Before Brains:
Spatial Specialization and Communication in Bacterial Biofilms Herald Brain Morphology

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Abstract. Data from paleontology, geo- and biochemistry, genetics and other fields of knowledge allow one to consider the communities of the most ancient representatives of living beings, microorganisms, as prototype elements of nervous systems of "complex" animals. Based on geologically early spatial and functional specialization, like the morphological one, a new look on the basic architectonics of brains is proposed. The emergence of communications between individual units and their groups signaled by electrographic data on interaction between the units and the formation of communities cast light on the evolutionarily earliest mechanisms of social relations.

Keywords: *microorganisms, morphological heterogeneity, physiological diversity, population heterogeneity, specialization, communication, ion channels, electrical activity, synchronization*

The structure of the nervous system may be
an important component for the reconstruction of
a long-past evolutionary event, however an auxiliary one.
S.V. Saveliev. The Origin of the Brain

The origin of neurons and nervous systems remains one of the greatest mysteries in the evolution of life. How did such complex structures come about? Since the Darwin time, the long-standing interest in this issue has been continuously providing suggestions for another new look at the evolution of neurons, in particular, the study of the features of the cells of ancient creatures that stood at the origins of living matter, and the newer ones that have already passed a multimillion-year path of improvement [1, 4, 16]. The main question is when and in what form the first neurons appeared [40].

It is also important to understand the mechanism of the formation of organic molecules, since life developed in interaction with inorganic components of the environment. There are no direct data for the analysis of the physicochemical conditions necessary for the origin of life, i.e. on the types and presence of inorganic cations that are necessary for the emergence of a system that synthesizes protein. However, there are data from paleogeochemistry, petrochemical indicators of the content of these main life-forming elements, sodium and potassium, in various sediments and rocks from different periods of the history of Earth [28]. Life and environment are two parts of a single system. The evolution of biota is closely related to changes in the physical environment on a planetary scale, and together they make up a single self-developing system [46, 8, 19]. Living things arose from inanimate substances in the course of prebiotic evolution, the result of which was the emergence of organic compounds from inorganic ones with the necessary influence of external factors [27]. The

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culmination was the emergence of the original living cell, which has a minimum of components that ensured life and the possibility of its continuation. Modern studies in Artificial Life suggest that a “minimal life”, the proto-organismic physicochemical system, should be based on three constituents implementing the interrelated functions of a proto-container, proto-metabolism and a proto-gene [34]. The question of emergence of mind is also widely discussed, and it looks as we still lack convincing data to arrive at a reliable solution. One may connect mind’s “starting point” with the emergence of life itself, or with the formation of nervous systems, or place it somewhere in between. Since one may raise almost no objection to the connection between mind and generation and use of signs, we see a certain perspective for the development of the concept of proto-semiosis [40] as a minimum condition for the emergence of mind, or at least the proto-mind, no matter how one may interpret the latter term.

One of the most important conditions for the emergence of living matter is its physical separation from the environment with the help of a membrane that would not only protect it from the surrounding world, but also ensure the exchange of substances on which the preservation of life depends. Studies have shown that potassium ions usually dominate over sodium ions in surface rocks. It is assumed that the first cellular forms arose in “potassium” reservoirs, and not in the “sodium” ocean [28]. An indispensable condition for the intracellular electrolytic environment should have been the dominance of potassium ions. In reservoirs where sodium began to predominate, only cells that already had a plasmatic membrane with a functioning potassium-sodium pump could survive. Measurement of the concentration of ions in cells and in the extracellular fluid in modern animals shows that potassium ions are the main intracellular cation that provides the potential difference between the intracellular and external contents [30, 38, 17]. The conservatism of nature in relation to the basic principles of the operation of living systems manifests itself in the similarity of electrical processes that form the basis for the implementation of vital functions from the first forms of life to those presently living on Earth.

According to some theories of the origin of life on Earth, the first living creatures were the ancestral forms of modern cyanobacteria that created mats, the traces of which in the form of stromatolites have survived to the present day [43]. The idea that microorganisms exist in nature in the form of structured communities rather than individual free-floating (planktonic) cells was expressed by Costerton and coworkers [6]. The communities of cyanobacteria are the structures of various levels of organization, the architecture of which is based on different ways of functional unification of their constituent elements [45]. Microorganisms survived and developed because they formed communities, the associations that could withstand threats from the external environment, contribute to the preservation of the species [41, 31, 32). The interpretation of a microbial colony as an analogue of an integral multicellular organism was probably first expressed by J. Shapiro [39]. In addition, the tasks that arose before the community required the specialization of cells and the formation of specialized structures, which increased the viability of the microbial community [44]. S.G. Smirnov in the 80-ies considered a microbial colony as a spatio-temporal continuum consisting of cell clusters with different properties; at each stage of culture development one subcolonial cluster dominated [41]. In fact, a viable colony of microorganisms in terms of functionality and structural complexity is a prototype of the brain, consisting of many departments with a special internal structure and containing nerve cells of different types [7].

