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FURTHER CHARACTERIZATION OF SLEEP-ACTIVE nNOS NEURONS IN THE MOUSE BRAIN

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Abstract

We recently demonstrated that Fos is induced in a subpopulation of cortical neuronal nitric oxide synthase (nNOS)-immunoreactive neurons in three rodent species both during spontaneous sleep (SS) and recovery sleep (RS) after a period of sleep deprivation (SD); the proportion of cortical Fos⁺/nNOS neurons was significantly correlated with non-REM (NREM) sleep delta energy. The present study was undertaken to evaluate the specificity of this state-dependent activation of cortical nNOS cells. The percentage of nNOS neurons that expressed Fos during SD and RS was determined in nine subcortical brain regions and the cortex of the mouse brain; a significantly greater proportion of Fos⁺/nNOS neurons was observed during RS only in the cortex and in none of the nine subcortical regions. The proportion of calretinin-, calbindin- and parvalbumin-immunoreactive cortical interneurons that expressed Fos during SD and RS was also determined. In contrast to cortical nNOS neurons, a higher percentage of Fos⁺/calbindin neurons was found during SD than RS; there were no differences in the proportions of Fos-expressing parvalbumin or calretinin neurons between these conditions. Since the nNOS and calretinin cortical interneuron populations overlap extensively in the mouse brain, triple-labeling with these two phenotypic markers and Fos was undertaken in mice from the RS group to determine which combination of markers could best identify the rare “sleep-active” cortical interneuron population. The proportions of both Fos⁺/nNOS neurons and Fos⁺/nNOS/calretinin neurons far exceeded the proportion of Fos⁺/calretinin neurons during RS, but the proportions of these two cell types were not significantly different during RS. Thus, functional activation of nNOS neurons during sleep appears to be restricted to the cerebral cortex and cortical nNOS cells and nNOS/calretinin cells collectively define a cortical interneuron population that is activated during sleep.

Keywords

Sleep deprivation; calbindin; calretinin; parvalbumin

Nitric oxide (NO) is a signaling molecule produced from L-arginine and oxygen and its synthesis is mediated by the enzyme nitric oxide synthase (NOS). Three distinct isoforms of this enzyme are known: neuronal NOS (nNOS), found in neurons; inducible NOS (iNOS), found in macrophages; and endothelial NOS (eNOS), found in endothelial cells (Nathan and Xie, 1994).

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Accumulating evidence suggests that NO is an endogenous sleep-promoting substance involved in homeostatic sleep regulation. Intracerebroventricular (icv) injection of the NO precursor, L-arginine, induces an increase in NREM sleep and does not affect REM sleep when administered to rats during the dark phase of the light-dark cycle (Monti and Jantos, 2004a). Similar effects were seen following treatment with the NO donors, 3-morpholiniosydnonimine (molsidomine; SIN-1) or S-nitroso-N-acetyl-DLpenicillamine (SNAP) in rats (Kapas and Krueger, 1996, Monti and Jantos, 2004b) and cats (Datta et al., 1997). Other studies have shown that NOS inhibitors produce decreases in spontaneous sleep (Monti et al., 2001, Monti and Jantos, 2004b), reduce EEG δ -activity during NREM sleep (Kapas et al., 1994, Ribeiro et al., 2000), and attenuate sleep recovery that follows sleep deprivation (Kalinchuk et al., 2006).

Neurons that express nNOS are widely expressed in brain including the cerebral cortex and subcortical regions implicated in the control of sleep and wakefulness, such as the pedunculopontine tegmental (PPTg), laterodorsal tegmental (LDTg) and lateral parabrachial nuclei (LPB) (Bidmon et al., 1997, Gotti et al., 2005). Although many of the brain regions in which nNOS neurons are found are considered to be “wake-active” (i.e., exhibit the highest neuronal discharge rate during wakefulness), this need not indicate that nNOS neurons in these regions are themselves wake-active. Indeed, we recently demonstrated that nNOS neurons are activated during sleep in the cerebral cortex (Gerashchenko et al., 2008), a brain area that contains a large proportion of wake-active neurons. Within the cerebral cortex, nNOS neurons are the smallest currently known subset of GABAergic interneurons (Kubota et al., 1994) and comprise about 0.5– 2% of the interneuron population in the rat primary sensorimotor and occipital cortices (Gonchar and Burkhalter, 1997). Given these observations, one purpose of the present study was to determine whether subcortical nNOS neurons are also activated during sleep or whether this is a property unique to cortical nNOS interneurons.

