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BASIC SCIENCE

Region-Specific Dissociation between Cortical Noradrenaline Levels and the Sleep/Wake Cycle

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Study Objectives: The activity of the noradrenergic system of the locus coeruleus (LC) is high in wake and low in sleep. LC promotes arousal and EEG activation, as well as attention, working memory, and cognitive flexibility. These functions rely on prefrontal cortex and are impaired by sleep deprivation, but the extent to which LC activity changes during wake remains unclear. Moreover, it is unknown whether noradrenergic neurons can sustain elevated firing during extended wake. Recent studies show that relative to LC neurons targeting primary motor cortex (M1), those projecting to medial prefrontal cortex (mPFC) have higher spontaneous firing rates and are more excitable. These results suggest that noradrenaline (NA) levels should be higher in mPFC than M1, and that during prolonged wake LC cells targeting mPFC may fatigue more, but direct evidence is lacking.

Methods: We performed in vivo microdialysis experiments in adult (9–10 weeks old) C57BL/6 mice implanted for chronic electroencephalographic recordings. Cortical NA levels were measured during spontaneous sleep and wake (n = 8 mice), and in the course of sleep deprivation (n = 6).

Results: We found that absolute NA levels are higher in mPFC than in M1. Moreover, in both areas they decline during sleep and increase during wake, but these changes are faster in M1 than mPFC. Finally, by the end of sleep deprivation NA levels decline only in mPFC.

Conclusions: Locus coeruleus (LC) neurons targeting prefrontal cortex may fatigue more markedly, or earlier, than other LC cells, suggesting one of the mechanisms underlying the cognitive impairment and the increased sleep presure associated with sleep deprivation.

Commentary: A commentary on this article appears in this issue on page 11.

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Significance

For the first time we measured changes in noradrenaline levels in two mouse cortical areas across the physiological sleep/wake cycle and in response to sleep deprivation, and found that the locus coeruleus neurons projecting to prefrontal cortex fatigue during sleep deprivation. These findings suggest that the activity of at least some neurons in the locus coeruleus, one of the best characterized wake-promoting arousal systems, cannot be maintained at high levels endlessly. This wake-related fatigue may help explaining the cognitive impairment and the increased sleep presure associated with sleep deprivation.

INTRODUCTION

The activity of noradrenergic, cholinergic, orexinergic, histaminergic, and serotonergic neurons is strongly modulated by the sleep/wake cycle, being high during wake, low during NREM sleep and, with the exception of cholinergic neurons, even lower during REM sleep.[1] Electrophysiological recordings have singled out the noradrenergic and cholinergic systems as especially important in promoting arousal. In contrast to histaminergic neurons, noradrenergic and cholinergic cells in the pons increase firing hundreds of milliseconds or even a few seconds before the EEG switches from the synchronized pattern of NREM sleep to the activated pattern of wake.[2–5] Moreover, optogenetic experiments have shown the strong wake-promoting effect of the noradrenergic cells in the pontine nucleus of the locus coeruleus (LC) and of the cholinergic neurons in the basal forebrain, with signs of behavioral arousal from NREM sleep occurring just a few seconds after stimulation, much earlier than after optogenetic stimulation of orexin cells.[6–8] Further evidence also suggests that the arousal effects of LC neurons may be unique, even with respect to the cholinergic system. Thus, optogenetic stimulation of LC always wakes up a sleeping mouse,[6] while that of the cholinergic basal forebrain causes behavioral arousal from NREM sleep but not from REM sleep,[7] in line with the fact that cholinergic cells are active during both wake and REM sleep.[5,9] Moreover, cortical application of NA blockers in an awake rat is sufficient to trigger down states similar to those usually seen in NREM sleep, an effect not seen with cholinergic antagonists.[10]

In both animals and humans, sleep is under homeostatic control, with the pressure to go to sleep being higher at the end of the waking day than immediately after awakening, and increasing further during 6–12 hours of total sleep deprivation.[11] Sleep loss also strongly impairs vigilance, attention, working memory and cognitive flexibility, functions that depend on prefrontal cortex and are promoted by the noradrenergic system.[12–15] Thus, if LC activity is crucially linked to behavioral arousal and EEG activation, one would expect cortical NA levels to change as a function of wake duration. Surprisingly, however, this hypothesis has never been tested. Microdialysis studies in cats and rats have shown that NA levels in pons, amygdala, and hippocampus are high in active wake, lower in quiet wake, even lower in NREM sleep, and reach the lowest levels during REM sleep.[16–18] However, in all these studies NA levels were averaged within each behavioral state. Moreover, dynamic changes in NA levels in the course of sleep deprivation have not been measured, and thus the extent to which LC cells can sustain high firing during extended wake remains unknown. Similarly, the time course of the decline of NA during NREM sleep is not known.

The LC has long been considered a homogenous cell group with broad and uniform action on the cortex, but recent evidence suggests otherwise. Thus, there is little overlap among the LC projections to three subregions of the rat prefrontal

cortex including the medial prefrontal cortex (mPFC), or between the LC afferents to each of these prefrontal areas and the primary motor cortex (M1).[19] Moreover, relative to the neurons targeting M1, LC cells projecting to mPFC have spontaneous firing rate three times as high, and are more excitable.[19] Based on these findings, it was predicted that NA levels in mPFC should be higher than in M1, because cortical NA levels increase linearly with the increase in firing rate of LC neurons.[20] However, direct evidence for this hypothesis is missing.

Here we used in vivo microdialysis to determine how NA levels in mouse mPFC and M1 change during the physiological sleep/wake cycle, as well as during and after sleep deprivation. The goal was to determine whether cortical NA levels track wake duration and/or sleep pressure and if so, whether sleep/wake dependent changes in NA levels vary between mPFC and M1.

MATERIALS AND METHODS

Animals

Adult (9–10 weeks old) C57BL/6 mice were used in this study. All animal procedures followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals; facilities were reviewed and approved by the IACUC of the University of Wisconsin-Madison and were inspected and accredited by AAALAC.

Surgical Procedures

Under deep isoflurane anesthesia (1% to 1.5% volume), mice (n = 14) were implanted for chronic electroencephalographic (EEG) recordings with epidural screw electrodes over the parietal cortex (AP −2, ML +2) and cerebellum (reference electrode and ground). Electrodes were fixed to the skull with dental cement. Two stainless steel wires (diameter 0.4 mm) were inserted into neck muscles to record the electromyogram (EMG). Two microdialysis cannulas were inserted in mPFC (AP +1.8, ML +0.3) and in M1 (AP +0.8, ML −1) and fixed with dental cement. After surgery, mice were housed individually in transparent Plexiglas cages. Light and temperature were kept constant (LD 12:12, light on at 8 am, 23 ± 1°C; food and water were available ad libitum and replaced daily at 08:00). After the experiments, mice were perfused for histological confirmation of probe locations.

Experimental Design

About 7 days were allowed for recovery after surgery, and experiments started only after the sleep/wake cycle had completely normalized. The duration of the experiments was limited to 10 h, to avoid tissue washout effects and deterioration in probe quality. Both experiments (see below) started at 13:00 and ended at 23:00, and thus included mostly the light period and the first 3 h of the dark period, which is the time when mice are most likely to stay awake spontaneously. This specific timeline was the result of a compromise dictated by the need to stabilize the probe (2 h) and to obtain a stable condition of baseline during wake (2 h): since NA was expected to be affected by locomotor activity, both pre-baseline and baseline occurred on a slow moving platform, to standardize the baseline as much as possible. Moreover, our main goal was to assess NA dynamics in 3 conditions, in the course of spontaneous sleep, which occurs during the day, as well as during spontaneous wake, which occurs during the night, and during sleep deprivation. To reduce the number of animals and to be able to compare the results across groups, both experiments (sleep/wake, sleep deprivation) started at the same time of day (17:00) and were preceded by the same baseline condition. Note that measuring NA in the first hours after lights off was the only way to obtain long periods of spontaneous wake. Moreover, having several hours of sleep followed by spontaneous wake allowed us to rule out the possibility that a progressive NA decline during sleep, if present, was a nonspecific effect due to deterioration of the probe.

