Hypothesis: Possible Molecular Mechanisms for Paramecium Learning

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ABSTRACT

Learning is a cornerstone of intelligent behavior in animals. This behavior has been mostly studied in organisms with a fairly complex nervous system. However, recent reports of learning in unicellular organisms suggested the existence of associative learning in unicellular organisms. In particular, the capability to associate different light intensities with cathodal stimulation in paramecium is of special interest. We have investigated the previous reports on this phenomenon and proposed a molecular mechanism for learning behavior in paramecium. Specifically, we have used the existing evolutionary evidence in order to find the possible molecular pathways that may play a role in Paramecium's learning. Moreover, previous studies have been reviewed in order to propose new experiments that can verify the plausibility of the present hypothesis.

1. Introduction

Coordinated, adaptive and intelligent behaviors are considered as hallmarks of neural systems. These features are expected from any sufficiently evolved neural system. However, characteristics like learning and memory seem to require less intricacy and organization. It seems that the learning per se does not need a highly complex machinery. More specifically, learning is divided into two types of associative and nonassociative [1]. Sensitization and habituation are two forms of nonassociative learning and they are defined as an increase (sensitization) or decrease (habituation) in stimulus-driven responses due to repetitive stimulation. These phenomena have been shown to exist in different types of organisms from the ciliateStentor coeruleus [2] and cultured neurons of *Aplysia californica* [3] to human subjects [4, 5]. Intriguingly, habituation has been also observed in the plant species Mimosa pudicathat possesses a foldable compound leaf.

On the other hand, associative learning (defined as the capability to form associations between two stimuli [1]) have been reported to exist mostly in animals with a well-developed nervous system [6]. Specifically, associative learning is often assumed to require strengthening of synaptic connectivity between neurons [1]. The process of associative learning involves strengthening of synaptic connections between pre-synaptic and post-

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synaptic neurons through a molecular cascade [6]. This molecular cascade needs to be triggered by an interneuron that signals the presence of an unconditioned stimulus [1]. Accordingly, the central process in associative learning is changes of synaptic strength between two or more neurons. Therefore, it is usually assumed that the associative learning at least requires a simple nervous system and interaction between few neurons.

However, this assumption has been seriously challenged by several findings in the past decade. It is found that the *Physarum polycephalum* (the giant unicellular slim mold) can have learning capabilities alongside its other intelligent behaviors such as shortest path finding and solving transportation problems [7, 8]. When Physarum polycephalumis exposed to relatively cold and dry temperatures, it reduces its speed and acceleration. Upon periodic exposure of Physarum polycephalum to cold temperatures, it can learn to predict the periods of temperature reduction and reacts prior to the start of the next cold period [9]. In is noteworthy to mention that *Physarum polycephalumcan* grow to tens or hundreds of centimeters in size [10] which is larger than many multicellular organisms. Therefore, it might be argued that Physarum polycephalum is not a typical unicellur organism ant it has probably developed some complex mechanisms for learning. Paramecium, on the other hand, is a typical unicellular organism that can learn [11]. Presence of learning in paramecium has been suggested as early as 1911 [12].

According to Day and Bently, when paramecium is sucked into a capillary tube that is still connected to the swimming medium of paramecium for several times, its escape time reduces in subsequent trials. Accordingly, it was suggested that paramecium can learn. This observation was replicated again in 1940's and 1970's [13-15]. However, Applewhite and Gardner showed that this behavior in due to accumulation of ions in the capillary tube after consecutive trials and upon changing the capillary-tube in each trial, this behavior will be vanished [16]. Moreover, Hinkle and Wood proposed that this behavior can be explained through geotropism in paramecium [17]. Another attempt by Gelber in 1952 showed that paramecia that had been exposed to food sources (*Aerobacter aerogenes*) on tip of a platinum wire will learn to accumulate on the tip of platinum wire after a few trials [18]. Again, this study was challenged 5 years later by Jensen [19]. It was suggested that the so called learning was due to metabolite concentration gradient of the food source [19].

Consequently, the study of the paramecium learning behavior extended to 21st century when Armus et al. showed that paramecium can learn to remain in cathodal side of its swimming medium to receive cathodal shocks as an attractive stimulus [11]. This study was followed by a series of investigations by the same team to find the retention time in paramecium which demonstrated that paramecium can have a memory of conditioning up to 1 minute [20].

