Fos expression in the VLPO than control animals. This finding is in agreement with reports on nocturnal rodents, which show that 6 h of forced wakefulness suppresses Fos expression in the VLPO [13].

4.2.2. Patterns of Fos expression in circadian controlling areas—In nocturnal rodents, acute [38] or chronic [39] wakefulness during the rest period suppresses Fos expression in the SCN, both in constant darkness and in a light-dark cycle. In addition, in laboratory rats, 6 h of sleep deprivation during the resting phase reduces the amplitude of the neuronal firing rhythm of the SCN, and this effect persists even after 6–7 h of sleep recovery [40]. Based on these observations, effects on Fos expression in the SCN of grass rats as a result of forced wakefulness would not have been surprising. However, we did not detect any changes in the SCN of sleep deprived grass rats. This could be explained in part by the fact that the raphe nuclei, an area that appears to mediate effects of sleep deprivation on the circadian system in hamsters [41], did not show changes in Fos expression under our experimental conditions. It remains to be determined whether the discrepancies between our findings and the findings of studies with nocturnal species are due to general species and/or experimental procedural differences, or if they stem from fundamental differences between diurnal and nocturnal rodents.

Another important brain area for circadian control is the vSPZ. This area receives a heavy projection from the SCN [42, 43] and may play a role in the modulation of SCN output signals [44]. The vSPZ regulates rhythms in locomotor activity in both grass rats [45] and laboratory rats [46]. Moreover, in laboratory rats the vSPZ appears to play a role in the circadian regulation of sleep, although the available data are from only a few post-surgical days following vSPZ lesions [46]. Similar to the results for the SCN, forced sleep deprivation had no effects on Fos expression in the vSPZ of grass rats. These observations for the SCN and the vSPZ are remarkably similar to those seen when grass rats voluntarily stay active at night [23, 47], and suggest that in contrast to what is reported for nocturnal rodents, key components of the circadian system of grass rats are refractory to the effects of voluntary or induced sleep deprivation.

4.2.3. Patterns of Fos expression in stress related areas—In this study, we evaluated whether the procedure used to induce wakefulness was stressful to grass rats. To that aim, we analyzed the patterns of Fos expression in the rostral PVN, since conditions that are considered challenging to an organism, such as exposure to a predator's odor [30] or immobilization [31], elicit Fos expression in this nucleus. Two regions of the PVN - the pPVN and mPVN - were included in the analysis given that they both modulate a variety of physiological and behavioral variables that accompany the stress response [48–50]. Even though we did not find significant effects among the groups in the mPVN, we saw a non-significant trend in the pPVN with a relatively large effect size, with higher Fos expression in the sleep deprived group than in the control group. This is an indication that the procedure was likely stressful for grass rats. Moreover, these results suggest that the Fos expression observed here in the MS and VDB of sleep deprived grass rats could be secondary to the activation of the stress axis. This is because the BF of lab rats contains receptors for corticotrophin releasing hormone [CRH; 51], a hormone released by the pPVN upon exposure to a stressful stimulus, and because delivery of CRH to the BF alters neural firing of neurons in the MS and VDB [52]. Against this interpretation is the fact that grass rats that

are voluntarily awake at night [11], and supposedly not under stress, show similar patterns of Fos expression in the MS and VDB to those observed in this study.

4.3. Effects of 2 h of recovery following induced wakefulness

The MS, VDB, LH, LC, VLPO, and pPVN were the only areas that showed apparent changes in Fos expression following forced wakefulness (i.e., either significant differences or trends associated with relatively large effect sizes). Remarkably, in all these areas, with the LH as a possible exception (see section 3.1), the Fos expression elicited by the forced wakefulness procedure returned to baseline levels within 2 h after the animals were left undisturbed. A similar effect is seen in the forebrain of lab rats and mice after 1–2 h of recovery sleep following 3–6 h of induced wakefulness [13, 16]. Although electroencephalographic data were not collected in our study, it is likely that there was sleep displayed by grass rats during the recovery period following forced wakefulness, since these animals show increased bouts of sleep at this time, even without previous sleep deprivation [12]. In contrast, our results for the LH suggest that in this area 2 h of recovery may not be sufficient to return Fos expression to baseline levels in this area. Additionally, in other wakefulness-promoting areas that were not immediately affected by sleep deprivation, namely the VTM and IDR, we saw non-significant trends with relatively large effect sizes for increased Fos expression after 2 h of recovery, as if the increase had taken place after the deprivation period and had not completely dissipated during the recovery period. Together, these findings suggest that wakefulness-promoting areas of the brain respond differently to sleep deprivation, with some areas being able to respond and recover faster than others.

