Dynamics of Excitatory Synaptic Components in Sustained Firing at Low Rates

Claire Wyart, Simona Cocco, Laurent Bourdieu, Jean-Francois Léger, Catherine Herr, and Didier Chatenay

Laboratoire de Dynamique des Fluides Complexes, Unité 7506 Centre National de la Recherche Scientifique, Université Louis Pasteur, Institut de Physique, Strasbourg, France

Submitted 19 May 2004; accepted in final form 16 January 2005

Wyart, Claire, Simona Cocco, Laurent Bourdieu, Jean-Francois Léger, Catherine Herr, and Didier Chatenay. Dynamics of excitatory synaptic components in sustained firing at low rates. J Neurophysiol 93: 3370-3380, 2005. First published January 26, 2005 doi:10.1152/jn.00530.2004. Sustained firing is necessary for the persistent activity associated with working memory. The relative contributions of the reverberation of excitation and of the temporal dynamics of the excitatory postsynaptic potential (EPSP) to the maintenance of activity are difficult to evaluate in classical preparations. We used simplified models of synchronous excitatory networks, hippocampal autapses and pairs, to study the synaptic mechanisms underlying firing at low rates. Calcium imaging and cell attached recordings showed that these neurons spontaneously fired bursts of action potentials that lasted for seconds over a wide range of frequencies. In 2-wk-old cells, the median firing frequency was low (11 \pm 8.8 Hz), whereas in 3- to 4-wk-old cells, it decreased to a very low value (2 \pm 1.3 Hz). In both cases, we have shown that the slowest synaptic component supported firing. In 2-wk-old autapses, antagonists of N-methyl-D-aspartate receptors (NMDARs) induced rare isolated spikes showing that the NMDA component of the EPSP was essential for bursts at low frequency. In 3- to 4-wk-old neurons, the very low frequency firing was maintained without the NMDAR activation. However EGTA-AM or α -methyl-4-carboxyphenylglycine (MCPG) removed the very slow depolarizing component of the EPSP and prevented the sustained firing at very low rate. A metabotropic glutamate receptor (mGluR)activated calcium sensitive conductance is therefore responsible for a very slow synaptic component associated with firing at very low rate. In addition, our observations suggested that the asynchronous release of glutamate might participate also in the recurring bursting.

INTRODUCTION

Persistence of activity in neuronal networks occurs in vivo as shown by unit recordings in behaving monkeys during delayed response experiments (Fuster and Alexander 1971). To persist after a stimulus, the electrical activity has to be sustained in the absence of any external input and to be stimulusselective. We address the question of the mechanisms sustaining activity once initiated. The electrical activity is usually assumed to be sustained by the propagation of reverberating synaptic excitation through a neural network, thanks to the high efficiency of recurrent synapses (Wang 2001). Recent experiments (Egorov et al. 2002; Fransen et al. 2002) and models (Lisman et al. 1998; Tegner et al. 2002; Wang 1999, 2001) have emphasized the possible role of the intrinsic slow temporal decay of the excitatory postsynaptic potential (EPSP) in the maintenance of a stable persistent state at low physiological frequencies (10-50 Hz). The slow decay time of the

EPSP is attributed to the activation of slow synaptic or synaptic-dependant conductance. While negative feedback mechanisms following a spike forbid the short term reinitiation of a spike, refiring after a long time interval requires the activation of a slow depolarizing component. Therefore if neuronal firings are partially synchronous and if synaptic mechanisms are implied, models predict that their decay time needs to exceed the typical time interval between spikes (Wang 1999, 2001).

In this study, we analyzed synaptic-dependant mechanisms involved in sustaining activity at low firing rates. We used simple model systems of highly synchronous excitatory networks: hippocampal excitatory autapses and pairs. Reverberating processes through large networks were prevented since they were bound to follow a one (or 2) neuron(s) loop. It has been observed previously that neurons undergo great synaptogenesis in culture (Verderio et al. 1999) and that synapses develop characteristics comparable with synapses in the intact brain (Wilcox et al. 1994). Autaptic neurons were constrained to connect only to themselves so that each spike leads to a large EPSP (Bekkers and Stevens 1991; Segal and Furshpan 1990). The activation of all synapses was therefore highly synchronous allowing an easy discrimination of synaptic components according to their kinetics (Bekkers et al. 1990; Cummings et al. 1996).

Despite the fact that large reverberating pathways were hindered in autaptic neurons and pairs of neurons grown in vitro, bursts lasting for several seconds at low (10-20 Hz) or very low (1-2 Hz) frequencies were observed spontaneously or after a brief stimulation. This sustained firing occurred as bursts of spikes either briefly evoked or spontaneously occurring after a long period of silence. Bursts were very similar to those observed in larger neuronal networks in culture (Bacci et al. 1999; Segal and Furshpan 1990). They were synaptically driven since no more activity was observed when glutamate synapses were blocked (Bacci et al. 1999). Here, we analyze the nature and the role of slow synaptic dependant components of the EPSP in sustaining recurring bursting activity at low frequencies in absence of reverberating excitation through large ensemble of neurons.

METHODS

Cell culture

Pyramidal neurons from rat hippocampus were grown on the substrates according to the protocol derived from Banker (Goslin and

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: L. Bourdieu, Laboratoire de Neurobiologie Moléculaire et Cellulaire, UMR CNRS 8544, Ecole Normale Supérieure, 46 rue d'Ulm, 75005 Paris, France (E-mail: laurent.bourdieu@ens.fr).

Banker 1991). Patterned coverslips (see next paragraph) were incubated for 5 days in neuron-plating medium containing 10% horse serum (Invitrogen, Carlsbad, CA). Hippocampi from E18 rats embryos were dissociated chemically (0.25% trypsin, 20 min) and mechanically using fire-polished Pasteur pipettes. Neurons plated on the patterned substrates (densities ranging from 1,000 to 10,000 cells/cm²) were maintained in a 5% CO₂ atmosphere at 37°C. After 4 h, neuron-plating medium was replaced by a serum-free maintenance medium, and a feeding layer of glial cells was added to each dish. Glial cells proliferation in the culture was stopped by AraC after 2 days (1 μ g/ml, Sigma, St. Louis, MO).

Photolithography

The lithography protocol has been detailed previously (Wyart et al. 2002). In brief, cleaned coverslips were coated with hydrophobic fluorosilane C₈H₄Cl₃F₁₃Si (ABCR, Karlsruhe, Germany) in dichloromethane and n-decane, for one-half an hour, at 4°C. After rinsing in chloroform, the silanized surfaces were spin-coated with a positive photoresist. Each coverslip was pressed against a mask and exposed to UV light. Incubation in a development bath removed the exposed photoresist. The fluorosilane layer (no longer protected by the photoresist) was removed with an H₂O plasma, and the glass surface was coated with poly-L-lysine (Sigma P2636, 1 mg/ml for 3 h at 37°C). Unexposed photoresist was washed out with acetone. Patterned domains for autapses have been optimized to obtain on average a single neuron per disk with a large probability of survival. Patterns for pairs consisted in two 60- μ m-diam disks connected to each other by a thin line $(2-4 \ \mu m \text{ wide and } 60-100 \ \mu m \text{ long})$ to guide the growth of the neurites. Masks for lithography were prepared in the laboratory: after a standard metallization procedure using chromium, we obtained typically 100-1,000 patterns on a coverslip.

