

Research Paper

Microtubule Disruption Without Learning Impairment in the Unicellular Organism, Paramecium: Implications for Information Processing in Microtubules

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ABSTRACT

Introduction: Information processing in microtubules is an open question that has not been adequately addressed. It was suggested that microtubules could store and process information in the nervous system or even support consciousness. The unicellular organism, *Paramecium caudatum*, has a microtubular structure but lacks a neuron or neural network. However, it shows intelligent behaviors such as associative learning. This property may suggest that the microtubules are involved in intelligent behavior, information storage, or information processing in this organism.

Methods: To test this hypothesis and study the role of microtubules in *P. caudatum* learning, we utilized a learning task in which the organism associates brightness in its swimming medium with attractive cathodal shocks. To see if microtubules are an integral part of information storage and processing in *P. caudatum*, we disrupted the microtubular dynamics in the organism using an antimicrotubular agent (parbendazole).

Results: We observed that while a partial allosteric modulator of GABA (midazolam) could disrupt the learning process in *P. caudatum*, the antimicrotubular agent could not interfere with the learning.

Conclusion: Microtubules are probably not vital for the learning behavior in *P. caudatum*. Consequently, our results call for further investigation of the microtubular information processing hypothesis.

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Highlights

- Importance of Information processing in microtubules;
- Microtubules could store and process information in the nervous system;
- Unicellular organism, *Paramecium caudatum*, has a microtubular structure but lacks a neuron or neural network.

Plain Language Summary

Information processing in microtubules is an open question that has not been adequately addressed. It was suggested that microtubules could store and process information in the nervous system or even support consciousness. The unicellular organism, *Paramecium caudatum*, has a microtubular structure but lacks a neuron or neural network. However, it shows intelligent behaviors such as associative learning. This property may suggest that the microtubules are involved in intelligent behavior, information storage, or information processing in this organism. To test this hypothesis and study the role of microtubules in *P. caudatum* learning, we utilized a learning task in which the organism associates brightness in its swimming medium with attractive cathodal shocks. To see if microtubules are an integral part of information storage and processing in *P. caudatum*, we disrupted the microtubular dynamics in the organism using an antimicrotubular agent (parabendazole). We observed that while a partial allosteric modulator of GABA (midazolam) could disrupt the learning process in *P. caudatum*, the antimicrotubular agent could not interfere with the learning. Microtubules are probably not vital for the learning behavior in *P. caudatum*. Consequently, our results call for further investigation of the microtubular information processing hypothesis.

1. Introduction

Herbert Froehlich was one of the first researchers who suggested the feasibility of long-life macroscopic coherence (classical and quantum) in “ordered” biological structures such as microtubules at room temperature (Fröhlich, 1970, 1975). Furthermore, some researchers argued that coherence between microtubules in the nervous system could potentially explain consciousness (Ekert Jozsa Penrose & Stuart Hameroff, 1998; Hameroff & Penrose, 1996). While the issue of quantum effects on the human brain is a controversial topic of science (Beck & Eccles, 1992; Koch & Hepp, 2006) and decoherence may not let quantum states survive long enough to affect cognitive processing (Sala Moradi, Sajadi, Fazileh, & Shahbazi, 2015; Salari, Naeij, & Shafiee, 2017; Schlosshauer, 2007), the possibility of information processing in microtubules is still an open question.

In a series of studies by G Albrecht-Buehler, he demonstrated that cell intelligence in fibroblast cells is due to some cytoskeletal structures in the cell, and living cells possess a spatial orientation mechanism located in the centriole (i.e., a microtubular structure). Also, electromagnetic signals are the triggers for the cells repositioning (Albrecht-Buehler, 1997; Albrecht-Buehler, 1994; 1995). Meanwhile, the ability of the centriole to detect magnetic radio signals is still a topic of debate.