Further studies revealed the morphological and physiological heterogeneity of the cell composition of the colony, which manifests itself in the form of genotypic and population heterogeneity. Community heterogeneity is a supra-organismic property of bacteria that contributes to its adaptation to environmental conditions at the population level. The heterogeneity of a population is the result of the implementation of adaptive behavior inherent in a specific microorganism; it is a way of revealing new adaptive capabilities of the same bacterial genome [24]. Prokaryotes can change the level of heterogeneity of their populations, maintaining it adequate to environmental conditions. The morphological diversity of bacteria forms the basis of the adaptive behavior of microorganisms; this idea arose at the stage of early classical studies [3]. The formation of cells that are not identical in many traits is a consequence of random processes and leads to an increase in the phenotypic heterogeneity of cultures. The variety of cells of microorganisms results from the interaction of the external and internal environment, as well as due to random fluctuations in biochemical and physiological signs. Heterogeneity increases the survival rate of the bacterial population in heterogeneous or changing environmental conditions, as well as when exposed to stress factors.

The discovery of the heterogeneity of the morphological composition led to the idea of cell specialization. The initial historical stage in the study of the specialization of bacterial cells is associated with the discovery of their diverse forms and states in pure culture (the doctrine of the cellular heteromorphism of bacteria). At the beginning of the 20th century, colonies of microorganisms living on surfaces were discovered, and it was also found that bacteria that form surface fouling exhibit new properties that were previously lacking in them (for example, resistance to the action of antimicrobial substances) [6]. N. D. Ierusalimsky showed the existence of various cell types at different stages of culture development along with vegetative cells: differentiating spores, dying cells, filtering ("invisible in a microscope") cell forms [13]. To date, both in natural populations and in laboratory cultures, about 20 types of cells with specialized functions have been described. In some cases, these are cells for which the molecular genetic mechanisms of cell differentiation have been described, in other cases specialization is obvious as a phenomenon, the function of some cell types has not yet been fully determined. Using electron microscopic autoradiography, morphological variants have been found to differ in functions and possibilities of reproduction. In addition, it has been shown that cells with common functions form structural and functional clusters [14, 49]. The cells differ in shape and physiological state. An analogy arises with morphologically diverse cells of brain structures - the shape and size of neurons are formed and fixed in evolution (for example, Betz pyramids are commanding for performing movements, much smaller stellate ones distribute synaptic flows). Golgi cells are the main inhibitory interneuron of the granular layer of the cerebellum, play a central role in the functioning of the cerebellar network, the central element in the cerebellum is the Purkinje cell, and each neuron receives up to 500 thousand synapses from the axons of granular cells [2, 12].

The diversified composition of a microbial population in the process of creating dynamic functional groups required information exchange based not only on slow chemical methods such as "quorum sensing", but also on fast electrical processes [26], generation of action potentials, their distribution, as it takes place in the nervous system of highly developed organisms. Experimental data are convincing that electrical activity is not only one of the components, but also a necessary organizer of the formation of microbial communities, morphogenetic processes, a way of realizing functions of specialized cells, a dynamic means of communication [11, 15]. The basis for this kind of activity is the presence of a variety of ion channels that penetrate the membrane of even the most "ancient" in origin microorganisms. The properties of the electrical activity of cells of microorganisms create the prerequisites for communication to implement the specialized functions and processes of the dynamic organization of morphogenetic structures required to perform life support tasks. We obtained the characteristics of electrical activity in experiments on microorganisms of various evolutionary ages.

Method

The experiments involved recording electrical activity using glass microelectrodes filled with 1 M or 2.5 M (for multicellular organisms) KCl. To work with the cyanobacteria Oscillatoria terebriformis, a physiological solution of the following composition was used [in grams per liter]: $NaHCO_3$ - 3, Na_2CO_3 - 17, K_2HPO_4 - 0.5, NaCl - 30, KNO_3 - 2.5, $MgSO_4$ - 0.2, $CaCl_2$ - 0.04, $FeSO_4$ - 0.01. A fragment of a biofilm that included *Geitlerinema sp.* and *Halothece sp.* was studied in the natural environment (water sample from the salt lake Dus-Khol, Republic of Tyva, Russian Federation). Hay sticks *Bacillus subtilis*, medicinal preparation of *Bifidobacterium*, unicellular eukaryotic yeast cells of *Saccharomyces cerevisiae* and amoeba *Amoeba proteus* were placed in a liquid medium for registration, and social amoebae (myxomycetes) *Dictyostelium discoideum* were placed on a wooden plate in a Petri dish or on a wooden plate. During registration, ciliates *Paramecia caudatum* were placed in a solution of the following composition: KCl - 4 mM, $CaCl_2$ - 1 mM, $MgCl_2$ - 5 mM, Tris-HCl - 1 mM. pH - 7.2. The physiological solution for working with the nervous system of the mollusc *Helix lucorum* consisted of NaCl - 80 mM, KCl - 4 mM, CaCl2 - 7 mM, $MgCl_2$ - 4 mM, Tris-HCl - 10 mM, pH - 7.2–7.5. The milk mushroom *Zooaglea* at the time of registration was in a liquid medium containing milk and water in equal amounts. In some of the experiments, registration was exercised simultaneously with two electrodes placed at different loci of the object under study. More than 500 fragments of records of electrical activity belonging to the above organisms were analyzed.

