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Mechanisms of Sleep-Dependent Consolidation of Cortical Plasticity

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SUMMARY

Sleep is thought to consolidate changes in synaptic strength, but the underlying mechanisms are unknown. We investigated the cellular events involved in this process during ocular dominance plasticity (ODP)—a canonical form of in vivo cortical plasticity triggered by monocular deprivation (MD) and consolidated by sleep via undetermined, activity-dependent mechanisms. We find that sleep consolidates ODP primarily by strengthening cortical responses to nondeprived eye stimulation. Consolidation is inhibited by reversible, intracortical antagonism of NMDA receptors (NMDARs) or cAMP-dependent protein kinase (PKA) during post-MD sleep. Consolidation is also associated with sleep-dependent increases in the activity of remodeling neurons and in the phosphorylation of proteins required for potentiation of glutamatergic synapses. These findings demonstrate that synaptic strengthening via NMDAR and PKA activity is a key step in sleep-dependent consolidation of ODP.

INTRODUCTION

Consolidation refers to mechanisms that stabilize and enhance memories over time (McGaugh, 2000; Wiltgen et al., 2004). Two recent advances in our understanding of consolidation include the discovery that similar processes occur in various forms of synaptic plasticity (Lynch et al., 2007; Wang et al., 2006; Whitlock et al., 2006) and that consolidation can occur during sleep (Frank and Benington, 2006; Walker and Stickgold, 2004). There are, however, relatively few model systems where plasticity mechanisms and sleep can be examined simultaneously in vivo during naturally occurring forms of synaptic remodeling (Frank and Benington, 2006). Moreover, because most studies have focused on hippocampal forms of learning and synaptic plasticity, less is known about consolidation mechanisms that operate in the neocortex (Lynch, 2004; Wang et al., 2006; Wiltgen et al., 2004) and how they are modulated by sleep and wakefulness.

We have shown that ocular dominance plasticity (ODP) is consolidated by sleep (Frank et al., 2001). As little as 6 hr of sleep is sufficient to enhance the effects of a preceding period of monocular deprivation (MD) on visual cortical neurons; this process is blocked when animals are prevented from sleeping or when postsynaptic activity in V1 is reversibly silenced during sleep (Frank et al., 2001, 2006; Jha et al., 2005). We have also shown that the underlying mechanisms, though still unknown, may involve CREB-mediated gene expression and protein synthesis (Dadvand et al., 2006). In many parts of the brain, these latter mechanisms are regulated by NMDARs and intracellular kinases (Waltereit and Weller, 2003). Reactivation of these mechanisms during post-MD sleep may also promote a mechanism known as "synaptic reentry reinforcement," which is thought to mediate memory consolidation in the hippocampus and the neocortex (Shimizu et al., 2000; Wang et al., 2006). Therefore, we hypothesized that the sleep-dependent consolidation of ODP involves reactivation of NMDARs and kinase signaling pathways.

To determine if NMDAR and kinase activation during sleep governs consolidation of ODP, we performed three parallel experiments. First, we tested the role of NMDARs and PKA in this process by infusing the NMDAR antagonist APV or the PKA inhibitor Rp-8-Cl-cAMPS into V1 during post-MD sleep. ODP and neuronal visual response properties were measured in drug-infused animals using two independent techniques (intrinsic signal imaging and single-unit recording) and were compared with measurements from control animals infused with vehicle, animals receiving waking MD only, and animals with normal binocular vision. Second, using western blot analyses, we examined sleep-dependent changes in the activity of kinases downstream of NMDARs (ERK and CaMKII) and the phosphorylation of GluR1 AMPA receptor (AMPAR) subunits at sites known to mediate NMDAR-dependent long-term potentiation (LTP). Third, we determined whether remodeling neuronal circuits increase their activity during sleep—an event that might enhance NMDAR and kinase signaling. This was accomplished by chronically recording multiunit activity from V1 in freely behaving animals before, during, and after a period of MD. We find that nondeprived eye responses are selectively potentiated during sleep. This potentiation is dependent on NMDAR and PKA activity, involves phosphorylation events associated with LTP, and is associated with increased neuronal activity in V1.

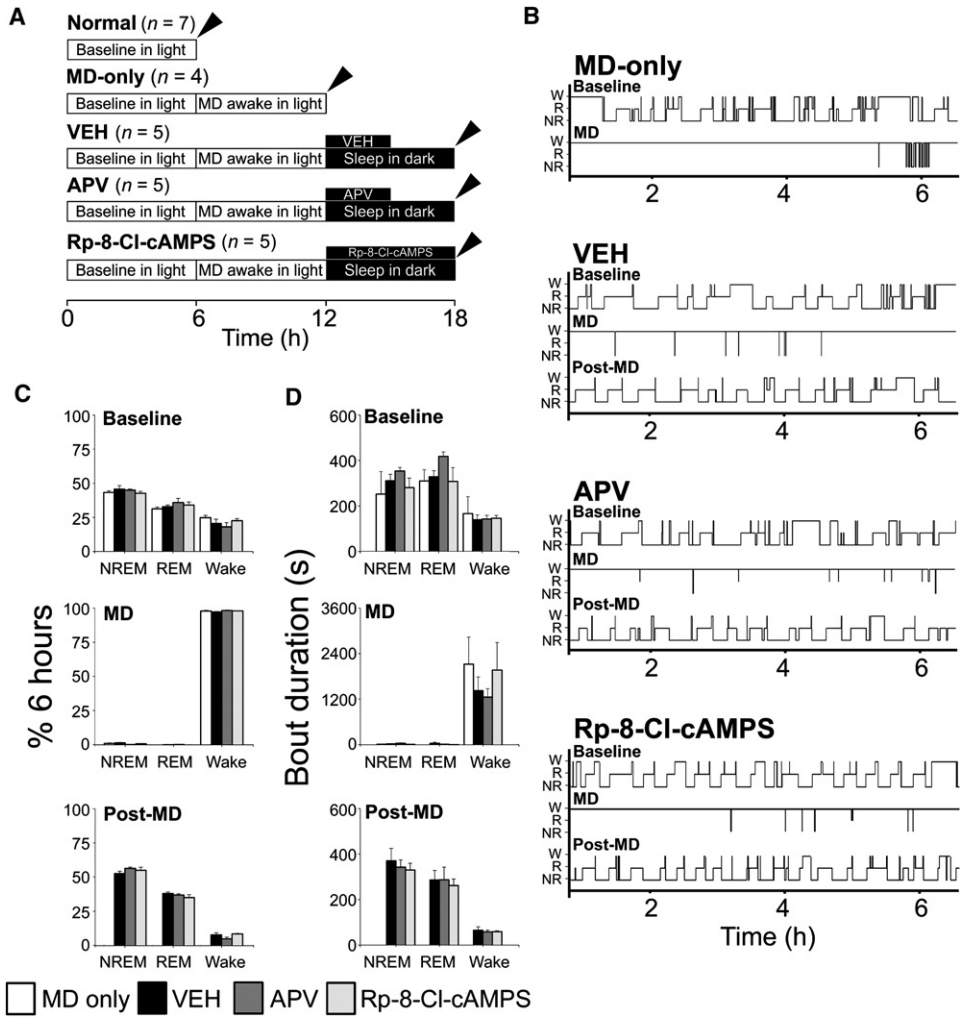

Figure 1. Sleep Data for Main Experimental Groups

(A) Experimental design. n = number of animals per group. Arrowheads indicate time at which measurements of OD were made.

(B) Hypnograms showing waking (W), REM sleep (R), and NREM sleep (N) for representative MD-only, VEH, APV, and Rp-8-Cl-cAMPS cats are shown for each phase of the experiment. Relative amounts (expressed as percentage of total recording time; mean ± SEM shown in [C]) and bout durations (in seconds [s]; mean ± SEM shown in [D]) for these vigilance states did not differ between the four groups during baseline or MD, or between the three sleeping groups during the post-MD recording period (N.S. for all measures, one-way ANOVA with Student-Newman-Keuls [SNK] post hoc test).