Moreover, some argued that learning in unicellular organisms such as *Paramecium* might support the hypoth-

esis that sub-cellular structures (such as microtubules) could contribute to intelligent behavior (Hameroff & Penrose, 2014) the mechanism by which it occurs in the brain, and its ultimate place in the universe are unknown. We proposed in the mid 1990’s that consciousness depends on biologically ‘orchestrated’ coherent quantum processes in collections of microtubules within brain neurons, that these quantum processes correlate with, and regulate, neuronal synaptic and membrane activity, and that the continuous Schrödinger evolution of each such process terminates in accordance with the specific Diósi–Penrose (DP). In a similar vein, it was suggested that microtubules process information and are also responsible for memory storage and learning. A modeling study showed that the structure of Ca^{2+} /calmodulin-dependent protein kinase II (CaM kinase II or CaMKII, which is essential for memory formation in neurons) fits into the phosphorylation sites on tubulins of microtubules (Craddock, Tuszyński, & Hameroff, 2012). This finding strengthens the possibility that tubulin phosphorylation in microtubules may encode information, as suggested elsewhere (Hameroff, Craddock, & Tuszyński, 2010).

Here, we sought to test the hypothesis that microtubules are involved in memory encoding and information storage in a unicellular organism that exhibits learning. We disrupted the microtubular dynamics in the organism to see if microtubules are involved in information storage in *Paramecium caudatum*. Our results suggested that while disrupting GABA receptor dynamics will impair *P. caudatum* learning, disruption of microtubular dynamics does not impair the learning behavior.

2. Methods

Experimental setup

To investigate the role of microtubules in *P. caudatum* learning, we used a previously developed behavioral learning paradigm (Alipour, Dorvash, Yeganeh, & Hatam, 2018; Armus, Montgomery, & Gurney, 2006; Armus, Montgomery, & Jellison, 2006) and combined it with a pharmacological manipulation approach (Zhou, Xia, Xu, Xin, Liu, & Zhang, 2012). Accordingly, we tested the effect of two different drugs on *P. caudatum*'s learning to investigate its underlying mechanisms. Specifically, we disrupted the dynamics of microtubules in the organism using parbendazole to investigate the role of microtubules in learning. Moreover, we investigated the role of GABA receptors through the administration of midazolam (a benzodiazepine and a GABA partial allosteric modulator [PAM]) and tested alternative mechanisms for this process.

Paramecium caudatum specimens

The experimental procedure was approved by the Ethics Committee of the Shiraz University of Medical Sciences (designated code: IR.SUMS.REC.1394.S999, available at <http://research.sums.ac.ir/fa/ethicrc/EthicsCodes.html>). Local samples of Khoshk River in Shiraz City, Iran, were gathered and incubated in hey infusion as the nutritious culture medium for paramecia with a volumetric ratio of 1 to 10 (i.e., 10 mL of a specimen with 100 mL hey infusion). After 3 days, the specimens were checked under 40× magnification for the presence of paramecia and isolated for further evaluation. *Paramecium caudatum* was identified based on its unique morphological features, i.e., the large size (~300 µm) and the presence of only one micronucleus beside the large macronucleus. Afterward, isolated paramecia were added into a new culture medium (20 mL of hey infusion) and incubated in culture flasks at room temperature. Cultures were passaged every 3 days by replacing 1/5 of the previous culture in a fresh culture medium. In this study, the culture medium was not axenic.

Learning behavior in *P. caudatum*

The methodology of Armus et al. (2006) was used to investigate the learning behavior of *P. caudatum*. In brief, the researchers showed that *P. caudatum* could learn to associate light intensity in its swimming medium with attractive cathodal shocks. In other words, if the organism receives the attractive cathodal shocks in the light side of its swimming medium, it learns to stay on the

light side to receive the cathodal shocks even though the shocks have been discontinued for several minutes. This observation was also replicated in our group (Alipour, Dorvash, Yeganeh, Hatam, & Seradj, 2018). Therefore, learning in *P. caudatum* results from the interaction between two mechanisms: photosensitivity and responses to cathodal stimulation.

While mechanisms of such photosensitivity in *P. caudatum* are not known yet, photosensitivity in other species of the *Paramecium* genus, such as *Paramecium multimicronucleatum*, *Paramecium bursaria*, and *Paramecium tetraurelia*, is well documented (Hinrichsen & Peters, 2013; Iwatsuki & Naitoh, 1983; Reisser & Häder, 1984; Saji & Oosawa, 1974). Interestingly, all these species show light avoidance, and *P. bursaria* can change its photosensitivity when it starts a symbiotic relationship with algae inside its cytosol. On the other hand, the sensitivity of *P. caudatum* to cathodal stimulation is a well-studied effect known as the Ludloff phenomenon. Ludloff reported that *Paramecium* moves toward the cathodal side when exposed to an electrical field in 1895, and hence, this phenomenon was named after him (Ludloff, 1895). This effect can be attributed to the fact that when *Paramecium* is placed inside an electrical field, the anodal side of the *Paramecium* will beat faster than its cathodal side and pushes the microorganism toward the cathode (Kamada, 1929).

