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Sensory Discrimination in Neural Networks of Dissociated Cortical Culture

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Abstract

For the acquisition and discrimination of sensory information, the nervous system comprises a variety of structural and functional instruments. These processes occur in accordance with the brain's physical nature and involve many of its labyrinths. On the other hand, the brain's complexity may pose a challenge in determining the correct mechanisms. Answering the question of why cerebral circuitry prefers some sensory stimuli over others could help us understand how we identify diversity in the world. Modeling the neuronal network in the dissociated cortical culture (DCC) homed on a multielectrode array (MEA) may help to tackle the problem and give an easier setting for research. This *in vivo-like in vitro* system of around 100000 neuronal and glial cells eventually build simplified but realistic neural structure and persist to exist for about two months on MEA. That allows to follow and measure structural and functional refinement and the potential for calculations and coding of information in the newly developed neural circuits as well as to understand the role of activity at the single neuron level that determines effective behavior. Following a month of *in vitro* development of DCC, pairs of electrodes were employed to simulate a variety of sensory inputs using various types of electric stimulation. Single, paired-pulse ((PP; 20 ms interstimulus interval) and 1, 5, 10, 20, and 50 Hz 300 mV stimuli of 1 s duration were repeated after every 20 s or at a random time interval. All channels that exhibited appropriate level of activity were monitored. Experiments revealed that registered channels tended to respond solely to one of the stimulus paradigms, creating or enhancing activity while suppressing responses to other stimuli. The most effective stimulus paradigm was PP stimuli in most cases, however specific cases indicated the efficiency of other stimulus paradigms as well. Even higher frequency stimuli had a chance to be beneficial in situations where low frequency stimuli were generally more effective. Both tonic and burst features were present in multicellular and single-unit responses. Many cases pointed to a phenomena that academics rarely pay attention to: replies that took more than 300 ms.

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They drew our attention since they showed selectivity to stimulator patterns as well. We revealed instant and delayed evoked responses that were not present before certain stimuli were administered, which may be regarded as gradual steps of sensory information processing by neural networks. Simultaneously, frequent exposure to the favored stimuli increased the occurrence of immediate reactions that demonstrated synaptic plasticity for memory formation. Data shows that DCC's small neural networks are highly sensitive to physical characteristics of sensory input, allowing sensory discrimination.

Keywords: Dissociated cortical culture; multielectrode array; sensory discrimination; in vivo-like in vitro; neural network.

1. Introduction

Human ambitions to decode the brain's intricate nature and develop similar artificial and cyborg systems are growing rapidly in our century. Brain-machine systems, in particular, are of particular interest because they have limitless potential in the medical area and in human life [1].

A modern effective scientific approach for registration of multiple electrophysiological signals, modeling brain to body feedback system, and even development of prototypes of neuroprostethic devices is an *in vivo like in vitro* system of dissociated cortical culture (DCC) grown on the multielectrode array (MEA). During development, bare neural cells from dissociated cortical tissue change shape and acquire axons and dendrites that connect to one another, forming neural networks that are comparable to those found in neural tissue. Action potentials are generated by neurons. Implanted on the glass surface electrodes designed for both stimulation and recording in the parallel regime allow to imitate sensory inputs over various areas of neural culture on the one hand, and to record a variety of evoked electric neural signals from the entire surface on the other hand, which reflect the complex nature of information processing [2].

They can be utilized as inputs to sophisticated algorithms that control body parts, robotic prosthesis, and virtual animations. It was possible in several works to develop feedback cyborg systems of DCC and robotic body, in which neural network received information about robotic body in permanent manner, could save information and could improve performance by experience, that actually depended on the neural plasticity processes in the neuronal culture. Similar cyborg systems enable to learn how to employ brain signals for managing machins, at one hand and to examine high nervous functions in the highly suitable long-term opened visible dinamic preparation of the forming neural networks, on the other hand [3].

