Removal of Synaptic Ca^{2+} -Permeable AMPA Receptors during Sleep

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There is accumulating evidence that sleep contributes to memory formation and learning, but the underlying cellular mechanisms are incompletely understood. To investigate the impact of sleep on excitatory synaptic transmission, we obtained whole-cell patch-clamp recordings from layer V pyramidal neurons in acute slices of somatosensory cortex of juvenile rats (postnatal days 21–25). In animals after the dark period, philanthotoxin 74 (PhTx)-sensitive calcium-permeable AMPA receptors (CP-AMPA receptors) accounted for ~25% of total EPSP size, and current–voltage (I – V) relationships of the underlying EPSCs showed inward rectification. In contrast, in similar experiments after the light period, EPSPs were PhTx insensitive with linear I – V characteristics, indicating that CP-AMPA receptors were less abundant. Combined EEG and EMG recordings confirmed that slow-wave sleep-associated delta wave power peaked at the onset of the more quiescent, lights-on phase of the cycle. Subsequently, we show that burst firing, a characteristic action potential discharge mode of layer V pyramidal neurons during slow-wave sleep has a dual impact on synaptic AMPA receptor composition: repetitive burst firing without synaptic stimulation eliminated CP-AMPA receptors by activating serine/threonine phosphatases. Additionally, repetitive burst-firing paired with EPSPs led to input-specific long-term depression (LTD), affecting Ca^{2+} impermeable AMPARs via protein kinase C signaling. In agreement with two parallel mechanisms, simple bursts were ineffective after the light period but paired bursts induced robust LTD. In contrast, incremental LTD was generated by both conditioning protocols after the dark cycle. Together, our results demonstrate qualitative changes at neocortical glutamatergic synapses that can be induced by discharge patterns characteristic of non-rapid eye movement sleep.

Introduction

A variety of experiments in humans and animals support a facilitating role for sleep in plasticity, learning, and development (Frank et al., 2001; Maquet, 2001; Hobson and Pace-Schott, 2002; Steriade and Timofeev, 2003; Walker and Stickgold, 2004; Rasch and Born, 2007). Plasticity processes in the brain are thought to result from the malleability of synaptic connections, in particular those involving the neurotransmitter glutamate (Martin et al., 2000). The most common receptors for glutamate are AMPA receptors (AMPA receptors), which can systematically vary in number and subunit composition during plasticity and learning (Kessels and Malinow, 2009). The absence of the glutamate receptor subunit GluR2 determines whether AMPA receptors are permeable to Ca^{2+} (CP-AMPA receptors) (Cull-Candy et al., 2006; Isaac et al., 2007; Liu and Zukin, 2007), and influx of Ca^{2+} is known to trigger processes associated with plasticity (Bliss and Collingridge, 1993). Previous experiments showed that CP-AMPA receptors can be incorporated into active synapses (Takahashi et al., 2003; Clem and Barth, 2006; Plant et al., 2006; Zhu 2009), which may tag those for additional modifications. In hippocampal CA3 pyramidal cells, CP-AMPA receptors are selectively removed at mossy fiber synapses by postsynaptic depolarizations (Ho et al., 2007). A recent

study showed that the slope of field potentials and the expression of AMPA receptor subunit proteins depend on the wake state of the animal (Vyazovskiy et al., 2008). These sleep–wake cycle-dependent changes at neocortical synapses were mainly attributed to non-rapid eye movement (NREM) sleep activity (Vyazovskiy et al., 2008). However, the cellular processes involved remain essentially unknown.

Sleep consists of alternating NREM and REM episodes, both of which have been implicated in contributing to memory formation and learning (Diekelmann and Born 2010). Sleep rhythms are the result of transiently synchronized discharges within dynamically interconnected groups of cells (Krueger et al., 2008). During such rhythms, cortical neurons discharge action potentials in characteristic patterns of which bursts are an elementary component (Steriade, 2004). Bursts consist of high-frequency clusters of action potentials that are riding on an underlying depolarizing potential (Connors and Gutnick, 1990). In many neuronal cell types, action potentials can backpropagate into dendrites and modify coincident synaptic inputs (Stuart and Sakmann, 1994; Markram et al., 1997). We have shown recently that the sign of synaptic plasticity also depends on the firing mode: pairings of EPSPs and delayed single spikes led to long-term potentiation, whereas similar pairings with bursts induced long-term depression (LTD) (Birtoli and Ulrich, 2004).

The aims of this study were to investigate the influence of the sleep–wake cycle on synaptic AMPA receptor composition and to assess whether these modifications could result from NREM sleep associated firing patterns.