Experimental procedure and apparatus

A U-shaped plastic trough (20×5×5 mm, length×width×depth) was filled with the original culture medium from which the test *Paramecium* was isolated (called swimming medium from now on). To exclude other microorganisms/impurities from the culture medium in the trough, the culture medium was filtered through a 0.22-µm filter. Copper-ended cathode and anode wires were placed at the center of the side walls at two ends of the trough. A length of 3-mm copper end of the wire was exposed to a culture medium at each end. The trough was divided into two dark and light sides using a dark transparent sheet placed underneath the plastic trough. The mean light intensity was set to 805±30 lx for the light side and 335±30 lx for the dark side of the trough (Figure 1). A total of 105 *P. caudatum* organisms were divided into four groups of experimental, control, parbendazole-treated, and midazolam-treated.

For the experiment, each *P. caudatum* in the experimental or control group underwent ten 90-s trials, 7 training trials, and 3 test trials without any inter-trial time intervals. The swimming medium between trials was not

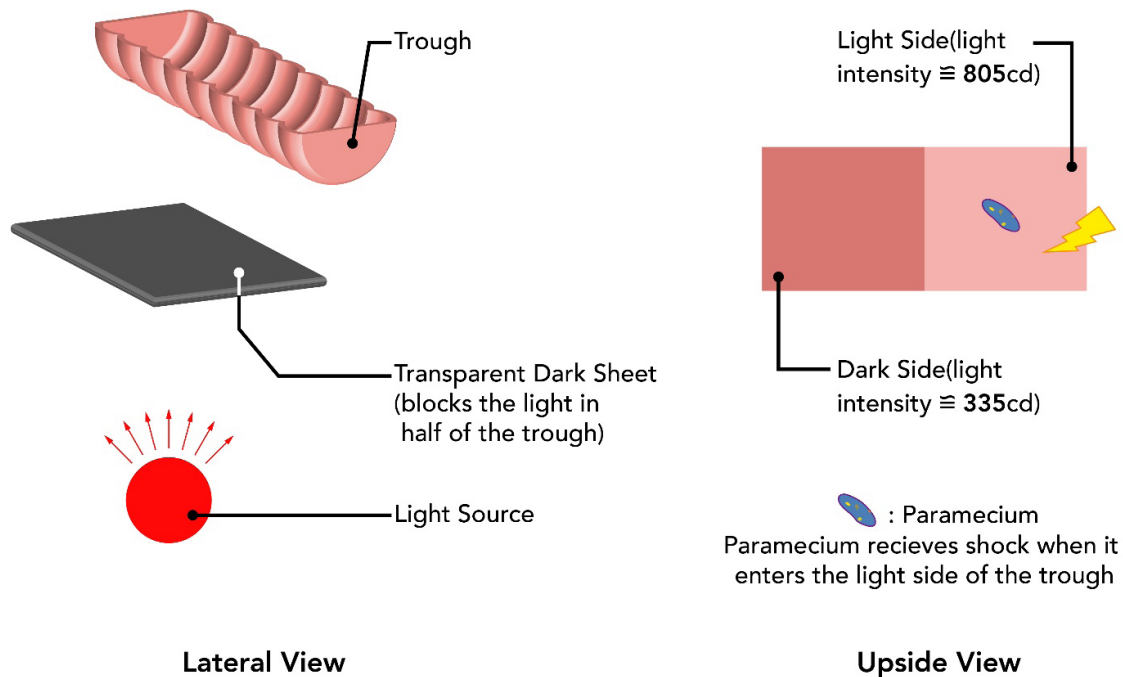


Figure 1. A schematic representation of the experimental setup

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changed (to prevent possible confounding factors such as changing *Paramecium*'s environmental cues, lighting conditions, interference with its free exploration, etc.), and individual *P. caudatum* was observed uninterrupted during the whole training and test trials. To place the *Paramecium* into the swimming medium, a few drops of culture flask containing the organism were placed on a microscope with 40X magnification, and a single *P. caudatum* was sucked into a mouth pipet tipped with a capillary tube (diameter of $\sim 500\ \mu\text{m}$). Subsequently, paramecia were injected into the swimming medium and observed under 10X magnification of a stereomicroscope. In the training trials of the experimental group (the first 7 trials), the experimenter manually started the shocks when the *Paramecium* entered the cathodal half of the trough (whether it was the dark or bright side), and shocks were turned off when the organism left that half. In the test trials, the total time that *P. caudatum* spent on the light side of the trough was recorded. In the control group, paramecia never received any shock, and the time spent in the dark and light sides of the trough in the test trials was recorded. The parabendazole-treated group underwent the same procedure as the experimental group, except that this group was treated with a $100\ \mu\text{M}$ solution of parabendazole for 24 h before starting the experiment. For the midazolam group, paramecia received the same treatment as the experimental group, with the difference that the swimming medium during the experiment contained $30\ \mu\text{g/mL}$ of midazolam.

Parabendazole

