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EFFECTS OF LOW FREQUENCY RECTANGULAR ELECTRIC PULSES ON *TRICHOPLAX* (PLACOZOA)

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The effect of extremely low frequency electric and magnetic fields (ELF-EMF) on plants and animals including humans is quite a contentious issue. Little is known about ELF-EMF effect on hydrobionts, too. We studied the effect of square voltage waves of various amplitude, duration, and duty cycle, passed through seawater, on *Trichoplax* organisms as a possible test laboratory model. Three Placozoa strains, such as *Trichoplax adhaerens* (H1), *Trichoplax* sp. (H2), and *Hoilungia hongkongensis* (H13), were used in experiments. They were picked at the stationary growth phase. Arduino Uno electronics platform was used to generate a sequence of rectangular pulses of given duration and duty cycle with a frequency up to 2 kHz. Average voltage up to 500 mV was regulated by voltage divider circuit. Amlodipine, an inhibitor of calcium channel activity, was used to check the specificity of electrical pulse effect on voltage-gated calcium channels in *Trichoplax*. Experimental animals were investigated under a stereo microscope and stimulated by current-carrying electrodes placed close to a *Trichoplax* body. Variations in behavior and morphological characteristics of *Trichoplax* plate were studied. Stimulating and suppressing effects were identified. Experimental observations were recorded using photo and video techniques. Motion trajectories of individual animals were tracked. Increasing voltage pulses with fixed frequency of 20 Hz caused H2 haplotype individuals to leave “electrode zone” within several minutes at a voltage of 25 mV. They lost mobility in proportion to voltage rise and were paralyzed at a voltage of 500 mV. Therefore, a voltage of 50 mV was used in further experiments. An animal had more chance to move in various directions in experiments with two electrodes located on one side instead of both sides of *Trichoplax*. Direction of motion was used as a characteristic feature. *Trichoplax* were observed to migrate to areas with low density of electric field lines, which are far from electrodes or behind them. Animals from old culture were less sensitive to electrical stimulus. H2 strain was more reactive than H1 strain and especially than H13 strain; it demonstrated stronger physiological responses at frequencies of 2 Hz and 2 kHz with a voltage of 50 mV. Motion patterns and animal morphology depended on the duration of rectangular stimulation pulses, their number, amplitude, and frequency. Effects observed varied over a wide range: from direct or stochastic migration of animals to the anode or the cathode or away from it to their immobility, an increase of optical density around and in the middle of *Trichoplax* plate, and finally to *Trichoplax* folding and detach from the substrate. Additional experiments on *Trichoplax* sp. H2 with pulse duration of 35 ms and pulse delay of 1 ms to 10 s showed that the fraction of paralyzed animals increased up to 80 % with minimum delay. Nevertheless, in the presence of amlodipine with a concentration of 25 nM, almost all *Trichoplax* remained fast-moving for several minutes despite exposure to voltage waves. Experimental animals showed a total discoordination of motion and could not leave an “electrode trap”, when amlodipine with a concentration of 250 nM was used. Further, *Trichoplax* plate

became rigid, which appeared in animal shape invariability during motion. Finally, amlodipine with a concentration of 50 μM caused a rapid folding of animal plate-like body into a pan in the ventral-dorsal direction and subsequent dissociation of *Trichoplax* plate into individual cells. In general, the electrical exposure applied demonstrated a cumulative but a reversible physiological effect, which, as expected, is associated with activity of voltage-gated calcium channels. Amlodipine at high concentration (50 μM) caused *Trichoplax* disintegration; at moderate concentration (250 nM), it disrupted the propagation of activation waves that led to discoordination of animal motion; at low concentration (25 nM), it prevented an electric shock.

Keywords: rectangular electric pulses, *Trichoplax*, Placozoa, voltage-gated calcium channels

Electromagnetic radiation in the range from units to several thousand Hz does not have a direct thermal effect on living tissue, but influences indirectly on certain cellular mechanisms and causes corresponding physiological effects [18 ; 29]. It was found that extremely low frequency electric and magnetic fields (hereinafter ELF-EMF) can induce gene expression [41 ; 48] and cause cell proliferation [43]. Laboratory studies showed that ELF-EMF affect cell membranes and ion channels [22], especially voltage-gated calcium channels [12].

It is of interest that calcium channel blockers significantly reduce various effects of ELF-EMF [26]. In addition, biophysical properties of voltage-gated channels can explain molecular mechanisms of ELF-EMF biological effects. For example, a downward regulatory cellular response to such effects can be mediated through Ca^{2+}/calmodulin stimulation of nitric oxide (NO) synthesis, while physiological reactions may be a result of stimulation of NO-dependent cGMP protein kinase G, and pathophysiological processes may result from NO-peroxynitrite oxidative stress. Other Ca^{2+}-mediated regulatory pathways, non dependent of nitric oxide, have been also described [11 ; 16 ; 25].

There are several types of calcium channels: high-, intermediate-, and low-voltage-activated calcium channels [27]. L-, P- and N-type calcium channels are activated at high values of membrane potential. Four proteins with many isoforms $Ca_v1.1$–$Ca_v1.4$ belong to L-type channels, encoded in humans by *CACNA1S*, *CACNA1C*, *CACNA1D*, and *CACNA1F* genes; they are expressed mainly in skeletal muscle and are responsible for the contraction of cardiac and smooth muscles [21]. P- and N-type channels are represented in neurons by $Ca_v2.1$ and $Ca_v2.2$ proteins, respectively; they are responsible for the release of neurotransmitters. R-type calcium channels, intermediate-voltage-activated ones, include $Ca_v2.3$ protein family [24 ; 35]. Low-voltage-activated channels are of T-type. Cells with pacemaker activity possess them, for example human pacemakers [5]. These channels are represented by $Ca_v3.1$–$Ca_v3.3$ proteins and are encoded by *CACNA1G*, *CACNA1H*, and *CACNA1I* genes, respectively [46]. The obvious role of T-type Ca_v3 channels is manifested in cellular excitability, where their low activation voltages make it easy to depolarize the membrane. T-type channels also play a role in triggering exocytosis mechanism in vertebrates and invertebrates [32 ; 45]. Ca_v3 channels are present in primitive animals and in unicellular organisms [23 ; 40].

