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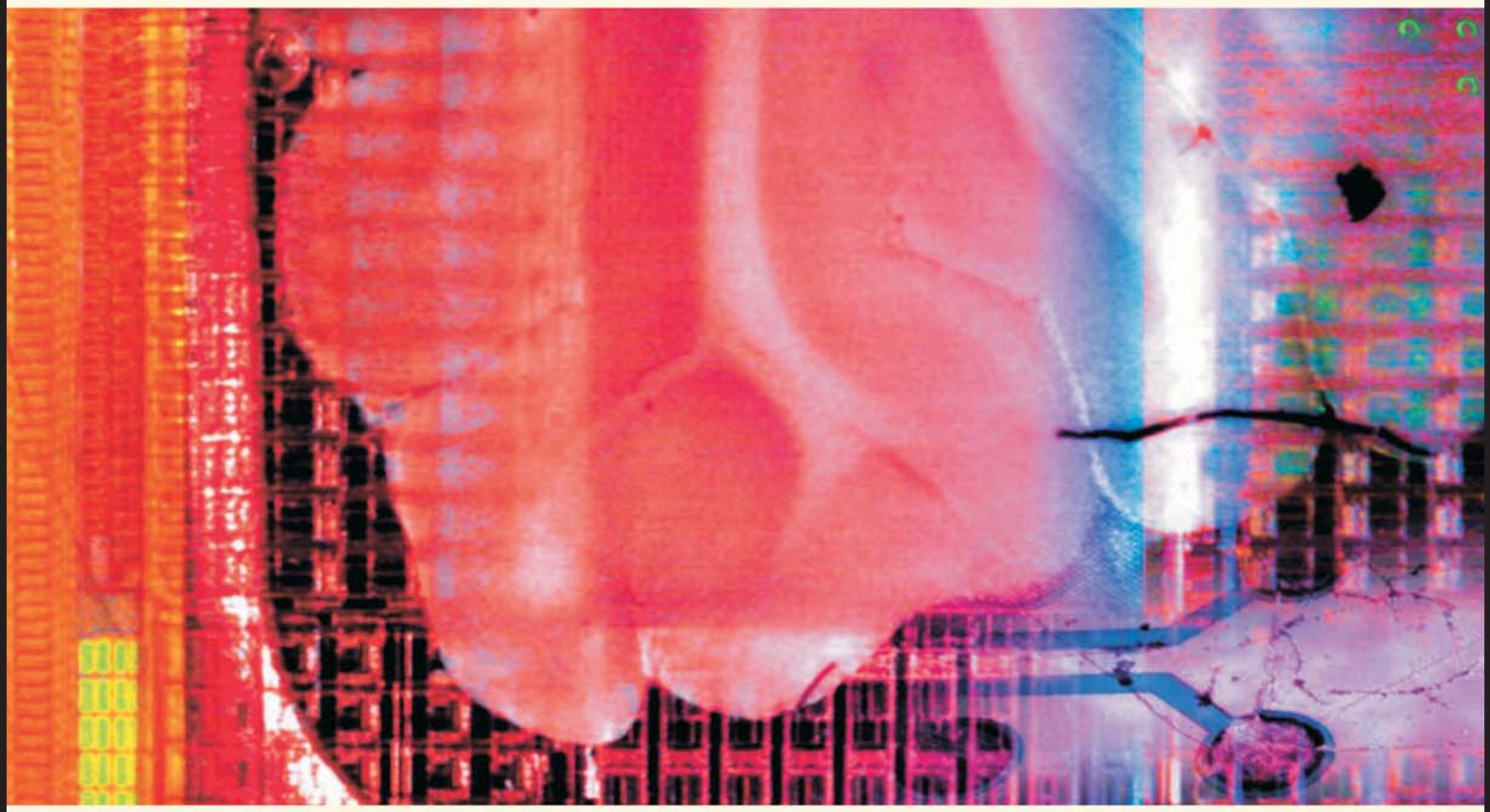

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Structure-function relations in generic neuronal networks

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Higher brain functions depend on the transmission and integration of information within cortical networks. We are interested in how far such activity dynamics are affected by the structural features of the underlying neuronal circuitry and vice versa. To investigate this relation, we performed simultaneous microelectrode array and patch-clamp recordings in dissociated cortical cultures. This work shows details on how neurons are embedded into the dynamics and circuitry of larger generic networks.

1 Methods

Cortical tissue obtained from neonatal wistar rats was dissociated and cultured on polyethylene imine-coated microelectrode arrays (MEA) following standard procedures [1,2] (Fig. 1). After maturation, networks had an average neuron density of about 2,000 cells per mm^2. Cultures were maintained in MEM supplemented with heat-inactivated horse serum (HS, 5%), L-glutamine (0.5 mM), and glucose (20 mM) in a humidified atmosphere at 37°C and 5% CO2. Medium was partially replaced twice per week.

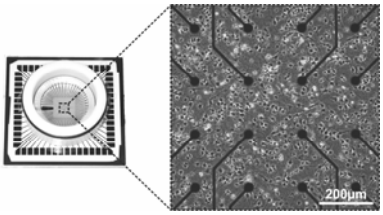

Fig. 1. Neuronal culture on MEA after 21 days in vitro.

Simultaneous MEA and paired patch-clamp recordings in whole-cell configuration were conducted outside the incubator at 35°C with a slow perfusion (100 µl/min^{-1}) of carbogenated (95% O2, 5% CO2) culture medium without HS supplement.

The neuron-to-neuron connectivity was analyzed based on subthreshold responses in a potential postsynaptic neuron to evoked action potentials (AP) in the potential pre-synaptic neuron. Array-wide burst detection was performed on all single MEA channel burst onset times. Participation in such network events was then detected in a window of max. 1 sec after network burst onset. Data was obtained from 21 cultures and 12 preparations. Analyses were performed with MATLAB using MEA-Tools [3] and FIND [4].

2 Results

Neuronal activity was almost exclusively comprised of synchronous network events. Individual cultures displayed subsets of bursts that had spatiotemporal patterns such as shorter bursts or phases of high-frequent bursting (Fig. 2).

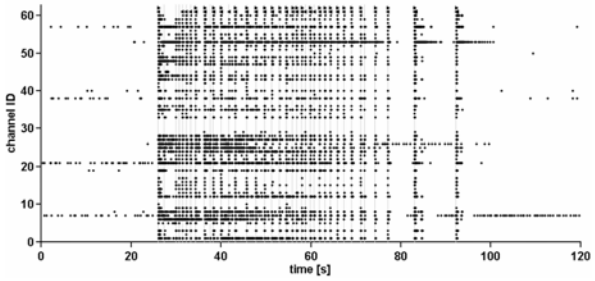

Fig. 2. Dot display of a characteristic network bursting phase in a simultaneous MEA and patch-clamp recording. (● APs on MEA channels (1-60), ◂ and ▸ APs on intracellular channels (61-62), | detected burst onsets).

The resting membrane potentials of the analyzed neurons (n=92) ranged from -46.2 mV to -78.3 mV (mean=-61.9 mV). A holding potential of -50 to -60 mV was applied to neurons with resting potential above -50 mV. The time constants ranged from 5.7 ms to 33.4 ms (mean=16.4 ms). Normal input-frequency properties were found for the analyzed neurons (data not shown).

Individual neuron pairs (n=10) were analyzed with respect to their embedding into network events (Fig. 3A). Activity was comprised of subthreshold responses to network input or single spikes and shorter bursts (Fig. 3B). No neurons were found to initiate network events. In general, activity was confined to network events only (mean=89%; mean MEA=81%; Fig. 3C). The first intracellular AP in network events had a mean delay of 43 ms (Fig. 3D). Mean rate of

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burst participation was about 56%, but we also found neurons that participated in all detected network events or that had a high failure rate of 91% (Fig. 3E).

