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ABSTRACT
Learning is a fundamental process that involves complex neural systems. However, microorganisms without a nervous system have also been shown to have learning abilities. Specifically, *Paramecium caudatum* has been reported to form associations between lighting conditions and cathodal shocks in its swimming medium. We replicated previous reports on this phenomenon and tested predictions of a molecular pathway hypothesis of paramecium learning. In contrast to previous reports, our results indicated that paramecia can only associate higher light intensities with cathodal stimulation and cannot associate lower light intensities with cathodal stimulation. These results support the predictions of the previously proposed model of the molecular mechanisms of learning in paramecia, which depends on the effects of cathodal shocks on the interplay between cyclic adenosine monophosphate levels and phototactic behavior in paramecia.

Keywords: *Paramecium caudatum*; learning; cAMP; phototaxis; associative learning

INTRODUCTION
Learning is a fundamental process in neural systems. Much effort has been devoted to elucidating its mechanisms. Learning in unicellular organisms is an intriguing observation that has not been investigated adequately. Examples of learning in unicellular organisms include learning in the giant slime mold *Physarum polycephalum* that can learn to predict subsequent cold shocks after periodic cold shock stimulation (Saigusa et al., 2008) or moving to colder areas to find food while warmer areas are generally preferred (Shirakawa et al., 2011). Additionally, in smaller organisms, such as *Escherichia coli*, the organism has been reported to be able to predict subsequent carbon sources through the proper expression of genes (Mitchell et al., 2009) and shift from fermentation to respiration based on environmental situation in yeast (Mitchell et al., 2009).

Paramecium caudatum is another unicellular organism that has been reported to exhibit intelligent behaviors, such as spontaneous alternation that requires remembering the previous choice in a T-maze (Harvey et al., 2006) and learning (Hennessey et al., 1979; Armus et al., 2006). These observations suggest that learning might not be restricted to the strengthening or weakening of synaptic connections, and "intracellular learning mechanisms" may exist in some organisms.

Therefore, the investigation of learning in unicellular organisms might reveal fundamental mechanisms of learning that have been preserved throughout evolution. Specifically, paramecia are an ideal organism for investigating this issue. Research on paramecium learning behavior dates back to 1911 (Day et al., 1911).

No consensus has been reached with regard to the existence of learning in paramecia. Different attempts to demonstrate learning in paramecia have resulted in contradictory and equivocal findings. Recent reports on paramecia suggested that they can learn to associate different light intensities in their swimming medium with attractive electrical shocks (Armus et al., 2006; Mingee, 2013). More specifically, individual paramecia were observed while swimming in a trough with two bright and dark sides. The organism received attractive cathodal shocks when it entered the bright/dark side of the trough, depending on the trial. At the end of the experiment, the paramecia were reported to remember the side of the trough where they received the attractive cathodal shocks, regardless of whether the shocks occurred in the dark or bright side (Armus et al., 2006). The authors concluded that paramecia can learn.

This observed phenomenon involves both phototactic and electrotactic behaviors in paramecia. Ciliary movement in paramecia is mainly coordinated through membrane potential and Ca^{2+} ions (Naitoh et al., 1969; Naitoh et al., 1973). *Paramecium bursaria* is known to exhibit phototactic behavior that is directly linked to its membrane potential (Matsuoka et al., 1988) and mediated by Ca^{2+} ions (Nakaoka et al., 1987). Interestingly, retinal molecules found to be present in *P. bursaria* and act as a possible chromophore in this microorganism (Tokioka et al., 1991).

Additionally, paramecium movement is essentially controlled by its membrane potential and can be affected by applying electrical or magnetic fields in the environment (Ludloff, 1895; Rosen et al., 1990; Nakaoka et al., 2000; Nakaoka et al., 2002). More specifically, negative galvanotaxis in paramecia (i.e. movement towards the cathodal side of an electric field) is known as the Ludloff phenomenon. Ludloff was the first to report galvanotaxis in paramecia. In an electrical field, paramecia move toward the cathode because the anodal end of the organism beats faster toward the cathodal end (Kamada, 1928) and consequently pushes the organism toward the cathode (Ludloff, 1895). Short-duration cathodal stimulation with relatively long intervals has been shown to be an attractive stimulus for paramecia (Armus et al., 2001). This paradigm has been used in paramecium learning experiments (Hennessey et al., 1979; Armus et al., 2006; Mingee 2013).