Members of the phylum Placozoa, in particular *Trichoplax adhaerens* [30], can be a useful model for studying ELF-EMF effects. *Trichoplax* has a body of irregular shape (its size is about 1 mm); it is formed by two layers of epithelium with a doughy cell layer between them. This tiny marine animal is built of six basic cell types [7 ; 39]. *Trichoplax* aroused interest in terms of minimum requirements for metazoa after F. E. Schulze made an initial description in 1883 [31]. The lack of organs symmetry, nerve and muscle cells, basal plate, and extracellular matrix did not leave doubts about the anciend origin of *Trichoplax*. However, despite the primitive structure, these animals are able to coordinate motion activity during feeding [37] and demonstrate chemotaxis [13], confirming the existence of complex mechanisms of intercellular interaction and integration. Rapid rhythmic contractions of *Trichoplax* dorsal epithelium were found [30], and animal motion model based on Voronoi diagrams was proposed [38].

Structure, function, and ionic selectivity of a single T-type calcium channel from *Trichoplax* (TCa_v3) were characterized using the patch-clamp technique after cloning it in HEK-293T human kidney embryonic cells [36]. Taking into account the fact that Ca_v channels play a decisive role both in intracellular and intercellular signal transmission [23] and the fact that *Trichoplax* has in its genome a complete set of Ca_v1, Ca_v2, and Ca_v3 genes encoding Ca_v channels [15 ; 40], it became possible to study molecular targets and mechanisms underlying ELF-EMF effects on multicellular organisms.

The aim of this work was to study *Trichoplax* response to sequences of square-shaped pulses that simulate the effects necessary for opening the calcium channels *in vivo*. Rectangular pulses were chosen to influence *Trichoplax* because they cause greater effects on biological objects than vibrations of any another shape. We also found it appropriate to use square waves as an idealized, "discrete" model of electromagnetic radiation.

MATERIAL AND METHODS

Cultivation. Three Placozoa strains were used in experiments, such as *Trichoplax adhaerens* (haplotype H1), *Trichoplax* sp. H2 (haplotype H2), and *Hoilungia hongkongensis* (haplotype H13), of which the last strain can be attributed to a separate species [8 ; 9]. Animals were cultivated in glass Petri dishes with a diameter of 90 cm on mats of unicellular green alga *Tetraselmis marina* in artificial seawater (ASW, Red Sea Salt, Red Sea Fish Pharm LTD, Israel) with a salinity of 35 ‰ at a temperature of +25 °C according to the standard protocol [17]. Seawater was replaced at least once a week. The pH value was maintained in the range of 7.8 to 8.0. One hour before the start of the experiment, animals were placed in ASW on plastic dishes without algae.

Amlodipine preparation. Dihydropyridine derivative amlodipine ($C_{20}H_{25}ClN_2O_5$, 3-*O*-ethyl 5-*O*-methyl 2-(2-aminoethoxymethyl)-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate as besylate, Teva) was added in 96 % ethanol or in water (ASW), and the concentration was calculated according to the solubility of this compound in those solvents.

Electrical stimulation. To generate a sequence of rectangular pulses of given duration and duty cycle, standard Arduino Uno R3 platform based on 8-bit AVR microcontroller ATmega328P was used. LED1 was used for visual control; a diode (D1, 1N4001) was connected in series with resistors R1 and R2 to prevent accidental short circuit. The average voltage value was adjusted from +10 mV to +1.5 V on voltage divider using potentiometer R2 (10 kΩ), as shown in Fig. 1. The electrodes were placed in seawater near the animal's body at a distance of about 1 mm from each other. We used plastic or wooden sticks, or metal electrodes disconnected from the controller as a base test of possible influence of foreign objects on animal behavior.

Software meander. Pulse duration and delay (duty cycle) were set by a program in the range of 0.5 ms to 10 s; the program was executed on the controller in an infinite loop (see supplementary file No. 1: https://doi.org/10.21072/mbj.2020.05.2.05). Conversion of time intervals (ms) into frequency (Hz) was carried out taking into account that 1 Hz corresponds to 1000 ms. To create packages of square-shaped pulses with changing duration and delay inside the package, incremental and decremental program cycles with a step of 1 ms were used (see supplementary file No. 2: https://doi.org/10.21072/mbj.2020.05.2.05).

Microscopy and data processing. The animals were stimulated with electric current under ZEISS Stemi 305 stereo microscope at magnifications ×8 and ×40. Variations in behavior (motion activity, direction, and trajectory) and morphological characteristics of the body (opalescence, shape) were evaluated. Stimulating and suppressing effects, *i. e.* leading to speed up of animal motion activity or to its retardation, shock, and paralysis, were identified. *Trichoplax* structure was studied under Nikon Eclipse Ts2R

inverted microscope with DIC optics at maximum magnification ×600. Images and behavioral activity of animals were documented using photo and video techniques. The video was processed using FFmpeg utility on Huawei FusionServer RH2288 V3. *Trichoplax* motion trajectories were tracked using ImageJ wrMTrck plugin (National Institutes of Health, USA) on Dell Precision T5810 graphics station.

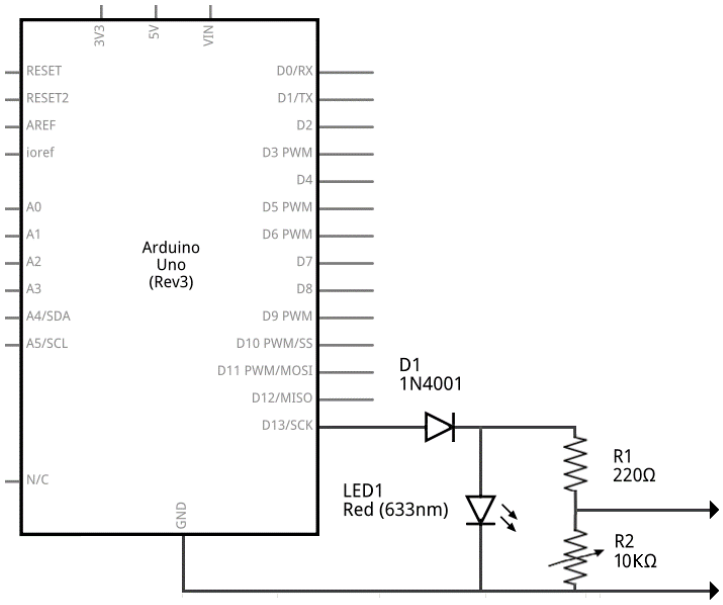

Fig. 1. Electronic circuit for *Trichoplax* electrical stimulation

RESULTS

Culture growth dynamics. *Trichoplax* culture was at the state of adaptation to new conditions for several days, showing no rise in number and size of individuals, after animals placement on fresh algal mats. *Trichoplax* began to grow with subsequent division after this lag phase. At the next stage, which corresponds to the exponential phase of the culture growth, *Trichoplax* grew actively and split in half once every 3–4 days by forming a constriction in cell division with a fall in the size of daughter individuals. Entry into the stationary phase of the culture growth was observed after 2–3 weeks, and it was accompanied by a twofold decrease in the average size of animals and by a sharp slowdown in the growth rate of their number. We observed decreasing size of individuals, change in their shape (elongation or, conversely, formation of small spheres detaching from the substrate and floating), as well as death of animals in old cultures. Therefore, *Trichoplax* were transplanted on fresh algal mats in the amount of 10–20 individuals per dish after 5–6 weeks of growth, and the cultivation process was repeated. In common, animals selected at the stationary phase of the culture growth were used.