Electrophysiological recordings

Cell-attached and whole cell patch-clamp recordings were obtained at room temperature from 2- to 4-wk-old cells. All recordings were performed using Axopatch 200B (Axon Instruments, Foster City, CA). Patch pipettes were made of borosilicate tubes (Clarks) and had a resistance of $3-4 \text{ M}\Omega$ when filled with the standard pipette solution. In cell-attached recordings, a 5-mV pulse was regularly applied to check that the perforation of the cell membrane did not occur. To monitor the recording characteristics in whole cell experiments, leak resistance was measured periodically during the recordings and ranged between 250 M Ω and 1 G Ω for a given cell. Leak current, monitored in voltage clamp, ranged from 10 to 200 pA. Cells older than 3 wk with a larger surface could sometimes not be clamped in voltage mode and would fire a spike on the edge of the excitatory postsynaptic current (EPSC) in this configuration. We have discarded these cells for our analysis of synaptic properties. Collection of data were interrupted if the recording showed a significant change in leak resistance. Fast and slow capacitance and series resistance compensation were performed in the whole cell mode. Series resistance in whole cell configuration was <10-12 M Ω and was compensated \leq 60%. Recording data were acquired at 5 kHz in real-time with an Axon Digidata 1320A (Axon Instruments).

Recording solutions

The bath solution contained (in mM) 145 NaCl, 3 KCl, 3 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES, pH = 7.25, and its osmolarity was adjusted to 315 mOsm. The pipette solution contained (in mM) 9 NaCl, 136.5 KGlu, 17.5 KCl, 0.5 CaCl₂, 1 MgCl₂, 10 HEPES, and 0.2 EGTA (pH = 7.25), and its osmolarity was equal to 310 mOsm. In our conditions, the reversal potential for glutamatergic currents was 0 mV, allowing us to distinguish them from GABAergic currents (reversal potential of -60 mV). Bath solution was superfused locally at 0.5–1

ml/min with a microperfusion tube inlet and outlet from a peristaltic pump. All experiments were performed at fixed temperature (22–25°C).

Drugs

In some experiments, the following transmitter antagonists (from Sigma) were applied in the bath: 100 μ M 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) for non–*N*-methyl-D-aspartate receptors (NMDARs); 50–100 μ M 2-amino-5-phosphopentanoic acid (APV) and 10 μ M MK801 for NMDARs; and 250 μ M α -methyl-4-carboxyphenylgly-cine (MCPG) for metabotropic glutamate receptors. EGTA-AM (Molecular Probes, Eugene, OR) was dissolved in 0.5% dimethyl sulfoxide before dilution at 50 μ M in the bath solution. Cells were incubated for 15 min. A solution of 1 mM EGTA was also used as a comparison to the EGTA-AM experiments. EGTA is a slow calcium buffer (Feller et al. 1996) that modifies the shape of a calcium transient by providing a faster initial decay while producing a smaller and slower subsequent phase.

Calcium imaging

Cultures were loaded with 5 μ M of the membrane-permeant acetoxymethyl ester of Fura-2 AM (Molecular Probes) for 15 min at room temperature and rinsed for 30 min. A 100-W Xenon lamp filtered at 380 nm ensured the excitation of the probe, and the emission was filtered at 510 nm. Binned images (8 × 8) obtained with a CCD (CoolSnap HQ, Roper Scientific, Duluth, GA) were acquired at 20 Hz, stored, and analyzed using Metamorph to measure the fluorescence intensity variation in a cell body. Each spike in a Fura-2 AM–loaded neuron induced a large calcium entry, associated with a decrease of the fluorescence emission (Mao et al. 2001). Therefore the variations of fluorescence intensity in the soma reflected the occurrence of spikes with the time resolution of our acquisition system (50 ms). The concentration of Fura-2 in the soma was estimated to be of the order of 50 μ M.

Detection of spikes in cell attached recordings

Spikes were detected above a threshold equal at least to three times the peak-to-peak electrical noise of the recording. By combining cell-attached recordings with spike detection by calcium imaging, we checked that no spikes were missed. A limitation of cell attached recordings is the ambiguity to distinguish the signal due to a spike from a signal due to large EPSPs. A large volley of EPSPs arriving very synchronously in the case of an autapse has a rising phase lasting for only a few milliseconds. For high-frequency signals, the cellattached technique provides a measurement proportional to the derivative of the neuronal membrane potential. Thus large autaptic EPSPs gave rise often to a negative peak in their early phase that is similar to a spike. For this reason, we did not consider higher firing rates than 50 Hz, and we limited our analysis to interspike intervals (ISIs) >20 ms.

Analysis

Statistics on burst duration and on median intraburst frequency were obtained with the criterion of 5 s as the maximal ISI within a burst and after suppression of ISI inferior to 20 ms. ISI distributions were normalized for each cell to compensate for differences in the duration of recordings or in burst frequencies between distinct cells.

ESTIMATION OF THE INTEGRATED CHARGE ASSOCIATED WITH THE AUTAPTIC RESPONSE. We evoked a spike in voltage clamp by 2-ms depolarizing pulses of current at 0.05 Hz to monitor a stable autaptic EPSC. The total integrated charge was estimated by integrating the EPSC from 4 to 600 ms after the evoked spike. To distinguish between the very slow and the slow components of the EPSC, we used

also the partial integrated charges corresponding to the integration of the EPSC from 4 to 200 ms and from 200 to 600 ms.

ESTIMATION OF THE FREQUENCY OF ASYNCHRONOUS MINIATURE EVENTS. Discrete asynchronous miniature EPSCs (mEPSCs) can be detected 200 ms after a spike on the autaptic response. These mEPSCs occur for ~ 1 s at higher frequency than spontaneous mEPSCs at rest. We estimated their mean frequency in a 500-ms time window beginning 200 ms after a spike. The slow component of the EPSC was fitted to a single exponential that was subtracted to the recording trace before the detection of mEPSCs with the MiniAnalysis software (Synaptosoft). For comparison, we show the mean frequency of miniature events at rest measured in TTX at -60 mV. Results are always presented as means \pm SD. The experiments followed European Community guidelines on the care and use of animals (86/609/ CEE, CE official journal L358, 18 December 1986), French legislation (decree no. 97/748, 19 October 1987, J. O. République française, 20 October 1987), and the recommendations of the CNRS.

