Ethological Response to Periodic Stimulation in *Chara* and *Blepharisma*

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Abstract. To study how organism responds to periodic stimulation is meaningful since it may be an approach to an elementary capacity of time memory and learning in chronological events. We reported here that the ability of time memory found in true slime mold *Physarum* was also found in a protozoan ciliate, *Blepharisma japonicum* and a green plant *Chara*. Stimulation of temperature or light was repeated several times in a regular period, and the creature anticipated the next timing of stimulation. After the anticipatory behavior disappeared some time later, another single stimulation triggered recalling of periodicity of the previous stimulation. We discuss that the observed capacity is expected to be common in a range of species as the similar capacity has been reported in true slime mold *Physarum*. The observed responses were, however, dependent of individual of organism and a wide range of different responses was observed. We need an extensive study of both experimental characterization and mathematical modeling of ethological dynamics. *abstract environment*.

Keywords: *Physarum*, cell memory, subcellular computing, primitive intelligence.

1 Introduction

Organisms in the wild life are exposed to various kinds of stimulation from their environments. Stimulations are not merely single but repeated many times in time sequence[1–4]. The time sequence can be in general described by a function of time. Such a function is properly approximated by a finite Fourier series of trigonometric functions. To study response to stimulation of regular single frequency is elementary since response to each single frequency contributes to organization of complex behavior induced by realistic time sequence of stimulation in the wild. Although the original time sequence is much more complicated,

here we study the elementary character of organism behavior in response to regularly periodic stimulation.

In 2008, an interesting response to a periodic stimulation was reported in a true slime mold, plasmodium of *Physarum polycephalum*. Plasmodium showed a kind of time memory[5]. Stimulation of low temperature and low humidity were applied three times in a regular period, and the creature anticipated the next timing of stimulation. After the anticipatory behavior disappeared some time later, another single stimulation triggered recalling of periodicity of the previous stimulation. The authors proposed a possible mechanism of observed behavior. An idea to be emphasized is that these behaviors can be realized in dynamics for collective motion of intracellular chemical oscillators. The model equation is simple and generic. This finding may give a hint at evolutionary origin of capacity of time memory[6].

However, it is unclear whether the ability of time memory is specific to *Physarum* only or common to a range of different species. If it is common, the capacity is expected to be general more or less. So seeking for the similar ability in a different organism from *Physarum* is meaningful. We reported here the similar ability of time memory in a protozoan ciliate, *Blepharisma japonicum* and a green algae *Chara*.

In this study, in addition to involvement of those different species, three kinds of physical nature of stimulation, temperature, light and electric current, were tested. If the similar capacity is observed independently from the difference of physical nature of stimulation, the capacity can be organized somewhat in a central unit of information processing, rather than peripheral sensory activity.

Discussion was made on general features that was observed among species and among three kinds of stimulation. Lastly we examined if the model equation proposed in the previous paper[5] was still applicable to *Blepharisma* and *Chara*.

2 Method

2.1 Organism and Culture

A protozoan ciliate, *Blepharisma Japonicum* (wild type), was purchased from Kyoto Kagaku Co. (Kyoto, Japan). The ciliate was cultured at 24 °C in dark, in a Petri-dish (12 cm in the diameter) with the culture medium of 100 times diluted Chokley solution (0.1 g/l NaCl, 0.004 g/l KCl, 0.006 g/l CaCl₂, in the final concentration) and several grains of rice. As the medium was not sterilized, some bacteria and protozoa co-existed. About twenty to thirty organisms were collected from the culture dish, and put them in the smaller arena with disk-shape (10 mm in the diameter and 1 mm in the thickness), which was served for experimental observation. The arena was immersed in the water bath (24 °C in dark) and fixed in order to keep the temperature constant and stable (see Fig. 1a).