Single electrode experiments. No pronounced reaction of animals to foreign objects was observed in tests in which small plastic, wood, or metal rods were applied. The animal usually moved away from an electric stimulus by a distance of one to three sizes of its body (1–3 mm) and continued to move slowly in a random direction, when electrodes connected to Arduino Uno controller, one of which (anode or cathode) setting near *Trichoplax* (H2 strain) and the other one at a considerable distance (more than 1 cm), were used. *Trichoplax* ran away immediately with pulse duration of 100 ms and a delay of 1 ms; they crawled away from the electrodes with a pause of several tens of seconds in the case of a delay of 1 s. It was noted that experimental animals taken from the old culture were less sensitive to an electric stimulus, which appeared in an increase in time of reaction delay and in a decrease in the distance they moved.

Two electrodes experiments. *Trichoplax* showed motion activity: it began to "explore" the space, approaching the electrodes and moving away from them, until it found a "comfortable" position in resulting weak electric field, when two rods made of metals with different electrochemical potential, forming a galvanic pair near the animal, were placed (Fig. 2).

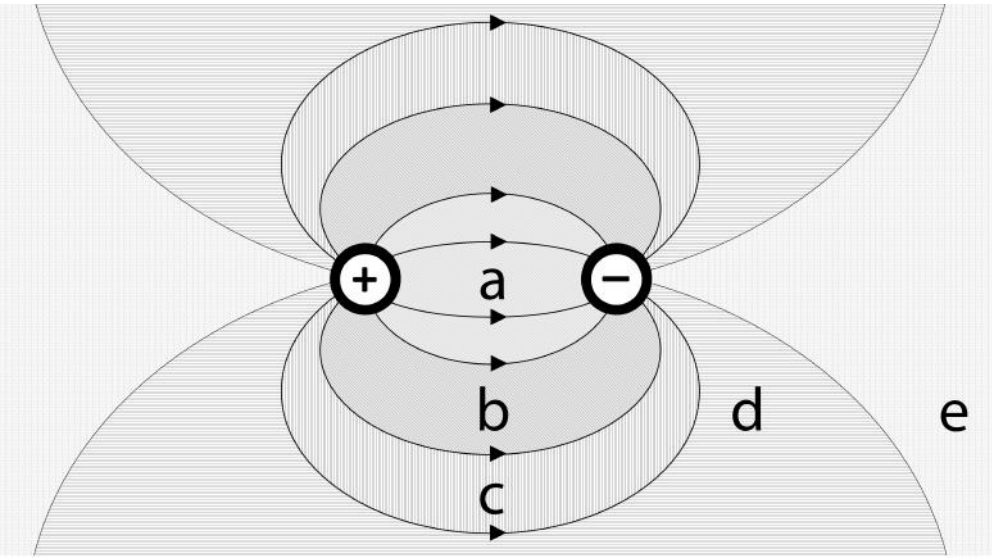

Fig. 2. Electric field lines in "electrode trap" for *Trichoplax*: (a) "electrode zone" with electrode gap no more than 1 mm; (b) immobilization zone corresponds to one *Trichoplax* body size (1 mm) from the center of the trap; (c) intermediate zone; (d) zone away from electrodes; (e) "comfort zone" just behind the electrodes (see the text for details)

Trichoplax avoided the cathode, when a direct current source (of 200 mV) was used, but did not approach the anode, crawling to the side and sometimes wandering between the electrodes. In case the animals crawled too close to the anode, they remained near it and wrinkled after a while. Reaction rate and motion direction depended largely on the initial position of the animal and the distance between the electrodes. A voltage of 1.2 V was lethal for the animals, *i. e.* they were paralyzed, radically changed their morphology, detached from the substrate, and subsequently desagregated.

Test studies were carried out in seawater with a salinity of 35 ‰, using pulse duration and delay of 35 ms (~ 30 Hz) with an average voltage of 50 mV. It is of interest that in the case of an industrial frequency (of 50 Hz), *Trichoplax* did not substantially change their morphology; they began to move, preferred the anode, crawled away from the electrodes, sometimes came back, and again moved away from the electrodes.

Voltage effect on *Trichoplax* was estimated by varying pulse amplitude using voltage divider at a fixed frequency of 20 Hz (see supplementary file No. 1: https://doi.org/10.21072/mbj.2020.05.2.05). The main evaluation criterion was the fraction of immobilized animals that did not leave the "electrode zone" within a few minutes. An additional criterion was pathological changes in the morphology of animals, such as the thickness of the rim of plate, rounding of the shape and reduction of opalescence. Their fraction increased in proportion to voltage rise (Fig. 3). Eventually, almost all animals left the "electrode zone" at a voltage of 25 mV, and they remained, on the contrary, within its boundaries, acquiring pathological signs at a voltage of 500 mV, although the intact features were recovered the next day after exposure withdrawal. A voltage of 50 mV was used in further experiments based on the results obtained.

We used mostly two active electrodes located in close proximity on both sides of *Trichoplax* plate. As one of effective options, pulses with a duration of 100 ms and a delay of 1 ms to 10 s were applied. For the experiments, 139 animals of H2 strain were used. We observed immobilization of animals, opacity