GABAergic cortical interneurons are classified by morphological, electrophysiological and neurochemical criteria (Ascoli et al., 2008). Among neurochemical criteria, the expression of neuropeptides and the calcium-binding proteins calbindin, calretinin and parvalbumin have been used to identify subtypes of cortical interneurons (Kubota et al., 1994, Gonchar and Burkhalter, 1997). Although relatively few neurons in the rat cortex express calbindin and parvalbumin, at least 90% of the GABAergic cortical interneuron population express either of these proteins (DeFelipe, 1993). In the rat cerebral cortex, nNOS neurons express calbindin D28K but not parvalbumin (Kubota et al., 1994, Bertini et al., 1996, Gonchar and Burkhalter, 1997) or calretinin (Kubota et al., 1994, Gonchar and Burkhalter, 1997). By contrast, in the mouse visual cortex, more than half of the nNOS neurons express calretinin, 25% express parvalbumin, and 17% express calbindin (Lee and Jeon, 2005). Thus, a second purpose of the present study was to determine whether the expression of calcium binding proteins, either alone or in conjunction with nNOS, would provide a better marker than nNOS alone to identify the sleep-active cortical neuron population. We find that functional activation of nNOS neurons during sleep appears to be restricted to the cerebral cortex and that cortical nNOS/calretinin cells as well as cortical nNOS cells identify the distinct cortical interneuron population that is activated during sleep.

EXPERIMENTAL PROCEDURES

Animals and experimental procedures

All studies were conducted in accordance with the principles and procedures described in the National Institutes of Health Guide for the Care and Use of Laboratory Animals, were approved by the Animal Care and Use Committees at SRI International, and conformed to international guidelines on the ethical use of animals. Male C57BL/6 mice, aged 8 weeks,

were housed in separate cages under a 12:12 dark/light cycle with food and water *ad libitum* and used for these studies. The minimum number of animals was used to obtain statistically meaningful results and all attempts were made to mitigate any suffering.

For the double-label studies of Fos expression in subcortical nNOS neurons and Fos expression in calbindin D28K-, calretinin- and parvalbumin-immunoreactive cortical neurons described below, sections from the SD and RS groups of Experiment 2 described in (Gerashchenko et al., 2008) were used. Thus, one group (n=6) was subjected to 6 h of SD from Zeitgeber Time (ZT)2.5 to ZT8.5 (SD group) whereas the second group (n=6) was subjected to 6 h SD from ZT0 to ZT6 and then allowed 2.5 h of recovery sleep (RS group). By convention, ZT0 refers to lights on whereas ZT12 refers to lights off. The SD procedure involved lightly tapping the cage or introducing novel objects into the cage, as described previously (Gerashchenko et al., 2008). At ZT8.5, mice were deeply anesthetized with pentobarbital (150 mg/kg i.p.; Butler, San Fernando, CA) and transcardially perfused with 20 ml of phosphate buffered saline (PBS; Sigma-Aldrich, Saint Louis, MO), followed by 20 ml of phosphate-buffered 10% formalin. All mice were perfused within a 30 min interval so that the median time of perfusion was ZT8.5 for both groups.

For the triple-labelling studies of Fos, nNOS and calretinin described below, an additional 5 mice were subjected to 6 h SD from ZT0 to ZT6 and then allowed 2.5 h of RS. The mice were then deeply anesthetized and perfused as described above. Although we did not conduct sleep/wake recordings in this experiment, we have previously reported that six-hour SD from ZT 0-6 resulted in a 96% reduction of total sleep time (TST) relative to the baseline and that sleep intensity, as measured by EEG delta power activity during NREM sleep, increased significantly throughout the four-hour recovery period (ZT6-10) relative to the same period on the baseline day (Terao et al., 2000, Terao et al., 2003).

Immunohistochemistry

Brains were removed and fixed in phosphate-buffered 10% formalin (Sigma-Aldrich) for 4 h and then transferred to 30% sucrose (Sigma-Aldrich) and stored at 4°C. Brains were sliced into 40 µm thick coronal sections using a freezing microtome and collected in five separate sets for subsequent immunostaining.