Fig. 1. Experimental methods for *Blepharisma*. (a) Schematic illustration for top and side views of experimental arena in order to observe swimming behavior in *Blepharisma*. The organisms were allowed to swim in the arena with a disc shape (diameter: 10 mm, height: 1 mm). In order to change temperature of water in the arena, coolant was poured on the arena but the coolant was isolated from water of arena with a thin plastic. PL: plastic plate, CL: coolant, OG: organism in the arena, RR: rubber ring. (b) Characterization of swimming behavior. From a real trajectory of swimming (thick solid line), the position of organism, $p(t_i)$, was measured every one second. Swimming distance was approximated by the straight distance every one second, $|p(t_i) - p(t_{i+1})|$ as indicated by the dashed line.

2.2 Observation of Swimming Behavior in Response to Periodic Stimulation in *Blepharisma*

The arena was put on the stage for observing behaviors of free swimming by the CCD video camera, under the infra-red light only. The video camera setup was placed in the incubator, in which temperature was controlled and fixed at 24 °C. The video image was recorded and served for image analysis.

After the ciliates in the arena were left gentle on the observation stage without any agitation for a few hours, swimming behavior was recorded. Two kinds of stimulation were applied. One was the stimulation of low temperature. The procedures were the following. The 24 °C-water in the water bath was replaced by the −18°C-coolant (saturated NaCl aqueous solution), and it was kept for five minutes and 24 °C-water was set back again. This was the low temperature stimulation. The other was stimulation of white-light illumination with the light source of fluorescent tube (20 lux).

One of the two types of stimulation was repeated multiple times in a regular interval of time, and a single stimulation was applied again after a long time was passed enough for behavioral response to the previous periodic stimulation to be negligible.

The trajectory of free swimming was traced at the video rate by the computer software, previously developed[7]. Along the trajectory, we picked up the position of creature every one second, and the position was expressed by $p(t_i)$ at time

Fig. 2. Typical trajectories of swimming in *Blepharisma*. Upper panels are superimposed four-second-long trajectories in the observation arena. Lower panels are characterization of swimming trajectory, measured by swimming speed and angle of turning at every second. (a) in normal conditions before the cold stimulation, (b) in cold stimulation of PS_n , (c) in the anticipatory occasion of A_n . Swimming was slower and the direction often turned in the cold stimulation. Similar tendency was observed in the anticipatory occasion although the response was much weaker. This tendency was quantitated by the quantity DS/RG (see the method in detail).

t_i discretized in second. The trajectory was approximated by a straight line segment that connected two positions of $p(t_i)$ and $p(t_{i+1})$. Thus the original trajectory was replaced by combination of line segments (see Figs. 1b,2).

Every four second, the distance of swimming (DS) was calculated as

$$DS(t_i) = \sum_{j=0}^3 |p(t_{i+j}) - p(t_{i+j+1})|.$$

We defined the range (RG) in order to characterize spatial extent of swimming trajectory as

$$RG(t_i) = \max |p(t_{i+j}) - p(t_{i+k})|,$$

where $j, k = 0, 1, 2, 3, 4$. RG takes a large and a low values when the swimming trajectory is straight and localized, respectively. In other words, RG is the largest distance between any two positions p in the period of four seconds. The ratio of two measures, DS/RG , is a non-dimensional quantity, which indicates features of swimming behavior. This index DS/RG is larger as the ciliates turns more frequently. The minimum value is one when it swims straight.

2.3 Measurement for Slowdown in Protoplasmic Flow, Induced by Periodic Stimulation in *Chara*

Chara, a green plant in fresh water like pond and river, was cultured in natural pond water with natural pond soil in 20 to 23 °C. A main stem of the plant with about five nodes was cut and served for experiment. A whole piece of the cut stem was exposed to an external stimulation (see Fig. 3).

Two kinds of stimulation were applied. One was the stimulation of low temperature. The procedures were the following. The specimen was soaked into water (23 °C) in a petri dish. The 23 °C-water in the dish was replaced by the 0°C-water, and it was kept for one minute and 23 °C-water was set back again. This was a single stimulation of cold water. This stimulation was repeated periodically.

The other was stimulation of electric current through the stem of specimen. Voltage of 1.5 volt was loaded with a resistor 60 Ω that was connected parallel to the specimen (see Fig.1c). Period of load lasted for one minute. This was the single stimulation and it was repeated periodically.