RESULTS

Spontaneous activity of glutamatergic autapses as a model of sustained firing at low and very low frequencies

Single isolated neurons having only autaptic synapses were obtained with neuronal cultures on patterned surfaces (see METHODS). This protocol allowed for the proper maturation of cells \leq 5 wk in vitro (Wyart et al. 2002). Autapses grew most of their neurites along the border of the poly-L-lysine disks (Fig. 1A) and were constrained to connect only to themselves. We studied only excitatory neurons exhibiting highly ramified



FIG. 1. Isolated excitatory neurons in culture show spontaneously synaptically driven bursts of action potentials. *A*: differential interference contrast (DIC) image of a typical autapse after 19 days in vitro. *B*: cell attached recording showing bursts of spikes. *C*: fluorescence image of an autapse loaded with Fura-2 AM. *D*: relative variation $(-\Delta F/F)$ of the fluorescence intensity (*F*) averaged over the soma reveals large calcium transients. *E*: spontaneous firing in a cell (*top*) is abolished by bath application of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 100 μ M; *bottom*). Scale bar on images is 50 μ m (*A* and *C*).

dendritic trees. Their excitatory nature was confirmed in whole cell voltage clamp by measuring the reversal potential of the autaptic EPSC (0 mV in our conditions, see METHODS). After 10 days in vitro (DIV; 10-31 DIV; age = 19.2 ± 8.5 DIV), spontaneous activity was detected in two-thirds of the neurons (80 among 126 cells) in cell-attached recordings (Fig. 1B) but not in whole cell recordings, probably because of the rapid dialysis of the intracellular components. Activity was also revealed by calcium imaging as large calcium transients occurring spontaneously (Fig. 1, C and D). Calcium transients were always abolished by bath application of TTX (0.5 μ M; n = 7; age = 20.1 \pm 5.7 DIV, data not shown) indicating that they arose from sodium action potentials. All spontaneously spiking cells fired bursts, i.e., groups of spikes separated by less than a few seconds. Interburst intervals had a widespread distributions with a mean in the order of tens of seconds (97 \pm 43 s; Fig. 1B). We set the following 5 s as the maximum ISI to define a burst. Burst detection was usually unambiguous since interburst intervals usually exceeded 10 s (Fig. 1, B and E, top).

In 96% of the cells tested (n = 24; age = 19.6 ± 5.3 DIV), the bath application of CNQX (100 μ M) prevented spontaneous activity to occur (Fig. 1E). The effect of CNQX was reversible (data not shown, n = 5). The primary cause of spontaneous firing in most neurons was spontaneous release of glutamate (unpublished data). Bursts could also be evoked by a brief (2 ms) depolarizing pulse from the cell-attached pipette (Fig. 2). For a given cell, the distributions of ISIs in the cases of spontaneous (Fig. 2A) and evoked bursts (Fig. 2B) were similar. In 3- to 4-wk-old cells (22–31 DIV, n = 6), the median ISI (Fig. 2C) and the mean burst duration (Fig. 2D) were indeed never significantly different for spontaneous activity and evoked activity (P < 0.05). The bursting activity, either spontaneous or evoked by a brief stimulation, was therefore a self-sustained process, and initiation and maintenance of firing were likely to be independent processes.

We observed a difference in firing frequency with the age of the culture (Fig. 3, A and B). ISI distribution showed a peak below 100 ms, which constituted >70% of the distribution in 2-wk-old cells (16.0 \pm 1.2 DIV, n = 4, Fig. 3C) and <25% in 3- to 4-wk-old cells (24.9 \pm 3.1 DIV, n = 8, Fig. 3C). In the latter case, most of the intervals ranged between 250 ms and 1 s (Fig. 3C). The median intraburst frequency shows a significant (P < 0.01) decrease from 11 ± 8.8 (2 wk) to 2 ± 1.3 Hz (3-4 wk; Fig. 3D), but without a significant change in burst duration $(2 \text{ wk: } 10.6 \pm 13.4 \text{ s}, n = 4; 3-4 \text{ wk: } 20.2 \pm 14.6 \text{ s}, n = 8; \text{ Fig.}$ 3E). Therefore using glutamatergic autapses, the synaptic mechanisms, which could underlie the persistence of spiking activity during a burst, can be studied within different frequency regimes: at low (about 10 Hz) and very low (1-2 Hz) frequencies. We first tested if NMDAR activation was responsible for the maintenance of activity in these two regimes (Lisman et al. 1998; Wang 1999).

NMDAR activation controls the persistence of activity at low frequencies

In the case of cells firing at low frequencies, the EPSC exhibited two components (Fig. 4, A1 and A2). The main component had a decay time of about 10 ms and was suppressed by 100 μ M CNQX (data not shown), indicating the activation of AMPA receptors. The second component was



FIG. 2. Spontaneous (*A*) and evoked (*B*) firing of excitatory autapses share similar characteristics in terms of interspike interval (ISI) distribution and burst duration. Cell attached recordings of a cell after 22 days in vitro (DIV) and the corresponding ISI distributions are shown, respectively, in the *top* and *bottom panels*. Horizontal and vertical scale bars are 200 ms and 25 pA, respectively. A: spontaneous activity. B: activity evoked by a 2-ms depolarizing pulse (at the time indicated by S on the figure). Medians of the ISI distributions (*C*) and the mean burst durations (*D*) are not statistically (P < 0.05) different for spontaneous (S) and evoked (E) bursts at 3–4 wk in vitro.

reduced by bath application of NMDAR antagonists (MK801, 10 μ M with APV, 100 μ M; Fig. 4, A1 and A2), revealing the activation of NMDARs: the integrated charge (see METHODS) decreased from 67.3 \pm 11.9 to 32.4 \pm 11.0 pA s in presence of the drugs (n = 6, P < 0.05; Fig. 5A3). The application of APV+MK801 in neurons bursting at low frequencies (Fig. 4B1) prevented also the occurrence of bursts (Fig. 4B2). Only single spikes or single pairs of spikes were detected (n = 6, Fig. 4B2). This effect was reversed by washing out the drugs (Fig. 4B3). This shows that the NMDA component of the EPSC (with a decay time of about 100–200 ms) was mainly responsible for sustained firing at ~10 Hz within bursts. On the contrary, the AMPA component of the EPSC (with a decay time of about 10 ms) was not lasting long enough to sustain recurring bursting.

Postsynaptic calcium-dependant mechanisms sustaining recurring firing at very low frequency

Neurons with sustained activity at very low frequencies exhibited a very slow component of the autaptic response that lasted for several hundreds of milliseconds (both in voltage and current clamp; see Fig. 5, A1 and A2). Application of MK801 and APV (Fig. 5, A1 and A2) modified only slightly the EPSC and the EPSP: the integrated charge (see METHODS) decreased only from 170.3 \pm 98.3 to 167.1 \pm 93.8 pA·s in presence of the drugs (n = 9, not significant; P < 0.05; Fig. 5A3). Application of the drugs did not abolish either the spontaneous sustained firing (n = 3, Fig. 5, B1 - B3). Moreover, firing within the bursts was less regular: ISIs shorter than 5 s exhibited a larger dispersion (P < 0.05; see Fig. 5C) in the presence of MK801-APV (1.6 \pm 2.9 s) than under control conditions $(1.3 \pm 0.7 \text{ s})$. This observation shows that, at very low rates, the NMDA component was important for the regularity of firing, but was not necessary to sustain firing.

