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Molecular and electrophysiological evidence for net synaptic potentiation in wake and depression in sleep

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Plastic changes occurring during wakefulness aid in the acquisition and consolidation of memories. For some memories, further consolidation requires sleep, but whether plastic processes during wakefulness and sleep differ is unclear. We show that, in rat cortex and hippocampus, GluR1-containing AMPA receptor (AMPAR) levels are high during wakefulness and low during sleep, and changes in the phosphorylation states of AMPARs, CamKII and GSK3β are consistent with synaptic potentiation during wakefulness and depression during sleep. Furthermore, slope and amplitude of cortical evoked responses increase after wakefulness, decrease after sleep and correlate with changes in slow-wave activity, a marker of sleep pressure. Changes in molecular and electrophysiological indicators of synaptic strength are largely independent of the time of day. Finally, cortical long-term potentiation can be easily induced after sleep, but not after wakefulness. Thus, wakefulness appears to be associated with net synaptic potentiation, whereas sleep may favor global synaptic depression, thereby preserving an overall balance of synaptic strength.

The brain is remarkably plastic, and plastic changes occur not only as a result of experience and learning, but even in response to variations in spontaneous activity[1,2]. Persistent plastic changes in neural circuits, such as long-term potentiation (LTP) and depression (LTD) of synaptic strength, involve a complex series of molecular and cellular mechanisms, including receptor delivery and phosphorylation. These changes are associated in turn with an increased or decreased efficacy of synapses, as measured by the strength of postsynaptic currents[3].

In principle, synaptic potentiation and depression could occur in parallel, ensuring that total synaptic strength remains balanced at all times. Indeed, large imbalances, especially in the direction of potentiation, could be detrimental because of known constraints related to energy, space, cellular supplies and saturation of the ability to learn[4,5]. Alternatively, a homeostasis of synaptic strength could be maintained over time by alternating phases of predominant potentiation with phases of predominant depression. It was suggested recently that, at least in adult animals, waking plasticity may be associated preferentially with net synaptic potentiation in the cerebral cortex and other brain regions, whereas sleep, especially non–rapid eye movement (NREM) sleep, would redress the balance by favoring global synaptic depression or downscaling[5]. However, this possibility has not been tested directly. Moreover, several learning studies that showed performance improvements after sleep have prompted the idea that sleep may favor the strengthening of neural circuits that are activated during the prior waking period[6,7]. Thus, it is currently a matter of great interest whether plastic processes during waking and sleep differ, and if so, in which way. Here we provide molecular and electrophysiological evidence in rats suggesting that periods of wakefulness are associated with a net increase in cortical synaptic strength and periods of sleep are associated with a net decrease.

RESULTS

GluR1-containing AMPAR levels

To shed light on these issues, we employed a combined molecular and electrophysiological approach. At the molecular level, the best established mechanism for the expression of LTP and LTD involves the trafficking of postsynaptic glutamatergic AMPARs containing the GluR1 subunit[8,9]. The delivery of these receptors to excitatory synapses is associated with increases in synaptic strength, whereas their removal from synapses is associated with synaptic depression[8,9]. These changes have been recently described *in vivo* in the cerebral cortex, hippocampus and amygdala, where they can be triggered by learning and natural experience[10–14]. More specifically, recent *in vivo* studies have shown that LTP and learning are associated with the delivery of AMPARs to synaptoneurosomes, a preparation that enriches for synaptic proteins, whereas LTD is associated with their removal[10,15]. To compare the density of GluR1-containing AMPARs between sleep and wakefulness, we implanted adult rats with bipolar concentric local field potential (LFP) electrodes for chronic polysomnographic recordings and assigned them to the waking group if they remained spontaneously awake for >75% of the first 6 h of the dark period (**Fig. 1a**; see Methods). Other rats were assigned to the sleep group if they remained asleep for >75% of the first 6 h of the light period (**Fig. 1a**). We measured protein levels of GluR1-containing AMPARs in the cerebral cortex by western blot in both total homogenates and cortical synaptoneurosomes (**Fig. 1b**). The

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entire left cortical hemisphere was sampled, as we found in previous experiments that, when rats wake up, the expression of activity-dependent, plasticity-related genes such as *Fos, Egr1* (also known as *Ngfi-A*), *Arc*, phosphorylated *Creb1* and *Bdnf* increase over most cortical regions, including frontal, parietal, temporal and occipitals areas[16]. Compared with the sleep group, the waking group showed a nearly 50% increase in total GluR1 levels in synaptoneurosomes (46 ± 12%, mean ± s.e.m., paired *t*-test, $P = 0.0015$; **Fig. 1b**). In contrast, no changes were observed in total homogenates between sleeping and waking animals in cortical GluR1 levels (data not shown), which is consistent with previous reports *in vivo* (for example, see refs. 10,15). For this reason, all of the remaining western blots were run in synaptoneurosomes. Protein levels of glutamatergic NMDA NR2A subunits, which can promote surface expression of GluR1-containing AMPARs[17], also showed a trend toward higher values in the waking group (**Fig. 1b**).

The net increase of GluR1-containing AMPARs shown here is similar in magnitude to increases triggered by learning and plasticity procedures *in vivo*[10–13,15]. This suggests that, on average, cortical glutamatergic synapses may be stronger after periods of wakefulness and weaker after periods of sleep.

AMPA and CaMKII phosphorylation

Is the net change in synaptic GluR1-containing AMPARs a result of the occurrence of synaptic potentiation during waking, the occurrence of synaptic depression during sleep or of both factors? To shed some light on these possibilities, we studied specific changes in the phosphorylation levels of the GluR1 subunit. These changes are known to affect the surface incorporation and the channel properties of AMPARs. *In vivo*, synaptic potentiation is usually associated with an increase in GluR1 phosphorylation at Ser831 (refs. 10,18), which enhances single-channel conductance[8]. Compared with the sleeping group, the waking group showed increased absolute levels of GluR1 phosphorylation at Ser831 (63 ± 11%, $P = 0.0001$; **Fig. 1b**), whereas the relative levels (normalized to total GluR1) did not change (20 ± 15%, $P = 0.77$). Ser831 is a substrate for calcium/calmodulin-dependent protein kinase II (CaMKII), and increased levels of CaMKII and CamKII phosphorylation at Thr286 are also associated with synaptic potentiation and LTP, both *in vitro* and *in vivo*[19]. Compared with the sleeping group, the waking group showed increased levels of CamKII that was phosphorylated at Thr286 (39 ± 13%, $P = 0.016$; **Fig. 1b**), whereas the increase in total CamKII protein levels did not reach significance (waking, 16 ± 10% of sleeping, $P = 0.34$). Consistent with previous studies[16], we also found that waking animals had higher levels of BDNF, Arc, NGFI-A and P-CREB, which are usually associated *in vivo* with LTP (data not shown).

