Long-term effects of sleep deprivation on neuronal activity in four hypothalamic areas

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A B S T R A C T

Lack of adequate sleep has become increasingly common in our 24/7 society. Unfortunately diminished sleep has significant health consequences including metabolic and cardiovascular disease and mental disorders including depression. The pathways by which reduced sleep adversely affects physiology and behavior are unknown. We found that 6 h of sleep deprivation in adult male rats induces changes in neuronal activity in the lateral hypothalamus, the paraventricular nucleus, the arcuate nucleus and the mammillary bodies. Surprisingly, these alterations last for up to 48 h. The data show that sleep loss has prolonged effects on the activity of multiple hypothalamic areas. Our data indicate also that measuring electroencephalographic slow wave activity underestimates the amount of time that the hypothalamus requires to recover from episodes of sleep deprivation. We propose that these hypothalamic changes underlie the well-established relationship between sleep loss and several diseases such as metabolic disorders, stress and depression and that sufficient sleep is vital for autonomic functions controlled by the hypothalamus.

1. Introduction

In humans, sleep loss can have widespread detrimental effects on health and is shown to be a risk factor for several diseases (Lim and Dinges, 2010; Grandner et al., 2010; Ma et al., 2015; Schmid et al., 2015; Aguirre, 2016). Chronic sleep loss is associated with deficits in attention, cognition, immune function, metabolism, mood, and cardiovascular function (Grandner et al., 2010; Ma et al., 2015; Schmid et al., 2015). For example, insufficient sleep is associated with decreases in the satiety hormone leptin, increases in the hunger-stimulating hormone ghrelin and increases in appetite (Spiegel et al., 2009; Hanlon and Van Cauter, 2011; Schmid et al., 2015). Combined with our modern obesogenic environment, this physiological state leads to overeating and ultimately overweight and obesity (Schmid et al., 2015). Even one night of sleep loss can acutely impair both executive functions and cognitive learning (Lim and Dinges, 2010) and alter metabolic hormonal balance (Leproult et al., 1997; Spiegel et al., 2009; Hanlon and Van Cauter, 2011). On the other hand, sleep loss can have a profound positive effect on mood in clinically depressed patients (Dallaspezia and Benedetti, 2015).

fMRI studies in humans investigating the effects of sleep deprivation on health have focused mainly on cortical structures leaving the potential contribution of subcortical brain areas largely unexplored (Ma et al., 2015). The hypothalamus plays a major role in regulating physiological and behavioral responses by sensing the body’s internal state and altering the electrical activity of hypothalamic neurons, thereby regulating the activity of other brain areas in order to maintain homeostasis (Stensson, 2013). Within the hypothalamus, the lateral hypothalamus (LH) as well as the arcuate nucleus (ARC) integrate diverse systemic and neuronal inputs in order to regulate metabolism and feeding behavior (Berthoud and Münzberg, 2011) while the paraventricular nucleus (PVN) and the mammillary bodies (MB) have been involved respectively in the control of the endocrine component of the stress response (Füzesi et al., 2016) and learning and memory (Vann and Aggleton, 2004). Because all the physiological functions controlled by these hypothalamic nuclei are known to be adversely affected by sleep loss (Lim and Dinges, 2010; Grandner et al., 2010; Ma et al., 2015; Schmid et al., 2015; Aguirre, 2016), we investigated in adult Wistar rats the acute and long-term effects of a 6-h of sleep deprivation (SD) on electrical multi-unit activity (MUA), as a measure of neuronal activity in the LH (n = 20 animals), the PVN (n = 7), the ARC (n = 8) and the MB (n = 7; Fig. S1). In addition, electroencephalogram (EEG) and electromyogram (EMG) recordings were performed simultaneously in order to investigate changes in sleep and waking before, during, and after SD.
2. Materials and methods

2.1. Animals

A total of 63 male Wistar rats (approximately 300 g at the time of surgery) were used for this study. The animals were purchased from Charles river. All animal experiments were approved by the Ethics Committee of the Leiden University Medical Center and were carried out in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