To determine Fos expression in nNOS neurons in subcortical brain regions during SD and RS, one set of tissue sections from each of six mice from the SD group and six mice from the RS group was processed for immunohistochemistry with Fos and nNOS antisera as described previously (Gerashchenko et al., 2008). Sections were treated with 1% H₂O₂ (Sigma-Aldrich) for 15 min to quench endogenous peroxidases and then incubated overnight in rabbit-anti-cFos antisera (1:15,000; Calbiochem, San Diego, CA) at room temperature (RT). Sections were then rinsed in PBS, incubated in biotinylated donkey anti-rabbit IgG (1:500; Jackson ImmunoResearch, West Grove, PA) for 2 h at RT, incubated with peroxidase-conjugated avidin-biotin complex (1:200; ABC, Vector Laboratories, Burlingame, CA) for 1 h, followed by the addition of 0.05% diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, Saint Louis, MO) and 0.01% H₂O₂ with 1% NiSO₄ (Sigma-Aldrich) to produce a black reaction product in cell nuclei. The sections were then incubated in mouse anti-nNOS monoclonal antibody (1:5,000; Sigma-Aldrich, N2280), rinsed in PBS, incubated in biotinylated donkey anti-mouse IgG (1:500; Jackson ImmunoResearch) for 1 h at RT and then incubated with peroxidaseconjugated avidin-biotin complex (1:200; Vector Laboratories) for 2 h. Finally, sections were developed using 0.05% DAB and 0.01% H₂O₂ to produce brown-colored cell bodies. Sections were mounted on gelatin-coated slides and coverslipped using VectaMount (Vector Laboratories). All antibodies were diluted in 5% donkey serum (Jackson ImmunoResearch), PBS, and 1% Triton X-100.

To determine whether calcium binding proteins would be useful phenotypic markers to identify the sleep-active cortical neuron population, double-labeling was performed on three separate sets of tissue sections from each of six mice from the SD group and six mice from the RS group to produce the following combinations of labeling: Fos/calbindin, Fos/calretinin and Fos/parvalbumin. The purpose of this experiment was to assess Fos activation in those cortical interneurons that expressed calcium-binding proteins but did not express nNOS. Therefore, we used the mouse-anti-nNOS antiserum to identify calbindin, calretinin and parvalbumin neurons that co-localized with nNOS and eliminated those cells from subsequent analysis. Thus, brain sections were initially incubated with a combination of rabbit-anti-cFos antisera (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) and mouse-anti-nNOS antisera (1:1000; Sigma-Aldrich) and stained using DAB-Ni (black label), and secondary labeling was then performed similar to the above by incubation of one set of sections either with mouse-anti-calbindin (1:300; Sigma-Aldrich), rabbit-anti-calretinin (1:2000; Neomarkers, Fremont, CA) or mouse-anti-parvalbumin (1:3000; Chemicon, Billerica, Massachusetts) and stained using DAB (brown label).

For the triple-label study, only sections from the additional RS group (n=5) described above were used. Sections were initially stained for Fos using the ABC/DAB-Ni method as described above and then processed for nNOS/calretinin double-fluorescence labeling. For this step, sections were incubated overnight in mouse anti-nNOS (1:5000; Sigma-Aldrich) antibody at RT. Sections were then rinsed in PBS, incubated in biotinylated donkey anti-mouse IgG (1:500; Jackson ImmunoResearch), again rinsed in PBS, and incubated in Alexa Fluor 594 streptavidin conjugate (1:500; Invitrogen, Frederick, MD). Sections were then rinsed in PBS again and incubated in the rabbit anti-calretinin (1:1000; Neomarkers) antiserum. After rinsing in PBS, sections were incubated in Alexa Fluor 488 donkey anti-rabbit IgG (1:500; Invitrogen) for 2 h at RT. All sections were then mounted on gelatin-coated slides and coverslipped using Fluoromount mounting media (Electron Microscopy Sciences, Hatfield, PA).

Cell Counts

Brain sections were examined under a light microscope (Leica DM5000B, Deerfield, IL) equipped with a CCD video camera operating with a computer-based, anatomical-mapping and image-analysis system (Stereo Investigator; Microbrightfield Bioscience, Williston, VT). Cell profiles were counted by a single examiner blind to treatment conditions. Profile counts of both single-labeled nNOS and Fos⁺/nNOS double-labeled neurons in subcortical brain regions were performed initially by outlining them at low power magnification (5X objective) and then counting at high magnification (20X objective). Neurons were considered to be Fos⁺ if they contained black Ni⁺-DAB reaction product in the nucleus. Cell profile counts were conducted on two different sections from each animal in nine subcortical brain regions (anterior-posterior coordinates relative to bregma according to a mouse brain atlas (Paxinos and Franklin, 2001): horizontal diagonal band (HDB) from +0.62 to +0.02 mm; anterior cortical amygdaloid nucleus (ACo) from -0.22 to -1.70 mm; periventricular nucleus of the hypothalamus (PVH) from -0.58 to -1.22 mm; medial amygdala (MeA) from -1.06 to -2.06 mm; dorsolateral periaqueductal gray (DLPAG) and interpeduncular nucleus (IPN) from -3.40 to -4.04 mm; PPTg from -4.24 to -4.96 mm; LDTg from -4.84 to -5.20 mm; and LPB from -4.84 to -5.40 mm. The counts were averaged and used for further statistical analysis. For the cerebral cortex, cell counts were conducted in five cortical areas (cingulate, motor, somatosensory, insular and piriform cortices) and an unweighted average was calculated. This unweighted cortical average is presented in Fig. 2 and was used for statistical comparisons.

