Spontaneous Development of Synchronous Oscillatory Activity During Maturation of Cortical Networks In Vitro

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Opitz, Thoralf, Ana D. De Lima, and Thomas Voigt. Spontaneous development of synchronous oscillatory activity during maturation of cortical networks in vitro. J Neurophysiol 88: 2196–2206, 2002; 10.1152/jn.00316.2002. Recent studies have focused attention on mechanisms of spontaneous large-scale wavelike activity during early development of the neocortex. In this study, we describe and characterize synchronous neuronal activity that occurs in cultured cortical networks naturally without pharmacological intervention. The synchronous activity that can be detected by means of Fluo-3 fluorescence imaging starts to develop at the beginning of the second week in culture and eventually includes the entire neuronal population about 1 wk later. A synchronous increase of [Ca^{2+}]_i in the neuronal population is associated with a burst of action potentials riding on a long-lasting depolarization recorded in a single cell. It is suggested that this depolarization results directly from synaptic current, which was comprised of at least three different components mediated by AMPA, N-methyl-D-aspartate (NMDA), and GABA_\alpha receptor. We never observed a gradually depolarizing pacemaker potential and found no evidence for a change of excitability during inter-burst periods. However, we found evidence for a period of synaptic depression after bursts. Network excitability recovers gradually over seconds from this depression that can explain the episodic nature of spontaneous network activity. Using pharmacological manipulation to investigate the propagation of activity in the network, we show that synchronous activity depends on both glutamatergic and GABAergic neurotransmission during a brief period. Reversal potential of GABA_\alpha receptor-mediated current was found to be significantly more positive than resting membrane potential both at 1 and 2 wk in culture, suggesting depolarizing action of GABA. However, in cultures older than 2 wk, inhibition of GABA_\alpha receptors does not result in block of synchronous network activity but in modulation of burst width and frequency.

INTRODUCTION

Development of highly organized structures and connections in the CNS depends on both activity-independent and activity-dependent mechanisms. In recent years, the occurrence of spontaneous synchronous neuronal activity during defined periods of early CNS development has been described in a wide range of structures and species (for review see Ben-Ari 2001; Feller 1999; O’Donovan 1999). This rather primitive form of network activity is suggested to be an essential step in the formation of functional networks. It coincides with the depolarizing action of GABA and glycine (Ben-Ari 2001), which act as excitatory transmitters via chloride permeable receptor channels in young neurons (Cherubini et al. 1991; Leinekugel et al. 1995; Owens et al. 1996), although the activity may be carried by different neurotransmitters and modulators varying with structure and age. In the retina, excitation is initially provided by GABA and acetylcholine and later switches to glutamate (Wong 1999). Similarly, in the spinal cord, GABA and acetylcholine start out as the dominating transmitters in the generation of patterned spontaneous activity (Milner and Landmesser 1999) but are relieved later by glutamate (O’Donovan 1999). Thus in most structures, GABA or glycine contribute initially to the generation of spontaneous synchronous neuronal activity in developing networks.

Very recently, large-scale spontaneous correlated neuronal activity has been described in the immature rat neocortex (Garaschuk et al. 2000; Peinado 2000, 2001), which differs from oscillations in local cortical domains (Yuste et al. 1992) in the requirement of action potential firing. Although it is still a matter of debate if this activity is carried by glutamatergic transmission (Garaschuk et al. 2000) or dendro-dendritic gap junctions (Peinado 2001), there is no compelling evidence for the need of GABA_\alpha receptor activation in the neonatal cortex.

As in the neonatal cerebral cortex, synchronized rhythmic activity also develops spontaneously in neuronal networks formed by embryonic neurons in cell culture. It can be observed as slowly rhythmic synchronous bursting of larger numbers of neurons accompanied by Ca^{2+} transients (Maeda et al. 1995; Murphy et al. 1992; Robinson et al. 1993), thus resembling hallmark features of spontaneous correlated activity recorded in cortical slices. Interestingly, failure of neurons to participate in the spontaneously generated activity of a network resulted in their elimination from the network (Voigt et al. 1997). In most cases, however, spontaneous activity of cultured cortical neurons was described under conditions facilitating glutamatergic synaptic transmission, for instance by use of Mg^{2+}-free extracellular solution, which prevents observations on the role of GABA. In a recent study, we found evidence that the development of synchronous calcium oscillations in neocortical cultures depends on the presence of a certain type of GABAergic neuron originating in the cortical preplate (Voigt et al. 2001). This suggests the necessity of GABAergic transmission for the expression of early synchro-
rous network activity also in networks of neocortical neurons. Here we describe in detail the development of synchronized activity in unstimulated cultures of rat cerebral cortex in conditions as similar as possible to the standard culturing conditions. This activity appears comparable to large scale activity recorded from acute brain slice preparations in that it involves action potential firing and correlated increase of intracellular Ca²⁺ concentration ([Ca²⁺]). We provide evidence for synaptic origin of synchronized network activity and find a period of synaptic depression after bursts. Furthermore, we show that GABAergic transmission is necessary for synchronized activity in young cultures but rather modulating oscillatory network activity of older ones although GABA still acts depolarizing.

METHODS

Cell culture

Cortical cells were cultivated on poly-d-lysine coated coverslips in serum free N2 medium in the presence of a glial feeding layer (for detailed description of the method see de Lima and Voigt 1999). Neocortical cells from rats at embryonic day 16 were prepared by trypsin treatment followed by mechanical dissociation and were plated with a density of 200 cells/mm². Excessive proliferation of glial cells was prevented by adding cytosine arabinoside (Ara-C) to the cultures at a final concentration of 5 μM at the 4th day in vitro (DIV). One third of the medium volume was then changed after 24 h and no further medium change was performed for the rest of the cultivation period.