Learning in unicellular organisms can have implications beyond basic science research. Particularly, development of unicellular models of memory impairments has been suggested to provide promising opportunities to study diseases like Alzheimer's [21, 22]. Therefore, understanding the underpinning mechanisms of memory formation in unicellular organisms will create new avenues in both clinical and basic research on memory. It might be possible to use paramecium as a new model for these types of studies. In addition, brain theories that assume a subcellular origin for intelligent behavior (such as orchestrated objective reduction for consciousness [23]) have suggested that the presence of learning in unicellular organisms can potentially support their assumptions [24].Therefore the main goal of the present study is to propose a plausible mechanism for learning behavior in paramecium.

2. Learning in Paramecium; Latest Reports

Paramecium learning has been addressed mostly by Armus et al. in the recent years. We will use their experiment series as the basis for our proposed learning mechanism in paramecium. In the experimental setup of the Armus et al, one paramecium is introduced to a trough filled with culture media (20*5*5 millimeters) while the trough is divided into two separate dark and bright sides with the light intensity of 7 ft-c and 30 ft-c, respectively.

For the experiment, each paramecium underwent ten 90-second trials, 7 training trials and 3 test trials for all groups. In training trials of the experimental group, each paramecium received electrical shock only when it was in the cathodal side of the trough. Members of the control group did not receive any shock in either sides of the trough. In test trials, paramecium did not receive any shocks in any of the groups. There was a third group where the paramecium received electrical shocks in both sides of the trough during the training period. Finally, the total time that paramecium spent in the light and dark sides of the trough in each of the test trials was recorded for all of the groups.

Armus et al. have found that the experimental group spends more time in the cathodal half of the trough as compared to the control groups. Accordingly, they concluded that this behavior can be considered as learning.

On the other hand, it would be difficult to make sense of this behavior without knowing its possible mechanism. Therefore, we will try to propose a possible mechanism for this behavior based on evolutionary evidence and known physiology of the paramecim.

3. Limitations of Armus et al. Report

There are some key points that need to be properly addressed before drawing a conclusion on learning in paramecium. The most important point is that in Armus et al. report, there was not a distinction between the paramecia who supposedly learned to associate "dark side" with the cathodal shock and "light side" with cathodal shock. The relationship between light and dark with cathodal shock is simply counterbalanced in their study. Accordingly, a closer look at Armus et al. report suggests that the data for the control group is probably invalid (Figure 1).

The control group had spent on average 30 seconds of a 90-second trial in "cathodal" side of the trough. Considering the fact that the time spent in the cathodal half is time spent in dark side of it for 50% of the time and light side for the other 50% of the time, paramecia should spend on average 45 seconds of the time in the cathodal side. Interestingly, the difference between experimental and control group lies exactly within this 10 second time window [11].

Moreover, there are some other considerations that should be taken into account to address learning in paramecium. First, the tendency of paramecium to spend a significantly longer time in the cathodal side of the trough can happen due to accumulation of unknown substances by cathodal shocks. In order to address this issue, Armus et al. have used a second control group in which the paramecium was under constant cathodal stimulation regardless of its position in the trough. If cathodal shocks could cause accumulation of unknown substances in the cathodal half of the trough, this control group should show the same behavior as experimental group. Interestingly, this control group showed the same behavior as the no-shock control group. Therefore this possibility seems to be ruled out.

Second, the tendency of paramecium to spend more time in the bright side of the trough may form due to excretion of ions or metabolites from paramecium after electrical stimulation. In order to address this issue, Armus et al. have shown that exchanging the bright and dark side of the trough for test trials does not change the tendency of paramecium to spend time in the bright side of the trough and the learning effect seems to be still in work (Figure 2).

