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Sleep active cortical neurons expressing neuronal nitric oxide synthase are active after both acute sleep deprivation and chronic sleep restriction

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Abstract

Non-rapid eye movement (NREM) sleep electroencephalographic (EEG) delta power (~0.5 to 4 Hz), also known as slow wave activity (SWA), is typically enhanced after acute sleep deprivation (SD) but not after chronic sleep restriction (CSR). Recently, sleep-active cortical neurons expressing neuronal nitric oxide synthase (nNOS) were identified and associated with enhanced SWA after short acute bouts of SD (i.e., 6 h). However, the relationship between cortical nNOS neuronal activity and SWA during CSR is unknown. We compared the activity of cortical neurons expressing nNOS (via c-Fos and nNOS immunoreactivity, respectively) and sleep in rats in 3 conditions: 1) after 18 h of acute SD; 2) after 5 consecutive days of sleep restriction (SR) (18 h SD per day with 6 h *ad libitum* sleep opportunity per day); 3) and time-of-day matched *ad libitum* sleep controls. Cortical nNOS neuronal activity was enhanced during sleep after both 18 h SD and 5 days of SR treatments compared to control treatments. SWA and NREM sleep delta energy (the product of NREM sleep duration and SWA) were positively correlated with enhanced cortical nNOS neuronal activity after 18 h SD but not 5 days of SR. That neurons expressing nNOS were active after longer amounts acute SD (18 h vs. 6 h reported in the literature) and were correlated with SWA further suggests that these cells might regulate SWA. However, since these neurons were active after CSR when SWA was not enhanced, these findings suggest that mechanisms downstream of their activation are altered during CSR.

Keywords

nNOS; slow wave activity; EEG; sleep restriction; sleep deprivation; c-Fos

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1. Introduction

Chronic sleep restriction (CSR) can have negative effects on human health leading to significant impairment in cardiovascular, immune, endocrine, and cognitive functions (Faraut et al., 2012). In animals models, CSR also produces changes in a variety of neurobiological systems [e.g., serotonergic signaling (Roman et al., 2005; Roman et al., 2006), neuroendocrine regulation (Meerlo et al., 2002; Novati et al., 2008), metabolic processes (Barf et al., 2010; Barf et al., 2012), and spatial memory (McCoy et al., 2013)]. Understanding changes that occur in the brain during CSR could lead to ways to avoid sleep loss-induced negative health and cognitive consequences.

Generally, two types of physiological/behavioral responses occur from CSR— homeostatic and allostatic. Whereas homeostatic responses maintain equilibrium within an organism by compensating for disrupting changes, allostatic responses maintain stability within the organism through change, a process that allows for the integration of physiology and behavior in response to a changing environment (McEwen and Wingfield, 2003). More specifically, when Sprague-Dawley rats are deprived of sleep for 18 h and then allowed to sleep *ad libitum* for 6 h, they have compensatory homeostatic responses of enhanced sleepiness, sleep duration, and non-rapid eye movement (NREM) sleep electroencephalogram (EEG) delta power [(~ 0.5-4 Hz range; also known as slow wave activity (SWA)] (Kim et al., 2012). When these rats are sleep deprived (SD) for 18 h per day for 5 consecutive days, sleepiness continues to increase. Using this CSR experimental design, we observed that the time pattern change in basal forebrain adenosine A1 receptor and frontal cortex adenosine A2a receptor mRNA levels resembled that of sleepiness. However, these rats do not exhibit enhanced sleep duration or SWA responses during the available sleep opportunities from the second sleep restriction (SR) day indicating an adaptation occurs (i.e., allostatic response) (Kim et al., 2012).

In the present study, we used the same experimental design to study responses of cortical gamma-aminobutyric acid-ergic neurons expressing neuronal nitric oxide synthase (nNOS) to CSR. Neuronal expression of c-Fos occurs as early as 20 minutes to several hours after activation of the cells and thus is often used to assess the activity of neurons after behavioral treatments including sleep deprivation (Cirelli and Tonnoni, 2000). The activity of neurons expressing nNOS, as assessed by the presence of both c-Fos and nNOS immuno-reactivity, is enhanced during sleep after acute SD and is positively correlated with NREM sleep delta energy (a measure of sleep intensity that is the product of NREM sleep duration and SWA) (Gerashchenko et al., 2008). Whether the activity of cortical nNOS neurons shows homeostatic or allostatic responses to CSR is unknown. Therefore, the present study was designed to determine the relationship between the activity of cortical nNOS neurons and SWA during CSR. Herein, we found that nNOS neurons were activated during sleep after an 18 h acute bout of SD (18 h SD) and that these cells had similar enhanced activity during sleep after CSR. We also report that the activity of cells expressing nNOS in the cerebral cortex positively correlated with SWA after 18 h SD, although there was a lack of correlation between SWA and the activity of these cells after CSR.