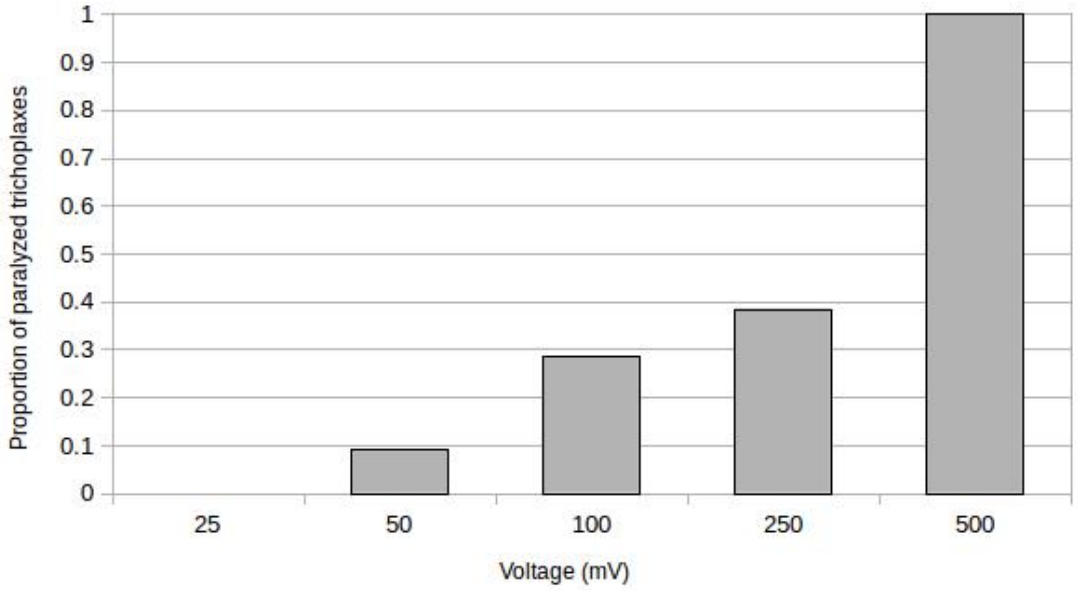

Fig. 3. Fraction of immobilized *Trichoplax* sp. H2 *versus* active animals at various voltage values of rectangular pulses at a frequency of 20 Hz. Totally 59 animals were used in the experiment

along the plate periphery and in its center with a subsequent reduction in size and wrinkling or folding of *Trichoplax* for different duty cycle values in 67 % of cases on average. These changes in physiological state and morphology of animal were not fatal. Trichoplaxes recovered several tens of minutes after the stimulus, which was became apparent in their flattening on the substrate and acquiring motion activity.

Probability of leaving the zone of stimulating electrodes increased in experiments with two electrodes located on one side of the plate. Moreover, the animal had the opportunity to move in different directions. This fact was later used as a characteristic feature for different strains. An average of 56 % of *Trichoplax* remained in immobilization zone near the electrodes.

In order to clarify the data obtained, an additional series of experiments was conducted on 121 animals using two significantly different duty cycle values, namely a delay of 1 ms and 1 s, with a pulse duration of 100 ms. In the case of placing the electrodes on both sides of *Trichoplax* plate, the fraction of animals crawled out from the zone of exposure was insignificant and amounted to 30 % with a delay of 1 ms and 52 % – with a delay of 1 s. The fraction of runaway animals was greater and amounted to 47 % for each pulse delay of 1 ms and 76 % – of 1 s, when the electrodes were placed on one side of *Trichoplax* plate (Fig. 4). It should be noted that in the experiments with two electrodes on both sides of the animal, *Trichoplax* took time to "decide" in which direction to crawl out of the "electrode trap". More often *Trichoplax* moved toward greater part of its body, located outside the electrodes.

Different *Trichoplax* strains testing. Various animal behavior patterns were revealed for different *Trichoplax* strains in comparative experiments on the effects of rectangular pulses with an average voltage value of 50 mV, a duty cycle of 0.5, and a frequency of 2 Hz and 2 kHz. The number of individuals moving from the anode to the cathode or *vice versa* – from the cathode to the anode – was counted. In the experiments, 143 individuals of *Trichoplax* were used. Of them: H1 strain – 51; H2 strain – 47; H13 strain – 45 individuals. The animal had to get out of the "electrode trap" – two electrodes on opposite sides of the body – in each experiment (Fig. 22). Long-term monitoring of shape change and *Trichoplax* trajectory was carried out (see supplementary file No. 3: https://doi.org/10.21072/mbj.2020.05.2.05).

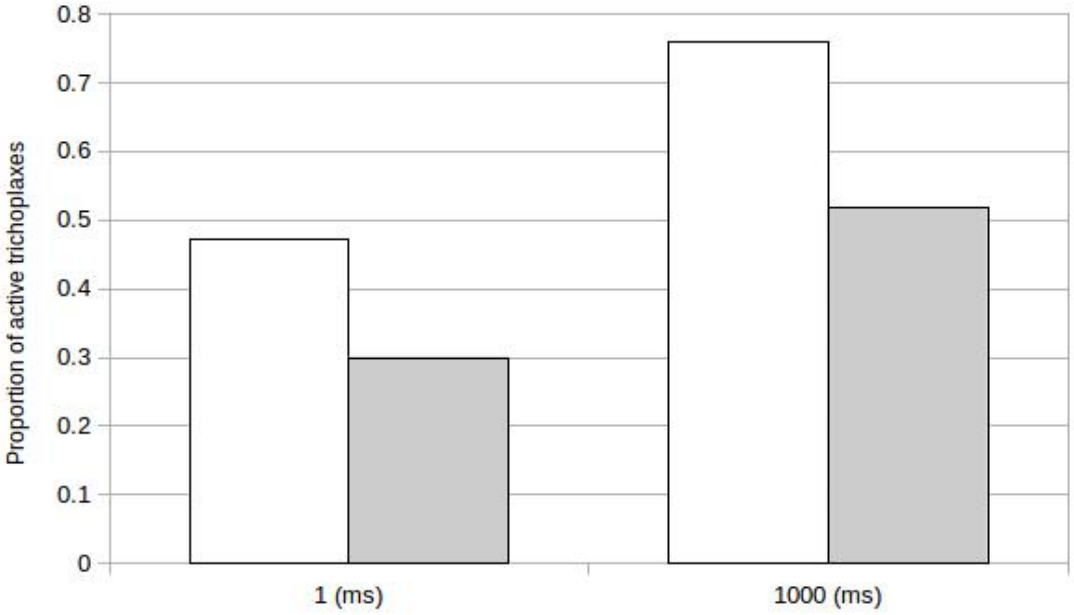

Fig. 4. Fraction of active *Trichoplax* sp. H2 *versus* immobilized animals at pulse duration of 100 ms and delay of 1 ms or 1 s; white color indicates the electrodes on one side of *Trichoplax* plate; gray color indicates the electrodes on opposite sides of *Trichoplax* plate. Totally 121 animals were used in the experiment

Trichoplaxes of H1 and H13 strains moved toward a low density of the lines of electric field intensity and sometimes stretched out along these imaginary lines (Fig. 5). Animals of H2 strain crawled to the opposite side of electrodes – to the "comfort zone" with a low density of lines of electric field strength – without significant moving away from the electrodes (Table 1). In most cases, individuals of H1 strain leaved the electrodes along an elongated path and often crawled behind one of the electrodes into the "comfort zone" at a low frequency (of 2 Hz). Animals of H2 strain preferred to move beyond the cathode, although sometimes they wandered between the electrodes and eventually crawled a short distance or left the anode. Representatives of H13 strain more often leaved the anode towards the cathode and moved beyond it. When using a high frequency (of 2 kHz), individuals of H1 strain preferred the anode, often remained in the "electrode zone", lost their motion activity, and wrinkled. Individuals of H2 strain quickly crawled to the anode or remained close to the electrodes, which often led to their immobilization. Individuals of H13 strain usually leaved the anode and moved to the cathode and crawled behind the cathode. Reaction lag, immobilization, and wrinkling at a frequency of 2 kHz were also observed for them.

