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LOW-FREQUENCY STIMULATION ENHANCES BURST ACTIVITY IN CORTICAL CULTURES DURING DEVELOPMENT

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Abstract–The intact brain is continuously targeted by a wealth of stimuli with distinct spatio-temporal patterns which modify, since the very beginning of development, the activity and the connectivity of neuronal networks. In this paper, we used dissociated neuronal cultures coupled to microelectrode arrays (MEAs) to study the response of cortical neuron assemblies to low-frequency stimuli constantly delivered over weeks *in vitro*. We monitored the spontaneous activity of the cultures before and after the stimulation sessions, as well as their evoked response to the stimulus. During *in vitro* development, the vast majority of the cultures responded to the stimulation by significantly increasing the bursting activity and a widespread stabilization of electrical activity was observed after the third week of age. A similar trend was present between the spontaneous activity of the networks observed over 30 min after the stimulus and the responses evoked by the stimulus itself, although no significant differences in spontaneous activity were detected between stimulated and non-stimulated cultures belonging to the same preparations. The data indicate that the stimulation had a delayed effect modulating responsiveness capability of the network without directly affecting its intrinsic *in vitro* development.

Key words: micro-electrode array, rat cortical neurons, multisite stimulation, network burst.

The use of dissociated cortical neurons cultured onto microelectrode arrays (MEAs) allows for the investigation of neuronal network dynamics at a mesoscopic scale. Cultures represent a useful experimental model to characterize both the spontaneous behaviour of neuronal populations and their activity in response to pharmacological and/or electrical conditioning. Several studies performed long-term monitoring of the electrical activity of cortical primary neurons by exploiting the distinctive features of extracellular non-invasive multielectrode recordings. By using this technique it was shown that the primary networks self-organize and rewire following plating (Pasquale et al., 2008), adapt to external stimuli (Eytan et al., 2003), respond to pharmacological manipulations (Eytan et al., 2004), learn through a set of external stimuli (Shahaf and Marom, 2001) or act as an effective computational device (Ruaro et al., 2005). However, most studies were focused on the analysis of neuronal dynamics in mature cultures once achieved a stable morphological and functional connectivity, while only few investigations were aimed at the characterization of the network spontaneous activity over time (Van Pelt et al., 2004a; Chiappalone et al., 2006; Wagenaar et al., 2006).

During development, while synapses and neural connectivity build up, neuronal network activity starts to organize in synchronized patterns. The latter activity is observed since the early stages of development and accounts for the majority of the spontaneous electrical activity for a period extending from days to weeks (Ben-Ari, 2001; Corner et al., 2002; Corner, 2008). As brain circuits develop through processes of synapse formation and elimination (Goodman, 1996; Katz and Shatz, 1996; Feller, 1999), the early activity shown during network development is believed to play a paramount role in the structuring of its functionality (Opitz et al., 2002). Developing *ex-vivo* cultured neurons show the same properties of brain tissue, although at a simplified level of organization (Fields and Nelson, 1992; Chub and O'Donovan, 1998; Chiappalone et al., 2006).

Virtually all previous studies, focusing on the spontaneous activity during development of cultured networks, left a number of unanswered questions such as: (i) How does a culture react if stimulated at different stages of its growth? (ii) When does it acquire the capability of responding in a stable way? (iii) Is the development of a neuronal culture affected by an early delivered electrical conditioning? (iv) Does a culture stimulated over time differ from a never stimulated mate? To address these questions, in the present work we monitored a population of rat neocortical primary cultures for at least 4 weeks from early stages to *in vitro* maturation and chronically administered constant low-frequency stimuli twice a week starting from the second week *in vitro*. To understand the effects of electrical stim-

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Abbreviations: COM, centre of mass; DIV, days *in vitro*; DT, differential threshold; IBI, interburst interval; II, increment index; MBR, mean bursting rate; MEA, micro-electrode array; MFR, mean firing rate; *mrd*, mean relative deviation; NB, network burst; NBD, network burst duration; NBFP, network burst falling phase; NBPV, network burst peak value; NBRP, network burst rising phase; PLP, peak lifetime period; *pmrd*, population mean relative deviation; PSTH, post stimulus time histogram; *rd*, relative deviation; RP, refractory period.