We tested pairs of neurons (n=46) for their interconnectivity by detecting potential postsynaptic responses to evoked presynaptic AP (Fig. 4).

Of all neuron pairs, 54% were connected. 17% of the tested pairs had unidirectional excitatory connections, 9% unidirectional inhibitory ones. 28% had reciprocal connections. 46% of all pairs were not connected (Fig. 5).

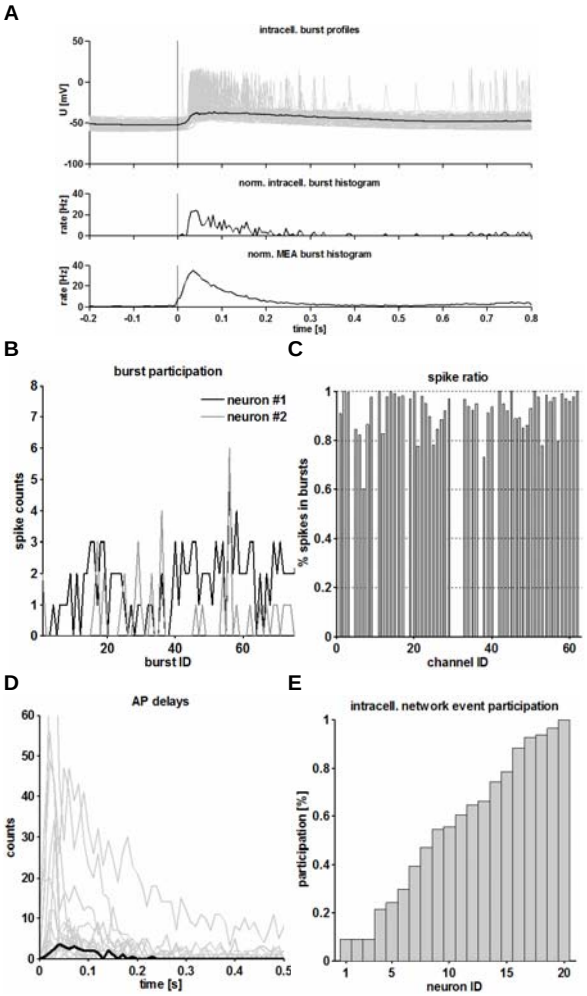

Fig. 3. A. Top: Burst profile of a patch-clamped neuron relative to network burst onset (black=mean). Mid: Normalized intracell. spike histogram. Bottom: Normalized extracellular rate profile. **B.** Participation of two neurons in network events. **C.** Ratio of spikes within network events for MEA (1-60) and two intracellular Channels (61-62). **D.** Delay distribution of intracellular APs relative to network event onset (black=median). **E.** Participation (i.e. min. 1 AP) in network events in intracellular recordings.

3 Conclusions

Our results show how neurons structurally and functionally integrate into larger generic networks. In particular, we found that individual neurons variably contribute to synchronized network events, which might explain the spatiotemporal patterns of bursting. Furthermore, we identified a high degree of connectivity on a range of lesser than 250 µm. Compared to the intact cortex, this indicates a high recurrent connectivity which might foster the synchronized bursting characteristic for such networks.

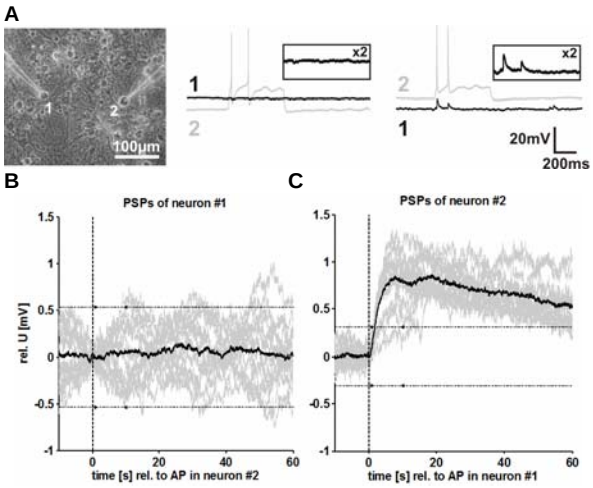

Fig. 4. A. Neuron-to-neuron connectivity of patched neuron pairs. Presynaptic APs were evoked by current stimulation (100-200 pA, 25-500 ms). After spike-triggered averaging, postsynaptic potentials (PSP) were detected based on a threshold criterion (5x SD of mean pre-AP signal amplitude; 1-11 ms after evoked AP). **B.** No PSP detection; neuron #2 did not synapse with neuron #1. **C.** PSP detection; neuron #1 had an excitatory connection with neuron #2.

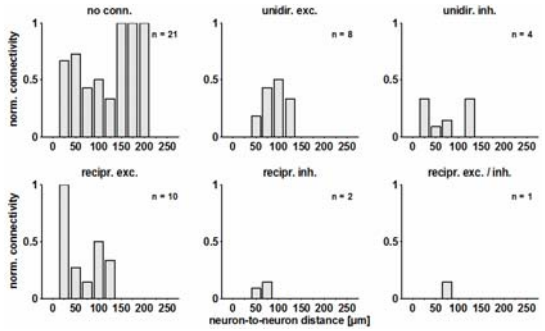

Fig. 5. Normalized unidirectional and reciprocal synaptic connections with respect to the linear distance between patched neuron pairs.

Acknowledgement

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Homeostatic regulation of activity in cortical networks

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In absence of complex architecture, central parameters of connectivity in neuronal networks are the size of dendrites and axons in conjunction with the spatial distribution of cell bodies. We study the effects of connectivity on activity dynamics in cultures of dissociated cortex by pharmacologically manipulating neuronal differentiation processes. This work shows that neurons developing under inhibited PKC activity display enhanced neurite outgrowth and decreased cell migration and pruning. Despite of changes in the connectivity statistics we found no profound changes in level and structure of activity of single neurons indicating homeostatic regulation mechanisms. Effects on network dynamics that are currently investigated however indicate functional consequences of altered connectivity statistics.

1 Background

Central parameters of connectivity in random neuronal networks are the spatial distribution of neurons in conjunction with the size and branching complexity of axonal and dendritic fields. We investigate these features and their functional consequences in cultures of dissociated cortical neurons. These generic random networks display a self-regulated maturation process characterized by cell migration, neurite outgrowth and pruning, similar to the critical period in developing cortex. Within this period we modulated neuronal connectivity by pharmacologically interfering with structural differentiation processes. Previous studies demonstrated that inhibition of the protein kinase C (PKC) prevented cell migration in granule cell cultures (Kobayashi, 1995), increased dendritic arborization of Purkinje cells in organotypic cerebellar slices (Metzger, 2001) and impaired pruning in climbing fibers of the cerebellum (Kano, 1995). Following these findings, cortical cell cultures were chronically treated with PKC inhibitors and morphological effects investigated and correlated to the activity dynamics in the developing networks.

2 Methods

Primary cell cultures were prepared from newborn rat cortices following a standard protocol adapted from S. Marom. Cells were plated at densities ranging between 1000-9000 cells per mm^2 onto PEI coated MEAs and coverslips. Cultures were incubated at 5% CO_2 and 37°C. One third of medium was replaced twice a week. PKC inhibitors (Goe6976 0.3 & 1 µM, K252a 0.15 µM; Sigma-Aldrich) were applied with the first medium exchange at DIV1. For morphological characterization, cultures were stained against microtubule-associated protein 2 (Abcam). Recordings were performed under culture conditions (MEA1600-BC system, MCS, Germany).

