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Networks of neurons coupled to microelectrode arrays: a neuronal sensory system for pharmacological applications

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Abstract

Two main features make microelectrode arrays (MEAs) a valuable tool for electrophysiological measurements under the perspective of pharmacological applications, namely: (i) they are non-invasive and permit, under appropriate conditions, to monitor the electrophysiological activity of neurons for a long period of time (i.e. from several hours up to months); (ii) they allow a multi-site recording (up to tens of channels). Thus, they should allow a high-throughput screening while reducing the need for animal experiments. In this paper, by taking advantages of these features, we analyze the changes in activity pattern induced by the treatment with specific substances, applied on dissociated neurons coming from the chick-embryo spinal cord. Following pioneering works by Gross and co-workers (see e.g. Gross and Kowalski, 1991. Neural Networks, Concepts, Application and Implementation, vol. 4. Prentice Hall, NJ, pp. 47–110; Gross et al., 1992. Sensors Actuators, 6, 1–8.), in this paper analysis of the drugs' effects (e.g. NBQX, CTZ, MK801) to the collective electrophysiological behavior of the neuronal network in terms of burst activity, will be presented. Data are simultaneously recorded from eight electrodes and besides variations induced by the drugs also the correlation between different channels (i.e. different area in the neural network) with respect to the chemical stimuli will be introduced (Bove et al., 1997. IEEE Trans. Biomed. Eng., 44, 964–977.). Cultured spinal neurons from the chick embryo were chosen as a neurobiological system for their relative simplicity and for their reproducible spontaneous electrophysiological behavior. It is well known that neuronal networks in the developing spinal cord are spontaneously active and that the presence of a significant and reproducible bursting activity is essential for the proper formation of muscles and joints (Chub and O'Donovan, 1998. J. Neurosci., 1, 294–306.). This fact, beside a natural variability among different biological preparations, allows a comparison also among different experimental session giving reliable results and envisaging a definition of a bioelectronic 'neuronal sensory system'.

Keywords: Whole cell-based biosensor; Micro electrode arrays; Electrophysiological monitoring; In-vitro neuronal networks

1. Introduction

Neural networks in the developing spinal cord are spontaneously active and able to generate a rhythmic activity, consisting in alternate patterns called 'bursts', directed to flexor and extensor muscles and used for locomotion (Chub and O'Donovan 1998; Tscherter et al., 2001). It is known that different pharmacological manipulations can induce various types of rhythmic behavior in the spinal cord; studies on the neonatal rats spinal cord showed that fictive locomotion can be induced by the application of NMDA (Kudo and Yamada, 1987) or by increasing the extracellular K^+ concentration (Bracci et al., 1996).

Using dissociated spinal neurons from the chick embryos, we analyzed the changes in activity pattern induced by the treatment with specific drugs acting on the glutamate receptors (NMDA and non-NMDA) in order to possibly identify different 'functional states' of the network or changes in the network dynamics.

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Neuron cells can be cultured and kept alive in vitro for a long time (Potter and DeMarse, 2001), giving birth to a very stable system. For this reason in vitro preparations are a good model to study how the electrophysiological behavior of neural tissue can be influenced by pharmacological (toxic) compounds.

Moreover in-vitro cultures of spinal cord neurons show, during basal conditions, repeatable and stable activity and therefore seem to be good candidates for such applications.

To monitor the changes into the network, we utilized microelectroede arrays (MEAs) that, after pioneering works by Gross et al. (Gross and Kowalski, 1991; Gross et al., 1992, 1993), are likely to become a powerful tool to investigate the potential time-dependent interactions among neurons (Nicolelis et al., 1997). In particular, two main features make MEAs a valuable tool for electrophysiological measurements under the perspective of pharmacological applications: first, they are non-invasive and, therefore, can allow long-term recordings and, second, they perform a multi-site recording (from 8 up to 60 channels). Taking advantage of such properties, our approach suggests that functional measures related to biosensor applications could be employed to facilitate the evaluation of pharmacological test substances in industrial research (Egert and Hämmerle, 2002), allowing a high-throughput screening and reducing the need for animal experiments. Moreover, it can be envisaged that chronic exposure studies and investigations will be possible for the demonstrated longevity of these biological preparations(Gross and Schwalm, 1994; Kamioka et al., 1996). It should be also noted that the collective electrophysiological activity modulated by pharmaceutical agents, can give useful information on primary mechanism involved in altered neuronal network responses.