RESULTS

Experiment 1: NMDAR and PKA Signaling Are Necessary for Sleep-Dependent Consolidation of ODP

Our experimental design is summarized in Figure 1A. Five groups of cats were formed (Normal, MD-only, VEH, APV, and Rp-8-Cl-cAMPS). Normal cats had unmanipulated visual experience and sleep. For MD-only, drug-infused, and vehicle-infused animals, each experiment began with a 6 hr ad lib baseline sleep period prior to MD. Cats then underwent 6 hr of continuous waking combined with right-eye MD as previously described (Frank et al., 2001). MD-only cats were then immediately prepared for assays of ocular dominance (OD). In antagonist- and vehicle-infused cats, MD was followed by a 6 hr post-MD ad lib sleep period in complete darkness with either bilateral aCSF vehicle (VEH), APV (5 mM), or Rp-8-Cl-cAMPS (1 mM) infusion into V1. Following the ad lib sleep period, these cats were immediately prepared for acute measurements of OD (intrinsic signal imaging and single-unit recording).

Sleep/Wake Architecture

All cats receiving MD were awake for >98% of the 6 hr MD period (Figure 1C) and had similar sleep/wake architecture during all phases of the experiment (Figure 1B). There were no significant differences in the amounts (as percentage total recording time; Figure 1C) or durations (Figure 1D) of wakefulness, rapid-eye-movement (REM) or non-REM (NREM) sleep bouts at any phase of the experiment (baseline, MD, post-MD periods) between the different groups. EEG analyses also showed that intracortical drug infusions had relatively modest effects. Compared to VEH-infused animals, Rp-8-Cl-cAMPS had no significant effects

on anterior/parietal (AP) or V1 EEG activity in NREM or REM sleep (at any frequency between 0 and 40 Hz). APV had limited effects on ongoing cortical activity that were restricted to a single EEG band (delta; 0.5–4.0 Hz) in REM sleep and only at the infusion site (Figure S1 available online). It is highly unlikely that this solitary change in REM sleep EEG delta power affected ODP, because increases in ODP are highly correlated with post-MD NREM sleep ($r = 0.95$) (Frank et al., 2001). We have also recently shown that profoundly inhibiting post-MD REM sleep by administering hypnotic drugs does not impair ODP (Seibt et al., 2008). To further verify that APV-induced changes in REM sleep do not impact ODP, we performed two supplementary experiments. As shown in Figure S2, APV-induced elevations in REM sleep delta power were not associated with abnormal OD or visual response properties in cats with normal vision. Furthermore, as shown in Figure S3, reducing post-MD REM sleep (with targeted awakenings) has no effect on ODP. Therefore, we conclude that (1) neither drug affected overall sleep/wake amounts or durations and that (2) changes in EEG activity caused by APV were restricted to a sleep state that does not contribute to ODP.

Sleep Enhances ODP

Both intrinsic signal imaging and single-unit electrophysiology confirmed our previous findings (Frank et al., 2001) that the effects of MD are enhanced by post-MD sleep. Intrinsic signal maps (Figure 2A) showed that, following MD, there was a loss of binocularity and a shift in OD in favor of the nondeprived eye (relative to Normal cats) that was significantly enhanced after a period of sleep (pixel distributions from representative V1 maps are shown in red in Figure 2B; see Experimental Procedures and Supplemental Data for details on imaging procedures). Quantitative assessments using previously described scalar measures (Frank et al., 2001; Jha et al., 2005) verified these changes. There were significant increases in OD shift, nondeprived eye bias, and monocularity indices (SIs, NBIs, and MIs; Figures 2C–2E) in MD-only cats relative to Normal cats. These values were further significantly enhanced by post-MD sleep. The relative increase in these parameters after sleep versus the effects of MD alone was comparable to previously reported values (e.g., in combined hemisphere data, 72%, 72%, and 116% increase over the effects of MD alone on SI, NBI, and MI, respectively).

The results of single-unit measurements were in agreement with those obtained with intrinsic signal imaging. The OD distribution in MD-only hemispheres showed a pronounced reduction in binocular responses. This was consistent with previous microelectrode studies showing that a loss of binocularity occurs during the initial stages of MD (Freeman, 1979; Mioche and Singer, 1989) (Figure 3A). This was verified by statistical evaluation of MIs from MD-only hemispheres, which were greater than those of Normal cats. However, a significant shift in OD relative to Normal V1 was observed only after post-MD sleep (Figures 3A–3D). This was evident in OD histograms from single-unit recordings and verified by scalar measures of OD, which were all significantly greater in VEH-infused V1 compared to Normal V1 and enhanced relative to MD-only (57%, 58%, and 26% increase in SI, NBI, and MI values, respectively, over effects of MD alone). In sum, while there were slight differences in some measures between intrinsic signal and single-unit recording as shown previously (Dadvand et al., 2006), both techniques confirmed our previous finding that ODP is consolidated by post-MD sleep (Frank et al., 2001).

Sleep/Wake-Dependent Changes in Synaptic Strength

We analyzed additional V1 neuronal response properties from the main treatment groups to determine how waking MD and post-MD sleep affect deprived eye (DE) and nondeprived eye (NDE) pathways. These included comparisons of normalized spike rates at the preferred stimulus orientation for both eyes (Figure 4A; see Supplemental Data for description of normalization procedures) and assessments of orientation selectivity (OSI45 and OSI90; Figures 4B and 4C) using previously published methods (Kaneko et al., 2008; Liao et al., 2004). We found that MD alone altered several visual response properties, but only within DE pathways. For example, after MD there were significant reductions in neuronal firing rates and orientation selectivity in response to DE stimulation (relative to Normal right eye responses), but there were no changes in these response parameters for NDE stimulation. However, after sleep, NDE firing rates at the preferred orientation and NDE orientation selectivity were significantly enhanced (Figures 4A–4C)—with no further changes in DE pathways (Kolmogorov-Smirnov [K-S] test, N.S.). Together these findings demonstrate that MD alone is sufficient to depress DE visual responses, while NDE responses are strengthened specifically during subsequent sleep.

NMDAR or PKA Antagonism during Sleep Blocks ODP Consolidation

Both intrinsic signal imaging and single-unit electrophysiology showed that APV or Rp-8-Cl-cAMPS infusion during the post-MD sleep period blocked sleep-dependent consolidation of ODP. Intrinsic signal measurements near (<3 mm from) APV or Rp-8-Cl-cAMPS infusion sites showed that OD shifts in favor of the NDE were blocked (Figures 2A and 2B). Intrinsic signal SI, NBI, and MI values were all reduced relative to the VEH group and were not significantly different from Normal values (Figures 2C–2E). Similar results were obtained from single-unit recordings. As shown in Figure 3A, APV or Rp-8-Cl-cAMPS infusion abolished the normal shift in the OD distribution observed after sleep. Instead, the OD distributions from APV and Rp-8-Cl-cAMPS cats were comparable to those from MD-only cats; i.e., the loss of binocularity was preserved, but there was no additional OD shift in favor of the NDE. This was confirmed by statistical comparisons of the resulting scalar measures, which showed that unit NBIs and SIs near APV or Rp-8-Cl-cAMPS infusion sites did not differ from those of Normal cats (Figures 3B and 3C). In contrast, unit MI values were similar to MI values observed after MD alone (Figure 3D). In agreement with previous infusion studies (Beaver et al., 2001), we also found that the effects of APV and Rp-8-Cl-cAMPS were restricted to areas near the infusion cannulae (Figure S4).

NMDAR or PKA Antagonism during Sleep Blocks Changes in DE and NDE Response Properties

As was true for ODP, antagonizing NMDARs or PKA during post-MD sleep largely inhibited the changes in DE and NDE response properties observed after MD and post-MD sleep. Both APV and Rp-8-Cl-cAMPS infusion prevented the sleep-dependent

