Ongoing Intrinsic Synchronous Activity Is Required for the Functional Maturation of CA3-CA1 Glutamatergic Synapses

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Fine-tuning of synaptic connectivity during development is guided by intrinsic activity of the immature networks characteristically consisting of intermittent bursts of synchronous activity. However, the role of synchronous versus asynchronous activity in synapse maturation in the brain is unclear. Here, we have pharmacologically prevented generation of synchronous activity in the immature rat CA3-CA1 circuitry in a manner that preserves unitary activity. Long-term desynchronization of the network resulted in weakening of AMPA-receptor-mediated glutamatergic transmission in CA1 pyramidal cells. This weakening was dependent on protein phosphatases and mGluR activity, associated with an increase in the proportion of silent synapses and a decrease in the protein levels of GluA4 suggesting postsynaptic mechanisms of expression. The findings demonstrate that synchronous activity in the immature CA3-CA1 circuitry is critical for the induction and maintenance of glutamatergic synapses and underscores the importance of temporal activity patterns in shaping the synaptic circuitry during development.

Keywords: GluA4, hippocampus, LTD, plasticity, silent synapse

Introduction

During development, activity-dependent mechanisms contribute to the formation of synaptic contacts. Intrinsic electrical activity in neuronal networks can either strengthen or weaken the nascent synapses in Hebbian-type processes such as long-term potentiation (LTP) and silent synapse induction or long-term depression (LTD), or balance the overall level of neuronal outputs in a homeostatic manner (Zhang and Poo 2001; Turrigiano and Nelson 2004; Kerchner and Nicoll 2008; Hanse et al. 2009; Pozo and Goda 2010). All these forms of activity-driven plasticity are utilized during the synaptic development, but their relative contributions in a given network are elusive.

In the hippocampal CA3-CA1 synapses, both postsynaptic and presynaptic mechanisms for activity-dependent synapse induction have been found. The postsynaptic mechanism involves insertion of new α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) on postsynaptic sites in an N-methyl-D-aspartate receptor (NMDAR) dependent, LTP-like process (Isaac et al. 1995; Durand et al. 1996). The presynaptic mechanism involves inactivation of tonically active kainate receptors and a change in the release probability, and seems to be strictly developmentally limited (Gasparini et al. 2000; Lauri et al. 2006; Hanse et al. 2009). Moreover, both NMDA-dependent and -independent forms of LTD exist in the developing CA3-CA1 (Bolshakov and Siegelbaum 1994; Pavlov et al. 2004). The Hebbian types of plasticity are thought to contribute to the synapse-specific strengthening or erasure of functional contacts, whereas homeostatic plasticity mechanisms maintain the overall stability in the neuronal networks. A critical factor in the induction of activity-dependent plasticity is the temporal pattern and the level of synchrony of the electrical activity in the neuronal network (Katz 1993). In general, coherent bursts of activity are the most efficient way to rapidly induce synapse-specific, long-term changes in synaptic efficacy, namely LTP or LTD (Bliss and Collingridge 1993). Synchronous activity in the developing hippocampus both in vitro and in vivo consists of intermittent, highly synchronous bursts (Palva et al. 2000; Lahtinen et al. 2002; Leinekugel et al. 2002; Karlsson et al. 2006). Gap junctional coupling in the developing hippocampal network can accentuate the neuronal synchronization thus allowing immature neuronal ensembles to oscillate at frequencies ranging from 20 to 200 Hz (Palva et al. 2000; Lamsa and Taira 2003; Crépel et al. 2007). In CA3-CA1 synapses, the synchronous discharging of CA3 circuitry produces a strong barrage of glutamatergic currents to CA1 target neurons (Lamsa et al. 2000). By virtue of this, the CA3 network bursts could serve to induce Hebbian type of plasticity at glutamatergic synapses in CA1. However, despite the frequent speculations on the role of these high-frequency bursts versus uncorrelated activity in the hippocampal development, no experimental approaches have been taken to directly address this question.

We now show that 15–20 h desynchronization of the CA3 network leads to weakening of glutamatergic transmission in the area CA1. In the absence of synchronous activity, the immature CA3-CA1 synapses are depressed in a manner that depends on metabotropic glutamate receptor (mGluR) and phosphatase activity. This depression parallels with reduced expression of the AMPAR subunit GluA4, and manifests as less efficient AMPAR-mediated transmission, presumably due to decrease in postsynaptic GluA4-subunit containing AMPA receptors. Our results show for the first time that the distinctive developmental activity in the hippocampal circuitry is critical for the maintenance of the CA3-CA1 synapses.