Experiment 1 (Sleep/Wake)

A group of mice (n = 8) was used for studying NA dynamics during sleep and wake. The day before the experiment, microdialysis probes were inserted under light isoflurane anesthesia (0.5% volume for few min). The next day mice were allowed to sleep normally for the first ~5 h of the light period, and then placed on a slowly moving platform (speed = ~0.7 cm/sec) at 13:00, when microdialysis started (flow rate = 1 μL/min). On the platform mice were kept awake by forced locomotion for 4 h; the first 2 h were used to allow microdialysis stabilization (pre-baseline), while the second 2 h were used as wake baseline. At 17:00, mice were returned to their home cages and allowed to sleep for 3 h (sleep opportunity period). At 20:00, at lights off, mice were given access to novel objects and stayed awake in their home cages for 3 h (wake period). As expected, very little if any intervention (a new object) was necessary to keep the animals awake at this time.

Experiment 2 (Sleep deprivation)

A different group of mice (n = 6) was used for studying NA dynamics during sleep deprivation. As in Experiment 1 microdialysis probes were inserted the day before the experiment under light isoflurane anesthesia (0.5% volume for few min). The next day, consistent with Experiment 1, microdialysis started at 13:00 (flow rate = 1 μL/min), when mice were put on the slowly moving platform and kept awake by forced locomotion for 4 h (speed = ~0.7 cm/sec). As before, the first 2 h were used for microdialysis stabilization, and the second 2 h served as wake baseline. Mice were returned to their home cages at 17:00 and kept awake for 6 additional h by exposure to novel objects (sleep deprivation period). Mice were never disturbed when they were spontaneously awake, feeding or drinking. Experiment 2 was performed at the same circadian time as Experiment 1, to control for time of day effects.

Electroencephalographic (EEG) Recordings

Mice were connected by a flexible cable to a commutator and recorded continuously for ~2 weeks after the surgery using the Multichannel Neurophysiology Recording and Stimulation System (Tucker-Davis Technologies Inc., TDT). EEG and EMG signals were amplified and filtered as follows: EEG, high-pass filter at 0.1 Hz; low-pass filter at 100 Hz; EMG, high-pass filter

at 10 Hz; low-pass filter at 100 Hz. All signals were sampled and stored at 256 Hz resolution.

Microdialysis Probe Construction and Calibration

The design for removable microdialysis probes coupled with implantable cortical cannulas was developed in house, to obtain high recovery performance while reducing skull damage. A single concentric probe was made by using a section of a 10 μL micropipette tip (ART 10, 10 μL pipet tip, Thermo Scientific) combined with 2 sections of small-inner-diameter FEP tubing (CMA Microdialysis) and a plastic-coated silica tubing (outer-diameter 150 μm; Scientific Glass Engineering). The silica tubing was placed in the center of semipermeable polyacrylonitrile/sodium methallyl sulphonate dialysis fiber (AN69; molecular cut-off: 12 KD; Gambro-Hospal). Quick-drying epoxy glue was used to seal the tip of the hollow dialysis fiber and to join and seal the other components. The semi-permeable membrane was partially shielded with a thin layer of epoxy glue, leaving an active length of 1.5 mm. The diameter of the final probe was approximately 300 μm. The implantable cortical cannula was made by gluing a thin-wall Polyimide tubing (Vention Medical Advanced Components) with a section of a 10 μL micropipette tip. The dummy cannula was assembled using a small-diameter stainless steel tubing (Cadence Technology Inc.) sealed with epoxy and inserted inside a section of a 10 μL micropipette tip. The in vitro percent recovery of NA was studied at room temperature as previously described,[21] by perfusing the microdialysis probe (1 μL/min) with a solution of artificial extracellular fluid (aECF; composition in mM: NaCL 147, KCl 2.7, $CaCl_2$ 1.2, $MgCl_2$ 0.85). The probe was calibrated in a beaker containing a standard solution of NA dissolved in aECF. The NA recovery was around 17%.

Microdialysis Procedure

Microdialysis experiments were carried out about 7 days after the implantation of cortical probes. The day before the experiment, the dummy cannulas were removed and the microdialysis probes gently lowered into the target cortical regions under light isoflurane anesthesia (0.5% volume for few min). Each of the 2 probes was then connected to the microfluidic circuit and perfused with aECF by a microinfusion pump (CMA/400, CMA Microdialysis) using a flow rate of 1 μL/min; after the filling of the circuit, the pump was stopped until the following day. Small-inner-diameter FEP tubing was used for the connections of the inlets and outlets; this setup allowed the recovery of the microdialysis samples without interfering with the mouse behavior. The dead space of the outlet FEP tubing was calculated to estimate the delay between microdialysis sampling and EEG recordings (15 min). The EEG data were then resynchronized in post hoc analysis. During the experiment, 16 microdialysis samples per probe were collected (one sample every 30 min, 30 μL each) in 250 μL plastic vials (ESA, PN 70-1695; ESA, Inc.) starting at the beginning of the wake baseline at 15:00 until 23:00. The 32 samples were stored at −80°C until High Performance Liquid Chromatography (HPLC) analysis. Note that sampling every 30 min was dictated by our specific experimental design. Specifically, we needed to acquire NA samples for many hours, and during spontaneous conditions without the use of brief stimulation, while microdialysis studies usually report phasic changes in NA after acute stimuli. Moreover, our goal was to detect not only spontaneous increases in NA, as usually done in microdialysis studies, but also spontaneous decreases (during sleep). Sampling at higher temporal resolution (10–15 min^{22}) requires high flow rates (high sample volume, high absolute recovery, low relative recovery) that cannot be maintained for many hours.

HPLC-CD Analysis of NA

NA was quantified in microdialysis samples by reverse-phase ion-paired HPLC with coulometric detection (HPLC-CD). The HPLC apparatus used in this study was composed by an isocratic solvent delivery module (ESA, Model 582; ESA, Inc.), an autosampler (ESA, Model 542) equipped with a Rheodyne-compatible injector, sample tray cooler and column oven, and a coulometric detector (ESA, Coulochem II) coupled with a Guard Cell (ESA, Model 5020) and a Microdialysis Cell (ESA, Model 5014B). The entire high-pressure circuit was metal free and assembled using Peek tubing; a graphite filter element (ESA, PN 70-0898) was inserted before the injector while a Peek filter (ESA, PN 70-3824) was positioned before every electrochemical cell. The separation column was a C18 (ODS, 5 μm packing, 250 mm × 4.6 mm i.d.; Spheri-5; Perkin Elmer) and the mobile phase was composed by: 1.36 g/L KH_2PO4, 1.74 g/L K_2HPO4, 100 μM EDTA, 10% (vol/vol) MeOH and sodium octyl sulphate (pairing agent) 520 mg/L (pH = 2.85); the flow rate was fixed at 1.0 mL/min. One hour before injection the sample tray cooler was stabilized at +4°C and the column oven at +30°C. Microdialysis samples were thawed immediately before analysis. The 50-microliter Peek loop was partially filled with 27 μL of sample while the mobile phase was used as transport liquid. The electrochemical potentials were fixed (*vs*. Palladium reference electrode) as follows: +300 mV (Guard Cell), −175 mV (Microdialysis Cell; electrode #1) and +175 mV (Microdialysis Cell; electrode #2). Chromatograms were analyzed by PC using ESA Chromatography Software (Ver. 5.01; ESA).