A deeper understanding of the reported learning behavior in paramecia requires elucidation of the molecular basis of phototaxis and galvanotaxis in this microorganism. We previously proposed a molecular model (Alipour et al., 2017) to explain this behavior based on molecular pathways that link cyclic adenosine monophosphate (cAMP) levels to

phototactic behavior in paramecia. However, our model predicted that paramecia cannot learn to associate lower light intensities (i.e., the dark side) with cathodal shocks. As such, the main goals of the present study were to first replicate the previous findings of Armus et al. (2006) and then test predictions of the learning hypothesis in paramecia and determine the possible mechanisms of such learning.

MATERIALS AND METHODS

Culture media

Hay infusion was used as the culture medium for the paramecia. The hay was boiled in purified water for 45 min, and the resulting extract was used for paramecium culture as described below.

Paramecium caudatum specimens

Local samples of the Khoshk River in Shiraz, Iran, were gathered and incubated in hay infusion as the nutritious culture medium for paramecia. After 3 days, the specimens were checked for the presence of paramecia, which were then isolated for further evaluation. *Paramecium caudatum* was identified based on its unique morphological features, including its relatively large size (300 μm) and the presence of only one micronucleus beside the large macronucleus.

Electrical shock device

A microcontroller device was used to deliver shocks to the culture medium (AT-MEGA 16 AVR controller). The microcontroller was programmed to deliver 60-ms shocks with 500-ms intershock intervals. Cathodal shocks (5 V, 1 mA) were delivered to the culture medium.

Paramecium learning experiment (see Fig. 1)

The methodology of Armus et al. (2006) was used to investigate learning behavior in *P. caudatum*. A U-shaped plastic trough (20 mm length, 5 mm width, and 5 mm depth) was filled with the culture medium that was filtered through a 0.22-μm filter. The trough was divided into two dark and light sides using a dark transparent sheet that was placed under the trough. Copper-ended cathode and anode wires were placed on the middle of the side walls at two ends of the trough. The light intensity was set to 805 ± 30 cd and 335 ± 30 cd for the bright and dark sides of the trough, respectively. A total of 84 paramecia (*P. caudatum*) were divided into three groups: light association (n = 23), dark association (n = 26), and control (n = 36).

For the experiment, each paramecium underwent ten 90-s trials, seven training trials, and three test trials for all groups without any intertrial time intervals. To avoid possible confounding factors, the swimming medium was not changed between trials, and individual paramecia were watched uninterrupted during the entire training and test trials. The testing trough was rinsed and dried at the end of each experiment with a single paramecium. In the training trials in the light association group, each paramecium received an electrical shock

only when it was on the bright side of the trough. In the training trials in the dark association group, each paramecium received an electrical shock only when it was in the dark side of the trough. Individual paramecia were observed under a stereomicroscope at 10× magnification. The experimenter manually started the shocks when the paramecium entered the cathodal half of the trough, and the shocks were turned off when the organism left that half. Paramecia in the control group did not receive any shocks in either side of the trough. In the test trials, the paramecia did not receive any shocks in any of the groups. The total time that the paramecia spent in the light and dark sides of the trough was recorded for all of the groups.

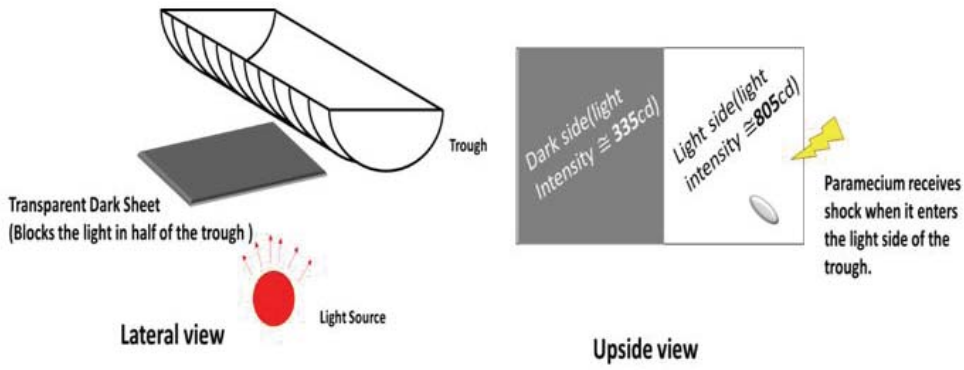

Fig. 1. Schematic representation of the experimental setup.

Statistical analysis

In the light association group, the total time spent in the cathodal half of the trough (i.e., the bright side) was compared with the total time that the control group spent in the bright side of the trough. In the dark association group, the total time spent in the cathodal half of the trough (i.e., the dark side) was compared with the total time that the control group spent in the dark side of the trough. To determine whether pooling the data from the light association group and dark association group produces spurious results, we pooled data from both the light association and dark association groups and compared the pooled data with the average time that the control group spent in either the dark or bright side of the trough.