Parabendazole is a member of the benzimidazole anthelmintic drug family used to treat parasitic infections in veterinary settings (Al-Hadiya, 2010). Even though parabendazole has been reported to interfere with cell metabolism as an inhibitor of glucose uptake and fumarate reductase and to disrupt secretory processes of acetylcholine (Al-Hadiya, 2010; Davidse, 1986), its main mechanism of action is to inhibit microtubule polymerization inside the cytoplasm (Havercroft, Quinlan, & Gull, 1981). Consequently, due to the depolymerization of microtubules during the so-called dynamic instability of microtubules, parabendazole effectively makes microtubular depolymerization irreversible. This condition disintegrates microtubular networks inside the cytoplasm over time. For example, the application of $20\ \mu\text{M}$ parabendazole in Vero cells for 21 h leaves only centrioles intact and causes an almost complete disappearance of microtubules (Havercroft et al., 1981).

More importantly, parabendazole is an ideal agent to study the role of microtubular dynamics in *Paramecium* since it does not kill cells even at high concentrations (Pape, Kissmehl, Glas-Albrecht, & Plattner, 1991) we screened a wide variety of such compounds for their effects on the growth of *Paramecium tetraurelia* cell cultures. Compounds tested include agents of widely different chemical composition and with reported effects on

widely different cell types. We can differentiate between different drug effects: (a. Pape et al. tested the effect of a wide variety of antimicrotubular agents on *Paramecium*'s growth. They found that although parbendazole can effectively inhibit cell growth, it does not show cytotoxic effects (Pape et al., 1991) we screened a wide variety of such compounds for their effects on the growth of *Paramecium* tetraurelia cell cultures. Compounds tested include agents of widely different chemical composition and with reported effects on widely different cell types. We can differentiate between different drug effects: (a. Therefore, we have chosen parbendazole to study the relationship between microtubular dynamics disruption and *Paramecium* learning while avoiding the possible cytotoxic effects of antimicrotubular agents. To ensure that parbendazole affects microtubules in *Paramecium*, we used a dose 2 orders of magnitude higher than the effective dose reported by Pape et al. (Pape et al., 1991) we screened a wide variety of such compounds for their effects on the growth of *Paramecium* tetraurelia cell cultures. Compounds tested include agents of widely different chemical composition and with reported effects on widely different cell types. We can differentiate between different drug effects: (a and 10 times higher than what has been reported to virtually inhibit all microtubular assembly in vitro (Havercroft et al., 1981).

Parbendazole treatment

Parbendazole was acquired from Sigma-Aldrich, USA. We incubated *Paramecium* in a culture solution with a 100 μ M concentration of parbendazole for 24 h prior to the experimental test for the parbendazole group. This time interval was selected based on the Pape et al. report (Pape et al., 1991) we screened a wide variety of such compounds for their effects on the growth of *Paramecium* tetraurelia cell cultures. Compounds tested include agents of widely different chemical composition and with reported effects on widely different cell types. We can differentiate between different drug effects: (a, indicating the presence of parbendazole's effect after 24 h. Parbendazole disrupts the microtubular dynamics of the *Paramecium*, so it should negatively affect its learning behavior if microtubules are involved in *Paramecium*'s learning.