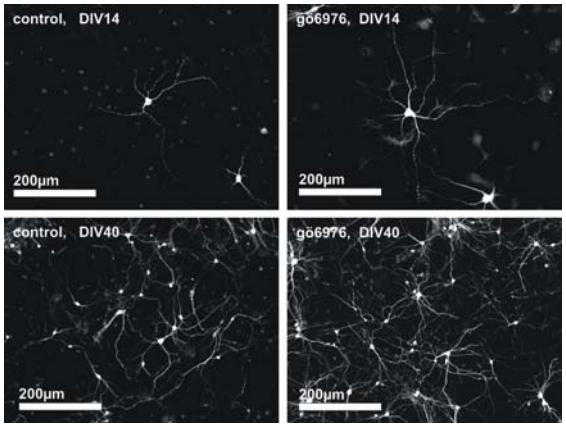

Fig. 1. Visualization of the dendritic network. Immunohistochemical staining against MAP2 expressed in dendrites and cell bodies. Chronic treatment with the PKC inhibitor Goe6976 resulted in enhanced neurite outgrowth in isolated neurons and in neurons embedded in a network

3 Results

Dendritic fields were characterized with a modified Scholl analysis (Scholl, 1953) describing the radial dendritic field density of neurons. To account for the overlap of dendritic fields of spatially non-uniformly distributed neurons and for the influence of neuronal neighborhood relations on neuritic differentiation, neurons were grouped into classes of comparable local neuron density. For this we determined the number of neighboring neurons within a range of

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100µm according to a measure for spatial clustering (Prodanov, 2007). Blocking PKC activity significantly enhanced maximal radial dendritic arborization up to +20% at intermediate (DIV14) and about +75% at later stages (DIV40) of development (Fig. 3). Typically, neurite density decreased in untreated cultures but slightly increased with PKC inhibition between DIV14 and DIV40. This suggests that blocking PKC activity prevents pruning and prolongs the neurite elongation phase. Neurons in sparse cultures with negligible dendrite field overlap were traced manually and likewise showed significantly enhanced dendritic field extents under PKC inhibition at DIV14 (Fig .2).

Although the overall neuron density revealed no differences across conditions (data not shown), control cultures had a higher proportion of neurons in regions with higher local neuron density. This indicates that cell migration was inhibited under PKC inhibition, resulting in the persistence of the homogenous initial spatial distribution of neurons. Comparable neuron distributions within one condition after DIV14 indicate that neuronal migration is largely accomplished in the initial phase of network maturation.

Electrophysiological recordings performed in the course of development revealed no significant changes in global level of activity, firing rates of single neurons or regularity of spiking. Changes in the size distribution of neuronal avalanches (Beggs, 2003) during development, however, followed the course predicted by a model of homeostatic network maturation and revealed an accelerated development of the network under PKC inhibition consistently with the prediction of a model for enhanced neurite outgrowth (Tetzlaff et al., Cosyne 2008).

4 Summary

Our work shows that pharmacological inhibition of PKC activity enhances neurite outgrowth and prevents cell migration and pruning in cortical cell cultures. This establishes modified connectivity statistics in the developing networks. The lack of profound changes in level and structure of activity of single neurons points towards homeostatic mechanisms that regulate these dynamics independent of neuronal connectivity statistics. Changes in the network dynamics that are currently investigated, however, indicate functional consequences of altered connectivity statistics.

Acknowledgements

We thank Christian Tetzlaff and Markus Butz for their helpful collaboration and discussion. Patrick Pauli is kindly acknowledged for technical assistance. Funding by EU (NEURO, # 012788) and the BMBF (01GQ0420)

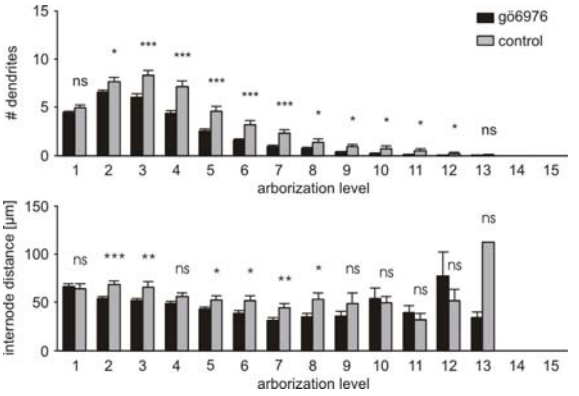

Fig. 2. Dendrite characteristics of single neurons. Dendrites of neurons in sparse cultures at DIV14 were traced manually and internode characteristics determined in dependence of the arborization level. Cultures chronically treated with the PKC inhibitor Goe6976 (1muM) revealed significantly more dendrites and slightly longer internode distances at intermediate arborization levels revealing enhanced bifurcation probabilities and neurite elongation. Bars depict mean values ±SEM. Significance levels were determined using student's t-test (5, 1, and 0.1 %)

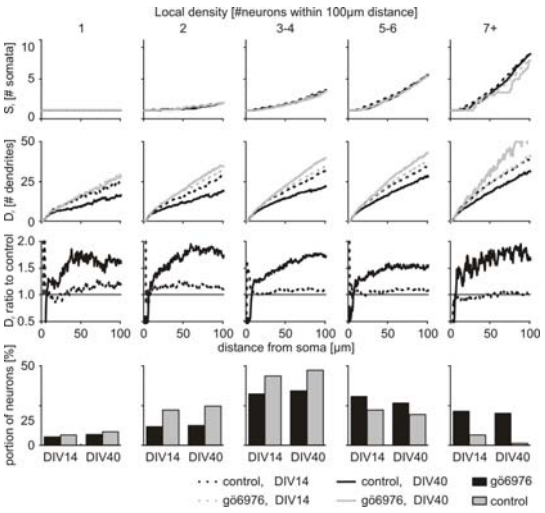

Fig. 3. Modified Scholl analysis. Neurons were grouped according to their local neuron density (columns) and the radial dendritic density (D_r) determined for these groups (2nd row). The ratio of D_r between treatment and control cultures emphasizes that PKC inhibition results in a higher radial dendrite density. This effect is markedly increased in older cultures. The number of somata within a certain distance from the soma (S_r) (1st row) shows that grouping according to local neuron density results in similar neuronal neighborhood relations in the comparison across pharmacological conditions. The bottom row depicts the percentage of neurons in a density class. Control cultures show a tendency towards denser classes indicating stronger clustering due to cell migration.

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Dynamics of Spontaneous Activity in Long-Term Measurements

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The focus of the experiments done using MEAs has traditionally been in repeated short-term measurements. However, in this study we utilize a specific setup to measure the activity of cell cultures over a longer term, in order to develop new methods for analysis of quantitative changes in the measurements and to provide insight on the dynamic shifts in baseline during long-term measurements.

1 Background and Aims

Microelectrode arrays (MEAs) have well established themselves as a dextrous tool for measuring the behaviour of networks consisting of electrically active cells. The focus of the experiments done using MEAs has traditionally been in repeated short-term measurements (see, e.g. [1]). However, in this study we utilize a specific setup to measure the activity of cell cultures over a longer term. The aim of this study with long-term measurements of cultured neurons and neuron-like cells is both to develop new methods for analysis of quantitative changes in the measurements and to provide insight on the dynamic shifts in baseline during long-term measurements. The former provides new points of comparison for such long-term measurements where the cells are stimulated, either electrically or chemically, while the latter is beneficial in extracting new information considering baseline changes over a longer period of time.

2 Methods

Cells were obtained from Wistar rat prefrontal cortex within 24 hr after birth (P0) and mechanically and enzymatically dissociated using standard cell biology protocol modified from [2]. 10^6 cells were plated on substrate-integrated MEAs coated with polyethylene imine. The cultures were maintained in an atmosphere of 37°C, 5% CO2 and 95% air in an incubator. Quarter of the medium was exchanged twice per week. The basic measurement system was an MCS microelectrode array (MEA) system. This system was modified to enable uninterrupted measurements over extended periods of time, ranging from days to weeks (with an additional perfusion system). The utilization of long recordings prevented the involuntary stimuli, and enabled the monitoring of changes in the baseline. Thereafter, the spontaneous activity was recorded for at least 12 hours, and the instances of activity were saved for offline analysis with Matlab®.

