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Structural/Functional Characteristics of Organotypic Spinal Cord Slices under Conditions of Long-Lasting Culturing

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Organotypic cultures of the spinal cord possess significant advantages, as compared with routine cell systems *in vitro*; the cytoarchitectonics, cytospecificity of the cells, cell-to-cell connections, and other characteristics of spinal cord tissues are preserved to a considerable extent. We analyzed structural/functional characteristics of organotypic spinal cord slices of mice under conditions of long-lasting (one to three weeks) culturing. Immunohistochemical staining and patch-clamp recordings from cells of the *substantia gelatinosa* showed that the morphology of different cell types and cell-to-cell connections typical of normal spinal nerve tissue are clearly manifested in such organotypic spinal cord cultures. This is why that such a technique of spinal cord culturing can be successfully used in subsequent experimental studies, in particular, in the examination of different-type damages of the spinal cord.

Keywords: spinal cord, organotypic culture, neurons, gliocytes, immunohistochemistry, action potential, background activities.

INTRODUCTION

Among *in vitro* cell systems extensively used at present in studies of the CNS, organotypic cultures of nerve tissues, in particular such cultures of the spinal cord (SC), occupy a special position. Organotypic SC slices of rats (postnatal days, P, 5–7) were used for evaluation of the viability level of stem cells in the microenvironment of a damaged SC tissue under conditions of prolonged culturing [2]. In studies with modeling of trauma of the SC (sections at a lumbar level), organotypic SC slices of rat embryos (E 13–14) [1] were used. Estimations of restorations of the connections of motoneurons were obtained in organotypic SC slices of rats (P 7–9) cultured using a fibrin hydrogel [3]. Such methods open, in general, significant possibilities for obtaining quantitative estimates of damage to the nerve tissue [4]. Culturing *in vitro* allows experimenters to examine SC cell systems within long time intervals while providing a relatively stabilized state of these cell complexes. The tissue organization and cell-to-cell contacts existing under natural conditions are preserved much better in organotypic slices, as compared with cell co-cultures (e.g., neurons, astrocytes, and oligodendrocytes). In contrast to routine cultured cell systems *in vitro*, the native cytoarchitectonics, cytospecificity, neuronal activity, and functional synaptic systems may be preserved in organotypic slices during rather long time intervals. Organotypic cultures of SC cells *in vitro* are a prospective technical approach for identification of primary manifestations of mechanical damage to neurons, estimation of possible consequences of traumatization of nerve tissues, and determining possible directions for the development of the respective therapeutic interventions.

We tried to obtain structural/functional estimates for organotypic SC slices subjected to long-lasting culturing; we postulate that this will allow us to estimate the possibilities for further use of the respective systems, in particular, in experiments with modeling of spinal traumas of various nature.

METHODS

Preparing and culturing of organotypic SC slices. Embryos of mice of the FVB strain (E16-17)

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were used for preparing the SM slices. The lumbar region of the SC of such animals was cut off in sterile cooled Neurobasal-A medium (Gibco, USA); transverse 375 μm-thick slices were prepared using a chopper (McIlwain, Great Britain). Culturing of the slices during a period up to three weeks was performed in a CO_2 incubator (5% CO_2 , at +37°C) on semipermeable membranes using the Stoppini technique.

Immunohistochemical labeling of cells in the SC slices. For identification of SC cells, we used double immunohistochemical labeling of these units by antibodies against NeuN (1:200), Iba-1 (1:200), olig-2 (1:200; all – Abcam, Great Britain), GFAP (1:1000, NB, Great Britain), and caspase-3 (1:200; Sigma, USA). After washing out in a phosphate buffer, slices were processed during 1 h in a mixture of secondary antibodies conjugated with Alexa Fluor 555, Alexa Fluor 488, and Alexa Fluor 647 (all 1:1000; Invitrogen, USA). Immunohystochemical staining of the SC slices was examined using a confocal microscope (FluoView FV1000, Olympus, Japan) supplied with a digital photocamera and a computer.

Electrophysiological experiments. For functional verification of the viability of neurons, we obtained patch-clamp recording from cells of the SC slices in the full-cell mode. A MultiClamp 700B amplifier, a DAC/ADC Digidata 1320A digitizer (Axon Instruments, USA), and pClamp 9.2 software (Molecular Devices, USA) were used. In the course of the experiments, slices were in a chamber perfused at a 1.5–2 ml/min rate by Krebs solution preliminarily saturated with a mixture of O_2 (95%) and CO_2 (5%). The composition of the solution was the following (mM): NaCl, 125; KCl, 2.5; glucose, 10; $NaHCO_3$, 26; NaH_2PO_4, 1.25; $CaCl_2$, 2.0, and $MgCl_2$, 1.0 (pH 7.3, osmolarity 310 mOsm). Patch-pipettes were prepared from borosilicate glass (Sutter Instruments, USA) on a P-87 puller (Sutter Instruments, USA); their resistance was 3–5 MΩ when filled with a solution of the following composition (mM): K gluconate, 145; $MgCl_2$, 2.5; HEPES, 10; Na_2-ATP, 2.0; Na-GTP, 0.5, and EGTA, 0.5 (pH 7.2, osmolarity 290 mOsm).