Because bursts were associated with large calcium transients (Fig. 1, C and D), we tested if this very slow autaptic component was dependent on the intracellular calcium concentration. Fifteen-minute bath incubation in 50 μ M EGTA-AM was sufficient to suppress the slow autaptic response (Fig. 6A1). The same result was obtained using 1 mM EGTA in the pipette intracellular medium (data not shown). The slow component, estimated as the 200- to 600-ms integrated charge (see METH-ODS), decreased by 85% in EGTA-AM (Fig. 6C), whereas the 0- to 200-ms integrated charge was only lowered by $\sim 30\%$ (Fig. 6C). EGTA-AM incubation also modified the maintenance of activity in 3- to 4-wk-old cells (n = 9, Fig. 6A2). In four cells, it prevented bursts to occur, and cells showed only single spikes (data not shown). In five of nine cells, bursts of three to six spikes separated by 200-ms intervals on average were still observed after long periods of silence. In these cells, burst duration decreased remarkably under 1 s (680 \pm 130 ms, n = 4, see Fig. 6A3). ISI distribution shifted to lower values; long ISIs (>300 ms) were abolished (Fig. 6A4). Thus it mimicked the ISI distribution of 2-wk-old cells (Figs. 3C and 6A4).

Next we attempted to determine if the large calcium increase during the bursts may be due to the activation of metabotropic glutamate receptors (mGluRs) (Woodhall et al. 1999). Bath application of MCPG (250 μ M), a nonselective mGluR antagonist, greatly reduced the slow component of the EPSP (Fig. 6B1). In six cells, the 200- to 600-ms integrated charge was decreased by $61 \pm 13\%$ in MCPG (Fig. 6C), whereas the 0- to 200-ms integrated charge was only reduced by $\sim 8 \pm 3\%$ (Fig. 6C). Therefore the very slow component of the EPSP corresponded to a calcium-sensitive conductance, activated by metabotropic glutamate receptors. We measured moreover its reversal potential (3 ± 4 mV), which was very close from the reversal potential for all cations calculated with the Nernst equation (-2.5 mV) in our conditions $([\text{cations}]_{\text{ext}} = 148 \text{ mM},$ $[cations]_{in} = 163$ mM). This indicates that this conductance was a calcium-sensitive nonselective cationic conductance activated by mGluRs. The application of MCPG (250 μ M) altered also the maintenance of firing (see Fig. 6B2): cells fired only single spikes or few (2-3) spikes (n = 6), separated by short (<300 ms) intervals. This is consistent with the observation that the autaptic responses of 3- to 4-wk-old cells incubated in EGTA-AM (Fig. 6A1) or with MCPG (Fig. 6B1)



FIG. 3. Dynamics of spontaneous activity in autapses cultured for 2–4 wk in vitro. *A* and *B*: cell attached recordings showing bursts in distinct autapses after 2 (*A*) and 3–4 wk (*B*). *C*: intraburst ISI distributions for 2-wk-old cells (15–17 DIV, n = 4) and 3- to 4-wk-old cells (21–31 DIV, n = 8). Bin is 100 ms, and distributions are shown only for ISI <2.5 s. *D* and *E*: median of the distribution of the intraburst frequencies (*D*) decreases from 2 to 3–4 wk of culture while the corresponding average bursts duration (*E*) does not show a significant change (P < 0.05).

and of 2-wk-old cells (Fig. 4AI) had a similar relatively short timescale (about 100 ms) and did not allow sustained firing at very low frequencies (i.e., with ISIs > 500 ms).

Presynaptic calcium-dependant mechanisms sustaining recurring firing at very low frequency

Neurons, showing sustained bursting activity at very low frequencies, exhibited, on top of the very slow component of the autaptic response, delayed release of glutamate. Asynchronous miniatures occurred at a very high frequency in a time window of 200 ms to 1 s after a spike (Fig. 7*A*). The frequency

of asynchronous miniature events increased noticeably with the number of DIV (Fig. 7*B1*) and significantly with the firing frequency, reaching a plateau level after a train of action potentials at 4 Hz (Fig. 7*B1*). Fifteen-minute bath incubation in 50 μ M EGTA-AM was sufficient to decrease the frequency of the asynchronous miniature events associated with the continuous component of the slow autaptic response (Fig. 7*B2*).

To enhance specifically the delayed release of glutamate, extracellular calcium was replaced at the same concentration by strontium. It increased the asynchronous miniature event frequency (Fig. 7B2) and modified significantly the dynamics



FIG. 4. N-methyl-D-aspartate (NMDA) component of the synaptic response allows sustained firing in 2-wk-old cells. A: effect of 100 µM 2-amino-5-phosphopentanoic acid (APV) and 10 µM MK801 on the excitatory postsynaptic potential (EPSP; A1) and on the excitatory postsynaptic current (EPSC; A2) in 2-wk-old cells is compared with the control conditions (CONT). A3: integrated charge of autaptic currents significantly decreases after application of the drugs compared with control conditions (n = 6, P < 0.05). B: bath application of APV + MK801 prevents reversibly sustained bursting recorded in cell attached configuration (top: long recording; bottom: zoom of the burst). B1: control. B2: after bath application of APV + MK801. B3: wash out.

Downloaded from www.physiology.org/journal/jn (195.005.242.180) on January 30, 2020.



FIG. 5. NMDA component of the synaptic response has a weak role on sustained bursting in 3- to 4-wk-old cells. A: effect of 100 µM APV and 10 μ M MK801 on the EPSP (A1) and on the EPSC (A2) in 3- to 4-wk-old cells is shown by comparing the response in the presence (APV MK801) and absence (CONT) of the drugs. A3: integrated charge of autaptic currents does not change significantly after application of the drugs compared with control conditions (n = 9, P < 0.05). B: bath application of APV + MK801 does not prevent sustained bursting recorded in cell attached configuration, but makes firing more irregular. B1: control. B2: after bath application of APV + MK801. B3: wash out. C: corresponding histograms of ISI before (CONT), during (APV MK801), and after (WASH OUT) bath application of the drugs.

of recurring bursting (Fig. 7, *C1* and *C2*). The ISI distribution was shifted to longer time intervals (Fig. 7*D*): long ISIs were favored when the late release of glutamate was enhanced. It also largely increased the burst duration (Fig. 7, *C1*, *C2*, and *E*), from 3.9 ± 2.4 to 18.3 ± 5.6 s (n = 7; age = 23.1 ± 3.2 day; P < 0.05). This set of evidence strongly indicates that the stochastic delayed release of glutamate, which relies on residual calcium in the presynaptic terminal (Atluri and Regehr 1998; Cummings et al. 1996; Feller et al. 1996; Goda and Stevens 1994; Hagler and Goda 2001; Zucker 1999), intervenes in the suprathreshold reactivation of action potentials within a burst. The stochastic nature of these asynchronous events might also explain the irregular firing that was observed with APV + MK801 in the bath.