3 Results

During the long-term measurements conducted in this study, the baseline was monitored for spontaneous changes in temporal and spatial activation patterns. Recordings with the presented setup demonstrated that the neuronal cultures have increased activity for up to 10 minutes after mechanical disturbances. Furthermore, overnight experiments demonstrated changes in the baseline activity with emerging high activity states and turn-over of active channels. Our results demonstrate the need for more detailed questions on the interpretation of "control" or "baseline" analysis and their possible consequences on the interpretation of the experimental results.

4 Conclusion

We report that there are clear changes in the baseline over a long-term measurement. Thus, it becomes evident that when analyzing long-term measurements, the above-mentioned baseline changes must be taken into account. This requires further development of the analysis methodology for long-term cultures. The methods used here for analyzing the long-term measurement data provide new assets for future development of possible online analysis tools.

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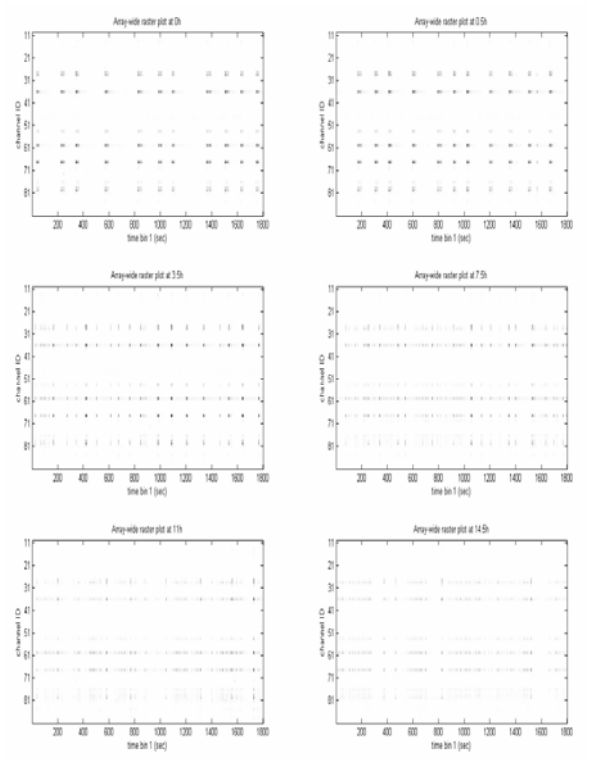

Fig. 1: Array-wide raster plots at different timepoints during one measurement.

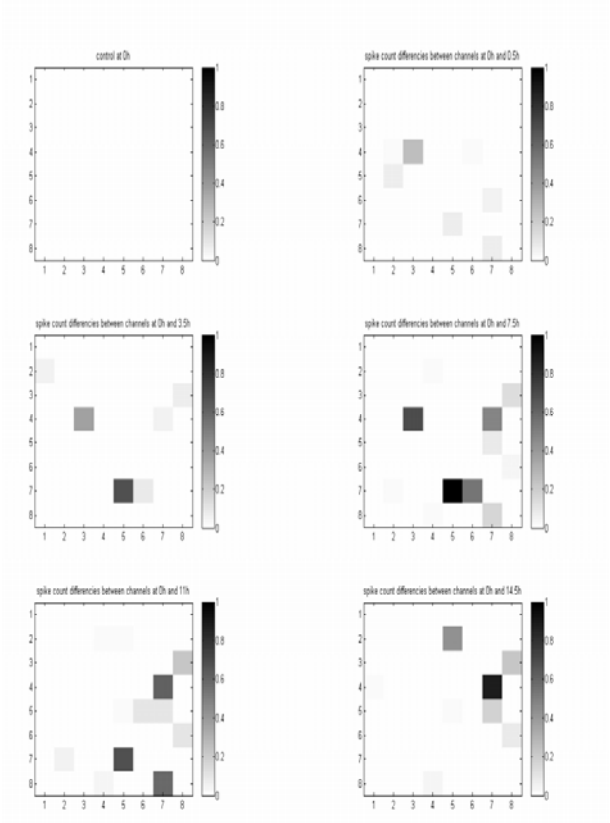

Fig. 2. Normalized spike count differences between channels at different timepoints during one measurement

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Network-state Dependent Stimulation Efficacy and Interaction with Bursting Activity in Neuronal Networks *in vitro*

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An understanding of the mechanisms that underlie information processing and storage in neuronal networks is largely missing. Dynamical interactions on a wide range of spatial and temporal scales are involved, but it is unclear how they arise and by which mechanisms they are governed. We are interested in how neuronal networks respond to incoming stimuli, which interactions arise and how this enables the processing and storage of information. We electrically stimulated cortical cell cultures grown on microelectrode arrays (MEAs) and characterized network responses during different activity states. Stimulation at fixed intervals without defined relation to an activity state ('random') resulted in decreasing response length with decreasing time since last synchronous network activity (*network burst*) and vice versa. Stimulation during spontaneously occurring, 20 - 60 second long periods of elevated network-wide bursting (*Superbursts)* yielded the longest responses. During the following refractory period, responses were shortest and stimulation even failed to induce spikes. Stimulation during burst-onsets ('intraburst') could either temporarily induce or inhibit spiking, but it could neither terminate nor shorten the burst progression. Stimulation during periods of no activity ('post-burst'), induced short- (≤ 50 – 100 ms post stimulus) and long-term (≥ 100 ms post stimulus) response components and increased response reproducibility. Different stimulation sites elicited distinguishable short-term response dynamics. Finally, low-frequency stimulation (0.1 – 0.4 Hz) at selected sites with any of the paradigms interfered with the bursting activity and reversibly suppressed Superbursts.

1 Introduction

Cortical cell cultures grown on MEAs give the possibility to study neuronal functions such as learning, memory, information processing and storage under controlled, easily accessible and experimentally manipulable conditions. Under the assumption that basic neuronal properties are maintained *in vitro*, various research groups in recent years were interested in revealing mechanisms that may underlie those functions. Shahaf *et al.* [1] for the first time showed learning and memory capabilities in *ex - vivo* networks by imprinting advanced stimulus/response associations. Eytan *et al.* [2] demonstrated stimulus-specific adaptation properties, possibly resulting from a selective gain control. Beggs & Plenz [3] investigated neuronal avalanches, i.e. network-wide propagating activity patterns, as a means for information transfer and storage. Bonifazi *et al.* [4] found fast and reliable stimulus decoding from *ensembles* of neurons along with a critical dependency on the network's balance between excitation and inhibition. Almost all those studies used electrical stimulation as network-input and the response was interpreted as the 'processed' output. Network responses are inherently variable, with large spatio-temporal fluctuations, reminiscent of chaotic systems [5]. Averaging over many trials and networks, or pooling different neurons [4] is necessary to reduce the noise in order to reliably conclude about the network's processing properties. In our work, we want to describe dynamic input/output relationships for neuronal networks *in vitro*. We aim at predictive models that allow a tight interaction with and control of neuronal activity by electrical stimulation.

Here, we show that electrical stimulation efficacy and spontaneous activity in neuronal networks *in vitro* are interdependent. The timing of stimulation with respect to oscillatory activity states determined over response lengths and stimulation could in turn interfere with spontaneous busting activity. Phase-coupled stimulation during always the same, predefined state of network activity had the advantage of a clearly defined and controlled stimulation environment. We obtained reproducible and stable responses, yielding a

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more unique description of network input/output relationships.