Protoplasmic flow was observed in a middle inter-nodal cell in the specimen under the microscope. Image of flow was recorded by the video-camera. Intracellular particles that flew in the cell were tracked by the personal computer and the

Fig. 3. Pictures of *Chara*. (a) Appearance of green stem of plant in the petri dish. (b) Inter-nodal cell in the stem. (c) Some flowing particles (indicated by arrows) in the inter-nodal cell. (d) Schematic illustration for periodic stimulation of electric current. CR: specimen, PW: pond water in dish. The specimen was soaked into three containers of left, center and right as connecting two containers of left and center, and center and right through the stem of plant. An inter-nodal cell in the center container was observed by a microscope.

originally developing software (particle tracking velocimetry). Flow velocity was calculated and averaged every 10 seconds. The original time variations of flow velocity was smoothed by the moving average (time window was two minutes).

3 Results

3.1 Spontaneous Increases of Swimming Measure at an Anticipatory and a Recalling Occasions in *Blepharisma*

Figure 4 shows a typical response to the repetitive stimulation of light (one minutes) once every five minutes. The occasions of stimulation were indicated by $PS1, PS2, \dots, PS10, TR$ and the behavior measure DS/RG increased by the stimulation. This means that the organism tends to frequently turn the swimming direction. After the stimulation, some spontaneous increases were periodically observed

Fig. 4. Responses to periodic light stimulation. (a) Typical time course in a collection of approximate thirty organisms in a single experiment. In response to the periodic stimulation, the behavior index (DS/RG , straightness of swimming) went down and up. After the stimulation, spontaneous down and up of index was observed. After the single stimulation at TS , the periodic spontaneous variations of index appeared again. PSn : the occasion of n th stimulation, An : the n th occasion of anticipatory response, TS : the occasion of single stimulation several hours after the periodic stimulation, Rn : the n th occasion of recalling response. (b) Statistical occurrence of spontaneous decrease in the swimming index with respect to time. The frequency was clearly higher at $A1$ than at the neighbors of $A1$. After the stimulation at TS , the frequency was higher at $R2, R3$ and $R4$ than at their neighbors. Anticipatory and recalling behaviors were observed although the responses were weak. $N=8$.

Fig. 5. Recalling responses to repetitive cold-stimulation in six times with the period of 20 minutes. Time courses were superimposed from four experiments. In each experiment, the time course was obtained from a collection of about thirty organisms. Almost 120 organisms were observed for this figure. Six hours after six times of stimulation, the single stimulation was given at TS. Significant increase of measure was observed every occasion of R1 to R4.

Fig. 6. Typical time course of protoplasmic streaming in response to the periodic electric stimulation. The speed of streaming slowed down at every occasion of stimulation (PS1 to PS10). Spontaneous slowdown was often observed just at the occasions of A_n (indicated by the arrows).

and the periodicity seemed similar to that of experienced stimulation (see the time section from A1 to A3), loosely speaking. The spontaneous increases happened just at A1, A2 and A3. Once this response of spontaneous increase disappeared around 70 min, the single stimulation was applied at TS.

Figure 4b shows the statistical occurrence of increase ($N=8$). The spontaneous increases were significant at A1 although environmental conditions were kept constant. After the single stimulation at TS, the periodic spontaneous increases clearly appeared again at R2, R3, R4 and R5 in the similar period to the previously experienced one although the single stimulation had no information

Fig. 7. Statistical examination of responses to the electric stimulation. (a) the flow speed averaged over five repeats. The speed shown in Figure was the difference from the time average of streaming over an entire experiment, so that deviations of flow speed in five organisms were canceled out. The spontaneous slowdown appeared around the occasion of An (indicated by the arrows). (b) Statistical frequency of spontaneous slowdown. A typical slowdown counted was indicated by the arrows in Figure a. High frequency was periodically observed at A1 and A2. $N=5$.

of periodicity. We say the spontaneous increases at An and at Rn 'anticipatory' and 'recalling' behavior, respectively, according to the previous paper[5].

Figure 5 shows the statistical time course of swimming measure after the single stimulation at TS, which was given six hours later from the periodic cold stimulation (period 20 min, 6 times). Data points obtained from four experiments were superimposed. The periodic increases were clearly observed at R1, R2, R3 and R4 while the stimulation was not given at all. Nonetheless, anticipatory response was not observed in the case.

