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SELF-ORGANIZATION AND NEURONAL AVALANCHES IN NETWORKS OF DISSOCIATED CORTICAL NEURONS

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Abstract—Dissociated cortical neurons from rat embryos cultured onto micro-electrode arrays exhibit characteristic patterns of electrophysiological activity, ranging from isolated spikes in the first days of development to highly synchronized bursts after 3–4 weeks *in vitro*. In this work we analyzed these features by considering the approach proposed by the self-organized criticality theory: we found that networks of dissociated cortical neurons also generate spontaneous events of spreading activity, previously observed in cortical slices, in the form of *neuronal avalanches*. Choosing an appropriate time scale of observation to detect such neuronal avalanches, we studied the dynamics by considering the spontaneous activity during acute recordings in mature cultures and following the development of the network. We observed different behaviors, i.e. sub-critical, critical or super-critical distributions of avalanche sizes and durations, depending on both the age and the development of cultures. In order to clarify this variability, neuronal avalanches were correlated with other statistical parameters describing the global activity of the network. Criticality was found in correspondence to medium synchronization among bursts and high ratio between bursting and spiking activity. Then, the action of specific drugs affecting global bursting dynamics (i.e. acetylcholine and bicuculline) was investigated to confirm the correlation between criticality and regulated balance between synchronization and variability in the bursting activity. Finally, a computational model of neuronal network was developed in order to interpret the experimental results and understand which parameters (e.g. connectivity, excitability) influence the distribution of avalanches.

In summary, cortical neurons preserve their capability to self-organize in an effective network even when dissociated and cultured *in vitro*. The distribution of avalanche features seems to be critical in those cultures displaying medium synchronization among bursts and poor random spiking activity, as confirmed by chemical manipulation experiments and modeling studies.

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Abbreviations: ACh, acetylcholine; AE, all electrodes shuffling; BIC, bicuculline; CI_0, coincidence index; DIV, days *in vitro*; IBI, interburst interval; IED, interelectrode distance; IEI, interevent interval; ISI, interspike interval; LFP, local field potential; MBR, mean bursting rate; MEA, micro-electrode array; RMSE, root mean squared error; SE, single electrode shuffling.

Key words: self-organized criticality, micro-electrode arrays, rat cortical neurons, *in vitro* development, scale-free network model.

Large random cortical networks developing *in vitro* and chronically coupled to micro-electrode arrays (MEAs) represent a valuable experimental model for studying the universal mechanisms governing the formation and conservation of neuronal cell assemblies (Marom and Shahaf, 2002). This preparation, unlike other experimental models such as acute and cultured cortical slices, is relatively free of predefined constraints and allows neurons to self-organize during development, creating a network that exhibits complex spatio-temporal patterns of activity (van Pelt et al., 2004a; Wagenaar et al., 2006a; Rolston et al., 2007).

Using this experimental framework, it is interesting to study how the spontaneous electrophysiological activity of the network changes and matures during development (van Pelt et al., 2005; Chiappalone et al., 2006a; Wagenaar et al., 2006b). A marked sensitiveness of the spatio-temporal firing patterns to structural changes in the network during the *in vitro* maturation has been extensively demonstrated, showing variations in the burst patterns and also in the cross-correlation among all pairs of electrodes. Mature cultures (between 21 and 35 days *in vitro* (DIV)) exhibit a synchronized and distributed bursting activity, mixed to a highly variable spiking activity. This network state has been associated to a stable condition of maturation of the culture, and synchronized bursting events have been named "network bursts" (van Pelt et al., 2004b, 2005).

Periods of synchronized electrophysiological activity are also present in other experimental models, such as acute and cultured cortical slices (Beggs and Plenz, 2003, 2004), where it has been demonstrated that within these synchronous epochs there exists a more sophisticated embedded form of dynamics, called *neuronal avalanche*. The presence of such neuronal avalanches, as an evidence of self-organized critical dynamics, has been often associated to the capability of the network to enhance the information transmission (Beggs and Plenz, 2003; Plenz and Thiagarajan, 2007). In other words, if a critical self-organization allows improvement of the transfer of information among cell assemblies, cultured systems should likely display a spontaneous tendency to move toward a critical state characterized by neuronal avalanches.

In our work, while trying to understand the phenomenon of self-organization in dissociated cortical networks, we asked whether and how neuronal avalanches are intrinsic to the network formation and stabilization. In what

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follows, we show that such critical states are found at a specific temporal resolution and they spontaneously appear in some cultures during the network development. Then, we describe in detail the analysis performed and the specific experiments devoted to clarify the variability of such dynamic organization. Finally, we introduce a computational model developed to interpret the experimental results and to demonstrate that networks showing spiking and bursting activity, similar to that observed in cultures of dissociated neurons, are able to reproduce neuronal avalanches at the spike-level with a power law distribution.

EXPERIMENTAL PROCEDURES

Cell culture technique

Dissociated neuronal cultures were obtained from cerebral cortices of embryonic rats, at gestational day 18. The embryos were delivered by cesarean section from deeply anesthetized rats and killed by decapitation. All experiments were carried out in accordance with the European Community Council Directive of November 24th 1986 (86/609/EEC) for the care and use of laboratory animals and approved by MIUR (Ministero dell'Universita' e Ricerca Scientifica). All efforts were made to minimize the number of animals used and their suffering.