2 Materials & Methods

2.1 Culture preparation

Cells from prefrontal cortical tissue of neonatal wistar rats were cultured on polyethylene imine-coated MEAs. Cultures were maintained in MEM supplemented with heat-inactivated horse serum (5%), L-glutamine (0.5 mM), and glucose (20 mM) at 37º C and 5% CO_2. Medium was partially replaced twice per week.

2.2 Recording and stimulation

A Multi Channel Systems MEA1060BC amplifier, STG2008 stimulus generator and meabench [6] were used for recording and stimulation inside the incubator. The criteria for single-electrode bursts were a minimum of three spikes, inter spike intervals ≤ 75 ms, with maximal one interval ≤ 150 ms allowed. Network burst criteria were burst onsets within ≤ 150 ms on at least three electrodes. For Superburts, global activity-periods clearly above baseline were manually extracted and network bursts and their respective on- and offsets assigned. Monophasic negative voltage pulses, width 400 µs, amplitudes ≥ 0.4 Volt were used for stimulation. Three stimulation paradigms were applied: 'Random' stimulation at fixed intervals (typically 20 sec), without relation to network activity. For 'intraburst' stimulation, the firing rate of a preselected electrode was calculated online by single-trial rate estimation [7]. Whenever a given threshold was crossed, a stimulus was triggered (Fig. 1a). In 'post-burst' stimulation, whenever a minimum period without spikes on pre selected electrode(s) passed, a stimulus was triggered (Fig. 1b).

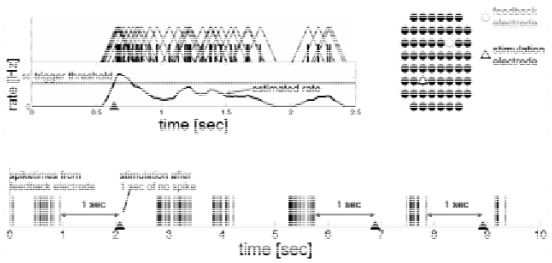

Fig. 1. a) left: Intraburst stimulation: online convolution of spike times and triangle kernel function yielded instantaneous rate estimate. Crossing of a threshold triggered a stimulus (marker). right: 8x8 MEA, with feedback (for phase-coupling) and stimulation electrode b) post-burst stimulation: feedback electrode's spike times were continuously monitored, after a fixed period without spikes, a stimulus was triggered (marker)

3 Results

3.1 Spontaneous activity modulates stimulation efficacy

Modulation by network bursts

After 'random' stimulation, responses were extracted according to the criteria for single-electrode bursts. Stimulation trials were sorted for increasing response length (in seconds). Pre-stimulus activity and response lengths were inversely correlated: high bursting activity immediately preceded short responses, periods of no or low bursting activity preceded long responses (Fig. 2a). Quantitative analysis, including examples from different networks shown in Fig. 2b. This effect was most clearly observed when stimulation elicited long- *and* short-term responses, as well as when the activity did not (yet) develop into Superbursts.

Modulation by Superbursts

During superbursting activity, stimulation elicited the longest responses. After the end of Superbursts, responses were shorter or stimulation even failed to elicit spikes (Fig. 3a). Response length distribution was broadest during Superbursts, distributions after Superbursts and during other activity phases were similar. Response reliability, however, was clearly diminished just after Superbursts.

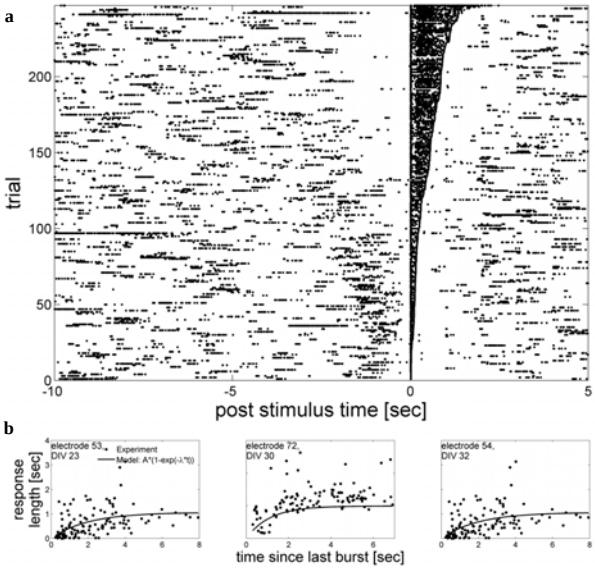

Fig. 2. a) raster plot, electrode 54, -10 to 5 seconds post stimulus, applied at electrodes 16 and 43. Trials sorted for response length. Note the increasing response length with decreasing pre-stimulus activity. b) Response length vs. time since last burst before stimulation for three electrodes, different networks. Experimental data (dots) and model A*(1-exp(-λ*t)) describe a saturating response length.

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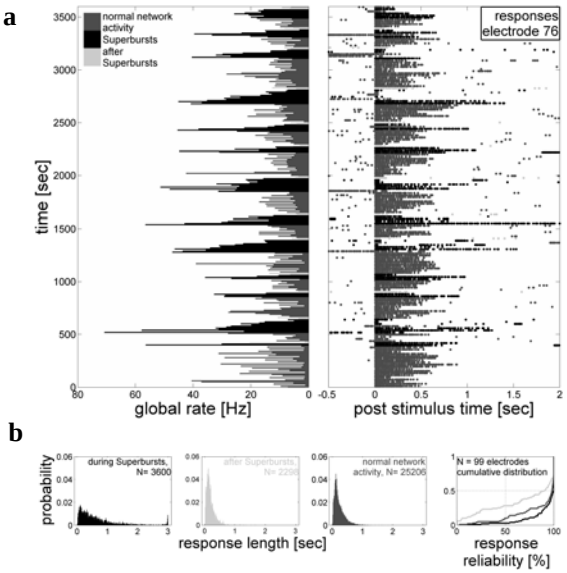

Fig. 3. a) left: 1-hr. global firing-rate, bin width 10 sec. right: responses from electrode 76 to 180 stimulation trials, applied at electrode 57, during the same period. Repeated increase and reset of response length, correlated with spontaneous Superbursts. b) response length distributions (99 electrodes). right: response reliability = (#responses)/(#stimuli)

3.2 Phase-coupled stimulation

Intraburst stimulation

Stimulation was triggered within 50 -100 ms after burst onset on the feedback electrode. The impact of stimulation on the burst dynamics was analyzed by comparing stimulation trials with control trials in which the trigger criterion was fulfilled, but no stimulus was applied. Stimulation could induce additional spikes on top of the ongoing burst or it could temporarily inhibit spiking during the burst (Fig. 4). Short-term responses were induced even when the ongoing spiking was inhibited. In general, intraburst stimulation could not terminate or curtail burst progression. A reduction of average burst length was observed only when stimulation suppressed superbursting activity (section 3.3).

Post-burst stimulation

Stimulation induced clearly separable short-and long-term response components. Stimulation at different sites elicited distinguishable delays in the reliable short-term response. Comparable dynamics could be reproduced 24 hrs. later, showing the stability of this effect (Fig. 5a).

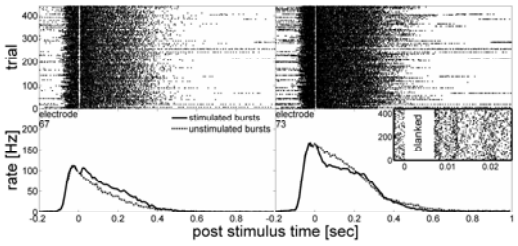

Fig. 4. top: raster plots, electrodes 67 (left) and 73 (right), 450 trials intraburst stimulation at electrode 64. Bursts started before stimulation. Burst progression did not markedly change during the experiment. inset: 7 ms blanking period that minimized artifacts, followed by short-term response spikes. bottom: corresponding PSTHs, including those for control trials (dashed). Additional spikes were induced (left), or spiking was temporarily inhibited (right).