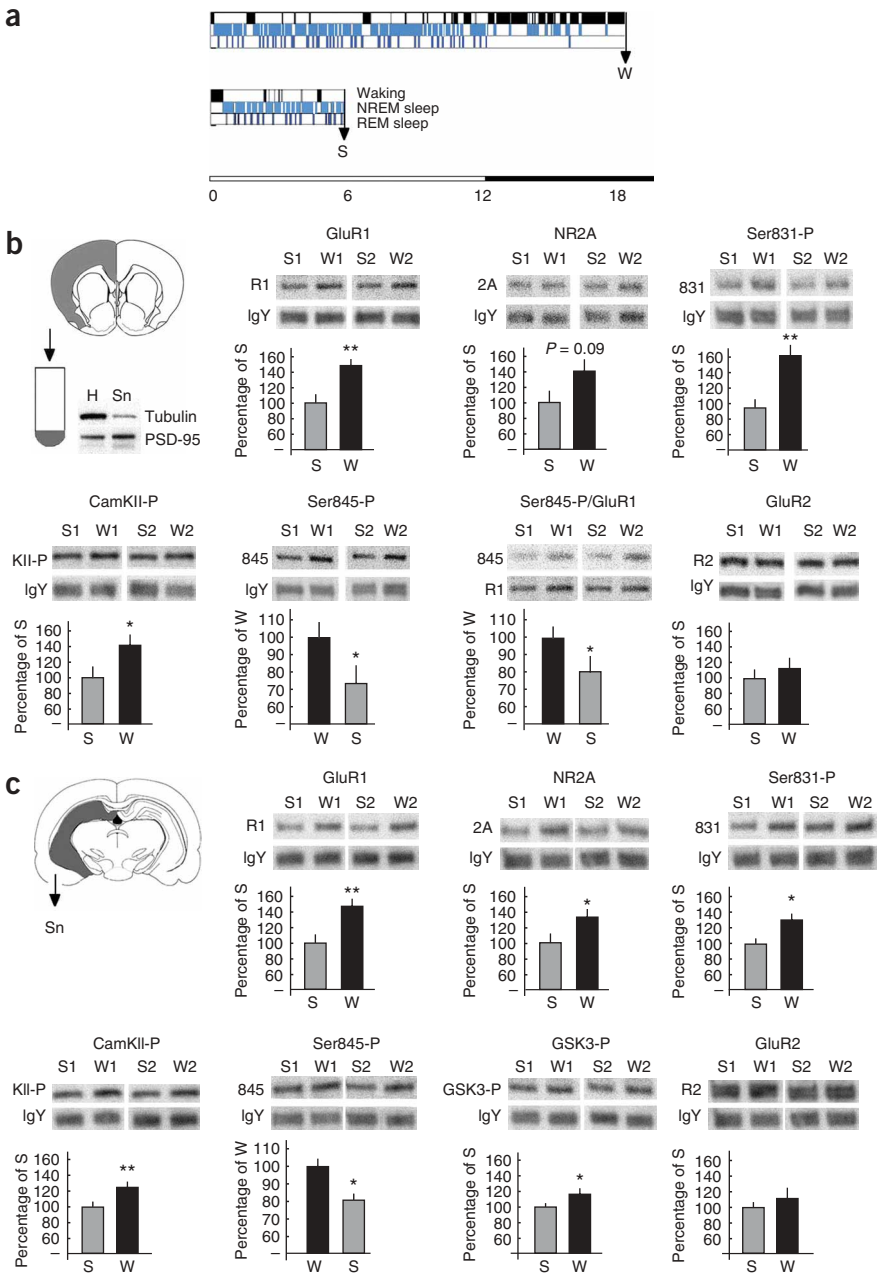

Figure 1 Molecular correlates of LTP/LTD in wakefulness and sleep. (**a**) Hypnograms from representative rats of the two experimental groups (S, sleep; W, wakefulness). In this and the following figures, the white and black bars indicate the light and dark period, respectively. Rats are spontaneously asleep for most of the light period and awake for most of the dark period. The last 6–18 h before killing (indicated by an arrow) are shown. (**b**) Cortical synaptoneurosomes (Sns) were prepared from the entire left cerebral cortex of each rat. Enrichment for synaptic proteins in Sns relative to homogenates (H) in one representative rat: tubulin levels decreased and PSD-95 levels increased. Representative immunoblots and quantification of the gels are shown. Values are mean ± s.e.m. ($n = 9$ rats per group). For display purposes, S (or W) values are expressed as 100% ± s.e.m. of average S (or W). IgY was used as loading control. $^*P < 0.05$; $^{**}P < 0.01$ (paired *t*-test). (**c**) Sns were prepared from the entire left hippocampus of each rat (same animals as in **b**). Immunoblots (1 and 2 represent two different representative samples) and quantification of the gels are shown. Values are mean ± s.e.m. ($n = 9$ rats per group). $^*P < 0.05$; $^{**}P < 0.01$ (paired *t*-test).