Materials and Methods

Postnatal day (P) 4-P5 Wistar rats (P0=the day of birth) were rapidly killed by decapitation without anesthesia in accordance with the University of Helsinki Animal Welfare Guidelines. Hippocampal slices (400 μm) were cut with a vibratome using standard methods (e.g., Lauri et al. 2005). The slices were used 1–4 h after cutting or prepared for incubation. In these experiments, the slices were washed with 1 mL incubation solution containing (in mM): 105 NaCl, 3 KCl, 1 MgSO4, 1.25 NaH2PO4, 26 NaHCO3, 2 CaCl2, 15 n-glucose, and 25 HEPES, and placed into Millicell CM 0.4-μm membrane inserts (Millipore) with 1 mL of the above solution. In some experiments, 1 μM tetrodotoxin...
(TTX), 50 μM carbenoxolone (CBX), 100 μM 2S-2-amino-2-(15,2S-2-carboxycyclopropyl-1-yl)-3-(xanth-9-yl)propanoic acid (LY 341495), 50, 140 nM fostriecin, 50 μM α-(−)-2-amino-5-phosphonopentanoic acid (α-API), 40 μM [(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate] (MK801), 300 μM sodium orthovanadate, or 500 μM (S)-α-methyl-4-carboxyphenylglycine (MCPG) were included in the incubation solution. Slices were kept in an incubator (35 °C, 5% CO2 in air) for 15–20 h.

For electrophysiological recordings, the slices were placed in a submerged chamber (except the recordings of synchronous burst frequency after incubation, which was done in the static interface chamber) and superfused with artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 3 KCl, 1.25 NaH2PO4, 1 MgSO4, 26 NaHCO3, 15 g glucose, and 2 CaCl2; 5% CO2–95% O2, at a rate of 1–2 mL/min (32 °C). Whole-cell recordings were obtained from CA1 or CA3 pyramidal neurons with 3–5 MΩ borosilicate glass microelectrodes using an Axopatch 200B amplifier (Axon Instruments).

Synchronous Network Bursts
Synchronous network bursts were recorded from CA3 pyramidal cells, which were patched with microelectrodes containing (in mM): 135 K gluconate, 10 HEPES, 5 EGTA, 2 KCl, 2 CaCl2, 4 Mg-ATP, 0.3 Na-GTP, pH7.2, and voltage clamped at −58 mV.

Spontaneous Action Currents
Spontaneous action currents were recorded in cell-attached configuration with ACSF-filled electrodes, and the amplifier set to “track” mode.

Recordings of AMPA-Receptor-Mediated Responses
For the recordings of AMPAR-mediated responses, cells were voltage-clamped at −70 mV with microelectrodes containing (in mM): 130 CsMeSO4, 10 HEPES, 0.5 EGTA, 4 Mg-ATP, 0.3 Na-GTP, 5 N-(2,6-dimethylphencylcarbamoylmethyl) triethylammonium chloride (QX 314), and 8 NaCl (285 mOsm), pH 7.2. AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs) were pharmacologically isolated by adding 1 μM TTX and 100 μM picrotoxin (PTX) to the ACSF, and evoked AMPAR-mediated responses were recorded in the presence of 100 μM PTX and 50 μM AP5 in response to stimulation of Schaffer collateral by a bipolar metal electrode.

Recordings of NMDA-Receptor-Mediated Currents
For recordings of NMDAR-mediated currents, cells were patched with microelectrodes containing 115 CsMeSO4, 10 HEPES, 10 BAPTA, 4 Mg-ATP, 0.3 Na-GTP, 5 QX 314, and 8 NaCl (285 mOsm), pH 7.2, and held at +40 mV. NMDA-R miniature events were recorded in the presence of 1 μM TTX, 100 μM PTX, and 10 μM 2,3-dihydroxy-6-nitro-7-

Figure 1. CBX (50 μM) and low concentration of TTX (50 nM) completely desynchronize endogenous activity in the developing hippocampus. (A and C) Sample recordings of spontaneous activity in CA3 pyramidal neurons and the effect of 50 μM CBX or 50 nM TTX. Whole-cell voltage clamp recordings were made using a low-chloride–containing filling solution at −58 mV and under these conditions, GABAergic activity is seen as outward currents while glutamatergic currents are inward. Synchronous network bursts contain a large GABAergic conductance with a barrage of EPSCs (1). Traces illustrate spontaneous synchronous activity before (1), during (2), and after (3) application of the drug. Asterisks indicate the part of the recordings that are shown on the expanded time scale (right). (B and D) Averaged data showing that acute application of 50 μM CBX (A, n = 7) or 50 nM TTX (C, n = 6) blocks synchronous network bursts recorded from CA3 pyramidal cells.
sulfanoylbenzof(f)quinazoline (NBQX). AMPA/NMDA ratio was calculated from the cells patched with the BAPTA-containing filling solution by recording the evoked currents at −70 and +40 mV in the presence of PTX. AMPAR–related component was calculated as the peak of the response at −70 mV, and NMDAR-mediated current was isolated by calculating the average amplitude of the response recorded at +40 mV 50–60 ms after stimulation. The compounds were from Tocris, Sigma-Aldrich, or Ascent Scientific.