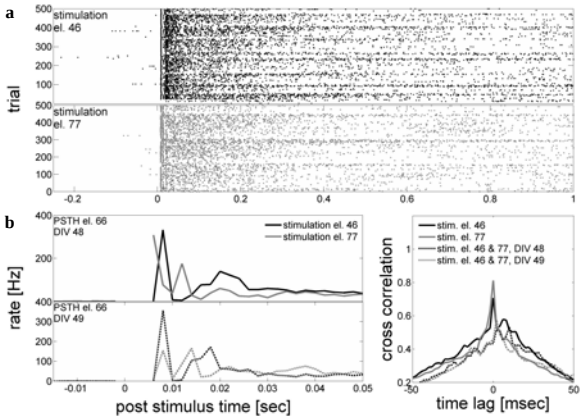

Fig. 5. a) raster plots, electrode 66, 500 post-burst stimulation trials at electrodes 46 (black) and 77 (gray). bottom left: corresponding PSTHs for -15 to 50 ms post stimulus at DIV 48 (solid lines) and for DIV 49 (dashed lines). Response-delays changed with different input sites, but were comparable for identical input sites at successive days (compare different colors vs. solid and dashed lines of same color). **b)** PSTH cross-correlation, normalized and averaged over 11 electrodes.

Different input sites (stimulation electrodes) could be distinguished by means of their induced short-term response. In cross-correlations between PSTHs, the normalized and averaged peak was higher and closer to 0-lag for same input sites compared to different input sites.

3.3 Interfering with Superbursting activity

Oscillatory superbursting activity with distinctive global rate fluctuations was terminated either directly or a few minutes after stimulation start. After the end of stimulation, Superbursts could reappear (Fig. 6). This effect was independent of the stimulation paradigm. Furthermore, effective stimulation electrodes consistently had early network burst onsets during spontaneous activity (Fig. 6 insets). The probability for very long network bursts was decreased during Superburst suppression. This means, from an initial bursting state, including abnormally long bursts during Superbursts, the network's activity was shifted to more regular burst firing without episodic Superburst patterns.

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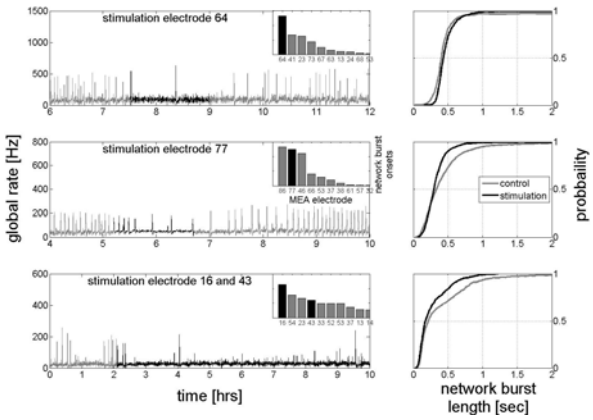

Fig. 6. left: global rate profiles, three different networks. 'intraburst' (top), 'post-burst' (middle) and 'random' (bottom) stimulation periods in black, control in gray. bottom: Superbursts reappeared during stimulation. insets: ranking of electrodes starting network bursts, used for stimulation electrode selection. right: cumulative distributions of network burst length. Probability for very long bursts decreased during stimulation.

4 Conclusions

Electrical stimulation efficacy and spontaneous bursting activity in neuronal networks *in vitro* are interdependent. Effective electrical stimulation does not merely need defined stimulus properties, e.g. amplitude, width or type, but also a consideration of the phase when the stimulus is applied. Phase-coupled stimulation places the stimulus into the same context of network activity, resulting in more reproducible and predictive responses. Furthermore, it enables a detailed assessment of the network's transfer function with reduced influence of interfering effects. Input/output relationships are defined for separate activity states, rather than for a grand average of different states. This would aid in the understanding how information imposed by electrical input patterns is processed by neuronal networks *in vitro* and physiologically realistic systems in general.

Acknowledgements

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High-Resolution CMOS-based Microelectrode Array and its Application to Acute Slice Preparations

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Recordings have been performed using a CMOS-based microelectrode array (MEA) featuring 11'016 metal electrodes and 126 channels, each of which comprises recording and stimulation electronics for extracellular, bidirectional communication with electrogenic cells. The important features of the device include (i) high spatial resolution at (sub)cellular level with 3'150 electrodes per mm^2 (diameter: 7 µm, pitch: 18 µm), (ii) a reconfigurable routing of the electrodes to the 126 channels, and (iii) low noise levels. Signals from neurons in an acute cerebellar slice preparation are presented.

1 Introduction

In many experiments with living tissue, it is desirable to adapt the locations of the recording sites with regard to the biological structure. One possibility is to simultaneously record from all electrodes of a high-density array [1, 2], which results in rather high noise levels due to the limited pixel area available for circuitry implementation. Instead of scanning the entire electrode array, the approach presented here provides a reconfigurable routing for an almost arbitrary set of electrodes to the readout channels, and enables low-noise signal amplification and filtering with the front-end circuitry placed outside the array.

Acute sagittal cerebellar slices have been used to assess the performance of the device. In these preparations, predominantly Purkinje cells are spontaneously active. The Purkinje cells are effectively disconnected from each other as the parallel fibers have been cut. Their electrical fields hence can be considered to be independent, which facilitates spike sorting and eases waveform interpretations.

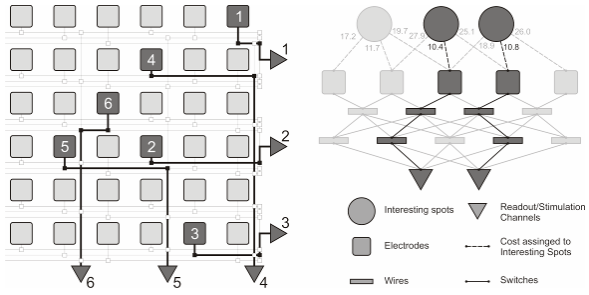

Fig. 1. Array wiring and routing scheme.

2 Results

The recorded signals are amplified and filtered in three stages. The gain is programmable via the digital interface from 0 to 80 dB.

The first stage provides a first-order high-pass-filter featuring a low cut-off frequency of 0.3 Hz. ADCs sample the signals at 20 kHz and 8 bit resolution. The stimulation capability is provided through an 8-bit flash DAC and stimulation buffers [3].

The equivalent-input noise of only the amplifiers is 2.4 μV_{rms} (1 Hz-100 kHz), it is 3.9 μV_{rms} for bare Pt-electrodes in physiological saline, and 3.0 μV_{rms} for dendritic Pt-black electrodes in physiological saline (+0.5 LSB quantization noise). The flexibility in electrode selection is attained by use of an analog switch matrix integrated underneath the electrode array. The switch matrix consists of 13k SRAM cells and analog switches to define the routing from the electrodes to the amplifiers as illustrated in Fig 1. The measurement setup, that includes an FPGA for data preprocessing, such as digital filtering and data compression/reduction is depicted in Fig 2. The fabricated chip is shown in Fig 3.

Measurements of spike activity in acute brain slices have been performed. The data shown in Fig 4 were obtained from a cerebellar slice of a Long-Evans rat shown in Fig 5. The preparation was performed at room temperature, as described in [4]. In the figure, six exemplary recordings from adjacent electrodes are shown: action potentials of single cells are visible on several neighboring electrodes.