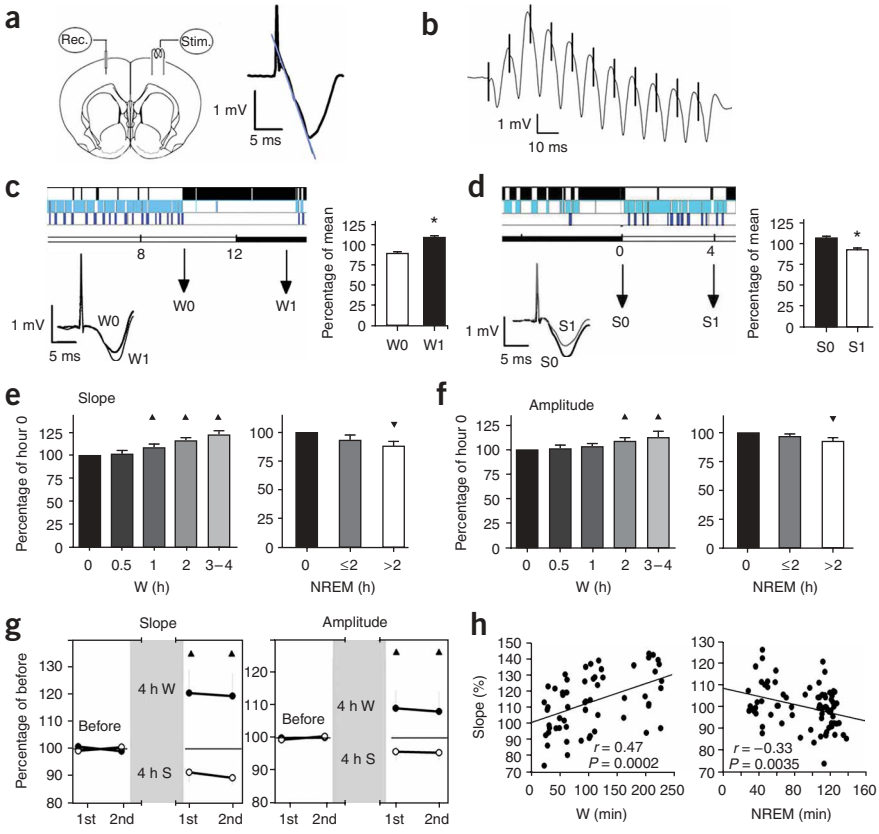

Figure 2 Electrophysiological correlates of LTP and LTD in wakefulness and sleep. (**a**) Left, setup for stimulation and EEG recordings. Right, a representative trace of the first component of the evoked LFP. (**b**) Example of the LFPs evoked by consecutive stimuli delivered every 10 ms (thick vertical bars). (**c**) Left, hypnogram from one representative rat depicting the light-to-dark transition. Arrows indicate the timing of LFP collection at the beginning of a spontaneous waking episode of ~4 h (W0) and at its end (W1). Inset, individual representative LFPs from one rat. Right, slope of evoked responses at W0 and W1 (always in quiet wakefulness). Values are mean ± s.e.m. ($n = 13$, $P < 0.05$, two-tailed paired *t*-test; each bar is shown as the percentage of the mean between the two bars). Percentages of behavioral states (mean ± s.e.m.): light period, wakefulness = 23.6 ± 1.5, NREM sleep = 60.1 ± 1.5, REM sleep = 15.5 ± 0.9; dark period, wakefulness = 80.6 ± 5.4, NREM sleep = 17.0 ± 4.7, REM sleep = 2.3 ± 0.7. (**d**) Left, representative hypnogram as in **c**. Evoked responses were collected at the beginning of a spontaneous sleep period (S0) and after a ~4-h period consisting of at least 2 h of NREM sleep (S1). Right, mean values (± s.e.m.) of the slope of LFPs at S0 and S1 expressed as the percentage of the mean between S0 and S1 ($n = 13$). *$P < 0.05$, paired two-tailed *t*-test. Inset, individual representative LFPs from one rat. The magnitude of change after waking and after sleep compared with the corresponding 0 h was similar (S0 – S1 versus W1 – W0, $P = 0.26$, unpaired *t*-test). (**e**) Mean values of the LFP slopes (± s.e.m.) recorded after 0.5, 1, 2 and 3–4 h of continuous wakefulness ($n = 12$–19 rats per group), and after ≤2 or >2 h of NREM sleep ($n = 10$–13). Triangles indicate significant differences from the first session (0 h = 100%). (**f**) Mean values (± s.e.m.) of LFP amplitudes recorded after 0.5–4 h of continuous wakefulness and after ≤2 or >2 h of NREM sleep (same rats as in **e**). Triangles indicate significant differences relative to the first session (0 h = 100%). (**g**) Mean values (± s.e.m.) of the slope and amplitude of evoked responses before and after 4 h of wakefulness (filled circles, $n = 13$) or 4 h of sleep (open circles, $n = 23$). LFPs collected during the first (1st, responses 1–20) and the second half (2nd, responses 21–40) of each session are plotted separately to show their intrasession stability. Values are percentage changes relative to the average of the first and the second half of the 'before' session (100%). Triangles indicate significant differences between slopes after wakefulness relative to slopes after sleep ($P < 0.05$, unpaired two-tailed *t*-test). The increase in slope and amplitude after wakefulness, and their decrease after sleep, showed a significant interaction (vigilance state × time point) in a two-way ANOVA (slopes: *F* value, 19.73, $P < 0.001$; amplitudes: *F* value, 6.38, $P < 0.05$). (**h**) Left, relationship between the slopes of evoked responses and the duration of the preceding period of continuous wakefulness ($n = 25$ rats). Slopes are expressed as percentage of the first session (hour 0) as in **e**. Right, relationship between the slopes of evoked responses and the amount of NREM sleep during the preceding 3-h period ($n = 26$ rats). The slopes for each time point are expressed as percentages of the mean value between all the time points in each rat. Lines depict a linear regression (Pearson correlation).

Suggested molecular fingerprints of synaptic depression, on the other hand, include the dephosphorylation of GluR1 at Ser845, which occurs both *in vitro* and *in vivo*[15,20] and leads to a decrease in the channel open probability and to AMPARs internalization[8]. Both absolute (74 ± 10% of waking, $P < 0.026$) and relative (80 ± 9% of waking, $P < 0.036$) levels of GluR1 phosphorylation at Ser845 were lower in the sleeping group relative to the waking group (**Fig. 1b**).

Similar changes in cerebral cortex and hippocampus

Wakefulness and sleep are global phenomena associated with changes in firing patterns and neuromodulation in most forebrain structures, including the hippocampal formation[21]. Do molecular correlates of synaptic potentiation in wakefulness and depression in sleep extend to brain areas other than the cerebral cortex? To find out, we measured the density and phosphorylation levels of AMPARs in hippocampal synaptoneurosomes that were prepared from the same animals in which we tested cortical synapsoneurosomes. We confirmed most of the results that we obtained in the cerebral cortex in the hippocampus, with a few notable differences (**Fig. 1c**). For example, NR2A levels in the hippocampus showed a significant increase in waking relative to sleeping ($P < 0.05$), whereas in the cerebral cortex there was only a trend ($P = 0.09$). We also examined the phosphorylation at Ser9 of glycogen synthase kinase–3β (GSK3β) in the hippocampus, an enzyme associated with native AMPARs. GSK3β phosphorylation at Ser9 has recently been shown to increase during hippocampal LTP and decrease during LTD, with no change in total expression[22]. We found that hippocampal protein levels of GSK3β phosphorylated at Ser9 were significantly higher in waking than in sleeping rats (18 ± 5%, $P = 0.018$; **Fig. 1c**), whereas total GSK3β levels were similar (data not shown).

It should be noted that, in both cerebral cortex and hippocampus, changes in GluR1 levels between wakefulness and sleep were not associated with similar changes in GluR2 levels (**Fig. 1b,c**). Unlike GluR2-containing AMPARs, GluR2-lacking AMPARs are permeable to calcium, and may be crucial for LTP maintenance[23]. Although some previous *in vivo* studies found parallel changes in both subunits[10,15,20], others observed a selective increase in GluR1-containing AMPARs[11–13,24]. In the hippocampus, this increase disappeared 25 min after electrically induced LTP[24], whereas in the cerebral cortex it was

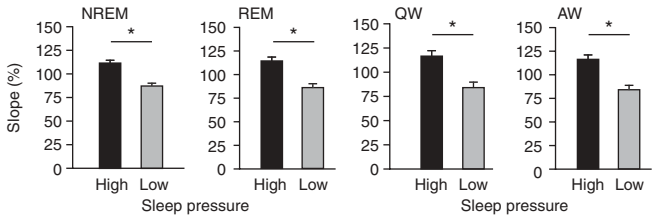

Figure 3 Effect of behavioral state on the LFP responses. LFPs were collected during spontaneous NREM sleep, REM sleep, quiet waking (QW) and active waking (AW). Mean values of LFPs slopes recorded in the first 1–2 h after light onset (high sleep pressure) and after 4–6 h of undisturbed sleep (low sleep pressure) in the four behavioral states. For each individual animal, the values of the slopes for each of the two conditions (High and Low) are expressed as the percengate of the mean between them before averaging among animals. Values are mean ± s.e.m. ($n = 9$ rats, $^*P < 0.05$, two-tailed paired *t*-test).

still present after 1 d of single-whisker stimulation[11], or after 7 d of dark rearing[13]. Thus, whether and when GluR1-containing AMPARs are eventually replaced by GluR2-containing AMPARs remains an open question and may depend on experimental conditions[23]. In summary, wakefulness is associated with an increased number of AMPAR GluR1 subunits and with an increased expression of phosphorylated CamKII. Sleep is associated instead with a decreased number of AMPAR GluR1 subunits and with the dephosphorylation of GluR1 at Ser845. These data provide molecular evidence that is consistent with the occurrence of net synaptic potentiation during wakefulness and synaptic depression during sleep in two large forebrain areas, the cerebral cortex and the hippocampus.