Approaches of prior cyborg systems of the DCC and robotic body (or virtual body) depended on the evoked responses of selected stimuli that were utilized for coding information and decoding patterns of spike responses for driving cybernetic parts [4]. We came up with the idea of using this technology to imitate various types of sensory information and investigate the fundamentals of sensory discrimination processes in such a small colony of roughly 100000 neural cells that eventually grow and construct neural networks and resemble neural tissue. We wanted to know if it's conceivable in small neural networks, and if so, how and when it happens. What kind of kinetics may it have?

We mastered the production of long-living DCCs for these purposes, which were allowed to mature for one month in MEA. At one month of *in vitro* cultivation, DCC was subjected to a variety of electric stimuli, employing the same and/or distinct inputs (pair of electrodes) to simulate varied sensory patterns. During stimulations, continuous registration of neuronal responses was carried out, revealing varied patterns of early and late responses across the culture area.

Data show that neuronal networks of DCC contain great selectivity to physical attributes and spatial position of sensory input, allowing for sensory discrimination. Instant and delayed evoked responses should reflect instant and delayed information processing, with the likelihood of rapid responses increasing during training sessions with preferred stimuli, indicating engaged memory formation neural plasticity mechanisms. Simultaneously, it highlights some of the complexity of sensory discrimination by emphasizing the gradual nature of information processing.

2. Materials and Methods

Experiments were carried out according to the norms of the International animal care and use committee (IACUC) and bioethics committee of Free University of Tbilisi.

Preparation of DCC. DCCs were prepared according to the standard approaches with significant modifications to meet our experimental demands. The major manual belong to the competent group of Potter [5] that define techniques concerning the preparation, care and electrophysiological registration from the DCC on the multielectrode array. At the 18th day of pregnancy, cortical peaces were taken from the brain of a rat fetus (total number 36 from the 12 litters). The use of a biosafety cabinet class 2 and 70% alcohol helped to ensure sterility. To insert cortical pieces and clear neural tissue from blood and arachnoid tissue, a cold Henks balanced salt solution was utilized. In order to inhibit excitatory processes linked to seisures, excitotoxicity and apoptosis in a mechanically dissociated brain tissue Kynurenic acid (final concentation 1 mM) and selective agonist of NMDA receptors, AP5 (0.025 mM final concentration) were added to solution. For tissue fermentive digestion, a mixture of active papain (15 units) and DNase (50 μM) was utilized for 20 minutes. Digested tissue remains were rinsed 3 times with culture medium that was CO2 independent hibernation medium (from Thermofisher) with B27 plus supplement (2%) and fetal bovine serum (10 %). Special microfilters with 40 μm phores were employed to filter tissue remains in order to get clear colony of dissociated cells (neurons and glial cells together) and to clean solution from the remains of arachnoid tissue. After, bovine serum albumin was added to solution. It was centrifuged at 200x speed and rinsed out again with a final medium in order to remove toxins released during a process of tissue digestion and dissociation from the cells. Using a cytometer grid and a microscope, the concentration of cells was measured to be around 1000-2000 cells per μl. Dilution or centrifugation were used to adjust for outreging of a standard concentration. We didn't try to separate neurons from glial cells because glial cells aid in the survival of neurons and provide natural space for development. 10 μl of cell suspension was poured over the surface of MEA central region, which itself was previously couted with polyethilen imine (0.05 %) and laminin (0.001 %) for 30 s. The cells were allowed to attach to the surface for 30 s before the final media was introduced to the culture. HEPES (hydroxy-ethil-hyperazin-ethan-solphonic acid) solution (0.01 M final concentration) was used to ensure extra buffer characteristics of the surrounding