2. Materials and methods

2.1. Spinal cord cultures

Dissociated neurons were obtained from the spinal cord of 7–8-day-old chick embryos, according to a modified version of the protocol described elsewhere (Dichter and Fischbach, 1977).

Eggs were whipped with 70% ethanol, cracked and embryos were transferred into a sterile plastic Petri dish. Spinal cords were approached from ventral aspect, severed at cephalic-end and finally dissected, free of attached dorsal root ganglia, meninges and connective tissue.

The cord was minced in small pieces by using microdissecting scissors, transferred to a centrifuge tube containing 3 ml per cord of trypsin solution (0.05% trypsin, 0.3% BSA, 0.005% DNAse typeI in CMF-HBSS) for 30 min at 37 °C. After enzymatic dissociation, 'the pellet' was washed twice with a solution of 0.025% trypsin inhibitor, 0.3% BSA, 0.005% DNAse typeI in CMF-HBSS or 'plating medium' consisting of DMEM, F12 90%, 5% HS, and 5% FCS with Glutamax (Gibco).

Neuron cells were gently disaggregated and the resultant single cell suspension was centrifuged for 10 min and the pellet re-suspended in the plating medium. Cell yield by this procedure were about $2.8–3 \times 10^6$ per cord and plating densities were about $6–7 \times 10^5$ per device. Then, neurons were seeded on microelectrode arrays (MEAs, Fig. 1) covered with adhesion promoting molecules (poly-lysine or laminin). The culture was maintained at 37 °C in an atmosphere of 5% CO_2 and 95% air, saturated with water vapor. Twenty-four hours after plating, the medium was completely aspirated and replaced with maintenance medium, consisting of NeuroBasal medium 96% (Sigma), 2% B-27, 1% HS, 1% L-glutamine 2 mM, Pen-Strepto 10^4 U.I. 0.1%.

Electrophysiological signals were recorded after 14–15 days in vitro (DIV), a time sufficient to allow neurite-promoting and spontaneous activity related to the development of glutamate receptors (Köller et al., 1990).

2.2. Pharmacological protocols

Two different pharmacological protocols were adopted in order to characterize the network under different drug conditions and to better understand what kind of glutamate receptors are involved in the modulation of spontaneous activity.

Protocol 1 was studied with the aim to define contribute of non-NMDA (AMPA) receptors in the developing activity of the network. To be sure that could be no intervention by NMDA receptor, we added a NMDA antagonist. Protocol 1 consists of four steps:

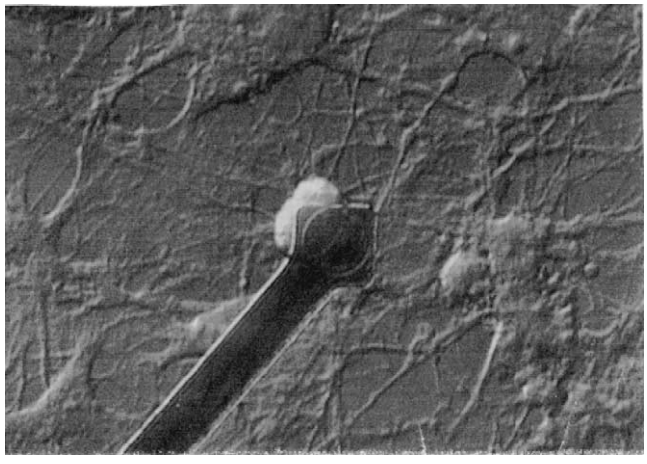

Fig. 1. A cell body placed above a single microelectrode.

1) Control condition, corresponding to the spontaneous activity in culture medium (NeuroBasal medium, Sigma);
2) Cyclothiazide (CTZ), which acts blocking AMPA receptor desensitization, added at a final concentration of 30 μM;
3) MK-801, antagonist of the NMDA receptor, added at a final concentration of 50 μM;
4) NBQX, an antagonist of the AMPA receptor, added at a final concentration of 20 μM.

Protocol 2 was studied with the aim to define the contribute of both NMDA and non-NMDA receptors in the developing activity of the network; it consists of four steps:

1) Control condition, corresponding to the spontaneous activity in culture medium (NeuroBasal medium, Sigma);
2) NMDA, agonist of the NMDA receptor, added at a final concentration of 30 μM;
3) MK-801, antagonist of the NMDA receptor, added at a final concentration of 100 μM;
4) NBQX, an antagonist of the AMPA receptor, added at a final concentration of 20 μM.