2. Experimental Procedures

2.1. Animals

Twenty-four 3-month-old male Sprague-Dawley rats (Jackson Laboratories, Bar Harbor, ME, USA) were used for these experiments. Rats were housed individually and provided food and water *ad libitum*. Rats were maintained on a 12:12 h light dark cycle (light onset = 10 am (zeitgeber time (ZT) = 0) at 22 ± 3 °C. All experimental protocols were approved by

Harvard University and Veteran Affairs Boston Healthcare system Animal Care and Use Committee and were in compliance with the National Institutes of Health guidelines.

2.2. Experimental treatment groups and sleep deprivation protocols

Rats ($N = 8$ per treatment group) were randomly selected into three experimental treatment groups: 1) 18 h SD; 2) CSR (5 consecutive days of 18 h SD followed by a 6 h *ad libitum* sleep opportunity per day); 3) baseline (time-of-day matched *ad libitum* sleep controls). For SD, rats were placed in a periodically rotating wheel (35.5 cm in diameter $\times$ 11 cm in width) that continuously rotated for 3 m/min for 4 seconds followed by 12 seconds of cessation for 18 h that deprived them of sleep (Lafayette Instrument Company, Lafayette, IN, USA) as described previously (Kim et al., 2012). Using the same paradigm, Kim et al. (2012) reported that rats had 93% wakefulness occurring within the 18 h SD time period during the CSR protocol, and rats had 94% wakefulness during the SD time period after 18 h of acute SD. Rats had *ad libitum* access to food and water at all times and were allowed daily *ad libitum* sleep in their home cages for 6 h from the beginning of the light period.

2.3. Polysomnography surgeries

Rats were anesthetized with isoflurane for surgery for polysomnography analysis 7 days prior to experimental treatments. Briefly, rats were implanted with a stainless steel EEG electrode above the frontal cortex (2 mm anterior to bregma and 2 mm lateral to the central suture) and a reference electrode over the cerebellum (2 mm posterior to lambda) as previously described (Kim et al., 2012). Further, rats were implanted with two electromyogram (EMG) stainless steel wires in the nuchal muscles to assess muscle activity. The EEG and EMG electrodes were secured to a pedestal on the rat skull with dental cement.

2.4. Polysomnography data analysis

Rats were tethered to a commutator that was connected to an amplifier (Grass15A94 Quad Neuroamplifiers, Astro-Med Inc., West Warwick, RI, USA). EEG and EMG signals were filtered with high and low pass filters (EEG: 0.3 and 100 Hz; EMG: 30 and 300 Hz, respectively) and recorded with VitalRecorder data acquisition system (Kissei Comtec Co., Japan). All rats were connected with the tether, habituated within the recording cage, and provided *ad libitum* sleep at least 1 week before the experiment began. NREM sleep, rapid eye movement (REM) sleep, and waking vigilance states were determined in 10 second epochs off-line using SleepSign (Kissei Comtec Co., Japan.) as described previously (Kim et al., 2012; Zielinski et al., 2012). Briefly, NREM sleep was determined by high-amplitude EEG and low amplitude EMG, REM sleep was identified by low amplitude regular EEG and little EMG activity, and waking was recognized by low amplitude EEG and robust EMG activity. Vigilance states were determined and NREM sleep and REM sleep episode durations and frequencies were calculated during a 12 h dark period of spontaneous sleep prior to experimental treatments and during the first 3 h of available sleep opportunity immediately before the animal sacrifice after 18 h SD, 5 days of SR, or baseline sleep. NREM sleep EEG power (0-30 Hz) was also analyzed during this time and normalized as a percentage of power spectra occurring during the 12 h dark period of spontaneous sleep for each individual animal. Further, NREM sleep EEG delta power (0.5-4 Hz) (i.e., SWA) during these sleep opportunity times was normalized as a percentage of the 12 h dark period of spontaneous sleep for each individual animal. NREM sleep delta energy was also determined and calculated as the product of NREM sleep duration and SWA normalized to spontaneous sleep during the dark period.