tissue media. In the incubator, the temperature was 36°C and the humidity was 65 %. To ensure tissue with proper intrinsic cell survival factors, half of the medium was changed twice a week and the other half was left. To maintain sterile and hermetic conditions, a suitable glass cover was adapted to the ring of MEA. Neural culture was kept in those settings for around 2 months, that for our situation was an excellent duration during that tussue appeared healthy and it could generate action potentials. Morphological control. Under the microscope, morphological control of DCCs was done once during cytometric adjustment of cell concentration and twice weekly during the experiment. For it, an inversive digital microscope with magnification of 100X to 200X was used. Pictures were taken with the AMCAP program that came with the digital camera. Concentration of cells, cleanness of around area, cell position related to electrodes and development degree of the neural network was noticed during the microscopy. Pictures were collected from the same two areas in order to have better comparison conditions: One was the square area of 9 electrodes from electrode N22; the other was the square area of 9 electrodes from electrode N77. Differentiation quality of observed neural networks were evaluated by the length of neural fibers that was simply estimated by geometric approaches compared to the diameter of electrodes (30 μm). Recording. Multichannelsystems Co. is responsible for the setup of 60 channel systems for electrophysiological research (Germany). The main device, the MEA1060-UP-BC, is a preamplifier suited for 60 electrode MEA recordings and is capable of ensuring blanking circuitry allowing registration from the stimulating electrodes with the others; the preamplifier, stimulator STG4002, and the computer (with the supplied proper cardboard) are synchronized with each other to ensure coordinated work of the system; for registration, stimulation, and selection of the sets of stimulating, recording, and grounding electrodes and the Company's free softwares: MC_Rack, MC_Stimulus, and MEA_Select were utilized.

From a methodological standpoint, the stimulation techniques were perhaps the most crucial aspect of the experiment because different types of stimuli were connected with a diversity of sensory information. For single, paired-pulse stimulations with a 20 ms interstimulus interval and varying frequencies of stimulations of 1, 5, 10, 20, 50, and 100 Hz of stimuli of 1 s duration, rectangular impulses with two phases of 100 μs duration and tension of 300 mV were utilized. All types of stimulations were applied once every 20 s (or at a close random time interval) from a pair of electrodes, with the duration of the stimulation session changing according to the experimental protocols and recorded responses.

To begin registration sessions, the phases of spontaneous multi-neuronal activity, which served as a background level for evoked activity, were always used. The signals were amplified 10000 times and filtered with a high-pass filter (>200, with the function of Butterworth 2nd order). After that, stimulation operations were carried out, and registration was carried out for an appropriate amount of time. The multichannel layout of the registering software, as well as the used system, allowed for dimensional dispersion of the signal processing.

Analyzes. The Python programming language was utilized to develop a data analysis program application pack (NeuroSpace) that was used to move data from MC_Rack files (mcd format) to appropriate graphical and numerical datafiles, which were then processed in the SPSS statistical program. NeuroSpace enabled the presentation of real-time signals from a specific channel, the separation of appropriate frequencies, the processing of specified partitions, the separation of single levels of neurons, the detection of neuronal vs network bursts, and the generation of appropriate datafiles. In total, 40 MC_Rack electrophysiological

recordings were examined using this method. From cultures ranging in age from 25 to 35 days *in vitro* (DIV), electrophysiological data was compiled for this work. On the other hand, data were categorized according to the stimuli used: 300 mV single, paired-pulse with a 20 ms interstimulus interval and varying stimulation frequencies of 1, 5, 10, 20, 50, and 100 Hz for 1 s stimuli. The characteristics of interest for evaluation were the spike frequencies of multi-units and single-units, which were estimated before and after the applied stimulus paradigms. These comparisons were carried out in three different ways: 1. In short-term (up to 300 ms) periods; 2. In longer-term (up to 1000 ms) periods; and 3. In general activity levels generated by specific stimuli.

One-way ANOVA analyses were used to compare activity levels before and after stimuli (for both multiunit and single unit data). If early and later post-stimulus response areas were evaluated, Multifactorial ANOVA analyses were applied; and Multifactorial ANOVA analyses were utilized as well when pre- and post-stimulus activity levels were compared with different stimuli types.