A similar procedure was adapted for counting of the single-labeled calbindin, calretinin and parvalbumin cells and the double-labeled Fos/calbindin, Fos/calretinin and Fos/parvalbumin

neurons in the cortex at +1.0 mm, -1.5 mm, and -3.0 mm from bregma. For the triple label studies, cell profile counts were performed using a combination of light and fluorescence microscopy at these same locations.

Statistical Evaluation

For each brain region, the number of single- and double-labelled cells were compared between conditions by the Mann-Whitney U test. Since the triple-labeling study produced data from related samples (i.e., the same neurons), the Wilcoxon Signed Ranks was used to analyze the results of this experiment. Differences were considered significant at $P < 0.05$.

RESULTS

Fos expression in subcortical nNOS neurons

Using morphological features and staining intensity as criteria, different types of nNOS neurons were found in subcortical regions of the mouse brain as described in previous studies (Gotti et al., 2005). Dense clusters of intensely-stained, medium-size (10-20 μm) nNOS neurons were found in HDB, ACo, MeA, IPN, DLPAG and PVH whereas larger, intensely-stained nNOS neurons (15-30 μm) were observed in the PPTg, LDTg, LPB and cortex (Fig. 1).

Table 1 presents the number of single- and double-labelled cells counted among the 10 regions studied. The number of nNOS cells did not significantly differ between the SD and RS groups in any brain region. The cerebral cortex was the only brain region to exhibit a significant difference in the number of Fos⁺/nNOS neurons between the SD and RS groups ($p=0.004$). The percentage of double-labeled cells in subcortical areas ranged from 4-11% in both the SD and RS groups (Fig. 2). In contrast, the percentage of double-labeled neurons in the cortical areas that contributed to the unweighted cortical average ranged from 46.7% in the piriform cortex to 68.5% in the cingulate cortex of the RS group, consistent with our previous observations (Gerashchenko et al., 2008). The elevated Fos expression in nNOS neurons evident in the LPB and IPN during SD in Fig. 2 did not reach statistical significance, although Mann-Whitney indicated a strong trend toward increased Fos expression in nNOS IPN cells ($p=0.054$).

Fos expression in calbindin, calretinin and parvalbumin cortical interneurons

Calcium-binding cortical interneurons differed in size and intensity of staining as described previously (Park et al., 2002, Lee and Jeon, 2005). Calbindin-labeled neurons were found to be darkly stained, 7-15 μm in size, oval or round in shape with a single axon (Fig. 3A) whereas calretinin interneurons were fusiform, 9-15 μm size, with medium staining intensity and had long-range projecting axons (Fig. 3B). In contrast, parvalbumin interneurons were intensely-stained and larger (15-20 μm) with a short axonal projection (Fig. 3C) and were thus distinct from the other two populations.

Whereas the percentage of Fos⁺/parvalbumin cortical neuronal populations did not differ between SD and RS groups, the number of Fos⁺/calbindin cortical neurons was significantly greater in the SD group than the RS group ($p=0.01$; Fig. 4). The percentage of Fos⁺/calretinin neurons exhibited a trend in the same direction that did not achieve statistical significance ($p=0.08$). The number of single-labeled calbindin, calretinin and parvalbumin cells did not differ between the experimental groups.

Colocalization studies of cortical nNOS and calretinin following RS

Although the experiment described above indicated that the proportion of calretinin cells that expressed Fos during SD vs. RS did not reach statistical significance, the calretinin