RESULTS

Light association group

The total time spent in the light side of the trough was 152.7 ± 12.8 s and 105.3 ± 8.1 s in the experimental and control groups, respectively. The independent *t*-test revealed a significant difference between the time spent in the light side of the trough in the light association group compared with the control group ($p < 0.01$; Fig. 2).

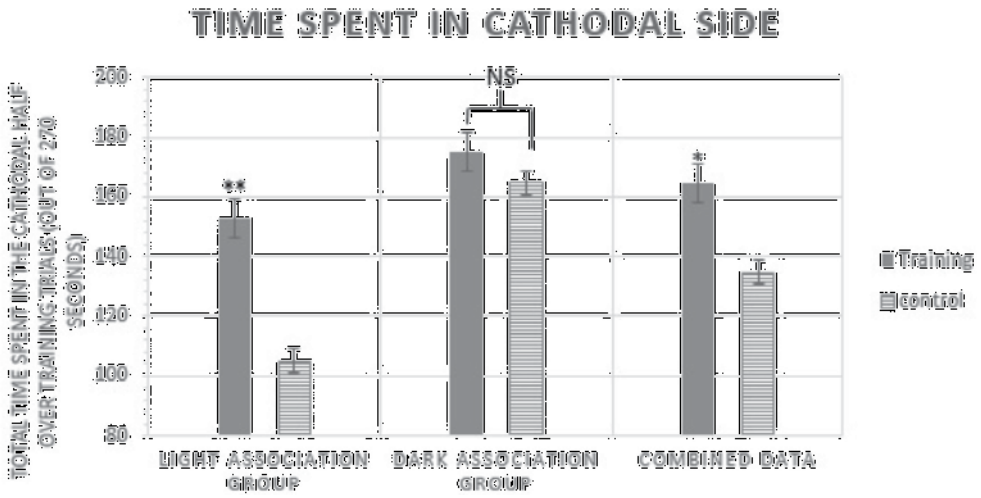

Fig. 2. Comparison between the total time spent in the cathodal side of the trough. (*Left*) Total time spent in the cathodal half of the trough in the light association and control groups. The cathodal half of the trough was the *bright side* in this group. A significant difference was found between these two groups ($p < 0.01$, paired *t*-test). (*Middle*) Total time spent in the cathodal half of the trough in the dark association and control groups. The cathodal half of the trough was the *dark side* in this group. No significant difference was found between these two groups ($p > 0.05$, paired *t*-test). (*Right*) Average time spent in the cathodal half of the trough in both the light association and dark association groups (i.e., the data were pooled) compared with the average time that the control group spent in either the dark or bright side of the trough. The comparison shows a spurious significant difference between the two averages. This underscores the importance of separately analyzing the dark and light association groups.

Dark association group

The total time spent in the dark side of the trough was 175.2 ± 11.7 s and 164.6 ± 8.1 s in the experimental and control groups, respectively. The independent *t*-test revealed that the time spent in the dark side of the trough in the dark association group was not significantly different from the control group ($p > 0.05$; Fig. 2).

Pooled data

Comparisons of the pooled data from both the dark and light association groups with the average time that the control group spent in either the dark or light side of the trough revealed a significant difference ($p < 0.05$, independent *t*-test), which was obviously a spurious result (Fig. 2).

DISCUSSION

The present study confirmed the existence of learning capabilities in *P. caudatum*. Our study replicated only the core finding of Armus et al. (2006) and not all of the previously

reported observations. Some key points need to be mentioned before definitive conclusions can be drawn concerning learning in paramecia. The most important is that Armus et al. (2006) made no distinction between paramecia that supposedly learned to associate the "dark side" with the cathodal shock and "light side" with the cathodal shock. The relationship between the light and dark sides and cathodal shocks was simply counterbalanced in their study. Accordingly, the present study made a distinction between dark-cathode and light-cathode associations and found that learning occurred only in the light-cathode condition. The theoretical aspects of this issue are discussed from the perspective of our previous hypothesis of learning in paramecia (Alipour et al., 2017). The data from the control group in Armus et al. (2006) might also be unreliable, in which their control group spent approximately 30 s of a 90-s trial in the "cathodal" side of the trough. The time spent in the cathodal half presumably reflects the time spent in the dark side 50% of the time, and the paramecia should have spent the other 50% of the time in the light side. Therefore, the paramecia in the control group should have spent an average of 45 s (± standard deviation) in the cathodal side, without a statistical trend toward spending more time in either side. However, Fig. 1 in Armus et al. (2006) shows that the paramecia in the control group presented a tendency toward spending less time in the cathodal side, and they spent only ~30 s in the cathodal side in each trial, thus casting doubt on the validity of the data from the control group. Interestingly, the difference between the experimental and control groups was within this 15 s time window (Armus et al., 2006).