2.2. In vivo multi-unit activity, EEG, and EMG recordings

In vivo multiunit activity (MUA) and EEG and EMG were recorded as described previously (Meijer et al., 1998; Deboer et al., 2003, 2007). In brief, for the MUA recordings, stainless steel tripolar electrodes (0.125 mm diameter; Plastics One, Inc., Roanoke, VA) were implanted in each animal under deep anesthesia. For differential recordings, two electrodes were directed toward the targeted hypothalamic structure with 0.4-mm space between the electrodes. The third electrode was placed in the cortex as a reference electrode. The electrodes were placed to record from the LH (relative to Bregma: 1.8 mm posterior and 2 mm lateral; depth: 8.15 mm), PVN (relative to Bregma: 2 mm posterior and 0.84 mm lateral; depth: 7.67 mm; 5 degrees relative to vertical), ARC (relative to Bregma: 3.8 mm posterior and 0.26 mm lateral; depth: 9.8 mm; 5° relative to vertical), and MB (relative to Bregma: 3.8 or 4.8 mm posterior and 1 mm lateral; depth: 9.1 mm). The coordinates were adapted from (Paxinos and Watson, 1982).

For EEG, electrodes were screwed into the skull above the dura over the right cortex (2 mm lateral to the midline and 3.5 mm posterior to Bregma) and cerebellum (at the midline and 1.5 mm posterior to lambda). For EMG recordings, two wires with suture patches were inserted in the tissue between the skin and the neck muscle.

The animals were connected to the recording system via a flexible cable and counterbalanced swivel system, and the animals were acclimated to the setup in continuous darkness for at least one week prior to the start of the recording. The animals’ behavioral activity (drinking and locomotion) was recorded continuously in order to obtain an estimate of the circadian rhythm.

Neuronal activity in the hypothalamic structures was amplified approximately 40,000 ×, band-pass filtered (500–5000 Hz, – 40 dB/decade), and processed further offline. Online, a window discriminator converted the action potentials into electronic pulses. A second window discriminator was set at a higher level to detect artifacts caused by the animal’s movements. Action potentials and movement-related artifacts were counted in 10-s epochs. The analog EEG and EMG signals, which were recorded continuously, were amplified approximately 2000 ×, band-pass filtered (0.5–30 Hz, – 40 dB/decade), and digitized at 100 Hz. All data were recorded simultaneously and stored on a computer hard disk. The stability of the multi-unit signal and EEG recording was evaluated daily by visually inspecting the signal using an oscilloscope; the circadian rhythm in the signal and the amplitude of the EEG were monitored for 7 days before the baseline data were collected. After the experiments, the animals were sacrificed in order to verify the recording sites. To mark the location of the electrode tip, current was passed through the electrode, and the brain was perfused with a buffered solution containing 4% paraformaldehyde and 8% potassium ferrocyanide.

The brains were removed, post-fixed overnight in 4% paraformaldehyde, and cryo-protected in 30% sucrose solution. Free-floating coronal sections (40 μm thickness) were cut on a freezing microtome. The sections were stained with cresyl violet, mounted on gelatinized slides, dried, dehydrated in increasing gradients of ethanol, cleared in toluene, and cover-slipped with Depex. Of the 63 animals used, the recording electrodes were positioned in the correct location as follows: 20 in the LH, 7 in the PVN, 8 in the ARC, and 7 in the MB (2 in the medial mammillary nucleus, 1 in the lateral mammillary nucleus, and 4 in the pre-mammillary nucleus).

Offline, the EEG power density spectra were calculated in 10-s epochs corresponding to the 10-s epochs of the action potentials of the targeted hypothalamic structure using a fast Fourier transform (FFT) routine within the frequency range of 0.25–25.0 Hz in 0.1 – Hz bins. EMG signals were integrated over 10-s epochs. Three vigilance states—wakefulness, NREM sleep, and REM sleep—were determined visually based on standardized EEG/EMG criteria for rodents (Deboer et al., 2003, 2007; Stensvers et al., 2016). Wakefulness was scored when the EMG showed an irregular, high-amplitude pattern and the EEG signal was low in amplitude with relatively high activity in the theta band (6–9 Hz). NREM sleep was scored when EMG amplitude was low and the EEG amplitude was higher than during wakefulness, with high values in the slow wave range (1–4 Hz). REM sleep was scored when the amplitude of the EMG and EEG were low and the EEG showed relatively high values in the theta range. Epochs containing artifacts in the SCN electrical signal or in the EEG signal (observed during the scoring of the vigilance states) were excluded from our analysis of the neuronal activity and EEG spectral analysis.