In the case of very low frequency firing, NMDAR activation was not necessary for maintenance of activity in a burst. Our data suggest that a postsynaptic calcium-sensitive nonselective cationic conductance activated by mGluRs and the presynaptic delayed release of glutamate were contributing together to the firing at very low frequency within a burst.

Origin of the long relative refractory period in 3- to 4-wkold cells

To control spiking in a defined range of frequencies, negative and positive feedback mechanisms are necessary, respectively, to forbid spiking shortly after a spike and to allow reactivation of a spike after a long ISI (Wang 1999, 2001). In 2-wk-old cells, we observed a classical refractory period (10–20 ms). On the contrary, in 3- to 4-wk-old cells, a relative refractory period lasted for about 150 ms (see Fig. 8). This long refractory period was associated with an enormous (about 50%) decrease of the membrane resistance during the EPSP (see Fig. 8 for the determination of the membrane resistance). This huge shunt was likely responsible for the inhibition of the spike reactivation. It could also explain the fact that 3- to 4-wk-old cells did not fire at low frequencies exactly as 2-wk-old cells when mGluR activation or intracellular calcium increase was inhibited: their large EPSP was responsible for an abnormally long shunt, superior to the time decay of the NMDA component.

Calcium-dependant mechanisms are also involved in the maintenance of activity in small excitatory networks

The synaptic mechanisms previously described could consist in specific properties of an autaptic system with only homosynapses (synapses connected to the neuron itself), deprived of heterosynapses (synapses connecting distinct neurons). To test this issue, we carried out similar experiments on pairs of isolated neurons (n = 9) in double cell-attached configuration. Spontaneous activity of 2-wk-old cells in a pair consisted in synchronous bursts with large ISIs separated by long silences (Fig. 9A1) and was suppressed by bath application of CNQX (100 μ M). Figure 9A2 shows that any cell could spike first (e.g., cell 1 for the 1st 2 spikes and cell 2 for the last 2 spikes on Fig. 9A2) and systematically activate the other neuron in a 10-ms time window (Fig. 9A2). Presumably, both cells remained silent for a few hundred of milliseconds due to the long



FIG. 6. Pharmacological characterization of the very slow synaptic component responsible for sustained firing at very low frequency in 3- to 4-wk-old cells. A: effects of EGTA-AM incubation. A1: EGTA-AM incubation reveals that the very slow component is a calcium sensitive conductance. A2: sustained firing is prevented. A3: bursts duration is greatly decreased (P < 0.01). A4: ISI distribution is shifted toward shorter intervals (as a comparison, the ISI distribution for 2-wk-old cells is depicted in control conditions). B: effect of α -methyl-4-carboxyphenylglycine (MCPG) on the EPSP (B1) and on the spontaneous firing (B2, cell attached recording) in a 26 DIV cell. MCPG reduces the very slow component of the EPSP and prevents the cells from sustained firing. C: very slow component is a calcium sensitive conductance activated by the mGluR receptors: EGTA-AM and MCPG mainly reduce the 200- to 600-ms component of the synaptic response. Integrated charge of the synaptic response is depicted 15 min after bath application of EGTA-AM (n = 4) and during bath application of MCPG (n = 5) compared with control conditions (n = 9). Total integrated charge is divided between 0–200 (white bars) and 200–600 ms (gray bars).

relative refractory period. Therefore there was no asynchronous spiking in the paired neuron configuration.

This synchronous activity was occurring at very low frequency in 2- and 3- to 4-wk-old cells (Fig. 9B), showing ISIs as large as 500 ms to 1 s. We tested the hypothesis that calcium-dependant mechanism(s) might be involved in recurring activity. We studied specifically the properties of heterosynaptic responses when homosynapses were not activated. We recorded one cell (cell 1) in the voltage-clamp mode (hold at -60 mV) while the spontaneous activity of the second cell (cell 2) was recorded in the cell-attached configuration (Fig. 9C). Cell 1 received only currents from cell 2 heterosynapses. After a spike of cell 2, a very slow continuous inward current associated with high-frequency asynchronous miniature events were observed, as in autapses (Fig. 9C). Moreover, incubation in 50 μ M EGTA-AM for 15 min (n = 5, Fig. 9D), as application of MCPG (250 μ M, n = 2, data not shown), strongly prevented sustained firing at low rates: only short bursts were found. Typical ISIs were of the order of 200 ms, and ISIs larger than 400 ms were never observed. These observations suggest that the maintenance of activity at very low frequencies in pairs of excitatory neurons relies, as in

autapses, on a slow calcium-sensitive conductance activated by mGluR associated with the delayed release.

DISCUSSION

We analyzed the nature and the kinetics of synaptic components involved in the low-frequency bursting of glutamatergic cells with a model system consisting in single or pairs of neurons. The use of such model has been used to analyze theoretically short-term analog memory (Seung et al. 2000). We studied experimentally the mechanisms underlying the recurring bursting. Spontaneously or evoked bursts showed the same characteristics, indicating that firing was self-sustained in a burst. Autapses after 2 wk fired bursts of action potentials, with a gradual decrease of the median interspike frequency from 11 (2 wk) to 2 Hz (3-4 wk). The median ISIs (90 and 500 ms, respectively) were always longer than the decay time of the AMPA component of the EPSC. We showed that the activation of these receptors was not sufficient to sustain firing. Instead, we showed that slower synaptic components, dependent on the activation of NMDARs and mGluRs, were necessary for sustained firing at low frequencies.



FIG. 7. Delayed release of glutamate is involved in sustain firing of 3- to 4-wk-old cells. A: voltage-clamp recording of a 21 DIV autapse shows delayed release after a triggered spike: delayed release is estimated as the mean frequency of asynchronous miniature events in a 500-ms time window 200 ms after a spike. B1: delayed release increases with time in vitro and firing frequency (white squares: after a single action potential; black diamonds: after a train of 4 action potentials at 4 Hz; filled circles: the spontaneous release, given as a comparison). Mean miniatures frequency was evaluated at -60 mV in TTX. B2: delayed release is sensitive to the bath application of EGTA-AM and enhanced when extracellular calcium (3 mM) is replaced by strontium (3 mM). C: sustained firing at very low frequency is prolonged when replacing calcium by strontium in the bath. Cell attached recordings showing spontaneous firing of a 3-wk-old autapse in 3 mM calcium (C1) and 3 mM strontium (C2). D: median frequency of ISI shifts from 250 ms in calcium (black bars) to 450 ms in strontium (white bars) for 3- to 4-wk-old cells (n = 7). E: burst duration is significantly increased in 3 mM strontium (n = 7, P < 0.05).