Table 1. Behavioral patterns of *Trichoplax* between electrodes when exposed to rectangular pulses of various frequencies

Haplotype	**Rectangular pulse frequency**	
	2 Hz	**2 kHz**
H1	moves remotely behind one of the electrodes	moves away from the cathode towards the anode or remains in the area of the electrodes
H2	wanders between the electrodes, moves behind the cathode or anode, crawls away	approaches the anode quickly, wanders, stays close to the electrodes
H13	crawls off the anode and sometimes behind the cathode	distinctly leaves the anode, crawls behind the cathode

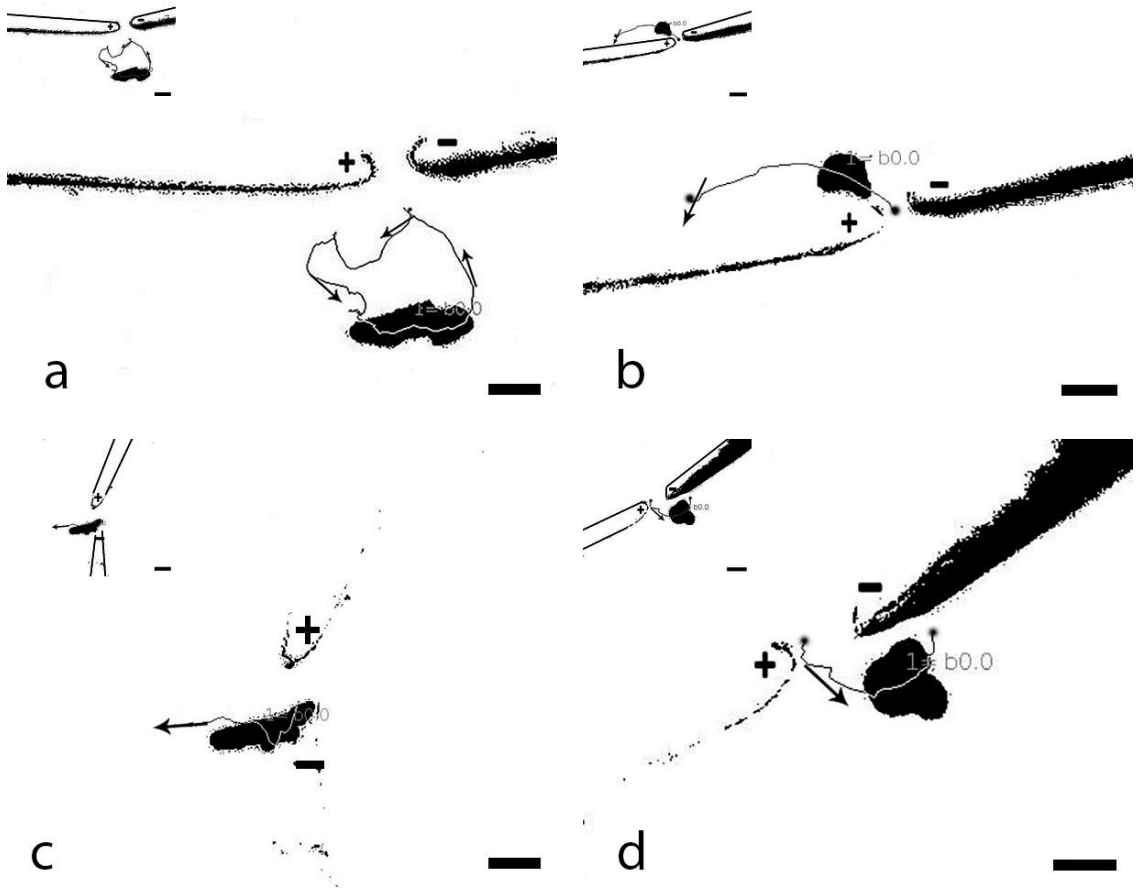

Fig. 5. Change of *Trichoplax adhaerens* H1 shape and motion trajectory from the "electrode trap": (a) seeking activity and elongation of *Trichoplax* along the line of electric field intensity; the directed motion of *Trichoplax* into the "comfort zone" located: (b) behind the anode, (c) far from the electrodes, and (d) behind the cathode. Arrows demonstrate *Trichoplax* motion direction; numbers in the figure indicate identification code assigned to the animal; "+" is the anode; "–" is the cathode; every bar length = 1 mm; graphic insert in the upper left corner explains the image

Experiments in the modes of calcium channels functioning. In order to presumably open *Trichoplax* calcium channels TCa_v3 *in vivo*, we used rectangular pulses with an average voltage of 50 mV, a duty cycle of 0.5, and frequencies of 2.5 Hz, 5 Hz, and 2 kHz. Frequencies are borrowed from [3 ; 20 ; 33]. Significant differences in the effects were found at frequencies of 2.5 Hz and 2 kHz. *Trichoplax* did not change the morphology, slowly crawled from the anode toward the cathode and moved beyond the cathode at a frequency of 2.5 Hz. On the contrary, *Trichoplax* mainly moved to the anode at a frequency of 2 kHz, but did not move far from the electrodes, changed the bulk tissue from transparent and shiny to opaque and dark, ultimately decreased in size, twisted, and detached from the substrate.

When applying the frequencies used in the patch-clamp technique [33], that is pulse duration and delay of 2 ms (500 Hz) or 500 ms (2 Hz), *Trichoplax* in the case of a frequency of 2 Hz did not change visible morphology and crawled from anode to the cathode, and in the case of a frequency of 500 Hz, they mainly moved from the cathode behind the anode or remained stationary and twisted.