3. Results

Morphology. Morphological monitoring of neuronal culture began on 0 DIV, when the cells had reached a sufficient concentration. At the time, cells represented oval/round shaped white entities with no fiber descendants. That also reveals that through enzymatic digestion neural tissue lost all properties adhering cell together that form neural tissue and we acquired dissociated healthy cells (Pic. 1, A). In the next time, morphological control was performed twice a week. Differentiated cell bodies were already observed at 3 DIV and axo-dendritic fibers reached about 40 $\pm$ 15 μm after 7 DIV (n=30). Gradual increase of cell fibers were noticed in following weeks of cultivation: 60 $\pm$ 17 at 14 DIV, 80 $\pm$ 28 at 21 DIV that did not change significantly later (Pic. 1, B). After 3-4 weeks, not all cells showed fiber extension, but some did, as well as a consistent decrease in cell number over the counted region.

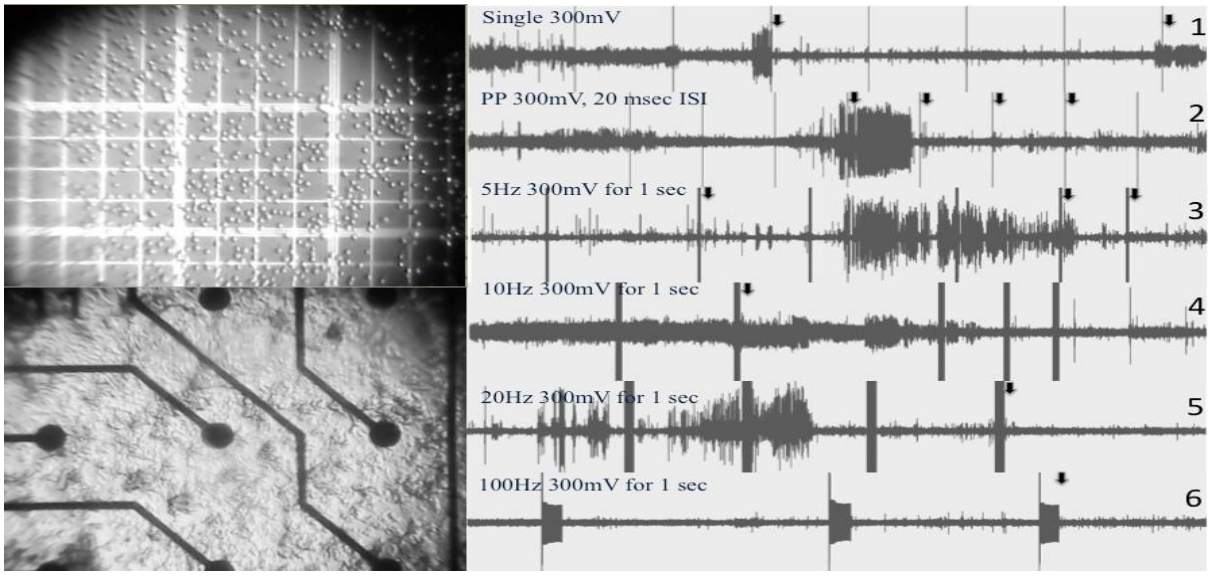

Figure 1

Pic. 1. A – DCC on the hemocytometer, exhibiting adequate dispersion in the suspension and healthy cells. **B** –

DCC at the day 25DIV, showing developed neuronal cells with axonal and dendritic fibers. **C** - Discrimination of multi-unit neuronal evoked responses to a variety of electric stimuli. Above the recordings applied paradigms are indicated. Black arrows show location of evoked responses generated in a short duration (<=300 ms) of applied stimulation. There is a change of quantity of activity in response to low frequency stimuli and particularly PP stimuli.