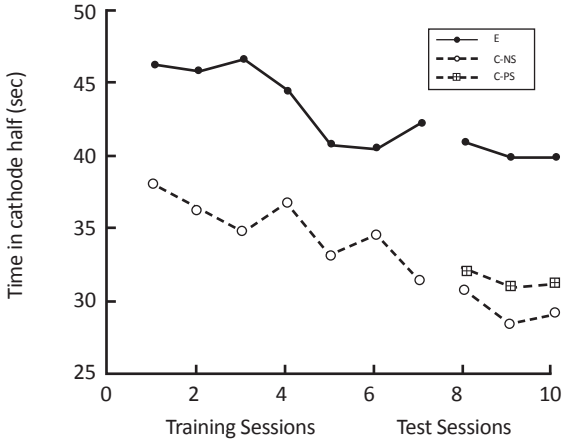

Figure 1. Data from Armus et al. [11]. Each data point indicates time spent in "cathodal half" of the trough in different trials for different groups. The difference between control group and experiment group lies within a 15-second time interval (the test sessions' data). However, since the data for control group is the average of time spent in both dark and bright halves of the trough, it should equal to a number close to 45± a possible SEM. Taking this consideration into account,the control group's data seems to be invalid. Adapted with permission from [11]

Existence of primordial light detection systems in P. caudatum

Previous studies by Armus et al. [11] and its replication by dorvash et al. [26], suggests that there should be a light detection system in P. caudatum. In fact, it is known that light exposure can induce or modulate biological processes in cellular structures that do not possess a

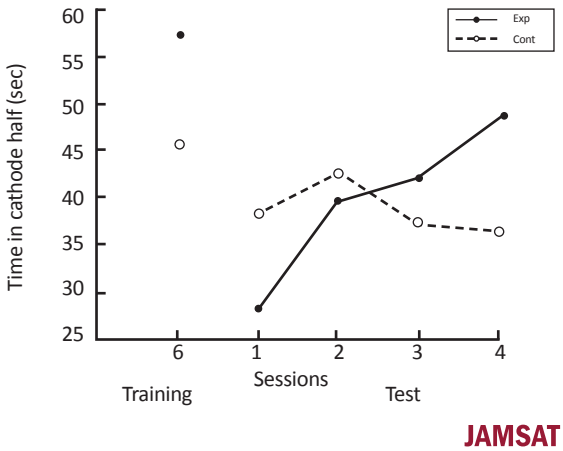

Figure 2. Adapted with permission from Armus et al. [11]. Mean time spent in the cathodal half of the trough in test trials (and the last training trial) when the place of the cathodal half changed for test trials. Data suggests that the paramecia still shows a tendency to spend time in the cathodal halfof the trough even though the location of the cathodal half was changed

structurally distinct light detection system. This includes growth induction in yeast cells [27], activation of pig's neutrophils [28] and growth modulation in paramecium itself [29]. There is also a theoretical model to explain this phenomenon [30]. Therefore, the existence of light detection molecules in paramecium seems to be possible. However, the inner machinery of such a system –if it exists- is still unknown. We believe that an evolutionary explanation can be useful in here.

Some unicellular photosynthetic organisms and motile green algae possess an eyespot apparatus usually called "stigma". In flagellated green algae, the Eyespot Apparatus (EA) helps the organism to either avoid light sources or navigate towards them. When light exposure is sudden and intensive, the organism shows a photophobic behavior and escapes the light. On the other hand, when the light is not intensive, the organism swims towards the light source and exerts a phototactic behavior [31]. It is known that in flagellated alga *Chlamydomonas reinhardtii*, light activates a signaling cascade involving archaeal-type rhodopsin [32]. In euglena, which is a unicellular photosynthetic organism, it has been shown that light avoidance is mediated through a blue-light-activated adenylyl cyclase and cAMP [33]. This blue-light receptor flavoprotein is the light receptor in euglena. Therefore, cAMP is an integral part of the photoorientation processes in many unicellular organisms.

The other major player in the eye spot apparatus is Ca^{2+} which is one the most important signaling agents in plants and animals [34-36]. It has been reported that Ca^{2+} involved in the light modulated movement of green algae and particularly in Chlamydomonas [37-39].

It is evident that eukaryotes have achieved the capability of phototaxis independently for at least eight times [40] and it is not difficult to achieve such a capability [40]. It has been suggested that chromalveolates (the eukaryotic supergroup that includes ciliates) were ancestrally photosynthesic and lost their red algal symbiont during their evolution [41]. Many of the Alveolates (superphylum of the protozoan phylum Cilliophora) exert phototactic behavior and almost 5% of the species in this superphylum possess the stigma and they are mostly fresh water organisms [40] and even some have phototactic activity without any stigma [42]. In ciliates, phototactic behavior evolved independently from other chromalveolates [40].