In order to presumably open *Trichoplax* calcium channels TCa_v3 *in vivo*, we used rectangular pulses with average voltage of 10 to 120 mV, corresponding to delays of 10 s to 1 ms. Pulse duration was of 35 ms, as in [36]. To assess cumulative TCa_v3 channel-mediated effect of square-shaped pulses on *Trichoplax* sp. H2, delays of 1 ms to 10 s were used. Physiological effect of total pulses action was revealed after several

tens of seconds and looked like a dependence of animal mobility on the duty cycle in pulses sequence. The fractions of paralyzed animals were high – 78 and 80 % for delays of 1 and 10 ms, respectively, at low duty cycle, *i. e.* high frequency and large number of incoming signals (Fig. 6). As the duty cycle increased, the fractions of paralyzed *Trichoplax* decreased up to 44 % and 17 % in the case of one pulse per 1 s and one pulse per 10 s, respectively. Immobilized *Trichoplax* were absent in control experiments without electrical exposure.

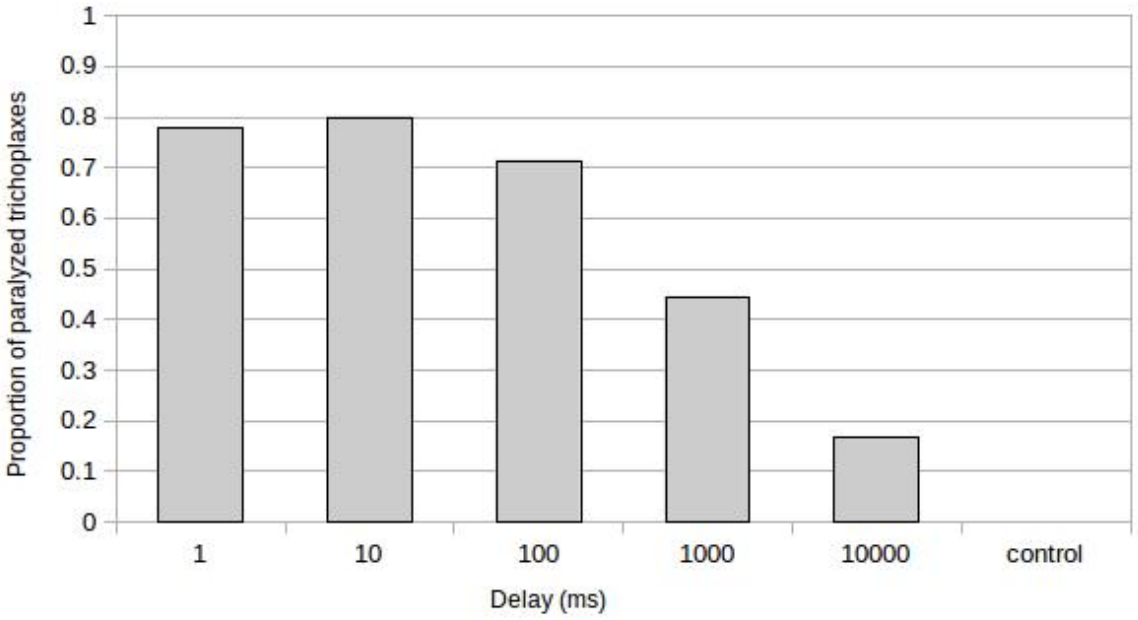

Fig. 6. Fraction of immobilized *Trichoplax* sp. H2 *versus* active animals at pulse duration of 35 ms and delay of 1 ms to 10 s. Totally 65 animals were used in the experiment

Calcium channels blocking. Amlodipine, an inhibitor of the activity of *Trichoplax* voltage-gated calcium channels TCa_v3, was used to prove the specificity of pulse currents effect on these channels.

In the presence of a small amount of amlodipine (25 nM), despite exposure to electric pulses of a duration of 35 ms with a delay of 10 ms, almost all *Trichoplax* (H2 strain) retained their native morphology and mobility for several minutes, preferring to move to the anode and then to leave it, which indicated the prevention of electric shock, observed at low duty cycle (Fig. 6). To identify possible targets, we exposed *Trichoplax* to rectangular pulse packages of variable width and duty cycle with a step of 1 ms, covering a frequency range of 1 Hz to 1 kHz (see supplementary file No. 2: https://doi.org/10.21072/mbj.2020.05.2.05). Immobilization of animals and formation of pans were observed, and their fraction decreased in the presence of 25 nM of amlodipine. However, all *Trichoplax* dissociated on individual cells under amlodipine treatment after several hours.

Effect of large doses of amlodipine on *Trichoplax* was studied in final series of experiments. Amlodipine at a concentration of 50 μM caused a rapid folding of *Trichoplax* plate-like body into a pan in the ventral-dorsal direction and subsequent dissociation of the plate into individual cells (Fig. 7). The pan formation continued for several minutes with amlodipine concentration reducing to 2.5 μM, which made it possible to register motion of the animals to the anode with a duration and a delay of pulses of 35 ms each [36] that corresponds to a frequency of 28.57 Hz. When using amlodipine with a concentration of 250 nM, gradual darkening of *Trichoplax* was observed – first along the periphery, then in the plate center. The outer rim thickened, *Trichoplax* rounded, forming a rugged scalloped edge, the blades of which were torn off the substrate, bent upward, and formed a rosette. Being in an “electrode trap”, animals moved discoordinated

and could not leave it. Further, rigidity of *Trichoplax* plate occurred, which was stated in the rigidity of the shape of the animals when moving. Nonetheless, motion patterns of individuals in electric field persisted: *Trichoplax* moved mainly to the anode. Later, plate rims resembling blades bent up and in. Animals dissociated into individual cells approximately 1 h after adding amlodipine, first along the periphery, then throughout the body.

Trichoplax H1 and H13 strains showed, like H2 strain, a change in morphology under the influence of 25 nM of amlodipine and a violation of amoeboid motion over time. In experiments without amlodipine, no similar phenomena were observed. Animals wandered between the electrodes, placed in the "comfort zone" or moved away at a safe distance from the electrodes in the absence of chemical effects. It should be noted that non-dihydropyridine Ca-channel blockers, such as verapamil and diltiazem with a concentration of 100 μM, did not significantly affect *Trichoplax* H2 strain, which remained viable in the presence of these substances within one day [data is not given].