**Western Blot Assays**

For Western blot assays, the CA1 area was isolated after the overnight incubation and homogenized in 30-µL ice-cold lysis buffer (150 mm NaCl, 50 mm Tris, 1 mm EDTA, 1% NP-40, 0.5% deoxycholate, and 0.1% SDS). Protein concentrations were measured using Bio-Rad DC protein assay (Bio-Rad Laboratories) with bovine serum albumin as the standard. Samples were boiled (except VGLUT-1 samples) at 95 °C in Laemmli buffer (62.5 mm Tris, 1.8% sodium dodecyl sulfate (SDS), 7.75% glycerol, and 4.4% 2-mercaptoethanol, pH 6.8) for 5 min. The proteins (15–30 µg per well) were separated on 4–15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to Hybond nitrocellulose membrane (GE Healthcare) by a semidry blotting technique. Membranes were blocked for 1 h with 5% milk (w/v) prepared in PBS and incubated overnight at 4 °C with primary antibodies anti-VGLUT1 (1:3000, polyclonal, Synaptic Systems), or anti-GluA4ctd (1:2000, rabbit, gift from Kari Keinänen). Membranes were incubated 1 h at room temperature with secondary antibodies Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:10,000, GE Healthcare), anti-rat IgG (1:1000, Millipore), or anti-GluA2 (1:1000, rabbit polyclonal, Synaptic Systems), anti-synaptophysin-1 (1:10,000, mouse monoclonal, Synaptic Systems), or anti-GluA4ctd (1:2000, rabbit, gift from Kari Keinänen). Membranes were incubated 1 h at room temperature with secondary antibodies Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:10,000, GE Healthcare), anti-rat IgG (1:1000, Millipore), or anti-mouse IgG (1:5000, Amershams Biosciences). The antibody complexes were detected using Pierce ECL Western Blotting substrate (Thermo Scientific). Developed films were scanned and the integrated optical density (IOD) of protein bands was measured using ImageJ software. To control for differences in total protein concentration between samples, the filters were stripped and rebotted with anti-α-tubulin antibodies, and the IOD values for synaptophysin, VGLUT1, GluA2, and GluA4 were normalized against the level of α-tubulin in the same sample. These corrected IOD values were used for calculation of statistical differences between drug-treated and parallel control samples (Student’s paired, two tailed *t*-test). For the histograms, the corrected IOD values were further normalized to corresponding controls and averaged (average ± standard error of the mean (SEM); 100% = no change).

**Data Analysis**

WinLTP (0.95b or 0.96, www.winltp.com, Anderson and Collingridge, 2007) or Axoscope 9.2 (Axon Instruments) was used for data acquisition. Offline analysis was done using WinLTP or MiniAnalysis 6.0.3 program (Synaptosoft Inc.). Spontaneous events were detected using a peak detector algorithm, and all events were confirmed visually. The time-course plots of burst frequency were constructed using a bin width of 2 min and normalized to the average baseline. The histograms of mEPSCs were constructed from at least 10 min of recording (at least 50 events) from each cell. The pooled data are given as mean ± SEM for the number of cells indicated. Data are expressed as percentage of control, obtained by dividing the values of drug-treated samples by matching control group average (i.e., 100% equals no change). Student’s two-tailed *t*-test was used for statistical analysis using the raw (non-normalized) data. The level of significance was set as *P* < 0.05. Membrane capacitance (*Cm*) was estimated using the equation: 

$$C_m = \frac{\tau}{1 + R_c/R_m}$$

where $\tau$ was obtained by exponential fitting of the decay of the transient current in response to a 5-mV pulse step using pCLAMP software (Clampfit 9.2, Axon Instruments). Only cells that had membrane capacitance higher than 50 pF were used for recordings.