Data Analysis

EEG power spectra were computed by a Fast Fourier Transform routine for 4-s epochs (0.25 Hz resolution). For staging, signals were loaded with custom-made Matlab programs using standard TDT routines, and subsequently transformed into the EDF (European Data Format) with Neurotraces software. Wake, NREM sleep, and REM sleep were manually scored offline (SleepSign, Kissei COMTEC) in 4-s epochs according to standard criteria. Epochs containing artifacts, predominantly during active wake, were excluded from spectral analysis. Vigilance state could always be determined. EEG data (vigilance states and spectral EEG data) were averaged in 30-min bins to match the temporal resolution of the microdialysis data. To allow comparisons across animals EEG data were normalized to the first 30 min of baseline (t_1). EMG activity was calculated by averaging the absolute EMG amplitude in 30-min bins and normalizing it to the first 30 min of EMG baseline (t_1). The number of objects given to each mouse was

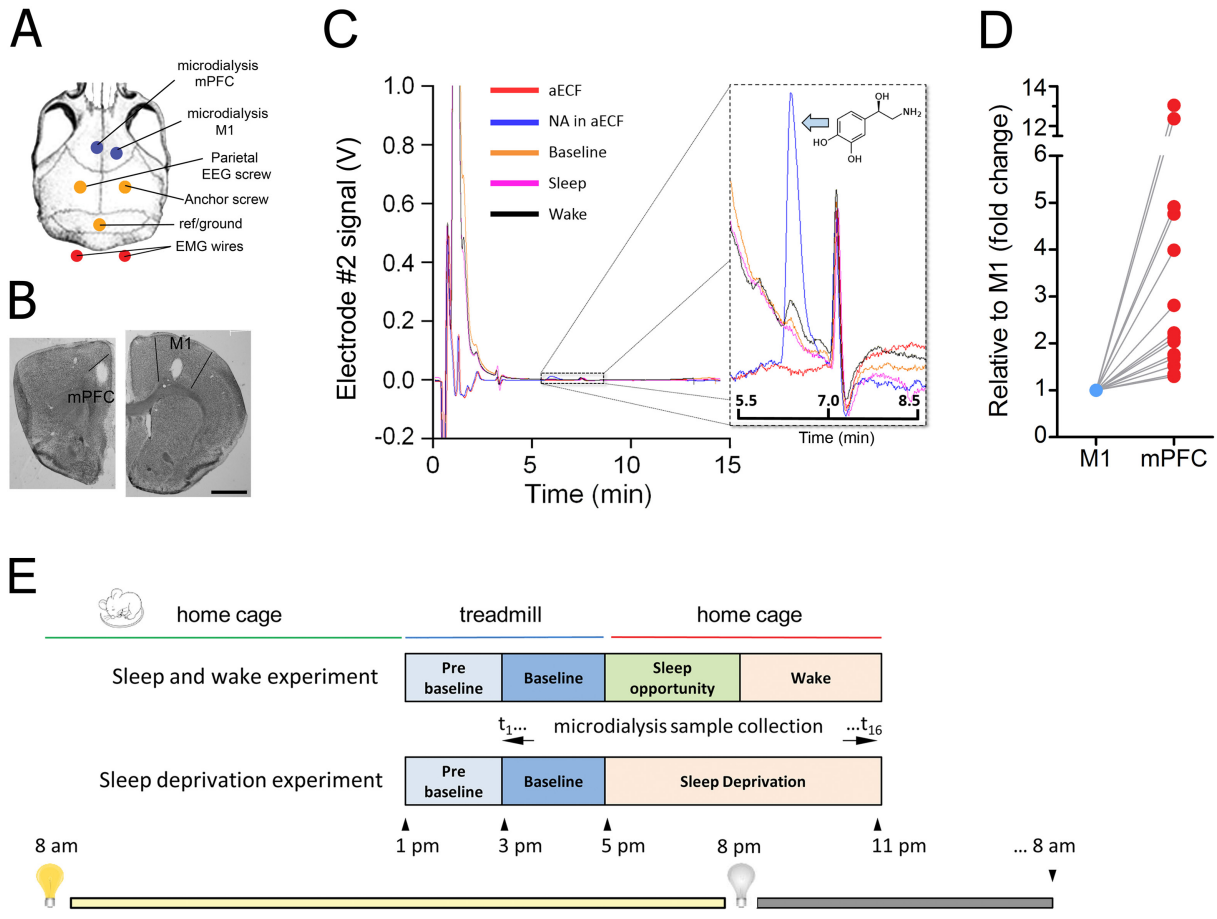

Figure 1—Experimental design. **(A)** Scheme of the location of microdialysis probes (blue), EEG electrodes (yellow), and EMG wires (red). **(B)** Representative pictures of coronal sections showing the location of the microdialysis probes. Black lines mark the borders of mPFC and M1.[51] Scale bar = 1 mm. **(C)** Representative chromatograms obtained by microdialysis, during baseline (orange line), sleep (magenta line), and active wake with exploration (black line) are shown superimposed on a typical chromatogram generated from a known quantity of NA dissolved in aECF (blue line, NA standard solution) and on a chromatogram generated from the injection of aECF (red line). Retention time (RT) of the NA peak in both NA standard solution and microdialysis samples is around 6.5 min; no detectable peaks are present at the same RT in the aECF chromatogram. **(D)** NA levels in mPFC of each of the 14 mice expressed as fold change of those in M1. **(E)** Experimental design for sleep/wake and sleep deprivation experiments.

counted and summed across 30-min bins. The EEG power in the 2 to 6 Hz range was averaged in each of 2 time windows and then expressed as ratio window 2 / window 1. Window 1 spanned the hour before NA levels reached the peak in mPFC, while window 2 was set to coincide with the duration of the NA decline in mPFC, and thus its length varied across mice.

All statistical tests were performed using Matlab and all data are presented as mean ± standard error of the mean. Parametric statistics were used to perform all pair-wise comparisons and correlations. Alpha was set to 0.05.

RESULTS

Cortical NA concentrations are higher in mPFC than in M1

All mice were implanted with parietal electrodes for chronic EEG recordings and 2 microdialysis cannulas, one aimed at mPFC and the other at M1 (Figure 1A). Histological examination confirmed that all microdialysis probes were successfully implanted in the 2 target areas (Figure 1B). All mice used for Experiment 1 and 2 (see below) were first placed on a slowly moving platform for 4 h to obtain a condition of sustained and stereotyped wake that would result in stable baseline NA levels, and then allowed to sleep and/or kept awake. Chromatograms from both cortical areas showed a distinct NA peak, which appeared in correspondence with the NA peak of the standard sample. As expected, the NA peak was larger during baseline wake than during sleep, and largest during active wake with exploration (Figure 1C). During the standardized baseline, all 14 mice showed higher absolute NA levels in mPFC than in M1. The mean increase across mice was ~4 fold (paired t-test, P = 0.012), but the range was wide, from ~0.5 to 14 fold (Figure 1D).

Cortical NA levels decline during sleep, and do so earlier in M1 than in mPFC

Starting at 13:00, mice in Experiment 1 (sleep and wake, n = 8) were placed on the platform for 4 h and then returned to their home cage, where they had a 3-h window opportunity to