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Materials and Methods

Slice preparation. Three- to 4-week-old Wistar rats were kept on a 12 h light/dark cycle with lights on (8:00 A.M.) and lights off (8:00 P.M.). Animals were killed at 8:00 A.M., 12:00 P.M., or 5:00 P.M. In one set of experiments, rats were kept on a reversed light/dark cycle for >14 d, and slices were prepared at 12:00 P.M., i.e., mid-dark cycle. Individual animals were killed within 5 min after collection at the times and light conditions indicated. Parasagittal slices of 300 μ m thickness were prepared at 4°C and incubated at 35°C in standard artificial CSF containing the following (in mM): 125 NaCl, 1.25 NaH_2PO_4 , 25 NaHCO_3 , 2.5 KCl, 1 MgCl_2 , 2 CaCl_2 , and 10 glucose (equilibrated with 5% CO_2 /95% O_2). All experimental procedures were in line with institutional guidelines for animal research.

In vitro electrophysiology. Whole-cell recordings were obtained with BVC-700A amplifiers (Dagan Corporation), low-pass filtered at 1 kHz, and digitized at 2 kHz with a Digidata 1440A analog-to-digital converter (Molecular Devices). For current-clamp recordings, patch pipettes were filled with the following (in mM): 125 K-gluconate, 5 NaCl, 0.1 EGTA, 10 HEPES, 4 ATP, and 0.4 GTP. For voltage-clamp experiments, K^+ was replaced by Cs^+ , and, to further improve space-clamp conditions, sodium and low-voltage-activated T-type calcium channels were blocked with QX-314 (2 mM intracellular) and Ni^{2+} (0.1 mM), respectively. Series resistance ($\sim 10 \text{ M}\Omega$) was compensated for by adjusting the bridge and unstable recordings were discarded. A liquid junction potential of -10 mV was left uncorrected. EPSPs/EPSCs were evoked every 5 s by brief extracellular current steps with an isolated pulse stimulator (model 2100; A-M Systems) through insulated bipolar nickel–chromium wires (0.025 mm; Goodfellow Corp.), placed in layers II/III. In the simple-burst protocol, 70 triplets of action potentials with an interspike frequency of 150–300 Hz were elicited by brief somatic current injections (10–15 ms, 1–2 nA, 0.2 Hz). For the paired-burst protocol, EPSPs were costimulated with similar bursts delayed by +10 ms. Small hyperpolarizing step current injections at the end of each trace were used to determine the input resistance of the neurons. Drugs were prepared as stock solutions (1000 $\times$) in H_2O and kept in aliquots at -20°C .

We measured the contribution of CP-AMPA receptors to synaptic transmission with philanthotoxin 74 (PhTx) (5 μM) (Eldefrawi et al., 1988; Washburn and Dingledine, 1996). Occasional augmenting responses to PhTx were considered unspecific, and the data from these cells were excluded from additional analysis (Brackley et al., 1990). We expressed the effects of PhTx relative to the original baseline. Similar findings were obtained in the plasticity studies if the effects of PhTx were quantified relative to the new baseline before PhTx wash-in.

Orthovanadate was prepared as described by Gordon (1991). Drugs were either intracellularly applied for >20 min via the pipette or added to the bath as indicated. Okadaic acid and orthovanadate were from Sigma-Aldrich. All other drugs were from Tocris Bioscience.

In vivo EEG/EMG recordings. Animals (at 3–4 weeks) were anesthetized with ketamine–xylazine (0.2 ml, i.p.). Screw electrodes (soldered to pin con-

Figure 1. Circadian changes of AMPA receptor subtypes. **A, B,** The EPSP amplitude time course (mean $\pm$ SEM) is shown in control and after bath application of PhTx (5 μM). Sample EPSPs are illustrated for two representative time points in control (1) and PhTx (2). Experiments are shown after the dark (**A**) and light (**B**) episodes. EPSPs were significantly depressed by PhTx only after the dark episode ($p < 0.05$). **C, D,** Sample EPSC current–voltage relationships are depicted with the underlying EPSCs at different holding potentials after the dark (**C**) and light (**D**) episodes. Straight lines were fitted by linear regression. Note the deviation of the data points from linearity in **C, E.** Summary histogram of RI (mean $\pm$ SEM) after the dark (gray bar) and light (white bar) phases. ****** $p < 0.0005$. **F,** Graphical representation of the relative time that the rats spent in wakefulness, NREM sleep, and REM sleep (mean $\pm$ SEM, $n = 9$) over a full light/dark cycle.

nectors) were implanted in the parietal and frontal skull (2 mm anterior and 2 mm lateral to bregma, 2 mm posterior and 2 mm lateral to bregma ipsilateral, respectively). For EMG recordings, two wire electrodes were implanted in the neck muscle. After the surgery, the animals were given intraperitoneal analgesic (buprenorphine) and left to recover for 40 h. The pin connectors for EEG and EMG recordings were connected to the wire leads of a telemetric transducer (TL11M2-F40-EET; Data Sciences International) located outside the cage. The signals were processed using Dataquest ART Acquisition Software (Data Sciences International) and analyzed using Sleep Sign Software (Kissei Comtec). Vigilance stages were automatically scored in 30 s epochs and classified as wakefulness (high-frequency low-amplitude EEG and high