Electrophysiology. – Electrophysiological recordings of multiunit activity were done using the MC Rack program supplied by multichannelsystems Co., and single-unit responses were isolated during analysis using the NeuroSpace software, which was built by us in Python. For this experiment, electrophysiological registration of DCC preparations was done exclusively from the 25 to 35 DIV samples in order to eliminate variable level in electrophysiological characteristics.

Neuronal signals of the spontaneous responses were registered initially and permitted for recording for around 10 minutes to develop a background level of activity before stimulation sessions were commenced. DCC preparations were characterized by substantial variation in development that lead some channels to be more active compared to others, from which some channels never got activated. At the same time, spontaneous activity was characterized by a wide range of responses, from infrequent spikes to strong bursts that became more prominent with age. The essential condition for registration, however, was that the channel must have been active for long enough to have registered for both spontaneous and stimulation sessions (at least 30 min). After a spontaneous activity registration session, the pair of electrodes were used to elicit a range of electric stimulus sessions using random time intervals. Stimulation sessions revealed that a variety of electric stimuli elicited particular tonic and burst responses in DCC and that these responses were very variable. In most cases they displayed preferred responsiveness to low frequency and specifically, to 300 mV paired-pulse stimuli, when other forms of electric stimuli were neglected or decreased level of activity (Pic. 1, C). However, in unusual conditions, greater susceptibility to other types of stimuli was also observed (Pic. 1, C 4, 5, 6). At the same time, it was detected that when an electric stimulus coincided with enhanced network activity, especially bursts, inhibition was typically followed (Pic. 1, C 1).

Changes in stimulation frequency or the relocation of stimulatory electrodes were other factors that caused alterations in the spatial distribution of electric responses. This frequently resulted in the activation of additional channels and/or a decrease of reactions in active channels. Furthermore, it was found that stimulation tension had a significant effect. When greater or weaker stimuli were largely neglected or had an inhibitory impact, an intermediate tension of about 300 mV was optimum for producing electric responses. At the same time, discrimination properties were so particular that even doubled version of PP stimuli were far from the effect that was shown with the PP stimuli.

In most cases, a rise in activity did not occur immediately, and some training sessions were required before the network was engaged. Unexpectedly, network activation started before occurrence of evoked responses, which only became prevalent after training sessions (Pic. 1, C 2). Typically, such registrations showed gradual activation of neural circuits that reached an optimal level before being inhibited again by the network. These stages were usually performed several times. Evoked responses largely related to PP stimuli. However, training

sessions were necessary in order to increase the likelihood of its success (Pic. 1, C 2). Accordingly, data analyses required a method for distributing specific examples and distinguishing between a background, training, and trained phases. As a result, trained networks still showed different probability of the evoked responses that reached approximately 60 % of cases for PP stimuli, 35-40 % for single or 5 Hz stimuli, about 15-20 % for 10 Hz stimuli, up to 10 % for 20 Hz and as low as 5 % for 50 and 100 Hz stimuli.

Delayed responses to certain stimuli with a latency of more than 300 ms were also the subject of interest. The data showed that they were largely tied to the specified favored stimuli and had little to do with other stimuli or random cases of spontaneous activity.

Data analyze at the neuronal level with NeuroSpace revealed that some of DCC single neurons in multiunit recordings reacted differently than the network as a whole, generating varying levels of responses on different types of stimuli (Pic. 1 C). The antagonistic nature of neuron populations was frequently highlighted by the spatial position of active channels, which promoted activity in some channels while inhibiting activity in others.

4. Discussion

MEA and related multichannel system setup make it possible to interface with DCC's neural networks in a near to the natural way, to stimulate any collection of electrodes, to record responses from the entire surface, and to analyze according long-term morpho-functional adjustments. This presents a perfect setting for studying sensory perception processes at the neural network level. In order to investigate the basics of sensory discrimination, we used a variety of electric stimuli from the same and different sources to imitate a variety of sensory inputs, recorded responses, and analyzed the potential of these reactions for coding and decoding a variety of information. Experiments revealed that DCC's newly built neural networks showed indications of basic level of sensory discrimination: active DCC regions responded to one of the supplied stimuli, establishing or raising levels of activity, while ignoring or lowering reactions to other stimuli. We found immediate and delayed evoked responses that were not present prior to the introduction of particular stimuli, indicating various steps of information processing. Simultaneously, training with the preferred stimuli resulted induced more frequent immediate responses, demonstrating triggered neuronal plasticity in the relevant neural circuits.

