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Population Bursts of Parvalbumin-Positive Interneurons Inhibit Spiking Pyramidal Cells in Spontaneously Active Cortical *in Vitro* Networks

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Abstract: Author present the interplay between different neuron types in the spontaneous electrical activity of low density cortical *in vitro* networks grown on MEA (multielectrode arrays) of glass neurochips. In 10% of the networks, the continuously spiking activity of some neurons was inhibited by synchronous bursts or superbursts of the majority of the other neurons. Immunohistochemical staining subsequent to MEA recordings suggest that the synchronously bursting neurons are parvalbumin-positive interneurons with abundant axonal ramifications. Blocking chemical synaptic transmission by Ca^{2+}-free medium revealed that the curbed spiking neurons are intrinsically active. It is assumed that these neurons are pyramidal cells which may be inhibited by groups of synchronously bursting interneurons. It is propose that the observed burst-induced inhibition is an important principle in the temporal organization of neuronal activity as well as in the restriction of excitation, and thus essential for information processing in the cerebral cortex.

Key words: Neurochip, MEA, cerebral cortex, cortical networks, spontaneous activity, inhibitory interaction, parvalbumin, interneurons.

1. Introduction

The jungle of the native cortex has remained pague, despite the efforts of *in vivo* recordings and circuit reconstruction techniques [1, 2]. In the mouse cortex, its very dense neuropil is mainly made of 4.1 km axons per mm^3 [3]. In addition to the density, the diversity of cortical neuron types and their complex connectedness hinders easy comprehension [4-6]. For these reasons, the native cortex is hardly accessible for detailed structural and electrophysiological analysis.

Self-organized *in vitro* networks, consisting of approximate. 100 interconnected neurons per mm^2, offer a significantly simpler structure than the native cortex, making them more accessible for microscopy and electrophysiological recording techniques. Since the initial recordings of the *in vitro* activity of neuronal networks more than 30 years ago, MEAs (microelectrode arrays) have found many research applications [7-10]. Nevertheless, their potential is not yet fully exploited. One challenge is the understanding of the "universals underlying formation and conservation of neural activity groups in networks" [11].

The networks exhibit autonomous spontaneous activity that is ubiquitous to mammalian brains. Understanding this activity is one of the most important challenges in neuroscience [12]. Spontaneous activity of *in vitro* networks has often been described as synchronized population bursts [13-15]. A burst is a sudden increase and subsequent decrease in firing frequencies [16]. Population bursts are characterized by the collective synchronized bursting of numerous neurons. The phenomenon of neuronal synchronization has been found *in vitro* as well as *in vivo* but its function is elusive [9]. The origins of spontaneous

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activity are believed to be intrinsically active neurons which probably belong to one of the two main neuron classes, pyramidal and Martinotti cells [17, 18]. Often, excitatory pyramidal cells are identified as the sources of regular spiking and bursting. This identification was based on current-injection experiments for example [19, 20].

The cellular composition of the *in vitro* networks of inhibitory and excitatory neurons is similar to that found in the intact cortex [21]. Different cortical neuron types can be distinguished by staining with specific markers [22-24]. One of the most suitable methods to mark specific interneuron types is the immunocytochemical staining of the calcium-binding protein parvalbumin. It is expressed in basket cells, which represent about 50% of all interneurons and also in chandelier cells [23-25]. Nevertheless, the heterogeneity of the interneuron family and the limitation of cell type-specific markers impair the analysis of their connectedness and their functions in cortical processing. It has been shown that interneurons play a central role in generating synchronous network activity and they can act as rhythm generators [26, 27]. The role of these rhythms for the neuronal communication is not clear.

In this study, cortical networks of low cellular density were grown on MEAs as a reductionist approach. It is focused at the interplay of basic cortical neuron types, i.e., pyramidal cells and interneurons in the spontaneous network activity. It is hypothesized that synchronized bursting represents the activity of specific cortical neuron types and that different patterns of neuronal activity as single-neuron activities, synchronized bursts or avalanches may coexist within a certain *in vitro* network based on the diversity of the cortical neurons forming the networks. To identify different cortical neuron types, author used immunocytochemical parvalbumin staining and the blockage of the chemical synaptic transmission by Ca^{2+}-free medium. It is report that synchronous bursts of parvalbumin-positive interneurons can interrupt the spike sequence of pyramidal cells.