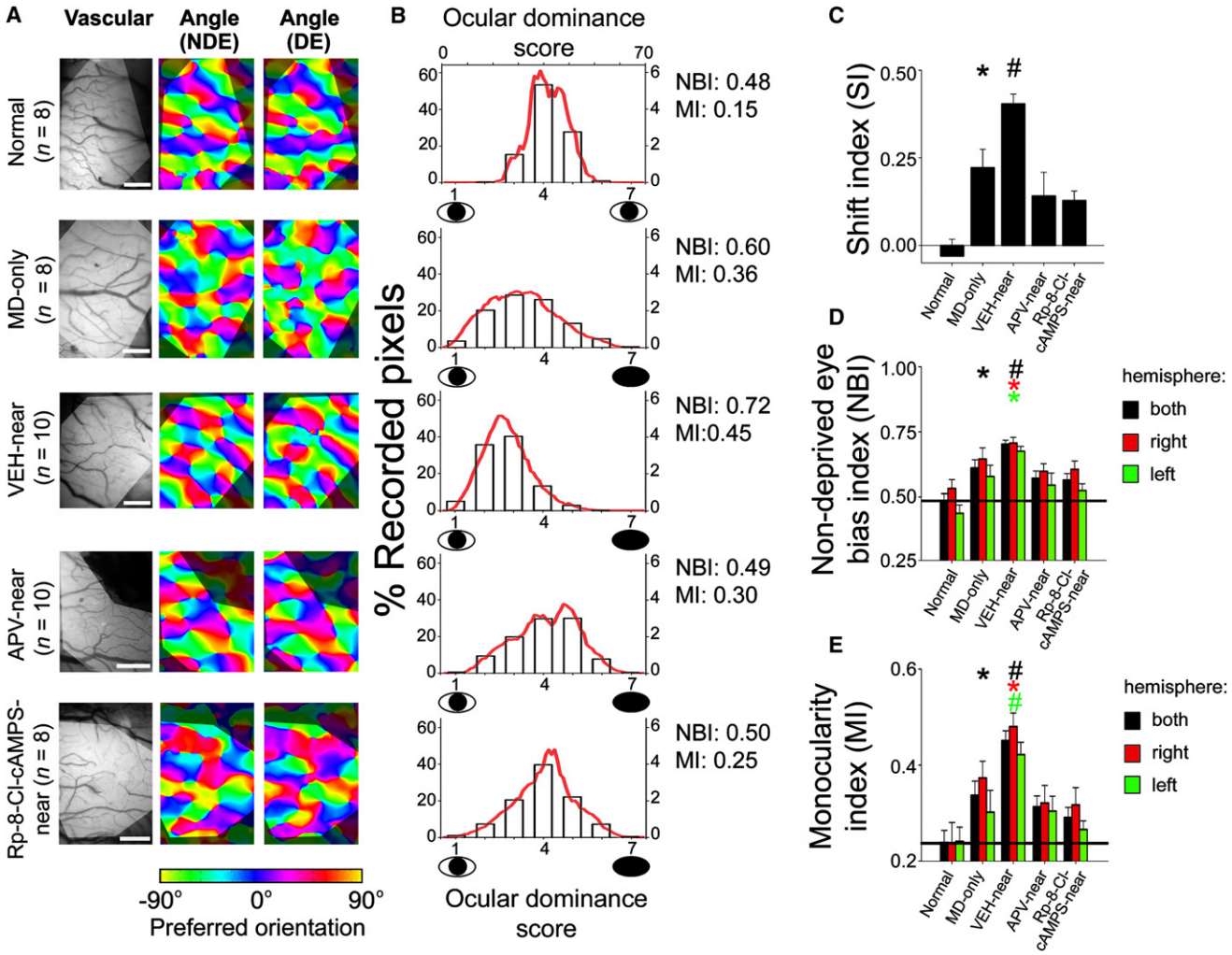

Figure 2. Sleep-Dependent Consolidation of ODP Is Disrupted by NMDAR and PKA Antagonism: Intrinsic Signal Imaging

(A) Representative visual response maps of V1 contralateral to the right (or deprived: DE) eye (i.e., left hemisphere) are shown for a normally sighted critical period cat (Normal), a MD-only cat, and cats infused with VEH, APV, or Rp-8-Cl-cAMPS during post-MD sleep. For infusions, maps are shown in areas near (<3 mm from) the infusion cannula site. n = number of imaged hemispheres in each condition. From left to right are vascular maps (highlighted areas indicate optimally focused, vascular artifact-free areas used for analysis), angle maps produced for left eye (NDE) stimulation, and angle maps produced for right eye (DE) stimulation. In angle maps, the hue indicates the preferred stimulus orientation at each pixel (see inset key). White scale bars in vascular maps = 1 mm.

(B) Pixel-by-pixel OD histograms were computed for each map by comparing the relative NDE and DE response magnitudes at each imaged pixel. The pixel distribution was collapsed into a classic seven-point OD measurement scale (Hubel and Wiesel, 1970) as described previously (Jha et al., 2005). Values to the right are the corresponding NBI and MI scores for each map.

(C–E) Quantitative measurements of OD. One-way ANOVA showed significant main effects of treatment group for SIs ($F = 10.8$, $p < 0.001$), NBIs ($NBI_{both\ hemispheres}$: $F = 10.0$, $p < 0.001$, $NBI_{right\ hemisphere}$: $F = 4.1$, $p = 0.017$; $NBI_{left\ hemisphere}$: $F = 6.1$, $p = 0.003$), and MI measures ($MI_{both\ hemispheres}$: $F = 11.4$, $p < 0.001$, $MI_{right\ hemisphere}$: $F = 6.4$, $p = 0.003$; $MI_{left\ hemisphere}$: $F = 5.3$, $p = 0.006$). SIs (mean ± SEM shown in [C]) were significantly increased following waking MD alone (*$p < 0.05$ versus Normal V1, SNK post hoc test), but were increased further by subsequent sleep (#$p < 0.05$ versus MD-only V1). SIs were not different from Normal V1 in areas near sites where APV or Rp-8-Cl-cAMPS was infused during post-MD sleep. NBIs (mean ± SEM shown in [D]) and MIs (mean ± SEM shown in [E]) were similarly affected by waking MD, subsequent sleep, and drug infusion. Effects were similar when right- and left-hemisphere measurements were combined (black bars in [D] and [E]) or compared separately (red and green bars, respectively). Black lines indicate mean combined hemisphere values for Normal V1; *$p < 0.05$ versus same-hemisphere measurements from Normal V1, SNK post hoc test; #$p < 0.05$ versus same-hemisphere measurements from MD-only V1. For SI and NBI measures, a value of 1 indicates complete dominance by the NDE. For MI measures, a value of 1 indicates complete loss of binocularity.

potentiation of peak firing in response to NDE stimulation (Figure 4A). APV also reversed the MD-induced depression of peak firing during DE stimulation. More complex changes were observed for orientation selectivity (Figures 4B and 4C). For example, Rp-8-Cl-cAMPS reduced orientation selectivity for stimuli presented to both eyes, in agreement with previous reports (Beaver et al., 2002, 2001). This finding was partially replicated in APV-infused V1, where we found similar effects on DE and NDE OSI90 measurements, as described in earlier studies (Bear et al., 1990).

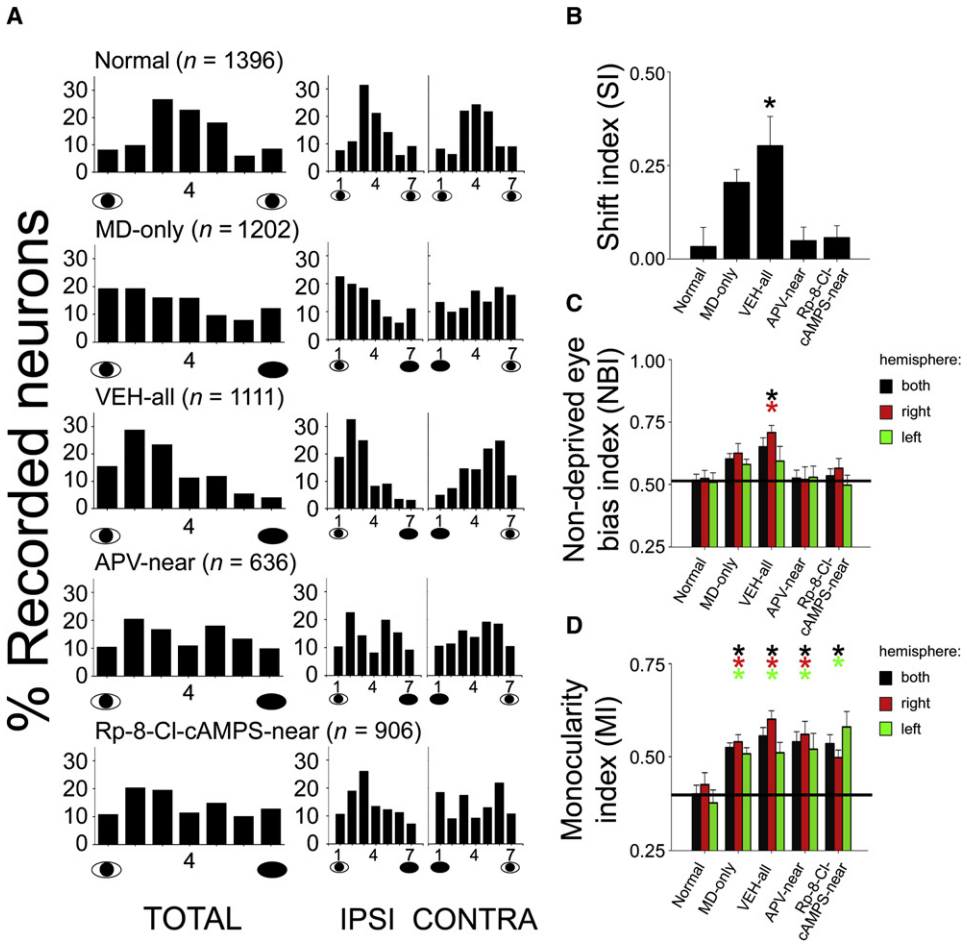

Figure 3. Sleep-Dependent Consolidation of ODP Is Disrupted by NMDAR and PKA Antagonism: Microelectrode Recordings

(A) OD histograms for single neurons recorded from both hemispheres (TOTAL) and in hemispheres ipsilateral (IPSI) and contralateral (CONTRA) to the DE are shown for the main groups. OD scores were ranked on a seven-point scale as described previously (Jha et al., 2005). n = number of neurons recorded in each condition.