Fluo-3 videomicroscopy

Fifty micrograms of Fluo-3 pentacetoxy-methylester (Molecular Probes, purchased from MoBiTec, Goettingen, Germany) were dissolved in 45 μl DMSO. Of this stock, 15 μl were added to 3 ml N2 culture medium to load the cells with dye (final concentration: 4.9 μM Fluo-3, 0.5% DMSO). For recordings in N2 culture medium, one-half of the medium was saved for later use before Fluo-3 was added. After 1 h of dye loading, cultures were washed twice with DMEM, and the saved portion of the original medium was restored. Dishes were tightly sealed to prevent degassing during recording that would lead to major pH changes. In most cases, however, fluorescence recording was performed in HEPES-buffered artificial cerebrospinal fluid (aCSF). The ionic composition of this aCSF was (in mM): 140 NaCl, 5 KCl, 1.5 CaCl₂, 0.75 MgCl₂, 2.5 NaH₂PO₄, 20 d-glucose, and 15 Hepes/NaOH (pH 7.4) and resembled the main components of the N2 culture medium with the exception of the pH buffering system (HEPES vs. bicarbonate). After ≥30 min to allow deesterification of the dye, culture dishes were transferred to an inverted microscope (Axiovert S100 TV; Zeiss) equipped with a cooled charge coupled device camera (Princeton Instruments). Fields for recording were chosen randomly and sometimes marked with a diamond tool when repeated imaging or later identification was required. We used 20× or 40× objectives (Plan-NeoFluar; Zeiss), resulting in images covering an area of 380 × 380 μm² or 190 × 190 μm², respectively. Frames were recorded at different intervals with sample time adjusted to cell loading (typically 200–400 ms). Excitation wavelength was 470 ± 20 nm (Chroma Technology, Brattleboro, VT). A differential interference contrast (DIC) image of each field was also acquired for later cell identification. Frames were stored on computer and processed off-line using MetaMorph software (version 3.5; Universal Imaging, West Chester, PA). To recognize changes in the fluorescence, we subtracted from each frame the one preceding it, and shifted zero (i.e., no change) to a value representing a medium gray level (=2046 for a 12-bit image). This procedure yields images as shown in Fig. 1, where white depicts increased and black represents decreased fluorescence.

These differential images enabled us to identify regions of interest (we used circles of 7.5 μm diam placed on neuronal somata) that corresponded to active neurons as confirmed with the help of DIC images. Ca²⁺ signals from astrocytes, when present, appeared as slowly propagating waves along processes. They could be clearly distinguished from signals of neuronal origin and were excluded from analysis. Average gray values of regions of interest were calculated, and a change in [Ca²⁺], was considered significant when the absolute difference of gray values exceeded five times the SD of background noise measured in cell-free areas. Neuronal density was calculated using DIC images of the recorded fields and usually expressed as number of neurons per square millimeter. Neurons could be clearly identified and distinguished from nonneuronal cells in these images.

Drugs and drug application

All drugs were dissolved to 100×–1000× stocks, stored at −20°C, and diluted to final concentration just before application. We purchased tetrodotoxin (TTx) from Alomone Labs (ICS Clinical Service, Munich, Germany), 5-aminoethyl-3-hydroxyisoxazole hydrobromide (muscimol) and (−)-bicuculline methide (BMI) from RBI (RBI/Sigma, Deisenhofen, Germany), and d-2-amino-5-phosphono-pentanoic acid (APV) and 6-cyano-7-nitroquinoxaline-2,3-dione di-sodium (CNQX) from Tocris Cookson (Biotrend, Cologne, Germany). Drugs were applied either directly from the stocks in case of static bath recordings (some of the Fluo-3 fluorescence imaging experiments) or via a gravity-fed perfusion system. For stimulation of a smaller part of the network or a single cell, we used a multichannel device that was controlled by magnetic valves (ALA Scientific Instruments, New York, NY) and had an inner tip diameter of 250 μm.