2. Materials and Methods

Cortices were prepared from embryonic mice (E13-E16) following standard procedures [13]. Author used this period of neurogenesis to derive networks with different cellular compositions in order to scan for possible neuronal interactions. Pregnant NMRI-mice were obtained from Charles River Laboratory (Sulzfeld, Germany). All the procedures were approved by the local animal care committee and are in accordance with the European Council Directive of November 24, 1986 (86/609/EEC). In short: after enzymatic dissociation by papain cells were plated at densities of 1,000-3,000 cells/mm^2 on poly-D-lysine/laminin coated glass neurochips. The glass neurochip was developed at the Department of Biophysics, University of Rostock. It is of a small size with a side length of 16 mm and an integrated array of 52 platinum microelectrodes [28]. The chip's culture area was 20 mm^2. For comparison, cells were cultivated on coverslips (Menzel GmbH & Co. KG, Braunschweig, Germany).

Cultured cells were grown in DMEM (Dulbecco's modified Eagle's medium) with 10% horse serum (both from Biochrom AG, Berlin, Germany), 5.5 g/L glucose and 580 mg/L L-glutamine in a carbogen atmosphere (10% CO_2) at 37 °C for four weeks. Half the medium was replaced three times a week.

Electrophysiological recordings from the neurochip were performed with a home-made headstage coupled to an amplifier/filter (Plexon Inc., Dallas, TX, USA) and data acquisition software (Nex Technologies, Littleton, USA) [28]. The software allowed us to separate the activity of four units, i.e., four different signal sequences per electrode. The unit separation is based on signal shape. It is generally assumed that the signals within a sequence possess similar shapes when they are generated by the same cell or cellular structure. The analyzed time period of the recording was two hours per network. In some experiments, chemical synaptic transmission was blocked by a complete

exchange of the cell-culture DMEM with Ca^{2+}-free/Mg^{2+}-high DMEM supplemented with 5 mM $MgSO_4$ (Biochrom AG, Berlin, Germany). During recording, the networks were supplied with carbogen (10% CO_2 + synthetic air, Westfalen AG, Münster, Germany) and heated at 37 °C.

For morphological characterization, networks were fixed with paraformaldehyde, immunohistochemically labelled with primary antibodies against β-3 tubulin, parvalbumin, and neurofilament 200 kDa (Sigma Aldrich, St. Louis, MO, USA; diluted 1:500, 1:500, and 1:100, respectively), and subsequently stained with AlexaFluor 594 or 488-conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA; diluted 1:100). The purchased primary and secondary antibody solutions were diluted in PBS (phosphate buffered saline) containing 0.2% gelatine. Incubation times were 2 h for primary and 45 min for secondary antibodies. Nuclei were stained with Hoechst Bisbenzimide H33258 (Sigma Aldrich, St. Louis, MO, USA) diluted 1:1,000 in PBS for 15 min. For confocal laser-scanning microscopy a Leica TCS SP2 (true-confocal system spectral-detector, generation 2; Leica Microsystems GmbH, Wetzlar, Germany) microscope was used with two objectives (10 × 0.4 NA HCX PlanApo and 63 × 1.4 NA DIC Oil HCX Plan-Apo), a camera resolution of 1,024 × 1,024 and three lasers (blue solid-state diode 405 nm, Arg/Kr 488 nm, and HeNe 594 nm).

3. Results

Embryonic cortical cells self-organized into neuronal networks (Fig. 1). The first spontaneous electric activity could typically be observed after 5 (DIV) days *in vitro* in the neurochips. 50 out of 55 networks (approximate 90% as known from other experiments) developed electric activity. These 50 networks were investigated after 28 DIV.

