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Cortical cultures coupled to Micro-Electrode Arrays: A novel approach to perform in vitro excitotoxicity testing

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ABSTRACT

In vitro neuronal cultures exhibit spontaneous electrophysiological activity that can be modulated by chemical stimulation and can be monitored over time by using Micro-Electrode Arrays (MEAs), devices composed by a glass substrate and metal electrodes. Dissociated networks respond to transmitters, their blockers and many other pharmacological substances, including neurotoxic compounds. In this paper we present results related to the effects, both acute (i.e. 1 hour after the treatment) and chronic (3 days after the treatment), of increasing glutamatergic transmission induced by the application of rising concentrations of glutamate and its agonists (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid — AMPA, N-methyl-D-aspartate — NMDA and AMPA together with cyclothiazide — CTZ). Increase of available glutamate was obtained in two ways: 1) by direct application of exogenous glutamate and 2) by inhibiting the clearance of the endogenously released glutamate through DL-threo-β-benzyloxyaspartate (TBOA). Our findings show that fine modulations (i.e. low concentrations of drug) of the excitatory synaptic transmission are reflected in the electrophysiological activation of the network, while intervention leading to excessive direct stimulation of glutamatergic pathways (i.e. medium and high concentrations of drug) results in the abolishment of the electrophysiological activity and eventually cell death. The results obtained by means of the MEA recordings have been compared to the analysis of cell viability to confirm the excitotoxic effect of the applied drug. In conclusion, our study demonstrates that MEA-coupled cortical networks are very sensitive to pharmacological manipulation of the excitatory ionotropic glutamatergic transmission and might provide sensitive endpoints to detect acute and chronic neurotoxic effects of chemicals and drugs for predictive toxicity testing.

1. Introduction

Primary neuronal cultures dissociated from different regions of the Central Nervous System (CNS) have been a classical model for in vitro electrophysiological studies of single neuron's neurobiological mechanisms (Dichter and Fischbach, 1977; Sakmann and Neher, 1984). Developed at the beginning of the '80s (Gross et al., 1977; Pine, 1980), the Micro-Electrode Array (MEA) technique nowadays offers a useful experimental approach for in vitro electrophysiological investigations. MEA technology and culture methods, in parallel, have continuously improved during these years. To date, MEAs find several applications in many research fields, such as neuroscience, pharmacology, physiology, biophysics and cardiac electrophysiology. In particular, the pioneering works by Gross and co-workers demonstrated the possibility to use dissociated neuronal networks coupled to MEAs as a first prototype of cell-based biosensor (Gross et al., 1977, 1992). This system showed both high sensitivity to neuroactive and neurotoxic compounds and reproducible results (Gramowski et al., 2000). The biocompatibility of the used materials (i.e. titanium nitride for the electrodes and glass for the substrate) and the non invasive nature of the extracellular measurement, make this system a perfect candidate to routinely record and evaluate the dynamics of the network behavior, both in spontaneous condition and under chemical manipulation (Chiappalone et al., 2006; Martinoia et al., 2005), either on short or long time-scales. This experimental system, that constitutes on a mesoscopic level a simplified model of a cortical tissue, allows to explore network properties, while preserving the morphological, molecular and functional properties of the individual neurons in the cortex (Marom and Shahaf, 2002).

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Excitatory neurotransmission in the brain is predominantly mediated by glutamate release via synaptic transmission. Glutamate activates ionotropic (ligand-gated) receptors of both NMDA and AMPA/kainate subtypes (Collingridge and Lester, 1989) and metabotropic (G-protein coupled) receptors. While synaptic glutamatergic transmission onto ionotropic receptors plays a critical role during brain development and in plasticity, memory, learning processes and cognition in adult brain (Antzoulatos and Byrne, 2004; Meldrum, 2000; Riedel et al., 2003), stimulation of ionotropic glutamate receptors may also activate neuro-excitotoxic cascades (Rothman and Olney, 1995, 1987).

In this study, we used cortical cultures coupled to MEAs to investigate the short/long term effects of increasing concentrations of ionotropic glutamate's agonists, which could cause excitotoxicity. Cell viability at different time points after glutamate agonist treatment was evaluated by fluorescein diacetate uptake by viable cells and propidium iodine staining of dead cells. In the attempt to get insight on the effects directly dependent on activation of a specific ionotropic glutamate receptor subtype, we selectively activated ligand-gated glutamatergic receptor subtypes by using glutamate agonists (NMDA, AMPA and AMPA together with cyclothiazide — CTZ) or added exogenous glutamate, at different concentrations to modulate the dynamics of cortical networks. Then, in order to rapidly enhance the extracellular concentration of endogenous glutamate, we applied DL-threo-β-benzyloxyaspartate (TBOA), a glutamate uptake inhibitor (Jabaudon et al., 1999; Shimamoto et al., 1998); moreover, to contribute to elucidation of mechanistic issues, the glutamate concentration in the extracellular medium (basal and drug phase) was evaluated by high-performance liquid chromatography (HPLC) analysis, as a measure of the activation of glutamatergic transmission.