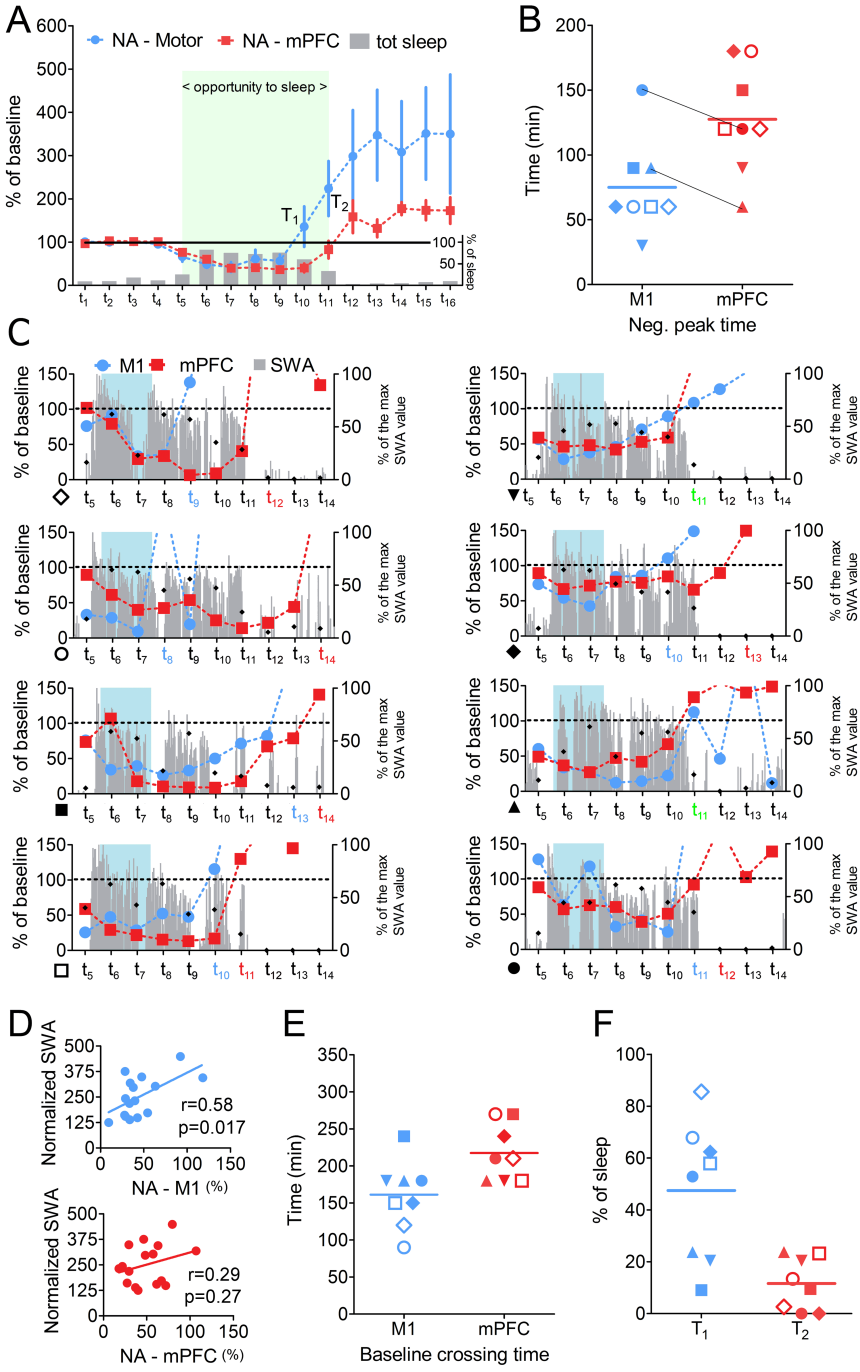

Figure 2—Changes in NA levels during sleep and wake. **(A)** NA levels (mean ± SEM, n = 8) relative to baseline (average t_1–t_4) during the sleep and wake experiment in M1 (blue) and mPFC (red). NA levels decline during the sleep opportunity period (highlighted in light green) in M1 (rmANOVA, $F_{6,42}$ = 2.6, P = 0.03, with t_6 and t_7 significantly lower than baseline; Bonferroni corrected P < 0.0083) and mPFC (rmANOVA, $F_{6,42}$ = 10.2, P < 0.0001, with t_6–t_{10} significantly lower than baseline; Bonferroni corrected P < 0.0083). Gray bars show average percentage of total sleep time for each 30-min time point. T_1 and T_2 identify the 30-min bin in which NA levels return above baseline in M1 and mPFC, respectively. **(B)** Negative peak (lowest NA level) times from the onset of the sleep opportunity period in M1 (blue) and mPFC (red). Each animal is represented with a different symbol. Note that mice (▲) and (●) show an opposite trend. **(C)** Changes in NA levels between t_5 and t_{14} for each individual mouse. SWA is computed over the entire period, displayed at 1-min resolution as % of the maximum SWA value within the t_5–t_{14} interval, and depicted in gray. For each time point the amount of total sleep is expressed as percentage of total time (see left Y axis for reference) and indicated by a black diamond. The light blue box underlines the two time points (t_6 and t_7) roughly corresponding to the first hour of consolidated sleep in all mice. Time points when NA levels return above baseline wake are indicated on the X axis in blue (T_1, M1) and red (T_2, mPFC). Time points depicted in green indicate that T_1 and T_2 coincide. Note that since the left Y axis only reaches 150%, in most cases NA levels go off scale at the end of the sleep opportunity window, and thus are not plotted. **(D)** Correlation between relative NA levels and normalized SWA during the two time points (t_6 and t_7) roughly corresponding to the first hour of consolidated sleep in M1 (upper panel) and mPFC (lower panel). **(E)** Time between the onset of the sleep opportunity window and T_1 (M1, blue) and T_2 (mPFC, red). **(F)** Percentage of total sleep during the 30-min bin corresponding to T_1 and T_2. Each animal is represented with a different symbol.

sleep, followed by 3 h of wake with exposure to novel objects (Figure 1E). Figure 2A shows changes in NA levels and time spent asleep for the entire duration of the experiment, averaged across the 8 mice and expressed in 30-min bins, the microdialysis sampling rate. Throughout the entire paper, we always refer to relative NA levels, which are expressed as % of baseline wake, when mice were on the platform. NA levels were very stable in both cortical areas as long as the mice stayed on the platform, and declined within the first hour of sleep opportunity, reaching levels significantly lower than during baseline within the first 30 min of consolidated sleep. NA remained lower than during baseline for 60 min in M1, and for 150 minutes in mPFC. In 6 of 8 mice NA reached its lowest levels in M1 earlier than in mPFC, while in 2 mice the opposite was true (Figure 2B). Across the entire group, the nadir occurred ~50 min earlier in M1 than mPFC (75 ± 14.6 min vs. 127.5 ± 17 min, paired t-test, P = 0.035).

The exact timing of the NA decline varied across animals depending on their sleep pattern. This can be seen in Figure 2C, which shows changes in relative NA levels, time spent asleep for each 30-min bin, and slow wave activity (SWA) during NREM sleep (averaged NREM EEG power between 0.5 and 4 Hz). SWA reflects the intensity of NREM sleep, being high at sleep onset and declining in the course of sleep.[11] Indeed, as expected, throughout the entire sleep opportunity period (t_5–t_{11}) mean SWA progressively declined (mean % relative to the first 30 min of baseline (t_1); 1h: 251.5% ± 33.5%; 2h: 216.6% ± 28.8%; 3h: 176% ± 9.2%). During this entire period, however, NA and SWA levels were not correlated, either in M1 (r = −0.03, P = 0.85) or in mPFC (r = −0.12, P = 0.44). When the analysis was restricted to t_6–t_7, or t_6–t_9, roughly corresponding in all mice to the first hour and the first 2 h of consolidated sleep, respectively, a positive correlation was found in M1 during the first hour (M1 hour 1, r = 0.58, P = 0.017, Figure 2D upper panel) but not when hours 1 and 2 were combined (hours 1+2, r = 0.12, P = 0.5). In mPFC there was no correlation, neither in hour 1 (r = 0.29, P = 0.27, Figure 2D lower panel) nor when hours 1 and 2 were combined (hours 1+2, r = 0.05, P = 0.78).

Upon awakening, cortical NA levels increase earlier than M1 than in mPFC

Since we found that during sleep NA levels decline with a different time course in M1 and mPFC, we then calculated when they reverted back to the level of baseline wake at the end of the sleep opportunity window. For each individual mouse, we identified the 30-min bin when baseline crossing occurred, which we called T_1 for M1 and T_2 for mPFC, and calculated the percentage of total sleep (NREM + REM sleep) during the same period. Surprisingly, we found that in 6 of 8 mice, baseline crossing occurred at different times in the 2 areas, with T_1 preceding T_2, while in the remaining 2 animals T_1 and T_2 coincided. Thus, on average across all animals T_1 preceded T_2 by ~1 h (time from the sleep opportunity window onset: 161.25 ± 16 min for M1 *vs*. 217.5 ± 13.6 min for mPFC, paired t-test, P = 0.035; Figure 2E). Of note, in one of the 2 mice in which T_1 and T_2 coincided the NA decline also showed the opposite trend, reaching the nadir earlier in mPFC than M1 (▲ in Figure 2B, 2C). However, we found no obvious reason to exclude this mouse (misplacement of the microdialysis probes, abnormal sleep/wake cycle, etc.), and thus its data were used for the grouped analysis.

In 5 of 8 mice T_1 occurred when the animals were spending asleep more than 50% of the 30-min bin (47.5% ± 9.4% for all mice; Figure 2C), but none of them was asleep during this entire time window (Figure 2F). By contrast, when T_2 occurred mice had spent on average only 11.6% ± 3.6% of the 30-min asleep (% of sleep at T_1 *vs* T_2, paired t-test, P = 0.017), and 3 animals had been awake almost during the entire time (Figure 2F). To analyze in more detail the temporal relationship between the relative increase in NA levels and behavioral state, for each animal we plotted sleep and wake in 4-sec epochs for the 30 min that preceded T_1 and T_2 (pre-crossing bin), and for the 30 min when T_1 and T_2 occurred (Figure 3). Despite inter-animal variability, it is clear that T_1 could occur after short wake bouts, in some cases lasting no more than 10 min (Figure 3, top 2 mice on the left), while in all cases T_2 occurred after ≥ 15 min of wake in the crossing bin, and in all but one case it was also preceded by ≥ 10 min of wake in the pre-crossing bin. Thus, after consolidated sleep mPFC requires longer wake bouts than M1 to return to pre-sleep baseline levels.