The cerebral cortices of four to five rat embryos were chopped into small pieces and exposed to a 0.125% trypsin solution for 25–30 min at 37 °C. Then, they were mechanically dissociated by trituration through fine-tipped pipettes. The resulting tissue was resuspended in 10 ml Neurobasal medium (purchased from Invitrogen, Carlsbad, CA, USA) supplemented with 2% B27 (Brewer et al., 1993; Brewer, 1997) and 1% Glutamax-I (both Invitrogen) and diluted at the final concentration of 800,000 cells/ml. No antimitotic drug was added to prevent glia proliferation, since glial cells are known to be fundamental for the healthy development of neuronal populations (Nedergaard, 1994; Pfrieger and Barres, 1997; Araque et al., 1999). Cells were then plated on 60-channel MEAs, precoated with adhesion promoting molecules (poly-D-lysine and laminin), at the final density of $5–8\times10^4$ cells/device (Fig. 1A). They were maintained in culture dishes, each containing 1 ml of nutrient medium (i.e. serum-free Neurobasal medium supplemented with B27 and Glutamax-I) and placed in a humidified incubator having an atmosphere of 5% CO_2 and 95% O_2 at 37 °C. Half of the medium was changed weekly. Under these environmental conditions, cortical neurons showed excellent growth and robust synaptic connections that allowed us to record spontaneous electrical activity from 7 DIV up to 5–6 weeks *in vitro*.

MEAs and experimental setup

Primary cultures of cortical neurons were plated over arrays (MEA 1060, Multi Channel Systems, Reutlingen, Germany) of 60 planar TiN/SiN micro-electrodes (30 μm diameter, 200 μm spaced) and kept alive in healthy conditions for several weeks.

The experimental setup is based on the MEA 60 system, consisting of a MEA, a mounting support with integrated 60 channels pre- and filter amplifier (MEA 1060, gain 1200×) and a personal computer equipped with a PCI data acquisition board for real time signal monitoring and recording. Commercial software, MC-Rack (Multi Channel Systems), was used for on-line visualization and raw data storage; then, data were processed by using specifically developed software tools as described in the next sections.

Experimental protocols

In order to detect the presence of neuronal avalanches, we considered different experimental conditions. Unless differently specified, each culture was tested after a period of rest, to allow for a stabilization of the network out of the incubator (Streit et al., 2001). The MEA device was maintained at 37 °C to avoid temperature shock out of the incubator and to preserve the metabolic kinetics of neurotransmission. Neuronal networks were kept in the culture medium during recording and electrophysiological signals were acquired at a sampling rate of 10 kHz.

Recordings during development. Spontaneous electrophysiological activity was recorded twice a week in six cultures starting from 7 DIV until 42 DIV. Every experimental session lasted 30 min.

Considering that cultures of dissociated cortical neurons reach a stable state of maturation after the 3rd week *in vitro* (Chiappalone et al., 2006a), we focused our attention on recordings from 21 to 42 DIV, although we also applied the avalanche analysis to the earlier stage of maturation.

Acute long-lasting recordings. Long-lasting recordings were performed on three cultures, obtained from different cell preparations, during the 4th week *in vitro* (25–28 DIV). Two cultures were recorded at a sampling rate of 10 kHz, whereas the last one was recorded both at 10 and 25 kHz. All recordings lasted 1 h.

The aim of these experiments was to expand our dataset in the key period of development (i.e. 4th week *in vitro*), and test how the acquisition parameters (e.g. sampling rate, duration) could affect avalanche analysis results.

Chemical stimulation experiments. Some cultures were treated with specific drugs affecting the bursting dynamics of *in vitro* cortical networks: we tested acetylcholine (ACh) in concentration 10 μM (Gross et al., 1995) on three cultures and bicuculline (BIC) in concentration 30 μM (Keefer et al., 2001; Lin et al., 2002; Gramowski et al., 2004) on other three cultures. All agents were purchased from Sigma Aldrich (St. Louis, MO, USA).

All the experiments started with a 20-min recording of the network spontaneous activity in physiological solution (NaCl 150 mM, KCl 2.8 mM, $CaCl_2$ 1.3 mM, $MgCl_2$ 0.7 mM, Hepes 10 mM, glucose 10 mM, pH 7.3), followed by 20 min under drug treatment. The aim of these experiments was to study whether and how chemical stimulation could affect network behavior and change avalanche dynamics.

Spike detection

Extracellularly recorded signals are embedded in biological and thermal noise (Fig. 1B) and spikes can be detected using a threshold-based detection algorithm (Perkel et al., 1967; Gross et al., 1995). A previously developed ad hoc algorithm (Chiappalone et al., 2003) calculates the peak-to-peak thresholds as multiple of the standard deviation (8×S.D.) of the baseline noise (Jimbo et al., 1999) for each electrode during the spontaneous activity phase (20.7 ± 0.8 μV, mean±S.D., one culture). In this study, no attempt was made to discriminate and sort the collected spikes (Eytan and Marom, 2006).

Burst detection

Developing cortical networks show spiking activity as well as bursting behavior (Robinson et al., 1993; Opitz et al., 2002). A population burst consists of episodes of activity occurring simultaneously at many channels, spread over the entire network (Fig. 1C). The spikes belonging to a burst are time-spaced in a range of a few milliseconds; these packages generally last from hundreds of milliseconds up to seconds with long quiescent periods. Bursts were identified and their features saved (i.e. duration, rate, etc.) according to a method previously presented in the literature (Chiappalone et al., 2005). Moreover, burst event trains, containing the sequence of the initial spike of each burst (Cozzi et al., 2006), were stored.