Cortical evoked responses

Although molecular markers of LTP and LTD are strongly indicative of corresponding changes in synaptic strength, it is important to determine directly whether synaptic efficacy is altered and whether it is altered *in vivo*. In freely behaving animals, this can be done by measuring LFP responses that are evoked by electrical stimulation. Specifically, the strength of population excitatory postsynaptic currents is reflected by the slope of LFPs evoked by electrical stimuli[25]. Accordingly, *in vivo* LTP-inducing procedures increase LFP slope[26,27], whereas LTD procedures reduce it (for example, see ref. 28). To evaluate changes in synaptic efficacy as a function of preceding waking and sleeping, we implanted two other groups of animals with bipolar concentric electrodes for electrical stimulation and chronic intracortical LFP recordings. We recorded LFPs from the left frontal cortex after electrical stimulation of the right frontal cortex (**Fig. 2a**). We then measured the slope of the first negative component of this transcallosal evoked response. We focused on the cerebral cortex because, together with the thalamus, it generates the characteristic electrical rhythms of mammalian sleep[1], and it has a central role in several hypotheses concerning the functions of sleep (see references in ref. 5). Also, the frontal cortex, relative to other cortical regions, shows the most substantial increase in sleep pressure after prolonged wakefulness, as measured by the increase in slow-wave activity (SWA) in the cortical electroencephalogram (EEG) (EEG power between 0.5 and 4 Hz)[29]. Furthermore, the impaired functioning of frontal cortical areas is thought to underlie several of the cognitive defects observed after sleep deprivation (see references in ref. 5). We focused on the transcallosal response because the corpus callosum consists of a distinct, isolated and homogenous bundle of excitatory fibers, and thus the early monosynaptic component of the evoked response can be identified. Indeed, this early component followed high-frequency stimulation rates up to 100 Hz (**Fig. 2b**) and met several other criteria used to define monosynaptic responses (**Supplementary Fig. 1** online). To avoid confounding effects of behavioral state on evoked responses, we carefully monitored the behavior of each rat using LFPs, muscle activity and direct visual observation, and recorded the evoked LFPs under standardized conditions of quiet wakefulness (see Methods). In this way, we could examine the effects of the preceding sleep-waking history on evoked LFPs that had been recorded in exactly the same behavioral state.

When sleep pressure is low, usually at the end of the light phase, some rats wake up and stay awake for a consolidated period of several hours. Conversely, at the beginning of the light phase, when sleep pressure is high, some animals enter a consolidated period of sleep that may last for 2 h or more. When we measured LFP responses in rats that had remained awake without interruption for 3–4 h between two LFPs recording sessions, we found that the slope of the first LFP component increased, on average, by 22% from the beginning to the end of the continuous waking period (**Fig. 2c**; for the distribution of individual responses, see **Supplementary Fig. 2** online). The increase in slope was also observed after equating for response amplitude (**Supplementary Fig. 3** online). In contrast, for rats that had been almost continuously asleep during the first 4 h of the light phase, the slope of the first LFP component decreased by almost 20% between the two recording sessions (**Fig. 2d**; for the distribution of individual responses, see **Supplementary Fig. 2**). Again, the decrease in slope was present after equating for response amplitude (**Supplementary Fig. 3**). Because some rats remained spontaneously awake for only 0.5–2 h, we recorded LFPs at shorter time intervals in these animals (after 0.5, 1 and 2 h of wakefulness). Overall, by analyzing all responses after spontaneous waking periods ranging from <30 min to ~ 4 h, we found a progressive increase in the slope that became significant after 1 h of wakefulness (**Fig. 2e**, one-way ANOVA, factor 'waking duration', $F = 10.0$, $P < 0.001$) and increased substantially by the end of the waking period. Conversely, the slope of the responses decreased significantly ($P = 0.019$) after at least 2 h of continuous sleep (**Fig. 2e**). Thus, an electrophysiological indicator of synaptic efficacy, the slope of the early component of cortical evoked potentials, was high after spontaneous wakefulness and low after spontaneous sleep. Moreover, we observed that the longer the preceding period of continuous wakefulness, the larger the increase in slope. In contrast, the longer the preceding period of sleep, the smaller the slope of the response. Notably, in our experiments, rats were never spontaneously awake for more than 3–4 h, as expected because they are polyphasic animals. These periods of continuous wakefulness, however, were sufficient to produce changes in the slope of the evoked response of $\sim 20\%$. Changes of similar magnitude have been reported in a recent *in vivo* study in the hippocampus after physiological learning[10] (*in vitro*, electrically induced hippocampal LTP is associated with slope increases of 100% or more; for example, see ref. 30). We also measured changes in the amplitude of the evoked responses, as amplitude is often used as an additional marker of synaptic efficacy[31]. We found that the amplitude of the evoked LFPs also increased after 4 h of wakefulness and decreased after 2 h of sleep, although to a lesser extent than the slope (**Fig. 2f**). Notably, both the slope and amplitude of LFPs were stable in each recording session, indicating that no measurable plastic changes occurred in relation to the stimulation procedure *per se* (**Fig. 2g**).

Finally, we examined whether the slope of the evoked responses and the duration of the periods of continuous wakefulness or the amount of NREM sleep during the preceding 3–4 h were correlated. To do so, we collected evoked responses approximately every 4 h during

the 12-h light period (**Fig. 2h**). We found a significant correlation in both cases, the slope of the response was highly positively correlated with the duration of the preceding period of continuous waking and negatively correlated with the amount of NREM sleep (waking, $r = 0.47$, $P = 0.0002$; NREM, r = –0.33, $P = 0.0035$; **Fig. 2h**). The slope of the response was also negatively correlated with the amount of the preceding REM sleep, but not as strongly as with NREM sleep ($r = –0.24$, $P = 0.037$). There was no significant correlation between the slope of the response and the ratio REM/NREM sleep in the preceding 3-h period ($r = –0.2$, $P = 0.09$; **Supplementary Fig. 4** online), suggesting that NREM is more directly related than REM sleep to the observed decrease in slope (NREM and REM sleep are strongly correlated with each other).