We found that pooling the data from the dark association and light association groups generated spurious results (Fig. 2, right). Therefore, not distinguishing between dark association and light association in paramecium learning can lead to false results. Moreover, some additional factors should be considered. First, the paramecia spent a significantly longer time in the cathodal half of the trough, but this could happen because of the accumulation of unknown substances at the tip of the cathode electrode. To examine this possibility, Armus et al. (2006) ran a second control group, in which the paramecia were continuously stimulated regardless of their location in the trough. If cathodal shocks cause the accumulation of unknown substances in the cathodal half of the trough, then this control group should exhibit the same behavior as the experimental group. Interestingly, this control group exhibited the same behavior as the no-shock control group, thus excluding this possibility.

Moreover, one possibility is that the mere presence of a paramecium in one side of the trough can cause the accumulation of its metabolites (e.g., carbon dioxide) that can attract the organism to one side of the trough through a decrease in pH that is attractive for paramecia because it can be a sign of a bacterial food source (Jennings 1904). To address this issue, Armus et al. (2006) showed that changing the bright and dark sides of the trough in the test trials did not alter the tendency of the paramecia to spend time in the bright side of the trough (Armus et al., 2006).

Light sensitivity in paramecia was reported by (Jennings 1904) more than a century ago. The study by Armus et al. (2006) confirmed this capability. Light exposure is known to induce or modulate biological processes in cellular structures that do not possess an anatomically distinct light detection system. This includes growth stimulation in yeast cells (Quickenden et al., 1976), porcine neutrophil activation (Shen et al., 1994), and growth modulation

in paramecia (Fels 2009). A molecular model was proposed to explain this phenomenon (Alipour 2015). We suggest that a photoreception system may exist in *P. caudatum*. However, the molecular pathways of such a system are still unknown, and exploration of the evolutionary relationship between photoreceptive unicellular organisms is necessary.

As described by (Jékely 2009), some motile photosynthetic organisms and green algae have an eyespot apparatus, called "stigma." The eyespot apparatus mediates phototactic movements of the organism through molecular cascades. For example, in the flagellated alga *Chlamydomonas reinhardtii*, light activates a signaling cascade that involves archaeal-type rhodopsin (Suzuki et al., 2003). In the unicellular photosynthetic organism euglena, light avoidance is mediated by blue-light-activated adenylyl cyclase and cAMP (Iseki et al., 2002). This blue-light receptor flavoprotein is the light receptor in euglena. Accordingly, cAMP appears to be an integral part of photo-orientation processes in several unicellular organisms

Another important signaling agent in phototaxis is Ca^{2+} ions, which are assumed to be a major signaling mediator in both plants and animals (Cohen 1989; Poovaiah et al., 1987; Roberts et al., 1992). Ca^{2+} is believed to be involved in the light-modulated movement of green algae, particularly Chlamydomonas (Harz et al., 1991; Kamiya et al., 1984; Litvin et al., 1978).

Different eukaryotic species achieved the capability of phototaxis independent from each other at least eight times over evolutionary history (Jékely, 2009). In Ciliates, phototactic activity can depend on the nutritional status of the organism such that under-fed organisms form stigma and a symbiotic relationship with green algae and exhibit phototaxis toward a light source. Well-fed organisms digest the stigma, loose the photoreceptors**,** and exhibit negative phototaxis. This likely helps the organism to provide light for its symbiont during under-fed conditions and lose it under well-fed conditions. Interestingly, *Paramecium bursaria* develops a similar symbiotic relationship with the green alga Zoochlorella. When the environment supports photosynthesis, *P. bursaria* forms a symbiotic relationship with Zoochlorella. When environmental conditions are unsuitable for photosynthesis, *P. bursaria* digests its symbiont. The mechanism of steering in ciliates is still unknown, but they have been suggested to have light-sensing vesicles that form an independent miniature stigma with their associated cilia (Jékely, 2009).

Based on the aforementioned lines of evidence, we argue that paramecia possess a similar light detection system that includes an unknown photoreceptor molecule and cAMP. Below we propose a molecular cascade model based on our data to explain light detection and learning capability in *P. caudatum*.