(B–D) Quantitative measurements of OD in both hemispheres. One-way ANOVA showed a significant effect of treatment for SI (F = 5.1, p = 0.006), NBI ($NBI_{both\ hemispheres}$: F = 4.1, p = 0.006, $NBI_{right\ hemisphere}$: F = 4.3, p = 0.011, $NBI_{left\ hemisphere}$: *N.S.*), and MI measures ($MI_{both\ hemispheres}$: F = 8.9, p < 0.001, $MI_{right\ hemisphere}$: F = 6.2, p = 0.002, $MI_{left\ hemisphere}$: F = 5.1, p = 0.005). SIs (mean ± SEM shown in [B]) were not significantly increased following MD alone (N.S. versus Normal V1, SNK post hoc test), but were greater in VEH-infused animals following post-MD sleep (*p < 0.05 versus Normal, SNK test). Effects of sleep on SI were blocked near sites of APV or Rp-8-Cl-cAMPS infusion. Similarly, NBIs (mean ± SEM shown in [C]) for neurons in MD-only animals and near APV or Rp-8-Cl-cAMPS infusion cannulae did not differ from Normal hemispheres, while neurons from VEH-infused animals showed a significant increase in NBI (*p < 0.05 versus same-hemisphere measurements from Normal V1, SNK test). MIs (mean ± SEM shown in [D]) were increased following MD alone, as well as after post-MD sleep (*p < 0.05 versus same-hemisphere measurements from Normal V1, SNK test). For (C) and (D), black horizontal lines indicate mean combined hemisphere values for Normal V1.

Supplemental Assessments of APV Effects on ODP Consolidation

We performed a number of supplementary experiments to address the temporal aspects of sleep-dependent consolidation and to determine if the effects of NMDAR blockade were comparable to sleep deprivation. As shown in Figures S5A–S5D, additional ad lib sleep following APV infusion did not rescue sleep-dependent changes in OD and response properties. Plasticity, however, was preserved when APV infusion began *after* an initial 6 hr ad lib sleep period (Figures S5A–S5D). We also found that the effects of NMDAR antagonism during sleep were statistically indistinguishable from the effects of sleep deprivation alone. For example, as shown in Figure S6, when sleep deprivation was combined with APV infusion (after MD), inhibition of plasticity near and far from the cannula was comparable (i.e., the effects of sleep deprivation and APV infusion were not additive).

Pharmacological Inhibition of ODP (Nonspecific Effects)

The loss of plasticity in the APV- and Rp-8-Cl-cAMPS-infused hemispheres was not due to gross defects in visual processing. For example, while there was an increase in spontaneous firing in neurons from Rp-8-Cl-cAMPS-infused V1 ([normalized] means [NDE and DE] ± SEM for VEH: 0.43 ± 0.03 and 0.45 ± 0.03, for APV: 0.43 ± 0.04 and 0.44 ± 0.04, and for Rp-8-Cl-cAMPS: 0.66 ± 0.04 and 0.64 ± 0.04: p < 0.0005 for Rp-8-Cl-cAMPS

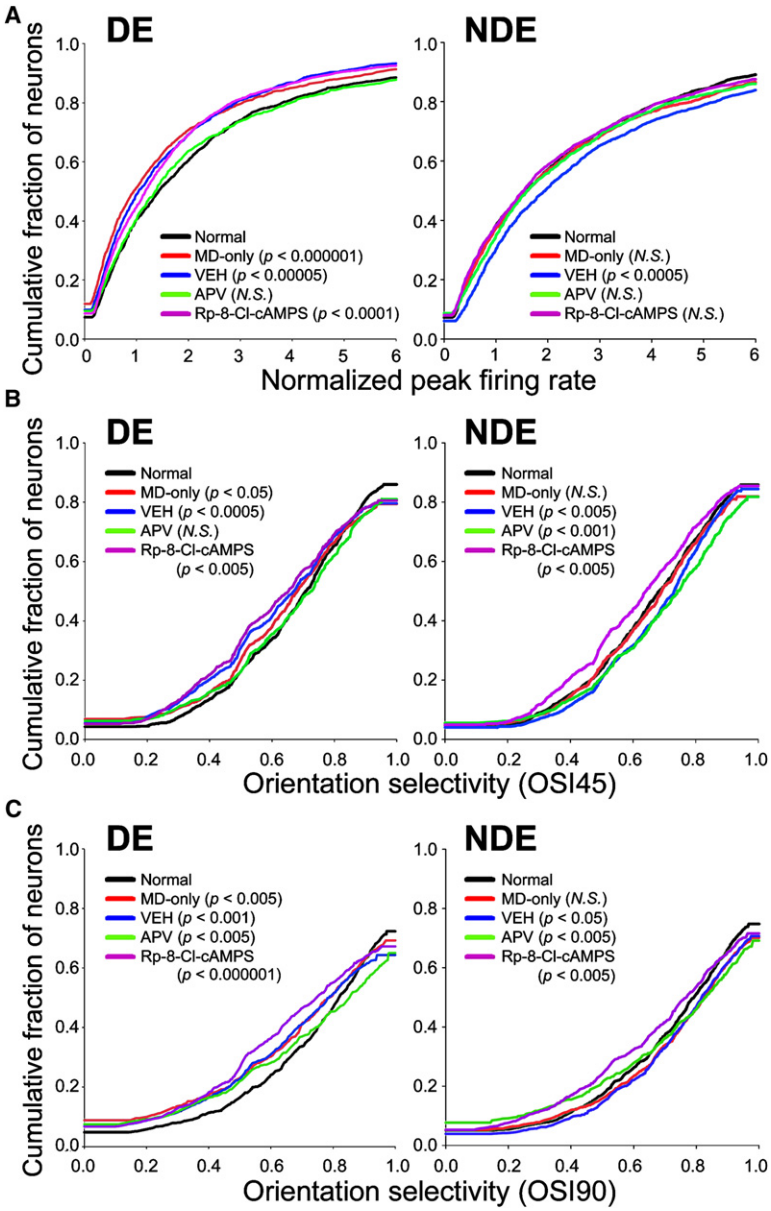

Figure 4. Post-MD Sleep Leads to Specific Augmentation of Nondeprived Eye Responses

(A) Cumulative distributions of normalized peak firing rates to stimulation of the right eye (DE) and left eye (NDE) at each neuron's preferred orientation. Firing rates were decreased following MD alone (versus Normal V1, Kolmogorov-Smirnov [K-S] test; p values shown) for the right eye (DE) and unchanged for the left eye (NDE). Following post-MD sleep, NDE firing rates were significantly augmented. APV and Rp-8-Cl-cAMPS infusion during post-MD sleep inhibited NDE augmentation, and APV also reversed DE depression.

(B and C) Cumulative distributions for two measures of orientation selectivity (OSI45 and OSI90) for DE and NDE responses. Both measures showed similar effects of MD and subsequent sleep—MD alone produced lower DE OSI values; post-MD sleep produced higher NDE OSI values. APV and Rp-8-Cl-cAMPS infusion both decreased orientation selectivity in both eyes (C), but this was more pronounced following Rp-8-Cl-cAMPS infusion (B).

versus VEH, K-S test), the proportion of recorded neurons that were visually responsive (i.e., showing higher firing rates during stimulus presentation than during blank screen presentation) was similar for all treatment groups (>97% of all neurons recorded). Examination of NDE responses (which do not normally degrade after MD) in APV and Rp-8-Cl-cAMPS-infused hemispheres showed no reductions in normalized firing rate at the preferred orientation (Figure 4A), and NDE angle maps were similar to those from VEH-infused hemispheres (Figure 2A). Rp-8-Cl-cAMPS (and to a lesser extent, APV) reduced orientation selectivity for both eyes. However, as discussed elsewhere (Bear et al., 1990; Beaver et al., 2002), this reflects overlapping critical periods that are differentially modulated by NMDARs and PKA (i.e., orientation selectivity versus OD), rather than a gross disruption in visual processing.