All MUA data and EEG power density data were calculated relative to the respective mean values recorded during NREM sleep over a 24-hour period.

2.3. Sleep deprivation

A previously validated method using an enriched, novel environment (Gompf et al., 2010; Zhang et al., 2014) was used to stimulate spontaneous exploratory wakefulness without inducing stress. SD was performed during the first 6 h in the subjective day. The experiments were performed in continuous darkness under constant temperature and humidity conditions. Clean bedding, food, water, climbing toys, and novel nesting materials were used as stimuli to stimulate wakefulness. During the 6-hour SD episode, the animals were monitored via their online EEG signal. Whenever the animals appeared to be entering NREM sleep—or if an increase in slow wave amplitude was observed—new toys were introduced to the cage of the animal.

2.4. Statistical analysis

Data were analyzed using SigmaStat version 12.0. All summary data are reported as the mean ± S.E.M. Statistical significance was determined using a repeated-measures ANOVA, with time, neuronal activity, sleep state, and power density considered as repeated measures coupled to Dunnett post hoc analysis in the case of significance (Fig. 1A, D, G, J; Fig. 2; Fig. 3A, D, G, J; Fig. 4A, C, E, G; Fig. 5), paired Student’s t-test (Fig. 1B, E, H, K; Fig. 3B, E, H, K), one-way ANOVA (Fig. 1C, F, I, J; Table S1; Fig. 3C, F, I, L; Fig. 4B, D, F, H) or simple linear regression (Figs. 6, 7; Fig. S2, Fig. S3). p-values are indicated in the text and the figure legends. Differences were considered significant when p < 0.05.

3. Results

To avoid any possible confounding effects of light and/or post-operative stress, the recordings were performed in continuous darkness at least one week following surgery. During the baseline 24 h, all four structures showed clear circadian rhythmicity, with high neuronal activity during the subjective night and low activity during the subjective day (Fig. 1A, D, G, J). After this initial 24-hour baseline period, the animals were gently sleep-deprived for 6 h and then recorded for an additional 42 h. During SD, all four structures had a sustained increase in neuronal firing (Fig. 1B, E, H, K). In contrast, after SD, each structure had a unique response. In the LH, a sustained decrease in activity was observed for 18 h (Fig. 1A) leading to an average 6.6% reduction in total MUA relative to baseline (Fig. 1C). After that, the normal circadian pattern recovered (Fig. 1A) with no significant alterations in the 2nd
However, subsequently, activity significantly increased over the last 24 h of the experiment (Fig. 1F). In the ARC, no significant up-regulation of activity was observed only after 17 h following SD and on the first day after SD (Fig. 1C). In the PVN, a sustained and significant change in activity following SD was evident primarily during NREM sleep (Fig. 1G). The average 24 h activity in the MB was significantly decreased in the first 8 h after SD (Fig. 1G). Finally, the MB had the most dramatic SD-induced changes in activity. Following the increase in MUA during SD, neuronal activity dropped to levels far below baseline values. This decrease in activity lasted up to 34 h following SD, and then returned to baseline levels (Fig. 1J). Consequently, relative to baseline, the average 24 h activity in the MB was significantly reduced by 9% and 10% respectively during the 1st and 2nd day post-SD (Fig. 1L). These results show that SD induced distinct, dynamically complex, and relatively long-lasting alterations in the neuronal activity of four crucial, and essentially different hypothalamic nuclei.