The networks exhibited various burst and spike patterns in the spontaneous activity: 34 networks showed synchronous bursting activity as the predominant feature in parallel to sparse spiking activity

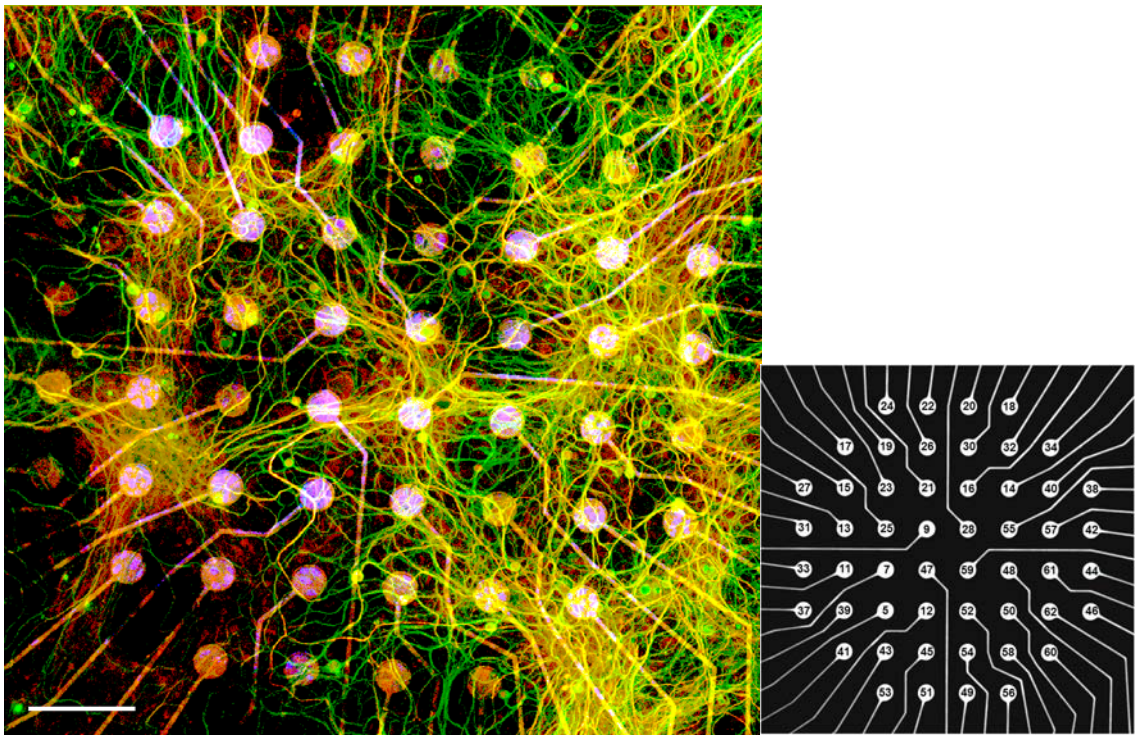

Fig. 1 Cortical neuronal network cultivated on the MEA of a glass neurochip.
Cells were paraformaldehyde fixed and immunohistochemically stained after 28DIV. Green: β-3 tubulin; red: neurofilament 200; bluish: electrodes; bar: 100 µm; right: electrode numbers of the MEA; electrode diameter: 35 µm.

of the same units (signal sequences at a specific electrode) in between the bursts. A number of other units in these networks exhibited purely spiking activity. Two of the networks showed exclusively super bursting activity, i.e., bursts with durations of several seconds, with very few spiking activity of some units in between the bursts. Four networks exclusively spiked at random. 10 networks drifted through different activity states of which the main ones were low random spiking, periodic bursting and super bursting, respectively (not shown).

A prominent kind of interaction between the synchronous bursting and continuously spiking activities co-existed in five of the 34 networks (10% of all 50 networks; two networks derived from embryonic cortices prepared at E13, three from E16) with predominantly synchronous bursts: an alternating activity of bursting and continuously spiking units. Fig. 2a shows action-potential trains of the network of Fig. 1. 32 of the 34 units of this network were synchronously active between 30 and 120 Hz while bursting and only two units showed continuous spiking activity between 4 and 12 Hz. Out of the five networks with co-existing synchronized bursting and continuously spiking activity, only in the network of Fig. 1 two interacting units were located at the same electrode (electrode 59). In all five networks, the continuously spiking activity rapidly ceased at the time of or a few milliseconds after the population bursts started and reappeared shortly after the burst ended.