Comparison with previous studies in culture and other preparations

Hippocampal glutamatergic cells in culture (Bacci et al. 1999; Maeda et al. 1995; Murphy et al. 1992; Opitz et al. 2002; Segal 1991), as in slices (Garaschuk et al. 1998; Menendez de la Prida and Sanchez-Andres 2000), fire bursts of spikes at low frequency (<10 Hz). Studies on recurring bursting in culture (Harris et al. 2002; Robinson et al. 1993; Nurphy et al. 1992; Opitz et al. 2002; Robinson et al. 1993; Voigt et al. 2001) have been achieved mainly at high density. Whereas the duration of bursts (2–5 s) is similar in our study, their high frequency (0.2 instead of 0.01 Hz) and regularity of occurrence (referred as "oscillatory" bursting) are distinct. This can be attributed to the higher density of cells (and certainly of synapses; Muramoto et al. 1993) and to the presence of inhibitory cells that modulate firing (Opitz et al. 2002; Siebler et al. 1993; Voigt et al. 2001).

On the contrary, Bacci et al. (1999), as in our study, report that bursts last for a few seconds and are separated by tens of seconds; they show also that spontaneous activity is abolished with CNQX and TTX but not with APV, which makes firing more irregular; the spontaneous activity of autapses is characterized by an interburst interval of about 1 min, and intracellular recordings show that action potentials occur on a very slow depolarizing component. Therefore our model system did not properly exhibit an "oscillatory bursting," since the occurrence frequency of burst differs. However, because bursts duration and intraburst frequencies are similar in all cases, mechanisms for sustained firing are likely to be identical.

Origin of the difference between cells firing at low and very low frequencies

The shift in firing rate between 2- and 3- to 4-wk-old cells could be due to a change in protein expression (for either mGluRs or the channels responsible for the slow inward current). By applying the agonist DHPG from group I mGluRs, we observed (for 2- and 3- to 4-wk-old cells) the induction of a slow inward current (n = 7, data not shown). This suggests that the difference in firing rate does not correspond to a difference in protein expression; it is probably due to the huge increase of the autaptic EPSP with the number of DIV.

Indeed we can draw a simple model (Fig. 10) that explains the sustained firing at low and very low rates. In Fig. 10, we approximated the slow synaptic inward currents and the membrane resistance $(R_{\rm m})$. We assumed that their product was proportional to the slow depolarization of the EPSP. Our scheme neglects many negative feedback mechanisms, such as calcium adaptation and potassium channels activation and deactivation, which probably play a role in the rate control in vivo. However, it provides a good explanation of the difference in firing rate with the number of DIV. In this scheme, the rate of the sustained firing is determined by the relative values of the decay time of the slow synaptic component and of the relative refractory period. The refractory period of 2-wk-old cells with a small and fast EPSP (10-20 mV) is 10-20 ms: the cell can fire, because of the NMDA component, 20-200 ms after the previous spike. In 3- to 4-wk-old cells with a large and



FIG. 8. A long relative refractory period is observed in 3- to 4-wk-old cells. A succession of 3 stimulations I(1)-S-I(2) is applied with 500 ms between I(1) and S and a stepwise increased delay (steps of 200 ms) between S and I(2). The suprathreshold stimulation S is the minimal 2-ms current pulse that always evokes a spike at 0.05 Hz. The infrathreshold stimulations I are set to two-thirds of S and never evoke a spike when applied at 0.05 Hz. Drop of the membrane resistance 200 ms after the spike is revealed by the reduced amplitude of the stimulation artifact (right star) compared with the artifact observed at rest (left star).



FIG. 9. Very slow calcium sensitive conductance associated with the delayed release of glutamate is involved in sustained firing for isolated pairs of neurons. *A1*: 2 connected excitatory neurons (cell 1 and cell 2, 28 DIV) show synchronized bursts of action potentials in cell attached recordings. *A2*: A zoom within the burst shows that the 2 cells always fire within a time window of 10 ms. For each pair of spikes, a cursor points to the 1st cell firing, indicating that both cells can fire 1st. *B*: as in autapses, ISI distribution shifts toward long ISI with time in vitro for pairs: white bars, 2-wk-old pairs; black bars, 4-wk-old pairs. Exponential fits have been added to the histograms (dashed line: 2-wk-old pairs; solid line: 4-wk-old pairs) showing that the distribution shifts to longer intervals for older cells. *C*: when cell 2 recorded in cell-attached mode fires a train of action potentials, the spontaneous currents received by cell 1 (clamped at -60 mV) show, at the end of the burst, a long-lasting slow component with high-frequency asynchronous events. *D*: incubation in EGTA-AM does not prevent spontaneous firing (recorded in 1 cell of the pair) but bursts are short and deprived of long ISI (>500 ms). *Bottom*: detail of a burst showing that a burst is made of a few spikes only.

slow EPSP (60 mV), the shunting effect lasts for about 150 ms: the cell can fire in a time window of 150–500 ms because of the calcium-sensitive cationic conductance and the delayed release of glutamate.

An analogy can be made between autapses and glutamatergic networks of different size and synchrony. Young autapses could correspond to small and weakly synchronized excitatory networks: the shunt of the membrane resistance due to the network activity is small. Old autapses with large EPSPs could correspond to highly synchronized and large excitatory networks, as in epilepsy: the large shunt of the membrane resistance is a consequence of the numerous and simultaneous synaptic inputs. Such a shunt has been evidenced in vivo (see Leger et al. 2005). The relative refractory period associated with the slow depolarizing conductance is critical for the firing rate control.

NMDAR activation necessary for 10-Hz firing

The suppression of bursts by APV + MK801 in 2-wk-old cells indicated that the NMDA component was necessary for sustaining activity at about 10 Hz. This corroborates previous observations in hippocampal cultures (Bacci et al. 1999; Mangan and Kapur 2004) and in hippocampal slices (Bonansco and Buno 2003; Dingledine et al. 1986; Lee and Hablitz 1990,



FIG. 10. Simple scheme accounting for the difference in the preferred refiring time window between 2- and 3- to 4-wk-old cells. The optimal window drawn on the membrane potential curve depends on the product of the membrane resistance and on the slowly decaying depolarizing current. *A*: in a 2-wk-old cell, synaptic response has a small (20 mV) and fast (\sim 10–100 ms) decay. Membrane resistance recovers to its normal value within a few tens of milliseconds, and the main slow depolarizing current due to NMDA receptor (NMDAR) activation lasts <200 ms. The optimal refiring window given by the product of the resistance by the current lies within 200 ms: the cell refires after short delays (<100 ms). *B*: in a 3- to 4-wk-old cell, the EPSP is larger (60 mV), due to the huge AMPA component, and slower (\sim 1s), due to the calcium sensitive cationic conductance activated by mGluRs. The optimal refiring window lies within 200 ms to 2 s: the cell fires with long ISIs (>200–500 ms)

1991; Schneiderman and MacDonald 1987; Wang and Jensen 1996; Williamson and Wheal 1992). At the same time, NMDAR antagonists abolish epileptiform discharges (Lee and Hablitz 1990, 1991), and NMDA induces rhythmic oscillations (Bonansco and Buno 2003).