Electrophysiological recordings

The feeder glia surrounding the neuronal culture was wiped off, and an acrylic ring was fixed to the culture dish with silicon grease, resulting in a chamber with a volume of 1–1.5 ml. This chamber was mounted to the stage of the inverted microscope and continuously superfused with aCSF at 1–2 ml/min. In some experiments (illustrated in Fig. 1, we used another aCSF to examine possible effects of HEPES versus bicarbonate buffering. This aCSF contained the following (in mM): 123 NaCl, 5 KCl, 1.5 CaCl₂, 0.75 MgCl₂, 1.25 NaH₂PO₄, 20 d-glucose, and 23 NaHCO₃ (pH 7.35 after adjusting with 95% O₂–5% CO₂). All electrophysiological recordings were performed at room temperature. Whole cell current-clamp and voltage-clamp recording was carried out with an Axoclamp-2B (Axon Instruments, Foster City, CA) or EPC-7 (HEKA Electronics, Darmstadt, Germany) amplifier. For most recordings of membrane potential or current, the patch pipette (tip resistance 3–5 MΩ) contained the following (in mM): 135 potassium glutonate (C₆H₁₂O₃K), 15 KCl, 2 MgCl₂, 10 HEPES, 0.2 EGTA, 2.5 Mg ATP, and 0.25 Na GTP (pH 7.2). For some voltage-clamp recording (Fig. 4C), another pipette filling solution was used (in mM): 120 cesium methanesulfonate (CH₃OSO₃Cs), 10 CsCl, 1 MgCl₂, 10 HEPES, 1 CaCl₂, 11 EGTA, 2.5 Mg ATP, and 0.25 Na GTP (pH 7.2). To monitor membrane potential in parallel to changes of [Ca²⁺], cultures were loaded with Fluo-3 AM as described above. The recording electrode was filled with the following solution (in mM): 135 potassium glutonate, 15 KCl, 2 MgCl₂, 10 HEPES, 2.5 Mg ATP, and 0.25 Na GTP (pH 7.2), supplemented with 10 μM Fluo-3 pentapotassium salt. While recording membrane potential continuously, sequences of images (20–60 frames at 1 Hz, exposure time 200–400 ms) were captured to disk, processed off-line as described above, and correlated with the electrophysiological signals. To determine the reversal potential of GABA<i>ₐ</i> receptor activated current (E<sub>GABAₐ</sub>) we employed the perfused patch method with gramicidin as the pore-forming agent because this prevents disturbance of intracellular [Ca²⁺] (Kyrozis and Reichling 1995). Recording electrodes were filled with the following solution (in
mM): 120 KCl, 1 CaCl₂, 11 EGTA, and 10 HEPES (pH 7.2), supplemented with 50 μg/ml gramicidin (Sigma). Although we did not use gramicidin-free solution in the electrode tip, no problems with gigaseal formation were encountered. After stabilization of series resistance (typically 30–50 MΩ within 10–20 min) we locally applied 30 μM muscimol. A voltage-ramp protocol (20 to 1100 mV within 800 s) was started when the current had reached steady state. After leak subtraction, I-V plots were constructed and reversal potential was computed by linear regression. Throughout this paper, means are ± SD.

RESULTS

To test if neocortical neuronal networks in vitro are capable to develop synchronous neuronal activity spontaneously without pharmacological intervention and under conditions present in the incubator, we recorded [Ca²⁺], in the original N2 culture medium. Because of the interference of phenol red in the N2 medium with Fluo-3 fluorescence measurements and the instability of the bicarbonate-based buffer system, however, we developed an aCSF that closely matched the ionic composition of the N2 medium used for culturing (see METHODS). Recordings were performed in 8-day-old cultures using both media subsequently in the same dish and yielded virtually the same results (Fig. 1), but the images in aCSF were crisper with no background bleaching, making the aCSF a suitable choice for the recordings described in this paper. In N2 medium, fluorescence image sequences revealed synchronous increase of [Ca²⁺], in 51 ± 10 (n = 6) neurons per 380 × 380-μm² field (Fig. 1A1). In aCSF, 55 ± 32 (n = 6) neurons per field showed synchronously elevated [Ca²⁺], (Fig. 1A2). Synchronous activity occurred at a fairly low frequency and was observed only once in each 1-min recording sequence (Fig. 1, B and C).

Rhythmic network activity could also be monitored with electrophysiological recording, which allowed observations over longer time periods at high time resolution. We employed this method to examine possible effects of the HEPES-buffered

FIG. 1. Cultured cortical neurons exhibit synchronous activity. A: differential images show increased (white) or decreased (black) [Ca²⁺], of cortical neurons that have been cultured for 8 days. Both image sequences were taken in the same dish, however, A₁ was recorded in the N2 culture medium in which the cells had been grown from the time of plating and A₂ was recorded in HEPES-buffered artificial cerebrospinal fluid (aCSF). Scale bar: 50 μm. B: traces of Ca²⁺ transients are shown derived from representative individual Fluo-3-AM loaded neurons recorded either in N2 culture medium (B₁) or aCSF (B₂). Traces span a time period of 1 min and are aligned to their accompanying histograms below. C: activity histograms of the recordings in N2 medium and aCSF are shown in C₁ and C₂, respectively. A neuron was considered “active” when fluorescence exceeded 5 times SD of background fluorescence in a cell-free area. In each field, a total of 60 neurons were active at least once during the 1-min recording period. D: whole cell current-clamp recording from a cortical neuron showing rhythmic burst activity. Extracellular medium was switched from HEPES- to bicarbonate-buffered aCSF at the time indicated by the arrowhead. Bottom traces: single bursts corresponding to the labels (a–c) above the top trace at expanded time scale. E: plot of inter-burst interval over time showing a transient decrease of burst frequency after the change from HEPES- to bicarbonate-buffered aCSF. Switch of the media is indicated by the arrowhead.
aCSF compared with bicarbonate buffering that is used in the culture media. At 13–16 DIV, neuronal networks showed robust rhythmic activity (Fig. 1D). On a switch from HEPES- to bicarbonate-buffered aCSF, network activity slowed down substantially and bursts appeared less coherent (Fig. 1D, a and b). A small (3–6 mV) transient hyperpolarization was found in most neurons (7 of 9). Input resistance did not change significantly (580 ± 188 vs. 555 ± 122 Ω in HEPES- and bicarbonate-buffered aCSF, respectively). However, network activity recovered to frequency and burst appearance observed in HEPES-buffered aCSF within 5–15 min (Fig. 1, D and E).