Because the effects of Rp-8-Cl-cAMPS on developing V1 neurons have been well-characterized (Beaver et al., 2002, 2001), we performed three additional experiments to verify that APV did not perturb normal visual responses. As shown in Figure S2, APV infusion in cats with normal binocular vision had no significant effects on OD or visual response properties; thus, APV by itself does not perturb normal visual processing in V1. This was further confirmed by examining the acute effects of APV on single neurons. As shown in Figure S7, acute application of APV (5 mM) had no appreciable effects on peak firing, spontaneous firing, or orientation selectivity in V1 neurons relative to baseline. In our final experiment, we began the APV infusion after the initial 6 hr post-MD sleep period. Although these cats underwent MD and subsequent APV infusion, plasticity was intact (Figure S5); thus, there was no evidence of a retrograde loss of plasticity due to APV. We conclude that other than the expected change in orientation selectivity when MD is combined with Rp-8-Cl-cAMPS or APV, neither drug produced gross abnormalities in visual processing.

To summarize, antagonizing NMDARs or PKA during post-MD sleep appeared to arrest the plastic processes initiated during waking MD and normally consolidated during subsequent sleep. These effects were restricted to areas near drug infusion sites, could not be reversed by additional sleep, and in order to be significant, had to occur in the sleep period immediately following MD. Sleep deprivation also occluded the effects of NMDAR antagonism during sleep, indicating that the inhibition of plasticity in both instances involves a common mechanism.

Experiment 2: NMDAR-Dependent Kinases Are Activated and GluR1 Is Phosphorylated during Sleep in Remodeling Visual Cortex

The results from experiment 1 suggested two primary conclusions. First, NMDAR- and PKA-mediated intracellular cascades

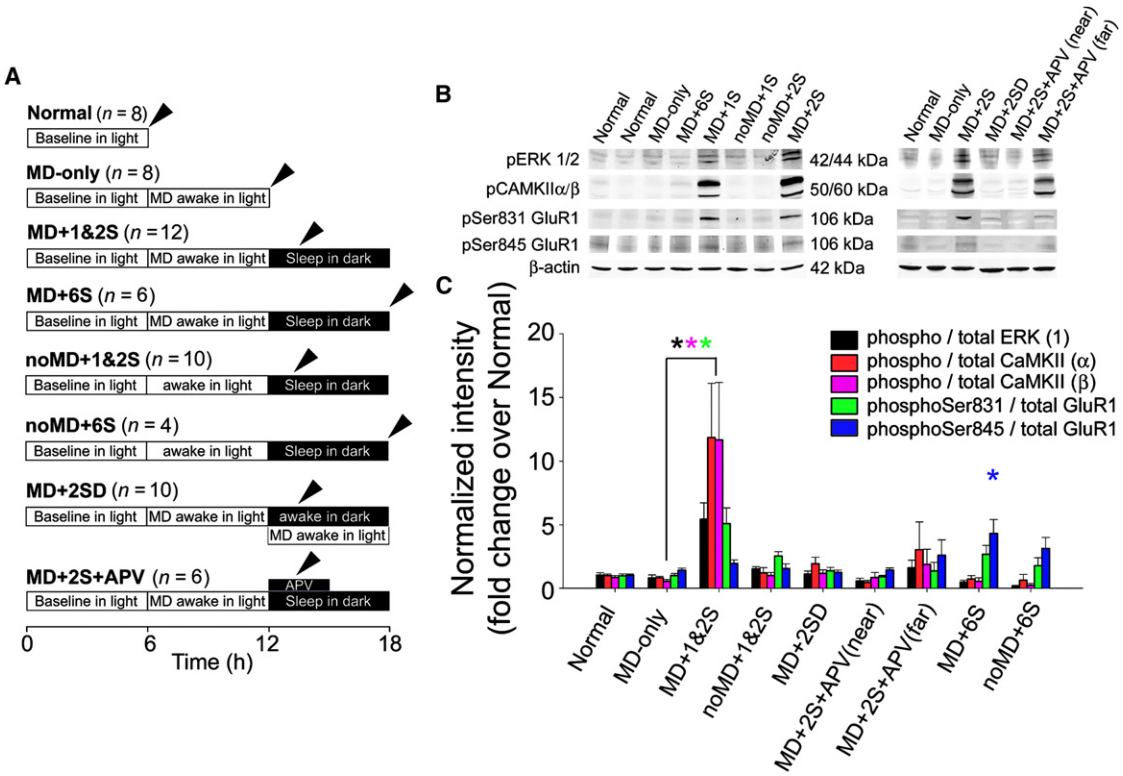

Figure 5. LTP-like Changes in Protein Phosphorylation Are Seen in V1 during Early Post-MD Sleep

(A) Experimental design. For details see Results and Experimental Procedures sections. n = number of hemispheres per group.

(B) Representative western blots of left-hemisphere V1 protein samples from the main groups run side by side, showing levels of phosphorylated ERK, CaMKII, and GluR1 with β-actin loading control.

(C) Quantification of phosphorylated/total levels of each protein for the main groups. Intensity data for each sample within individual blots were normalized to mean levels for Normal control samples run concurrently. This allowed for comparisons of intensity values across multiple blots; values are thus expressed as fold change over Normal (mean ± SEM). Significant effects of treatment on phosphorylation levels were found for ERK (H = 29.5, p = 0.001, Kruskal-Wallis ANOVA), CaMKIIα and -β (H = 16.9, $p < 0.05$, and H = 20.0, $p < 0.005$, respectively), and GluR1 (H = 26.7, $p < 0.001$ for pSer831, and H = 17.7, $p < 0.01$ for pSer845). Relative to MD alone (MD-only), phosphorylated ERK, CaMKII, and GluR1 (at Ser831, the site phosphorylated by CaMKII) were significantly higher after 1–2 hr of post-MD sleep (MD+1&2S; *$p < 0.05$, Dunn's post hoc test); these changes did not occur in animals that were sleep deprived following MD (MD+2SD) or had APV infused into V1 during the post-MD sleep period (MD+2S+APV). These increases in phosphorylation were sleep dependent (MD+1&2S versus MD+2SD) and transient (MD+1&2S versus MD+6S). However, phosphorylation of GluR1 at Ser845 (PKA site) was significantly increased after 6 hr of post-MD sleep (relative to Normal V1, *$p < 0.05$, Dunn's post hoc test).

are critical components of the cellular machinery governing sleep-dependent consolidation of ODP. Second, their activation during sleep promotes synaptic strengthening. To further explore this possibility, we examined changes in the activation state of selected kinases (indicated by phosphorylation of these kinases at specific amino acid residues—e.g., CaMKIIα and -β subunits at Thr286 and Thr287, respectively, and ERK1/2 at Thr202/Tyr204 and Thr185/Tyr187, respectively) and the relative phosphorylation of selected downstream targets that are known to mediate LTP (e.g., phosphorylation of AMPA receptor subunit GluR1 at Ser845 by PKA or at Ser831 by CaMKII), using an experimental design similar to the one employed in experiment 1 (Figure 5A). Normal and MD-only cats were treated as above, but separate groups of sleeping cats were formed: MD followed by 1, 2, or 6 hr of ad lib sleep (MD+S). These latter groups would determine the time course of any changes in target proteins. Three additional comparison groups were also formed: animals kept awake for 6 hr but not exposed to MD, with 1, 2, or 6 hr of subsequent ad lib sleep (noMD+S), MD followed by sleep deprivation (MD+SD), and MD with subsequent sleep and V1 APV infusion (MD+S+APV). The noMD+S groups were treated exactly as the MD+S groups, except that binocular vision was left intact—thus, any changes in the phosphorylation of target proteins in this group would be due to arousal and subsequent sleep only, but unrelated to ODP. The MD+SD group was formed to determine whether any changes seen in the MD+S groups were sleep dependent or merely time dependent. The MD+S+APV group was used to test whether any changes in the MD+S groups were dependent on NMDAR activation during sleep. Following these manipulations, the animals were deeply anesthetized and visual cortices were removed and prepared for western blot analyses (for additional details, see Experimental Procedures and Supplemental Data).