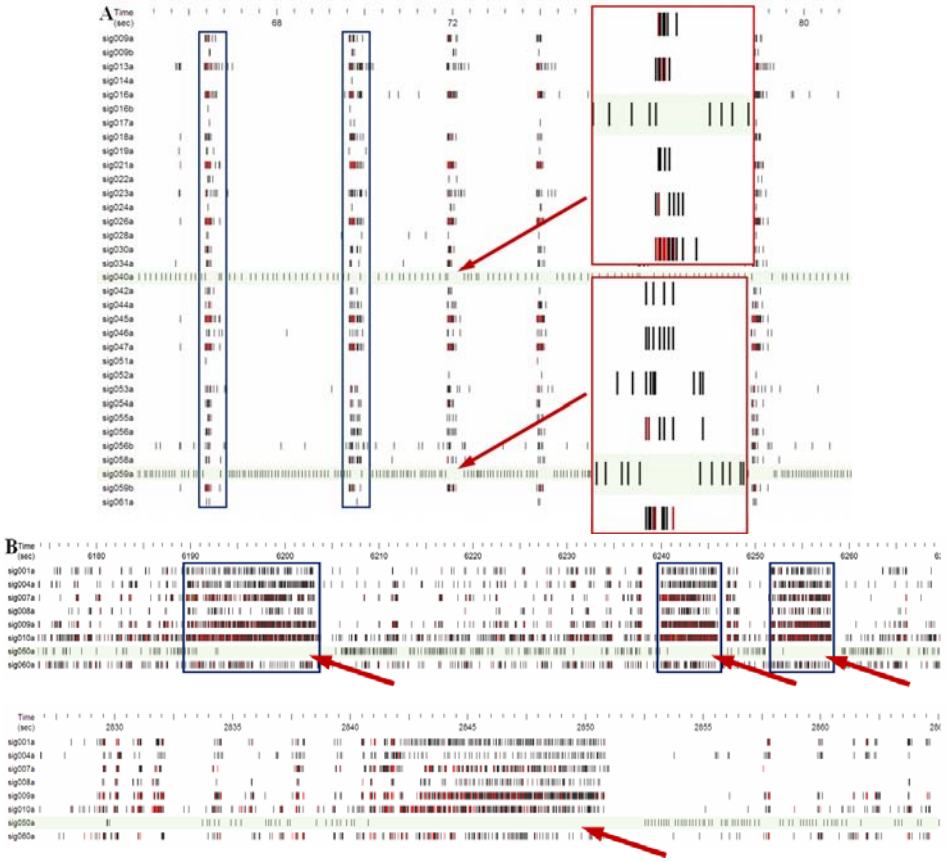

Fig. 2 Burst-induced inhibition.
(a) Spike and burst activity of the network in Fig. 1. over 15.6 seconds Each action potential is visualized by a timestamp bar. Each trace shows the activity of one detected unit. Synchronously bursting neurons (blue boxes) inhibit the activity of the spiking neurons (highlighted in green). Red boxes: zooms of the arrow-marked areas. The red arrows indicate the silent periods of the continuously spiking neurons during bursts. Their firing was restricted to the silent periods between the synchronous bursts; (b) Spike and burst activity of a network with superburst-induced inhibition. Superbursts inhibit the activity of the spiking neuron of unit 50a.

These observations led us to the assumption that the synchronously bursting neurons curbed the continuously spiking neurons, thereby imposing a rhythmic inhibition of their spiking activity (Fig. 2). The spiking activity was also curbed by superbursts (Fig. 2b).

To identify neurons subsequent to the electrophysiological recordings, authors analyzed 10 of the 50 networks by confocal laser scanning microscopy after immunohistochemical staining of parvalbumin and neurofilament 200. Two of the analyzed networks showed burst-induced inhibition. Morphologically, the parvalbumin-positive interneurons displayed abundant axonal ramifications (Fig. 3c). In these cells, parvalbumin was especially localized in the soma as well as in basal and several distal compartments of dendrites. Axons were stained only in rare cases.