3.2 Spontaneous Slowdown of Protoplasmic Flow at an Anticipatory Occasions in *Chara*

Figure 6 shows a typical time course of protoplasmic flow in response to the periodic stimulation of electric current (period 6 min, 10 times). The flow speed decreased with the stimulation and the spontaneous slowdown took place periodically at An (indicated by arrows) although the slowdown was sometimes not so clear at A2. Figure 7 shows statistical confirmation of anticipatory responses by measuring average speed of protoplasmic flow (a) and statistical occurrence of slowdown (b). The spontaneous slowdown was clearly periodic and corresponded to the occasion of An (indicated by the arrows) although there sometimes was a slight shift of time like A1 and A4. In the frequency, anticipatory response was significant at A1 and A2.

Fig. 8. Typical time course of protoplasmic streaming in response to the periodic cold stimulation with a period of 6 minutes. The speed of streaming slowed down at every occasion of stimulation (PS1 to PS10). Spontaneous slowdown was often observed just at the occasions of A_n (indicated by the arrows).

Fig. 9. Statistical examination of responses to the cold stimulation. (a) the flow speed averaged over five repeats. The speed shown in Figure was the difference from the time average of streaming over an entire experiment. The spontaneous slowdown appeared around the occasion of A_n (indicated by the arrows). (b) Statistical frequency of spontaneous slowdown. A typical slowdown counted was indicated by the arrows in Figure a. High frequency was periodically observed at A1 (weak), A2 (strong) and A3 (intermediate). $N=5$.

In the periodic cold stimulation (period 6min, 10 times), the response was similar to that in the electric stimulation as the typical time course was shown in Fig. 8. The protoplasmic flow spontaneously slowed down around the time of A_n , similarly to the response induced by the cold stimulation. The slowdown was, however, sometimes not clear at A1, for instance.

Figure 9 shows the statistical examination of anticipatory response. The averaged flow speed varied up and down between A1 and A2 and between A2 and A3 (Fig. 9a). The time of slowdown was nearly at An while there might be slight shift to just An . But the statistical frequency of slowdown increased just at An as shown in Fig. 9b.

4 Discussion

On the periodicity of stimulation, the period tested in this report was limited at 5, 6 and 20 min and shorter while the periods tested in *Physarum* were 30, 40, 50, 60, 70, 80 and 90 min. But the range of the shortest and the longest periods, $20/5(=4)$, was rather wider than that of $90/30(=3)$. In such new periods tested in the report, anticipatory and/or recalling behaviors are observed.

In this report, not only temperature stimulation but also light and electric stimulation were newly tested. The organisms tested were capable of the anticipatory and/ or recalling behaviors in response to those types of stimulation. This implies that the behaviors are processed not in a peripheral sensory system but in rather central (or common) system for information processing. A question arise then: what and where is the system for the information processing and how it works in Protozoa and plant?

Blepharisma is one of protozoa similar to *Physarum*. The capacity of time memory like the anticipatory and recalling behaviors may be widely common in protozoa. On the other hand, *Chara* is far from protozoa from a phylogenetic point of view. However, the similar capacity of time memory was found. What does this mean? We expect that the capacity might be common much widely in phylogenetic tree of organism as we remind the other suggestion that human can also have a similar capacity. It is attractive to continue to study the time memory found in this report since the study might give a hint at origin of memory capacity in organism.

In the future, we will examine the other species. It is also interesting to test a more complex time sequence of stimulation. A possible sequence can be multiply periodic and irregular. Then we may evaluate a level of capacity.

Because the responses seems not so simple as observed in this report, we need a powerful method to analyze oscillatory behaviors in general. Frequency analysis and mode analysis are of strong interest. Oscillatory behaviors in *Physarum* have been widely studied experimentally and theoretically from a dynamical system point of view[5, 8–14]. The dynamical system point of view is to be helpful as well. Namely it is promising to compare to dynamic behaviors of visco-elastic mechanics and biochemical reaction kinetics in response to external periodic forcing.

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