Figure 2. CP-AMPA receptors are removed during the light phase. **A, B**, EPSP amplitude time course (mean $\pm$ SEM) in control and PhTx ($5 \mu\text{M}$). Sample EPSPs are shown before (1) and after (2) PhTx application. Group data after 4 h of light (**A**, Noon) and 4 h of darkness [**B**, Noon (reversed)]. **C**, Summary histogram (mean $\pm$ SEM) of PhTx effects on EPSPs for the different experimental conditions indicated. Significant PhTx effects were seen at 8:00 A.M. and Noon (reversed). $*p < 0.05$. **D**, Summary histogram of the amount of total NREM sleep that rats had spent during the previous 4 h at various points during the light/dark phase. NREM sleep was significantly diminished in the 8:00 A.M. versus the Noon and 5:00 P.M. group. $**p < 0.01$, $***p < 0.001$.

amplitude EMG), NREM sleep (low-frequency high-amplitude EEG with low-amplitude EMG), or REM sleep (low-amplitude EEG with predominant theta waves and muscle atonia, or low-amplitude EMG). After being automatically scored, the stages were visually inspected and manually revised if necessary.

Data analysis. EPSP/EPSC amplitudes were obtained by subtracting two 2–3 ms time windows of baseline from peak. LTD magnitude is the ratio of 5 min. EPSP amplitude averages at the beginning and end of each experiment. EPSP time series were generated by local six-point averaging (i.e., 30 s averages), and ensemble time series are shown as mean $\pm$ SEM. Sample EPSPs are the average of 30 individual traces. To determine the rectification index (RI), a straight line was fitted to the data points for negative voltages. The RI was taken as the amplitude ratio of the actual current at 50 mV divided by the linearly extrapolated value (Ho et al., 2007).

Statistical analysis. Normalized data (LTD experiments) were compared with two-tailed nonparametric tests (Mann–Whitney U test) at a significance level of 0.05. Original (EEG/EMG) data were tested for normality (Shapiro–Wilk test, $p < 0.05$) and assessed with a two-sided Student's t test or ANOVA, followed by a Tukey–Kramer multiple comparisons test.

Results

Synaptic AMPAR composition changes during the sleep–wake cycle

Pyramidal cells in layer V of somatosensory cortex were visualized with infrared differential interference contrast video micros-

copy (Stuart et al., 1993), and whole-cell patch-clamp recordings were performed in current- or voltage-clamp mode. Composite EPSPs of 2–5 mV were elicited in layers 2/3, and stable control sequences were recorded for ≥ 10 min. GABA_A receptor-mediated inhibitory synaptic responses were routinely blocked by adding (–)bicuculline methochloride ($20 \mu\text{M}$) to the bath, and NMDA receptors were inhibited by ± 2 -amino-5-phosphono-valeric acid (0.1 mM). In a subset of experiments, bicuculline was replaced by intracellular picrotoxin ($10 \mu\text{M}$). In recordings from animals recruited at the end of the dark period, blockade of CP-AMPA receptors with PhTx ($5 \mu\text{M}$) (Eldefrawi et al., 1988; Washburn and Dingledine 1996) led to a significant decrease of EPSPs to $73 \pm 9\%$ of control ($n = 6$, $p < 0.05$) (Fig. 1A). In contrast, in equivalent experiments from rats after 9 h of the light period, PhTx left EPSPs unaltered ($105 \pm 8\%$, $p = 0.1$, $n = 7$) (Fig. 1B). The membrane resting potentials (V_m) and input resistances (R_i) were not different between animals after the light ($V_m = 64 \pm 1 \text{ mV}$, $R_i = 51 \pm 4 \text{ M}\Omega$, $n = 18$) or dark ($V_m = 62 \pm 1 \text{ mV}$, $R_i = 57 \pm 5 \text{ M}\Omega$, $n = 18$) episode ($p > 0.4$). In another series of experiments, we assessed the presence of CP-AMPA receptors by determining their current–voltage (I – V) relationship. CP-AMPA receptors are inhibited by intracellular spermine at depolarized membrane potentials, leading to inwardly rectifying I – V curves (Bowie and Mayer, 1995; Donevan and Rogawski, 1995). Evoked EPSCs were obtained in voltage-clamp mode with Cs⁺-based pipette solu-

tions that contained spermine (0.3 mM ; see Materials and Methods). Experiments from animals after the dark period showed inwardly rectifying I – V curves (Fig. 1C). In contrast, I – V plots of EPSCs in the light episode were linear (Fig. 1D). The RIs after the dark and light cycle were significantly different (dark, 0.54 ± 0.07 , $n = 12$; light, 0.97 ± 0.07 , $n = 7$, $p < 0.0005$) (Fig. 1E). Combined EEG/EMG recordings from a group of nine animals allowed us to characterize the sleep–wake activity pattern (Fig. 1F). There was a clear predominance of wakefulness during the dark episode ($61.6 \pm 2.6\%$; light phase, $46.4 \pm 3.7\%$, $p < 0.005$), as would be expected for the rat.