The presence of axons or cell bodies of parvalbumin-positive neurons on the MEA electrodes was examined under the microscope independent of the electrophysiological recordings. Afterwards, the microscopic results were correlated to the electric activity registered at each electrode. A close vicinity of the axons or the cell bodies of parvalbumin-positive neurons to a MEA electrode implied a high probability of a pickup of the electric activity. Nevertheless, the analysis was only possible for MEA electrodes which were not covered by a dense axonal clutter. Five parvalbumin positive interneurons could be correlated to their firing patterns. All of them exhibited bursting activity. Two parvalbumin-positive neurons and the respective electrodes are shown in Fig. 3. The cell body of the neuron in Fig. 3a is located at the edge of electrode 37 which detected synchronous bursting activity and interburst-spiking activity. Some axons of the neuron in Fig. 3b crossed electrode 31 which detected superbursting activity.

It is known that Ca^{2+}-free media cause a block of the chemical synaptic transmission [1, 29]. Five networks were tested before and after an exchange of the cell-culture medium by Ca^{2+}-free medium. Before the medium change, two networks exhibited predominantly synchronous bursts, two networks spiked at random and one network (the network of Fig. 2a) showed two continuously spiking units with burst-induced inhibition. The exchange of the culture medium caused a nearly complete ceasing of the activity in the two synchronously bursting networks, whereas the activity of the randomly spiking networks showed only a slight decrease in the spike rates. In the network depicted in Fig. 2a, the burst-induced inhibition disappeared. The spike-rate of the continuously spiking neurons increased and even changed to short bursts. In contrast, the activity of most of the synchronously bursting neurons ceased immediately after the medium change. Only some of the bursting neurons exhibited a residual, randomized or sparsely spiking activity (Fig. 4). This suggests that an intrinsic, Ca^{2+}-independent activity must be attributed predominantly to the spiking neurons, that is, they are capable of autonomous spike generation in the absence of chemical synaptic transmission. The majority of the formerly bursting neurons were not intrinsically active. As a rule, the alterations in activity were reversible for all units. After the re-exchange of the Ca^{2+}-free medium by the cell-culture medium, the original firing patterns reappeared (not shown).

4. Discussion

As early as the middle of the last century Donald Hebb [30] was aware of the synchronous firing of pacemaker neurons in the cortex. Voigt et al. [27] postulated that a population of large GABAergic interneurons forms an interconnected network as a key element in the generation of synchronous, oscillatory network activity. GABAergic, parvalbumin-positive neurons form a gap junction-coupled network in the cerebral cortex and may generate strong, synchronous activity in the gamma-frequency range [29, 31, 32]. As seen in rat hippocampal slices, the synchronous activity of these neurons is enhanced when gap junctions are open and is reduced after a gap junction block [33].

Gamma-frequency oscillations of neuronal groups have been hypothesized to play a role in timing and coordinating the activity of other neurons as shown in theoretical models and verified in brain-slice experiments [34, 35]. Accordingly, inhibitory interneurons, firing in the gamma-frequency range, may act as a network clock constraining the firing of pyramidal neurons. Interactions between gamma-