2.5. Tissue collection and immunohistochemical analysis

Immunohistochemical analysis of c-Fos and nNOS positive cortical cells was performed in rats that were killed at ZT 3 h (i.e., 3 h after the beginning of sleep opportunities after 18 h SD, 5 days of SR, or time-of-day matched baseline sleep controls). As previously described (Gerashchenko et al., 2008), rats were anesthetized with an intraperitoneal injection of sodium pentobarbital and perfused with 10% formalin in phosphate buffered saline (PBS). Rat brains were placed in 10% formalin overnight and then placed in a 30% sucrose solution at 4°C. Coronal sections (40 µm) (between 0 mm and -5 mm caudal from bregma) were made on a freezing microtome. The coronal sections were washed in PBS, treated with 1% hydrogen peroxide in PBS, washed in PBS, and blocked in 5 % donkey serum and 1% triton X-100 in PBS. The brain sections were incubated overnight in mouse anti-nNOS antibodies (Sigma Aldrich, St. Louis, MO, USA) in PBS (1:2000) and rabbit anti-c-Fos antibodies (Calbiochem, San Diego, CA, USA) in PBS (1:5000) at room temperature. Thereafter, sections were washed in PBS and incubated in biotinylated donkey anti-rabbit IgG (Jackson Immuno Research, Westgrove, PA, USA) in PBS (1:500) for 2 h. Then, sections were washed in PBS, and incubated in ABC (1:200) (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA) for 2 h. Sections were then washed in PBS and placed in a diaminobenzidine tetrahydrochloride and nickel chloride solution. The sections were then washed in PBS to stop the reaction. Next, the sections were incubated in biotinylated donkey anti-mouse IgG (Jackson Immuno Research, Westgrove, PA, USA) in PBS (1:500) for 2 h. The sections were washed in PBS and incubated in ABC-AP (1:200) for 2 h (Vectastain ABC-AP kit; Vector Laboratories, Burlingame, CA, USA). The samples were washed in PBS and then tris-hydrogen chloride (0.1 M, pH 8.0) and placed in Vector red substrate (Vector Red AP Substrate Kit I; Vector Laboratories, Burlingame, CA, USA). The reaction was stopped by placing the sections in PBS. The tissue sections were mounted onto gelatin-coated slides and coverslips were applied using Vector Mount (Vector Laboratories, Burlingame, CA, USA).

2.6. Cell counts

Brain sections were examined under a light microscope (Olympus EX51, Olympus, Tokyo, Japan) using Stereo Investigator 7 software (MicroBrightField, Inc., Williston, VT, USA). Cell counts were performed by an investigator that was double blinded to the procedures and treatment groups. Entire cortices of both hemispheres (0 and -5 mm caudal from bregma) were outlined at a low power magnification (5 ×). Thereafter the outlined region was analyzed for quantifying the numbers of single and double positive immuno-reactive cells against c-Fos and nNOS antibodies at a higher magnification (20 ×) as described previously (Gerashchenko et al., 2008). Cortical sections were selected after adjacent sections were aligned by reference to anatomical landmarks. Cells were only counted in cortical sections that were completely intact. The percentage of c-Fos and nNOS double positive cells to total nNOS positive cells was determined in each section ($N = 3-6$ per animal) and averaged for statistical analysis.

2.7. Statistical analysis

Three- and two-way ANOVAs were used to analyze sleep data. One-way ANOVA analysis was used to determine significant differences in the percentage of nNOS activated cells between treatment groups. Independent *t*-tests were used for post-hoc analysis when appropriate. Pearson's correlations were calculated between sleep data and the immunohistochemistry data and their significances were determined. Data are presented as means ± SEM. Significance differences were set at $p < 0.05$.

3. Results

3.1. Cortical neurons expressing nNOS

A main effect was found for sleep loss (i.e., 18 h SD and 5 days of SR) enhancing the percentage of double positive cells stained with anti-c-Fos and anti-nNOS antibodies to total positive anti-nNOS labeled cells during the available sleep opportunity after sleep loss (i.e., sleep-active cortical neurons expressing nNOS) [$F_{(2, 22)} = 4.556, p = 0.023$] (Fig. 1 and 2). Compared to baseline sleep control rats, rats that underwent h SD had increased percentages of sleep-active cortical neurons expressing nNOS [$t_{(1, 14)} = 2.347, p = 0.034$]. In addition, the percentage of sleep-active cortical neurons expressing nNOS was also greater in rats that underwent 5 days of SR compared baseline sleep controls [$t_{(1, 13)} = 2.176, p = 0.049$]. No significant difference in the percentage of activated cortical nNOS neurons was found between 18 h SD and 5 days of SR treatment groups.