Midazolam

Midazolam is a benzodiazepine drug that is widely used in medical procedures such as anesthesia, sedation, and amnesia (Reves, Fragen, Vinik, & Greenblatt, 1985). Midazolam is a partial allosteric modulator of GABAA receptors. While it does not cause a direct opening of the GABAA receptors, it increases the frequency of

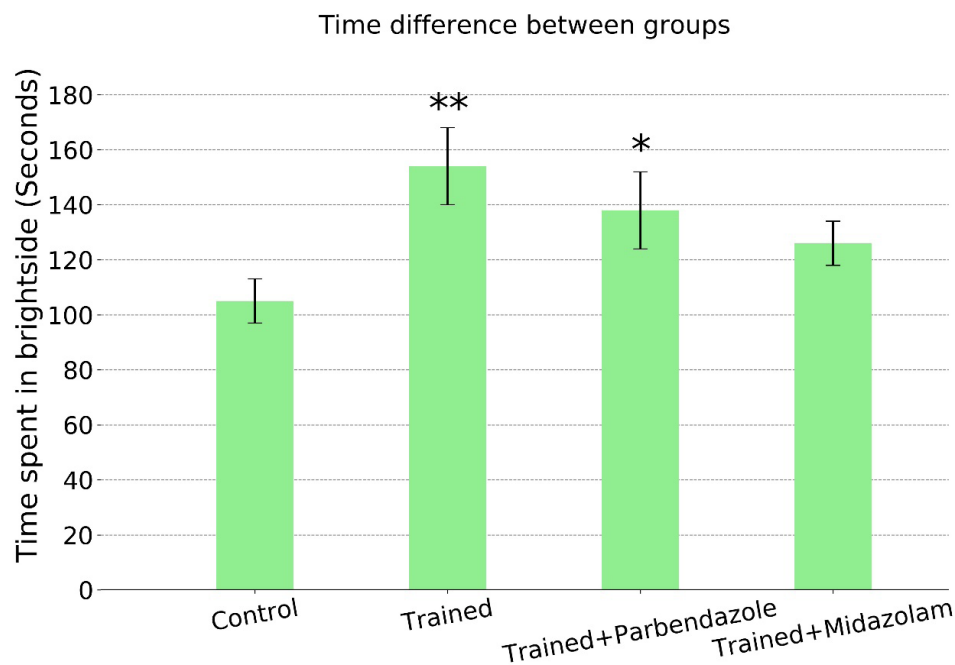
opening in these receptors upon agonist binding (Olkola & Ahonen, 2008) hypnosis, decreased anxiety, anterograde amnesia, centrally mediated muscle relaxation and anti-convulsant activity. In addition to their action on the central nervous system, benzodiazepines have a dose-dependent ventilatory depressant effect and they also cause a modest reduction in arterial blood pressure and an increase in heart rate as a result of a decrease of systemic vascular resistance. The four benzodiazepines, widely used in clinical anaesthesia, are the agonists midazolam, diazepam and lorazepam and the antagonist flumazenil. Midazolam, diazepam and flumazenil are metabolized by cytochrome P450 (CYP. This event results in an increased inflow of Cl^- and hyperpolarization of target cells. Inspired by the amnesic effects of the midazolam in human subjects, we sought to test the idea that similar effects can be observed in *Paramecium* due to evolutionary preserved pathways for memory formation across different species. To make the midazolam dosage comparable to parbendazole, we chose a dosage of midazolam that was 2 orders of magnitude higher than the previously reported plasma concentration of midazolam required for maintenance of anesthesia in human subjects (i.e., 30 $\mu\text{g/mL}$ in the experiment as compared to reported values of 259 to 353 ng/mL in human subjects (Crevat-Pisano, Dragana, Granthil, Coassolo, Cano, & Francois, 1986).

Midazolam treatment

A filtered cultured medium with a 30 $\mu\text{g/mL}$ (≈ 90 nM) concentration of midazolam was prepared and used as the swimming medium for *P. caudatum* in the learning trials of the midazolam group. To test the role of GABA receptors in the learning behavior of *Paramecium*, we used midazolam as it disrupts the normal functioning of GABA receptors. Midazolam was acquired from Exir pharmaceutical company, Iran.