Cortical evoked responses and behavioral state

Although all recordings were carried out under strictly controlled behavioral conditions of quiet wakefulness (animal immobile, eyes open, low muscle tone, low-voltage, high-frequency cortical EEG activity), it is possible that the sleep-waking history of the animal may affect evoked LFPs not only by modifying synaptic efficacy, but also through subtle changes in neuromodulation and neuronal excitability that might occur between high and low sleep-pressure conditions. To address this issue, we carried out additional experiments ($n = 9$ rats) by collecting evoked LFPs under both high and low sleep pressure in four different behavioral states (active wakefulness, quiet wakefulness, NREM sleep and REM sleep) that are associated with marked changes in neuromodulation and excitability[32]. The results showed that, although the amplitude of the early component of evoked LFPs increased as expected[33] in NREM sleep relative to all the other behavioral states (one-way ANOVA, factor 'behavioral state'; high sleep pressure $F = 3.2$, $P = 0.04$; *post hoc* *t*-test, $P < 0.005$), the average latency remained unchanged in all behavioral states (one-way ANOVA, factor 'behavioral state'; high sleep pressure. $F = 0.2$, $P = 0.90$; low sleep pressure, $F = 0.04$, $P = 0.99$). Crucially, the mean slope of the early component of evoked LFPs did not differ across the four behavioral states (one-way ANOVA, factor 'behavioral state'; high sleep pressure, $F = 0.3$, $P = 0.83$; low sleep pressure, $F = 0.3$, $P = 0.81$), indicating that major neuromodulatory changes had negligible effects on this parameter. In contrast, when we compared the LFPs collected under high sleep pressure (in the first 2 h after light onset) and under low sleep pressure (after 4–6 h of undisturbed sleep) separately for each of the four behavioral states, the slope of evoked LFPs decreased by 15–20% not only in quiet wakefulness, confirming our previous results, but also in active wakefulness, NREM sleep and REM sleep (two-tailed paired *t*-test, $P < 0.05$; **Fig. 3**). Thus, this experiment indicates that the decrease in the LFP slope after a sustained period of sleep is independent of the actual behavioral state during which the responses were collected, and is unlikely to be accounted for by changes in neuromodulation and excitability.

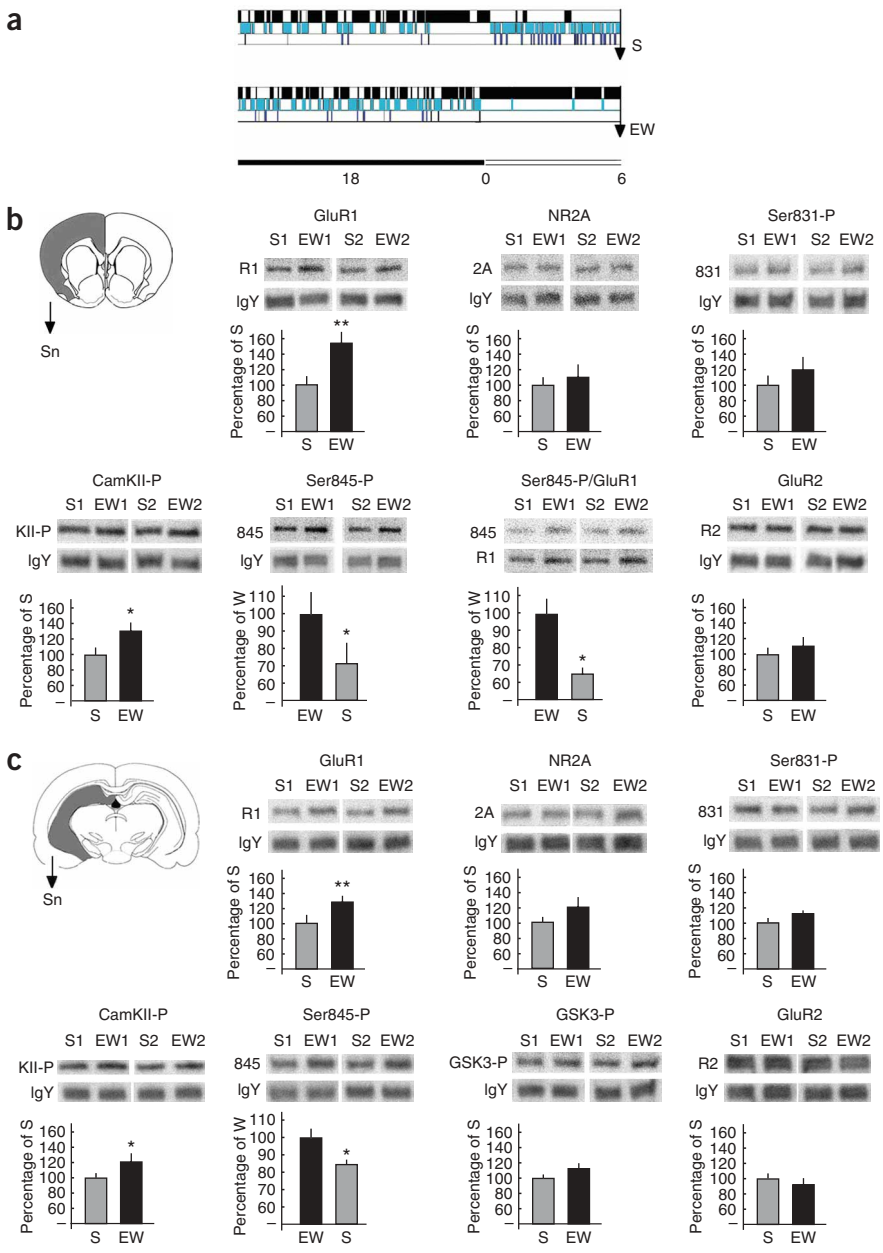

Figure 4 Molecular correlates of LTP/LTD after enforced wakefulness. (**a**) Hypnograms from representative rats of the two experimental groups (S, sleep; EW, enforced wakefulness). The last 18 h before sacrifice (indicated by an arrow) are shown. (**b**) Cortical synaptoneurosomes: representative immunoblots and quantification of the gels. Values are mean ± s.e.m. ($n = 10$ rats per group). For display purposes, S (or EW) values are expressed as 100% ± s.e.m. of average S (or EW). IgY was used as loading control. $^*P < 0.05$; $^{**}P < 0.01$ (paired *t*-test). (**c**) Hippocampal synaptoneurosomes. Immunoblots (1 and 2 represent two different representative samples) and quantification of the gels are shown. Values are mean ± s.e.m. ($n = 10$ rats per group; same animals as in **b**). $^*P < 0.05$; $^{**}P < 0.01$ (paired *t*-test).

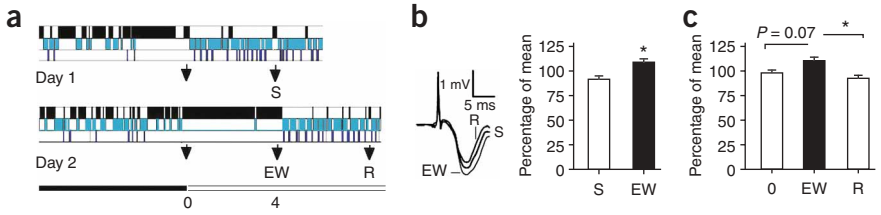

Figure 5 Electrophysiological correlates of LTP/LTD after enforced wakefulness. (**a**) Hypnogram from one representative rat recorded across 2 consecutive d (only 12–16 h per d are shown). Evoked responses were recorded (always in quiet wakefulness) during day 1 at light onset (0) and after 4 h of sleep (S), and during day 2 at light onset (0), after 4 h of enforced wakefulness (EW), and after 4 h of recovery sleep (R). (**b**) Left, individual representative LFPs from one rat. Right, changes in LFPs slope between S and EW. As in **Figure 2**, for each individual animal, the values of the slopes for each of the two conditions (S and EW) are expressed as the percentage of the mean between them before averaging among animals. Values are mean ± s.e.m. ($n = 13$ rats, $^*P < 0.05$, two-tailed paired *t*-test; the bars are shown as the percentage of the mean between S and EW). (**c**) Changes in LFPs slope between 0, EW and R. Values are mean ± s.e.m. ($n = 13$ rats, $^*P < 0.05$, two-tailed paired *t*-test).