To explore the development of synchronous activity in our culture system, we recorded spontaneous [Ca2+]i changes in cultures of the same preparation at 6, 9, 12, and 15 days after plating for a total of four different preparations. In each dish we recorded fluorescence images at a frequency of 1 Hz from four to five fields using a 20× objective, resulting in a field size of 380 × 380 μm². For each field, the total number of neurons as well as the number of active neurons was determined. Analysis of these experiments is summarized in Fig. 2. At six DIV (median, 28%; range, 14–42%) of all neurons were spontaneously active and the same portion of neurons remained active in the presence of 10 μM CNQX and 20 μM BMI (median, 27%; range, 17–41%). During this spontaneous intrinsic activity we found the maximal fraction of simultaneously active neurons to be 7.5%. Thus for further analysis of the developmental time course of synchronous activity, a significant rise of [Ca2+]i in more than 10% of the neurons in the field was considered a synchronous event. Such synchronous activity was first detected at 9 DIV in 10 of 20 examined fields. In these fields, 41% (median; range, 10–82%) of all neurons participated in synchronous events. At 12 DIV we observed synchronous increases of [Ca2+]i in only one-half of the examined fields (11 of 20). However, the fraction of neurons that were involved in the synchronous events now averaged 91% (median; range, 37–100%) of all neurons in the fields. Fifteen days after plating, synchronous activity could be recorded in almost all examined fields (15 of 16), and in these fields, 94% (median; range, 81–100%) of the neurons took part in those events. Over the 9-day observation period, the neuronal density declined steadily from 1804 ± 59 neurons/mm² at 6 DIV to 626 ± 31 neurons/mm² at 15 DIV (n = 16 fields), a phenomenon usually observed in cortical cultures over this time period (Voigt et al. 1997). At the same time, however, the number of active neurons as assessed by Fluo-3 fluorescence imaging remained relatively stable with 512 ± 24 neurons/mm² at 6 DIV and 568 ± 33 neurons/mm² at 15 DIV (n = 16 fields). Thus similar to cultures examined in Mg²⁺-free solution (Voigt et al., 1997), after 2 wk in culture, the steady decrease of neuronal density and the increasing number of neurons participating in synchronous activity lead to a network with virtually all members being synchronously active.

To correlate the changes in [Ca2+]i, with the actual changes in membrane potential patch-clamp recordings were combined with Fluo-3 fluorescence imaging in a 12-day-old culture. These recordings showed that a rise of [Ca2+]i, coincided with a pronounced depolarization and firing of action potentials (Fig. 3A) in all neurons that were measured in this set of experiments (n = 7). Importantly, when [Ca2+]i increased synchronously in a larger number of neurons in the field under observation, the electrophysiological recording displayed a burst of action potentials riding on top of a long-lasting depolarization. The electrophysiological response roughly correlated with the number of adjacent cells that displayed a significant rise of [Ca2+]i. When only a small number of neurons were active (i.e., showed a [Ca2+]i increase) at a time, single or compound excitatory postsynaptic potentials (EPSPs) were observed that did not reach action potential threshold. When a larger number of cells were synchronously active, the neuron under investigation fired a burst of action potentials (Fig. 3A). Thus although only one neuron at a time could be observed, electrophysiological recordings allowed registration of synchronous network activity, which could be investigated for longer time periods than with Fluo-3 fluorescence imaging. The inter-burst interval and hence the time between two synchronous network events was relatively stable over long time periods. In the example shown in Fig. 3B, the inter-burst intervals lasted 10.9 ± 2.2 s (n = 43) with no significant trend to slow down or accelerate during the 40 min of measurement (Fig. 3C) and followed a Gaussian distribution (Fig. 3D). However, the interval between the synchronous events was variable among different cultures and was found to be in the range of 9–87 s (22 cultures from 4 different platings ana-
lyzed). There was no apparent correlation between age of the network and frequency of synchronous activity ($R^2 < 0.1$).

A detailed characterization of the synchronous burst activity was carried out in neurons after about 2 wk of culture (12–16 DIV). When the recording mode was switched from current clamp to voltage clamp near resting membrane potential (RMP), large inward currents were observed at the same phase and frequency as the bursts (Fig. 4A). These currents appeared to be barrages of synaptic currents (Fig. 4B) of which three different components could be isolated. At a holding potential of −70 mV (which is close to the estimated Cl$^-$ reversal potential), the inward current could be partially inhibited by the N-methyl-D-aspartate (NMDA) receptor antagonist d-APV (50 µM), leaving a component most probably driven by AMPA receptors (Fig. 4C). The effect of d-APV was especially pronounced with regard to the kinetics. A time constant of the current decay was derived by a single exponential fit but was found to vary substantially between different preparations. However, it was relatively constant for bursts recorded from the same cell, making quantitative comparisons of current decay before and after d-APV application feasible. For the neuron illustrated in Fig. 4C, for example, $\tau_{\text{decay}}$ of the burst-associated currents was calculated to be 649 ± 268 ms under control conditions, but only 356 ± 166 ms after application of 50 µM d-APV ($n = 7$ bursts). In another five cells, $\tau_{\text{decay}}$ of currents recorded in the presence of d-APV was found to be one-half to one-tenth of the control values. At a holding potential of 0 mV, large outward currents were observed that could be totally blocked by the GABA$\text{\textsubscript{A}}$ receptor antagonist BMI (20 µM) and were assumed to represent GABAergic inputs during burst activity (Fig. 4C).