Effect of parbendazole on paramecium's growth

To ensure the parbendazole effect on *P. caudatum*'s microtubular dynamics, we measured the growth of paramecia after drug administration as a proxy for its effectiveness. Population growth directly relates to healthy microtubular dynamics, and other known pharmacological effects of parbendazole do not directly affect population growth and cell division. A sample population of paramecia was taken and divided into two groups of control and parbendazole (100 μM concentration). Then, the population density of paramecia were counted in each sample before and 24 h after parbendazole administration to the parbendazole group. To estimate the popu-



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Figure 2. Time difference between groups: control (n=30), trained without any drug (n=23), trained with parbendazole (n=26), and trained with midazolam (n=26)

lation density in each sample, we collected 28 samples (20 μ L each) from each culture medium and counted the number of paramecia in each sample using an optical microscope. As stated earlier, was recorded the population density both at the parbendazole inoculation time and 24 hours after inoculation.

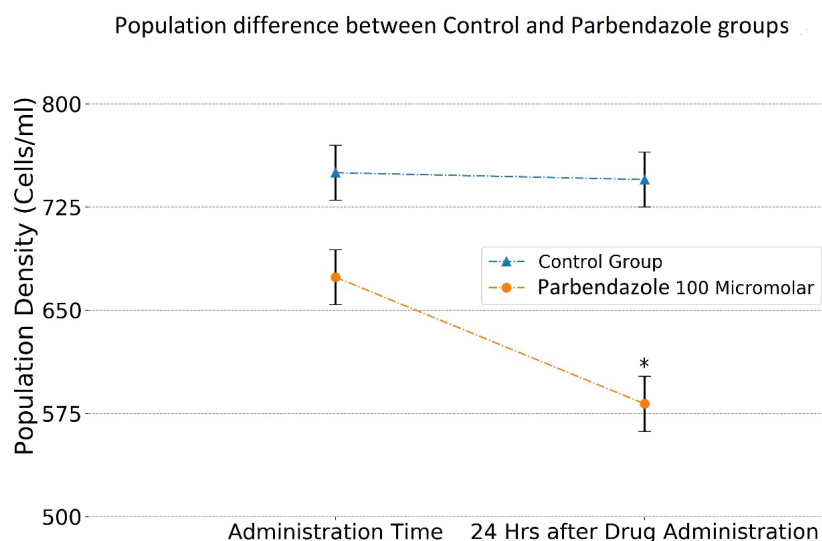
This procedure was performed in both logarithmic and stationary growth phases of paramecia. As reported before, the effect of antimicrotubular agents on the logarithmic growth phase can be studied by their administration 24 h after a new passage (Pape et al., 1991) we screened a wide variety of such compounds for their effects on the growth of *Paramecium tetraurelia* cell cultures. Compounds tested include agents of widely different chemical composition and with reported effects on widely different cell types. We can differentiate between different drug effects: (a. For the stationary phase, we evaluated the effect of parbendazole 72 h after a new passage. In other words, for the logarithmic phase, 24 h after a new passage, the culture medium was inoculated with parbendazole, and after another 24 h, the population density was measured to identify changes. Similarly, for the stationary phase, 72 h after a new passage, the culture medium was inoculated with parbendazole, and after 24 h, the population density was measured to identify changes.

Effect of parbendazole on *P. caudatum* swimming speed

The swimming speed of *P. caudatum* was recorded and measured using a microscope-mounted camera before and 24 h after parbendazole inoculation. Specifically, a sub-population of paramecia was randomly sampled, and the movement of paramecia was filmed for 10 min using the camera mounted on top of an eyepiece with a scale bar. Subsequently, the movement speed (μ m/s) of randomly selected paramecia was calculated by measuring the amount of movement of each *Paramecium* under the microscope, and a mean speed was calculated based on the population average (n=10 for both groups).

Electrical shock device

A microcontroller was used to adjust the shocks to the culture medium (ATMEGA 16 AVR controller). The circuit was designed to deliver cathodal shocks of 5 V (60-ms shocks with 500-ms no-shock intervals) as soon as the experimenter pressed the bottom. Therefore, when the *Paramecium* entered the bright side of the trough, the circuit was activated and delivered cathodal shocks every 500 ms. See learning behavior in the *Paramecium* section for more information.



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Figure 3. The population density of *P. caudatum* before and 24 h after the administration of parbendazole (dash-dotted line) compared to the control group (dotted line)

Statistical analysis

We used 1-way ANOVA (Tukey HSD for post hoc) to analyze the time spent in the light side of the trough. This procedure determined the time differences between four groups of control, experimental, parbendazole, and midazolam. SPSS software v. 16 was used for statistical analysis. The effect of parbendazole and midazolam on the population and swimming speed of *P. caudatum* was evaluated using the independent t test in the same program.