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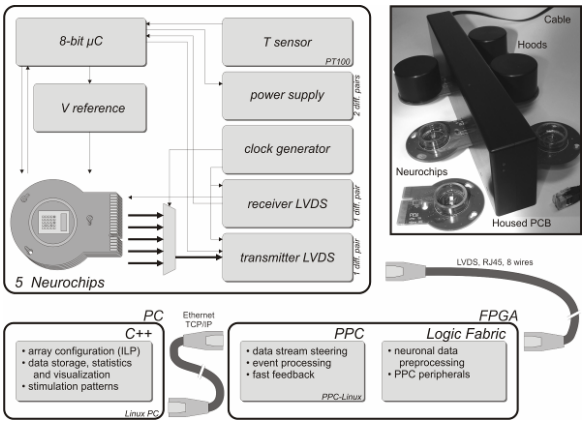

Fig. 2. Measurement setup.

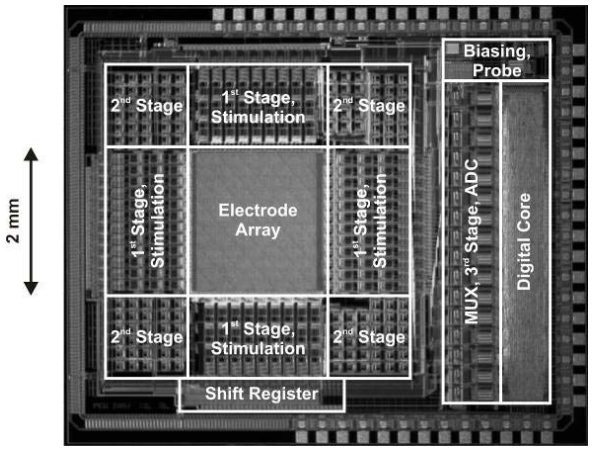

Fig. 3. Micrograph of the CMOS HD-MEA.

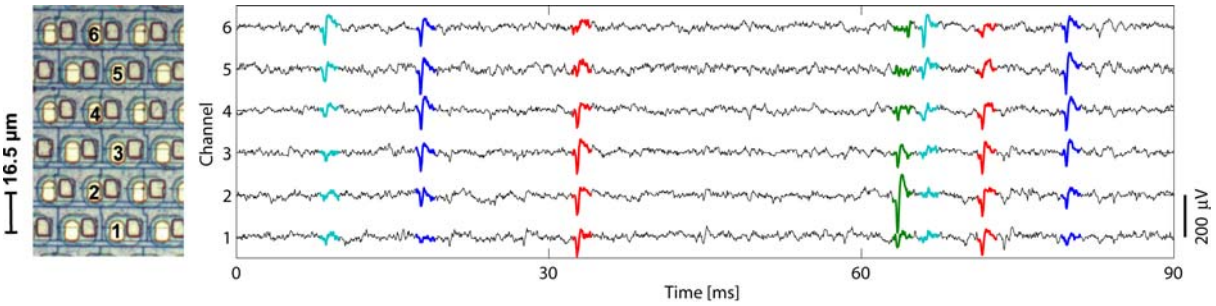

Fig.4. Recordings from an acute sagittal cerebellar slice of a Long-Evans rat.

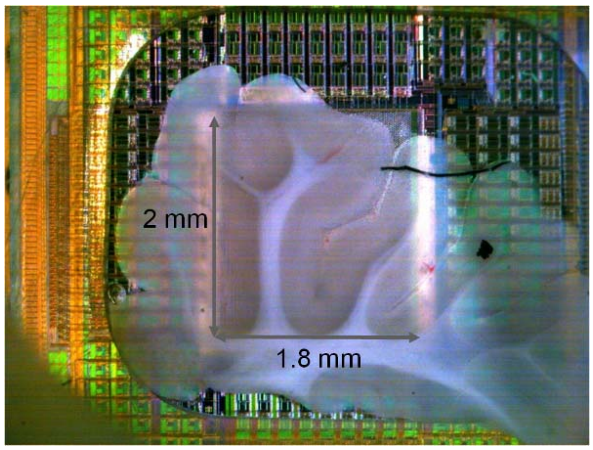

Fig. 5. HD-MEA with an acute cerebellar

3 Conclusion

We developed a CMOS-based microelectrode array with 11'016 metal electrodes and 126 on-chip channels. Each channel includes recording and stimulation electronics for bidirectional communication with electrogenic cells, such as neurons or cardiomyocytes. The new features of this chip include the possibility to perform high spatial resolution recordings with 3'150 electrodes per mm^2 at cellular or subcellular resolution (electrode diameter 7 µm, pitch 18 µm), a great flexibility in routing the 126 channels to the 11'016 recording sites, and low noise levels, since the large front-end amplifiers have been placed outside the electrode array.

Spike sorting allows for identifying single action potentials in the multi-unit recordings obtained from the acute cerebellar slices.

Acknowledgement

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Towards Interruptionless Experiments on MEAs

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Microelectrode array (MEA) systems are widely used to explore neuronal cells and their interactions in culture. They are suitable for long term cellular experiments and sustainable over months or even years. However, routine maintenance of the cultures introduces artificial disturbances, a human-made rhythmicity, to the cultures. We propose to circumvent or reduce the artificial break-points in culturing by providing a continuous maintenance system for the neurons cultured on MEAs.

1 Introduction

Neurons grown on MEAs offer a practical platform to explore small-scale biological networks. These networks maintain the essential features of brain-bound networks, but are recordable over weeks and can be influenced by extensive pharmacological or electrical manipulations [1]. For example, isolated cortical neurons plated on a MEA form monosynaptic connections within first week after plating, and a mature bursting network within four weeks [2, 3]. However, the state of the network, and thereby its response to electrical stimuli or pharmacological treatments, is strongly affected by any systematic changes. Typical changes are mechanical disturbances, i.e. moving of the MEA, heat induction through the measurement setup, CO_2 level fluctuations, and the exchange of the neuronal culture medium. We present here an approach towards interruption-free recordings of the neuronal culture.

2 Methods

Recordable cultures should reside in the MEA amplifier inside a dry incubator. Generally, the amplifier produces heat that damages the culture, if the incubator is operated at 37°C. One could lower the temperature of the incubator to compensate for the heat production of the amplifier. However, this is not feasible, since it limits other usage of the incubator. Therefore, we propose to couple the amplifier with a cooling body with water circulation which transfers the excess heat from the amplifier to a radiator outside of the incubator. With the above described setup the recording time of the cultures is limited only by the medium exchange. An additional coupling with a continuous perfusion system consisting of a peristaltic perfusion pump provides the culture continuous replacement of culture medium, and, in principle, enables temporally unlimited recordings. Most perfusion systems can be set to slow (20 to 100 µl/h) rates, thus providing the same daily volumes as bolus exchange thus circumventing the need to recalibrate the osmolarity and pH of the culture medium. In order to minimize the risk of over-floating the MEA, there must be a bias favouring medium suction over pumping of the fresh medium. This causes drying of the MEA and therefore the micro-climate of the MEA should be humidified. The incubator itself must be kept dry to protect the electrical equipment and to avoid contamination.