3.2. Sleep responses

Rats that underwent baseline control sleep, 18 h SD, or 5 days of SR exhibited similar amounts of NREM sleep duration ($186.3 \pm 7.7, 211.2 \pm 17.1, 185.0 \pm 9.2$ min, respectively) and REM sleep duration ($42.1 \pm 5.8, 49.1 \pm 10.9, 39.8 \pm 4.2$ min, respectively) during the baseline dark period that occurred prior to experimental treatments. The durations of NREM sleep and REM sleep obtained during the baseline dark period were similar to those previously reported in the literature (Roky et al., 1999; Baracchi et al., 2011). NREM sleep episode durations ($1.10 \pm 0.07; 1.22 \pm 0.14; 1.11 \pm 0.08$ min, respectively) and episode frequencies ($170.3 \pm 11.7; 178.3 \pm 15.8; 167.1 \pm 4.4$, respectively) did not significantly differ between treatment groups during spontaneous sleep during the baseline dark period. REM sleep episode durations ($1.01 \pm 0.13; 1.09 \pm 0.10; 1.03 \pm 0.08$ min, respectively) and episode frequencies ($41.3 \pm 0.5; 46.0 \pm 10.2; 38.3 \pm 3.8$, respectively) were also similar between treatment groups at this time. SWA and NREM sleep EEG power (0.5-30 Hz range) did not significantly differ between treatment groups during spontaneous sleep during the baseline dark period (data not shown).

NREM sleep, REM sleep, or total sleep (i.e., NREM sleep + REM sleep) durations immediately after experimental treatments during the first 3 h (ZT 0-3) sleep opportunity prior to tissue collection were not significantly different between treatment groups (Fig. 3A, 3B and 3C, respectively). Further, sleep occurred during the first hour of sleep opportunity after all experimental treatments, although no significant differences in NREM sleep (baseline: 30.7 ± 7.1 min; 18 h SD: 28.9 ± 3.5 min; 5 days of SR: 22.8 ± 3.1 min) or REM sleep (baseline: 2.2 ± 1.6 min; 18 h SD: 2.6 ± 0.9 min; 5 days of SR: 4.6 ± 1.0 min) duration within the first hour (ZT 0-1) after experimental treatments were found between treatment groups. There were also no significant differences in NREM sleep or REM sleep episode frequencies and episode durations between treatment groups during this time period (data not shown).

SWA was enhanced during the first 3 h (ZT 0-3) sleep opportunity after 18 h SD compared to control values [$t_{(1, 8)} = 2.866, p = 0.021$] (Fig. 4A), although this enhancement in SWA occurred largely within the first hour (ZT 0-1) of the sleep opportunity [$t_{(1, 8)} = 5.471, p = 0.001$] (Fig. 4B). SWA values during the sleep opportunity after 5 days of SR were also similar to control values, although reduced compared to those after 18 h SD [ZT 0-3: $t_{(1, 11)} = 3.752, p = 0.003$; ZT 0-1: $t_{(1, 11)} = 10.331, p = 0.001$]. There were no significant differences in SWA during the first hour (ZT 0-1) between 5 days of SR and control groups. No significant differences in REM sleep or waking EEG delta power were observed between experimental treatment groups during this time (data not shown). Similar to the SWA findings during this 3 h sleep opportunity time period, NREM sleep delta energy was

enhanced after 18 h SD compared to control values [$t_{(1, 8)} = 3.177, p = 0.013$] (Fig 4C). SWA values after 5 days of SR were reduced compared to those after 18 h SD, and were similar to control values. Rats that experienced CSR had attenuated NREM sleep delta energy during this time period compared to those that only underwent 18 h SD [$t_{(1, 11)} = 4.549, p = 0.001$]. Additionally, both SWA and NREM sleep delta energy during the first 3 h of sleep opportunity were not significantly correlated with cortical nNOS cell activity when including rats that experienced 5 days of SR, although significant positive correlations were found for these variables only when rats that underwent 18 h SD were included ($r = 0.636, p = 0.048$ and $r = 0.703, p = 0.023$, respectively).

A main effect was found for sleep loss enhancing NREM sleep EEG power (0.5-30 Hz range) during the first 3 h (ZT 0-3) sleep opportunity after experimental treatments [$F_{(1, 779)} = 270.276, p < 0.001$] (Fig 5). However, this effect differed depending upon sleep loss treatment and within frequency bands [treatment group: $F_{(2, 779)} = 18.204, p < 0.001$; treatment group $\times$ frequency: $F_{(118, 779)} = 3.417, p < 0.001$, respectively]. Rats that experienced 18 h SD had enhanced NREM sleep EEG power in the 2.0-8.0, 9.0, and 17.0-23.0 Hz frequency ranges compared to baseline sleep controls. Further, rats that underwent 5 days of SR had similar NREM sleep EEG power over the entire 30 Hz frequency range compared to the baseline group but attenuated NREM sleep EEG power in the 2.5-11.0 and 19.0-24.5 Hz frequency ranges compared to rats that experienced 18 h SD.