Relating the MUA recordings with the EEG and EMG recordings revealed a vigilance state-dependent modulation of neuronal activity in all four hypothalamic structures (Table S1; Fig. S2). During baseline day, the firing rate was higher during wakefulness and REM sleep than during non-REM (NREM) sleep, with the exception of the ARC, in which activity did not differ significantly between REM and NREM sleep (Table S1).

Because changes in vigilance states influenced neuronal firing rate and SD induced changes in sleep architecture (Fig. 2), the alterations observed in the MUA of the hypothalamic nuclei (Fig. 1) could be due to either changes in sleep/wake behavior or intrinsic changes in electrical activity. To disentangle these two factors, we analyzed the patterns of neuronal activity separately for waking and NREM sleep (Figs. 3, 4). All four structures showed clear circadian rhythmicity in their firing rate during wakefulness and NREM sleep. The decreased activity following SD in the LH was evident primarily during NREM sleep, with an average decrease of 4% during the first 6 h of the first day after SD (Fig. 4A, B) and no significant changes during waking (Fig. 3C). In contrast, the increased activity in the PVN occurred primarily during wakefulness, with 7% and 8.5% increases in activity on the 1st and 2nd days after SD (Fig. 4D, E) and no significant changes during NREM sleep (Fig. 4A, B). Although the ARC and MB had similar patterns during wakefulness (Fig. 3G, J) and NREM sleep (Fig. 4E, G), overall 24 h activity did not differ significantly within vigilance states (Figs. 3I, L, 4F, H). These results suggest that SD induced not only region-specific, but also state-dependent alterations in neuronal activity with LH activity being predominantly affected during NREM sleep and PVN during wakefulness. In contrast, the ARC and MB
were similarly affected in both states.

Because sleep is homeostatically regulated, sleep pressure increases in the course of SD (Borbély et al., 2016). As also shown in our data (Fig. 2), this homeostatic regulation of sleep/wakefulness behavior is reflected by the marked increase in NREM sleep after SD (Deboer et al., 2003; Vyazovskiy et al., 2009; Borbély et al., 2016). However, the best characterized physiological marker of sleep homeostasis is slow wave activity (SWA), which is comprised of the EEG power density of cortical slow (0.1–1 Hz) and delta wave (1–4 Hz) oscillations during NREM sleep (Borbély et al., 2016). Changes in SWA have been used to predict the effects of various SD paradigms and shift-work schedules on alertness and fatigue (Van Dongen et al., 2011; Borbély et al., 2016), and the duration of changes in SWA is thought to correlate with the duration of the recovery period after SD. Whether homeostatic changes in cortical SWA reflect dynamic changes in neuronal activity in the hypothalamus—as it does in the cortex (Vyazovskiy et al., 2009)—is currently unknown. Therefore, we examined whether the changes in SWA faithfully mirror the SD-induced changes in neuronal firing in the hypothalamus. To this end, we compared cortical EEG power density between 0 and 25 Hz with the neuronal activity recordings in the hypothalamic structures. During baseline, both delta wave (1–4 Hz) and slow wave (0.1–1 Hz) activity decreased during the subjective day (i.e., the sleep phase) and increased during the subjective night (i.e., the active phase) (Fig. 5A, B), consistent with previous studies (Deboer et al., 2003; Vyazovskiy et al., 2009; Borbély et al., 2016). After SD, however, both slow wave and delta wave activity were significantly higher than baseline for 14 and 7 h following SD respectively (Fig. 5A, B). Both delta wave and slow wave activity returned to baseline during the 2nd day following SD. Surprisingly, the dynamics of SWA did not reflect the electrophysiological responses measured in three of the four hypothalamic structures examined. The LH was the only structure that recovered in the same time frame as SWA (compare Fig. 5A with Fig 1A and Fig 4A). In the other three hypothalamic areas (the PVN, ARC, and MB), the changes in neuronal activity persisted long after SWA returned to baseline. With the exception of the ARC, NREM-related activity was inversely correlated with SWA in all hypothalamic structures (Fig. 6). These data indicate that SD modulates neuronal activity within the hypothalamus for remarkably long periods of time. These periods were much longer than expected based on the recovery of cortical SWA. Therefore, SWA is not a reliable marker for measuring the duration of changes in hypothalamic neuronal firing following SD.