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**Figure 2.** CBX and low concentration of TTX (50 nM) disrupt the burst firing in CA3 pyramidal cells. (A) Sample traces of cell-attached recordings from CA3 pyramidal cells during showing the effects of 50 µM CBX and 50 nM TTX on spike firing pattern. (B) Averaged data showing that acute application of CBX (50 µM; *n* = 5) and low concentration of TTX (50 nM, *n* = 5) increases the mean interevent interval of spontaneous action potentials. *P* < 0.05. (C) Averaged cumulative distribution of interevent intervals showing the effects of 50 µM CBX and 50 nM TTX on spontaneous AC. The insert shows the same distribution using expanded y-scale.
Results

Carbenoxolone and 50 nM TTX Block the Synchronous Network Bursts in the CA3-CA1 Circuitry in the Newborn Hippocampus

To start to study the role of synchronous spontaneous activity in synaptic induction, we first examined the acute effects of the broad-spectrum gap junction blocker CBX (50 μM) (Placantonakis et al. 2006; Bissiere et al. 2011) or a low concentration (50 nM) of TTX, on spontaneous activity in area CA3. As CBX is a nonspecific drug (Rouach et al. 2003; Vessey et al. 2004; Chepkova et al. 2008), the key experiments (Figs. 1–3, 5, 7, and 8) were repeated using 50 nMTTX in parallel with CBX. In TTX is a blocker of voltage-sensitive sodium channels, and at the low concentrations, a selective blocker of persistent sodium currents (Stasheff et al. 1993; Gasparini et al. 2000; Bean 2007) nanomolar concentrations TTX also blocks synchronous network bursts in CA3 yet by a different mechanism than CBX (Stasheff et al. 1993; Gasparini et al. 2000). Both CBX (n = 7) and 50 nM TTX (n = 6) fully blocked synchronous network bursts in CA3 pyramidal neurons in neonatal (P4–P6) hippocampal slices (Fig. 1).

To more closely examine the effects of CBX and 50 nM TTX on neuronal synchronization, we next performed cell-attached recordings of action currents (AC) in CA1 pyramidal cells. Both CBX (n = 5) and 50 nM TTX (n = 5) disrupted the firing pattern of the cells; in the presence of these drugs, the cells fired single spikes instead of the high-frequency trains of ACs typically observed under control recording conditions. Consequently, the mean AC frequency was significantly decreased (Fig. 2).

Figure 3. Chronic (15–20 h) desynchronization and inhibition of all action-potential–dependent activity have different effects on glutamatergic transmission. (A and C) Sample recordings of mEPSCs from CA3 (A) and CA1 (C) pyramidal cells after long-term treatment with 50 μM CBX, 1 μM or 50 nM TTX. The traces in expanded time scale depict average of 8 events from each recording. (B and D) Pooled data illustrating mean AMPAR–mediated mEPSC amplitude and inter-event interval from CA3 (B) (n = 12–15) and CA1 (D) (n = 10–15) pyramidal cells after long-term treatment with 50 μM CBX, 1 μM or 50 nM TTX. Error bars represent the SEM. *P < 0.05.
The long-term effect of desynchronizing the network bursts was then studied by incubating the hippocampal slices for 15–20 h with 50 μM CBX, or 50 nM TTX. As reported previously, the incubation by itself did not have any effect on the viability of pyramidal neurons or the excitability of the hippocampal network (Huupponen et al. 2007). Slices incubated under control conditions maintained their spontaneous network activity after the incubation period (frequency 1.46 ± 0.17 events/min, n = 4; (see also Lauri et al. 2003) recorded in a static interface chamber to maintain the incubation conditions) while following overnight incubation with CBX, no synchronous network bursts were detected (n = 7; not shown).

Overnight application of CBX did not have any toxic effect, as illustrated using propidium iodide staining to evaluate neuronal viability within the hippocampal slice following incubation (Supplementary Fig. 1A). Furthermore, acute application of CBX did not affect the amplitude or frequency of mEPSCs, or the amplitude of evoked EPSCs in hippocampal CA1 pyramidal neurons at P4–P6 (Supplementary Fig. 1B). In addition, CBX treatment did not influence passive membrane properties at this age (Supplementary Table).

These data suggest that CBX and 50 nM TTX can be used as tools to block selectively intrinsic network synchronization to study the consequences on synaptic development in the immature hippocampal network.