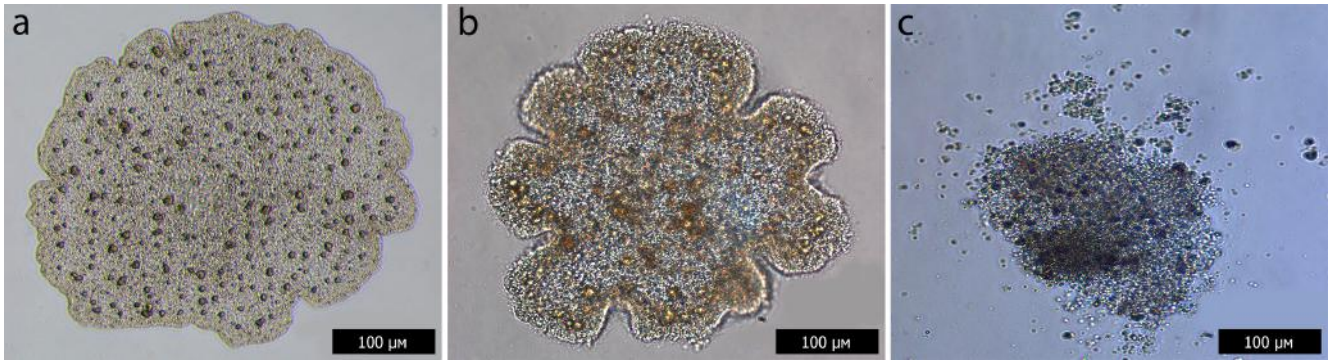

Fig. 7. Time-dependent effect of amlodipine (of 50 μM) on *Trichoplax* sp. H2: (a) intact animal; (b) folding into a pan after 30 minutes; (c) dissociation into individual cells after 60 minutes; bar length = 100 μm

DISCUSSION

Because of widespread distribution of extremely low frequency electric and magnetic fields, causing multiple physiological effects in humans [26 ; 44], the search for test objects for studying the mechanisms of ELF-EMF action is relevant. *Trichoplax adhaerens* was recently proposed as a test laboratory model [1]. We studied the effect of rectangular electric pulses of various amplitude, duration, and duty cycle on three laboratory *Trichoplax* strains (H1, H2, and H13).

In control tests using wood, plastic, or metal rods, no reaction of the animal to foreign objects placed near it was observed, except the cases of galvanic pair formation. Under the effect of a weak direct current with a voltage of 200 mV, *Trichoplax* H2 strain crawled away from the electrodes. However, reaction rate and motion trajectory were largely dependent on animal initial position in relation to the electrodes. When using active electrodes, one of which (the anode or the cathode) was placed near *Trichoplax*, the animal usually moved away from the stimulus. When placing both electrodes near *Trichoplax* plate, various motion patterns to the anode or the cathode were registered, depending on the stimulation mode and animal strain: "positive" migration to the anode, "negative" migration to the cathode, and "variable" migration when the animal changed preference anode – cathode several times. In the case of *Trichoplax* getting into the zone of close proximity to the electrodes, the animal was not always able to get out of the "electrode trap", which directly depended on the rise of amplitude and number of pulses. Far from the electrodes, *Trichoplax* sometimes stretched along the lines of electric field strength and headed to the hypothetical "comfort zone" with the lowest electric field intensity on back side of the electrodes.

In comparative experiments on the effect of rectangular pulses with a frequency of 2 Hz and 2 kHz on different *Trichoplax* strains, it was revealed that *Trichoplax* sp. H2 is more reactive and shows more pronounced physiological responses at frequencies of 2 Hz and 2 kHz compared with H1 strain and especially with H13 strain, which mainly migrates from the anode to the cathode. Therefore, most experiments were conducted with H2 strain. Despite preferable motion of *Trichoplax* H2 strain toward the cathode at low pulse current frequencies (about 2 Hz), a tendency was observed to gradually change a migration direction towards the anode with increasing pulse frequency (up to 2 kHz). Nevertheless, attention should be paid to unexpectedly wide individual variability in *Trichoplax* behavior, which complicates such interpretations and requires further research.

Sufficient attention was paid to time regimes previously used by other authors in detailed studies of L- and T-type calcium channels [3 ; 20 ; 33 ; 34 ; 36]. We investigated effects in frequency range of 2 Hz to 2 kHz. *Trichoplax* behavioral reactions were not unambiguous: extreme frequencies sometimes did not have an expected effect or led to an electric shock of the animal, which might be due to *Trichoplax* physiological state and/or initial position of the animal in the "electrode trap". The motion absence, plate clouding, size reduction, and wrinkling were reversible, and after a while or after the animal returned to the algal mat, *Trichoplax* revived their motion activity.

Depending on duration of stimulating pulses and their number, motion reactions and morphology of animals changed: from stochastic or directed migration to/from anode/cathode to immobilization of animals, optical density elevation, first along the periphery, then in plate center, to *Trichoplax* wrinkling, and even to separating it from the substrate. The effect applied was cumulative in its nature, which is probably related to the work of calcium channels and the activity of downstream regulatory cascades [10]. It is known that glandular cells located on *Trichoplax* periphery express voltage-gated calcium channels [32 ; 39]. Morphological changes observed in *Trichoplax* plate can be associated with calcium channel-mediated responses of secretory cells containing regulatory neuropeptides [42]. On the other hand, it was shown that ELF-EMF pathophysiological effects are associated at the molecular level with regulation of Ca^{2+}/nitric oxide/peroxynitrite, and positive ELF-EMF physiological effect is explained by the alternative pathway of Ca^{2+}/nitric oxide/cGMP/protein kinase G [19]. Mutually exclusive *Trichoplax* behavioral reactions, such as positive and negative electromigration, to varying modes of electrical exposure (Table 1) may be due to the various signaling pathways involving calcium ions in behavioral reactions.

It is believed that amlodipine, when binding to dihydropyridine receptors, blocks L- and T-type calcium channels, which leads to a drop-off in Ca^{2+} transfer to the cell. Amlodipine also has antioxidant properties and contributes to the production of the neurotransmitter nitric oxide due to regulation of Ca^{2+} ions concentration in the cell [4 ; 11 ; 14 ; 16 ; 25]. Additional experiments with amlodipine showed that this calcium channel blocker at low concentrations (of 25 nM) is capable of briefly neutralizing the shock effect of rectangular electric pulses in a duration of 35 ms and a delay of 10 ms, which usually leads to immobilization of *Trichoplax* sp. H2 without amlodipine. We also scanned potential cellular targets in frequency range of 1 Hz to 1 kHz and studied *Trichoplax* responses to packages of rectangular pulses using a software meander with a step of 1 ms. A decline in ELF-EMF negative effect within this range with amlodipine at a concentration of 25 nM may indicate that ELF-EMF affects L- and/or T-type calcium channels of *Trichoplax*.

In addition to silencing the effect of electric stimulus on the animals with amlodipine, we observed other effects. Thus, use of this calcium antagonist in high concentration (of > 2.5 μM) resulted in *Trichoplax* dissociation into individual cells, which is directly caused by the destruction of calcium bridges [28].