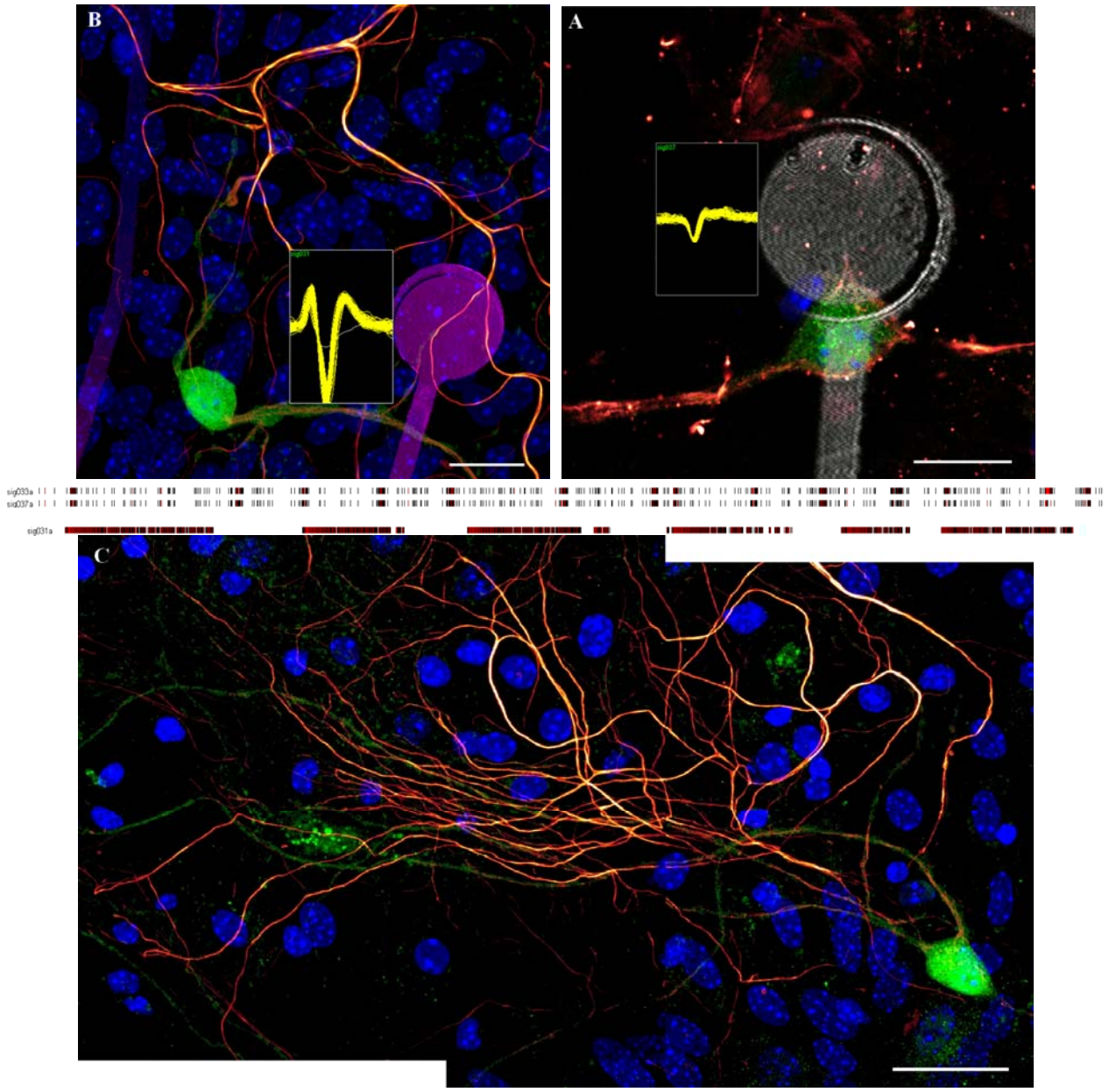

Fig. 3 Signal-to-cell correlation of parvalbumin-positive neurons.

(a) Neuron that exhibited bursting activity and some spiking activity at MEA electrode 37;

(b) Neuron of another network that exhibited superbursting activity at MEA electrode 31. Inserts: Superposed waveforms of detected action potentials. Sig37a and sig33a: synchronous bursting activity with interburst spiking activity as detected by MEA electrode 37 and its adjacent electrode 33 (not shown) pictured as timestamps over a period of 45 sec. Sig31a: superbursting activity with burst durations of up to 10 sec as detected by electrode 31 of another network over a period of 45 sec. Green: parvalbumin; red: neurofilament 200; blue: DNA; magenta: electrode; bars: 20 μm;

(c) Parvalbumin-positive interneuron with abundant axonal ramifications cultivated on a coverslip. The neuron is surrounded by glial cells (numerous blue nuclei) that form a glial carpet at the bottom of the coverslip. Bar: 30 μm.