Freely swimming paramecia spent ~39% of the time in the trials in the bright side of the trough (based on our data). Therefore, paramecia may be presumed to exhibit "photophobic behavior." Accordingly, we suggest that light exposure might increase cAMP levels. cAMP increases the ciliary beat frequency in paramecia (Nakaoka et al., 2009), and light exposure can potentially increase paramecia's swimming speed in bright areas. This causes the paramecium to leave the bright side of the trough faster than the dark side, which causes an overall reduction of the time spent in the bright side of the trough.

Another major player in paramecia's movement is voltage-gated Ca^{2+} channels (Hinrichsen et al., 1984; Machemer et al., 1979). Membrane depolarization causes a reversal of

paramecia's ciliary beating direction through Ca^{2+} (Brehm et al., 1978). The resting membrane potential of paramecia is around -25 mV (Nakaoka et al., 2009). Cathodal shocks can depolarize paramecia's membrane. Therefore, cathodal shocks can reduce paramecia's swimming speed through the aforementioned mechanisms and thus block the "light-induced speed increase" (Fig. 3). However, this does not explain the capability of paramecia to retain the learned information after the training trials. We propose that when paramecia spend more time in the bright side of the trough, more cAMP can accumulate in the cytosol through light exposure. Therefore, during the test trials, the accumulation of cAMP molecules will remain in the cytosol and boost the swimming speed of the paramecium, regardless of its position in the trough. This is consistent with the experimental finding that paramecia in the experimental group spent an almost equal amount of time in both halves of the trough during the test trials (56%; see Results section and Fig. 3 for more details).

According to our hypothesis, cathodal shocks counter the presumed cAMP-driven photophobic behavior in the bright side of the trough, and paramecia cannot learn to associate the dark side of the trough with cathodal shocks. This prediction was tested and validated by the data from the dark association group (Fig. 3).

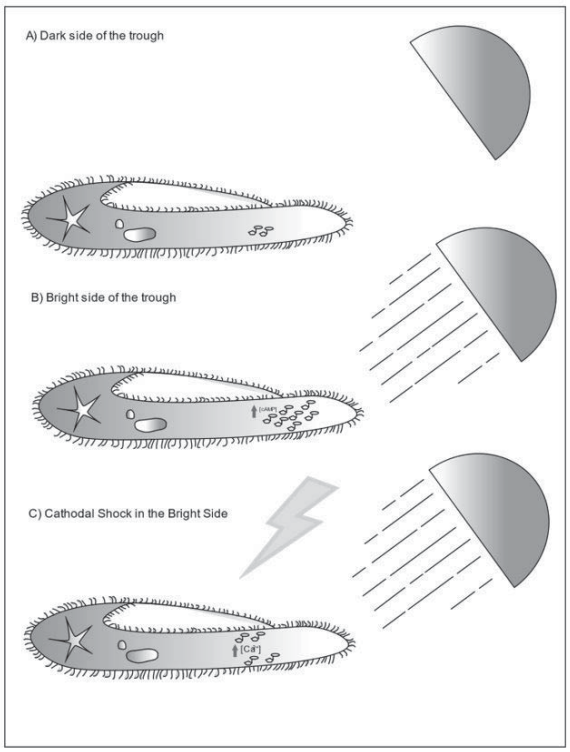

Fig. 3. Proposed learning mechanism in *Paramecium caudatum*. (A) Swimming in a relatively dark area maintains a minimal cAMP level. (B) When the paramecium enters the bright side of the trough, light exposure increases intracellular cAMP levels and swimming speed. (C) If the paramecium receives cathodal shocks when it swims in the bright side of the trough, then the electrical shocks will cause subtle and temporary backward movement through miniature depolarization of the membrane and the inward flow of Ca^{2+}. This will block the increase in swimming speed in the bright side of the trough. Additionally, this process causes the accumulation of cAMP in the intracellular environment, thus leading to the blockade of photophobic behavior in the paramecium during the test trials.

One of the major limitations of the current study is that we did not collect trial-by-trial data on the amount of time that was spent in the cathodal half of the trough in each trial. This makes direct comparisons between our results and Armus et al. (2006) less straight forward. We hope future studies can overcome this limitation.

In conclusion, the present results corroborate other studies that suggest that paramecia exhibit associative learning. Nonetheless, several issues about learning in paramecia remain unresolved, such as (*i*) the exact molecular pathway that governs this behavior and (*ii*) possible similarities between learning mechanisms in paramecia and other animals that can potentially translate to Alzheimer's disease research. These issues can be addressed through pharmacological manipulations of paramecium learning. Future studies of the mechanisms of paramecium learning will shed light on our understanding of learning at the molecular level in unicellular organisms.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

SUBMISSION DECLARATION AND VERIFICATION

The authors declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

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