The membrane potential during the bursts recorded in current-clamp measurements and the correspondent inward currents observed in voltage clamp shared approximately the same time course, suggesting the maintained synaptic current as the source of the long-lasting depolarization. To exclude that a voltage-dependent sodium or calcium "plateau" current is also activated, we applied short strongly hyperpolarizing current pulses during burst depolarizations, which should switch off a plateau current if present (Fig. 5A). This protocol did not affect the time course of the decay ($n = 5$ cells), suggesting that the depolarization results directly from synaptic current. A further indication that this suggestion is correct was that direct stimulation of the neuron by short (30–50 ms) depolarizing current pulses was able to trigger single action potentials but never induced bursts nor influenced the rhythm of the intrinsic bursts ($n = 10$ cells).

A common mechanism of rhythmic bursting is the appearance of pacemaker currents that lead to gradual depolarization of the neuron between discharges. However, in all neurons that were recorded in current-clamp mode during this study ($n = 36$ cells), the membrane potential remained relatively stable between bursts, and we never observed a pacemaker potential. Another mechanism of rhythmicity could employ a gradual increase of excitability after the bursts so that the neuron becomes increasingly prone for participation in a network activity. To explore this possibility we applied short (30–50 ms) depolarizing current pulses in regular intervals to bursting neurons (Fig. 5B), estimated the threshold potential to fire an action potential for every inter-burst current injection, and examined for correlation with time left until the next burst. The rationale of this protocol was to detect a decreased action potential threshold if excitability rises. However, in none of the five neurons studied with this protocol did we found evidence for a change of the threshold potential between two bursts (Fig. 5C). No strong correlation between temporal proximity to a burst and action potential threshold could be observed ($R^2 = 0.037$).

Another mechanism that has been suggested to render spontaneous activity an episodic nature is activity-dependent depression of network excitability. To test for a period after a burst during which the network is not able to trigger the next burst we recorded the membrane potential of a neuron and stimulated part of the network (about 2–5.5 mm away from the recording site in the direction of the aCSF flow to avoid direct effect on the recorded neuron) by brief pulses of aCSF containing elevated [K$^+$] ($1 s, 15$ mM KCl) at random times after each fifth spontaneous burst. Such a stimulus was able to...
induce neuronal activity that spread synaptically (tested by blocking with 10 μM CNQX, data not shown) through a large part of the network and could be recorded even >5 mm from the stimulation site as long-lasting depolarization with a burst of action potentials (Fig. 6A). Plotting the width of the evoked burst against the interval between stimulus and preceding spontaneous burst revealed a period during which the network could not be activated (Fig. 6B). The duration of this period of network depression appeared variable between different cells (3.7–9.3 s, n = 4 cells) and could not be determined accurately because after a number of stimuli (>10) the network did not respond reliably anymore.

To gain more insight into the involvement of activity-dependent depression in synchronous network activity, we analyzed the statistical relationship between burst width and inter-burst interval. If the network is depressed always to the same level after a burst and recovery is gradual, then the duration of the next burst should depend on the time of recovery. On the other hand, if the level of depression depends on the burst width, then there should be a correlation between burst duration and the following inter-burst interval. For this analysis we selected networks with slow and more irregular burst frequency (inter-burst intervals ≥1 min) for a larger range of values. Burst duration and inter-burst intervals were calculated from long-term membrane potential recordings of 6 neurons containing 27–52 bursts. Plotting the burst width against the interval preceding the burst revealed that the duration of a spontaneous burst became larger with longer recovery from the last synchronous network activity (Fig. 6C). On the contrary, there was no apparent dependence of the following inter-burst interval on the burst duration (Fig. 6D). Statistical analysis yielded a good correlation of the burst width with the preceding but not the following interval (Fig. 6E).

Voltage-clamp experiments suggested the presence of both glutamatergic and GABAergic neurotransmission during rhythmic burst activity (Fig. 4C). We investigated the influence of the two transmitter systems on synchronous network activity at various time points during the cultivation period. Fluo-3 fluorescence was recorded in fields of 190 × 190 μm² in a recording chamber perfused with aCSF for 10 min. During this time, transmitter receptor antagonists were applied for 2 min. Images were taken only every 3 s to prevent bleaching over the long recording period; however, this low sampling rate still...
allowed save detection of synchronous events that usually exceeded 5 s. When synchronous activity was first detected by Fluo-3 fluorescence imaging at the beginning of the second week of cultivation, it could be totally blocked by 10 μM CNQX, 50 μM D-APV, or by 20 μM BMI (Fig. 7A, left). Also, the Na⁺ channel blocker tetrodotoxin (1 μM) inhibited synchronous network activity (data not shown). It is noteworthy, however, that neither CNQX nor BMI were able to decrease the number of neurons in which a spontaneous increase of [Ca²⁺]ᵢ could be detected between synchronous events (see Fig. 2B: difference between the total number of active neurons and the number of synchronously active ones). This suggests that substantial spontaneous neuronal activity (as recorded by Fluo-3 imaging) does not depend on synaptic transmission at

FIG. 6. Origin of rhythmic bursts and long-lasting depolarization. A: spontaneous bursts of a neuron (16 DIV) followed by exogenous stimulation (arrowheads) with a local pulse of ACSF containing 15 mM KCl to a part of the network about 5.5 mm away from the recording site. Note the lack of evoked response at the short interval (bottom). B: summary of the experiment shown in A. Duration of evoked burst became longer with larger intervals between the spontaneously occurring burst and stimulus. At short intervals (~5 s or less) no burst could be evoked. C and D: data from a neuron of a 16-day-old culture illustrating the statistical relationship between the duration of spontaneous bursts and the preceding (C) or the following (D) inter-burst interval. For each set of data the correlation coefficient is given. E: summary of the analysis of correlation between the duration of spontaneous bursts and preceding or following inter-burst interval. The bar chart depicts mean ± SD for 6 cells.