Statistical analysis

Fragments of the recording of electrical activity were digitized and subjected to spectral analysis in the R 3.0 statistical processing environment (R Development Core Team). Spectral analysis was performed on the original recording by constructing a periodogram using the fast Fourier transform. To identify the features of oscillatory activity, autocorrelation analysis was carried out. To analyze the interactions, we used the calculation of the cross-correlation coefficient and coherence. The duration of the digitized chunks was 3 seconds.

Results

The introduction of a microelectrode into the object under study makes it possible to determine the level of its membrane potential. All cells of microorganisms that have been in the experiments, as a rule, had a negative charge and the membrane potential (MP) reached -65 mV (Fig. 1). The MP of microorganisms varied within wide limits, for example, in Paramecia it was not possible to determine it accurately enough due to the mobility of these creatures; in cyanobacteria, the MF level could depend on the position of the ME, which it occupies in a separate filament (if it is located along the course of the filament, then the MF stable, and if along its diameter, then oscillations are inevitable, up to the exit of the microelectrode (ME) from the cell). For comparison, the level of the resting membrane potential when working with neurons of the mollusk *Helix lucorum* remained stable for many hours [42].

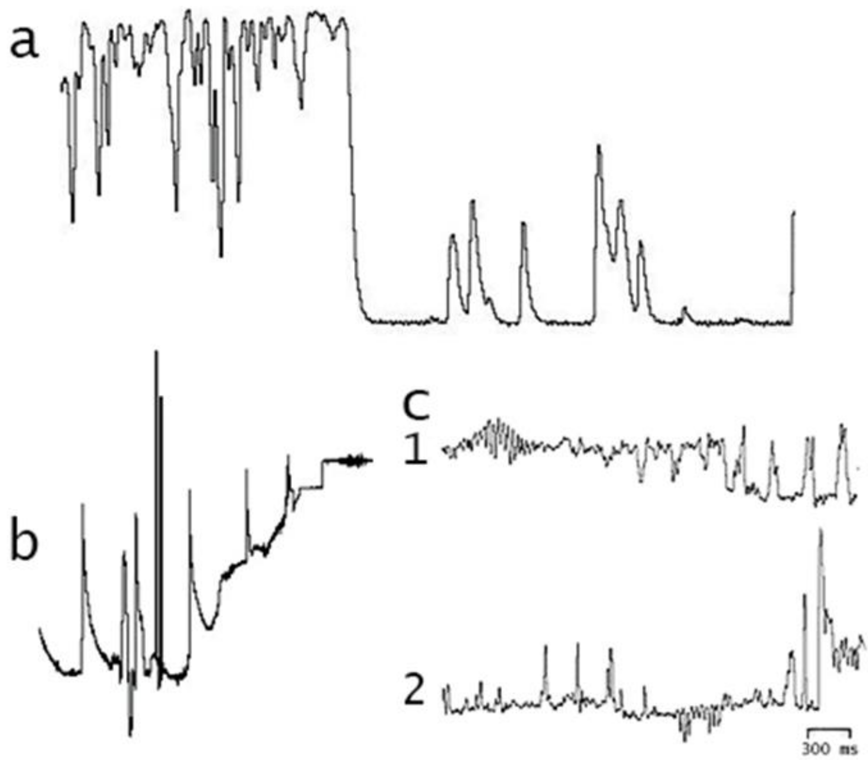

Fig. 1. Change in the resting membrane potential (MP) of individual units upon intracellular introduction of a microelectrode (ME). a - introduction of ME into the filament of cyanobacteria; b - exit of ME from the cell of the yeast *Saccharomyces cerevisiae;* c - introduction (1) and output (2) of ME in the social amoeba *Dictyostelium discoideum*. Calibration: 300 ms, 20 mV.

The introduction of a microelectrode into some microorganisms was technically difficult due to the microscopic size of these creatures and their rapid movement - for example, the amoeba. In other cases, the elasticity of the cell membrane could be an obstacle to the successful introduction of ME, for example, in yeast cells.