Long-Term CA3-CA1 Network Desynchronization and Total Activity Blockade Have Different Effects on Glutamatergic Transmission in the Areas CA3 and CA1

Long-term total activity blockade results in a homeostatic increase in the mEPSCs in the immature CA3 pyramidal neurons (Lauri et al. 2003; Huupponen et al. 2007). Accordingly, here the long-term incubation of hippocampal slices with 1 μM TTX resulted in an upregulation of both mEPSC frequency and amplitude in CA3 pyramidal cells (n = 6, P < 0.05, Fig. 3A, B). However, selective blockade of the synchronous bursts only with CBX or with 50 nM TTX had no effect on the mEPSCs in the area CA3 (n = 12–15, Fig. 3A,B). In contrast, the long-term total activity blockade with 1 μM TTX induced no changes in mEPSC frequency or amplitude in area CA1 (n = 10–15, Fig. 3C,D), suggesting that in CA1 pyramidal cells the induction threshold for the homeostatic regulation of

Figure 4. Desynchronization by long-term application of CBX (50 μM) treatment depresses evoked AMPAR–mediated transmission in area CA1 and does not change the probability of release at CA3-CA1 synapses. (A) Sample recordings of EPSCs from the control- and carbenoxolone-treated slices, in response to stimulation with 3 different intensities. Traces are averages of 5 sweeps. Trace numbers refer to the corresponding time points shown in B. (B) Input–output characteristics of EPSCs, evoked by Schaffer collateral stimulation under different stimulation strength in control (n = 8) and CBX (n = 8) treated slices. X-Axis: relative stimulation voltage, the last subthreshold no-response voltage is denoted as zero. (C) Sample traces of paired-pulse responses in control- and CBX-treated slices at 50-ms interpulse interval. Traces represent average of 10 sweeps. (D) Paired pulse ratio of EPSCs. Data are presented as the distribution of individual cell responses (black circles) and mean values (white circles). n = 24 and 20 for control and CBX, respectively. (E) Sample traces of EPSCs evoked by 5 pulse stimulation at 50 Hz in control- and CBX-treated slices (average of 10 sweeps). (F) Facilitation ratio (amplitude of fifth/first EPSC at 50 Hz) of EPSCs. Data are presented as the distribution of individual cell responses (black circles) and mean values (white circles). n = 12 and 10 for control and CBX, respectively. *P < 0.05. Error bars represent the SEM.
mEPSCs is different to that seen in CA3 at this developmental stage (Huupponen et al. 2007). It is possible that any putative effect of CBX treatment on mEPSCs in area CA3 is masked by the pronounced homeostatic regulation, thus resulting in no net effect on transmission. Consistent with this idea, long-term blockade of the synchronous activity in CA1 pyramidal cells with either CBX or with 50 nM TTX resulted in a decrease in the amplitude and frequency of mEPSCs (n = 10–15, P < 0.01, Fig. 5C, D).

To further characterize the downregulation of synaptic transmission induced by network desynchronization, we measured EPSCs evoked by Schaffer-collateral stimulation in CA1 pyramidal cells in control and CBX incubated slices. The mean slope of the input–output curve for evoked EPSCs in CA1 pyramidal neurons was significantly lower in CBX-treated slices than in control slices (n = 8, P < 0.05, Fig. 4A). Thus, higher stimulation intensity was needed to evoke EPSCs in CA1 after overnight blockade of synchronous activity, and the maximum amplitude of the evoked EPSCs was smaller after CBX incubation (Fig. 4B). Together, these data show that ongoing synchronous activity is instrumental in the establishment and/or maintenance of glutamatergic transmission in area CA1.

CA3 Network Desynchronization Does Not Change the Release Probability in CA3-CA1 Synapses

We then went on to investigate the mechanisms underlyng the desynchronization-associated downregulation of EPSCs in CA1 neurons. It has been shown that in the newborn CA3-CA1 synapse, activity-dependent switch from low to high transmitter release probability contributes to the functional development of glutamatergic synapses (Lauri et al. 2006; Sallert et al. 2009). Here, we saw no change in paired-pulse ratio of evoked EPSCs after the CBX incubation (Fig. 4C, D, n = 20–24, P = 0.3). Further, the synaptic dynamics in response to high-frequency stimulation trains (50 Hz) were not affected by the chronic network desynchronization (n = 10–12, Fig. 4E, F; see Lauri et al. 2006; Sallert et al. 2009). There were also no changes in the NMDAR-mediated mEPSCs after the blockade of synchronous bursts (n = 8–14, Fig. 5). Together, these data indicate that the depressed glutamatergic transmission in CA3-CA1 synapses was not due to altered release probability.