To investigate the temporal evolution of the CP-AMPA receptor removal during the light phase, additional experiments were performed. EPSPs were insensitive to PhTx at 12:00 P.M., i.e., 4 h after light onset ($107 \pm 7\%$, $n = 9$, $p > 0.5$) (Fig. 2A). In contrast, EPSPs were significantly reduced by PhTx to $82.5 \pm 5.7\%$ ($n = 9$, $p < 0.05$) at 12:00 P.M. in rats on a reversed light/dark cycle (i.e., 4 h after dark onset; see Materials and Methods). In the reverse cycle animals, the wake–sleep pattern was fully inverted with a predominance of wakefulness during the dark episode ($59.6 \pm 4.9\%$) compared with $46.2 \pm 5.7\%$ in the light phase ($p < 0.01$), i.e., not different from normal cycle animals ($p > 0.5$). Figure 2C shows a summary graph of PhTx effects on EPSPs under the various conditions tested. A significant PhTx effect was detect-

able only for the experimental groups at 8:00 A.M. (i.e., end of dark phase) and 12:00 P.M. (reversed cycle).

We next compared the amount of sleep–wakefulness in the preceding 4 h for each of the three different experimental conditions in normal cycle animals (Fig. 2D). Overall, there was a significant effect of time on sleep–wake states (one-way ANOVA, $F_{(3,27)} = 11.64$, $p < 0.0001$, $n = 9$). *Post hoc* analysis revealed that the preceding total amount of wakefulness decreased whereas that of NREM sleep increased significantly between the end of the dark episode and 4 h later ($p < 0.01$) (Fig. 2D), a time when CP-AMPA were no longer detectable.

In addition, power spectrum analysis was used to quantify slow-wave activity (SWA) (EEG power between 1.0 and 4.0 Hz) during NREM sleep, providing a means of measuring sleep homeostatic pressure, which varies on top of circadian processes. SWA showed a significant change over the 24 h cycle (repeated-measures ANOVA, $p < 0.05$, $n = 9$), peaking during the first hour ($143.9 \pm 17.3\%$ total NREM SWA) after onset of the more quiescent, lights-on phase of the cycle and declining thereafter to reach $76.3 \pm 6.9\%$ in the last hour of this phase. Similar to the rats maintained on the normal light/dark cycle, animals entrained to the reverse timing had a significant variation in sleep pressure as measured by the power of SWA during NREM ($p < 0.05$, $n = 8$). Again, the peak SWA activity was observed in the first hour of the relatively quiescent lights-on phase ($125.4 \pm 6.6\%$) and declined to $96.0 \pm 5.9\%$ at the end of this phase.

Selective removal of AMPAR subtypes through burst firing

During slow-wave sleep, pyramidal cells generate action potentials in burst mode, i.e., high-frequency clusters of spikes that occur repetitively at approximately delta frequencies (Amzica and Steriade, 1998). Bursts can occur synchronously or asynchronously with synaptic activity. We have shown previously that repetitive pairings of EPSPs and bursts (burst pairings), mimicking synchronous burst firing, lead to LTD (Birtoli and Ulrich, 2004; Czarniecki et al., 2007). First, we investigated whether the burst-pairing protocol affected CP-AMPA by conditioning EPSPs with action potential bursts. These and subsequent experiments were done in rats after the dark period. Figure 3A shows the EPSP amplitude time course after burst pairings that regularly led to robust LTD (average, $44 \pm 6\%$ of control, $p = 0.001$, $n = 7$). In these and the following experiments, the stability of V_m and R_i was monitored throughout the experiment, and unstable recordings were discarded (Fig. 3A). After the burst pairings, conditioned EPSPs were insensitive

Figure 3. CP-AMPA are eliminated by NREM sleep discharge patterns. **A**, EPSP amplitude time course (mean $\pm$ SEM) in control and after EPSP burst pairings (■). The CP-AMPA-selective inhibitor PhTx ($5 \mu\text{M}$) was bath applied toward the end of the experiment as indicated. Sample EPSPs above are in control (1), after conditioning (2), and in PhTx (3). A sample EPSP burst is illustrated (■). Bottom, V_m and R_i of a representative cell remained stable throughout the experiment. **B**, Representative EPSC current–voltage relationship and corresponding EPSCs obtained by repatching the neuron with a Cs^+ -based solution that contained 0.3 mM spermine. A straight line was fitted by linear regression. **C**, Summary histogram of RI (mean $\pm$ SEM) in control (black bar) and after conditioning (gray bar). **D**, EPSP amplitudes (mean $\pm$ SEM) in control and after burst pairings (■). PhTx was bath applied toward the end as indicated. Sample traces are shown for control (1), after conditioning (2), and in PhTx (3). **E**, The tyrosine phosphatase inhibitor orthovanadate (1 mM) was bath applied throughout the experiment. LTD was still significant ($p < 0.05$), but PhTx was without effect ($p > 0.5$). **F**, The PP1/2A inhibitor okadaic acid ($0.1 \mu\text{M}$) was added to the pipette solution. Both burst pairings and PhTx led to a significant EPSP reduction ($p < 0.005$ and $p < 0.05$, respectively).

to PhTx ($46 \pm 7\%$, $p > 0.05$, $n = 7$), indicating that CP-AMPA were absent (Fig. 3A). In agreement, when pyramidal cells were repatched after the burst pairings with Cs^+ containing pipettes, I – V curves of conditioned EPSCs were linear (RI = 1.02 ± 0.11 , $n = 7$) (Fig. 3B), with RI values significantly different from control ($p < 0.0005$) (Fig. 3C). This suggests that NREM sleep-related discharge patterns are capable of removing CP-AMPA from excitatory synapses.