We show that high concentrations of either glutamate agonists or glutamate uptake inhibitor are responsible of a complete loss of activity in our cortical cultures (due to the excitotoxic effect of glutamate which brings to cell death, as proven by the live/dead assay results), while low concentrations of the same compounds bring network activity to a stable level. These results will be discussed in the view of homeostatic mechanisms such as receptor desensitization and glutamate clearance, responsible of the maintenance of the balance between excitatory and inhibitory activity (Watt et al., 2004). On the other hand, due to high sensitivity to ionotropic glutamate receptor activation and to the ability to detect interference with glutamatergic transmission, suitability of MEA-coupled cortical cultures as a candidate for *in vitro* neurotoxicity testing would deserve to be evaluated.

2. Materials and methods

2.1. Cell culture

Dissociated neuronal cultures were obtained from cortices of embryonic rats. Pregnant rats (Sprague–Dawley derived by Charles River in 1955, IGS) were deeply anesthetized and sacrificed by inhalation of CO_2: 18-day embryos (E18) were removed immediately by cesarean section, and killed by decapitation. All experimental procedures and animal care were conducted in conformity with institutional guidelines, in accordance with the European legislation (European Communities Directive of 24 November 1986, 86/609/EEC) and with the NIH Guide for the Care and Use of Laboratory Animals. Culture preparation was performed as previously described (Bologna et al., 2010a). Briefly, the cerebral cortices of 4–5 embryos were dissected out from the brain and dissociated first by enzymatic digestion in trypsin solution 0.125% (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, 1% Glutamax-I, 1% Pen-Strep solution and 10% Fetal Bovine Serum (Invitrogen, Carlsbad, CA), at the final concentration of 36–40 k cells/ml. Cells were afterwards plated onto 60-channel MEAs previously coated with poly-D-lysine and laminin to promote cell adhesion (final density around 1200 cells/mm^2) and maintained with 1 ml of nutrient medium (i.e. serum-free Neurobasal medium supplemented with B27 and Glutamax-I). They were then placed in a humidified incubator having an atmosphere of 5% CO_2-95% air at 37 °C. Half of the medium was changed weekly.

2.2. Micro-Electrode Array recordings

Microelectrode arrays (Multichannel Systems, MCS, Reutlingen, Germany) consisted of 59 TiN/SiN planar round electrodes (30 μm diameter; 200 μm center-to-center interelectrode distance) arranged in an 8×8 square grid excluding corners. One recording electrode was replaced by a bigger ground electrode. The activity of all cultures was recorded by means of the MEA60 System (MCS). After 1200× amplification, signals were sampled at 10 kHz and acquired through the data acquisition card and MC_Rack software (MCS). To reduce thermal stress of the cells during the experiment, MEAs were kept at 37 °C by means of a controlled thermostat (MCS) and covered by PTFE lid (ALA Scientific Instruments, NY, USA) to avoid evaporation and prevent changes in osmolarity.

2.3. Experimental protocols

The general protocol adopted for chemical stimulation included 30 minutes of recording in culture solution (Neurobasal (Invitrogen cat.no. 21103049) + 2% B27 (Invitrogen cat.no. 17504044) + 1% Glutamax-I 200 mM (Invitrogen cat.no. 35050038) + 1% Penicillin-Streptomycin sol. (Invitrogen cat. no. 15140122)), defined as control condition. All drugs were added to the bath solution at increasing concentrations by directly pipetting in the medium. For each concentration, the electrophysiological activity was recorded for 30 min. Since we noticed that mechanical perturbation due to the pipette injection in the medium could cause a temporary instability of the firing rate, we discarded the first 10 min of each recording phase. According to this choice, the presented data refer to a recording period of 20 min for each experimental phase. All tested networks were measured between the 3rd and the 4th week in vitro.

2.3.1. Glutamate and agonists of the glutamatergic receptors

α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) are agonists of ionotropic glutamatergic receptors at the post-synaptic site. We performed experiments by adding to the culture medium progressively increasing concentrations of each drug at intervals of 30 min. More specifically, we tested low (i.e. 0.05–0.2 to 1–5 μM), medium (i.e. 10 – 30 μM) and high (i.e. 50–100 μM) concentrations of AMPA/NMDA to different sets of cultures (N = 4 cultures for AMPA and N = 5 cultures for NMDA). At the same time, we tested the effects of increasing doses of glutamate (0.05 - 0.2 - 1 – 5–10 – 30 – 50–100 – 500 μM, N = 4 cultures). We mainly based our choices on the available literature by ours and other groups in the MEA field (Gramowski et al., 2006, 2000; Gross et al., 1992; Martinoia et al., 2005).

2.3.2. Inhibitor of AMPA receptor desensitization

Following activation, many ligand-gated ion channels enter in a desensitized state in which the neurotransmitter remains bound but the ion channel is closed (Sun et al., 2002). Both AMPA and NMDA receptors are subject to desensitization, with a different degree also related to the activating agonist. To prevent the desensitization of AMPA receptors, we used cyclothiazide (CTZ) at different concentrations (Trussell et al., 1993). We treated a subset of our cultures with either low (i.e. 1–3 μM) or high (i.e. 10–30 μM) concentrations of CTZ, combined with AMPA at a fixed concentration of 0.2 μM (N = 3 cultures).