All agents were applied by adding them to the culture medium, following the order according to protocol 1 or 2.

2.3. Microelectrode array and experimental set-up

The microelectrode array used for our experiments was a glass substrate with 60 gold microelectrodes of which 40 used for acquisition (10 μm diameter). The 40 recording electrodes were subdivided into five groups of eight electrodes each, directly connected with the amplifier via a microdin cable.

Data were obtained by using a custom-made experimental set-up based on a PC and on a long-term recording instrument. The acquisition devices is a Digital Tape Recorder (BioLogic DTR 1802) with a maximum of eight recording channels at sampling frequency of 12 KHz per channel. The utilized experimental set-up for non-conventional electrophysiological investigations is shown in Fig. 2. It consists of several functional elements:

- The MEA, which provides an interface between the biological and the electrical environment;
- A Faraday cage and an antivibrating table
- 8–16 channels amplifier and filtering stage (total gain = 1000).
- Long term acquisition instrumentation: Digital Tape Recorder (BioLogic DTR-1802) with a maximum of eight recording channels, 14 bits of resolution and a GPIB parallel interface.
- Digital oscilloscope for real time monitoring of neuronal signals;
- System for network electrical stimulation (Scanner, Stimulation Interface, and Isolator);
- PC for data management equipped with National Instruments PCI 6071E board, used for acquisition and real-time signal visualization.

Moreover, during each experiment a TV camera connected with a microscope and positioned in proximity of the MEA, is used to verify the position of the cells on the microelectrodes and to correlate the recorded signals with images of the neurons on the array.

2.4. Signal processing tools

Signals coming from microelectrode arrays have typical amplitudes in the range of 0.1–0.4 mV and are embedded in biological and thermal noise ranging from 10 up to 40 μV peak to peak. Each microelectrode records signals of one or a few neurons, but no attempt was made to discriminate and sort spikes collected by one channel, considering each signal the result of a time-spatial summation of the responses from a few cells and providing information of the 'micro' network related to that recording site.

Local spiking behavior is defined as one or few spikes seen at one or more microelectrodes; bursts consist of episodes of activity lasting from 50 ms up to several seconds and occurring simultaneously in many channels, spread over the entire network (Streit et al., 2001).

Although, due to variability of the preparations and to the differences in DIV, our cultures showed spontaneous activity ranging from stochastic local spiking to organized bursting, a sort of temporal structure constituted by the presence of synchronized activity, organized in bursts, were found as characterizing behavior of our preparations.

Therefore, from a quantitative point of view, we chose to describe the activity of the network at burst level, analyzing burst patterns in different pharmacological conditions and thus providing information about network behavior without having to deal with the complexities of spike analysis (Gross, 1994). According to this goal, we developed a custom-made algorithm for burst automatic detection using data pre-processed by the peak-detection algorithm. A sliding window, sized to contain at most a single spike, is shifted over the signal until the difference between the maximum and the minimum inside the window is under the peak-to-peak threshold, and, when the difference is over this threshold, a spike is found. The algorithm for burst detection works in the same way but with a larger sliding window used to add the peak-to-peak values of the spikes inside; if the sum is over a defined threshold a burst is detected.