To elucidate the underlying signaling mechanism, we first blocked tyrosine phosphatases that were shown to mediate group I metabotropic glutamate receptor (mGluR)-dependent LTD and the removal of AMPARs in the hippocampus (Moult et al.,

2006). However, when tyrosine phosphatases were blocked with orthovanadate in the bath (1 mM), we could still elicit comparable LTD ($58 \pm 9\%$, $p < 0.05$, $n = 6$) and CP-AMPA removal as revealed by the lack of PhTx sensitivity of the conditioned EPSP ($57 \pm 9\%$, $p > 0.5$) (Fig. 3D). It is well established that dephosphorylation of the GluR1 subunit of AMPARs at serine 831 and 845 attenuates their activity, leading to LTD (Lee et al., 1998). When we blocked serine/threonine phosphatases (PP) 1/2A with okadaic acid (0.1 μM intracellular) (Cohen et al., 1990; Mansuy and Shenolikar, 2006), significant LTD was still inducible ($68 \pm 5\%$, $p < 0.005$, $n = 6$). However, PhTx now further reduced the conditioned EPSPs to $47 \pm 6\%$ of control ($p < 0.05$, $n = 6$) (Fig. 3D). This suggests that the elimination of CP-AMPA depends on serine/threonine phosphatases and that the paired-burst protocol also affects receptors other than CP-AMPA. In agreement, when paired bursts were applied after CP-AMPA were blocked with PhTx, which decreased the EPSP to $70 \pm 10\%$ of control ($p = 0.01$, $n = 6$), burst pairings were still capable of inducing LTD ($43 \pm 8\%$, $p < 0.05$, $n = 6$) (Fig. 4A). We then tested whether this residual LTD was dependent on Ca^{2+} -impermeable AMPARs (CI-AMPA). It has been shown that activated PKC phosphorylates AMPAR on the GluR2 subunit, which triggers their endocytosis thus promoting LTD (Chung et al., 2003). In line with this finding, when we added the protein kinase C inhibitory peptide 19–31 (RFARKGALRQKNV, 0.5 μM) to the pipette solution in the presence of PhTx, LTD was now fully blocked ($100 \pm 10\%$, $n = 6$, $p > 0.5$) (Fig. 4B). Similar results were also obtained with the PKC inhibitor bisindolylmaleimide (0.2 μM ; data not shown). After phosphorylation by PKC, GluR2/3-containing CI-AMPA are internalized via PICK1 (Kim et al., 2001). In accordance, intracellular application of the GluR2 C-terminal synthetic peptide p-SVKI (YNYVGIESVKI, 0.25 mM) (Kim et al., 2001), which disrupts GluR2 interaction with PICK1, inhibited paired-burst-induced LTD ($96 \pm 7\%$, $n = 6$, $p > 0.1$) (Fig. 4C). However, in contrast to similar experiments in the hippocampus, p-SVKI did not affect basal synaptic transmission (Fig. 4C) (Kim et al., 2001). Overall, these experiments show that EPSP burst pairings are capable of concomitantly removing both CP-AMPA and CI-AMPA from neocortical synapses.

During slow-wave sleep, bursts are not only synchronized among cells but can also occur asynchronously. To assess the impact of asynchronous burst discharges on synaptic plasticity, we recorded EPSPs, followed by a simple-burst protocol without synaptic stimulation (Fig. 5A). Burst conditioning routinely led to robust LTD ($74 \pm 8\%$, $n = 9$, $p < 0.005$) (Fig. 5A). The insensitivity of burst-conditioned EPSPs to PhTx ($74 \pm 11\%$, $p = 0.9$) indicated that this LTD was associated with a predominant removal of CP-AMPA (Fig. 5A). In agreement, I – V relationships of conditioned EPSCs obtained by repatching the neurons with Cs^+ -based solutions were linear after burst conditioning (Fig. 5B). The average RI was close to unity (1.1 ± 0.1 , $n = 7$) (Fig. 5C) and significantly enhanced versus control ($p < 0.0005$). Interestingly, this form of LTD was insensitive to blockade of PKC because EPSPs were still considerably depressed after burst conditioning in the presence of the inhibitory peptide 19–31 ($71 \pm 9\%$, $n = 6$, $p < 0.005$) (Fig. 5D). Similarly, robust LTD ($60 \pm 4\%$, $n = 6$, $p < 0.005$) was inducible after blocking CI-AMPA endocytosis with p-SVKI (Fig. 5E). The simple-burst protocol also remained effective in inducing LTD ($67 \pm 6\%$, $n = 6$, $p < 0.005$) in the presence of the broad-spectrum protein kinase blocker H9 [N -(2-aminoethyl)-5-isoquinolinesulfonamide dihydrochloride] (0.5 mM intracellular) (Fig. 5F). In contrast, the simple-burst