Phosphorylation of Target Proteins during Post-MD Sleep Mimics Changes Underlying LTP

As shown in Figures 5B and 5C, 1 or 2 hr of sleep after MD led to a substantial increase in the phosphorylated forms of ERK and

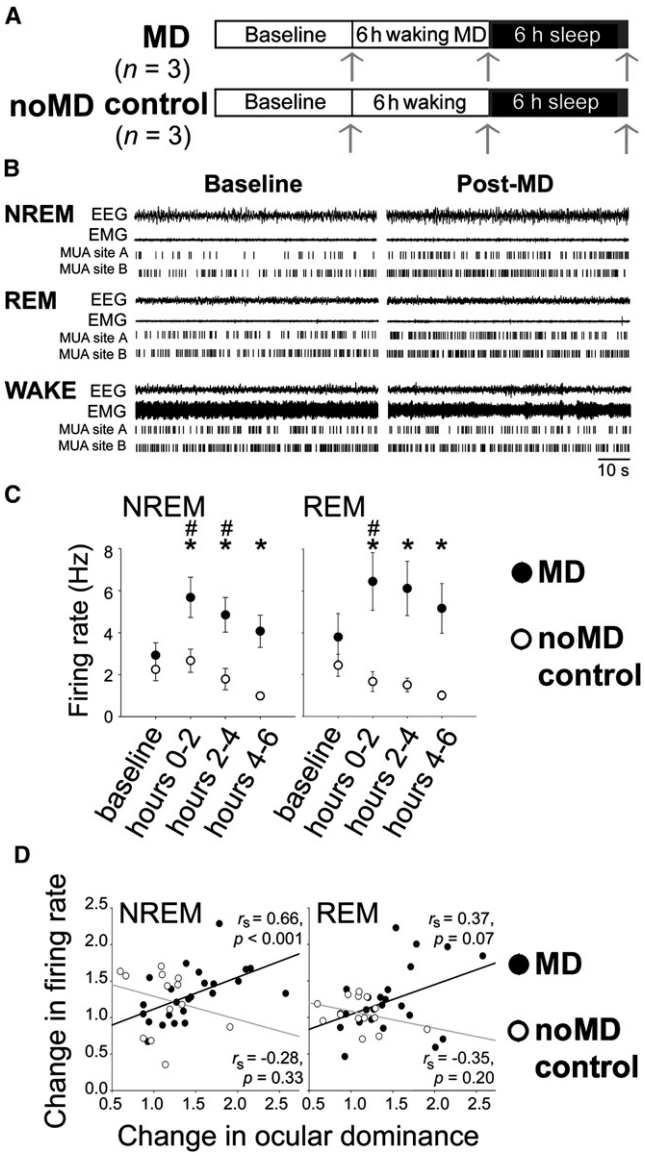

Figure 6. Remodeling Neurons Show Increased Firing Rate during Early Post-MD Sleep

(A) Longitudinal V1 multiunit recordings were made in cats before, during, and after MD and in control cats that underwent the same procedures without MD. Changes in OD were measured during periodic assessments (time indicated by arrows). n = number of cats per group.

(B–D) Raster plots showing MUA from representative V1 sites (B) during baseline recording (left) and post-MD sleep (right). Mean firing rates during the first 2–4 hr of post-MD NREM and REM sleep (black filled circles; ±SEM shown in [C]) were significantly increased relative to baseline (main effect of time for NREM: χ^2 = 31.7, p < 0.001; for REM: χ^2 = 19.6, p < 0.001, Friedman's repeated-measures ANOVA on ranks; #p < 0.05 versus baseline, SNK post hoc test). In contrast, firing rates during sleep for control (noMD, open circles; mean ± SEM) animals did not change relative to baseline (repeated-measures ANOVA on ranks, N.S.), and were significantly lower (relative to animals receiving MD) during ad lib sleep (two-way ANOVA for effects of treatment × time, main effect of treatment, p < 0.001 for both REM and NREM, *p < 0.05 versus MD condition, SNK test). On a site-by-site basis, changes in normalized firing rates during NREM from baseline to post-MD sleep (D) were positively correlated in MD cats with changes in OD following post-MD sleep (r_s and p values shown, black line, filled circles, Spearman rank order correlation test). There was a similar positive relationship between REM firing rate changes and OD changes, but this did not reach statistical significance. In noMD cats, there were no significant correlations in these parameters (r_s and p values shown, gray line, open circles; Spearman rank order correlation test).

CaMKII, as well as phosphorylation of Glur1 at Ser831. This appeared to be a transient event because these increases in phosphorylation had subsided after 6 hr of ad lib sleep. Phosphorylation of GluR1 at Ser845 on the other hand was more protracted and only significantly elevated at the end of the post-MD sleep period. These changes were not observed in animals sacrificed after MD only or in sleeping cats without prior MD. Moreover, increases in phosphorylation of these proteins did not occur in cats kept awake during the initial 2 hr period following MD (either in complete darkness or with normal illumination) or whose visual cortices were infused with APV during this time period. These findings demonstrate that in the remodeling visual cortex, there is a sleep-dependent activation of several LTP-related kinases known to act downstream of NMDARs. These changes are accompanied by phosphorylation events specifically associated with LTP (Lee et al., 2000)—i.e., CaMKII- and PKA-mediated phosphorylation of GluR1 AMPA receptor subunits.

Experiment 3: Chronic Recording of Remodeling Circuits Reveals Sleep-Specific Elevations in Cortical Activity

Neurons (or brain regions) activated during waking tasks are reported to reactivate in subsequent sleep (Ji and Wilson, 2007; Maquet et al., 2000; Pavlides and Winson, 1989; Wilson and McNaughton, 1994). We hypothesized that a similar increase in activity might occur during sleep in remodeling cortical neurons, leading to activation of NMDARs and kinase pathways (Nadasky et al., 1999). To investigate this possibility, we performed chronic extracellular multiunit recordings in freely behaving cats before, during, and after a period of MD (see Figure 6A for experimental design). Because fine-grained analyses of neuronal ensembles cannot be performed with this technique, we instead addressed two basic questions: do remodeling neurons increase their activity during sleep, and is this activation proportional to changes in OD?

Six cats had micro-wire bundles placed deep into the medial bank of V1 ipsilateral to the deprived eye (see Experimental Procedures and Supplemental Data). Multiunit activity (MUA) recordings were made simultaneously with EEG and nuchal EMG recordings. The animals then underwent procedures similar to those used in our acute studies. Six hours of baseline EEG/EMG and MUA recordings were made in each cat, followed by 6 hr of waking MD (three cats) or sleep deprivation (SD) with normal, binocular vision ("noMD" controls, three cats). The animals were then allowed to sleep undisturbed in complete darkness for an additional 6 hr. After scoring the EEG/EMG data as wakefulness, REM, and NREM sleep, we then computed the mean MUA firing rates (in Hz) for each vigilance state in the baseline period and in 2 hr bins throughout the post-SD or post-MD sleep period.

OD was assessed using an eye-testing and ranking scheme modified for use in freely behaving animals. In each animal,

MUA was measured at fixed intervals (at baseline, immediately after MD or SD, after post-MD or post-SD sleep; Figure 6A) in a counterbalanced design as a light-proof patch was switched to each eye. A left eye/right eye mean firing ratio was calculated at each microwire using the same algorithm used for our acute single-unit assessments (Jha et al., 2005). As there were not enough recordings per animal to calculate scalar OD measures, we analyzed changes in pooled left eye/right eye ratios as a measure of ODP.

In agreement with our acute measurements (Figures 2 and 3) and previous findings (Frank et al., 2001), we found that MD alone caused a significant shift in OD that was enhanced further by a period of sleep (n = 24 total microwire recordings, mean (±SEM) left eye/right eye ratios, baseline: 1.02 ± 0.14 < MD only: 1.22 ± 0.13* < MDS: 1.56 ± 0.31*; repeated-measures ANOVA on ranks, $\chi^2 = 17.043$, $p < 0.001$; *SNK $p < 0.05$). As expected, there were no significant changes in OD in the control cats with normal vision (n = 15 total microwire recordings, baseline: 1.17 ± 0.08, SD only: 1.1 ± 0.05, SDS: 1.23 ± 0.05; repeated-measures ANOVA on ranks, N.S.).