Ciliates' phototactic activity can depend on the nutritional status of the organism. In under-fed *Chlamydodon mnemosyne*, the organism forms a stigma and shows phototaxis towards light source. On the other hand, well-fed organisms digest the stigma, hold the photoreceptors and exert a negative phototaxis. This probably helps the organism to feed its symbiont during under-fed situations and lose it in well-fed situations. Interestingly, Paramecium bursaria forms a similar symbiotic relationship with the green alga Zoochlorella i.e. when the environment suits photosynthesis, P. bursaria forms a symbiotic relationship with the Zoochlorella and when environmental parameters are not suitable for photosynthesis, P. bursaria digests its symbiont. There are also different types of ciliates that exert phototactic behavior without the presence of stigma [43-45]. The mechanism of steering in ciliates is still unknown but it has been suggested that there are light sensing vesicles that form an independent miniature stigma with their associated cilia [40].

Based on the aforementioned evolutionary evidence, we argue that paramecium possesses a similar light detection system that includes an unknown photoreceptor molecule and cAMP. In the following, we will suggest a molecular cascade based on the existing literature to explain the light detection and learning capability in P. caudatum.

A molecular cascade can explain learning in paramecium

We propose that a similar molecular cascade mentioned earlier can be responsible for learning in *P. caudatum*. Since freely swimming paramecia spend only 39% of their time in the bright side of the trough (based on our data), it is conceivable that paramecia have a natural tendency to the dark side of the trough and it is exerting a "photophobic behavior". Based on the present hypothesis, light exposure increases cAMP concentration and cAMP will increase the cilliary beat frequency in paramecium as a consequence [46]. We suggest that photophobic behavior of paramecia is mediated through the same mechanism. In other words, light exposure increases cAMP concentration and paramecium's swimming speed consequently. This causes the paramecium to leave the bright side of the trough faster than its dark side which results in spending less time in bright side of the trough by default.

On the other hand, It has been previously shown that voltage gated Ca^{2+} channels exist in paramecium [47] and they are extensively involved in the movement behaviors of this organism [48-50]. Moreover, it is known that membrane depolarization can cause a reversal in ciliary beating direction of paramecium and this effect is mediated through Ca^{2+} ions [51]. Since the resting membrane potential of paramecium is around -25 millivolts [52], it is fair to assume that successive cathodal shocks

