The Use of Neuronal Networks, Cultured on Microelectrode Arrays, to Explore the Pharmacological and Neurotoxicological Effects of Different Compounds

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Microelectrode arrays (MEAs) have been in use over the past decade and a half to study multiple aspects of electrically excitable cells. In particular, MEAs have been applied to explore the pharmacological and toxicological effects of numerous compounds on spontaneous activity of neuronal and cardiac cell networks. The MEA system enables simultaneous extracellular recordings from multiple sites in the network in real time, increasing spatial resolution and thereby providing a robust measure of network activity.

Key words: Microelectrode arrays, amyloid-beta peptides, neuronal networks, Alzheimer's disease.

Introduction

In the mid-70s the young neurobiologists Guenter Gross and Dieter Weiss, who worked under the guidance of the renowned neuropathologist Georg Kreuzberg at the Max Planck Institute for Psychiatry in Martinsried, Munich, shared the idea of "eavesdropping" on the "conversations" between cultured neuronal cells. For this purpose, however, they lacked the appropriate spyware.

In 1979, Gross published an article [4] describing a tissue culture chamber which bottom was covered by a dense grid of microelectrodes, created by photo-etching and electroplating. He considered that such a camera can be used for simultaneous long-term recordings of the extracellular electrical activity of more than 30 individual neurons in culture. However, a number of problems had to be solved - the substrate must not be toxic to the culture, the electrodes must be well insulated, with only their tips allowed to communicate with the neurons. And he had an additional goal – to find electrodes which are completely transparent to allow light-microscopic observation of the morphological characteristics of neurons during treatment.

In the late 70s Gross left Germany and returned to the USA where he continued his work on the creation of the so-called MEA (microelectrode array – array of microelectrodes on which neuronal cells are cultured).

Methods

In 1985 Gross published an article in which he described the newly created MEA [5], with transparent electrodes from indium tin oxide on a 5×5 cm glass plate (chip). The bottom of the chip was isolated with a layer of polysiloxane. The isolation of the contact tips of the electrodes was removed by a single laser pulse and the tip was coated with colloidal gold to reduce impedance. The recording end of the electrode is located on the periphery of the chip where it is connected to an amplifier. On the chip there are 64 electrodes. Before seeding the MEAs are processed so as to create conditions for maximum adhesion of cells. For this purpose the central part of the MEA is flamed through a mask of stainless steel, whereby the methyl groups of the polysiloxane are oxidized to hydroxyl groups, which creates a hydrophilic surface that provides good adhesion of the cells. Polylysine and laminine stick only to the flamed area of the MEA.

Monolayer tissue cultures are grown on MEA from dissociated tissues that are prepared from certain areas of the brain or spinal cord of 14-15-day-old mouse embryos (**Fig. 1**).
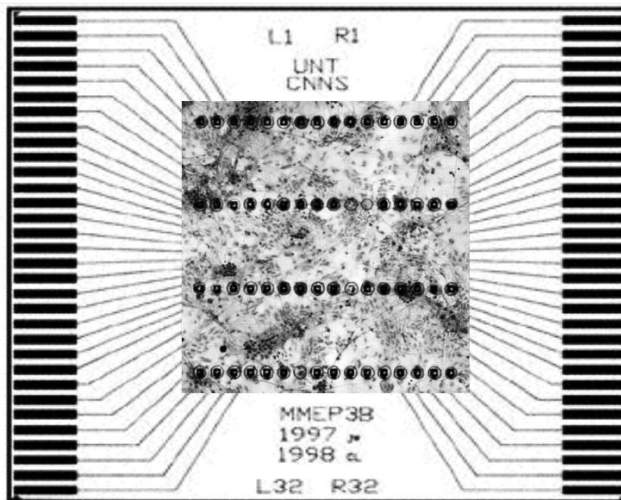

Fig. 1. Microelectrode array with neuronal network cultured on the microelectrodes

The spatiotemporal pattern of electrical activity (extracellular action potentials) provides important information regarding network structure and function that is difficult or impossible to obtain using other electrophysiological techniques. The electrical activity of the individual neurons is recorded on the tip of the electrode and is fed to its terminus at the periphery of the chip and then through an external amplifier into a computer with specialized software.

Around the end of the first week after seeding the cultures develop spontaneous electrical activity, which is stabilized about the third week in culture. Each tissue cul-

ture, which is a network of interconnected neurons, acquires characteristic electrical activity, which is considered native. This native activity is used as a basis for comparison in experimental treatments of the cultures with various agents.