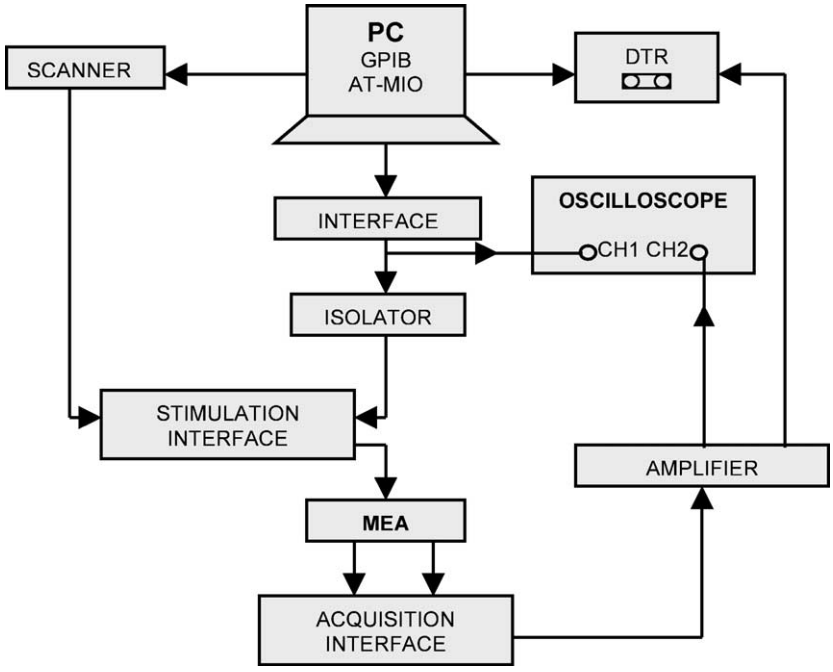

Fig. 2. Scheme of the acquisition system used for experiments with MEAs.

The extracted features for each detected burst are the following:

- Time of occurrence (ms), defined as the moment of occurrence of the first spike inside the burst.
- Burst duration (ms), defined as the time length between the first spike and the last one inside the same burst.
- InterBurst Interval—IBI (s), defined as the time length between the end of a burst and the beginning of the next one;
- Burst amplitude (arbitrary units), an indication of the power of the burst.

3. Results

Experimental data are obtained from neuronal cultures, following two different protocols (see Section 2.2). Experiments were carried out for several hours and activity recorded from a set of eight channels chosen among the 5×8 channels of the MEA.

The first step of our protocols always consists in the recording of spontaneous activity, in order to compare it with the behavior of the network under drug treatment.

Fig. 3a shows an example of typical spontaneous activity recorded from one channel (i.e. one microelectrode) for about 12 minutes: simply by eye inspection, it is possible to identify bursts and single, isolated spikes. Mean burst frequency (number of bursts/minute) in this condition, for all the channels, was evaluated (see Fig. 7) according to the methods explained in Section 2.4.

Adopting Protocol 1, we observed that spinal neurons respond to the presence of CTZ with significantly different signal, in which burst frequency seems to be higher than in case of spontaneous activity (Fig. 3b), as confirmed by the average bursting rate shown in Fig. 8. This is an expected behavior, because the blockade of AMPA receptor desensitization by cyclothiazide makes the responses of spinal cord neurons potentiated (Dai et al., 2001) and therefore much more evident the synchronized activity.

After adding MK-801 we assist to a very fast depression of the network in term of burst frequency (Fig. 3c). The bursting rate reaches values of 0.841 ± 0.033 burst/min (see Fig. 8) obviously paralleled by the increasing in the Inter Burst Interval, while the mean burst duration, indicatively, becomes longer than in basal condition.

The treatment with NBQX after MK-801 seems to indicate a burst frequency still lower than the initial CTZ or NMDA conditions (see Fig. 8), as presented in Fig. 3d. This behavior is not clearly confirmed by the obtained results and it might be explained by considering that the blockade of AMPA receptors by the competitive antagonist NBQX inhibits the responses of spinal cord neurons (Turrigiano, et al., 1998) and therefore makes the synchronized activity much less evident.

Application of NMDA, during the second step in Protocol 2, shows signal not so different from the previous spontaneous activity in the same experiment (Fig. 7), with a mean bursting frequency of 1.682 ± 0.038 burst/min (see also Fig. 8).

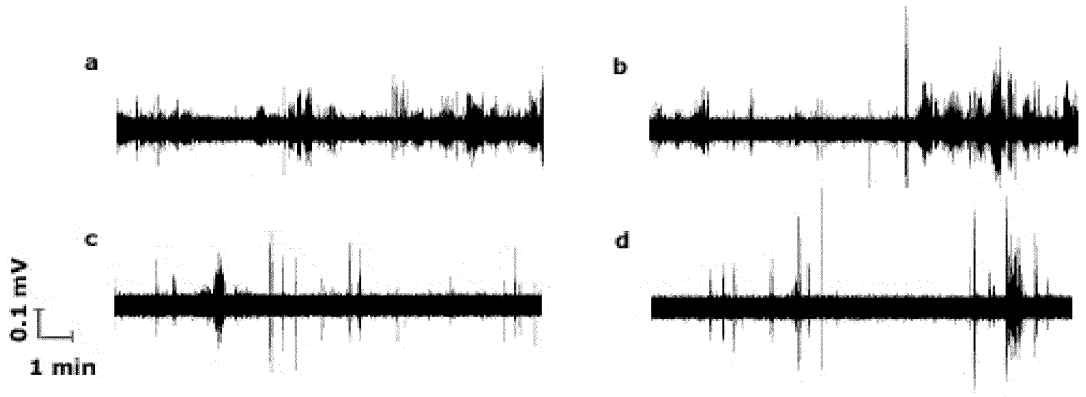

Fig. 3. Electrophysiological activity recorded from one microelectrode of the array during the experiment; (a) typical spontaneous activity; (b) note the increasing of burst number and duration during CTZ treatment; (c) activity recorded during MK-801; and (d) NBQX treatment.