FIG. 7. Influence of glutamatergic and GABAergic transmission on synchronous network activity. A: fluorescence traces each of 5 neurons recorded in the same field and demonstrating synchronous increase of [Ca²⁺]ᵢ. Application of various selective transmitter receptor antagonists is indicated by horizontal bars spanning the time of application (2 min). Left: recordings from a 12-day-old. Right: one from a 16-day-old culture. Note the different ΔF/F scale for the traces of the 2 ages. B: averaged Ca²⁺ transients recorded from 78 neurons in the same field at a higher time resolution than in A (1 vs. 0.33 Hz). Fluo-3 fluorescence was monitored first in the absence (control) and then in the presence (BMI) of 20 μM BMI. For comparison of the kinetics, control transient was also scaled to the amplitude of the BMI transient. C: single bursts of a cell in a 15-day-old culture recorded before (control) and after (BMI) extensive wash-in of 20 μM BMI. Dashed line marks the level of the neuron’s resting membrane potential (~61 mV). D: whole cell currents recorded from a neuron in a 19-day-old culture during rhythmic burst activity. Each of the traces shown represents an average of 5 consecutive burst events under the designated conditions: no antagonists (control), 20 μM BMI (BMI), 50 μM D-APV (APV), or 50 μM D-APV and 20 μM BMI (APV + BMI). The dashed line marks the level of 0 pA.
this early age. One week later, in cultures at the beginning of the third week of cultivation, the effects of the same three transmitter receptor antagonists differed from each other. While CNQX (10 μM) still blocked synchronous activity completely, d-APV (50 μM) significantly reduced but did not completely abolish the recorded Ca²⁺ transients (Fig. 7A, right). Blockade of GABAₐ receptors with 20 μM BMI did not prevent synchronous network activity. In fact an increase of the Ca²⁺ transients was observed in 11 of 12 fields (380 × 380 μm², 90 ± 30 neurons/field participated in synchronous events) when recorded at higher time resolution (1 Hz, Fig. 7B), whereas the frequency of events was reduced. For quantification the baseline-subtracted area under the Ca²⁺ transients was calculated and found to be increased 2.6-fold in BMI compared with control conditions (range: 1.3- to 4-fold). It should be noted that the above described effects of CNQX, d-APV, and BMI were stable for ≥30 min when examined with longer-lasting drug application (data not shown). Also, in electrophysiological recordings, we did not find notable effects on RMP of the transmitter receptor antagonists.

We employed electrophysiological recordings to seek possible causes of the larger Ca²⁺ transients in the presence of BMI. A first assumption was a possible developmental transition of GABA to become an inhibitory transmitter. We had demonstrated that E₉GABA in neurons of 8- to 12-day-old cultures is significantly more positive than the RMP (E₉GABA = −44.6 ± 1.4 mV, RMP = −54.8 ± 1.1 mV, n = 13; Voigt et al. 2001). Now we determined E₉GABA in neurons of 14- to 16-day-old cultures but found it still significantly more positive than the RMP measured in the same neurons (E₉GABA = −38.1 ± 4.7 mV, RMP = −55.7 ± 6 mV, n = 9). In current-clamp recordings from neurons in 15- to 19 day-old cultures (Fig. 7C), we observed that application of 20 μM BMI tended to decrease the number of spikes per burst from which one would rather predict a decrease of Ca²⁺ transients. However, another very robust effect of BMI on rhythmic bursts appeared to be a substantially prolonged depolarization. This finding could be verified in voltage-clamp experiments (Fig. 7D). Application of 20 μM BMI significantly prolonged the burst-associated inward current recorded at a holding potential of V₉H = −70 mV but clearly reduced its amplitude (241 ± 64 vs. 145 ± 18 pA, n = 5). Interestingly, calculation of charge transfer during burst activity revealed no significant difference between control condition and BMI treatment (535 ± 81 vs. 564 ± 236 nA s, n = 5). Assuming that in these older cultures virtually all neurons participate in synchronous network activity (Fig. 2), the number of inputs per burst should be relatively constant and should not increase by BMI application. A possible explanation for the prolonged depolarization is that the activity of neurons during rhythmic bursting becomes less coherent when GABAₐergic transmission is blocked. This view is supported by voltage-clamp measurements at V₉H = −70 mV in 50 μM d-APV (Fig. 7D), a condition when AMPA receptors are the major contributors to the recorded currents. Burst-associated currents appear very brief compared with recordings in the absence of d-APV. However, after application of 20 μM BMI, the currents became substantially prolonged with smaller amplitude (342 ± 35 vs. 114 ± 16 pA, n = 5) but equal charge transfer (58 ± 10 vs. 54 ± 6 nA s, n = 5; Fig. 7D).

**Discussion**

In this paper, we describe and characterize synchronous neuronal activity that occurs in cultured cortical networks naturally without pharmacological intervention. The synchronous activity that can be detected by means of Fluo-3 fluorescence imaging developed at the beginning of the second week in culture and included virtually the entire neuronal population about 1 wk later. Ca²⁺ signals arising from astrocytes could be observed only sporadically and in no temporal relation to the neuronal synchronous activity. It has been reported that astrocytes are required for oscillatory activity in cultured hippocampal neurons (Verderio et al. 1999). Although we cannot rule out that astrocytes influence synchronous neuronal activity in our cortical cultures, there are indications against their role as a permissive element. We found synchronous oscillatory activity in cultures that were treated with Ara-C at the second DIV. Such early termination of mitotic activity yields cortical networks that are virtually free of astroglia. Furthermore, preliminary experiments with a broad-spectrum glutamate transporter antagonist showed no profound influence on synchronous activity as reported by Verderio et al. (1999) for hippocampal cultures.