4.4. Fos expression in the LH in relation to other brain regions

Even though we did not evaluate the phenotype of the LH neurons that expressed Fos, we expect that many of these neurons were orexinergic. This is because in grass rats that are voluntarily awake during the rest phase there is a significant increase in Fos expression in the OXA and OXB cells of this region [20]. Moreover, Fos expression in these cells is positively correlated with Fos expression in ACh and nACh cells of the MS and VDB, but only in animals that are voluntarily awake during the night [11]. Thus, given the role that LH cells appear to play in the modulation of neural activation in other wakefulness promoting areas of the brain [reviewed in 15], we predicted concordance between Fos expression in cells of the LH and Fos expression of other wakefulness promoting areas. We obtained strong positive correlations between Fos expression in all the wakefulness-promoting areas analyzed and Fos expression in the LH, with the exception of the NI, ACh cells of the VDB, and nTH cells of the LC. Although we are lacking a causal link, our observations are consistent with the view that in grass rats cellular populations of the LH modulate neural activity of other wakefulness promoting areas, as described for nocturnal rodents [reviewed in 15].

In addition, we found strong associations between Fos expression in the LH and Fos expression in other areas of the brain linked to arousal as well as reward, such as the SUM, aVTA, and in one area associated with stress responses i.e., the pPVN. The association between the LH and the pPVN is of particular interest given the activating effects that LH neurons have on the stress axis [53, 54].

5. Summary and conclusions

Overall our results suggest that induced and voluntary wakefulness during the resting phase has different effects on Fos expression in neural systems involved in wakefulness and reward. However, this claim needs to be tested further since the data for voluntary wakefulness were obtained with a chronic experimental paradigm [11] and the present data

came from an acute manipulation. Since changes in Fos expression may indicate changes in neural activation in wakefulness and reward systems (see section 4), different behavioral and physiological outcomes are expected to occur depending upon the particular protocols used to keep animals awake during their rest phase. These observations are important for the evaluation of the animal models used to study sleep deprivation. Also, it remains to be investigated whether voluntary or induced wakefulness has differential effects on Fos expression in arousal and reward areas of the brain depending on age, sex, and hormonal conditions [see for example 55], and if the ability to return to baseline after a sleep recovery period is affected by those variables. Further work on the effects of sleep recovery on wakefulness promoting areas is also needed given that, as shown in this study, wakefulness promoting areas appear to recover differently after sleep deprivation.

Our results also revealed that the effects of forced wakefulness in the brain of grass rats are different from the ones reported for nocturnal rodents [13, 14, 17]. This finding strongly suggests that we must be cautious when generalizing to situations involving humans, the results of experiments with nocturnal animals that are forced to be awake. The finding also points to the importance of comparative studies in the examination of how organisms respond to temporal shifts in wakefulness.

Finally, we found that in the grass rat Fos expression in most neural groups that promote wakefulness shows robust correlations with Fos expression in the arousal system located in the LH. In nocturnal rodents, this system has been postulated to play an important role in the modulation of neural activity in other wake-promoting neural groups [reviewed in 15]. The present study is the first to provide evidence for that hypothesis in a diurnal species.

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Figure 1.

Schematic representation of the experimental design. Two groups of male grass rats were sleep deprived (indicated by the lighting symbol) starting at ZT 10 and ending at ZT 16. One group was euthanized immediately after the sleep deprivation episode (sleep deprived group, SD), and the other group was left undisturbed for 2 h (recovery group, R) before euthanasia at ZT 18. Two additional groups were used as controls for the SD and R groups: CSD and CR, respectively. Time of euthanasia is represented by a thick vertical line at the end of the behavioral manipulations. The bar above the groups illustrates the 12:12 light-dark cycle.

Figure 2.