Microorganisms generate action potentials (APs). High-amplitude discharges of an individual cell were found in all investigated objects (see: Method). AP could be observed both with intracellular injection of ME (Fig. 2) and with its extracellular placement.

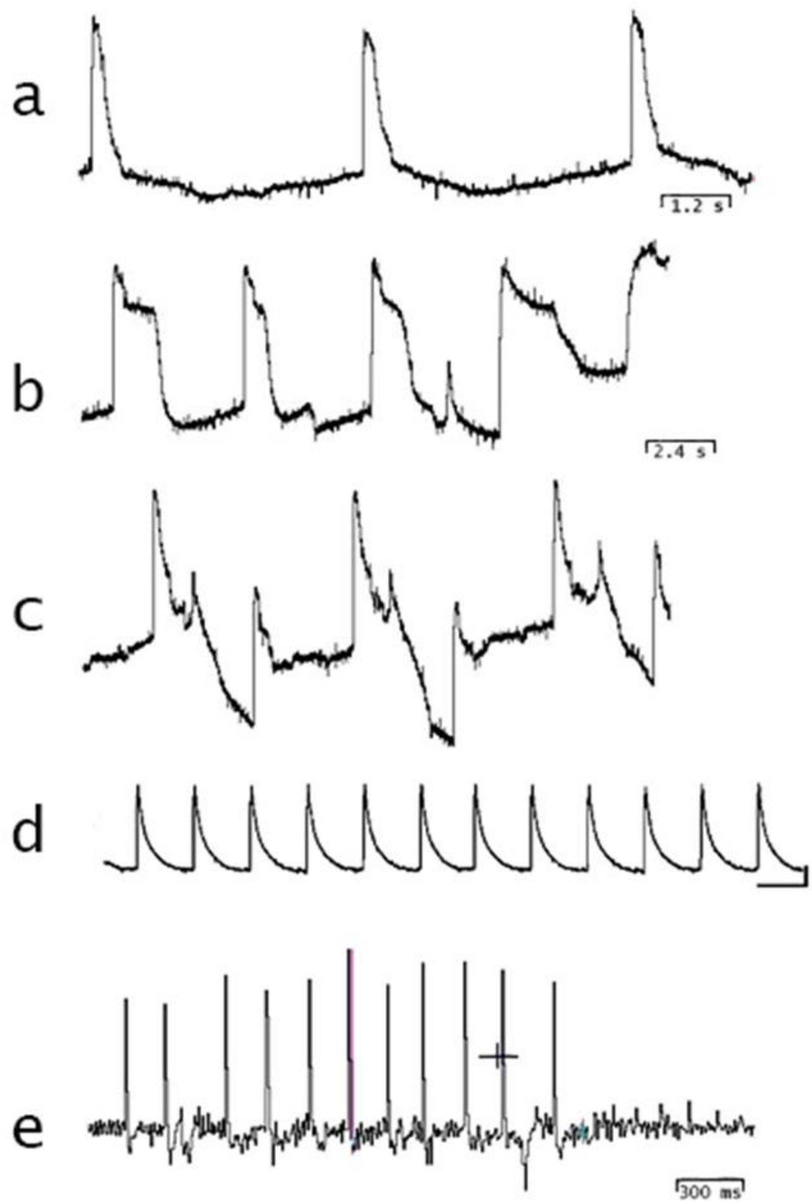

Fig. 2. Types of electrical activity of cyanobacterial filaments recorded by intracellular microelectrodes (ME). Calibration: a - 1.2 ms; b, c - 2.4 ms, d, e -300 ms; 20 mV.

The amplitude reached 50 mV (with intracellular registration); a feature of the AP of some microorganisms is the rate of development (Fig. 2). For example, in cyanobacteria, high-amplitude discharges to reach the maximum took an order of magnitude longer than in mollusks. The variety of electrical activity recorded by intracellular electrodes from different elements of the same type of microorganisms indicates a variety of functional states of cells and, apparently, their different functional capabilities. For example, the electrical activity removed from single cyanobacteria differs not only in the rate of development, but also in the shape (Fig. 2, a-c), the organization of the temporal sequence of discharges (Fig. 2, d-e), and the absolute value of the amplitude.

Intracellular oscillatory electrical potentials of microorganisms form patterns - AP, distributed in a special way in time (Fig. 2, a, d, e, Fig. 3, a-c). For many cells of the microbial community, the active manifestation of the endogenic mechanism is normal: at least the following tasks can be distinguished that these cells solve: initiating movements, measuring time, coordinating the work of various cell ensembles located in spatially separated loci of the community, generators of rhythms of the functional state. The functional purpose of endogenous oscillations is different, therefore, the working modes of cells, the distribution of AP in time are different. The generation of certain discharge patterns creates the prerequisites for cell specialization.