CA3 Network Desynchronization Increases the Number of Silent Synapses in the Area CA1

Functional development of CA1 glutamatergic synapses involves a decrease in the number of silent synapses, i.e., synapses having NMDAR-mediated EPSCs only (Isaac et al. 1995; Durand et al. 1996). We thus next examined whether CA3 desynchronization results in changes in the AMPA/NMDA ratio which is used as an index of the number of silent synapses. When subthreshold stimulation intensity for evoking AMPAR-mediated currents was used, the NMDAR-mediated EPSC amplitude was found to be bigger in CA1 pyramidal neurons in CBX-incubated slices (Fig. 6A, n = 9–10; P < 0.05). With minimal stimulation strength the AMPA/NMDA ratio was significantly lower after CBX incubation (Fig. 6B, n = 9–10; P < 0.05).

CA3 Network Desynchronization Down-Regulates CA1 Glutamatergic Synapses via Mechanisms Involving Protein Phosphatase and mGluR Activity

To start to resolve the molecular signaling mechanisms underlying the synaptic depression in CA1 pyramidal neurons induced by the CA3 network desynchronization, the hippocampal slices were incubated for 15–20 h in CBX in the presence of the NMDAR antagonists AP5 (50 μM) and MK801 (40 μM). It has been previously shown that NMDAR activation might be needed for AMPAR delivery and synaptic induction driven by spontaneous activity in the developing hippocampus (Zhu et al. 2000). Further, LTD in newborn CA1 pyramidal cells is known to be at least partially dependent on the NMDAR activation (Pavlov et al. 2004). Here, however, blockade of NMDARs did not prevent the desynchronization-associated depression of mEPSCs (Fig. 7A, B, n = 9). In our hands, NMDAR blockade has little or variable effects on the spontaneous activity as also reported by Bolea et al. 1999 (see

**Figure 5.** Network desynchronization by long-term application of CBX (50 μM) or TTX (50 nM) has no effect on NMDAR-mediated transmission. (A) Example recordings of NMDAR–mediated mEPSCs in control-, CBX-, and 50 nM TTX-treated slices. Average of 8 events from each recording are shown in expanded time scale. (B) Mean NMDAR–mediated mEPSC amplitude and interevent interval from CA1 pyramidal cells after long-term treatment with 50 μM CBX (n = 14) or 50 nM TTX (n = 8). Error bars represent the SEM.
also Lamsa et al. 2000) and by itself has no effect on mEPSCs within this time scale (Fig. 7A, B, n = 5). Yet, there are also reports demonstrating that NMDARs can play a role in the maintenance of spontaneous hippocampal activity, this issue thus being somewhat unresolved and/or reflecting different experimental conditions between the laboratories (Ben-Ari et al. 1989; Garaschuk et al. 1998). mGluRs are required for hippocampal LTD (Massey and Bashir 2007). Accordingly, it was found here that both the broad-spectrum mGlur antagonist, LY 341495, at a concentration (100 μM) that blocks the activation of mGlur1–mGlur8 (Fitzjohn et al. 1998) and MCPG (500 μM), a nonselective group I/group II mGlur antagonist, blocked the depression of mEPSCs after the CA3 desynchronization by CBX or 50 nM TTX (Fig. 7A, B, n = 8–10 and 7, respectively). Neither LY 341495 nor MCPG alone had any effect on mEPSCs (Fig. 7A, B, n = 12 and 9), and have no or only slightly depressive effect on the occurrence of synchronous bursts (Strata et al. 1995, see also Wagner and Luhmann 2006). Hippocampal LTD is known to be also dependent on protein phosphatase activity (Mulkey et al. 1993). Therefore, we next tested the effects of the protein phosphatase inhibitors fostriecin (140 nM) and orthovanadate (300 μM) on the desynchronization-induced synaptic depression in CA1 pyramidal cells. Incubation of hippocampal slices with CBX or 50 nM TTX in the presence of fostriecin (n = 8 and 7, respectively) or orthovanadate (n = 8 and 9, respectively) prevented mEPSC depression thus indicating the involvement of protein phosphatases (Fig. 7A, B). Neither fostriecin or orthovanadate incubation alone had any effect on the mEPSCs (Fig. 7A, B; n = 11 and 10, respectively) or on the frequency of synchronous bursts (91.8 ± 1% and 118.7 ± 4%, respectively, not shown).