As an alternative marker, other EEG frequencies may better represent the long-term changes in neuronal activity within the hypothalamus. Theta power density (6–9 Hz) during wakefulness also increased following SD (Fig. 5D). This increase occurred primarily during the first 6 h following SD and during the subjective night. However, this pattern also did not mirror the changes in neuronal activity in the hypothalamus during wakefulness (Fig. 3). During baseline for example, the MUA during waking in the PVN was positively correlated with theta wave activity. After SD, this correlation was inverted so that with increased theta, lower discharge rates were found in the PVN (Fig. 7B). Nevertheless, after SD MUA was increased in the PVN during wakefulness (Fig. 3D). Similar results were obtained with respect to beta oscillations (12.5–25 Hz) during wakefulness (Fig. S3). Taken together, these findings indicate that although EEG power density is correlated with neuronal activity in hypothalamic nuclei, the changes in the EEG do not reliably reflect the long-lasting changes in neuronal activity induced by SD.
logical processes are a correlate from our animals. However, given that all of these physiological and behavioral responses (Füzesi et al., 2016). The SD-induced changes in PVN neuronal activity may therefore represent the pathophysiological correlates of the delayed increase in cortisol secretion (Leproutil et al., 1997), mood disturbances (Dallapiccola and Benedetti, 2015; Finan et al., 2016), and stereotypic behavior (Demos et al., 2016) that occur in humans following sleep loss. Both the LH and ARC nuclei play essential roles in processes that underlie feeding behavior (Sternson, 2016), it is likely that the changes in neuronal activity we measured in these hypothalamic structures contribute—at least in part—to the many disorders associated with SD, including obesity and cognitive impairment (Lim and Dinges, 2010; Grundner et al., 2010; Ma et al., 2015; Schmid et al., 2015; Aguirre, 2016). Given that the changes in neuronal activity outlast the SD-induced changes in the EEG, the recovery time in these brain areas is likely underestimated.

The neuronal activity changes precipitated by SD varied between 6.6 and 10% depending on the hypothalamic structures involved. Comparable changes have been observed after 4 h of SD in basal forebrain (Kostin et al., 2010) and in the cortex (Vyazovskiy et al., 2009). Although such changes appear to be of modest magnitude, their physiological significance will depend on the impact of such changes on physiology and behavior. Activation of a single cortical neuron can modulate behavior (Brecht et al., 2004; Houweling and Brecht, 2008) and even trigger a switch in the global brain state (Li et al., 2009). Furthermore, fMRI studies in humans have revealed that 24 h SD protocols induce relative activity changes in several brain areas which do not exceed the magnitude of 10% (range between 0.9 and 7%, Thomas et al., 2000). We therefore believe that the effects of SD on hypothalamic structures revealed in our study may hold important clinical importance.

In addition to the negative effects inherently associated with lack of sleep (Lim and Dinges, 2010; Grundner et al., 2010; Ma et al., 2015; Schmid et al., 2015; Aguirre, 2016), we did not measure any physiological or behavioral correlates from our animals. However, given that all of these physiological processes are affected by sleep loss (Lim and Dinges, 2010; Grundner et al., 2010; Ma et al., 2015; Schmid et al., 2015; Aguirre, 2016), it is likely that the changes in neuronal activity we measured in these hypothalamic structures contribute—at least in part—to the many disorders associated with SD, including obesity and cognitive impairment (Lim and Dinges, 2010; Grundner et al., 2010; Ma et al., 2015; Schmid et al., 2015; Aguirre, 2016). Given that the changes in neuronal activity outlast the SD-induced changes in the EEG, the recovery time in these brain areas is likely underestimated.