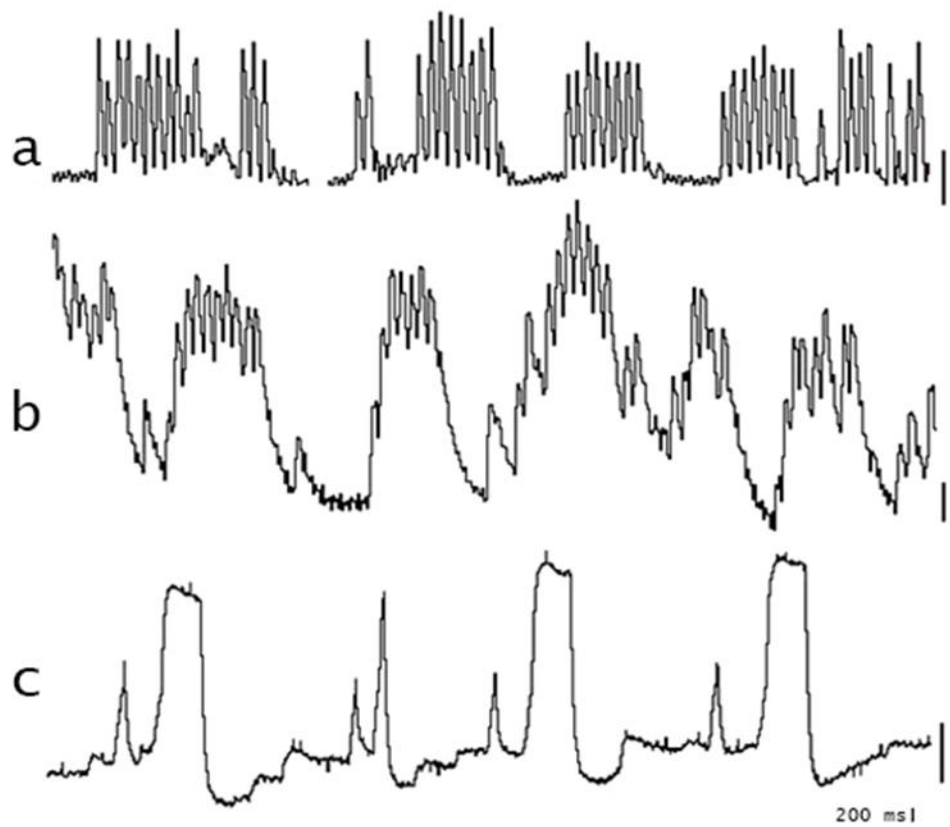

Fig. 3. Patterns of electrical activity of microorganisms: a - hay bacillus *Bacillus subtilis*; b - yeast cells *Saccharomyces cerevisiae*; c - ciliate *Paramecia caudatum*. Calibration: 200 ms, 20 mV.

The endogenous electrical activity of many cells may be synchronized, as shown by the registration of field potentials by means of macro-electrodes (Fig. 4).

Oscillatory activity has frequencies typical of the electrical activity of the brain of higher animals and humans. Periodograms have maxima at frequencies from 0.5 to 40 Hz, i.e. rhythms are present known as alpha, beta, delta and theta, which could be registered during the entire time of the experiment, i.e. 2-3 hours, or, on the contrary, disappear after 1-2 minutes. The oscillation amplitude varies from several μV to tens of μV (Fig. 4).

Simultaneous registration by several electrodes from different loci of the bacterial community showed that, depending on the conditions, the level of interaction between these regions can change significantly. The emergence of a common task enhances electrical activity and increases the interaction between organisms located in these places. This is reflected in the