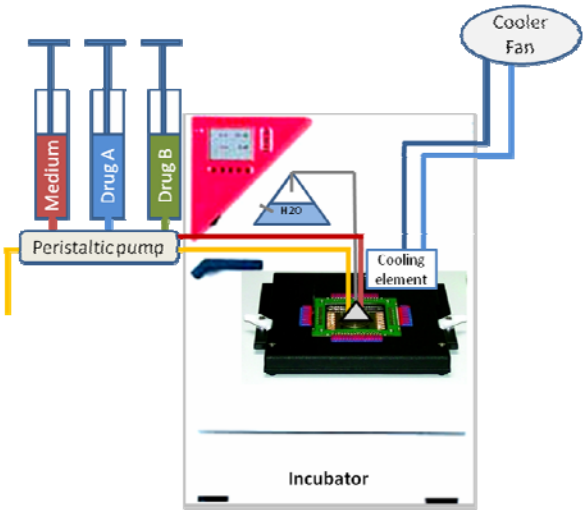

Fig. 1. Schematic illustration of the incubator embedded recording system, including water-based cooling system, air humidification for the culture and perfusion system with peristaltic syringe pump

3 Results and conclusion

We present here a system that enables recording and stimulation without involuntary stimulation to the neuronal culture. Furthermore, such a system could include slow infusion of pharmacological agents at rates resembling the situation *in vivo*. Our approach

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enables the design of long term experiments with repetitive stimulations or adaptations beyond artificial experiment endpoints.

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Guided Adhesion and Outgrowth of a Constrained Network on Tailormade Surfaces

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Planar micro-electrode arrays (MEAs) have been used for some time to investigate the activity dynamics and response of neural networks to electrical or chemical stimuli. Constructing defined connectivity statistics under conditions that allow the maturation of the neuronal networks has thus far not been achieved. Such networks would be useful to understand the influence of the composition, connectivity statistics, and plasticity in a neuronal network on the dynamics of electrical activity. The key issue is the control over microscale, spatial and long-term stability of cell adhesion and neurite outgrowth by physicochemical surface modifications under cell culture conditions that allow long-term maintenance of these networks. We report a simple way to tailor the surface-chemistry using a photochemical approach, leading to the covalent attachment of polymer layers to glass and MEA surfaces. We examined the biocompatibility of polymer layers with different properties, such as hydrophilicity and charge, and their potential to promote or inhibit neuronal cell adhesion. Finally, patterns with cell attractive domains were obtained using pin printing or µ-contact printing (µ-CP) of polymer solutions. These patterns were able to guide neuronal cells in a constrained network and were stable over several weeks in a serum containing cell culture medium.

1 Biocompatibility of covalently attached polymer layers

To tailor the surface chemistry of glass slides and MEAs we used a surface-immobilized benzophenone derivative, which can be attached to any alkyl group photo-chemically (Figure 1) [1]. These surfaces were modified by attaching hydrophilic (poly(dimethyl acrylamide) - PDMAAm), hydrophobic (poly(methyl methacrylate) - PMMA, polystyrene - PS), ultra-hydrophobic (fluoropolymer - FP), and positively charged (poly(ethylene imine) - PEI) polymers (for

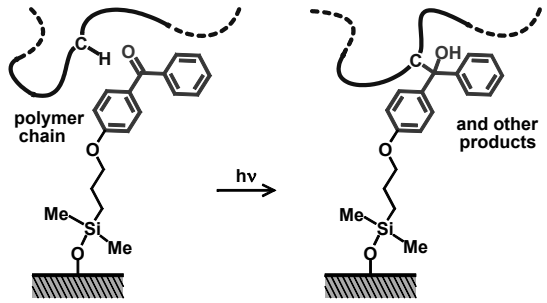

Fig. 1. Schematic illustration of the photo-chemical attachment of polymers via benzophenone units. Image taken from [1].

detailed polymer synthesis see [2]). Adhesion and outgrowth of neurons were studied in a serum containing medium. Dissociated neuronal cells from neonatal rat cortex (for details see [3]) were seeded onto the polymer coatings and incubated for several weeks. The number of adherent cells was reduced and the neuronal network morphology was degraded on the FP, PMMA, and PS coatings (indicated by clustering of cell somata). PEI (Figure 2, A) supported the initial adhesion of neurons forming a uniform neuronal network within three days in vitro. In contrast, neurons never adhered on PDMAAm (Figure 2, B).

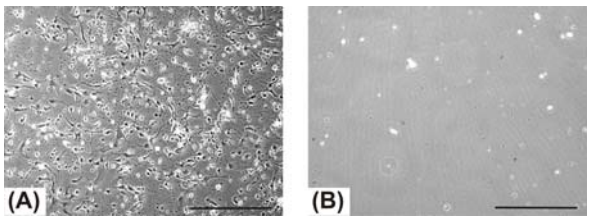

Fig. 2. Dissociated neurons from neonatal rat cortex grown on surface-attached PEI (A) and PDMAA (B) after two weeks of incubation in serum-containing medium. Scale bar 200 µm.

2 Guidance of neuronal cells on patterned polymer layers

To guide neuronal cells, long-term stable patterns of two polymers on a single substrate (MEA or cover slip) were developed. These polymers were chosen specifically such that the neurons should adhere to only one of the polymers but be repelled by the other. As described previously, PEI coatings were found to be cell attractive. PDMAAm, on the other hand, was

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cell repellent even after several weeks of incubation in serum-containing medium.

A grid like pattern with a minimum line-width of 100 µm was developed by a contact pin-printer, which deposited the cell attractive polymer on the cell repellent background. This structured substrate led to a patterned initial adhesion of neuronal cells. Even after incubation over a period of four weeks in serum-containing medium, neurons were confined to the PEI surface and formed a highly interconnected network (Figure 3, A). Since the grid resolution of 100 µm was insufficient for the detailed control of the network structure and the analysis of the neuronal network dynamics we used micro-contact printing (µ-CP) [4], where a silicone rubber stamp created a comparable pattern with smaller dimensions. After incubation with a neuronal cell suspension, the µ-CP patterns showed similar results (Figure 3, B). Neuronal cell bodies preferred to adhere on the circular area of the pattern (diameter 40 µm) and after several days of incubation, a network was formed along the patterned lines (width 5 µm).

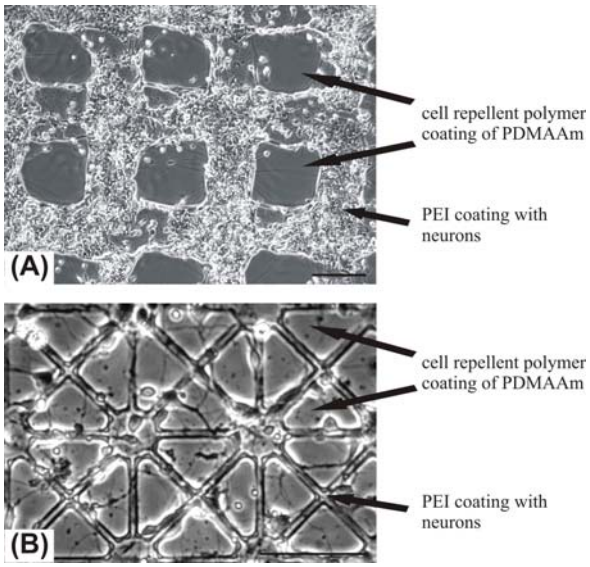

Fig. 3. Neurons grown on (A) printed or (B) stamped PEI on a PDMAAm background after incubation for (A) 32 days and (B) 2 days. Scale bar 100 µm.

Nevertheless it should be noted that the patterning techniques revealed specific limitations. Minute quantities of PEI, e.g. when washing off excess PEI, will firmly adhered to the PDMAAm surface outside the attachment regions. Ultimately, this led to neuronal growth violating the parent pattern. It appears that PEI physisorbs rather strongly to PDMAAm, which in many cases prohibits a quantitative removal of PEI through simple washing procedures.

3 Conclusions

We have shown that versatile surface coatings can be obtained with covalent attachment of polymers to solid substrates via a photochemical approach. Furthermore, a qualitative study of cell adhesion on certain polymer surfaces revealed the biocompatibility of these coatings. PEI and PDMAAm were found to induce initial neuronal cell adhesion with further network formation and no adhesion, respectively. The cells conform on patterns developed with the combination of these two polymers for several weeks in serum-containing medium.

Acknowledgment

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