Cortical evoked responses and brain temperature

Changes in brain temperature of as low as few degrees can alter evoked responses[34,35], raising the issue of whether the increase in slope that we observed after prolonged waking could be due to, at least in part, an increase in cortical temperature. Passive or active heating of the brain increases the slope and decreases the latency of the evoked response, and decreases the amplitude of the population spike[34]. However, in our experiments, both the slope and amplitude of the downward (**Fig. 2**) and upward (data not shown) segments of the LFP increased after prolonged wakefulness, and the change in the slope persisted after equating evoked responses on the basis of their latency (**Supplementary Fig. 3**). To investigate specifically whether brain temperature changes in parallel with the LFP slope, we recorded brain temperature in a subset of animals ($n = 7$). In all of them, temperature increased rapidly after the transition from sleep to wakefulness by 1.4 ± 0.2 °C, and remained stable for the entire duration of the waking episode (one-way ANOVA, factor 'waking duration', $F = 0.63$, n.s., **Supplementary Fig. 5** online). In contrast, the LFP slope showed a progressive increase during the 4-h waking period (**Supplementary Fig. 5**) and no significant correlation with brain temperature ($r = 0.03$, $P = 0.9$). In summary, the observed changes in the slope and amplitude of cortical evoked responses suggest that synaptic efficacy increases progressively during wakefulness and decreases progressively during sleep.

Controlling for time of day and light

To ensure that the observed molecular and electrophysiological changes are due to wakefulness and sleep, other confounding factors, such as time of day or lighting conditions, must be ruled out. For this purpose, additional animals were kept awake for several hours during the light phase, when they would normally have slept, by presenting them with novel objects that kept them busy and interested (enforced wakefulness group). For the molecular analysis, enforced wakefulness rats were killed at the same time of day as the sleeping group (**Fig. 4a**). Similar to waking rats, enforced wakefulness rats had increased levels of total GluR1 compared with sleeping rats (57 ± 16%, $P = 0.0059$), but no changes in the phosphorylation of GluR1 at Ser831 and in the expression of NR2A (**Fig. 4b**). Like waking rats, enforced wakefulness rats also showed significantly increased levels of CamKII phosphorylation at Thr286 (36 ± 9%, $P = 0.021$) and a trend toward an increase in total CamKII protein levels (enforced wakefulness, 20 ± 9% of S, $P = 0.10$). As in waking rats, no significant increases were seen in GluR2 ($P = 0.9$) and NR2A ($P = 0.5$) expression (**Fig. 4b**), whereas BDNF, Arc, NGFI-A and P-CREB expression increased, as previously demonstrated (data not shown and ref. 16). On the other hand, compared with the enforced wakefulness group, the sleeping group showed a marked GluR1 dephosphorylation at Ser845, which *in vivo* is usually associated with LTD (absolute levels, 73 ± 14% of enforced wakefulness, $P < 0.012$; relative levels, 64 ± 4% of enforced wakefulness, $P < 0.019$; **Fig. 4b**), just as observed in comparison to the waking group. Once again, the results obtained in the hippocampus were similar (**Fig. 4c**). Thus, several molecular correlates of LTP and LTD in cerebral cortex and hippocampus appear to change as a function of wake and sleep history, and not of time of day or light. On the other hand, time of day, light conditions, or the duration and quality of prior wakefulness may affect the phosphorylation of GluR1 at Ser831 and the expression of NR2A, which showed no consistent change in the enforced wakefulness and waking groups relative to the sleeping group.

For the electrophysiological analysis, another group of enforced wakefulness rats were kept awake with novel objects for 4 h beyond their usual sleep time (**Fig. 5a**). The slope of the first LFP component at the end of the enforced wakefulness period was ~20% higher than after an equivalent period of sleep during a baseline recording, though time of day and lighting conditions were the same (**Fig. 4b**; for the distribution of individual responses, see **Supplementary Fig. 2**). Again, the change in the slope persisted after equating evoked responses on the basis of their latency (**Supplementary Fig. 3**). The slope of the LFP was ~13% higher after 4 h of enforced wakefulness compared with the end of the dark phase, suggesting the possibility of a further increase in synaptic efficacy with prolonged wakefulness (**Fig. 5b**). Once again, LFP slope decreased significantly after recovery sleep (**Fig. 5b**). Thus, slope increases after wakefulness and decreases after sleep in a manner that is largely independent of time of day. Note, however, that our study was not specifically designed to test a possible role of the circadian system, as molecular and electrophysiological measurements were not taken across the 24-h cycle under constant conditions. Thus, we cannot rule out that, in addition to behavioral state, circadian time may also affect markers of synaptic strength.

Cortical evoked responses and slow wave activity

SWA in the cortical EEG (SWA, the EEG power between 0.5 and 4 Hz) increases in proportion to the time spent awake and decreases during sleep. For this reason, SWA is thought to reflect the accumulation of sleep need during wakefulness and its discharge during sleep[29] (**Fig. 6a**), although the underlying mechanisms are unknown. Computer simulations[36] have recently suggested that the level of sleep SWA may reflect overall cortical synaptic strength; because of the effects on neuronal recruitment and synchronization, stronger connections should lead to larger slow waves and weaker connections to smaller waves. Support for this hypothesis comes from experiments showing that sleep SWA is increased globally and locally[37] by procedures presumably associated with synaptic potentiation, and decreased by those presumably associated with synaptic depression[38]. We therefore predicted that the increase in the slope of the LFP response after a period of waking should be positively correlated with SWA at the