Up to 4 different single neuron activities can be distinguished from the electrical activity derived from one electrode.

Action potentials (impulses), also called spikes, are integrated according to certain criteria, such as frequency, maximum and minimum interval between single spikes etc., resulting in defined groups of pulses termed bursts. These two parameters – spikes and bursts characterize the electrical activity of every culture.

Hereafter we shall call the network of neuronal cells cultured on MEA simply MEA. This experimental model proved to be very suitable for testing the toxicity of various substances as it provides information about the chemical effects leading to disruption of the function of nerve cells, which allows to reveal the mechanisms of action of a particular compound.

Results

The neurotoxin trimethyl-tin (TMT) is one of the first substances whose effect was investigated on neurons cultured on MEA, as it is known that TMT causes acute changes in the function of nerve cells, which directly affect the transmission of signals (signal transduction) between the neurons [1].

It can be seen that the cells begin to react at low concentrations (2-3 µM) and at 4 µM one signal disappears completely. Removing TMT from the culture by medium change results in recovery of the native activity (**Fig. 2**).

When the amplitude and the form of the action potential remain unchanged upon treatment with a particular agent, it can be concluded that the metabolism of the cell is not impaired, and the effect is directly on the synapse. No morphological changes in
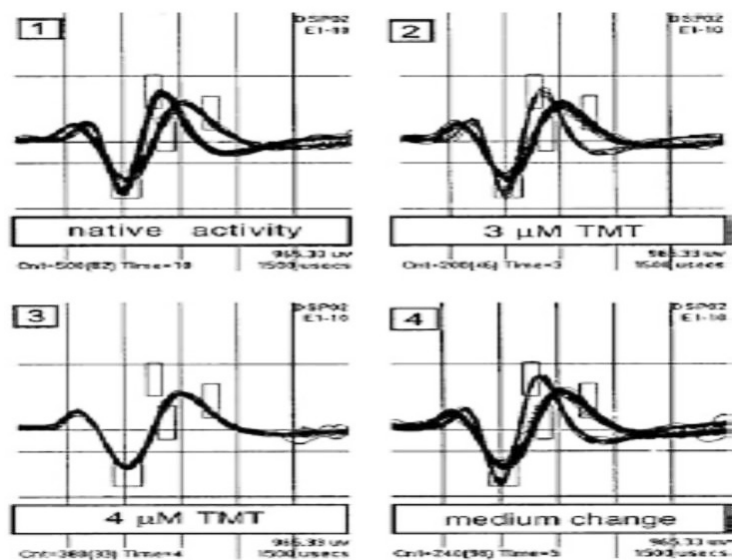

Fig. 2. Reversible inhibition of electrical activity by TMT

the cells have been observed at these concentrations. At higher concentrations (50-100 mM), there was total inhibition of electrical activity which cannot be restored with repeated medium changes. Such concentration of TMT are in the range of concentrations that are lethal to mice when administered *in vivo*. This and many other examples demonstrate the comparability of the results obtained *in vitro* with the MEA to those obtained *in vivo*.

Employing the MEA model system we investigated the effect of the amyloid β-peptides on the electrical activity of neuronal networks, cultured on MEA. The amyloid β-peptides are aberrant metabolic products of the amyloid precursor protein (APP) and are involved in the etiology of Alzheimer's disease. There are various assumptions about the mechanism of their toxicity. There are numerous publications that describe different effects of the amyloid β-peptides on neuronal cells. Some of the major ones are: inhibition of cell adhesion in cultures; inhibition of the outgrowth of neurites; disruption of calcium homeostasis; inducing oxidative stress; causing abnormal phosphorylation of the τ-protein; initiation of apoptotic mechanisms; participation in inflammatory mechanisms; effects on electrophysiological processes etc.