**CA3 Network Desynchronization Decreases the Amount of GluA4 and Increases the Amount of GluA2 in Area CA1**

To investigate further the possible pre- and postsynaptic mechanisms of depression in glutamatergic transmission in CA1 after long-term inhibition of synchronous activity, we

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**Figure 6.** Chronic desynchronization increases the number of silent synapses in CA1 pyramidal cells. (A) Sample traces (left) and pooled data and illustrating the amplitude of NMDAR response (gray line), evoked using a stimulation strength set at the subthreshold level for AMPAR–mediated response (black line). Sample traces are average of 10 sweeps, recorded at −70 mV (black) and at 40 mV (gray). n = 9 and 10 for control and CBX, respectively, P < 0.05. (B) Sample traces (left) and pooled data and showing the amplitude ratio of AMPAR and NMDAR–mediated EPSCs, evoked by minimal stimulation strength. n = 9 and 10 for control and CBX, respectively, P < 0.05. Error bars represent the SEM.
next quantified the protein levels of synaptophysin, VGLUT1, GluA2, and GluA4 protein levels in area CA1 isolated from control-, CBX-, and 50-nM TTX-treated slices. Synaptophysin, which is routinely used as a marker for synaptic density (see, e.g., Lauri et al. 2003; Vesikansa et al. 2007), was not altered in either CBX- or 50-nM TTX-treated slices (Fig. 8A–D). Similarly, no significant changes were found in the amount of VGLUT1 in CBX- or 50-nM TTX-treated slices (Fig. 8A–D). However, desynchronization resulted in a significant change in the levels of AMPAR subunits. A robust decrease in the amount of GluA4 was observed both in CBX- and 50-nM TTX-treated slices (CBX: Fig. 8A,B, n = 4, P < 0.05; TTX: Fig. 8C,D, n = 6, P < 0.05). Moreover, in the CBX-treated slices, there was also an increase in the amount of GluA2 (Fig. 8A, n = 7, P < 0.01).

These data support the idea that the lack of synchronous bursts in the CA3-CA1 circuitry perturbs the maintenance of AMPARs in area CA1, thus resulting in downregulation of synaptic transmission.

Discussion

Although intermittent bursts of synchronous activity provide most of the on-going activity in various areas of the brain during early development (Ben-Ari et al. 1989; Garaschuk et al. 1998; Palva et al. 2000), only a few studies concerning the role of endogenous activity in synapse formation exist (Lauri et al. 2003; Colin-Le Brun et al. 2004; Gonzalez-Islas et al. 2003). These tools allowed us to desynchronize pharmacologically the network without directly influencing the function of fast ionotropic receptors, and to study concomitantly the role of asynchronous versus synchronous spontaneous activity on the synaptic development and plasticity in the immature hippocampus.

In CA1 pyramidal neurons, mere desynchronization of network activity lead to a downregulation of AMPAR-mediated transmission and to an increase in the number of silent synapses as indicated by the decrease in the ratio of AMPA/NMDAR-mediated evoked EPSCs. It was somewhat surprising to find that chronic desynchronization of the CA3 network by CBX or 50 nM TTX resulted in no changes in mEPSCs in CA3 pyramidal neurons. However, these glutamatergic synapses in CA3 pyramidal cells were clearly capable of undergoing pronounced homeostatic upregulation following chronic activity block by 1 μM TTX. Thus, the most plausible explanation for the lack of apparent effect of desynchronization on mEPSCs in CA3 is that the homeostatic upregulation compensated for the weakened AMPA transmission, resulting in no net effect on mEPSCs at the level of CA3 synapse population. In contrast to that observed in area CA3, chronic activity block by 1 μM TTX did not induce any change either in mEPSC frequency or in amplitude in area CA1. It has been recently shown that homeostatic changes induced by activity blockade are not necessarily uniform at different synapses even within the same neuronal network (Echegoyen et al. 2007; Goel and Lee 2007; Cingolani and Goda 2008; Kim and
Tsien 2008) and at different stages of development (Huupponen et al. 2007). The distinct effects of activity blockade in immature CA1 and CA3 corroborates the emerging view that the mechanisms, as well as consequences, of inactivity-induced plasticity differ depending on the brain area in question, developmental stage, the cell type, and the mode of activity manipulation used (Turrigiano 2006; Pozo and Goda 2010).

Chronic network desynchronization lead to a decrease in mEPSC amplitude and frequency and to a shift in the input/output curve of stimulation evoked EPSCs in area CA1. These changes could be due to postsynaptic loss of AMPARs or decrease in presynaptic release. We could not observe any changes in paired pulse facilitation of EPSCs or in the frequency of NMDAR–mediated quantal transmission, indicating that presynaptic glutamate release probability was not altered in response to desynchronization. In contrast, the decrease in AMPA–NMDA ratio as well as a strong reduction in the expression of GluA4 protein point to a postsynaptic mechanism involving loss of functional AMPARs.