Rhythmic synchronized activity appears to be a prerequisite of developing neuronal networks (for review see Ben-Ari 2001; Feller 1999; O’Donovan 1999). It has been described in a number of different CNS regions of different species, and therefore, does not seem to depend on a special network architecture. Consequently synchronous activity has been observed even in cultures of dissociated neurons; however, in most cases after pharmacological intervention like prolonged inhibition of glutamatergic neurotransmission (Furshpan and Potter 1989), enhancement of synaptic transmission by means of Mg²⁺ removal from the aCSF (Higashi et al. 1999; Robinson et al. 1993; Streit et al. 2001), or block of inhibition (Misgeld et al. 1998; Murphy et al. 1992; Streit et al. 2001), but also in ventral horn neurons without pharmacological alterations (Latham et al. 2000b). In the present study, we found that synchronous activity in cultures older than 1 wk in vitro can be detected in aCSF closely resembling the ionic composition of the culture medium or even in N2 culturing medium itself. The most prominent difference between incubator conditions and recording conditions was the temperature, since the recordings were made at room temperature instead of 36°C. Because synaptic transmission is temperature dependent (Hardingham and Larkman 1998; Weight and Erulkar 1976), this could have resulted in an underestimation of network activity. However, experiments addressing this issue indicated that the frequency of synchronous events but not the number of participating neurons changes when temperature is raised from 20°C to 35°C (Opitz and Voigt, unpublished observation). Another difference to incubator conditions is the use of HEPES for pH buffering of the aCSF instead of bicarbonate. It has been reported that a switch from bicarbonate- to HEPES-buffered solutions or vice versa affects a number of functional aspects in neurons. A consistent finding is a change of RMP to more positive values when cells are bathed in HEPES-buffered solution (Church 1992; Cowan and Martin 1995; Gu et al. 2000). In agreement with this, we observed a small but transient hyperpolarization when the aCSF was switched from a HEPES- to a bicarbonate-buffered one. Changes of the neu-
ron’s input resistance was reported by some authors (Church 1992; Gu et al. 2000) but was not always observed (Cowan and Martin 1995; see also our data in here). The different pH buffers also affect Na\(^+\) channel kinetics (Gu et al. 2000) and possibly some Cl\(^-\) and K\(^+\) conductances (Cowan and Martin 1996; Stea and Nurse 1991). Taken together it appears that neurons show enhanced excitability on a switch from bicarbonate- to HEPES-buffered solutions that may also be of relevance for synchronous network activity described in this paper. However, our experiments showed only a transient effect of a pH buffer change on burst frequency. We hypothesize that the degree of recurrent excitation in cultured neocortical networks is high enough to provide strong inputs that override a possible drop of excitability. Alternatively, this drop may be only transient.

The correlation of synchronous increase of [Ca\(^{2+}\)]\(_i\) with firing bursts of action potentials superimposed on long-lasting depolarizations is in agreement with other reports on rhythmic activity in cortical cell cultures (Murphy et al. 1992; Robinson et al. 1993). Interestingly, there appear to be no major phenomenological differences between recordings with (this study; Murphy et al. 1992) or without Mg\(^{2+}\) (Robinson et al. 1993) in the extracellular solution: rhythmic burst activity was found to be mediated by synaptic excitation without the appearance of a gradually depolarizing pacemaker potential. If NMDA receptor channels are not a major source of Ca\(^{2+}\) influx during bursts (Robinson et al. 1993) and the generation and propagation of synchronous activity depend on the level of spontaneous presynaptic firing and the degree of connectivity of the network (Maeda et al. 1995), one would deduce that Mg\(^{2+}\)-free medium would simply facilitate burst generation and possibly prolong depolarization. Indeed we found an increase in burst frequency and number of action potentials per burst when the extracellular [Mg\(^{2+}\)] was changed from 2 to 0 mM (Opitz and Voigt, unpublished observation).

In the immature neocortex, several kinds of coordinated network activity have been described. A locally, rather restricted one, appears to be a biochemically mediated coactivation that involves gap junctional coupling and has been termed “neuronal domains” (Kandler and Katz 1998; Yuste et al. 1992). Only recently, waves of activity that involve large neuronal populations and can span long distances have been described in the neonatal cortex (Garaschuk et al. 2000; Peinado 2000, 2001). Peinado (2001) reported two types of wave-like neuronal activity: one that initiates in infragranular layers and spreads toward upper cortical layers requires glutamatergic transmission, and another type propagating along the longitudinal axis of the cortex that is not blocked by antagonists of glutamate receptors but relies on the presence of dendrodendritic gap junctional connections. In contrast, Garaschuk et al. (2000) found oscillatory Ca\(^{2+}\) waves traveling along the longitudinal axis of the cortex that are totally blocked by the AMPA receptor antagonist CNQX, suggesting spread of activity by glutamatergic neurotransmission. This pharmacology is in agreement with our findings in cultures of dissociated cortical neurons. It has been noted, however, that glutamate could be only involved in neuronal excitation but not in the mechanism of wave propagation per se (Peinado 2001). Indeed, compared with the situation in neuronal domains where activity is transmitted biochemically (Kandler and Katz 1998), long-range waves supposed to involve gap junctions require strong depolarization and action potential firing. Thus the fact that synchronous activity in cortical cultures can be blocked effectively by CNQX or TTx would still leave the possibility for gap junctional contribution. On the other hand, in whole cell voltage-clamp recordings, we found barrages of glutamatergic synaptic currents associated with rhythmic bursts but never attenuated unclamped spikes from supposedly coupled cells. This suggests that the synchronous activity in the network of cultured cortical neurons spreads via synaptic transmission.