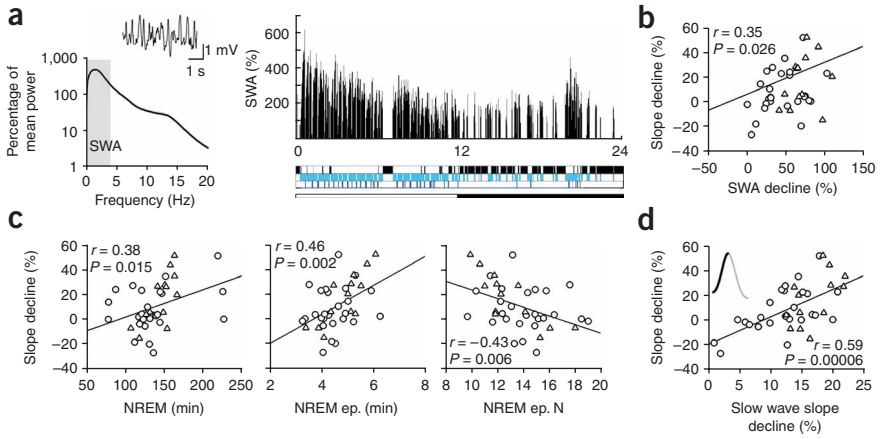

Figure 6 Relationship between LFP response slope and sleep slow-wave homeostasis. (**a**) Left, representative examples of slow waves and SWA, which comprises slow waves. Right, 24-h time course of relative SWA (% of 24-h mean SWA in NREM) in one representative rat (the hypnogram from the same rat is shown below). (**b**) Relationship between SWA decline after ~4 h of baseline sleep (% difference between 1-h mean SWA after S0 and after S1, circles, $n = 23$ rats), or after the first 4 h of recovery sleep after EW (% difference between 1-h mean SWA after EW and after R, triangles, $n = 13$ rats), and the decline of LFPs slope from the beginning to the end of the corresponding 4-h interval (% difference between S0 and S1 or between EW and R). Line depicts a linear regression (Pearson correlation). (**c**) Relationship between the decline of LFPs slope (same as in **b**) and amount of NREM sleep, mean duration of NREM episodes (>6 s) and number of NREM episodes per hour of NREM sleep in the corresponding 4-h interval (S0-S1, circles, $n = 23$ rats; EW-R, triangles, $n = 13$ rats). Line depicts a linear regression (Pearson correlation). (**d**) Relationship between the percentage decline of the slope of the first segment of NREM slow waves (in bold) after ~4 h of baseline sleep (circles, $n = 23$ rats), or after the first 4 h of recovery sleep after enforced wakefulness (triangles, $n = 13$ rats), and the percentage decline of the LFPs slope from the beginning to the end of the corresponding 4-h interval. Slow waves during the first 1-h period of NREM sleep after S0 and S1 or EW and R were equated based on their amplitude[39,40], the percent difference of their slopes was computed for each match and then averaged between all the matches in each rat.

beginning of the subsequent sleep period. On the other hand, the decrease in the slope of the waking LFPs evoked before and after sleep should be positively correlated with the decrease in SWA during sleep. To test the first prediction, a subset of animals were constantly observed during the 12-h dark period and presented with novel objects if they showed signs of drowsiness. Evoked LFPs were collected every 4 h during the preceding light period, as well as at the end of the 12-h waking period, after which the rats were allowed to obtain undisturbed sleep. As expected, at the end of the 12-h period of waking, slopes were 15.8 ± 3.7% above the mean over the baseline light period, when the animals predominantly slept ($n = 5$ rats, $P = 0.014$, paired t-test). Moreover, the increase in slope after the 12-h waking period was highly correlated with both the mean ($r = 0.97$, $P = 0.006$) and the peak ($r = 0.98$, $P = 0.003$) SWA of the first hour of NREM sleep (**Supplementary Fig. 6** online); thus, the larger the increase in slope after waking, the larger the SWA at sleep onset. To test the second prediction, we examined the relationship between the decline of LFP slope across 4 h of sleep (baseline sleep, $n = 23$ rats; recovery sleep after enforced wakefulness, $n = 13$ rats) and the decline of SWA. We found that the larger the decline in the waking LFP slope, the larger the decline in sleep SWA ($r = 0.36$, $P = 0.03$; **Fig. 6b**). Moreover, the decline in slope was positively correlated with the total amount of NREM sleep ($P = 0.015$; **Fig. 6c**, left), as well as with the mean (**Fig. 6c**, middle) and maximal (data not shown) duration of NREM sleep episodes ($P = 0.002$ and $P = 0.0008$, respectively), and negatively correlated with the number of NREM sleep episodes ($P = 0.006$; **Fig. 6c**, right). In contrast, we did not find any correlation between the decline in slope and the amount of REM sleep ($r = 0.17$, $P = 0.31$) or theta (6–9 Hz) power in REM sleep ($r = -0.20$, $P = 0.21$; **Supplementary Fig. 7** online).

Computer simulations also predicted that a decrease in net synaptic strength in cortical circuits should be reflected in a decrease in the slope of spontaneous sleep slow waves (as opposed to the slope of evoked responses)[36] between early and late sleep. Experiments in both rats[39] and humans[40] have confirmed these predictions and have shown that the decline in slow-wave slope is evident even when wave amplitude does not change. We therefore examined the relationship between the decline in the slope of evoked LFPs (collected in quiet wakefulness) and changes in the slope of spontaneous sleep slow waves. We found a strong positive correlation ($P = 0.00006$; **Fig. 6d**), suggesting that changes in synaptic efficacy can be revealed by both electrically evoked volleys and spontaneously generated volleys.

Altogether, these results indicate that the occurrence of consolidated periods of NREM sleep, accompanied by a large decline in NREM SWA, strongly predicts the ensuing decrease in the slope of evoked LFPs, an *in vivo* marker of synaptic strength. Although these results are correlative, they raise the intriguing possibility that slow waves, which fall in a frequency range usually leading to synaptic depression, may not just reflect synaptic strength, but may actively contribute to the weakening of connections during sleep[5,41].

Cortical evoked responses and LTP occlusion

If our finding that AMPAR density and the slope and amplitude of field potential responses increase after periods of waking is indeed indicative of a net increase in synaptic strength, and if the converse is the case after periods of sleep, then it should be more difficult to induce LTP in animals that have been awake for several hours than in animals that have been awake for just a few minutes after a long period of sleep. In

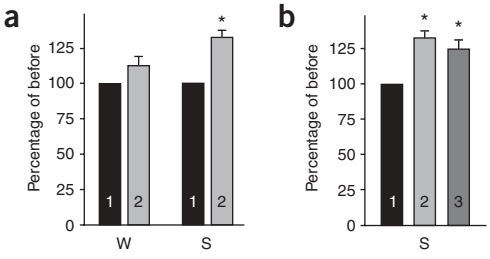

Figure 7 Partial LTP occlusion after wakefulness. (**a**) LFPs were collected at light onset (W) or after 4 h of sleep (S). Bars 1 and 2 represent slope values immediately before and after the tetanic stimulation used to induce LTP, respectively. All responses were collected during quiet wakefulness. Values are mean ± s.e.m. ($n = 7$ rats), computed as the mean first derivative of the first down-going segment, and expressed as the percentage of the corresponding 'before' condition (100%). (**b**) LFPs in S rats were collected immediately before (bar 1), 1 min (2) and 1 h (3) after LTP induction. Rats remained awake, mostly in quiet wakefulness, for the entire hour. *$P < 0.05$ (paired t-test).

other words, there should be evidence for partial occlusion of LTP after prolonged wakefulness. In contrast, after sleep, LTP-induction should be facilitated. To test this prediction, an additional group of rats ($n = 7$) were implanted, handled and recorded as before. The animals then received high-frequency electrical stimulation in the frontal motor cortex to induce LTP using an established protocol[42]. Each rat was subjected to the LTP-inducing protocol twice, at light onset and after 4 h of sleep, always in a standardized state of quiet wakefulness. At light onset, after several hours of wakefulness, no consistent change in the LFP slope was elicited (**Fig. 7a**). In contrast, LTP could easily be induced after a period of sleep (**Fig. 7a**) and persisted for at least 1 h (**Fig. 7b**). These results suggest that the induction of LTP is partially occluded after a period of wakefulness and restored after sleep.