Rostro-caudal illustrations depicting the sampling areas used to quantify overall Fos expression in the IDR, MR, mDR3 (A), mDR4 (B), mDR5 (C), NI (E), PVN (F), as well as Fos expression in TH and nTH cells of the LC (D and E; note that for this area the two sampling levels were averaged per section and level). See text for sampling box dimensions. The placement of boxes for the areas not depicted in the figure has been described previously (see text). Abbreviations: LC1-2, locus coeruleus levels 1–2; IDR, lateral dorsal raphe; mDR3-5, medial dorsal raphe levels 3-5; mPVN, magnocellular subnucleus of the rostral paraventricular nucleus of the hypothalamus; MR, median raphe; NI, nucleus incertus; pPVN, parvocellular subnucleus of the rostral paraventricular nucleus of the hypothalamus. (A–E) scale bar = 1 mm, (F) scale bar = 400 μm.

Figure 3.

Patterns of Fos expression (mean $\pm$ SEM) for areas where significant effects and trends towards significance were observed across groups. The data are for overall Fos expression (C–D, and F–H), Fos in nACh cells (A and B), and Fos in TH cells (E). Single asterisks (*) represent significant differences within ZT ($p < 0.05$). Note that SD and R groups were not compared to each other due to the confounding effect of ZT. Abbreviations: LC, locus coeruleus; IDR, lateral dorsal raphe; LH, lateral hypothalamus; MS, medial septum; pPVN, parvocellular subnucleus of the rostral paraventricular nucleus of the hypothalamus; SEM, standard error of the mean; VDB, vertical diagonal band of Broca; VLPO, ventro lateral preoptic area; VTM, ventral tuberomammillary nucleus.

Figure 4.

Photomicrographs of cells expressing ChAT (i.e., ACh cells; brown cell body staining) or Fos (blue nuclear staining) in the MS of representative grass rats from the SD group (A) and CSD group (B). Note that double-labeled cells are absent in both animals. Abbreviations: ChAT, choline acetyltransferase; MS, medial septum. Scale bar = 100 μ m.

Table 1

Fos protein levels (mean $\pm$ SEM) averaged across groups for the brain areas where significant effects or trends towards significance were not observed.

		Mean $\pm$ SEM	p value SD vs. CSD	p value R vs. CR
Wakefulness controlling areas				
MS	% ACh cells containing Fos	1.23 $\pm$ 0.45	0.62	0.23
VDB	% ACh cells containing Fos	0.97 $\pm$ 0.24	0.40	0.81
HDB	% ACh cells containing Fos	1.06 $\pm$ 0.16	1.00	0.23
	# of nACh cells containing Fos	10.37 $\pm$ 0.91	0.21	0.28
DTM	# of Fos-ir cells	6.72 $\pm$ 0.75	0.84	0.32
mDR3	# of Fos-ir cells/mm ²	139.65 $\pm$ 19.48	0.39	0.90
mDR4	# of Fos-ir cells/mm ²	359.01 $\pm$ 48.10	0.43	0.41
mDR5	# of Fos-ir cells	5.00 $\pm$ 0.62	0.84	0.17
MR	# of Fos-ir cells	23.00 $\pm$ 2.59	1.00	0.62
LC	# of nTH cells containing Fos	14.51 $\pm$ 2.30	0.08	0.73
NI	# of Fos-ir cells	22.24 $\pm$ 1.76	1.00	0.46
Reward related areas				
SUM	# of nTH cells containing Fos	8.24 $\pm$ 0.94	0.17	0.61
aVTA	% TH cells containing Fos	0.11 $\pm$ 0.08	1.00	0.46
	# of nTH cells containing Fos	1.48 $\pm$ 0.38	0.23	0.46
mVTA	% TH cells containing Fos	0.05 $\pm$ 0.05	1.00	0.71
	# of nTH cells containing Fos	0.37 $\pm$ 0.08	0.23	0.46
pVTA	% TH cells containing Fos	0.93 $\pm$ 0.38	1.00	0.78
	# of nTH cells containing Fos	1.22 $\pm$ 0.16	0.71	0.46
Circadian controlling areas				
SCN	# of Fos-ir cells	34.48 $\pm$ 2.45	0.81	0.54
vSPZ	# of Fos-ir cells	39.38 $\pm$ 2.37	0.54	0.41
Areas related to stress responses				
mPVN	# of Fos-ir cells	13.46 $\pm$ 1.64	0.95	0.57