neuronal population in the mouse cortex is heterogeneous since some neurons express nNOS and others do not (Lee and Jeon, 2005). To determine whether the cortical interneuron population that expressed both calretinin and nNOS might be a better marker for sleep-active cortical neurons than nNOS-immunoreactivity alone, we undertook triple-labeling using antisera to Fos, nNOS, and calretinin on sections from the RS group (Fig. 5). Fig. 6A presents the average number of single-, double-, and triple-labelled cells counted in the mouse cortex at +1.0 mm, -1.5 mm, and -3.0 mm from bregma. Although there were 5-6x as many calretinin-IR neurons as nNOS-IR neurons in this sample of the mouse cortex, the number of Fos⁺/calretinin and Fos⁺/nNOS cells were comparable, indicating that nNOS was a better marker than calretinin for the Fos⁺ cortical population during RS (Fig. 6A). Indeed, as illustrated in Fig. 6B, the Wilcoxon Signed Ranks test indicated that the percentage of both Fos⁺/nNOS and Fos⁺/nNOS/calretinin⁺ cells was much greater than the percentage of Fos⁺/calretinin cells in RS mice ($p < 0.05$). Although the proportion of Fos⁺/nNOS/calretinin⁺ cells exceeded that of Fos⁺/nNOS/calretinin⁻ cells (Fig. 6B), this trend ($p=0.08$) did not reach statistical significance.

DISCUSSION

The present study was undertaken with two aims: to determine (1) whether nNOS neuronal populations in subcortical brain regions are sleep-active as they are in the cerebral cortex (Gerashchenko et al., 2008), and (2) whether calcium-binding proteins might provide another means to identify sleep-active cortical neurons in addition to nNOS immunohistochemistry. Our comparison of Fos⁺/nNOS-immunoreactive cells between SD and RS failed to identify any sleep-active subcortical region in the mouse brain, despite the fact that some of the brain regions examined (e.g., the LPB, the PPTg and the LDTg) have previously been implicated in sleep/wake control. Furthermore, none of the three calcium binding proteins examined – neither parvalbumin, calbindin nor calretinin – were useful to identify the sleep-active cortical neurons alone. Lastly, calretinin-immunostaining, when used in conjunction with nNOS-immunostaining, was not statistically superior to nNOS alone to identify the “sleep-active” cortical neurons, although there was a trend in that direction. Collectively, these results underscore the unique nature of the sleep-active nNOS population in the cerebral cortex.

Fos expression in nNOS neurons in subcortical brain regions

The nine subcortical brain regions examined were selected based on a medium to high density of nNOS cells in a previous study of the mouse brain (Gotti et al., 2005). The percentage of Fos⁺/nNOS cells did not differ between the SD and RS groups in any subcortical brain region, although Mann-Whitney indicated a strong trend toward increased Fos expression in nNOS IPN cells ($p=0.054$) during SD (Fig. 2). These results are particularly surprising given that nNOS knockout mice have less REM sleep and greater slow wave activity during NREM sleep than controls (Chen et al., 2003) and suggests a role for cortical nNOS neurons in the normal expression of these states.

Calcium binding proteins and nNOS neurons

Two methods have typically been used to detect NOS neurons in the cerebral cortex: immunohistochemistry for nNOS and NADPH diaphorase (NADPH-d) histochemistry. NADPH-d-positive neurons in the mouse cortex have been classified as Type I or Type II. Type I neurons have a large soma, intense NADPH-d activity as well as intense nNOS immunoreactivity, and are located in the deeper layers of the cortex and even in the white matter. In contrast, Type II neurons have a small soma, weak NADPH-d activity and nNOS immunoreactivity. The distribution of intensely-stained nNOS-immunoreactive neurons and NADPH-d-positive neurons in the cortex typically overlaps (Oermann et al., 1999), although

minor differences have been found in some studies, which have been attributed to different antibody specificities, the presence of oxidoreductase-associated mitochondrial staining in otherwise nNOS negative cells, or the presence of different splice variants of isoforms of nNOS (Wiencken and Casagrande, 2000).

Since cortical Type I nNOS neurons are so rare, few studies have reported on the electrophysiological properties of these cells. In a combined patch clamp, morphological and molecular study, Cauli et al. recorded from cortical GABAergic interneurons and then determined the expression of specific phenotypic markers in these neurons by single-cell PCR (Cauli et al., 2004). In response to current injection, four of the 5 neurons that expressed nNOS were described as “regular spiking nonpyramidal type” whereas the remaining cell that coexpressed Neuropeptide Y (NPY) was a “late spiking neuron”. The interneurons that expressed nNOS were multipolar or bitufted, and one NPY/NOS cell without somatostatin (SST) had a neurogliaform morphology. These cells were proposed to be involved in neurovascular coupling (Cauli et al., 2004). A subsequent study (Karagiannis et al., 2009) used similar techniques to characterize cortical interneurons from NPY-EGFP mice. Of the 15 nNOS/NPY neurons encountered in this study, 80% adapted to current injection and had a morphology similar to neurogliaform or of nNOS-containing “ivy” cells described in the hippocampus (Fuentelba et al., 2008). In contrast, cells expressing nNOS, NPY and SST were rare and had larger somata suggestive of Type I neurons.