In our initial experiments with the peptide which is the carrier of the biological activity, consisting of the amino acid sequence of Aβ25-35 [6], we found a concentration-dependent inhibition of the electrical activity of cultured neuronal networks (**Fig. 3**).
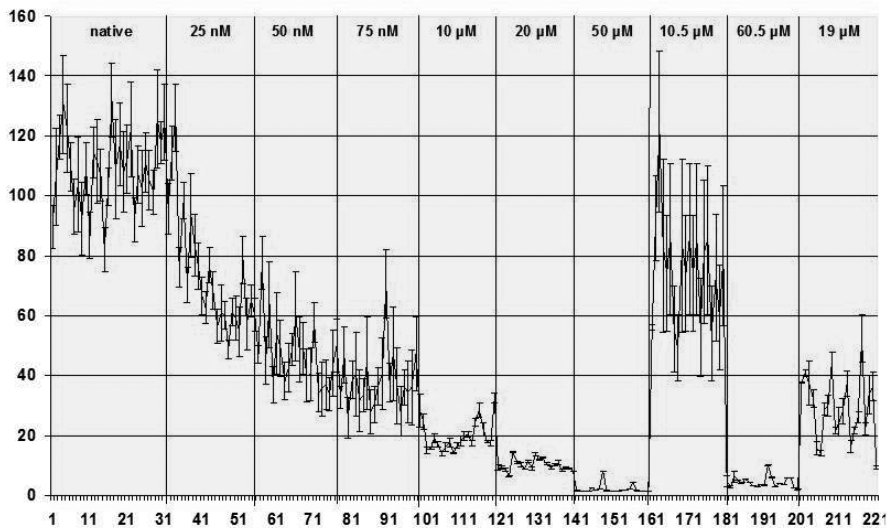

Fig. 3. Effect of Aβ25-35 on the spike rate of a spinal cord network

The effect is rapid, in the first three to five minutes after the addition of peptide, and reversible. Upon lowering of the concentration of the peptide by medium changes, the electrical activity of the neurons recovers.

Treatment with Aβ25-35 influences neither the amplitude nor the shape of action potentials, which indicates that the energy metabolism of neurons is not affected.

In summary, the effects of Aβ25-35 on the electrical activity of the neuronal networks are as follows: fast – 3-5 min after addition of the peptide; concentration-dependent; reversible; do not influence the amplitude or the shape of the action potentials; Central Nervous System region-specific.

Having established the inhibitory effect of Aβ25-35, we naturally had to think about the mechanism of action of this peptide. One of the most frequently reported effects of Aβ-peptides is the induction of oxidative stress. Therefore, we compared the effect of Aβ25-35 with that of the well-known agent causing peroxidation of membrane lipids – divalent iron ions (Fe^{2+}) [7].

We found that in comparison to the effect of the amyloid peptide, the effects of Fe^{2+} ions was slower (90 min after addition) and irreversible (**Fig. 4**). On the basis of these results we can suggest that unlike the non-specific general toxic effect of lipid peroxidation, the effect of Aβ has a specific site of action.
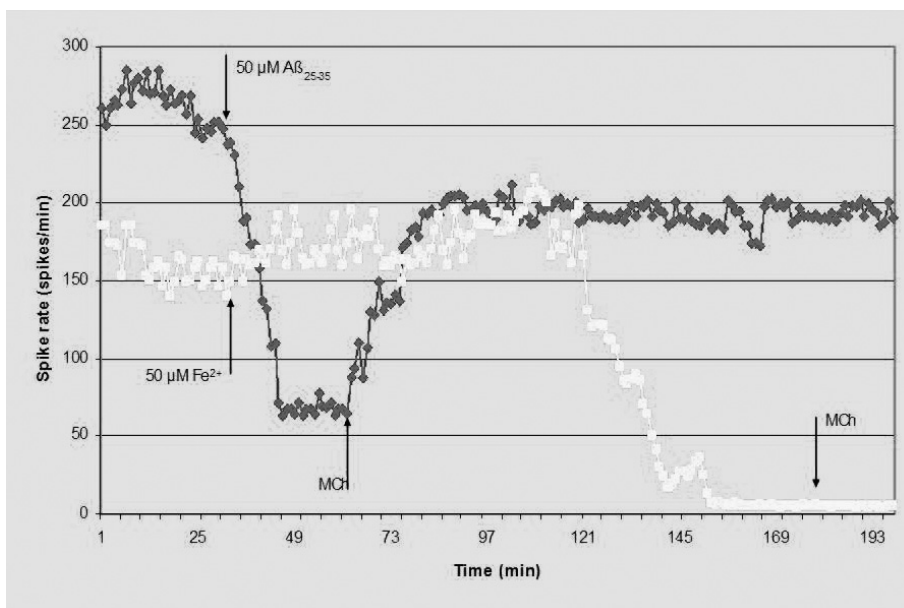

Fig. 4. Differential effects of Aβ25-35 and Fe^{2+} ions on the spike rate of cultured neuronal networks

This conclusion is supported further by additional experiments in which the effects of Aβ25-35 were compared with that of the well-known oxidants – divalent iron ions and hydrogen peroxide on the redox activity and viability of cultured neuronal cells. The effect of Aβ is always the fastest and most prominent one. It is not affected by the antioxidants vitamin E and propyl gallate, unlike the protective effect these antioxidants exhibit against the effects of the iron ions and hydrogen peroxide.