Modeling studies have shown that it is sufficient for the expression of synchronous oscillatory network activity if there are recurrent, functionally excitatory connections rendering hyper-excitability to the network, and activity-dependent depression of network excitability (Tabak et al. 2000). Our cortical cultures appear to fulfill these two requirements. We found that the two major transmitters glutamate and GABA are both causing excitation. Furthermore, we demonstrated a period of network depression lasting seconds after a synchronous event (Fig. 6). Synchronous bursting has been characterized by a positive correlation between burst duration and the preceding interval (Staley et al. 1998; Streit et al. 2001; Tabak et al. 2001), suggesting that burst duration is controlled by parameters recovering from depression after the previous burst. The same kind of correlation could be observed in neocortical cultures (Fig. 6). Thus it is possible that the synchronous oscillatory activity is a feature of the network without the need for a specific pacemaker. Indeed we never observed a gradually depolarizing pacemaker potential and found no evidence for a change of excitability during inter-burst periods (Fig. 5). Instead bursts could be initiated by spontaneous activity. Very recently it was demonstrated that burst generation induced by disinhibition in spinal cord cultures is controlled by intrinsic spiking of some neurons (Darbon et al. 2002). Theoretical considerations showed that the firing patterns in a recurrent network are controlled largely by the fraction of endogenously active cells (Latham et al. 2000a). It was predicted and experimentally shown that networks with a larger fraction of spontaneously active neurons fire at low rates, whereas lowering the number of endogenously active cells led to bursting (Latham et al. 2000b). In line with this we registered a much higher number of neurons with spontaneous Ca\(^{2+}\) transients in young cultures (6–9 DIV) than in older ones (12–16 DIV) under conditions of blocked synaptic transmission.

In this study, GABAergic transmission was found to be involved in generation of synchronous activity in cultured neocortical neurons. During the first 3–4 days after their emergence, synchronous events could be blocked totally by application of the GABA\(_A\) receptor antagonist BMI, suggesting that GABA acts as an excitatory neurotransmitter in these young networks. Perforated-patch recording that avoid disturbance of intracellular chloride concentration and [Ca\(^{2+}\)] imaging during specific activation of GABA\(_A\) receptors showed that this is indeed feasible (Voigt et al. 2001). Depolarizing action of GABA and even excitation have been described in immature CNS structures and are based on a high intracellular [Cl\(^-\)] which results in a shift of E\(_{GABA}\) to more positive values (Cherubini et al. 1991; Leinekugel et al. 1995; Owens et al. 1996). Accordingly GABA\(_A\) receptor antagonists block rhythmic synchronous activity in neonatal hippocampus (Ben-Ari et al. 1989; Garaschuk et al. 1998), but they fail to do so in...
immature cerebral cortex (Garaschuk et al. 2000). There are, however, striking similarities in the action of BMI between cortical slices of newborn rats and our culture system. In cortical cultures older than 2 wk, synchronous neuronal activity persisted despite the presence of BMI, but we found that inhibition of GABAergic transmission decreases burst frequency, prolongs depolarization, and increases burst-associated Ca\(^{2+}\) transients (Fig. 7). A robust decrease of burst frequency and an increase of Ca\(^{2+}\) transients (but only in 5 of 9 cases) has been also described in immature neocortical slices. In consideration of the difference to the situation in neonatal hippocampus, it was suggested that maturation of excitatory glutamatergic transmission in the cortex occurs earlier than in hippocampus (Garaschuk et al. 2000). Our results in culture suggest that there is a period in cortical development when GABAergic neurotransmission is necessary for synchronous network activity (Fig. 7) and a specific type of preplate neuron was shown to be sufficient for the initiation of synchronous oscillatory network activity in culture (Voigt et al. 2001). Neurons of the preplate, however, mature far earlier than those of the cortical plate. Thus with ongoing development and synaptogenesis (de Lima et al. 1997), GABAergic cells will lose their decisive role to the now well-developed glutamatergic neurons. In the rat, this might happen before birth; a blockade of synchronous activity by GABA\(_A\) receptor antagonists could not be observed in neonatal slices (Garaschuk et al. 2000). Our findings regarding the prolonged depolarization and increased burst-associated Ca\(^{2+}\) transients during inhibition of GABA\(_A\)ergic transmission in older cortical cultures (Fig. 7) are also consistent with those of Murphy et al. (1992), who observed larger Ca\(^{2+}\) transients after picrotoxin-treatment in neocortical cultures older than 3 wk. However, the increase of Ca\(^{2+}\) transients in picrotoxin was explained with the block of inhibitory GABAergic transmission. Thus one would assume a larger excitatory input in BMI or picrotoxin. Voltage-clamp experiments revealed a significant longer inward current but unchanged charge transfer in the presence of BMI, suggesting a change of excitatory input in time but not size. Furthermore, we found that GABA\(_A\) transmission in immature rat CA3 hippocampal neurones in vitro. GABAergic transmission to the soma (Jaffe and Brown 1994).

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