In the present study, we only examined the anatomical properties of the intensely-stained Type I nNOS neurons in the mouse cortex. These neurons were round or oval, bipolar or multipolar, medium-sized, with many processes of various lengths. Similar morphological properties of Type I nNOS neurons have been reported in other species (Gonchar and Burkhalter, 1997, Lee et al., 2004, Lee and Jeon, 2005). Such morphological similarities would suggest that nNOS neurons in the cerebral cortex represent a homogeneous group of neurons. However, co-localization studies of nNOS with different neurotransmitters and calcium binding proteins indicate a diversity of these neurons among species. For example, in the mouse visual cortex, 16.7% of the NOS-immunoreactive cells were double-labeled with calbindin, more than half of the NOS-immunoreactive cells colocalize with calretinin, and a quarter of the NOS-immunoreactive cells were also labeled with parvalbumin (Lee and Jeon, 2005). Although these percentages differ in the mouse cortex in the present study, our results are consistent with previous studies of the mouse cortex in that the proportion of nNOS/calretinin double-labeled cells is greater than the proportion of nNOS/calbindin or nNOS/parvalbumin double-labeled cells.

There is a surprising degree of intraspecies variation in the extent of nNOS colocalization with calcium binding proteins. Differences have been reported depending on the strain or cortical area within an animal. Whereas 50% of nNOS-immunoreactive cortical interneurons contain calbindin in Sprague-Dawley rats, there is only 10% colocalization of these markers in Wistar rats (Bertini et al., 1996) and, in the visual cortex of the Long-Evans rat, only 1% of the nNOS neurons express calbindin and none of the nNOS neurons co-express calretinin or parvalbumin (Gonchar and Burkhalter, 1997). Furthermore, there appears to be an anterior-posterior gradient of nNOS/calbindin co-localization within the cortex of the Sprague-Dawley rat: whereas >50% of nNOS-immunoreactive neurons are also calbindin-immunoreactive at anterior levels (medial prefrontal, frontal and cingulate areas), <20% of nNOS-immunoreactive neurons are calbindin-immunoreactive in the occipital cortex and posterior portion of the temporal cortex (Bertini et al., 1996). Based on the biochemical heterogeneity of nNOS cortical interneurons, it has been suggested that NADPH-d/nNOS neurons in the neocortex do not represent a single morphological class, but rather comprise a small population of several types of interneurons (Estrada and DeFelipe, 1998). Indeed, some of the variation described above may result from the failure to distinguish between

Type I and Type II nNOS neurons. Our results indicate that only cortical and not subcortical nNOS neurons are activated during sleep and that, within the cortex, only Type I nNOS neurons are activated. These observations add functional support to the concept of a diversity of nNOS neurons.

Fos expression in cortical interneurons during sleep

In the present and a previously published study (Gerashchenko et al., 2008), we observed that the majority of nNOS neurons in all cortical regions expressed Fos during RS after SD. In rats, mice or hamsters, about 60-80% of nNOS neurons expressed Fos during RS, indicating a physiologically similar response of the majority of nNOS neurons in the cortex. However, we also observed that about 20 to 40% of Type I nNOS neurons did not express Fos during RS. This result suggests the possibility that there is a group of Type I cortical nNOS neurons that are not sleep active. One way to test this possibility is to subdivide nNOS neurons based on the chemical markers that they express and to then assess Fos expression in the resultant subgroups of nNOS neurons. We chose calretinin as such a marker because this protein has been reported to be expressed in more than half of nNOS neurons in the mouse cortex (Lee and Jeon, 2005). Calcium-binding proteins are thought to play important roles in controlling intracellular calcium levels, thereby affecting physiological properties of neurons (Baimbridge et al., 1992, Heizmann et al., 2002, Grateron et al., 2003). Therefore, we hypothesized that the neurons containing both calretinin and nNOS may respond differently than neurons containing only nNOS by differentially expressing Fos during RS. We observed that the presence of calretinin in nNOS neurons did not have a statistically significant effect on Fos expression during RS, although there was a strong trend in that direction (Fig. 6B). Thus, despite the fact that Type I nNOS neurons in the cerebral cortex are biochemically heterogeneous, they seem to have a common pattern of activity during sleep and wakefulness. This similarity in the activity of nNOS neurons may be due to a common receptor expression pattern on these cells and/or to a common afferent input which would ensure activation of nNOS neurons during sleep and inhibition of nNOS neurons during wakefulness. However, given the variation in cortical neurons expressing calcium binding proteins among mammals, it is conceivable that we would have formed different conclusions had we conducted these experiments in a different species.