Different dynamics of NA concentration levels during prolonged wake in mPFC and M1

In Experiment 2 (sleep deprivation) a different group of 6 mice was placed on the slowly moving platform for 4 h as before, to obtain a stable baseline, and then kept awake in their home cages for 6 additional h by exposure to novel objects (Figure 1E). In all mice NA levels during the 6 h of sleep deprivation showed a large and progressive increase above baseline levels in both mPFC and M1 (Figure 4A). When averaged across all mice for the entire 6 h, the relative increase in NA levels was similar in the 2 areas. However, NA levels in mPFC peaked earlier (Figure 4B) and declined during the last 1–2 h of sleep deprivation (Figure 4A), while in M1 they remained high until the end of the experiment. Specifically, across all mice NA peaked 230 ± 22.4 min after the sleep deprivation onset in mPFC (paired t-test, P = 0.042, Figure 4B), while in M1 the highest levels of NA were reached after 270 ± 23.2 min.

As in Experiment 1, the extent and the timing of NA changes varied across animals and differed between mPFC and M1 (Figure 4C). Thus, for each 30-min bin we calculated the average amount of muscle activity (EMG) and the number of objects presented to each mouse, and tested whether they were correlated with NA changes. In M1, relative NA levels correlated positively with both measures (EMG, r = 0.44, P = 0.0005; N of objects, r = 0.38, P = 0.001, Figure 4D, 4E upper panels). In mPFC, by contrast, relative NA levels were correlated with the number of objects (r = 0.38, P = 0.001), but not with muscle activity per se (r = 0.22, P = 0.09, Figure 4D, 4E lower panels).

NA in mPFC started to decline at different times in different mice (Figure 4C). To test whether inter-individual differences in sleep need could account for this finding we measured the EEG power in the 2 to 6 Hz range, a reliable marker of sleep pressure that increases with the duration of sleep deprivation.[23]

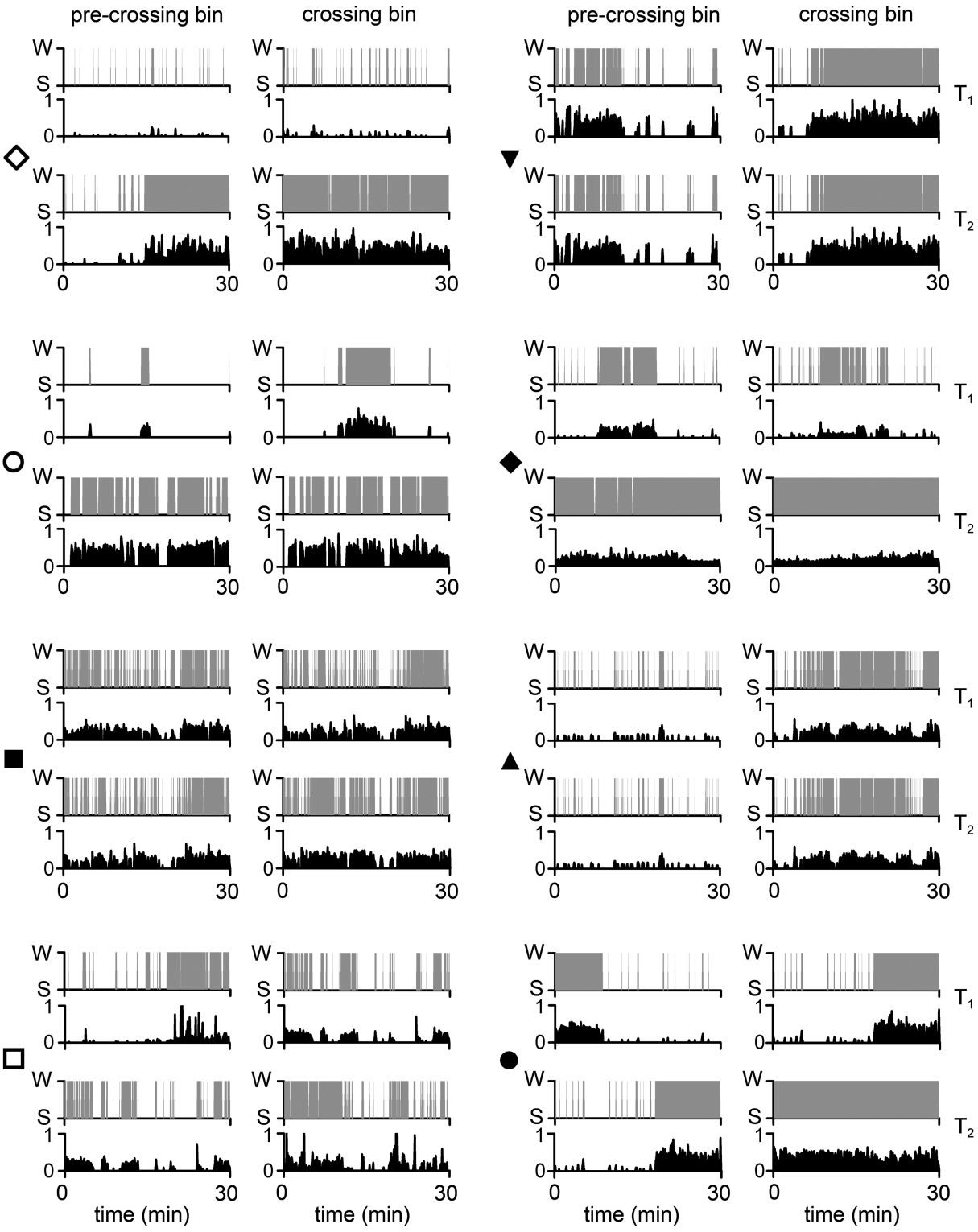

Figure 3—Individual differences in sleep/wake and cortical NA dynamics. Plots for each mouse showing wake (W) and sleep (S) activity (upper graphs) and normalized EMG activity (lower graphs) during the 30-min bin preceding baseline crossing and the 30-min bin in which baseline crossing occurred for M1 (upper rows) and mPFC (lower rows). Note that T_1 and T_2 coincide for mice (▲) and (▼).

We calculated the ratio between the 2–6 Hz EEG power after and before NA peaked in mPFC (see Methods for details), and tested whether it correlated with the time of the NA peak. We found a negative correlation, that is, NA levels started to decline earlier in those mice that showed larger (positive) ratios, indicating an increase in 2–6 Hz power ($r = -0.86$, $P = 0.02$, Figure 4F). Of note, the 2 mice with the earliest progressive decline in NA spent ~20% of the time asleep by the end of the