Bursts were selected according to 'burst detection' algorithm, and both IBI (see Section 2.4) and burst duration was calculated for each recording channel. Usually not all the channels were employed for the analysis because of a not good coupling of the correspondent microelectrode with the cells; for this reason in the following reported data the noisy channels are missing.

In Fig. 4 two bar graphs show the IBI mean value (+S.E.) and the burst duration mean value (+S.E.) for six channels, during the four experimental phases of Protocol 1 applied to a 14 DIV culture. For each channel the presence of CTZ causes a decreasing of the mean IBI and an increasing of the mean burst duration, facts that prove an increasing of the whole electrophysiological activity.

After adding the inhibitory drugs, we observe a faster increasing of IBI, obtaining a quite different response from the control and CTZ conditions. The burst duration graph shows that, during the NBQX phase, bursts are shorter than in the first two conditions, while during MK801 treatment, the channels' responses are not homogeneous, showing both increasing and decreasing of this parameter.

We can also note a very small variability in the IBI values among the channels for CTZ condition: this could suggest a high correlation between channels in the presence of the drug and a more stable state of the whole network. On the contrary, during inhibitory treatments, there is a high variability of IBI values over the whole network, proving that these drugs actually change the status of the network, bringing perturbations to its rhythmic activity.

Results reported in Fig. 5 come from a 15 DIV culture, treated with Protocol 2. Also in this case we can observe an increasing of IBI values in the two last

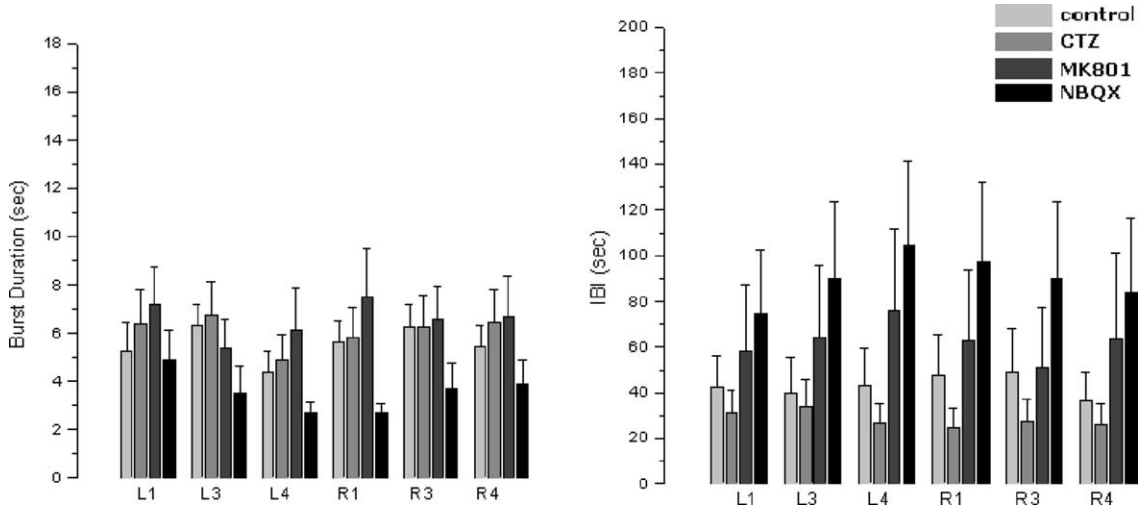

Fig. 4. Mean value (+S.E.) of burst duration (left) and IBI (right) calculated for the four phases of protocol 1 used for a 14 DIV culture.