Fos expression in cortical neurons during wakefulness

Several studies have previously assessed Fos expression in interneurons in the cerebral cortex. Fos-expressing cell types were determined in the rat barrel cortex after exploration of an enriched environment (Staiger et al., 2002). Inhibitory interneurons, identified by the glutamic acid decarboxylase, parvalbumin, calbindin and vasoactive intestinal polypeptide immunostaining, were found to contain Fos-IR nuclei. The absence of double staining of Fos and glial fibrillary acidic protein excluded astrocytes as a possible cell type activated under these conditions (Staiger et al., 2002). Other studies demonstrated that stimulation by novel environmental cues induced Fos expression in the calbindin and parvalbumin cell populations in the rat cingulate cortex (Bertini et al., 1996). However, the vast majority of Fos-positive cells in the Bertini et al. study did not express either parvalbumin or calbindin. Since cells that express parvalbumin or calbindin represent more than 90% of cortical GABAergic interneurons (DeFelipe, 1993), these results suggest that the majority of cells expressing Fos in the cortex during wakefulness are excitatory neurons. This conclusion is consistent with the results of the present study in which we determined the proportions of Fos⁺ parvalbumin, calbindin and calretinin cells in the cortex of mice subjected to 6 h of SD. Overall, we observed a low number of Fos-positive parvalbumin, calbindin and calretinin cells in the cortex compared to the total number of Fos-positive cells. The proportion of Fos-positive calbindin cells was significantly higher in the SD group than in the RS group of

mice, whereas there were no significant differences in the percentage of Fos-positive parvalbumin and calretinin cells between these groups (Fig. 4).

Conclusions

Our comparison of the percentage of Fos⁺/nNOS-immunoreactive cells between SD and RS failed to identify any sleep-active subcortical region in the mouse brain. Furthermore, none of the three calcium binding proteins examined – neither parvalbumin, calbindin nor calretinin – were useful to identify the sleep-active cortical neurons alone. Finally, although calretinin-immunostaining when used in conjunction with nNOS-immunostaining was not statistically superior to nNOS alone to identify the “sleep-active” cortical neurons, there was a trend in that direction. These studies underscore that activation during sleep is a unique property of cortical interneurons that are collectively defined by expression of nNOS and nNOS in conjunction with calretinin.

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Abbreviations

ABC	avidin-biotin complex
ACo	anterior cortical amygdaloid nucleus
DAB	diaminobenzidine tetrahydrochloride
DLPAG	dorso-lateral periaqueductal gray
eNOS	endothelial nitric oxide synthase
HDB	horizontal diagonal band
ICV	intracerebroventricular
iNOS	inducible nitric oxide synthase
IPN	interpeduncular nucleus
IR	immunoreactive
LDTg	laterodorsal tegmental nucleus
LPB	lateral parabrachial nucleus
MeA	medial amygdala
NO	nitric oxide
NOS	nitric oxide synthase
nNOS	neuronal nitric oxide synthase
NREM	non-rapid eye movement (sleep)
PBS	phosphate buffered saline
PPTg	pedunculopontine tegmental nucleus
PVH	periventricular nucleus of hypothalamus
RS	recovery sleep
RT	room temperature

SD	sleep deprivation
SNAP	S-nitroso-N-acetyl-DL-penicillamine
SS	spontaneous sleep

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Fig. 1.

Representative sections of mouse brain illustrating subcortical regions immunostained for Fos and nNOS and that were used for cell counting in the SD and RS groups. **A.** Double-labeled immunohistochemistry of Fos and nNOS. Single-labeled Fos black nucleus (thin arrow), nNOS brown-labeled neuron (thick arrow) and double-labeled Fos/nNOS neuron (arrowhead). **B-H.** Subcortical brain regions counted across SD and RS groups. Abbreviations: ACo, anterior cortical amygdaloid nucleus; Aq, Aqueduct of Sylvius; CxA, cortex-amygdala transition zone; DLPAG, dorso-lateral periaqueductal gray; HDB, horizontal diagonal band; IPN, interpeduncular nucleus; LDTg, laterodorsal tegmental nucleus; LPB, lateral parabrachial nucleus; MeA, medial amygdala; MnPo, median preoptic nucleus; MPA, medial preoptic area; PPTg, pedunculopontine tegmental nucleus; PVH, periventricular nucleus of the hypothalamus; scp, superior cerebellar peduncle.

Fig. 2.