4. Discussion

We report that cortical neurons expressing nNOS were activated (i.e., enhanced presence of c-Fos immune-reactivity) during sleep within the first 3 h of available sleep opportunity after 18 h SD and had similar activation levels during the available sleep opportunity after CSR. As previously reported by our group and others (Kim et al., 2007; 2012; Deurveilher et al., 2012), we found that acute SD enhances SWA, although this enhancement was reduced after CSR. Further, we found that NREM sleep delta energy was correlated with the activity of cortical nNOS neurons only after acute SD but not CSR. Overall, our findings are consistent with the hypothesis that sleep-active cortical neurons expressing nNOS are involved in SWA regulation after acute SD. Our findings indicate that certain neurophysiologic mechanisms or measures, such as SWA, change during CSR, while other mechanisms or measures, including sleep-active cortical nNOS neuronal activity, purine type 1 receptor expression (Kim et al., 2012), and spatial memory (McCoy et al., 2013), do not.

Our present findings showing enhanced SWA after 18 h SD are consistent with the large literature indicating that cortical SWA is considered a marker of sleep intensity after acute SD (Brown et al., 2012). Much evidence indicates that waking activity and local use of neurons and glia in the cortex induce sleep and SWA (Zielinski and Krueger, 2011). Sleep regulatory substances are produced by these cells during wakefulness and accumulate to induce sleep and enhance sleep duration SWA. SWA is a known marker of sleep-wake history, which is high after prolonged wakefulness and low after consolidated sleep. SWA has thus been used to build mathematical models that may explain empirical data on sleep propensity under a variety of experimental conditions, such as acute SD (McCauley et al., 2009; Achermann and Borbély, 2003; Dijk and Kronauer, 1999).

There are, however, multiple instances when SWA does not reflect sleep history or sleep propensity (Davis et al., 2011). For example, mice injected systemically with inflammatory stimuli have enhanced sleep duration but attenuated SWA, pro-inflammatory cytokines applied to one hemisphere enhance SWA in that particular hemisphere but not sleep, and benzodiazepines can enhance sleep but attenuate SWA. In relation to the present study, CSR

induces high sleep pressure but it is not associated with increased SWA (Kim et al., 2007; Deurveilher et al., 2012). Recently, Kim and colleagues demonstrated that CSR induced an allostatic response in sleep duration and intensity (Kim et al., 2007; Kim et al., 2012), which was evidenced in the present study by the absence of increased SWA and NREM sleep duration after CSR. Unlike that found after acute SD, rats do not sleep longer or deeper after 2-5 days of SR even though they exhibit significant elevations of behavioral sleepiness throughout 5 consecutive days of SR (Kim et al., 2012). Similar results were observed in another model of CSR in which rats underwent continuous 3 h cycles of SD followed by 1 h of sleep opportunity for 4 consecutive days at the onset of the 12 h light phase (i.e., “3/1” CSR protocol). The “3/1” CSR protocol triggered both homeostatic (increased sleep amounts and SWA during sleep opportunities after acute sleep loss) and allostatic sleep responses (gradual decline in SWA during sleep opportunities across CSR days) (Deurveilher et al., 2012). Nevertheless, rats that are hypersensitive to stress (Pearson et al., 2006), Wistar-Kyoto rats, have enhanced SWA responses throughout 5 days of SR (Leemburg et al., 2010).

Gerashchenko and colleagues previously demonstrated that nNOS-immuno-reactive neurons in the cerebral cortex have activity profiles that parallel the changes of SWA in different species (Gerashchenko et al., 2008). In addition, previous studies in both rats and mice showed increased numbers of c-Fos-positive nNOS neurons after acute sleep loss (Gerashchenko et al., 2008; Pasumarthi et al., 2010). The highest increase in both SWA and the activity of cortical nNOS neurons occurs within the first 2 to 3 hours of recovery sleep after a period of acute SD, which is the time when homeostatic sleep pressure is the greatest (Gerashchenko et al., 2008). Our current findings indicated that 18 h of acute SD enhanced SWA mostly within the first hour of sleep opportunity after acute SD, which is consistent with a wide literature indicating that SWA declines exponentially during sleep (Borbély and Achermann, 2005). We also found that rats had increased SWA and NREM sleep delta energy during the first 3 h of recovery sleep immediately after 18 h of SD that was associated with high cortical nNOS neuronal activity. Our current findings are consistent with those reported in mice and rats after shorter durations of acute sleep loss (6 h vs. 18 h) and suggest that the typical homeostatic sleep responses of these nNOS neurons and SWA remain intact after longer periods of acute sleep loss (Gerashchenko et al., 2008).