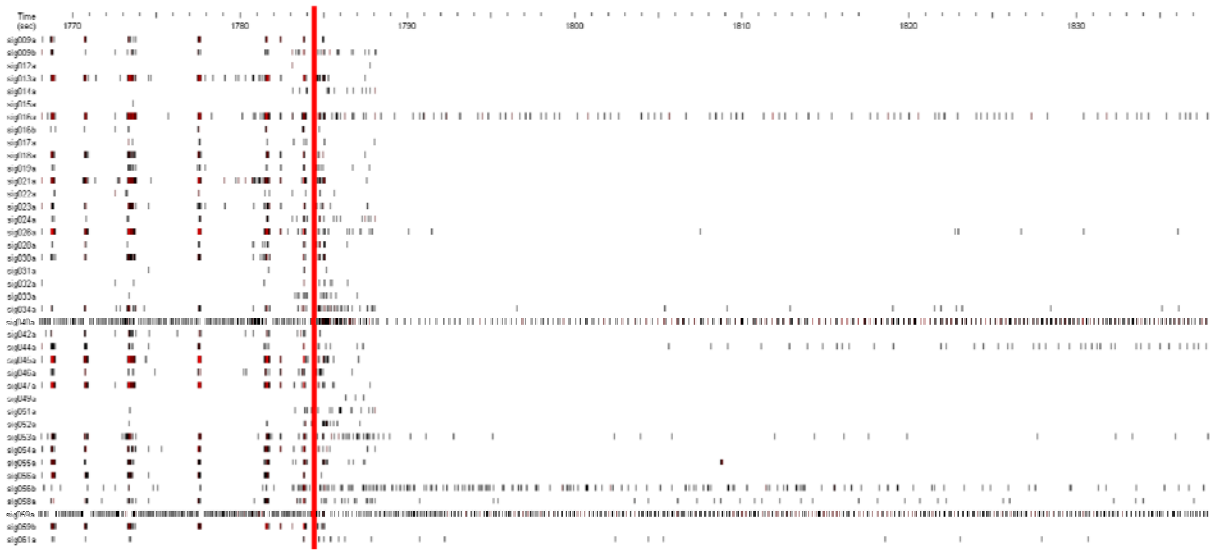

Fig. 4 Activity pattern of the network in Fig. 1 before (left of the red line) and after (right of the red line) blockage of chemical synaptic transmission.

Directly after the exchange of the cell-culture medium with Ca^{2+} free medium the bursting activity ceased and neurons changed their activity patterns. Spiking neurons changed from continuously-spiking to short-bursting activity whereas some of the synchronously bursting neurons changed their activity to low random spiking. The activity of most of the previously bursting neurons ceased completely.

frequency and theta-frequency rhythms engender the critical conditions underlying learning and memory formation [36].

The identified parvalbumin-positive interneurons were capable of burst or superburst generation (Figs. 3a and 3b). Their widespread axonal ramifications with many thin (< 0.5 µm) axonal branches are a possible reason why the electric activity of these cells may be detected with a higher probability than that of other neuron types, as possibly spiking pyramidal cells. This, in turn, may result in a biased detection of the electrical activity in the network in favour of bursting interneurons, even though they only account for a minority (10%-30%) of the neurons present in our networks. This percentage is also found in cortical *in vivo* circuits [3]. Because most of the electric activities in our system stemmed from interneurons, authors suggest that the ratio between interneurons and pyramidal cells is not a sufficient criterion for predicting the sources of electric activity within *in vitro* networks. Our result that parvalbumin positive neurons account for the synchronized burst activity is also supported by studies suggest that the generation of synchronized bursts depend on a sub-network composed of a small number of neurons [18, 27, 37].