We observed that NMDAR activation was not necessary for sustained firing in 3- to 4-wk-old cells. Similarly, it has been shown to play a weaker role in inducing ictal discharge in adult slices compared with immature ones (Wang and Jensen 1996). It was also sufficient but not necessary for burst generation in the CA3 region (Neuman et al. 1988), indicating that other conductance might be involved.

Moreover, we observed that NMDAR activation reinforces regular firing within burst when the calcium-sensitive cationic conductance and the delayed release are playing a key role. Similar observations were reported in experiments performed on hippocampal cultures (Bacci et al. 1999) and on slices of rat visual cortex (Harsch and Robinson 2000).

A slow depolarizing conductance activated by mGluRs necessary for 2-Hz firing

The autaptic response of 3- to 4-wk-old cells had a very slow depolarizing component (\sim 500 ms to 1 s), attributed to a nonselective cationic calcium-sensitive conductance activated by mGluRs. Similar conductance have been described in the hippocampus (Congar et al. 1997; Crepel et al. 1994) and in the entorhinal cortex (Egorov et al. 2002; Fransen et al. 2002). Three arguments indicate that this slow conductance sustained activity within a burst: *1*) its kinetics matched long ISIs; *2*) application of EGTA-AM or MCPG suppressed long ISIs; and *3*) it also decreased the burst duration.

Some arguments indicate that such calcium-sensitive slow conductance operate in other systems. In pairs of excitatory cells, the synaptic response and the firing were sensitive to EGTA-AM and MCPG. In standard culture, a slow depolarizing current has also been noticed in spontaneous current-clamp recordings (Bacci et al. 1999). In slices, a slow depolarizing current induced by group I mGluRs in CA1 (Crepel et al. 1994) has been described after high-frequency stimulations (Congar et al. 1997). Finally, several experiments have emphasized the possible role of a calcium-sensitive cationic current, activated by cholinergic muscarinic receptors, in the graded persistent activity in entorhinal cortex neurons (Egorov et al. 2002; Fransen et al. 2002). Therefore calcium-sensitive nonspecific cationic conductance might be involved in an ubiquitous manner in self-sustained activity.

Delayed release of glutamate necessary for retriggering a spike

Our data suggest that the delayed release of glutamate (Cummings et al. 1996; Goda and Stevens 1994; Hagler and Goda 2001; Van der Kloot and Molgo 1993) facilitated the bursting activity. We observed that the asynchronous release of glutamate was widely present in autapses as in pairs of excitatory cells after 3 wk in vitro. When the NMDAR was blocked, cell firing became irregular as if a stochastic mechanism was contributing to firing. Finally, the replacement of extracellular calcium by strontium increased the burst duration and favored long ISIs within a burst. This suggests that delayed

release through large asynchronous events could trigger a spike after a long delay (hundreds of milliseconds) within a burst in glutamatergic networks.

Using an in vitro model of synchronous glutamatergic networks, we were able to identify that the nature and temporal dynamics of synaptic or synaptic-dependant conductance play a key role in recurring bursting. Our study reveals new mechanisms that may be involved in the sustained firing of glutamatergic neuronal networks. Further experiments are necessary to show their implication in vivo.

A C K N O W L E D G M E N T S

We thank B. Poulain for carefully reading the manuscript and for invaluable advice and comments and A. Feltz, who provided useful advice during this work. We also thank C. Girit for corrections and commenting on this manuscript, and R. Monasson for helpful discussions about the experiments.

G R A N T S

This work was supported by the "Université Louis Pasteur, Strasbourg" (contract exceptionnel 2001), the "Ministère de la Recherche" (ACI jeune chercheur 1999), the Centre National de la Recherche Scientifique (Projet jeune chercheur 1999 and ATIP jeune chercheur 2002, département SPM), and the Direction des Recherches Etudes et Techniques (contract 961179, 1996).

REFERENCES

- Atluri PP and Regehr WG. Delayed release of neurotransmitter from cerebellar granule cells. J Neurosci 18: 8214–8227, 1998.
- Bacci A, Verderio C, Pravettoni E, and Matteoli M. Synaptic and intrinsic mechanisms shape synchronous oscillations in hippocampal neurons in culture. *Eur J Neurosci* 11: 389–397, 1999.
- Bekkers JM, Richerson GB, and Stevens CF. Origin of variability in quantal size in cultured hippocampal neurons and hippocampal slices. *Proc Natl Acad Sci USA* 87: 5359–5362, 1990.
- Bekkers JM and Stevens CF. Excitatory and inhibitory autaptic currents in isolated hippocampal neurons maintained in cell culture. *Proc Natl Acad Sci* USA 88: 7834–7838, 1991.
- **Bonansco C and Buno W.** Cellular mechanisms underlying the rhythmic bursts induced by NMDA microiontophoresis at the apical dendrites of CA1 pyramidal neurons. *Hippocampus* 13: 150–163, 2003.
- Congar P, Leinekugel X, Ben-Ari Y, and Crepel V. A long-lasting calciumactivated nonselective cationic current is generated by synaptic stimulation or exogenous activation of group I metabotropic glutamate receptors in CA1 pyramidal neurons. J Neurosci 17: 5366–5379, 1997.
- **Crepel V, Aniksztejn L, Ben-Ari Y, and Hammond C.** Glutamate metabotropic receptors increase a Ca(2+)-activated nonspecific cationic current in CA1 hippocampal neurons. *J Neurophysiol* 72: 1561–1569, 1994.
- Cummings DD, Wilcox KS, and Dichter MA. Calcium-dependent pairedpulse facilitation of miniature EPSC frequency accompanies depression of EPSCs at hippocampal synapses in culture. J Neurosci 16: 5312–5323, 1996.
- **Dingledine R, Hynes MA, and King GL.** Involvement of *N*-methyl-D-aspartate receptors in epileptiform bursting in the rat hippocampal slice. *J Physiol* 380: 175–189, 1986.
- **Egorov AV, Hamam BN, Fransen E, Hasselmo ME, and Alonso AA.** Graded persistent activity in entorhinal cortex neurons. *Nature* 420: 173–178, 2002.
- Feller MB, Delaney KR, and Tank DW. Presynaptic calcium dynamics at the frog retinotectal synapse. J Neurophysiol 76: 381–400, 1996.
- Fransen E, Alonso AA, and Hasselmo ME. Simulations of the role of the muscarinic-activated calcium-sensitive nonspecific cation current INCM in entorhinal neuronal activity during delayed matching tasks. *J Neurosci* 22: 1081–1097, 2002.
- Fuster JM and Alexander GE. Neuron activity related to short-term memory. Science 173: 652–654, 1971.
- **Garaschuk O, Hanse E, and Konnerth A.** Developmental profile and synaptic origin of early network oscillations in the CA1 region of rat neonatal hippocampus. *J Physiol* 507: 219–236, 1998.
- Goda Y and Stevens CF. Two components of transmitter release at a central synapse. *Proc Natl Acad Sci USA* 91: 12942–12946, 1994.