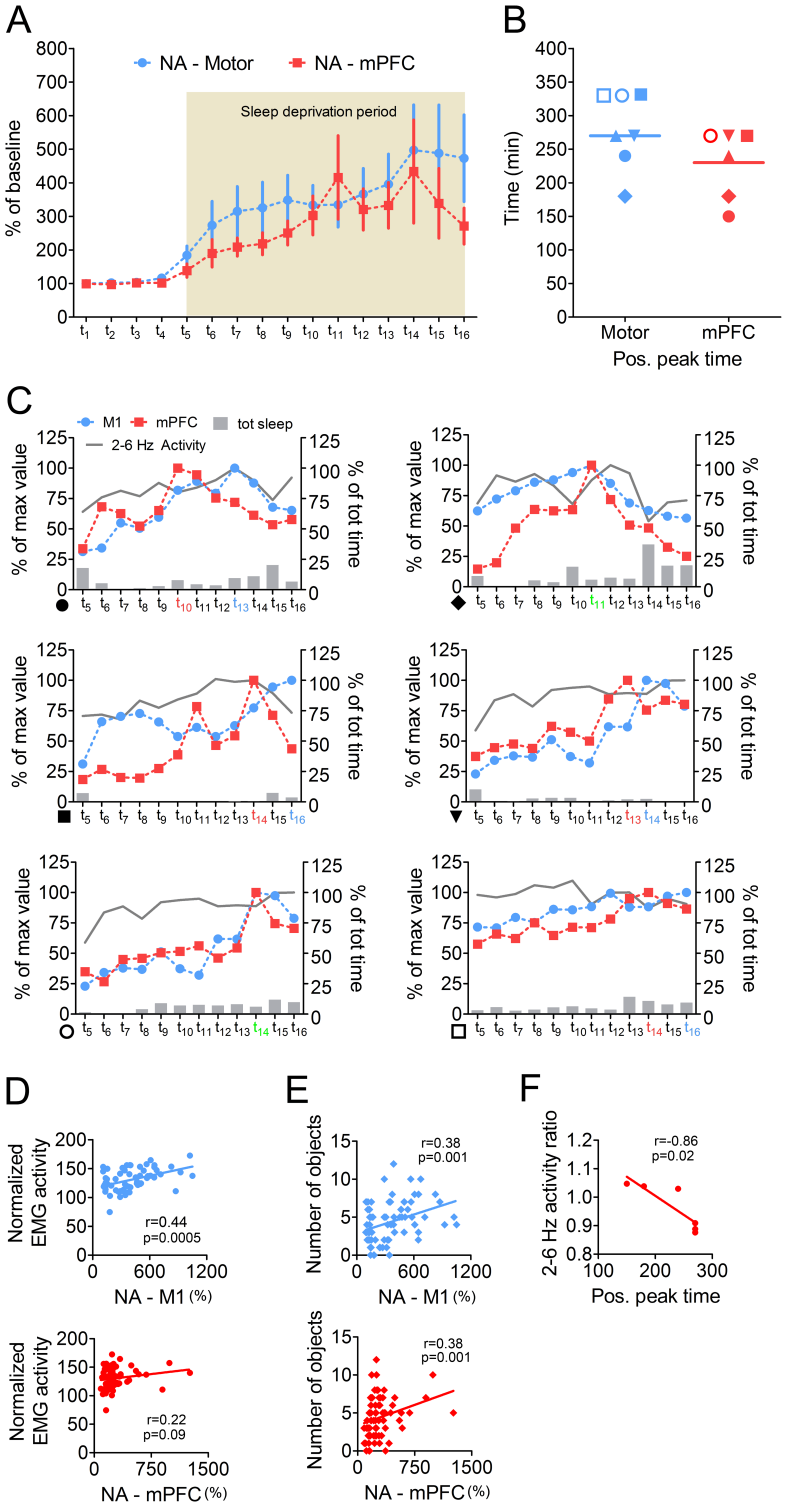

Figure 4—Changes in NA levels during sleep deprivation. **(A)** NA levels (mean ± SEM, n = 6) relative to baseline (average t_1–t_4) during the sleep deprivation experiment in M1 (blue) and mPFC (red). NA levels progressively increase during sleep deprivation (highlighted in light brown) in M1 (rmANOVA, $F_{12,60} = 28.25$, $P < 0.0001$, with t_9–t_{16} significantly higher than baseline; Bonferroni corrected $P < 0.0042$) and in mPFC (rmANOVA, $F_{12,60} = 13.18$, $P = 0.0019$, with t_{11} and t_{14} significantly higher than baseline; Bonferroni corrected $P < 0.0042$). **(B)** Positive peak (highest NA level) time from the onset of sleep deprivation in M1 (blue) and mPFC (red). **(C)** Changes in NA levels from t_5 to t_{16} for each individual mouse. Low frequency (2–6 Hz) EEG activity was computed over the entire period, averaged in 30-min bins, and depicted as a dark gray line. Total sleep is expressed as percentage of total time and indicated by gray bars. Time points indicating the highest NA levels for M1 and mPFC are indicated in the X axis in blue and red, respectively. Green is used when the highest NA levels coincide in M1 and mPFC. (D-E) Correlation between the relative NA levels and normalized EMG activity **(D)** or the number of presented objects **(E)** during the sleep deprivation in M1 (upper panel) and mPFC (lower panel). **(F)** Correlation between positive peak time in mPFC (corresponding to the onset of NA decline) and the 2–6 Hz EEG power ratio.

sleep deprivation, despite continuous attempts to keep them awake. The other mice instead, in which NA started to decline much later in the course of sleep deprivation, never spent more than 10% of the latest bins awake. Thus, in mPFC the decline in NA levels may reflect the build-up of sleep pressure during wake and may herald an irresistible need to go to sleep.

DISCUSSION

In this study we monitored changes in NA levels in two mouse cortical areas across the physiological sleep/wake cycle and in response to sleep deprivation, and correlated these changes with several behavioral and EEG parameters. LC neurons projecting to mPFC have increased excitability and higher firing rates than those targeting M1, and there is little overlap in their afferents.[19] These findings led to the prediction that NA levels should be higher in mPFC than M1.[19] To test this hypothesis we focused on NA levels during wake baseline, when behavior was standardized by keeping the mice on a slowly moving platform. All 14 animals included in Experiments 1 and 2 showed higher absolute levels of NA in mPFC than in M1, although fold increases ranged widely across mice. This variability may reflect to some extent true inter-individual biological differences. However, it may also be of technical origin, due to small variations in the recovery rate of each probe and/or its exact anatomical location. Independent of the exact cause, lower absolute NA levels in M1 may partly explain why the relative increase during wake was larger in this area than in mPFC.

As expected, we found that NA levels declined with sleep in both areas. The exact rate of this decline is difficult to assess and to relate to the rapid changes in behavioral state, given the difference in sampling rate between microdialysis (30 min) and EEG (4 sec). Despite this limitation, however, it is clear that in most mice (6 of 8) NA levels reached their nadir earlier in M1 than in mPFC. In addition, after declining in early sleep, NA levels started to rise much faster in M1 than in mPFC (~1 h earlier). Similarly, at the end of the sleep opportunity window NA levels returned to values typical of baseline wake earlier in M1 than in mPFC, and in some mice this increase occurred after periods of wake as short as 10 min. The quick dynamics in M1 is not surprising if one assumes that NA levels in this region are closely linked to locomotor activity, as suggested by the positive correlation between muscle activity and NA levels during sleep deprivation. More difficult to explain is why NA changes were slower in mPFC. For the slow build-up during wake, one clue is provided by the fact that NA levels in mPFC did not correlate with muscle activity but with the number of novel objects presented during sleep deprivation. This finding suggests that they may reflect the level of attention induced by exploratory activity, in line with the role of mPFC in attention.[15]

We previously found that the sleep-related decline in cortical levels of glutamate and lactate is positively correlated with the intensity of NREM sleep as measured by SWA.[24,25] Those studies used voltammetry, which has a temporal resolution of ~1 sec. Here, we found little evidence for a correlation between NA and SWA levels (a correlation was found only in M1 during the first hour of sleep), but because of the large difference in sampling rate between EEG and microdialysis these data should be interpreted with caution. Still, the data show that during the third (last) hour of sleep SWA levels were still declining as expected, while NA levels in both areas had reached the nadir and/or started to increase. It has been proposed that the decline in SWA during sleep reflects the occurrence of synaptic renormalization.[26] An alternative hypothesis, however, is that SWA progressively drops during sleep due to an increase in neuromodulatory tone, leading to an increase in neuronal excitability, but this interpretation is difficult to reconcile with our data.