Figure 4. LTD of CI-AMPA. **A**, EPSP amplitude time course (mean $\pm$ SEM) and sample EPSPs (1–3). After a control period of 5 min (1), PhTx (5 μM) was bath applied, leading to a significant reduction of the EPSP (2) ($p < 0.05$). Subsequent burst pairings (■) lead to additional LTD (3) ($p < 0.05$). **B**, Similar experiment with PhTx in the bath and the PKC inhibitory peptide 19–31 (0.5 μM) in the pipette (1). Burst pairings (■) left EPSPs unaltered (2) ($p > 0.5$). **C**, Similar experiment with PhTx in the bath and the inhibitory peptide p-SVKI (0.2 mM) added to the pipette. EPSPs in control (1) and after burst pairings (■) were not significantly different (2) ($p > 0.1$).

LTD was fully blocked by adding the PP1/2A inhibitor okadaic acid to the pipette ($101 \pm 9\%$, $n = 6$, $p = 0.9$) (Fig. 5G), and EPSPs remained sensitive to PhTx. With PhTx, EPSPs declined to $66 \pm 11\%$ of control ($n = 6$, $p < 0.05$) (Fig. 5G). Additional control experiments in which bursts were replaced by single spikes left EPSPs unaltered (data not shown). Together, these

results indicate that asynchronous postsynaptic burst firing is capable of removing CP-AMPA receptors via activation of PP1/2A.

PP1/2A is activated by elevations of intracellular Ca^{2+} that may be a consequence of burst-firing (Dell'Acqua et al., 2006). To discriminate between several sources of Ca^{2+} , we performed burst conditioning after blocking different types of Ca^{2+} channels (Fig. 6). When L-type Ca^{2+} currents were inhibited with $10 \mu\text{M}$ nifedipine, burst firing was still capable of eliciting LTD ($61 \pm 7\%$, $n = 6$, $p < 0.005$) (Fig. 6A). In contrast, when T- or R-type Ca^{2+} channels were blocked with $10 \mu\text{M}$ mibefradil or $0.1 \mu\text{M}$ SNX482 (GVDDKA-GCYRMFGGCSVNDCCPRLGCHSLF-SYCAWDLTFSD-OH), respectively, LTD was abolished (mibefradil, $101 \pm 8\%$, $n = 6$; SNX482, $103 \pm 9\%$, $n = 7$, $p > 0.1$) (Fig. 6B,C). Similar findings were obtained with the T-/R-type channel blocker Ni^{2+} (0.1 mM ; data not shown). This result identifies T- and R-type Ca^{2+} channels as a main source for extracellular Ca^{2+} associated with burst LTD. However, because of a reported action of mibefradil on R-type channels, the involvement of T-channels remains less certain (Randall and Tsien, 1997).

Specificity of burst-firing-induced LTD
Synaptic plasticity phenomena may be input specific or global with different functional implications on neural computations. We next analyzed whether the two synaptic plasticity events, i.e., paired-burst-induced and simple-burst-induced LTD could occur independently of each other in the same neuron. We assessed the input specificity of both forms of LTD by stimulating two synaptic inputs on opposite sides of the recorded neuron in layers 2/3 (Fig. 7). The simple-burst protocol led to similar LTD of both pathways (74 ± 4 and $76 \pm 5\%$, $p < 0.05$, $n = 6$). In contrast, in the same neurons, a subsequent paired-burst protocol induced significant LTD only at the conditioned input ($48 \pm 9\%$, $p < 0.05$, $n = 6$) with the unconditioned not significantly altered ($69 \pm 5\%$, $p > 0.5$) (Fig. 7). Overall, the data indicate that burst pairings are specific for homosynaptic inputs, whereas burst conditioning affects synapses indiscriminately.

State-dependent forms of burst-firing-mediated LTD

If the simple-burst protocol selectively affects CP-AMPA receptors, which are absent after sleep, then burst conditioning should depend on the phase of the sleep–wake cycle. In agreement, in animals at the end of the dark period, the simple-burst protocol regularly depressed EPSPs to $76 \pm 6.9\%$ of control ($n = 7$, $p <$