Almost identical results were obtained in studies of the effect of two Aβ-peptides with different amino acid chain length - Aβ1-40 and Aβ1-42 [8].

Starting in 2001, colleagues from the Universities in Rostock and Leipzig have taken upon themselves the laborious task of creating a database, encompassing the effects of numerous neuro-active substances, with a known mechanism of action, on neuronal networks, cultured on MEA. They record about 100 characteristics of the effects of these substances on the electrical activity of cultured neuronal networks. In this way they create a kind of pharmacological "fingerprint" of each substance. These features are introduced in the database, which is then used to compare the effects of unknown substances with those of well-known and characterized ones. This allows to get direc-

tion to search for the mechanism of action of the unknown substance and significantly shortens the period of experimental search [2, 3].

Using this approach in the database the characteristics of the effects of the investigated by us Aβ-peptides have been introduced and compared with those of neuroactive substances with known mechanisms of action [9]. The observed effects are illustrated with 5 characteristics of neuronal electrical activity:

– Burst shape: the average duration of plateaus in bursts (burst plateau);
– Synchronisation: the number of coordinated spikes within 1 ms (simplex);
– General activity in bursts: mean burst amplitude (burst amplitude);
– General spiking activity: mean spike rate (spike rate).

Employing the data analyzing tool for pattern recognition – Pattern Expert, we compared the influence of four "fingerprinted" compounds to the effects of Aβ peptides. The compounds are as follows: baclofen (a direct agonist of GABAB receptors), levetiracetam (opposes the activity of negative modulators of GABA and glycine-gated currents and partially inhibits N-type calcium currents in neuronal cells), fentanyl (a strong agonist at μ- and kappa-opiate receptors), and diazepam (a GABAA receptor agonist). The effects of the first three compounds on cultured neuronal networks are shown in **Fig. 5**.
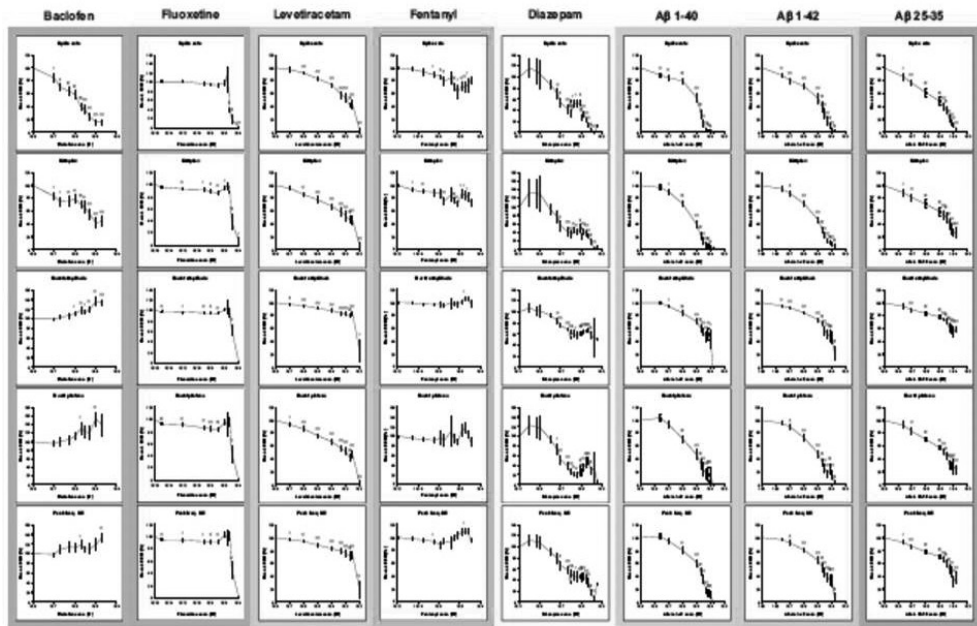