The question remains: to which neuron class do the continuously spiking neurons belong-pyramidal cells fire relatively slowly and irregularly-as do the curbed cells in our cultures. In the rat, parvalbumin-positive neurons are mainly found in cortical layer 5 where the cell bodies of large pyramidal cells are located [38]. In cell cultures, GABAergic stellate-shaped neurons cover extensive areas and encircle the somata of non-GABAergic neurons in a basket-like fashion [39]. In our cultures, parvalbumin-positive interneurons show abundant axonal ramifications and form a widespread network. In the native cortex, large-scale GABAergic oscillatory networks can exert a powerful block for the discharge of pyramidal cells [29]. Specific types of interneurons may control the activity of pyramidal cells in the hippocampus and they are capable of timing and synchronizing the discharge of these cells [6]. Inhibitory interneurons are known to be very effective in determining the moment at which a pyramidal cell fires [26]. These facts are consistent with our conclusion that the curbed spiking neurons are

pyramidal cells in our *in vitro* networks. Nevertheless, there are other neuron types that can be inhibited in a similar fashion. For example, O-LM (oriens-lacunosum molecular) cells may be suppressed during ripple episodes in the hippocampus [40].

The Ca^{2+}-free medium caused the suppression of bursts in the synchronously bursting parvalbumin-positive interneurons. Their bursting activity was either turned into sparse spiking or ceased altogether after medium change. Evidently, Ca^{2+} was essential for the burst generation in these neurons. The medium change also induced a change in the firing frequencies and patterns in the continuously spiking neurons. Their activity increased, and no burst-induced inhibition was observed after medium change.

Fast-spiking parvalbumin-expressing basket cells show a high Ca^{2+} buffer capacity, which are known to express Ca^{2+}-permeable glutamate receptors and to generate Ca^{2+} synchronization [41]. This suggests their strong dependence on Ca^{2+} and may explain the inhibitory effect of Ca^{2+} depletion on these neurons.

Neurons showing action potentials in the absence of Ca^{2+}-mediated, chemical synaptic transmission are considered to be intrinsically active. Many such neurons have been found in neocortical slices. They belong to a subclass of pyramidal cells [18] and require persistent sodium currents for their activity [17]. In our *in vitro* networks, the continuously spiking neurons exhibit a strong intrinsic activity. This finding also supports our assumption that these neurons are pyramidal cells. The increase in their activity after Ca^{2+} depletion may have its origin in a disinhibition caused by the suppression of synchronized bursts. Their ability to burst, which became visible only after the medium exchange, was probably silenced by the synchronous bursts in the presence of Ca^{2+}.

To our knowledge, inhibitory interactions between different neuron types have not been described before in the spontaneous activity of cortical *in vitro* networks grown on MEA neurochips. Usually, the *in vitro*-network activities were described by synchronized population bursts spreading throughout the networks [13-15, 42]. This was also observed in the majority of our networks. Nevertheless, the spontaneous activity in mature cortical neuronal networks is more complex and not solely characterized by periodic synchronized bursts with some asynchronous components. In some of our 50 network, authors detected drifts between various activity states, pure spiking activity or burst-induced inhibition. The differences in the cellular composition between the networks may be causal for the differences in spontaneous activity patterns.

It is probable that not all cortical-cell types seeded into the chips contribute to the network activity seen in our experiments after four weeks *in vitro*. Possible reasons may include the electrical anergy or even apoptosis of neurons excluded from the synchronized *in vitro* activity [27]. Anergy or apoptosis may be induced either by a loss of connectivity and subsequent change in cellular architectonics caused by the randomization of neurons during preparation, the low density of the *in vitro* networks, the culture conditions or the lack of an adequate global electrical input. Naturally, a reduced number of active cell types will result in a reduced number of different activity patterns in comparison to that of the native cortex. Therefore, very high numbers of *in vitro* networks have to be examined in order to reveal many of the possible *in vivo* interactions.

5. Conclusions

In our experiments, population bursts which were generated by neurons synchronously bursting at 30-120 Hz (i.e., the gamma and fast oscillations-frequency bands in the brain) inhibited the activity of continuously spiking neurons firing between 4 and 12 Hz (i.e., the theta and beta-frequency bands). The synchronously bursting neurons acted as a network clock subdividing the spiking activity and thereby imposing a rhythm on the network activity. It is concluded that the synchronously bursting neurons

curb the spiking neurons (and not vice versa). The basis of our conclusion is that inhibition of the spiking neurons was observed a few milliseconds after the bursting neurons started to discharge and the spiking units' activity reappeared several milliseconds after the population burst ended.

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