When using this calcium blocker in moderate concentration (of < 250 nM), a violation of amoeboid motion of *Trichoplax* was noted, which can result from a decrease in the functional activity of the cells initiating motion, a distortion of activation waves propagation, or a violation of nitric oxide synthesis, which may play a role in rapid contractions of the dorsal epithelium [2 ; 11 ; 16 ; 25]. It should be noted that at the same time, the residual mobility of rigid animals provided by the cilia was observed, which indicates independence of the mentioned process from regulation by calcium ions.

Our data show that amlodipine inhibits *Trichoplax* calcium channels functioning, which is manifested both in a decrease in animals' reactivity at a low concentration of Ca^{2+} channel blocker and in the dissociation of the cells, that make up the animal, at a high concentration of calcium antagonist. It should be noted that amlodipine effect is similar to that of a compound ML218 – a specific blocker of T-type calcium channels in humans. Thus, electrophysiological studies of neurons of the subthalamic nucleus in the presence of ML218 revealed the inhibitory effect of ML218 on T-type calcium channels, suppression of the low-voltage-activated response, and inhibition of neuron activity burst [47].

The assumption in favor of T-type Ca^{2+} channels was confirmed in additional experiments on *Trichoplax* H2 strain, where besides amlodipine, one of dihydropyridine calcium channel blockers, non-dihydropyridine calcium channel blockers, such as verapamil and diltiazem, were tested. Amlodipine led to *Trichoplax* dissociation into individual cells, while verapamil and diltiazem did not have such an effect on animals. This fact confirms that amlodipine blocks *Trichoplax* low-voltage-activated Ca^{2+} channel TCa_v3, because amlodipine is a blocker of L- and T-type calcium channels, while verapamil and diltiazem are only blockers of high-voltage-activated L-type calcium channels.

It should be noted that *Trichoplax* motion was not strictly targeted, but resembled a "stochastic" taxis [38], kinesis, or motion to a target by trial and error. It points out that *Trichoplax* has no central regulator and indicates, possibly, distributed control and collective decision making between cells, which leads in some cases to a delay in system response to stimulus [6].

Conclusion. The study of *Trichoplax* electrophysiology is important in connection with the prevalence of ELF-EMF and is of interest because of simple structure of the animal and ease of cultivation, which makes it possible to understand the mechanisms of its behavior and motion in the future. The diverse responses of *Trichoplax* to electrical stimulus discovered in our experiments indicate latent possibilities of this organism, based on the collective action of its cells.

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ДЕЙСТВИЕ ПРЯМОУГОЛЬНЫХ ЭЛЕКТРИЧЕСКИХ ИМПУЛЬСОВ НИЗКОЙ ЧАСТОТЫ НА ТРИХОПЛАКСА (ТИП PLACOZOA)

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Влияние низкочастотного электромагнитного излучения (НЭМИ) на растения и животных, включая человека, достаточно спорно. Мало известно и о воздействии НЭМИ на гидробионтов. Мы изучили действие прямоугольных импульсов напряжения различной амплитуды, длительности и скважности, пропущенных через морскую воду, на трихоплакса (тип Placozoa) как на возможную тестовую лабораторную модель. В опытах использовали три штамма Placozoa, *Trichoplax adhaerens* (H1), *Trichoplax* sp. (H2) и *Hoilungia hongkongensis* (H13), отобранных на стационарной стадии роста культуры. Для генерации последовательности прямоугольных импульсов заданной длительности и скважности с частотой до 2 кГц применяли аппаратную платформу Arduino Uno. Среднее значение напряжения до 500 мВ регулировали с помощью схемы делителя напряжения. Для доказательства специфичности действия электрических импульсов на потенциалзависимые кальциевые каналы трихоплакса использовали ингибитор активности кальциевых каналов амлодипин. Животных стимулировали электрическим током под стереомикроскопом. Электроды располагали в непосредственной близости от животного. Исследовали сопутствующие изменения поведения и морфологии пластинки трихоплакса. Выделяли стимулирующие и подавляющие воздействия. Наблюдения документировали с помощью фото- и видеосъёмки. Отслеживали траектории движения отдельных особей. Увеличение напряжения на электродах при фиксированной частоте 20 Гц приводило к тому, что животные штамма H2 покидали «зону электродов» в течение нескольких минут при 25 мВ, однако теряли подвижность пропорционально росту напряжения и обездвиживались при 500 мВ. Именно поэтому в дальнейших опытах применяли напряжение 50 мВ. В экспериментах с двумя электродами, находящимися с одной стороны трихоплакса, у животного было больше возможностей перемещаться в разных направлениях, чем в случае расположения электродов по обеим сторонам пластинки. Направление движения использовали как характеристический признак. Отмечено, что трихоплаксы мигрируют в области с низкой плотностью линий электрического поля, которые расположены вдали или за электродами. Животные из старой культуры отличались меньшей чувствительностью к электрическому раздражителю. Штамм H2 был наиболее чувствительным и демонстрировал более выраженные физиологические реакции на частотах 2 Гц и 2 кГц с напряжением 50 мВ, чем штамм H1 и особенно штамм H13. В зависимости от длительности стимулирующих прямоугольных импульсов, их числа, амплитуды и варьирующей частоты менялись двигательные реакции и морфология животных: от направленной или стохастической миграции в сторону анода/катода или от него до обездвиживания животных, увеличения оптической плотности по периферии и в центре пластинки и до сворачивания трихоплакса и отделения его от субстрата. В дополнительных опытах на *Trichoplax* sp. H2 показано, что при длительности импульсов 35 мс и задержке импульсов

от 1 мс до 10 с доля обездвиженных животных увеличивается до 80 % при минимальной задержке. Тем не менее в случае применения амлодипина в концентрации 25 нМ практически все трихоплаксы в течение нескольких минут сохраняли подвижность несмотря на обработку электрическими импульсами. Между тем при использовании амлодипина в концентрации 250 нМ животные двигались дискоординированно и не могли покинуть «электродную ловушку». Далее пластинка трихоплакса становилась ригидной, что выражалось в неизменности формы животного при движении. Наконец, амлодипин в концентрации 50 мкМ вызывал быстрое сворачивание краёв трихоплакса в розетку в вентрально-дорсальном направлении и последующую диссоциацию пластинки на отдельные клетки. В целом применяемое электрическое воздействие имело кумулятивный, но обратимый эффект, который, как предполагается, может быть связан с работой потенциалзависимых кальциевых каналов. Амлодипин в большой концентрации (50 мкМ) вызывал разрушение трихоплакса, в умеренной (250 нМ) он нарушал, вероятно, распространение волн активации, что приводило к дискоординации движений животного, а в малой (25 нМ) предотвращал электрошок.

Ключевые слова: прямоугольные электрические импульсы, трихоплакс, пластинчатые, потенциалзависимые кальциевые каналы