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Schmid et al., 2015; Aguirre, 2016), the classical methodologies used to sleep deprive laboratory animals might induce stressful responses which introduce confounding factors to the interpretation of the results (Rechtschaffen et al., 1999). To minimize such biases, we used a previously validated method that promotes spontaneous exploratory wakefulness using enriched and novel environment (Gompf et al., 2010; Zhang et al., 2014). As shown by the levels of plasma corticosterone, this methodology does not stress the animals (Zant et al., 2011; Zhang et al., 2014). As shown by the levels of plasma corticosterone, this methodology does not stress the animals (Zant et al., 2011; Zhang et al., 2014). In our setting, this is corroborated by the firing rates of the PVN throughout the 6 h of SD which were not significantly increased relative to baseline (Fig. 3D). Therefore, it is unlikely that the SD-induced changes in neuronal activity of the hypothalamic structures found in the present experiments can be attributed to stress. A remaining confounding factor for the interpretation of SD results is the exposure of animals to new objects. To fully disentangle the response to new objects from the response to SD, future studies should contrast the impact of SD in familiar vs new and enriched environments.

Our recordings consist of multi-unit activities from the population of neurons surrounding the tip of the electrode regardless of the neurochemical identity of neurons. All the hypothalamic nuclei investigated contain several and intermixed neuronal types (Berthoud and Münzberg, 2011; Sternson, 2013). Therefore, the neuronal responses we recorded should be regarded as an estimation of the average activity of all neurons residing within the nuclei. Future studies using targeted single cell recordings will be necessary to investigate the impact of SD on different neuronal population of each hypothalamic nucleus.

There is ongoing discussion on the function and role of sleep (Siegel, 2005). Sleep is common among all organisms and lack of sleep is clearly devastating for proper functioning and may be lethal in the most extreme case. Theories on the function of sleep pinpoint to the importance for i. neuronal plasticity, ii. memory consolidation (Stickgold, 2013) and iii. Energy conservation (Berger and Phillips, 1995). By showing that a lack of sleep affects the deep hypothalamic nuclei, sleep seems also essential for the vital autonomic functions in addition to the previously described functions (Siegel, 2005).

The duration of the observed effects on the hypothalamus has significant implications with respect to the current health and safety regulations designed to minimize fatigue and reduce the occurrence of workplace accidents. Such regulations are based on current models predicting the effects of perturbing sleep patterns (Barger et al., 2009)
and are relevant to professions in which mistakes may have tragic consequences (Barger et al., 2009; Brown and Masor, 2017). For example, NASA imposes a minimum amount of time between shifts, ranging from 8 to 24 h, depending on the duration of the previous work period (Brown and Masor, 2017). Similarly, the Accreditation Council for Graduate Medical Education requires that each 24-hour shift (which is the equivalent of 8 h of sleep loss) be followed by at least 14 off-duty hours (Barger et al., 2009; Blum et al., 2011; Brown and Masor, 2017). Although these regulations are associated with a reduced prevalence of errors in the workplace, our results suggest that full recovery from sleep loss takes significantly more time.

Author contributions

J.H.M. and T.D. designed the experiments. K.F. performed the experiments and analyzed the data. K.F., T.D. and J.H.M. wrote the paper.

Fig. 5. Changes in cortical EEG power density following 6 h SD. (A–E) Time course of mean percentages of EEG slow oscillations activity (A), Delta wave activity (B), spindle frequency activity (C) during NREM sleep, and theta activity (D) and beta activity (E) during waking over the 72 h experimental protocol. Data are presented as a percentage of the mean activity (± S.E.M.) over the 24 h baseline day and averaged in 1 h bins. The traces of the baseline day are triple-plotted in gray for easy comparison. The subjective day and subjective night are indicated by white and black bars (top). Values during the 6 h SD in (A, B and C) are omitted because the remaining ≈ 10% of NREM during this period are influenced by the SD intervention. *p < 0.05.
Conflict of interest

The authors declare no competing financial interests.

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Fig. 7. Neuronal activity in hypothalamic structures during wakefulness is correlated with theta activity measured by EEG. (A-D) Change in mean firing rates in the LH (A), PVN (B), ARC (C), and MB (D) measured in 1-min bins as a function of the power density of theta oscillations.

*p < 0.05, **p < 0.001.

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