Figure 5. CP- and CI-AMPA receptors are eliminated via different conditioning protocols. **A**, EPSP amplitude time course (mean $\pm$ SEM) before and after burst conditioning (□) that led to significant EPSP depression ($p < 0.005$). Bath-applied PhTx ($5 \mu\text{M}$) at the end of the experiment left EPSPs unaltered ($p > 0.5$). Top, Sample EPSPs in control (1) and after conditioning (2); sample burst (□). **B**, Current–voltage relationship and sample traces of conditioned EPSCs recorded in a representative experiment by repatching the neuron with a Cs^+ solution. **C**, Summary histogram for RI (mean $\pm$ SEM) in control and after conditioning. Both values were significantly different (** $p < 0.0005$). **D**, Similar experiment to that in **A** with the PKC inhibitor 19–31 ($0.5 \mu\text{M}$) added intracellularly. Burst conditioning (□) led to significant LTD ($p < 0.005$). PhTx was bath applied toward the end of the experiment without affecting the EPSPs ($p > 0.1$). Sample EPSPs for control (1), after conditioning (2), and PhTx (3) are illustrated above. **E**, Analogous experiment with the GluR2-specific inhibitory peptide SVKI (0.2 mM) in the pipette. Burst conditioning (□) still induced significant LTD ($p < 0.005$). **F**, Burst conditioning (□) in the presence of bath-applied H9 (0.5 mM), a broad-spectrum protein kinase inhibitor, induced significant LTD ($p < 0.005$). **G**, Burst conditioning (□) with the PP1/2A inhibitor okadaic acid ($0.1 \mu\text{M}$) in the pipette left EPSPs unaltered ($p > 0.5$). However, PhTx that was bath applied after the conditioning significantly diminished the EPSPs ($p < 0.05$). Above sample EPSPs in control (1), after conditioning (2), and in PhTx (3).

0.005) (Fig. 8A), indicative of burst-firing-induced LTD. In addition, after a second conditioning with paired bursts, EPSPs declined further to $53 \pm 8.7\%$ of control ($p < 0.05$), i.e., simple-bursts and paired-bursts led to incremental LTD (Fig. 8A). However, when the same protocol was repeated in animals after the light period (5:00 P.M.), simple bursts left EPSPs unchanged

Figure 6. Burst conditioning involves R- and T-type Ca^{2+} channels. **A–C**, EPSP amplitude time course (mean $\pm$ SEM) in control and after burst conditioning ($\square$) with control (1) and conditioned (2) sample EPSPs illustrated above. **A**, Experiments in the presence of the L-type channel blocker nifedipine (10 μ M) still showed significant LTD ($p < 0.005$). **B**, Similar experiments to that in **A** with the T-type channel blocker mibefradil (10 μ M), rendered burst conditioning ineffective ($p > 0.1$). **C**, Similar experiment to that in **A** and **B** with the R-type channel blocker SNX482 (0.1 μ M) in the bath. Burst conditioning was now again ineffective ($p > 0.1$).

Figure 7. Input specificity of burst pairing/conditioning. EPSP amplitude time series (mean $\pm$ SEM) and sample EPSPs in control (1), after burst conditionings ($\square$, 2), and after burst-pairings ($\blacksquare$, 3). Two synaptic inputs (I1, I2) were stimulated, and input 2 was switched off during the burst pairings. Significant LTD occurred in both pathways with burst conditioning ($p < 0.05$), but burst-pairing-induced LTD was specific for input 1 ($p < 0.05$, $n = 6$), whereas input 2 was not significantly affected ($p > 0.5$).

(100 $\pm$ 6.9%, $n = 6$, $p = 0.5$) whereas paired bursts depressed EPSPs to 67 $\pm$ 6.7% ($p < 0.05$) (Fig. 8B). Burst conditioning was also ineffective at 12:00 P.M. in line with the reported absence of CP-AMPA receptors at this time of the day ($p > 0.1$; data not shown) (compare with Fig. 2A). These findings demonstrate that, depending on the sleep–wake state, different forms of LTD can be induced at excitatory neocortical synapses.

Discussion

This study shows time-of-day fluctuations in AMPA receptor composition at excitatory cortical synapses with CP-AMPA receptors being present after the dark episode and being undetectable after the light phase. The CP-AMPA receptor removal occurred gradually over time and was significant after 4 h. Similarly, CP-AMPA receptors were again measurable 4 h after light offset. Combined EEG/EMG recordings revealed a clear relationship between sleep–wakefulness and the light/dark cycle at this developmental stage of the animals. It is thus reasonable to conclude that the selective loss of CP-AMPA receptors from cortical synapses is associated with sleep, although a role for clock-related parameters other than sleep cannot be ruled out completely. A previous report has revealed that sleep leads to a decrease in the GluR1 protein level (Vyazovskiy et al., 2008). Our results would be compatible with this finding because CP-AMPA receptors are devoid of GluR2 and most likely consist of GluR1 homomers and/or GluR1/3 heteromers (Geiger et al., 1995). In addition, our results suggest that the described decrease of GluR1 protein levels involves functional AMPA receptors participating in synaptic transmission.

At neocortical synapses, the number of CP-AMPA receptors steadily declines within the first weeks of development (Kumar et al., 2002). Because our animals were selected at random from a confined age group (postnatal days 21–25), ontogenetic factors are unlikely to be a main cause of the reported CP-AMPA receptor fluctuations. Notably, the study by Vyazovskiy et al. (2008) was performed in 11- to 12-week-old animals, indicating that GluR1 expression is still ongoing at later stages of development.