For cases involving double-labeling percentages are given, whereas for single-labeling for Fos either overall number of cells or number of cells/mm² are given. The same applies for the data used for the correlation coefficients of Table 2. Abbreviations: aVTA, anterior ventral tegmental area; DTM, dorsal tuberomammillary nucleus; Fos-ir, Fos immunoreactivity; HDB, horizontal diagonal band of Broca; LC, locus coeruleus; mDR3-5, medial dorsal raphe levels 3-5; mPVN, magnocellular subnucleus of the rostral paraventricular nucleus of the hypothalamus; MR, median raphe; MS, medial septum; mVTA, medial ventral tegmental area; NI, nucleus incertus; pVTA, posterior ventral tegmental area; SCN, suprachiasmatic nucleus; SEM, standard error of the mean; SUM, supramammillary nucleus; VDB, vertical diagonal band of Broca; vSPZ, ventral subparaventricular zone.

Table 2

Correlations between Fos expression in LH cells and all other areas analyzed in this study.

LH		
Wakefulness and sleep controlling areas		
MS	Fos in ACh cells	$r = 0.393^*$; $p = 0.047$
	Fos in nACh cells	$r = 0.676^*$; $p = 0.000$
VDB	Fos in ACh cells	$r = 0.105$; $p = 0.610$
	Fos in nACh cells	$r = 0.721^*$; $p = 0.000$
HDB	Fos in ACh cells	$r = 0.534^*$; $p = 0.005$
	Fos in nACh cells	$r = 0.724^*$; $p = 0.000$
VLPO	Overall Fos	$r = -0.164$; $p = 0.422$
DTM	Overall Fos	$r = 0.620^*$; $p = 0.001$
VTM	Overall Fos	$r = 0.508^*$; $p = 0.011$
mDR3	Overall Fos	$r = 0.508^*$; $p = 0.011$
mDR4	Overall Fos	$r = 0.627^*$; $p = 0.001$
mDR5	Overall Fos	$r = 0.491^*$; $p = 0.020$
IDR	Overall Fos	$r = 0.602^*$; $p = 0.002$
MR	Overall Fos	$r = 0.438^*$; $p = 0.032$
NI	Overall Fos	$r = 0.158$; $p = 0.471$
LC	Fos in TH cells	$r = 0.571^*$; $p = 0.013$
	Fos in nTH cells	$r = 0.437$; $p = 0.070$
Reward related areas		
SUM	Fos in nTH cells	$r = 0.617^*$; $p = 0.001$
aVTA	Fos in nTH cells	$r = 0.411^*$; $p = 0.037$
mVTA	Fos in nTH cells	$r = 0.049$; $p = 0.815$
pVTA	Fos in nTH cells	$r = 0.243$; $p = 0.232$
Circadian controlling areas		
SCN	Overall Fos	$r = 0.133$; $p = 0.526$
vSPZ	Overall Fos	$r = 0.331$; $p = 0.106$
Areas related to stress responses		
mPVN	Overall Fos	$r = 0.723^*$; $p = 0.000$
pPVN	Overall Fos	$r = 0.759^*$; $p = 0.000$

* Significant correlation ($p < 0.05$).

Abbreviations: aVTA, anterior ventral tegmental area; DTM, dorsal tuberomammillary nucleus; HDB, horizontal diagonal band of Broca; LC, locus coeruleus; IDR, lateral dorsal raphe; LH, lateral hypothalamus; mDR3-5, medial dorsal raphe levels 3-5; mPVN, magnocellular subnucleus of the rostral paraventricular nucleus of the hypothalamus; MR, median raphe; MS, medial septum; mVTA, medial ventral tegmental area; NI, nucleus incertus; pPVN, parvocellular subnucleus of the rostral paraventricular nucleus of the hypothalamus; pVTA, posterior ventral tegmental area; SCN, suprachiasmatic nucleus; SUM, supramammillary nucleus; VDB, vertical diagonal band of Broca; VLPO, ventro lateral preoptic area; vSPZ, ventral subparaventricular zone; VTM, ventral tuberomammillary nucleus.