We found that rats enduring CSR had enhanced nNOS cell activity during available sleep opportunities that was as high after the fifth day of SR as it was after an 18 h acute bout of SD. Thus, the homeostatic increase in activity of nNOS cells in rats occurred both during acute SD and CSR. Sleep-active cortical nNOS neurons were not further enhanced after CSR compared to that after 18 h SD, suggesting that CSR might alter neuromolecular mechanisms regulating the activity of these cells. However, the lack of enhanced cellular activity might occur, in part, because of ceiling effects in the high percentage (~70 %) of cortical neurons expressing nNOS after sleep loss. Although the mechanisms by which activity of cortical nNOS neurons is regulated are not fully understood, Gerashchenko and colleagues previously hypothesized that these mechanisms include interactions between inhibiting input to sleep-active cortical nNOS neurons from wake-active brain areas (e.g., cholinergic neurons in the basal forebrain) and activating input to nNOS neurons by sleep-regulatory pro-inflammatory cytokines and neuromodulators (e.g., adenosine) that are locally produced in the cerebral cortex during wakefulness (Kilduff et al., 2011; Gerashchenko et al., 2011). We speculate that the occurrence of sleep removes wake-related inhibition of cortical nNOS neurons but prior accumulation of sleep pressure is required for full activation of this neuronal population.

Because nNOS neurons have long-projections that even span hemispheres and that widely arborize within the cerebral cortex (Tricoire and Vitalis, 2012), their anatomical properties

are well suited for facilitation of long-range synchronization between neurons. The long-range synchronization between neurons is needed for SWA production (Timofeev et al., 2012). However, in the present study, we observed the activity of cortical nNOS neurons did not parallel SWA after CSR suggesting that nNOS cells participate in EEG synchronization and SWA production after acute sleep loss but not after repeated days of SR. Although speculative, a likely explanation of this result is that EEG synchronization is suppressed downstream of cortical nNOS neuronal activation after CSR. Several mechanisms contributing to EEG synchronization have been proposed (Timofeev et al., 2012). For example, synchronous oscillatory activities are generated by various types of neuronal interactions and controlled by cortico-thalamo-cortical feedback mechanisms and activities of cholinergic, norepinephrine and serotonergic systems (Timofeev et al., 2012). Additional studies will be needed to determine whether any of these mechanisms of EEG synchronization and SWA are affected in the cerebral cortex during CSR.

4.1. Conclusions

In conclusion, we demonstrate that cortical nNOS neurons are activated during sleep similarly after both acute SD and CSR and that SWA is enhanced after acute SD but not CSR. Our findings suggest that mechanisms downstream of the activation of these nNOS neurons are affected by CSR. Nonetheless, cortical neurons expressing nNOS remain active after CSR and could be involved with other homeostatic changes that persist with CSR including sleepiness or impairments in cognition.

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References

- Achermann P, Borbély AA. Mathematical models of sleep regulation. *Front Biosci.* 2003; 8:S683–S693. [PubMed: 12700054]
- Baracchi F, Ingiosi AM, Raymond RM Jr, Opp MR. Sepsis-induced alterations in sleep of rats. *Am J Physiol Regul Integr Comp Physiol.* 2011; 301:R1467–R1478. [PubMed: 21900639]
- Barf RP, Meerlo P, Scheurink AJ. Chronic sleep disturbance impairs glucose homeostasis in rats. *Int J Endocrinol.* 2010; 2010:819414. [PubMed: 20339560]
- Barf RP, Van Dijk G, Scheurink AJ, Hoffmann K, Novati A, Hulshof HJ, Fuchs E, Meerlo P. Metabolic consequences of chronic sleep restriction in rats: changes in body weight regulation and energy expenditure. *Physiol Behav.* 2012; 107:322–328. [PubMed: 22995976]
- Borbély, AA.; Achermann, P. Sleep homeostasis and models of sleep regulation. In: Kryger, MH.; Roth, T.; Dement, WC., editors. *Principles and Practice of Sleep Medicine*. 4th. Elsevier Saunders; Philadelphia: 2005. p. 405–417.
- Brown RE, Basheer R, McKenna JT, Strecker RE, McCarley RW. Control of sleep and wakefulness. *Physiol Rev.* 2012; 92:1087–1187. [PubMed: 22811426]
- Cirelli C, Tononi G. On the functional significance of c-fos induction during the sleep-wake cycle. *Sleep.* 2000; 23:453–469. [PubMed: 10875553]
- Davis CJ, Clinton JM, Jewett KA, Zielinski MR, Krueger JM. Delta wave power: an independent sleep phenotype or epiphenomenon? *J Clin Sleep Med.* 2011; 7:S16–S18. [PubMed: 22003323]
- Deurveilher S, Rusak B, Semba K. Time-of-day modulation of homeostatic and allostatic sleep responses to chronic sleep restriction in rats. *Am J Physiol Regul Integr Comp Physiol.* 2012; 302:R1411–R1425. [PubMed: 22492816]
- Dijk DJ, Kronauer RE. Commentary: models of sleep regulation: successes and continuing challenges. *J Biol Rhythms.* 1999; 14:569–573. [PubMed: 10643754]