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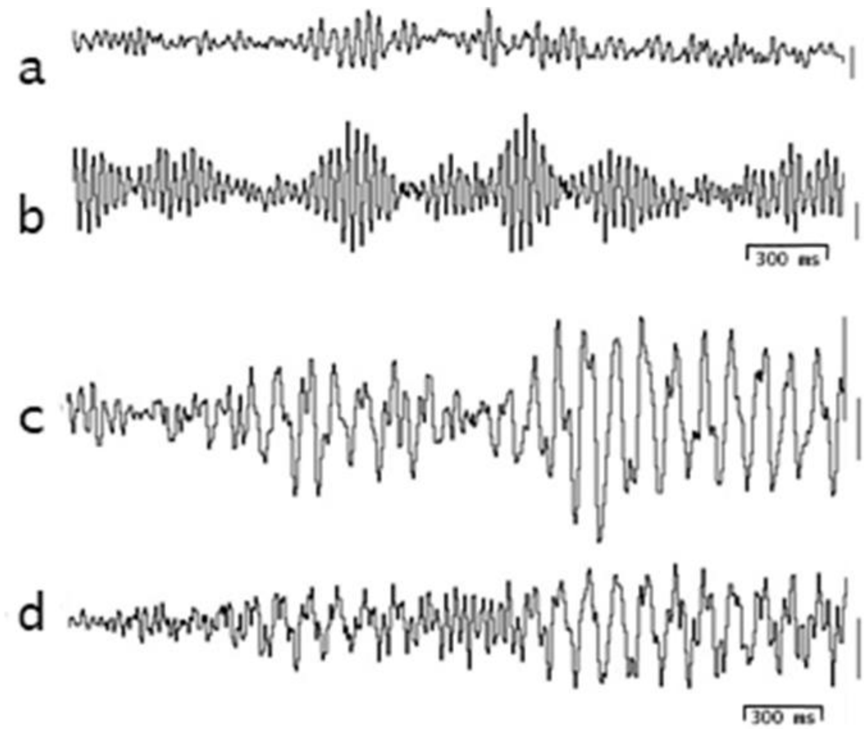

Fig. 4. Electric field potentials recorded in microorganisms; a - from yeast cells of Saccharomyces cerevisiae, b – from Bacillus subtilis rods, c, d - from cyanobacteria Oscillatoria terebriformis. simultaneously with two electrodes from different loci of the fruiting body. Calibration: 300 ms, 20 μV.

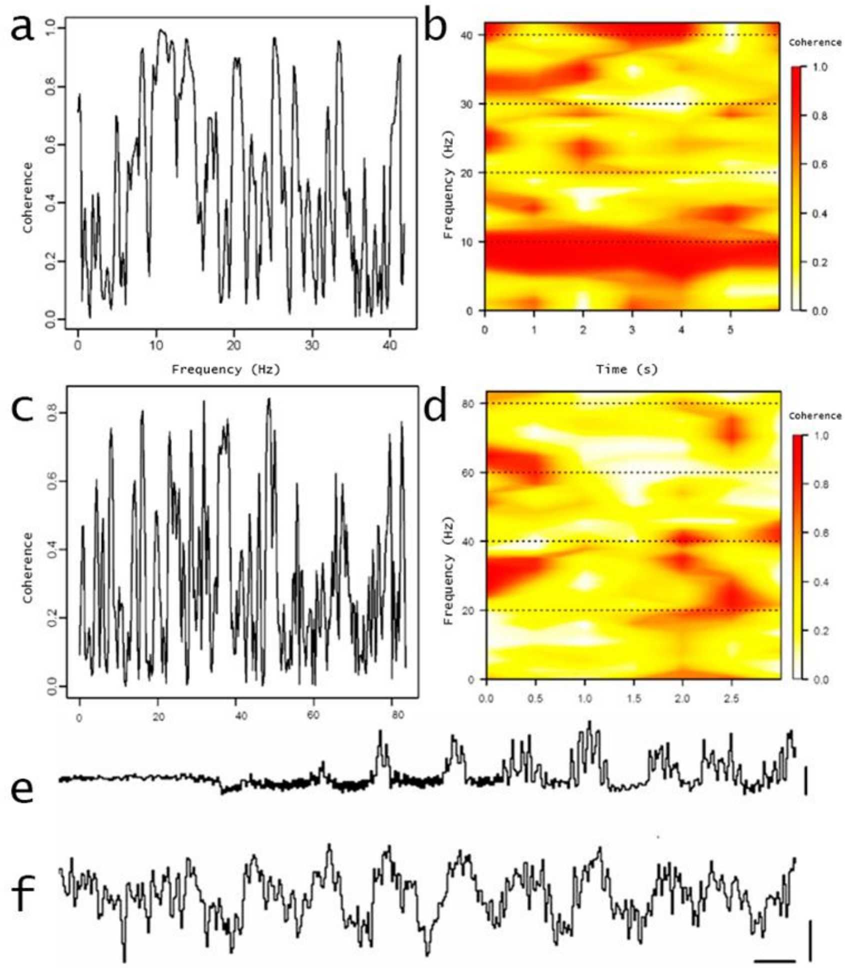

Fig. 4. Synchronization of electrical activity as recorded in two loci of the cyanobacterial biofilm at different stages of actions to preserve the integrity of the living space during active construction (a, b) and without interference in the process (c, d). a, c - graphs of phase-frequency coherence. The abscissa is the frequency in Hz, the ordinate is the coherence coefficient; b, d - time-frequency coherence at (a, c). The abscissa axis is the time in seconds, the ordinate axis is the frequency in Hz, the coherence coefficient (on the right axis); e, f are the field potentials recorded at the biofilm loci during active actions presented in (a, b). Calibration: 300 ms, 20 μV.

coherence coefficients characterizing the level of synchronization of electrical processes (Fig. 5).