There were no significant differences in mean firing rates between the MD and noMD cats during the baseline period in any vigilance state. However, cortical sites undergoing ODP (i.e., in cats exposed to MD) became more active in the first 2–4 hr of subsequent REM and NREM sleep (Figure 6C). In contrast, there were no significant changes in wake (data not shown, repeated-measures ANOVA on ranks, *N.S.*); nor were there any significant changes in MUA in noMD cats relative to baseline in any vigilance state. Further analyses of baseline and post-MD data from MD cats showed that there was a significant positive correlation between the local increases in overall (normalized) NREM firing rate and OD shifts at each recording site (Figure 6D). There was a similar trend for changes in MUA during REM sleep, but this did not reach statistical significance. Analyses in the noMD control cats showed no significant relationships between changes in firing rate and changes in OD over the course of the experiment.

DISCUSSION

We used three complementary approaches to test the hypothesis that reactivation of NMDARs and kinase pathways is a central mechanism in sleep-dependent consolidation of cortical plasticity in vivo. In agreement with previous findings (Frank et al., 2001), a period of ad lib sleep enhanced the effects of prior MD. This was primarily due to NMDAR and PKA-mediated strengthening of synaptic responses to NDE stimulation. Western blot analyses showed that kinases critical for LTP and activated by NMDAR activity were specifically activated in post-MD sleep, while chronic, longitudinal MUA recording in freely behaving animals showed that remodeling cortical circuits transiently increase their activity at times when this kinase activation is maximal. In sum, these findings strongly suggest that when the cortex is triggered to remodel in wakefulness, synaptic changes are further modified and consolidated by cortical reactivation and a secondary series of NMDAR and kinase-mediated signaling cascades during sleep.

Synaptic Strengthening and ODP

Our analysis of single-unit response properties and western blot data from V1 shows that ODP proceeds in two discrete stages that are divided across wakefulness and sleep. MD during wakefulness triggers depression in DE pathways, while NDE responses are potentiated specifically during subsequent sleep. These results are consistent with previous findings that MD initiates a two-step process leading to depression of DE responses and subsequent potentiation of NDE responses (Frenkel and Bear, 2004; Mioche and Singer, 1989). However, we now show that the latter process is specifically sleep dependent and much more rapid than previously reported. Differences between these studies and our own are likely due to at least two factors. First, these investigators did not quantitatively measure or control for sleep and wakefulness. Second, it is possible that similar state-dependent processes occur in mice, but over longer timescales—consistent with the fact that ODP is less robust in mice (Gordon and Stryker, 1996).

Sleep-dependent potentiation requires NMDAR and PKA activation. One important role of PKA in this process may be the phosphorylation of AMPAR subunit GluR1 at Ser845 (Figure 5C), which is critical for postsynaptic potentiation of glutamatergic synapses, both in vitro (Lee et al., 2000) and during naturally occurring plasticity in vivo (Hardingham et al., 2008). Our findings further suggest that PKA-mediated phosphorylation is paralleled by large increases in the activation of CaMKII and ERK, and the phosphorylation of GluR1 at Ser831 during the first 1–2 hr of post-MD sleep. These changes are particularly revealing because CaMKII and ERK activation mediate several forms of NMDAR-dependent LTP (Poser and Storm, 2001) and phosphorylation of GluR1 Ser831 by CaMKII results in the insertion and stabilization of AMPARs in the postsynaptic membrane—a critical event for the expression of LTP at glutamatergic synapses (Lee et al., 2000).

An important area of future investigation will be to determine the precise relationship between NMDARs and kinase pathways in sleep-dependent consolidation. The interaction between NMDARs and PKA, for example, may be indirect (i.e., via calcium-dependent adenylate cyclases) or reflect parallel mechanisms that must be activated in concert (Poser and Storm, 2001). However, as shown in Figure 5, NMDAR activation appears to be a rate-limiting step in the activation of ERK and CaMKII; APV infusions completely block the sleep-dependent phosphorylation of ERK and CaMKII. A related unanswered question is the precise role of ERK and CaMKII, as both kinases are massively activated during post-MD sleep. In addition to CaMKII's role in GluR1 regulation, both kinases (in addition to PKA) are involved with transcriptional events in remodeling neurons (Waltereit and Weller, 2003; Zieg et al., 2008). Although many genes necessary for synaptic plasticity are upregulated during waking, some are also upregulated during sleep (Cirelli et al., 2004; Mackiewicz et al., 2007; Ribeiro et al., 1999). Thus, transcriptional regulation may also play an important role in kinase-mediated synaptic potentiation during sleep.

Sleep-Dependent Plasticity: Current Theories

Our findings are consistent with a recent hypothesis (termed "synaptic reentry reinforcement") that consolidation of brain

plasticity involves secondary waves of NMDAR activation that occur after the initial induction of plasticity or encoding of experience (Shimizu et al., 2000; Wang et al., 2006). Our findings in combination with previous results now demonstrate that this occurs during sleep. For example, systemic NMDAR antagonist treatment immediately following a period of MD is sufficient to block ODP, but administration following a delay of 1–6 hr does not affect plasticity (Rauschecker et al., 1990; Rauschecker and Hahn, 1987); results comparable to our current findings (Figure S5). Similarly, systemic administration of NMDAR antagonists during sleep impairs memory consolidation on a visual discrimination task in humans (Gais et al., 2008). Finally, several studies show that brain areas engaged during learning reactivate in subsequent sleep (Ji and Wilson, 2007; Maquet et al., 2000; Pavlides and Winson, 1989; Wilson and McNaughton, 1994), which may provide sufficient neuronal depolarization to activate NMDARs (Nadasky et al., 1999). We did not determine if specific temporal or spatial patterns of neural activity present during MD "replayed" in subsequent sleep, but the increase in MUA in remodeling cortices is consistent with this general hypothesis. Interestingly, the effects of NMDAR blockade in our experiments were comparable to those of sleep deprivation alone (Figure S6), suggesting that dysfunction in NMDAR signaling may mediate the negative effects of sleep loss on cognition. Indeed, sleep deprivation has been shown to reduce NMDAR surface expression (Chen et al., 2005; McDermott et al., 2006) and NMDAR-dependent LTP (Kopp et al., 2006; Tartar et al., 2006).

On the other hand, our results are seemingly at odds with the recently proposed "synaptic homeostasis" hypothesis, according to which wakefulness is accompanied by net synaptic strengthening, while sleep promotes generalized homeostatic downscaling of synaptic strength (Tononi and Cirelli, 2006). As shown by these investigators, CaMKII and GluR1 phosphorylation is lower when adult rats are sacrificed after their normal rest period and higher when sacrificed during wakefulness. Changes in evoked responses to electrical stimulation of the cortex and tetanic LTP in vivo across sleep and wake were also consistent with the theory (Vyazovskiy et al., 2008). There are several important differences between our studies that may account for this apparent discrepancy.

First, we investigated a naturally occurring form of plasticity in vivo, whereas these authors examined artificial forms of induced plasticity (or electrically evoked responses) and gene and protein expression in animals whose brains were not stimulated to remodel (Cirelli et al., 2004; Vyazovskiy et al., 2008). While electrically induced responses and tetanic LTP are important experimental correlates and models of synaptic plasticity, respectively, they do not always reflect what naturally occurs in the intact brain (Albensi et al., 2007). Gene expression in sleep can also vary depending on whether or not synaptic plasticity has been induced (Ribeiro et al., 1999, 2002).

Second, it is possible that the cellular mechanisms involved in "synaptic homeostasis" are distinct from those involved in ODP. Recent findings suggest that the net synaptic potentiation that is proposed to trigger downscaling in sleep is mediated by the neurotrophin BDNF (Faraguna et al., 2008; Huber et al., 2007). However, MD-induced cortical plasticity does not require activation of BDNF TrkB receptors, while recovery from the effects of MD is dependent upon BDNF (Kaneko et al., 2008). We have shown that this latter form of BDNF-dependent plasticity is *not* consolidated by sleep (Dadvand et al., 2006).

Finally, previous studies examining "synaptic homeostasis" have focused entirely on adult animals and humans; therefore, differences between developmental and adult plasticity may also be a contributing factor. However, this explanation seems unlikely; while there are differences in cortical plasticity in developing and adult animals, these appear to be outweighed by the pool of shared cellular and molecular mechanisms (Berardi et al., 2003; He et al., 2006; Hofer et al., 2006). In summary, the most parsimonious explanation for differences between our results and previous findings is that the effects of sleep are highly dependent upon the types of plasticity under examination and the extent to which the brain is remodeling when measurements of synaptic plasticity are made.