Hebbian-type plasticity driven by correlated synchronous activity of neurons is generally thought to contribute to reinforcement or erasure of functional contacts at developing networks. AMPARs appear to be particularly mobile during synapse development (see Hanse et al. 2009) and regulated trafficking of AMPARs in and out from the postsynaptic site has been shown to contribute to several developmental plasticity phenomena. For example, activation of silent synapses (Isaac et al. 1995) as well as homeostatic plasticity in response to reduced activity at developing networks leads to insertion of new AMPARs on postsynaptic site (Goel et al. 2006; Pozo and Goda 2010). In contrast to mature stage, neonatal CA1 pyramidal neurons express GluA4 subunit of AMPARs (Zhu et al. 2000). It has been suggested that spontaneous activity in developing hippocampus initiates the formation of functional contacts by transient delivery of GluA4 (GluR4) AMPARs into synapses (Zhu et al. 2000). However, for the establishment of stable glutamatergic synapses high-frequency stimulation may be required, leading to incorporation of GluA2/3 subunit-containing AMPARs (Hayashi et al. 2000; Shi et al. 2001). Thus, the GluA4-containing synapses might represent immature contacts that are prone to elimination in the absence of synchronous activity.

Intriguingly, nascent synapses are particularly susceptible to LTD induction (Wasling et al. 2002; Pavlov et al. 2004; Lanté et al. 2006). Moreover, repeated “baseline” low-frequency stimulation (0.05–1 Hz) is sufficient to cause silencing of AMPAR-mediated transmission at neonatal area CA1 (Xiao et al. 2004) accentuating the labile nature of these receptors at immature contacts (Groc et al. 2006; Hanse et al. 2009). Our data shows that a similar silencing occurs in response to endogenous unitary activity if the synchronous high-frequency bursts of activity are pharmacologically
elimated. Similar to the low-frequency stimulation-induced AMPA silencing (Xiao et al. 2004) and some forms of neonatal LTD (Oliet et al. 1997; Pavlov et al. 2004), desynchronization-induced depression of AMPA transmission was independent of NMDA receptors. The depression was significantly diminished by broad spectrum antagonists of mGluRs and protein phosphatases, thus further resembling mechanisms of LTD (Mulkey et al. 1993; Gladding et al. 2009). Blockade of the depression by Ser/Thr protein phosphatase inhibitor fostriecin may indicate that this effect is not linked to mGluR LTD which is thought to be associated to tyrosine phosphatase activation. Finally, it has recently been shown in organotypic cultures that repeated low-frequency stimulation simultaneous with LTD induction is capable of restructuring synaptic contacts and could ultimately lead to synapse elimination in an mGluR-dependent manner (e.g., Bastrikova et al. 2008; Becker et al. 2008; Shinoda et al. 2010).

In conclusion, our data supports the idea that immature synapses that are not involved in the correlated network activity are silenced and ultimately eliminated (Goda and Davis 2003; Xiao et al. 2004; Hanse et al. 2009). The coherent high-frequency network activity that is endogenously generated in the developing CA3-CA1 network protects nascent synapses from depression that occurs in response to asynchronous activity or inactivity (Hanse et al. 2009), and thereby has a central role in fine-tuning of the CA3-CA1 circuitry.

Finally, how do these findings relate to the brain development in behaving animal? The distinct activity pattern in the in vitro hippocampus (i.e., 20–200-Hz synchronous bursts in CA3-CA1 local circuitry, see Palva et al. 2000) has an identical in vivo counterpart as demonstrated by intrahippocampal and facial nucleus activity in freely moving and anesthetized rats (Lathiönen et al. 2002; Leinekugel et al. 2002; Karlsson et al. 2006; see also Khazipov et al. 2004). In vivo the bursts are associated with muscle startles and frequent myoclonic twitching characterizing active sleep in newborns (Gramsbergen et al. 1970; Hilakivi and Taira 1995; Seelke et al. 2005). These early kinetic behaviors have been suggested to reflect the parallel intense formation of synaptic contacts and the simultaneous buildup of functional sensory-motor connections (Lauri et al. 2003; Kasyanov et al. 2004; Khazipov et al. 2004; Karlsson et al. 2006). Thus, our findings may provide a direct insight into the mechanisms sculpting synaptic circuitry during early sleep and associated motic behaviors.

**Supplementary Material**

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

**Funding**

This work was supported by the Academy of Finland (S.E.L., S.M.M., and T.T.), The Sigrid Juselius Foundation (J.H., S.E.L., and T.T.), The University of Helsinki research funds (S.E.L.).

**Notes**

The authors thank Dr. Vernon Clarke and Dr. Tiina-Kaisa Kukko-Lukjanov for their expert help and comments on the manuscript. Conflict of Interest: None declared.

**References**


Cerebral Cortex November 2013, V 23 N 11
Synchronous Activity Maintains Glutamate Synapses