- Faraut B, Boudjeltia KZ, Vanhamme L, Kerkhofs M. Immune, inflammatory and cardiovascular consequences of sleep restriction and recovery. *Sleep Med Rev.* 2012; 16:137–149. [PubMed: 21835655]
- Gerashchenko D, Wisor JP, Burns D, Reh RK, Shiromani PJ, Sakurai T, de la Iglesia HO, Kilduff TS. Identification of a population of sleep-active cerebral cortex neurons. *Proc Natl Acad Sci U S A.* 2008; 105:10227–10232. [PubMed: 18645184]
- Gerashchenko D, Wisor JP, Kilduff TS. Sleep-active cells in the cerebral cortex and their role in slow-wave activity. *Sleep Biol Rhythms.* 2011; 9:71–77. [PubMed: 21625335]
- Kilduff TS, Cauli B, Gerashchenko D. Activation of cortical interneurons during sleep: an anatomical link to homeostatic sleep regulation? *Trends Neurosci.* 2011; 34:10–19. [PubMed: 21030095]
- Kim Y, Bolortuya Y, Chen L, Basheer R, McCarley RW, Strecker RE. Decoupling of sleepiness from sleep time and intensity during chronic sleep restriction: evidence for a role of the adenosine system. *Sleep.* 2012; 35:861–869. [PubMed: 22654205]
- Kim Y, Laposky AD, Bergmann BM, Turek FW. Repeated sleep restriction in rats leads to homeostatic and allostatic responses during recovery sleep. *Proc Natl Acad Sci U S A.* 2007; 104:10697–10702. [PubMed: 17548824]
- Leemburg S, Vyazovkiy VV, Olcese U, Bassetti CL, Tononi G, Cirelli C. Sleep homeostasis in the rat is preserved during chronic sleep restriction. *Proc Natl Acad Sci U S A.* 2010; 107:15939–15944. [PubMed: 20696898]
- McCauley P, Kalachev LV, Smith AD, Belenky G, Dinges DF, Van Dongen HP. A new mathematical model for the homeostatic effects of sleep loss on neurobehavioral performance. *J Theor Biol.* 2009; 256:227–239. [PubMed: 18938181]
- McCoy JG, Christie MA, Kim Y, Brennan R, Poeta DL, McCarley RW, Strecker RE. Chronic sleep restriction impairs spatial memory in rats. *Neuroreport.* 2013; 24:91–95. [PubMed: 23238166]
- McEwen BS, Wingfield JC. The concept of allostasis in biology and biomedicine. *Horm Behav.* 2003; 43:2–15. [PubMed: 12614627]
- Meerlo P, Koehl M, van der BK, Turek FW. Sleep restriction alters the hypothalamic-pituitary-adrenal response to stress. *J Neuroendocrinol.* 2002; 14:397–402. [PubMed: 12000545]
- Novati A, Roman V, Cetin T, Hagewoud R, den Boer JA, Luiten PG, Meerlo P. Chronically restricted sleep leads to depression-like changes in neurotransmitter receptor sensitivity and neuroendocrine stress reactivity in rats. *Sleep.* 2008; 31:1579–1585. [PubMed: 19014078]
- Pasumarthi RK, Gerashchenko D, Kilduff TS. Further characterization of sleep-active neuronal nitric oxide synthase neurons in the mouse brain. *Neuroscience.* 2010; 169:149–157. [PubMed: 20438808]
- Pearson KA, Stephen A, Beck SG, Valentino RJ. Identifying genes in monoamine nuclei that may determine stress vulnerability and depressive behavior in Wistar-Kyoto rats. *Neuropsychopharmacology.* 2006; 31:2449–2461. [PubMed: 16710313]
- Roky R, Kapas L, Taishi TP, Fang J, Krueger JM. Food restriction alters the diurnal distribution of sleep in rats. *Physiol Behav.* 1999; 67:697–703. [PubMed: 10604840]
- Roman V, Hagewoud R, Luiten PG, Meerlo P. Differential effects of chronic partial sleep deprivation and stress on serotonin-1A and muscarinic acetylcholine receptor sensitivity. *J Sleep Res.* 2006; 15:386–394. [PubMed: 17118095]
- Roman V, Walstra I, Luiten PG, Meerlo P. Too little sleep gradually desensitizes the serotonin 1A receptor system. *Sleep.* 2005; 28:1505–1510. [PubMed: 16408408]
- Timofeev, I.; Bazhenov, M.; Seigne, J.; Sejnowski, T. Neuronal Synchronization and Thalamocortical Rhythms in Sleep, Wake and Epilepsy. In: Noebels, Jeffrey L.; Avoli, Massime; Rogawski, Michael A.; Olsen, Richard W.; Delgado-Escueta, Antonio V., editors. *Jasper's Basic Mechanisms of the Epilepsies*. National Center for Biotechnology Information (US); Bethesda (MD): 2012. p. 1p. 24
- Tricoire L, Vitalis T. Neuronal nitric oxide synthase expressing neurons: a journey from birth to neuronal circuits. *Front Neural Circuits.* 2012; 6:82. [PubMed: 23227003]
- Zielinski MR, Krueger JM. Sleep and innate immunity. *Front Biosci (Schol Ed).* 2011; 3:632–642. [PubMed: 21196401]