DISCUSSION

The present results provide both molecular and electrophysiological evidence that periods of wakefulness are associated with a net increase in cortical synaptic strength and periods of sleep are associated with a net decrease. At the molecular level, GluR1-containing AMPAR levels in synaptoneurosomes were high during waking and low during sleep in both cortex and hippocampus. Changes in AMPAR, CamKII and GSK3β phosphorylation were also consistent with synaptic potentiation during wakefulness and with depression during sleep. Moreover, an electrophysiological marker of synaptic efficacy, the slope of cortical evoked responses, increased after wakefulness and decreased after sleep. Molecular and electrophysiological indicators of synaptic strength changed in a similar manner after spontaneous wakefulness during the night and after enforced wakefulness during the day, suggesting that the underlying processes are largely independent of time of day, but depend instead on the sleep-waking history of the animal. Finally, consistent with a net increase in synaptic strength during wakefulness, LTP could easily be induced in animals after they had slept for several hours, but not after they had remained awake for a similar period of time.

By themselves, the observed molecular changes cannot prove conclusively that synaptic efficacy *in vivo* has changed, as molecular assays do not necessarily reflect the functional state of synapses; for example, synaptoneurosomes cannot distinguish between surface and internal receptors, or between pre- and postsynaptic pools. Similarly, *in vivo* field responses often include a polysynaptic component that is hard to isolate. The electrophysiological changes observed *in vivo* might reflect variations in factors besides synaptic strength that can affect neuronal excitability, such as inhibition and neuromodulation. However, the latter is unlikely to have a major role, as the slope did not change substantially across different behavioral states, but decreased consistently in each behavioral state between high and low sleep pressure. Altogether, alternative explanations that may not involve synaptic plasticity cannot be ruled out, but at present it is difficult to envision what other factors may offer an equally parsimonious account for our findings. Indeed, the observed molecular and electrophysiological changes support each other, offering complementary evidence, both structural and functional, for opposite changes in net synaptic strength between wakefulness and sleep. In the future, it will be essential to confirm and extend these studies in slice preparations; for example, by investigating pre- and postsynaptic changes as a function of prior sleep and wake history through the measurements of the surface expression of AMPARs and through the analysis of miniature synaptic potentials. Moreover, given the major changes observed after a few hours of wakefulness, it will be important to confirm and extend our initial results suggesting that the induction of LTP in the cerebral cortex is partially saturated after prolonged wakefulness and may be restored by sleep. Notably, *in vitro* studies in the hippocampus have shown that insufficient sleep impairs the induction of LTP, but favors the induction of LTD (for example, see refs. 30,43–45). Finally, it will be important to investigate whether NREM sleep, and specifically sleep slow waves, is actively responsible for decreasing synaptic strength[5,46] and to explore the underlying mechanisms. In this respect, it is notable that we found similar molecular changes in cortex and hippocampus, despite differences in sleep EEG activity and the lack of cellular slow oscillations in many hippocampal cells[47]. Both hippocampal sharp wave-ripple events and the membrane potential of hippocampal cells[47] are strongly modulated by the cortical slow oscillation[48]. Perhaps the occurrence of high-frequency bursts of activity in the appropriate neuromodulatory milieu is sufficient to produce a net downscaling synaptic strength in both structures[41].

The molecular and electrophysiological changes reported here occurred in freely behaving animals that had not been forced to engage in specific learning tasks, suggesting that net plastic changes in opposite directions accompany natural wakefulness and sleep[5]. On the other hand, synaptic strength increased further when wakefulness was extended by 4 h with exposure to novel objects, only to be restored to a low level by the following period of sleep. Altogether, net cortical synaptic strength appears to be homeostatically regulated in a way that is similar to sleep pressure, which grows as a function of waking duration and intensity and decreases with sleep[29]. Consistent with this observation, the slope of the evoked responses was positively correlated with the level of the sleep SWA, a well-established marker of sleep pressure[29].

The finding that wakefulness leads to a net potentiation of synaptic strength and that sleep leads to a net depression, if confirmed in other species and with different methodologies, has several implications. First, it indicates that, despite the existence of mechanisms that could enforce an ongoing homeostasis of plastic changes in a neuron[49], the overall balance of plasticity during wakefulness appears to lean toward potentiation. Indeed, there are indications that, in the awake adult brain under physiological conditions, it is easier to induce LTP than LTD[8]. Second, this finding implies that prolonged wakefulness may pose an increasing burden on plastic circuits, consistent with theoretical and computational analyses suggesting that a progressive strengthening of synapses with experience cannot continue indefinitely because of constraints on energy, space, supplies and saturation of the ability to learn[4,5]. Indeed, by triggering LTP-like mechanisms, learning can strengthen synapses to near the maximum of their modification range, impairing the further induction of LTP (for example, see refs. 10,50). Third, these results suggest that sleep may help to maintain synaptic homeostasis[5]; if wakefulness is associated with an increasing burden to neurons imposed by progressively stronger synapses, then sleep would be a good time to redress the balance. Indeed, a progressive downscaling of synaptic strength may represent a key function of NREM sleep, possibly through its characteristic firing patterns and neuromodulatory changes[41]. We emphasize that the present results cannot distinguish between a proportional renormalization of all synapses and the depression of a majority of synapses coupled with the preservation or even strengthening of select circuits, and they are certainly compatible with the stabilization, consolidation and integration of memory traces[7]. In future work, it will be important to extend these findings to other species, and to determine whether the likely consequences of net changes of synaptic strength, such as metabolic changes, show a similar relationship to preceding wakefulness and sleep.

METHODS

Surgery and chronic recording of sleep and wakefulness. Details are provided in the **Supplementary Methods** online. Briefly, male WKY rats that were used in

the electrophysiological experiments were implanted in the frontal cortex with bipolar concentric LFP electrodes for stimulation and chronic electroencephalographic recordings. Rats used for molecular studies were recorded using epidural screw electrodes. Sleep stages were scored off-line by visual inspection of 4-s epochs. All animal procedures followed the US National Institutes of Health Guide for the Care and Use of Laboratory Animals and facilities were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Wisconsin-Madison, and were inspected and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Synaptoneurosome preparation and quantitative immunoblotting. The protocol was optimized according to suggestions from M.F. Bear and his laboratory, and was essentially carried out as previously described[10]. Details are provided in the **Supplementary Methods**.

LFP evoked responses. In all rats, LFP and EMG signals were continuously recorded during a 24-h baseline period, sleep deprivation and recovery after sleep deprivation. The collection of LFP-evoked responses occurred at ~4-h intervals, usually starting at around light onset (10 a.m.) of the baseline day, and in some animals continued during sleep deprivation and the following 4 h of recovery. Prior to the experiment, input-output tests were performed on each rat. Details are provided in the **Supplementary Methods**. On completion of the experiments, the position of the LFP electrodes was verified by histology in all animals.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

C.C. and M.P.-G. carried out the molecular experiments. V.V.V. performed the electrophysiological experiments and wrote part of the manuscript. U.F. participated in some of the electrophysiological experiments. C.C. and G.T. designed the experiments, coordinated the development of the study and wrote most of the manuscript.

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