The results were obtained reflecting the dynamics of communication in the cyanobacteria *Oscillatoria terebriformis*, in the social amoebae (myxomycetes) *Dictyostelium discoideum* when creating fruiting bodies, as well as in hay sticks *Bacillus subtilis* and yeast *Saccharomyces cerevisiae* cells when solving problems whose meaning in experiments was not controlled

The participation of organisms located in different parts of the colony in the performance of any socially significant task can be different, and this is evidenced by the analysis of the coherence of electrical activity recorded at the corresponding loci (Fig. 5). Do cells of the same locus participate with the same zeal in the "work"? Long-term registration from the same place of the colony of microorganisms shows a change in the dominant frequency (Fig. 6, a, b), which can occur both naturally, at certain time intervals, or chaotically. In this case, the presence of both "high" and "low" frequencies is preserved in the frequency spectrum of the periodogram (Fig. 6).

Apparently, this means that there is a change in active elements, i.e. high and low frequency oscillations are generated by different units. Consequently, the assumption about the "division of labor" in the bacterial community in the form of generators providing high-frequency and low-frequency oscillatory activity is confirmed in experiments.

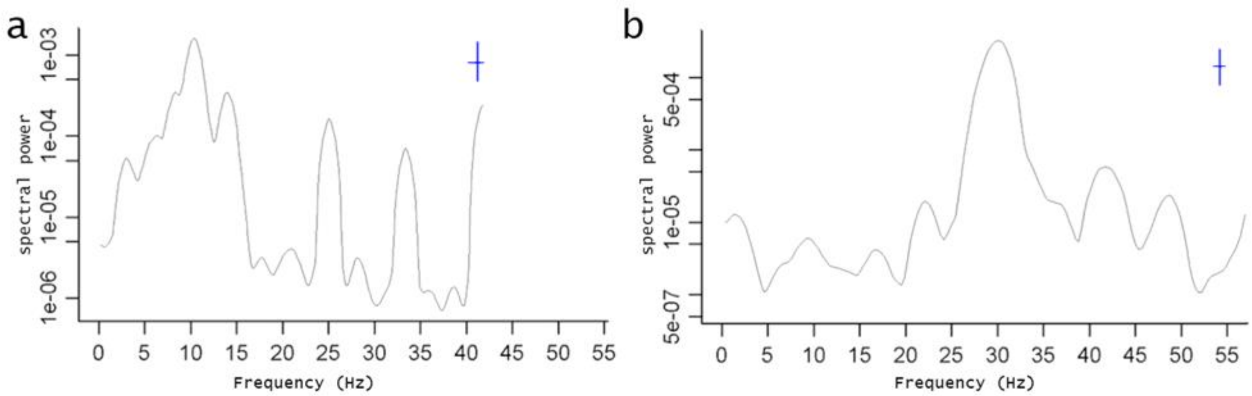

Fig. 6. "Division of labor" in a cyanobacterial film as registered from the same locus. The interval between registrations is 5 minutes. Designations: the abscissa axis – frequency in Hz; the ordinate axis – spectral density in a.u. The horizontal bar is the bandwidth, the vertical bar is the 95% confidence interval.

Discussion

Electrophysiological experiments were carried out, the purpose of which was to demonstrate, using objective indicators, the existence of qualities common to the most ancient microorganisms and nerve cells of highly organized animals and thereby strengthen the ideas about the common origin of both. Neurons and a complexly organized nervous system are a step up the evolutionary ladder from the same source.

Communication using electrical signals is very common in biological systems, and the electrical activity of microorganisms and highly organized creatures is very similar in its main forms of manifestation and physical characteristics. This may mean that the generation mechanisms have the same roots. The first intracellular electrical recordings from a living organism were obtained on a single-celled eukaryote, a paramecium, into which a glass microelectrode was inserted in 1934 to register the resting membrane potential [25]. It should be taken into account that the structure and activity of ion channels evolved long before the appearance of complex multicellular organisms on Earth [28, 25]. An example is the variety of ion channels existing on the cell membranes of prokaryotes. It was found that such classes of ion channels as sodium, chloride, calcium-dependent potassium, as well as ionotropic glutamate receptors, similar to those found in neurons, are involved in bacteria. The first information on the structure of the channels and their selective conductance was obtained in prokaryotes [5]. It is assumed that potassium ion channels work in bacteria, mediating electrical signals to coordinate biofilm metabolism: they conduct potassium waves propagating along the bacterial biofilm [33].

This depolarization wave coordinates the metabolic state of other cells located in different parts of the biofilm. Potassium channel blockade destroys this response.