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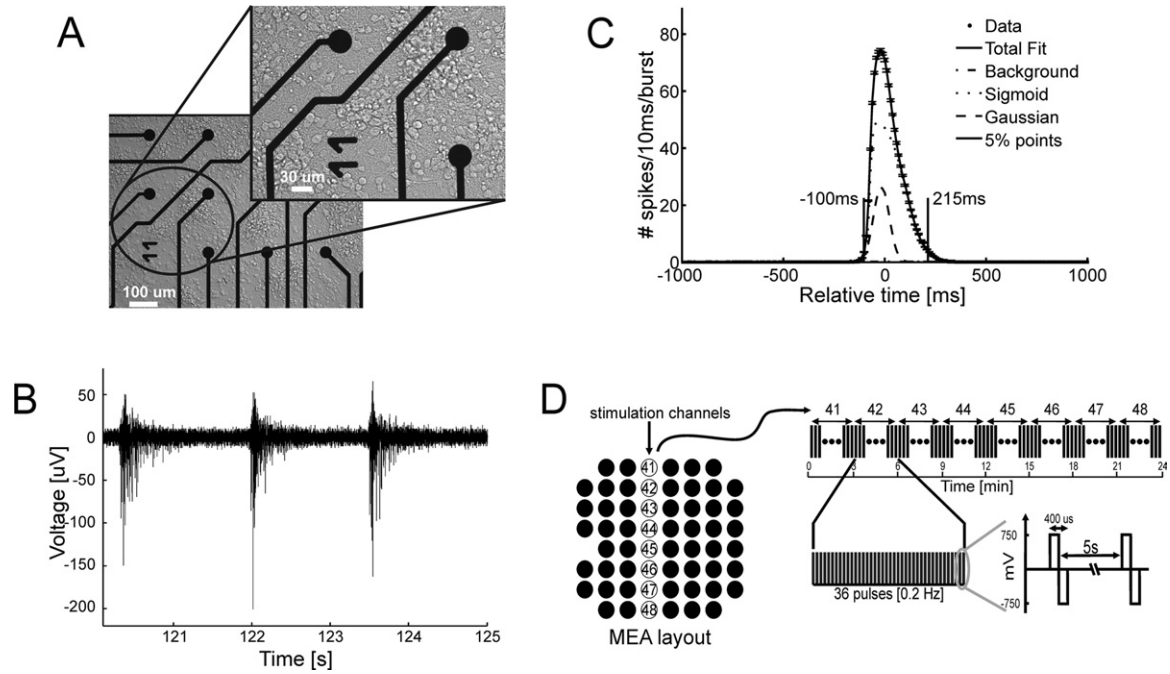

Fig. 1. Overview: (A) Example of neuronal cells coupled to MEAs. (B) An example of a burst recorded through a single channel. The early and central phase of the burst is clearly denser with spikes than the tail in which isolated spikes can be distinguished. (C) An example of the network burst fitting procedure: the network burst profile is fitted by using three curves, that is, a background, a double sigmoid and a gaussian. For further details, cf. *Experimental procedures*. (D) Sketch of the stimulation protocol: the central column of the MEA layout is used to deliver the stimuli from top to bottom (left). Every channel delivers a set of 36 stimuli at 0.2 Hz, for 3 min (top right). A single pulse was 1.5 V peak to peak and biphasic (positive phase first) and lasted 400 μs (bottom right).

ulation on the spontaneous activity of developing neuronal populations, we monitored spontaneous activity before and after the stimulation and compared stimulated and non-stimulated cultures under the same conditions.

Although the networks started to reliably respond to the electrical conditioning at various stages of the development, the stimulation paradigm drove the cultures to significantly burst more frequently than before stimulation. The changes in activity lasted for at least 30 min and all active electrodes were involved, showing that the effect impacted on the entire culture. In addition, a coherent behaviour was found between the changes in the bursting activity and the post-stimulus time histogram area, indicating a long-term effect of the stimulation.

EXPERIMENTAL PROCEDURES

Cell culture preparation and maintenance

Primary neuronal cultures were obtained from cortices of Sprague–Dawley rats at day 18 of gestation. Embryos were extracted by caesarian section from anesthetized pregnant dams in accordance with the European Community Council directive approved by the Italian Ministry of Health. Culture preparation was performed as previously described (Chiappalone et al., 2008). Briefly, rat embryonic cerebral cortices were dissected out from the brain and dissociated first by enzymatic digestion in trypsin solution (20 min at 37 °C) and subsequently by mechanical dissociation with a fine tipped Pasteur pipette. The resulting tissue was resuspended in Neurobasal medium supplemented with 2% B-27 and 1% Glutamax-I (Invitrogen, Carlsbad, CA, USA) at the final concentration of 1600∼2000 cells/μl. Cells were afterwards plated onto MEAs previously coated with poly-D-lysine and laminin to promote cell adhesion (see Fig. 1A). Cultures were kept in an incubator at 5% CO_2 at 37 °C and pulled out only during the experimental sessions or for medium change. To reduce thermal stress of the cells, MEAs were kept at 37.2 °C by means of a controlled thermostat (MCS, Reutlingen, Germany). Culture medium was changed weekly, immediately after the recording sessions. All the animal experiments were made in accordance with the Italian and UE legislation and special care was made to reduce animal suffering. Additionally the use of dissociated cultures intrinsically helped to reduce the number of animals used.

Culture dishes and experimental setup

Microelectrode arrays (Multichannel systems, MCS) consisted of 59 TiN/SiN planar round electrodes (30 μm diameter; 200 μm center-to-center interelectrode intervals) arranged in a square grid excluding corners (see Fig. 1D). One recording electrode was replaced by a bigger ground electrode. All dish chambers were sealed with a gas permeable Teflon membrane to prevent contamination and evaporation (Potter and DeMarse, 2001). The activity of all cultures was recorded by means of either a MEA60 or MEA120 System (MCS). After a 1200× amplification, signals were sampled at 10 kHz and acquired through the data acquisition card and MCRack software (MCS). Electrical stimuli were delivered through a four or eight channel stimulator (MCS STG2004 or 1008). Data analysis was performed off-line by a custom software tool developed in MATLAB© (The Mathworks, Natick, MA, USA; Vato et al., 2004).

Experimental protocols

(a) Spontaneous activity recording protocol. The spontaneous activity of 11 cultures belonging to three independent preparations was measured twice a week. All recordings started 20–30