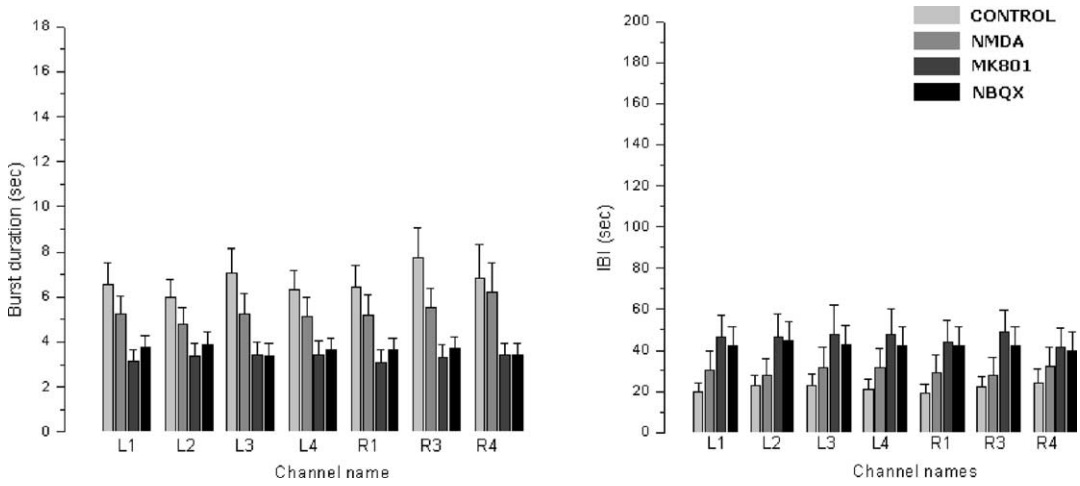

Fig. 5. Mean value (+S.E.) of burst duration (left) and IBI (right) calculated for the four phases of protocol 2 used for a 15 DIV culture.

conditions, while the NMDA treatment gives responses quite similar to the basal situation in term of both IBI and burst duration.

In parallel with the burst analysis, also a spike analysis was performed to characterize the phases from a different point of view and to prove the results obtained employing burst analysis.

We computed an Inter-Spike-Interval histogram (ISI) over a time window of 100 ms, with bin of 2.5 ms for the time scale. Fig. 6 shows the results, for one selected channel, for the ISI applied to the 14 DIV culture treated with Protocol 1. Control condition shows a very weak correlation among the cells, with a small number of spikes in the whole window.

Looking for a function to fit our data we found that, during CTZ treatment, ISI histogram looks like a well-shaped Poisson distribution, monotonically decreasing from the maximum value (in the very first bins) to the minimum: this behavior indicates a good synchronization in the network, as was also predicted by the burst analysis. Even during MK801 and NBQX treatment we can observe a good synchronization, but the number of