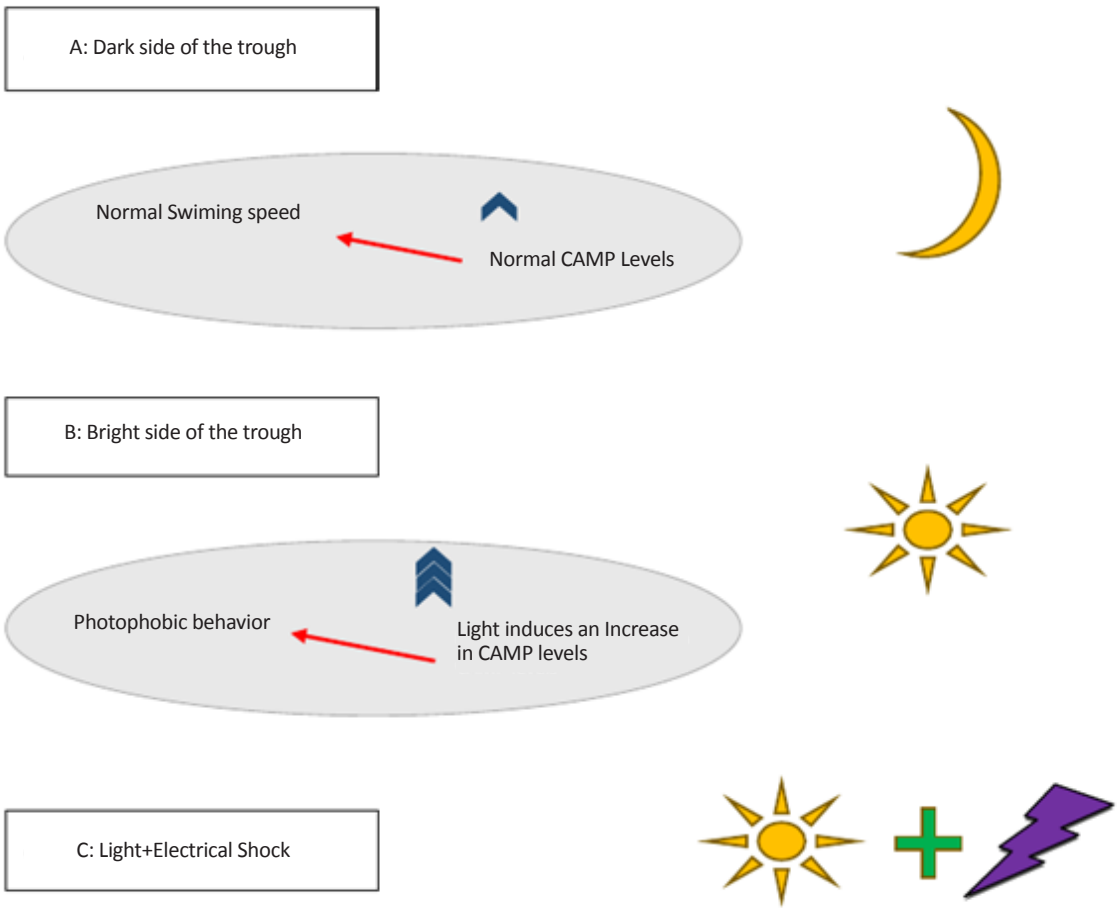

Figure 3. A schematic representation of the proposed model for learning in paramecium. A: When paramecium is swimming in the dark side of the trough, there is a baseline cAMP concentration which maintains a normal swimming speed. B: When paramecium enters the bright side of the trough, light exposure causes an increase in cAMP levels which increase the swimming speed consequently. C: When paramecium enters the bright side of the trough and receives successive electrical shocks, electrical shocks will cause subtle and temporary backward movements which leads to a normal swimming speed for paramecium similar to its swimming speed in the dark side. This causes accumulation of cAMP molecules in cytosol that eventually cancels the photophobic behavior of paramecium during test trials. Therefore, it seems that this mechanism is the basis of learning in paramecium

will depolarize paramecium's membrane. Therefore, we suggest that electrical shocks can reduce paramecium's swimming speed through abovementioned mechanisms and cancel the light-induced speed increase (Figure 3).

Yet, this assumption does not explain the mechanisms of "memory retention" in paramecium. A precise analysis might be useful in here. In light association group, light exposure produces a substantial amount of cAMP in the cytosol while reduction of Ca^{2+} in the cell -due to electrical shocks- opposes the effect of cAMP as a speed booster. In the test trials, when there is no electrical shock, the stored cAMP exists in excessive amounts and it speeds up paramecium movement regardless of its position in the bright or dark side of the trough.

Based on the present hypothesis, paramecium should not be able to associate darkness (lower light intensities) with cathodal shocks, Because cathodal shocks essentially counter the assumed cAMP driven photophobic behavior which only happen in the dark side of the trough. Therefore, lack of ability to associate darkness with cathodal shocks can happen due to this mechanism.

Implications of paramecium learning for Orchestrated Objective Reduction theory (Orch-OR) for consciousness

It is noteworthy to mention one of the important implication of learning in paramecium which is related to previously proposed quantum basis of consciousness. Particularly, Orchestrated Objective Reduction (Orch-

OR) [23] asserts that the presence of intelligent behavior in paramecium and Physarum polycephalumcontradicts the contemporary understandings of intelligent behavior in neuroscience [24]. Learning without synaptic interactions in paramecium points to the sub-cellular learning machinery in this organism. Penrose and Hameroff suggest that this subcellular machinery for light detection and learning is probably the microtubular network [25]. Confirmation of learning in paramecium through independent studies [26] paves the road for testing the Orch-OR's predictions by pharmacological manipulation of microtubular networks in paramecium.

4. Conclusion

In conclusion, presence of learning behavior in paramecium seems to be established after one century of debate. However, this phenomenon needs more experimental and theoretical explanation. These explanations can have implications for development of unicellular models of Alzheimer's disease, testing the Orch-OR theory and investigation of phototaxis in ciliates. While further studies on our proposed mechanisms for paramecium learning is necessary, it seems that simple molecular mechanisms based on evolutionary analyses can give good insights about this phenomenon. Additionally, this theoretical framework can be used to design new experiments on paramecium learning in order to elucidate other aspects of learning in paramecium.

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Conflict of Interest

The authors declared no conflict of interests.

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