Fig. 5. Selected spike train parameters for 5 compounds and the 3 Aβ peptides

Pattern recognition and classification experiments indicate a similarity of the effects of Aβ peptides with GABAA receptor agonists such as diazepam, clonazepam and propofol (**Fig. 6**). We observe a course of cessation, which is known for example from GABAA agonists. This allows us to suggest the involvement of at least one ion-channel receptor mechanism, as it is controversially discussed in literature.
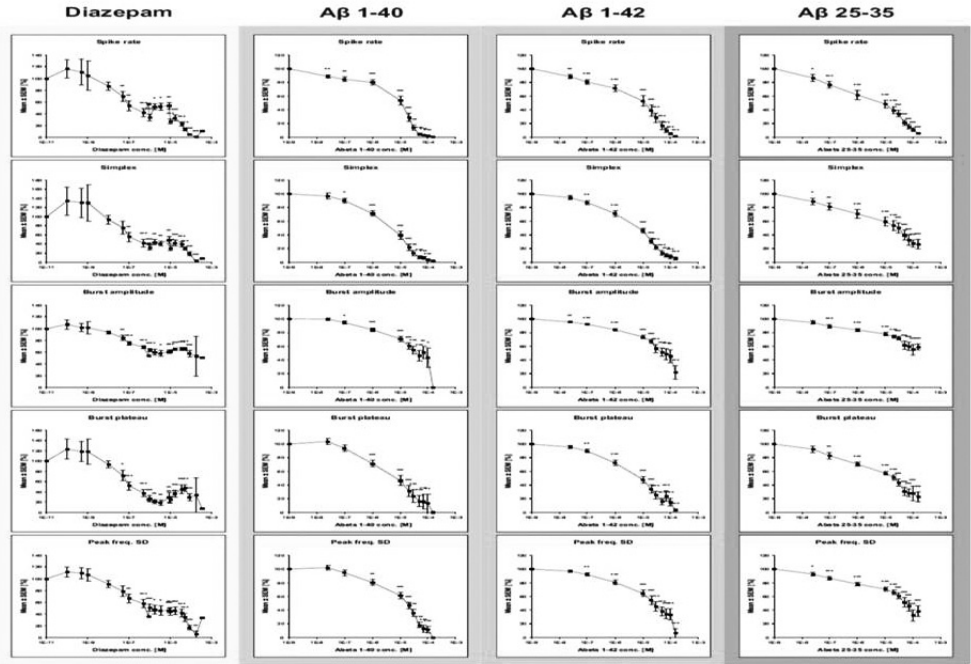
Fig. 6. Selected spike train parameters for diazepam and the 3 Aβ peptides

Discussion

These results lead us to the hypothesis that the rapid neurotoxic effect of increased amyloid beta peptide concentrations on the synaptic function could cause the early memory loss in Alzheimer's disease, preceding the synaptic loss and neuron degeneration. By hypothesis this mechanism seems to be a similar mechanism as it is described in literature for amnesia caused by benzodiazepine treatment.

At present modern MEA systems have different applications. There are numerous attempts MEA chips to be used for monitoring of toxic substances in the environment – air and water. For instance, water samples could be constantly fed to MEA chips. The presence of toxic substances in the water would change immediately the electrical activity of cells in the chip and will be reported instantly.

MEA systems offer greater flexibility in terms of biological tissue and experimental design. Electrodes of the MEA can be placed flat – for monolayer cultures, as the example discussed. A wide variety of electrically excitable biological tissues can be placed on the MEA. This includes tissue from the heart muscle, primary cultures of nervous system tissues from various regions of the central nervous system, whole tissue pieces – for example a slice of the hippocampus or the retina. MEA chips specialized for a particular tissue can be purchased from commercial suppliers.

MEAs may also have a three-dimensional structure, which allows the penetration of the electrodes through the outer layer of the damaged cells in tissue sections, thereby allowing the electrode to contact with the neighboring healthy cells. The spatial

46

arrangement of the electrodes can be changed, for example to place more electrodes in specific regions of the hippocampal slice or retina.

After use, the biological tissue can be removed from the MEA and can be used repeatedly. If treated carefully MEAs can be quite economical ($ 10-30 / per use).

The added value of this experimental model is significant. The approach to determining the toxicity is in the transition state from intensive *in vivo* testing the toxicity of various agents on animals, to approaches based on *in vitro* screening, which also allow the processing of large numbers of samples in a short time. The requirement for a better understanding of the potential hazards of tens of thousands of substances, currently used in everyday life, and the need to increase the number of substances whose potential toxicity is characterized, are the main driving force behind this change. In addition, the need to reduce the time, cost and number of animals used in modern test methods for toxicity also highlights the need for changes in the approach to hazard assessment.

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