RESULTS AND DISCUSSION

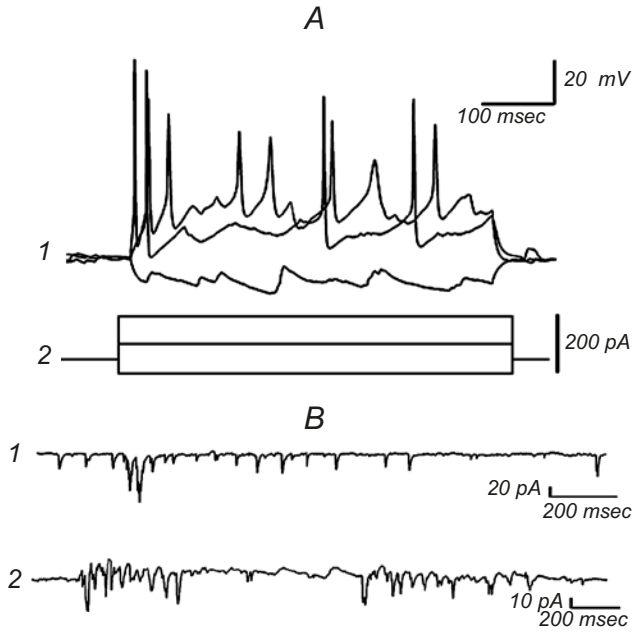
Up to the end of the first week, cultured SC slices were completely cleaned from cells damaged in the course of preparation; the state of slices remained stable during the entire culturing period, up to the third week inclusive. Cultured SC slices were spread out; their thickness decreased from 375 to about 250–270 μm.

Immunohistochemical labeling using antibodies against proteins NeuN (typical of the nuclei of neurons), GFAP (an astrocyte marker), olig-2 (a nuclear marker of oligodendrocytes), Iba-1 (a marker of microglial units), and caspase-3 (a marker of the cells that perished because apoptosis) demonstrated that the cultured slices preserved the cell composition typical of the nerve tissue during both the first and second/third culturing weeks. The density of caspase-3-positive cells remained practically unchanged within the entire culturing period.

To detect the capability of neurons in SC slices to generate action potentials (APs), cells of the superficial layers of the dorsal horn (*substantia gelatinosa*) were stimulated by long-lasting (500 msec) rectangular current pulses of increasing amplitude. Among 10 examined cells of the *substantia gelatinosa*, in 5 units we observed only mild changes of the potential on the plasma membrane induced by such stimulation, without any manifestations of the functioning of voltage-activated channels. Most probably, such cells could be qualified as gliocytes. At the same time, five other cells generated high-amplitude APs, which allowed us to qualify such units as neurons (Fig. 1 A).

To estimate whether neurons in cultured SC slices are involved in the formation of neuronal networks, we recorded ongoing synaptic activity in such cells in the absence of stimulation. At the holding potential of –60 mV, negatively directed synaptic currents were recorded in all five examined neurons. This fact is indicative of the presence of glutamatergic synaptic contacts between neurons in organotypic SC slices (Fig. 1 B1). In some cases, positively directed currents were recorded together with negative currents. This was observed mostly in the cases of generation of "bursts" of the synaptic events (Fig. 1 B2). Such a pattern allows us to conclude that functional inhibitory synapses are also present in the slices, and some neuronal networks are functioning in the latter.

Thus, definite modes of long-lasting culturing of organotypic SC slices allow experimenters to preserve in such preparations all the main types of



F i g. 1. Electrical activity of neurons in organotypic slices of the murine spinal cord. A) Generation of action potentials (1) evoked by stimulation of long-lasting current pulses (2). B) Examples of spontaneous excitatory synaptic currents (1) and "bursts" of excitatory and inhibitory currents (2) in two neurons of organotypic slices.

cells (neurons and cells of macro- and microglia) and synaptic connections present in the SC under physiological conditions. Such results confirm the possibility of using such a model (organotypic cultured slices of the murine SC) in subsequent experiments (e.g., with modeling of traumas of the SC of different genesis), for the development of novel methods of therapy of SC injuries and for testing the effects of various pharmaceutical agents on nerve tissues.

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