To our knowledge, this is the first study that used in vivo microdialysis to assess dynamic changes in NA levels during sleep deprivation, in the cerebral cortex or any other brain region. Not surprisingly perhaps, NA levels were stable during baseline, a period presumably not long enough to trigger LC fatigue and during which mice were forced to maintain a very stereotypical behavior with little or no "distractions." Later, by contrast, during the longer period of free exploratory behavior (17:00–23:00), mice showed as expected constantly changing levels of interest towards the novel objects, associated with different levels of locomotor activity. To stay awake, mice were presented with a new object as soon as they lost interest for the old one. Mean tonic firing of LC neurons is low during wake and increases phasically in response to salient stimuli.[27,28] Thus, our sleep deprivation protocol presumably forced LC cells to sustain a high level of activity, which is unusual for these neurons. There is evidence that LC cells may not be well equipped to sustain prolonged periods of activity. Continuous optogenetic LC stimulation at 10 Hz for 10 min leads to a decline in NA levels in frontal cortex.[6] Moreover, in a mouse model of sleep apnea, chronic hypoxia/reoxygenation leads to loss of NA and dopaminergic cells but spares cholinergic, orexinergic, histaminergic, and serotonergic neurons.[29] Finally, after 8 hours of sleep deprivation repeated in two consecutive days, LC cells show signs of apoptotic activation and degeneration and their mitochondria are unable to mount the adaptive antioxidant response normally seen after short wake.[30] The idea that sleep may serve to restore the function of neurons "fatigued" during wake is an old one. Moruzzi, for instance, proposed that sleep may not concern the whole brain, but specifically allow the recovery of those brain cells and synapses that during wake have undergone plastic changes while supporting wake conscious activity.[31] Other authors have focused on LC cells and proposed that during wake NA stores may be depleted,[32–34] and/or NA receptors may undergo desensitization/downregulation.[35] In this view sleep, and more specifically REM sleep when LC cells are completely silent, would be required to restore noradrenergic functions. Recently, it was also suggested that sleep restores mitochondrial metabolic homeostasis disrupted in LC cells by sustained wake.[30]

We found that NA levels started declining by the end of sleep deprivation in mPFC but not in M1, and this decline correlated with an increase in low EEG frequencies, a sign of increased sleep pressure.[23] Thus, our results specifically suggest that LC neurons projecting to prefrontal cortex may be under higher pressure and show signs of fatigue more markedly or earlier than other LC cells. A few previous experiments in rats measured NA tissue levels either at the end of 11–20 days of total sleep deprivation using the disk-over-water method,[36] or

at three time points (8, 24, 72 h) during sleep deprivation using the small platform method, a procedure that prevents REM sleep without large effects on NREM sleep.[37] Rats subjected to prolonged total sleep loss showed no changes in NA tissue levels in frontal cortex and other brain areas, at least relative to yoked controls housed on the same disk-over-water apparatus, which were chronically sleep restricted.[36] On the other hand, REM deprived rats showed an early (at 8–24 but not 72 h) decrease in NA tissue levels in frontal cortex relative to controls housed in large platforms, an effect that was not observed in hippocampus and hypothalamus.[37] These last results should be interpreted with caution because tissue levels only indirectly reflect NA turnover, and "frontal cortex" was not anatomically defined in the study. These limitations withstanding however, the findings are consistent with our results that pinpoint to the LC cells that target mPFC as especially sensitive to sleep loss. Of note, vigilance, sustained attention, working memory, and cognitive flexibility, which depend on prefrontal cortex and are promoted by NA,[14,15] are also highly sensitive to sleep deprivation.[12,13] On the other hand, motor performance per se, that is engaging in forced locomotion or other motor routines that do not require attention, does not seem to be impaired by sleep loss. In fact, for both animals and humans locomotion remains among the most effective way to stay awake.

Why do LC cells fatigue? It could be that the underlying mechanism is intrinsic to these neurons, and/or that its origin may lie outside the LC itself. LC is part of a complex, heavily interconnected neuronal network that controls arousal and the sleep/wake cycle: it receives inputs from arousal-promoting orexinergic, histaminergic, dopaminergic, cholinergic, and serotonergic cells, and inhibitory GABAergic projections from the ventrolateral preoptic (VLPO) nucleus of the anterior hypothalamus.[38] Thus, the decline in prefrontal NA levels during sleep deprivation could reflect at least in part the fatigue of other arousal systems, and/or the increased activity of the sleep promoting VLPO area.[39] Indeed, a subset of VLPO neurons increases its firing by the end of sleep deprivation and before the onset of recovery sleep.[40] However, LC also inhibits VLPO neurons,[38] and thus the causal chain of effects is difficult to establish. Other microdialysis studies that have measured changes in arousal promoting neurotransmitters in the course of sleep deprivation may provide a clue, although in most cases their focus was outside the cerebral cortex. In the rat basal forebrain, histamine levels are low during sleep, quickly increase upon awakening, and remain elevated throughout 6 hours of sleep deprivation.[41] In the same region the levels of serotonin and dopamine metabolites (5-HIAA, DOPAC, HVA) gradually build up during the first 3 hours of sleep deprivation, remain high during the following 3 hours of forced wake, and decline only after at least one hour of recovery sleep.[42] In the cat anterior hypothalamus, histamine levels are also high throughout 6 hours of sleep deprivation.[43] Moreover in the rat hippocampus, two studies reported that serotonin levels remain high when sleep deprivation was enforced by exposure to novel objects for 4 hours[44] or by forced locomotion for 24 hours,[45] while one study found declining levels in hippocampus and frontal cortex during sleep deprivation by gentle handling.[46] Overall, these results suggest that histaminergic, dopaminergic, and serotonergic neurons, at least those projecting to several subcortical regions, are not easily fatigued during sustained wake.

LC receives a strong projection from orexin cells,[47] and at least in the rat, this input targets specifically the part of the LC that projects to mPFC.[48] A microdialysis study found that orexin levels in the rat hypothalamus and thalamus remained high throughout 6 hours of sleep deprivation,[49] suggesting that the orexinergic cells that project to these two areas do not fatigue during prolonged wake. It remains possible, however, that like LC cells, orexinergic cells are also heterogeneous, with those targeting the LC being more sensitive to fatigue. LC is also the target of a cholinergic projection from the pedunculopontine and laterodorsal tegmental nuclei of the brainstem,[38] but we are not aware of any study that measured acetylcholine levels during sleep deprivation in the cortex or any other brain region. Finally, there are reciprocal monosynaptic pathways between LC and prefrontal cortex, and thus it is possible that wake-related fatigue starts in mPFC, although direct evidence is missing. Overall, the available data in animals are more consistent with the idea that at least some LC neurons may be intrinsically unable to maintain high activity during sustained wake. In humans, pupillary size instability increases with time spent awake, and is being tested as a promising objective measure of sleepiness.[50] Since pupillary size in the dark is primarily controlled by LC activity,[38] these findings also point to the noradrenergic system as a primary target of wake fatigue.

The reasons why sleep pressure becomes irresistible during sleep deprivation remain unclear. One possibility is that the activity of the wake-promoting arousal systems cannot be maintained at high levels endlessly, but surprisingly this hypothesis had not been tested before for LC. In this study we find that this is indeed the case, i.e., LC neurons projecting to frontal cortex fatigue during sleep deprivation. Of note, this result contrasts with all the published data for other arousal systems, including histaminergic, dopaminergic and orexinergic systems, for which there is no evidence of fatigue during sustained wake. Thus, these findings may suggest one of the mechanisms underlying both the cognitive impairment and the increased sleep presure associated with sleep deprivation.

REFERENCES

1. Brown RE, Basheer R, McKenna JT, Strecker RE, McCarley RW. Control of sleep and wakefulness. Physiol Rev 2012;92:1087–187.
2. Takahashi K, Lin JS, Sakai K. Neuronal activity of histaminergic tuberomammillary neurons during wake-sleep states in the mouse. J Neurosci 2006;26:10292–8.
3. Takahashi K, Kayama Y, Lin JS, Sakai K. Locus coeruleus neuronal activity during the sleep-waking cycle in mice. Neuroscience 2010;169:1115–26.
4. Eschenko O, Magri C, Panzeri S, Sara SJ. Noradrenergic neurons of the locus coeruleus are phase locked to cortical up-down states during sleep. Cereb Cortex 2012;22:426–35.
5. Boucetta S, Cisse Y, Mainville L, Morales M, Jones BE. Discharge profiles across the sleep-waking cycle of identified cholinergic, GABAergic, and glutamatergic neurons in the pontomesencephalic tegmentum of the rat. J Neurosci 2014;34:4708–27.
6. Carter ME, Yizhar O, Chikahisa S, et al. Tuning arousal with optogenetic modulation of locus coeruleus neurons. Nat Neurosci 2010;13:1526–33.