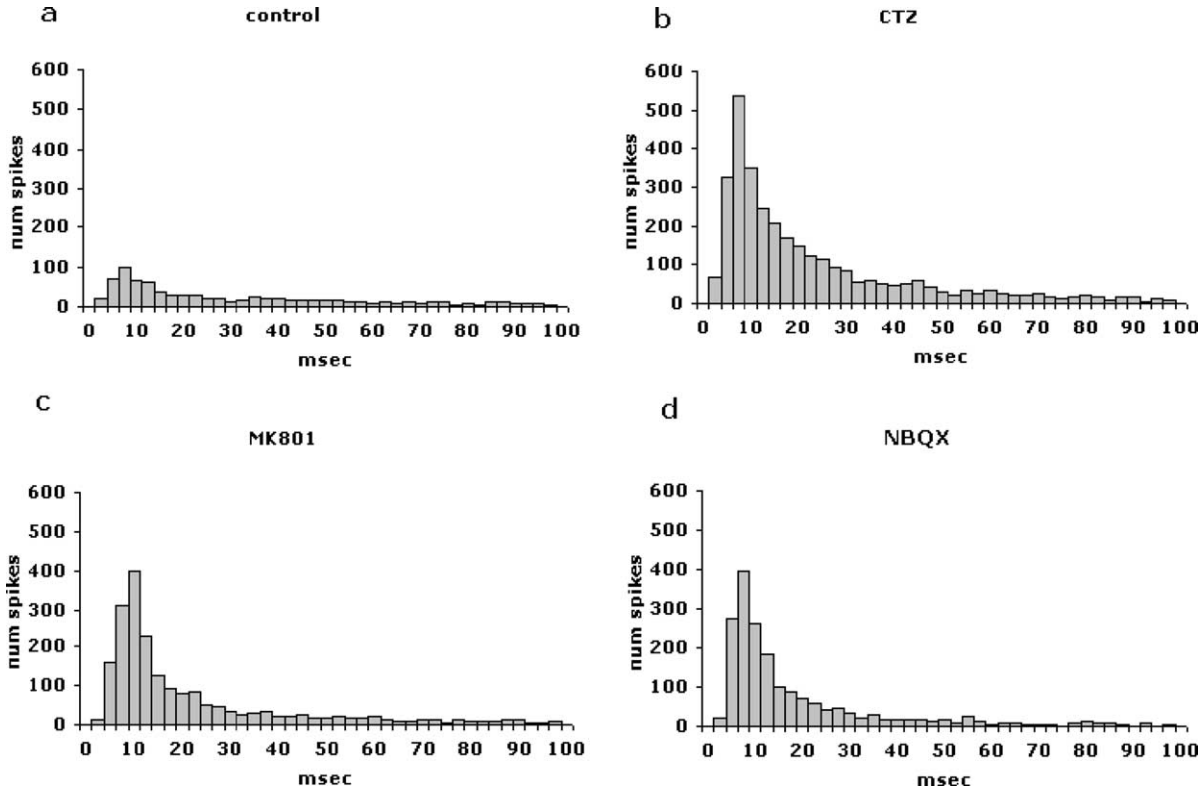

Fig. 6. ISI histogram for the four phases of protocol 1: (a) control, (b) CTZ, (c) MK801, (d) NBQX.

involved spikes is lower than in CTZ phase. This could mean that burst activity is still present but only high frequency spikes are involved, without a low frequency component inside the burst, that usually appear at its beginning and its the end. The range of spike periods for these two phases is between 5 and 15 ms, while in CTZ phase we can observe that spikes periods values are more distributed over the whole time window.

Fig. 7 shows the ISI histogram, for one selected channel for the four phases of Protocol 2.

Same results as in the previous protocol appear for control condition and NMDA treatment. During MK801 and NBQX we can notice the antagonist action of MK801 corresponding to a decrease in the total number of correlated spikes while for NBQX a spike distribution similar to that of NMDA treatment is observed.

4. Discussion and prospects

Recent advances in the ability of growing neuronal networks stable coupled to MEAs together with the availability of commercial system for the monitoring of the electrophysiological activity allow researcher to carry out experiments in a variety of fields ranging from basic research in neuroscience (see e.g. Shahaf and Marom, 2001) to neuropharmacological biosensing investigations.

The aim of this work is to make a step toward the direction of the use of such kind of experimental system as a new bioelectronic 'neuronal sensory system'.

As already reported in the literature, mainly by Gross et al. (Gross and Kowalski, 1991; Gross et al., 1992, 1995; Keefer et al., 2001) the possibility of using living neuronal network for pharmacological investigation is now well demonstrated. These systems show both high sensitivity to neuroactive-toxic compounds and reproducible results (Keefer et al., 2001).

Moreover is likely that more subtle changes induced by pharmaceutical agents can be studied looking at the dynamic of the network in terms of correlation among channels, spiking and bursting activity and electrically modulated activity. As in our work the possibility of investigating membrane receptor sub-sets, can constitute an important instrument also for pharmacological investigations.

The presented results show a modulation in the bursting activity and in spiking activity (inside a single burst) due to the agonist-antagonist and desensitization action of specific chemical compound on the glutamatergic receptors. The designed protocols and experimental system showed the possibility of investigating the intervention of NMDA and non-NMDA receptors to spontaneous activity present in the developing spinal networks, under the effect of different pharmacological substances that specifically act on glutamatergic synapses.