Summary

In conclusion, our study provides new insights into the cellular mechanisms governing the sleep-dependent consolidation of cortical plasticity. We find that consolidation of ODP involves reactivation of remodeling neurons and NMDAR and PKA-dependent intracellular cascades during sleep, which in turn trigger phosphorylation events and processes that promote synaptic strengthening. These findings thus support the hypothesis that a key function of sleep is the consolidation of waking experience.

EXPERIMENTAL PROCEDURES

Experiment 1

Formation of Groups

Experimental groups (Figure 1A) consisted of cats with either (1) normal binocular visual experience (Normal; n = 7 [age = 36.6 ± 1.5 days]), (2) 6 hr of MD without subsequent sleep (MD-only; n = 4 [age = 35.0 ± 0.8 days]), (3) 6 hr of MD with 3 hr vehicle infusion into V1 over the first half of a 6 hr post-MD sleep period (VEH; n = 5 [age = 35.2 ± 1.0 days]), (4) 6 hr MD with 3 hr APV infusion during the first half of post-MD sleep (APV; n = 5 [age = 33.8 ± 1.4 days]), or (5) 6 hr MD with Rp-8-Cl-cAMPS infusion during post-MD sleep (Rp-8-Cl-cAMPS; n = 5 [age = 35.4 ± 1.7 days]). Four of the Normal cats were previously used to provide normative data in an earlier study (reproduced with permission from Jha et al. [2005]). There was no difference in age between any of the groups ($F = 0.69$, $p = 0.70$, one-way ANOVA).

Surgical Procedures and Sleep/Wake Recording

All cats (except Normal) were implanted with EEG/EMG electrodes and cannulae for infusion into V1 as described previously (Jha et al., 2005). After 4–5 days of postoperative recovery, cats were placed in a light-proof, illuminated sleep-recording chamber with a revolving base. EEG/EMG signals were continuously recorded during a 6 hr baseline period, a 6 hr MD period, and a 6 hr post-MD sleep period in total darkness (except for MD-only cats: Figure 1A). Cats were provided with food and water ad lib at all times. Polygraphic signals were amplified with an Astro-Med (West Warwick, RI) amplifier system, filtered (high-pass at 0.3 Hz, low-pass at 100 Hz), digitized at 200 Hz, and recorded as previously described using SleepSign software (Kissei Comtec; Irvine, CA) (Jha et al., 2005).

MD Procedure

Following the baseline recording period, cats were anesthetized with isofluorane and had their right eyelids sutured closed (except for noMD+APV cats, which were anesthetized for an equal amount of time but underwent no further manipulation) as previously described (Frank et al., 2001). Following recovery, cats were returned to their recording chambers and were kept awake (through a combination of gentle handling, novel object exploration, vocalization, and floor rotation) under normal room illumination for the next 6 hr to provide

a common stimulus for remodeling in V1. An equal amount of wakefulness was provided by these means in the noMD+APV cats (Figure S2A). The deprived eye remained sutured closed in all cases until acute OD assessments.

Intrinsic Signal Imaging and Ocular Dominance Analysis

Intrinsic signal imaging was performed using previously described methods (Jha et al., 2005) (see Supplemental Data). Previously used scalar measures of OD in each hemisphere were then used to quantify the pixel distributions (Frank et al., 2001; Jha et al., 2005). To simplify our presentation, the traditional contralateral bias index (CBI) was modified so that scores of 1 indicated complete dominance by the nondeprived eye, 0 complete deprived eye dominance, and 0.5 equal representation of both eyes in the hemisphere under study (a metric hereafter referred to as the "nondeprived eye bias index": NBI). We also calculated monocularity indices (MI) for unihemispheric and combined hemisphere data and shift indices (SI) as previously described (Frank et al., 2001; Jha et al., 2005). MI values of 1 indicate a complete loss of binocular responses and 0 indicate that all pixels are activated equally by both eyes. The shift index (SI) measures overall changes in OD across both hemispheres ($CBI_{Ipsilateral\ to\ the\ DE} - CBI_{contralateral\ to\ the\ DE}$) with a value of 0 indicating complete binocularity, and 1 or −1 indicating complete shifts toward one eye or the other (Frank et al., 2001; Issa et al., 1999).

Single-Unit Recording

To provide a complementary and finer assessment of OD and neuronal response properties, after each intrinsic signal imaging session, microelectrode recordings of single neurons were performed in all groups as described previously (Jha et al., 2005). Neuronal responses to grating stimuli presented in either eye were recorded near and far from cannula sites using a 1 × 1 mm array of 16 electrodes (Frederick Haer; Bowdoinham, ME) which was placed near (<3 mm) or far (>3 mm) from the infusion site. Scalar measures of OD (SIs, CBIs, and MIs) were calculated from single unit data in a manner similar to that described for intrinsic signal imaging data, using methods described previously (Frank et al., 2001; Jha et al., 2005). See Supplemental Data for additional details.

Experiment 2

Formation of Groups

For all western blot experiments, a minimum of four V1 samples were used for each group, and each hemisphere was considered a single sample as described previously (Kaneko et al., 2008). Cats from individual litters were divided into the experimental groups as shown in Figure 5A. Animal ages at the time of sacrifice did not vary between groups ($H = 6.3$, $p = 0.50$, Kruskal-Wallis one-way ANOVA on ranks). Mean (±SEM) ages and animal numbers for the main groups were: Normal: n = 4 (32.5 ± 1.5 days), MD-only: n = 4 (31.8 ± 0.5 days), MD+1&2S: n = 6 total (31.7 ± 0.7 days), MD+6S: n = 3 (32.3 ± 0.7 days), noMD+1&2S: n = 5 total (32.8 ± 1.1 days), noMD+6S: n = 2 (34.0 ± 0.0 days), MD+2S+APV: n = 3 (33.7 ± 0.9 days), MD+2SD: n = 5 total (30.8 ± 1.3 days). These group sizes compare favorably with recent studies employing western blot analyses of visual cortex (Cnops et al., 2008; Kaneko et al., 2008).

Tissue Preparation and Western Blot Analysis

At the end of each experimental manipulation, animals were sacrificed with an overdose of pentobarbital. Right and left visual cortices were dissected and flash-frozen on dry ice, then stored at −70°C until use (see Supplemental Data for additional details).

Experiment 3

Formation of Groups

Six cats were divided into MD and noMD groups (n = 3 each), which compares favorably with group sizes used in two recent studies using chronic recording techniques (which used groups or four and two animals, respectively) (Euston et al., 2007; Ji and Wilson, 2007). MD cats were 36 ± 1.2 days old, and noMD control cats were 31 ± 2.3 days old at the time of recording (Student's t test, N.S.).

Chronic Neuronal Recording

An 8–14 microwire bundle was surgically implanted in the medial bank of the hemisphere ipsilateral to the deprived eye (along with reference and ground electrodes, and nuchal EMG electrodes). Following postoperative recovery, cats were placed in the same sleep/wake recording chambers used for acute studies. Electrophysiological signals acquired using MAP hardware and RASPUTIN software (Plexon Inc.; Dallas, TX). EEG recordings were made on subsets of microwires and collected with nuchal EMG recordings (see Supplemental Data for details).

MD and Modified Ocular Dominance Assessments in the Freely Behaving Cat

MD was achieved by covering the right eye with a light-proof patch for 6 hr while the animals were kept awake as described above. OD assessments were made by covering each eye with the same patch used in a counterbalanced design (left eye open, right eye open, both closed). Each test consisted of a 5 min exposure with one or both eyes patched while the animal explored its environment in the sleep chamber (i.e., a natural scene test; see Supplemental Data for details).

Spike Analyses during Sleep and Wakefulness

For both MD and control (noMD) cats, all episodes ≥1 min in length of artifact-free REM and NREM sleep and wakefulness were used to calculate mean baseline and post-MD or -SD MUA values. Changes in OD were calculated as [left eye/right eye ratio after post-MD or -SD sleep]/[left eye/right eye ratio during baseline]. A normalization procedure was used to scale the firing rate data in a manner comparable to the changes in OD, by dividing mean raw spike counts in each state by the mean spike rate averaged across vigilance states for that time bin. Changes in firing rate were then computed for REM and NREM sleep as normalized firing [post-MD sleep]/normalized firing [baseline sleep].

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures and eight figures and can be found with this article online at http://www.neuron.org/supplemental/S0896-6273(09)00040-3.

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