Zielinski MR, Taishi P, Clinton JM, Krueger JM. 5 Ectonucleotidase-knockout mice lack non-REM sleep responses to sleep deprivation. *Eur J Neurosci.* 2012; 35:1789–1798. [PubMed: 22540145]

Highlights

Cortical nNOS neurons were activated during sleep after 18 hours of acute sleep deprivation.

Cortical nNOS neurons are activated during sleep after chronic sleep restriction.

nNOS neuronal activity was correlated with slow wave activity after acute sleep deprivation.

nNOS neuronal activity was not correlated with slow wave activity after chronic sleep restriction.

Fig. 1. Representative immunohistochemically stained section of c-Fos positive and nNOS positive cells in the parietal cortex (10 × objective). The insert shows c-Fos positive nNOS cells at a higher magnification (40 × objective). c-Fos positive cells are stained black and nNOS positive cells are stained red. Black arrows = c-Fos and nNOS double positive cells. White arrows = nNOS positive and c-Fos negative cells.

Fig. 2.

Percentage of cortical c-Fos positive cells per total nNOS positive cells after the first 3 h (ZT 0-3 h) of the sleep opportunity after sleep loss treatments. Rats that underwent 18 h SD had enhanced percentages of activated nNOS cells in the cerebral cortex compared to time-of-day matched baseline sleep control rats. The percentage of activated nNOS cells in the cerebral cortex were similarly enhanced in rats after CSR compared to baseline control rats. * = significant difference between treatment groups. Significance was set at $p < 0.05$.

Fig. 3.

NREM sleep, REM sleep, total sleep time (i.e., NREM sleep + REM sleep) durations during the first 3 h (ZT 0-3 h) of available sleep opportunities after sleep deprivation treatments. NREM sleep (**A**), REM sleep (**B**), and total sleep time (**C**) durations after 18 h SD and after CSR were not statistically different to time-of-day matched baseline sleep values.

Fig. 4.

SWA and NREM sleep delta energy responses normalized to spontaneous sleep measures during the dark period during sleep opportunities after sleep loss treatments. During the first 3 h (ZT 0-3 h) of available sleep opportunity after sleep loss treatments (ZT 6-24). SWA (A) and NREM sleep delta energy (C) values were significantly enhanced after 18 h SD compared to both time-of-day matched baseline control and CSR values. SWA and NREM sleep delta energy values after CSR were not significantly different from baseline control values. However, the enhanced SWA found after 18 h SD was found to occur mostly within the first hour (ZT 0-1) of available sleep opportunity (B). (*) = significant difference between treatment groups. Significance was set at $p < 0.05$.

Fig. 5.

NREM sleep EEG power (0.5-30 Hz range) responses during the first 3 h (ZT 0-3 h) of available sleep opportunity after sleep loss treatments (ZT 6-24) normalized as a percentage of spontaneous sleep during the dark period. NREM sleep EEG power (0.5-30 Hz range) was largely enhanced in rats after 18 h SD compared to rats that underwent baseline sleep (2.0-8.0, 9.0, and 17.0-23.0 Hz frequency ranges) or CSR (2.5-11.0 and 19.0-24.5 Hz frequency ranges). NREM sleep EEG power (0.5-30 Hz range) was not significantly different after CSR when compared to baseline sleep controls. (—) and (*) = significant difference between 18 h SD and baseline sleep control groups. (—) and () = significant difference between 18 h SD and CSR treatment groups. Significance was set at $p < 0.05$.