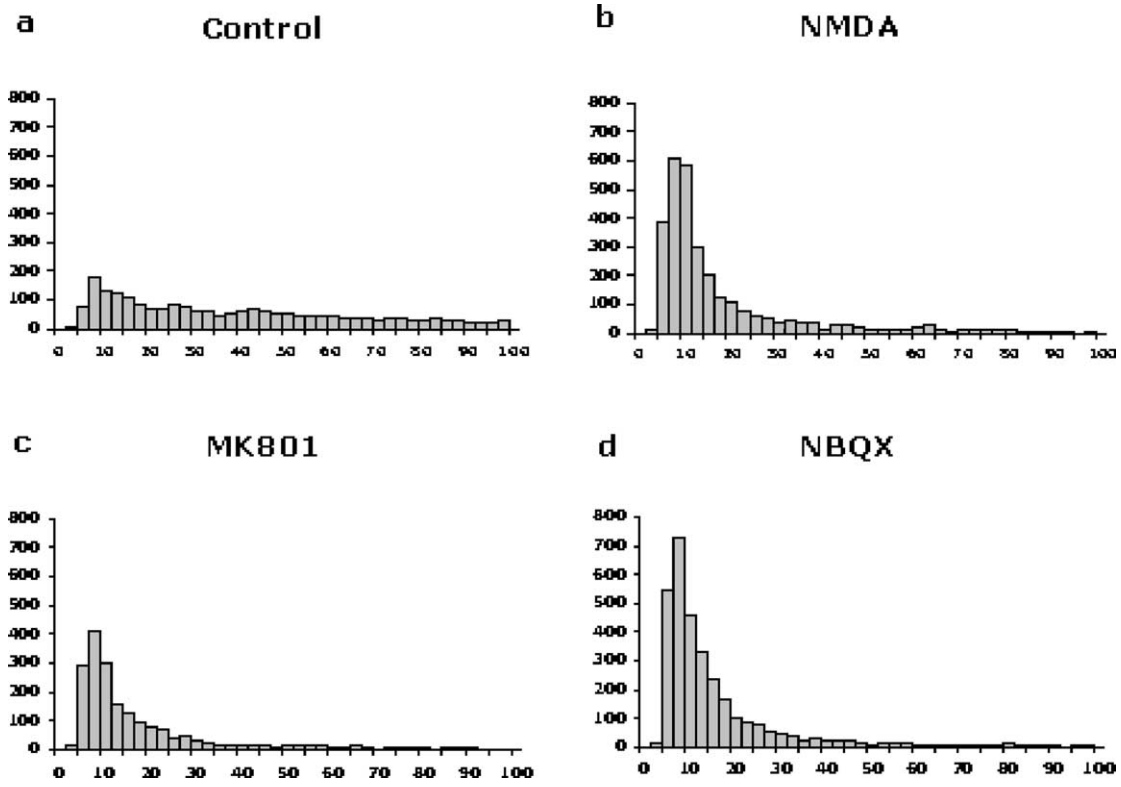

Fig. 7. ISI histogram for the four phases of protocol 2: (a) control, (b) NMDA, (c) MK801, (d) NBQX.

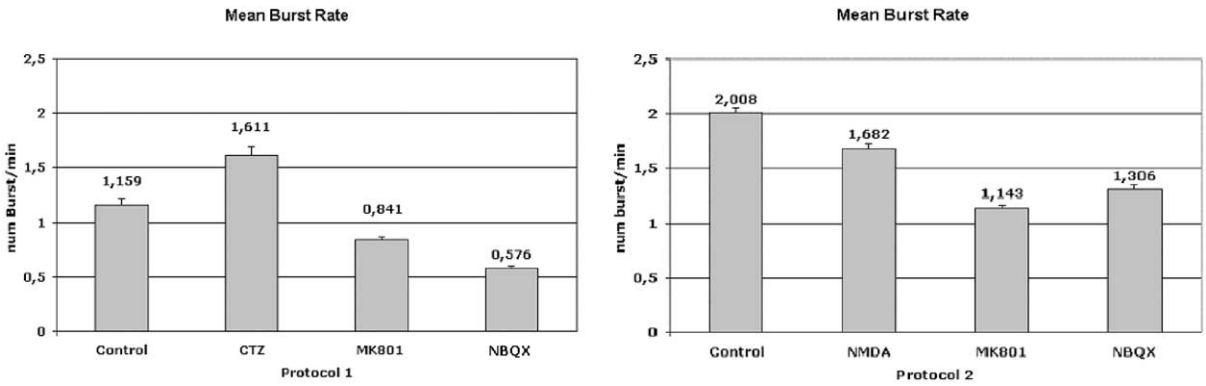

Fig. 8. Mean burst rate (burst/min) calculated over all channels for the four phases of protocol 1 (left) and protocol 2 (right).

As recently reported by IEEE spectrum (Biological warfare canaries, pp. 35, October 2001) on-field application can be foreseen using miniaturized microsystem and microincubators (Potter and DeMarse, 2001). Thus to build a stand-alone embedded system, based on microelectronic devices steadily coupled to a living (neuronal) cell population, which acts as a biosensor for neurotoxic detection. Kovacs and his research group presented a handheld biodetector which measures on-field the signal given by mammalian heart cells maintained alive for several days in a portable system.

Systematic investigations on this kind of bioelectronic systems can lead to further improvements related to possible biosensing application opening new opportunities of studies for rapid screening of pharmaceutical agents.

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