- **Goslin K and Banker G.** Rat hippocampal neurons in low density culture. In: *Culturing Nerve Cells*, edited by Banker G and Goslin K. Cambridge, MA: MIT Press, 1991, p. 251–281.
- Hagler DJ Jr and Goda Y. Properties of synchronous and asynchronous release during pulse train depression in cultured hippocampal neurons. *J Neurophysiol* 85: 2324–2334, 2001.
- Harris RE, Coulombe MG, and Feller MB. Dissociated retinal neurons form periodically active synaptic circuits. J Neurophysiol 88: 188–195, 2002.
- Harsch A and Robinson HP. Postsynaptic variability of firing in rat cortical neurons: the roles of input synchronization and synaptic NMDA receptor conductance. J Neurosci 20: 6181–6192, 2000.
- Lee WL and Hablitz JJ. Effect of APV and ketamine on epileptiform activity in the CA1 and CA3 regions of the hippocampus. *Epilepsy Res* 6: 87–94, 1990.
- Lee WL and Hablitz JJ. Excitatory synaptic involvement in epileptiform bursting in the immature rat neocortex. *J Neurophysiol* 66: 1894–1901, 1991.
- Leger JF, Stern EA, Aertsen A, and Heck D. Synaptic integration in rat frontal cortex shaped by network activity. J Neurophysiol 93: 281–293, 2005.
- Lisman JE, Fellous JM, and Wang XJ. A role for NMDA-receptor channels in working memory. *Nat Neurosci* 1: 273–275, 1998.
- Maeda E, Robinson HP, and Kawana A. The mechanisms of generation and propagation of synchronized bursting in developing networks of cortical neurons. J Neurosci 15: 6834–6845, 1995.
- Mangan PS and Kapur J. Factors underlying bursting behavior in a network of cultured hippocampal neurons exposed to zero magnesium. J Neurophysiol 91: 946–957, 2004.
- Mao BQ, Hamzei-Sichani F, Aronov D, Froemke RC, and Yuste R. Dynamics of spontaneous activity in neocortical slices. *Neuron* 32: 883–898, 2001.
- Menendez de la Prida L, and Sanchez-Andres JV. Heterogeneous populations of cells mediate spontaneous synchronous bursting in the developing hippocampus through a frequency-dependent mechanism. *Neuroscience* 97: 227–241, 2000.
- Muramoto K, Ichikawa M, Kawahara M, Kobayashi K, and Kuroda Y. Frequency of synchronous oscillations of neuronal activity increases during development and is correlated to the number of synapses in cultured cortical neuron networks. *Neurosci Lett* 163: 163–165, 1993.
- Murphy TH, Blatter LA, Wier WG, and Baraban JM. Spontaneous synchronous synaptic calcium transients in cultured cortical neurons. *J Neurosci* 12: 4834–4845, 1992.
- Neuman R, Cherubini E, and Ben-Ari Y. Epileptiform bursts elicited in CA3 hippocampal neurons by a variety of convulsants are not blocked by N-methyl-D-aspartate antagonists. *Brain Res* 459: 265–274, 1988.
- Opitz T, De Lima AD, and Voigt T. Spontaneous development of synchronous oscillatory activity during maturation of cortical networks in vitro. *J Neurophysiol* 88: 2196–2206, 2002.
- Robinson HP, Kawahara M, Jimbo Y, Torimitsu K, Kuroda Y, and Kawana A. Periodic synchronized bursting and intracellular calcium tran-

sients elicited by low magnesium in cultured cortical neurons. J Neurophysiol 70: 1606–1616, 1993.

- Schneiderman JH and MacDonald JF. Effects of reduced magnesium on hippocampal synchrony. Brain Res 410: 174–178, 1987.
- Segal MM. Epileptiform activity in microcultures containing one excitatory hippocampal neuron. J Neurophysiol 65: 761–770, 1991.
- Segal MM and Furshpan EJ. Epileptiform activity in microcultures containing small numbers of hippocampal neurons. J Neurophysiol 64: 1390–1399, 1990.
- Seung HS, Lee DD, Reis BY, and Tank DW. The autapse: a simple illustration of short-term analog memory storage by tuned synaptic feedback. J Comput Neurosci 9: 171–185, 2000.
- Siebler M, Koller H, Stichel CC, Muller HW, and Freund HJ. Spontaneous activity and recurrent inhibition in cultured hippocampal networks. *Synapse* 14: 206–213, 1993.
- Tegner J, Compte A, and Wang XJ. The dynamical stability of reverberatory neural circuits. *Biol Cybern* 87: 471–481, 2002.
- Van der Kloot W, and Molgo J. Facilitation and delayed release at about 0 degree C at the frog neuromuscular junction: effects of calcium chelators, calcium transport inhibitors, and okadaic acid. J Neurophysiol 69: 717–729, 1993.
- Verderio C, Coco S, Pravettoni E, Bacci A, and Matteoli M. Synaptogenesis in hippocampal cultures. *Cell Mol Life Sci* 55: 1448–1462, 1999.
- Voigt T, Opitz T, and de Lima AD. Synchronous oscillatory activity in immature cortical network is driven by GABAergic preplate neurons. *J Neurosci* 21: 8895–8905, 2001.
- Wang C and Jensen FE. Age dependence of NMDA receptor involvement in epileptiform activity in rat hippocampal slices. *Epilepsy Res* 23: 105–113, 1996.
- Wang XJ. Synaptic basis of cortical persistent activity: the importance of NMDA receptors to working memory. J Neurosci 19: 9587–9603, 1999.
- Wang XJ. Synaptic reverberation underlying mnemonic persistent activity. *Trends Neurosci* 24: 455–463, 2001.
- Wilcox KS, Buchhalter J, and Dichter MA. Properties of inhibitory and excitatory synapses between hippocampal neurons in very low density cultures. *Synapse* 18: 128–151, 1994.
- Williamson R and Wheal HV. The contribution of AMPA and NMDA receptors to graded bursting activity in the hippocampal CA1 region in an acute in vitro model of epilepsy. *Epilepsy Res* 12: 179–188, 1992.
- **Woodhall G, Gee CE, Robitaille R, and Lacaille JC.** Membrane potential and intracellular Ca²⁺ oscillations activated by mGluRs in hippocampal stratum oriens/alveus interneurons. *J Neurophysiol* 81: 371–382, 1999.
- Wyart C, Ybert C, Bourdieu L, Herr C, Prinz C, and Chatenay D. Constrained synaptic connectivity in functional mammalian neuronal networks grown on patterned surfaces. J Neurosci Methods 117: 123–131, 2002.
- Zucker RS. Calcium- and activity-dependent synaptic plasticity. *Curr Opin Neurobiol* 9: 305–313, 1999.

3380