Subtype-specific pathways of AMPAR elimination

Our data suggest parallel mechanisms by which NREM-sleep-related discharge patterns can induce LTD at glutamatergic synapses in neocortex. Unpaired bursts lead to the removal of CP-AMPA by activating PP1/2A that is not pathway specific. Paired bursts additionally trigger the pathway-specific elimination of CI-AMPA via a PKC-mediated signaling pathway. Postsynaptic spike trains without concomitant synaptic stimulation were shown previously to induce a mixture of long-term potentiation and LTD in layer 2/3 pyramidal neurons (Volgushev et al., 1994). This suggests that the precise spike pattern plays an important role in determining the synaptic plasticity outcome. Our result is reminiscent of a recently described form of plasticity in hippocampal CA3 pyramidal cells in which transient depolarizations of the membrane potential led to the removal of CP-AMPA (Ho et al., 2007, 2009). In the study by Ho et al. (2007), the Ca^{2+} channels involved in LTD were identified as L-type, whereas in this study they are of R-type and possibly T-type. Ni^{2+} -sensitive Ca^{2+} channels are the main participant in generating bursts in layer V pyramidal cells (Williams and Stuart, 1999), and Ni^{2+} preferentially blocks T- and R-type Ca^{2+} channels.

The role of PP1/2A in LTD via GluR1 removal is well established (Kameyama et al., 1998). In line with a main role of PP in sleep-related changes of AMPA receptor subunits, the reported decrease in GluR1 protein levels was associated with increased dephosphorylation (Vyazovskiy et al., 2008). In our study, PP were identified as type 1/2A by the specificity of the blocker applied (Cohen et al., 1990) as well as the exclusion of tyrosine phosphatases. The non-Hebbian character of burst conditioning is consistent with the lack of input specificity.

The role of PKC in mGluR-mediated LTD involving GluR2-containing AMPA receptors is well described in the cerebellum but to a lesser degree in cortical areas (Linden and Connor, 1993; Kim et al., 2001; Santos et al., 2009). We have shown previously that paired-burst-induced LTD involves mGluR-mediated activation of PKC and AMPAR endocytosis (Birtoli and Ulrich, 2004; Czarnecki et al., 2007). The current findings identify these AMPA receptors as GluR2 containing, i.e., CI-AMPA. Thus, burst-pairing LTD in neocortical pyramidal cells that we describe has at least partial similarities with complex spike-induced LTD in Purkinje cells. In contrast to cerebellar LTD (Reynolds and Hartell, 2000), we found input specificity for paired-burst-induced LTD in the majority of our recordings. However, stimulation electrodes were placed far apart to ensure that the pathways activated were independent. It can thus not be ruled out completely that input specificity will break down with increased proximity of synapses as reported for long-term potentiation (Engert and Bonhoeffer, 1997). Interestingly, input specificity of mGluR-dependent LTD has also been reported in hippocampal pyramidal neurons and may be the result of the restricted presence of endoplasmic reticulum in a subset of spines (Holbro et al., 2009). Ca^{2+} release from the endoplasmic reticulum appears to be essential for mGluR-dependent LTD and has also been implicated in burst LTD (Czarnecki et al., 2007).

Figure 8. Circadian efficacy of burst conditioning. **A**, **B**, EPSP amplitude time series and sample EPSPs in control (1), after burst conditioning (2), and after burst pairings (3) in animals after the dark (**A**) and light (**B**) episodes. Note the incremental LTD in **A** ($p < 0.05$), whereas LTD was absent after burst conditioning in **B** ($p = 0.5$) with only burst pairings significantly inducing LTD ($p < 0.05$).

Overall, our data provide additional evidence that NMDA-receptor-independent LTD can be mediated via multiple signaling pathways (Anwyl, 2006).

Functional implications

The strength of synaptic connections is known to decrease/increase during the sleep–wake cycle (Barnes et al., 1977). Our data suggest that glutamatergic synaptic transmission in neocortex is also altered qualitatively during the sleep–wake cycle. CP-AMPA receptors have been shown to be inserted into active synapses in which they are thought to act as tags. Sleep may temporally restrict this tagging mechanism by periodically removing CP-AMPA receptors from synapses in a non-Hebbian way. This process may reset cortical connections for subsequent plasticity induction. Superimposed would be another anti-Hebbian-like process that leads to a PKC-mediated LTD of GluR2-containing AMPARs. This may lead to a synapse-specific proportional reduction of synaptic weights of synchronously firing neurons (Birtoli and Ulrich, 2004; Czarnecki et al., 2007). NREM-sleep-related discharge patterns were also shown to enhance GABAergic synaptic transmission (Kurotani et al., 2008). Together, these processes may contribute to the overall decline in excitability observed during sleep with field potential recordings (Barnes et al., 1977; Vyazovskiy et al., 2008).

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