The proportion of Fos⁺/nNOS neurons in mouse subcortical brain regions of SD and RS groups. The percentage of Fos⁺/nNOS neurons did not differ between groups in any of the subcortical regions. Data are mean $\pm$ SEM. CX-cortex; other abbreviations – refer to Fig. 1 legend. * $p = 0.004$ when compared to SD by Mann-Whitney U test.

Fig. 3. Immunohistochemical labeling of calcium-binding proteins in cortical interneurons. Representative sections of cortical interneurons labeled by antisera to (A) calbindin, (B) calretinin and (C) parvalbumin at low power magnification (10x). (A'-C') Higher power magnification of the same sections illustrating cortical interneurons labelled for calbindin, calretinin, or parvalbumin (thick arrow), Fos-stained nuclei (thin arrow), nNOS (broken arrow) and double-labeled Fos⁺/calbindin cell (arrow head in A').

Fig. 4. Percentage of Fos⁺/calcium binding cortical neurons expressed in mouse cortex. The percentage of Fos⁺/calbindin cortical neurons was significantly greater in the SD group than in the RS group. The percentage of Fos⁺/calretinin cortical neurons exhibited a trend in the same direction that was not statistically significant. * $p < 0.05$ when compared to SD by Mann-Whitney U test.

Fig. 5.

Representative section from the cortex of a mouse from the RS group triple-stained for Fos (A), nNOS (B) and calretinin (C). “Sleep-active” neurons immunopositive for Fos and nNOS are indicated by the thick arrows in A and B; triple-labeled neurons, immunopositive for Fos, nNOS and calretinin, are indicated by arrowheads in all three sections.

Fig. 6.

Summary of cell counts obtained from triple label studies undertaken to determine the extent of colocalization between cortical nNOS and calretinin interneurons following RS. (A) Average number of different cell types expressed per square millimeter of mouse cortex. (B) Although the proportion of Fos⁺/nNOS neurons is significantly greater than the proportion of Fos⁺/calretinin neurons, the proportions of Fos⁺/nNOS⁺/calretinin⁺ and Fos⁺/nNOS⁺/calretinin⁻ cells were not significantly different from each other. [#]=*p* < 0.05 when compared to Fos⁺/calretinin cells by Wilcoxon Signed Ranks test.

Table 1Number of nNOS neurons and double-labelled Fos⁺ nNOS neurons in the ten brain regions examined

	SD (n=6)	RS (n=6)	Mann-Whitney p value
<i>Cerebral cortex</i>			
nNOS-IR neurons	209.5 ± 21.9	211 ± 19.8	NS
nNOS/Fos-IR neurons	25.5 ± 8.8	112.0 ± 12.50	0.004
<i>HDB</i>			
nNOS-IR neurons	136.3 ± 18.7	141.7 ± 11.3	NS
nNOS/Fos-IR neurons	14.3 ± 2.1	16.8 ± 2.4	NS
<i>Aco</i>			
nNOS-IR neurons	227.8 ± 37.7	156.2 ± 27.2	NS
nNOS/Fos-IR neurons	20.0 ± 5.6	10.8 ± 1.9	NS
<i>PVH</i>			
nNOS-IR neurons	143.0 ± 15.5	161.8 ± 19.2	NS
nNOS/Fos-IR neurons	8.7 ± 1.4	7.0 ± 0.9	NS
<i>MeA</i>			
nNOS-IR neurons	225.4 ± 41.0	171.5 ± 34.5	NS
nNOS/Fos-IR neurons	18.8 ± 3.9	10.2 ± 2.8	NS
<i>DLPAG</i>			
nNOS-IR neurons	258.7 ± 45.1	322.8 ± 37.5	NS
nNOS/Fos-IR neurons	19.3 ± 3.1	23.0 ± 3.9	NS
<i>IPN</i>			
nNOS-IR neurons	423.5 ± 31.3	314.8 ± 62.0	NS
nNOS/Fos-IR neurons	22.8 ± 2.1	13.0 ± 3.5	NS
<i>PPT_g</i>			
nNOS-IR neurons	217.7 ± 25.9	206.7 ± 14.1	NS
nNOS/Fos-IR neurons	8.7 ± 1.3	11.0 ± 1.3	NS
<i>LDT_g</i>			
nNOS-IR neurons	347.7 ± 40.2	408.7 ± 35.9	NS
nNOS/Fos-IR neurons	17.7 ± 4.0	18.7 ± 4.3	NS
<i>LPB</i>			
nNOS-IR neurons	142.0 ± 23.7	93.0 ± 12.0	NS
nNOS/Fos-IR neurons	16.6 ± 5.6	5.8 ± 1.9	NS