Previous research has shown that DCCs are responsive to some produced low frequency stimuli and inhibited by high frequency stimuli in independent investigations [6, 7, 8]. We gained ideas for simulation of numerous sensory inputs from that studies, which may be accomplished by varying the frequency, intensity, or sequence of impulses.

Our study's main finding was that individual cultures, or even particular portions within the same culture, show great selectivity for one of the many stimuli and increase activity to that stimulation while activating less effectively or decreasing activity to other stimuli. In spite of the preference to the PP stimulus or single stimuli or low frequency stimuli, the physical structure and features of the particular network establish individual nature for selection. Surely, particular selectivity to electric stimuli can hint about the individual properties of the supplied neural network. Another topic concerns how training with specific stimuli aids in the establishment of a

specific preference for those stimuli. That can be the focus of future investigations, however, we suggest that there should be an interplay of two related phenomena: The physical structure of certain neural circuits provides selective preference to the specified electric patterns, therefore various patterns of persistent electric stimuli can improve effectiveness to that stimuli. Furthermore, we agree with Nieus and colleagues' recent findings in hippocampal neural cultures [9] regarding state-dependent representation of stimulus-evoked activity, which was frequently indicated in our recordings and it could play an important role in seizure disorder management from a medical standpoint.

It was revealed that favored stimuli elicit gradually rising early and late responses, showing that diverse neural circuits involve gradually in information processing, on the one hand, and that early and late stages of sensory information processing may exist, on the other hand. Most research concentrate on short-term evoked responses, which reflect the features of fully established functional brain tissue. However, DCC symbolizes a developing neural circuitry at various phases, beginning with a colony of completely bare brain cells and progressing to a fully developed neural network. Certainly, the degree of activation of DCC's isolated neural networks differs significantly from that of *in vivo* neural tissue, as well as by some points for *in vitro* tissue culture, which is a major enviromental determinant for development. Newly created neural connections of DCC obtain outside information from the electric stimuli delivered in our experimental settings that may be considered as a training for the existing synaptic connections. Usually, Increase of responses occurs repeatedly that could be associated to the restricted amount of the released neurotransmitter that need a time for rebuilding full synaptic capacity. On the other hand, it alludes to the gradual activation of the systems of information processing. The establishment of synapses is aided by training, which is a key aspect in the correct development of neural tissue. Simultaneously, it increases the probability of evoked responses in the presence of favored stimuli, as demonstrated in our study.

Our experimental findings point to the importance of the later responses for the discrimination of stimuli as well that should be reflection of the prolonged neuroplasticity processes that could assist information coding especially in the developing neural circuits. Data show that generation of later responses also depend on the stimulus specifity and are not present before they are applied. Literature findings are rare for that responses and the main accent fall on the evoked responses of short latency. However, several works indicate to the importance of delayed responses in the neural plasticity processes [10].

Instant evoked and delayed responses should reflect instant and delayed information processing, with the chance of quick responses rising after training sessions with preferred stimuli. It demonstrates the effect of synaptic plasticity for memory formation in response to favorable sensory input. At the same time, it emphasizes the progressive nature of information processing in order to memorize it.

5. Conclusions

1. The newly developed neural networks of DCC structure contain sufficient mechanisms for implementing sensory discrimination.

2. Sensory discrimination in DCC's neural circuits is a complex process with both immediate and delayed components that continues with training and produces favorable conditions for neuronal plasticity to memorize preferred stimuli and produce quick responses with increased probabilities.

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