Calcium as a bivalent ion was chosen by evolution as a signaling molecule for both prokaryotes and eukaryotes. All living prokaryotes have a low concentration of cytosolic free calcium (80-100 nM). These channels are indeed widespread in prokaryotic organisms, and are possibly the oldest ion channels [50]. In eukaryotes, the signaling system becomes more complex. This is primarily due to the development of intracellular organelles with their specific signaling calcium mechanism [47]. The complexity of calcium signaling in eukaryotes is also associated with the emergence of several types of calcium permeability channels, with different gate mechanisms. Electrically excitable channels use a voltage-related influx of calcium with the ability to transmit intracellular signals usually studied in neurons.

Volt-gated sodium permeability channels provide the basis for electrical excitability in animals [5]. Na-channels evolved from calcium channels and was probably permeable to Na^+ and Ca_2^+ ions. Like many other ion channels and receptors, sodium channels predate neurons. About 500 million years ago, in early chordates, sodium channels formed a cluster on the initial segment of the axon, and 50 million years later, with the evolution of myelin, sodium channels consolidated this property and accumulated in Ranvier's interceptions. Sodium channels show the impact of evolution on increasing the diversity of communication signals (electric fish), on defense against toxins (snakes, newts, fish, insects). It was found that bacteria can change the membrane potential in seconds, but what ion fluxes create these changes is not known. Scattered calcium sensors show that calcium current is induced by depolarization similar to action potentials. These results demonstrate the function of ion channels in microorganisms and provide a prokaryotic paradigm for spatial signaling activity in cellular communication.

It was shown experimentally that the cells of microorganisms have not only electrophysiologically expressed manifestations of metabolism in the form of changing membrane potential, but also rhythmic processes similar to those found in evolutionarily newer living things, the multicellular eukaryotes and in animals with a highly developed nervous system. Endogenous activity, which plays an important role in the organization and implementation of many functions of the nervous system of higher animals, appeared for the first time in prokaryotes, which are actually the same age as the Earth. Individual microorganisms have intracellular electrical activity similar to pacemaker endoneuronal oscillations in brain structures [42, 9]. The electrical oscillations can be rhythmic as in neurons of the time of the suprachiasmatic nucleus, periodically rhythmic as in neurons of the upper olive, generate patterns, like command neurons [20, 21]. The endogenous origin of the pacemaker activity of neurons and their independence from the cellular ensemble of various structures of the nervous system was proved in special experiments: on cultured Purkinje cells of the cerebellum, neocortex, upper olives and many other structures [23, 18, 22, 9]. All types of endoneuronal activity have precursors at the level of prokaryotic microorganisms [10].

Brain structures are morphologically heterogeneous and functionally specialized [36]. The predecessor of such an architecture of a biological substance responsible for the life of a given creature is apparently morphogenesis in prokaryotic colonies, biofilms and bacterial mats [44, 15, 29]. Microorganisms create the necessary temporary specialized structures to perform a variety of functions. S.G. Smirnov suggested that multicellular creatures took the path of creating concentrations of specialized cells, and of one type of cells, for example, a liver was gradually formed, a heart of another, muscle tissue of a third type, etc. In prokaryotes, there are also groups of cells specialized in performing different functions. Another type of specialization emerged, the "persistent" cells that resist antimicrobial drugs. The assumption about specialized members of the community is based on the results of electron microscopy. A number of studies show the morphological heterogeneity of microbial populations. Regularities were established in changes in the structure of microbial communities at different stages of development, manifested in a change in the ratio of different types of cells, i.e. physiologically active, resting, autolyzed and involutionary [36, 48]. The functional division of the cells of a microbial community is also supported by the data illustrating that in any population, along with bacteria that have an ultrastructural organization characteristic of a given species, various morphological variants can be found that differ not only in structure, but also in physiological and genetic properties. Electrophysiological measurements prove that cells are functionally specialized in different ways. Specialization may be determined by frequency characteristics of the oscillators.

Conclusions

Brain neurons and individual microorganisms produce a resting potential of 0 to -65 mV, created by the asymmetric distribution of K + ions. Both neurons and individual microorganisms generate action potentials, in the development of which Ca ++ and Na + ions are involved. The presence of ion channels for these ions in neurons and microorganisms has been experimentally proved.

1. Both neurons and microorganisms have endogenous rhythmic activity.
2. The endogenous activity of cells may be synchronized and form field potentials with a rhythm of 0.5 to 40 Hz.
3. The electrical activity of microorganisms that form spatially separated loci of the microbial community is synchronized. The level of synchronization is characterized by coherence.
4. As in brain structures, in the communities of microorganisms clusters of morphologically and functionally specialized elements are created, one of the ways of communication between them is patterned electrical signals.
5. The structural and functional organization of microbial communities provide a prototype of the brain structure of evolutionarily advanced animals.

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