7. Han Y, Shi YF, Xi W, et al. Selective activation of cholinergic basal forebrain neurons induces immediate sleep-wake transitions. Curr Biol 2014;24:693–8.
8. Adamantidis AR, Zhang F, Aravanis AM, Deisseroth K, de Lecea L. Neural substrates of awakening probed with optogenetic control of hypocretin neurons. Nature 2007;450:420–4.
9. Lee MG, Hassani OK, Alonso A, Jones BE. Cholinergic basal forebrain neurons burst with theta during waking and paradoxical sleep. J Neurosci 2005;25:4365–9.
10. Constantinople CM, Bruno RM. Effects and mechanisms of wakefulness on local cortical networks. Neuron 2011;69:1061–8.
11. Vyazovskiy VV, Cirelli C, Tononi G. Electrophysiological correlates of sleep homeostasis in freely behaving rats. Prog Brain Res 2011;193:17–38.
12. Goel N, Rao H, Durmer JS, Dinges DF. Neurocognitive consequences of sleep deprivation. Semin Neurol 2009;29:320–39.
13. Killgore WD. Effects of sleep deprivation on cognition. Prog Brain Res 2010;185:105–29.
14. Chamberlain SR, Robbins TW. Noradrenergic modulation of cognition: therapeutic implications. J Psychopharmacol 2013;27:694–718.
15. Spencer RC, Devilbiss DM, Berridge CW. The cognition-enhancing effects of psychostimulants involve direct action in the prefrontal cortex. Biol Psychiatry 2015;77:940–50.
16. Kalen P, Rosegren E, Lindvall O, Bjorklund A. Hippocampal noradrenaline and serotonin release over 24 hours as measured by the dialysis technique in freely moving rats: correlation to behavioural activity state, effect of handling and tail-pinch. Eur J Neurosci 1989;1:181–8.
17. Shouse MN, Staba RJ, Saquib SF, Farber PR. Monoamines and sleep: microdialysis findings in pons and amygdala. Brain Res 2000;860:181–9.
18. Park SP. In vivo microdialysis measures of extracellular norepinephrine in the rat amygdala during sleep-wakefulness. J Korean Med Sci 2002;17:395–9.
19. Chandler DJ, Gao WJ, Waterhouse BD. Heterogeneous organization of the locus coeruleus projections to prefrontal and motor cortices. Proc Natl Acad Sci U S A 2014;111:6816–21.
20. Berridge CW, Abercrombie ED. Relationship between locus coeruleus discharge rates and rates of norepinephrine release within neocortex as assessed by in vivo microdialysis. Neuroscience 1999;93:1263–70.
21. Bazzu G, Biosa A, Farina D, et al. Dual asymmetric-flow microdialysis for in vivo monitoring of brain neurochemicals. Talanta 2011;85:1933–40.
22. Ihalainen JA, Riekkinen P Jr., Feenstra MG. Comparison of dopamine and noradrenaline release in mouse prefrontal cortex, striatum and hippocampus using microdialysis. Neurosci Lett 1999;277:71–4.
23. Vyazovskiy VV, Olcese U, Hanlon EC, Nir Y, Cirelli C, Tononi G. Local sleep in awake rats. Nature 2011;472:443–7.
24. Dash MB, Douglas CL, Vyazovskiy VV, Cirelli C, Tononi G. Long-term homeostasis of extracellular glutamate in the rat cerebral cortex across sleep and waking states. J Neurosci. 2009;29:620–9.
25. Dash MB, Tononi G, Cirelli C. Extracellular levels of lactate, but not oxygen, reflect sleep homeostasis in the rat cerebral cortex. Sleep 2012;35:909–19.
26. Tononi G, Cirelli C. Sleep and the price of plasticity: from synaptic and cellular homeostasis to memory consolidation and integration. Neuron 2014;81:12–34.
27. Aston-Jones G, Bloom F. Activity of norepinephrine-containing locus coeruleus neurons in behaving rats anticipates fluctuations in the sleep-waking cycle. J Neurosci 1981;1:876–86.
28. Aston-Jones G, Bloom FE. Nonrepinephrine-containing locus coeruleus neurons in behaving rats exhibit pronounced responses to non-noxious environmental stimuli. J Neurosci 1981;1:887–900.
29. Zhu Y, Fenik P, Zhan G, et al. Selective loss of catecholaminergic wake active neurons in a murine sleep apnea model. J Neurosci 2007;27:10060–71.
30. Zhang J, Zhu Y, Zhan G, et al. Extended wakefulness: compromised metabolics in and degeneration of locus ceruleus neurons. J Neurosci 2014;34:4418–31.
31. Moruzzi G. The functional significance of sleep with particular regard to the brain mechanisms underlying consciousness. In: Eccles JC, ed. Brain and conscious experience. New York: Springer-Verlag, 1966.
32. Hartmann E. The D-state and norepinephrine-dependent systems. International psychiatry clinics. 1970;7:308–28.
33. Hartmann EL. Functions of sleep. Yale University Press, 1973.
34. Stern WC, Morgane PJ. Theoretical view of REM sleep function: maintenance of catecholamine systems in the central nervous system. Behav Biol 1974;11:1–32.
35. Siegel JM, Rogawski MA. A function for REM sleep: regulation of noradrenergic receptor sensitivity. Brain Res 1988;472:213–33.
36. Bergmann BM, Seiden LS, Landis CA, Gilliland MA, Rechtschaffen A. Sleep deprivation in the rat: XVIII. Regional brain levels of monoamines and their metabolites. Sleep 1994;17:583–9.
37. Porkka-Heiskanen T, Smith SE, Taira T, et al. Noradrenergic activity in rat brain during rapid eye movement sleep deprivation and rebound sleep. Am J Physiol 1995;268:R1456–63.
38. Szabadi E. Functional neuroanatomy of the central noradrenergic system. J Psychopharmacol. 2013;27:659–93.
39. Sherin JE, Shiromani PJ, McCarley RW, Saper CB. Activation of ventrolateral preoptic neurons during sleep. Science 1996;271:216–9.
40. Alam MA, Kumar S, McGinty D, Alam MN, Szymusiak R. Neuronal activity in the preoptic hypothalamus during sleep deprivation and recovery sleep. J Neurophysiol 2014;111:287–99.
41. Zant JC, Rozov S, Wigren HK, Panula P, Porkka-Heiskanen T. Histamine release in the basal forebrain mediates cortical activation through cholinergic neurons. J Neurosci. 2012;32:13244–4.
42. Zant JC, Leenaars CH, Kostin A, Van Someren EJ, Porkka-Heiskanen T. Increases in extracellular serotonin and dopamine metabolite levels in the basal forebrain during sleep deprivation. Brain Res 2011;1399:40–8.
43. Strecker RE, Nalwalk J, Dauphin LJ, et al. Extracellular histamine levels in the feline preoptic/anterior hypothalamic area during natural sleep-wakefulness and prolonged wakefulness: an in vivo microdialysis study. Neuroscience 2002;113:663–70.
44. Penalva RG, Lancel M, Flachskamm C, Reul JM, Holsboer F, Linthorst AC. Effect of sleep and sleep deprivation on serotonergic neurotransmission in the hippocampus: a combined in vivo microdialysis/EEG study in rats. Eur J Neurosci 2003;17:1896–906.
45. Lopez-Rodriguez F, Wilson CL, Maidment NT, Poland RE, Engel J. Total sleep deprivation increases extracellular serotonin in the rat hippocampus. Neuroscience 2003;121:523–30.
46. Bjorvatn B, Gronli J, Hamre F, et al. Effects of sleep deprivation on extracellular serotonin in hippocampus and frontal cortex of the rat. Neuroscience 2002;113:323–30.
47. Peyron C, Tighe DK, van den Pol AN, et al. Neurons containing hypocretin (orexin) project to multiple neuronal systems. J Neurosci 1998;18:9996–10015.
48. Del Cid-Pellitero E, Garzon M. Hypocretin1/OrexinA-containing axons innervate locus coeruleus neurons that project to the Rat medial prefrontal cortex. Implication in the sleep-wakefulness cycle and cortical activation. Synapse 2011;65:843–57.
49. Yoshida Y, Fujiki N, Nakajima T, et al. Fluctuation of extracellular hypocretin-1 (orexin A) levels in the rat in relation to the light-dark cycle and sleep-wake activities. Eur J Neurosci 2001;14:1075–81.
50. Peters T, Gruner C, Durst W, Hutter C, Wilhelm B. Sleepiness in professional truck drivers measured with an objective alertness test during routine traffic controls. Int Arch Occup Envir Health 2014;87:881–8.
51. Franklin KBJ, Paxinos G. Paxinos and Franklin's the Mouse Brain in Stereotaxic Coordinates, Fourth Edition, 4th ed. Amsterdam: Academic Press, 2013.

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