3. Results

Differences in behavioral profiles

Out of 270 s of test time, the *P. caudatum* learning group and the parbendazole-treated group spent 152 ± 12.7 and 137 ± 12.5 s in the light side, respectively. This time turned out to be significantly longer when compared to 105 ± 8.1 s in the control group (1-way ANOVA, Tukey HSD test, $P < 0.01$ and $P < 0.05$ for the learning group and parbendazole group, respectively; Figure 2). For the midazolam group, however, we did not observe a significant increase in the time spent in the light side (126 ± 8.7 s, $P = 0.31$). All numbers are reported as Mean $\pm$ SEM.

While there was a significant difference between the time of the parbendazole-treated group and the control group (1-way ANOVA, Tukey HSD test, $P < 0.01$ and $P < 0.05$, respectively), the midazolam-treated group did not show a significant time difference compared to the control group. Error bars are $\pm$ SEM.

Drug effectiveness analysis for parbendazole

Drug effectiveness analysis was performed in both logarithmic and stationary phases. For the stationary phase, the parbendazole-treated group and the control group had the same population density at the time of the inoculation. However, parbendazole-treated paramecia had a significantly lower population density than the control group after 24 h (Figure 3). In the logarithmic growth phase, the control group population showed a significant increase in the number of cells, while the parbendazole-treated group demonstrated an inhibited growth (Figure 4).

We also tested the changes in population density before and after parbendazole administration within each group, i.e., comparing before and after population densities within each drug treatment. In the logarithmic growth phase, the control population showed a significant increase in its population density after 24 h ($P < 0.01$), while the parbendazole-treated group demonstrated no change after 24 h ($P > 0.05$), which showed the effectiveness of parbendazole to inhibit growth. In the stationary phase, however, neither the control nor parbendazole group showed a significant difference with their initial values ($P > 0.05$).

Since every single *Paramecium* was being tested individually, population density differences are unlikely to affect the learning behavior of *Paramecium*. While there was no significant difference between the two groups at the administration time, the t test indicated a significant difference after 24 hours between the two groups in the stationary phase ($P < 0.05$, $n = 28$), error bars are $\pm$ SEM.

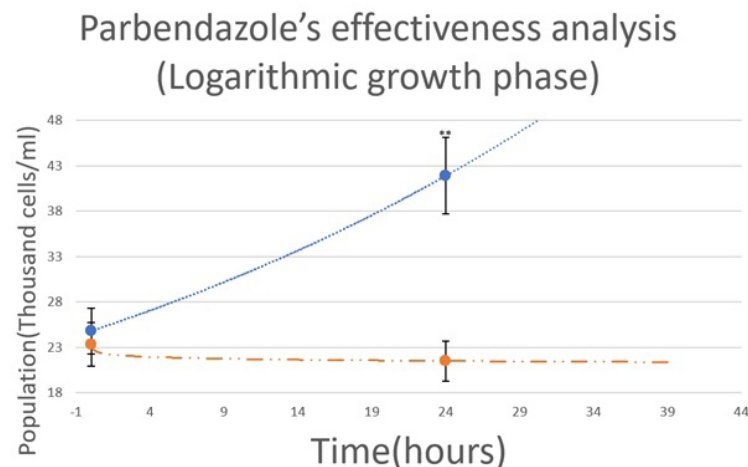


Figure 4. The population density of paramecium

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Note that the reduction in population density is due to the stationary phase of the growth and not cytotoxic effects. In other words, while the population stays the same in the control group due to equal division rates and cell death, inhibition of cell division in the parbendazole group causes a net reduction in population density. Similarly, the constant population density of the parbendazole group in Figure 4 shows that this effect is unlikely to be due to cytotoxicity.

In the logarithmic growth phase, the control group (dotted line) shows logarithmic growth while the parbendazole group (dash-dotted line) demonstrates a growth inhibition due to the parbendazole administration after 24 h ($P < 0.01$, $n = 28$), error bars are $\pm$ SEM. The lack of population density decline in the logarithmic phase suggests that parbendazole was not cytotoxic at this concentration.

Paramecium swimming speed analysis for parbendazole group

The independent t test indicated that the swimming speed difference of *P. caudatum* before and after parbendazole treatment was not significant (1031 ± 131 and 969 ± 107 μ m/s, respectively; Figure 5).

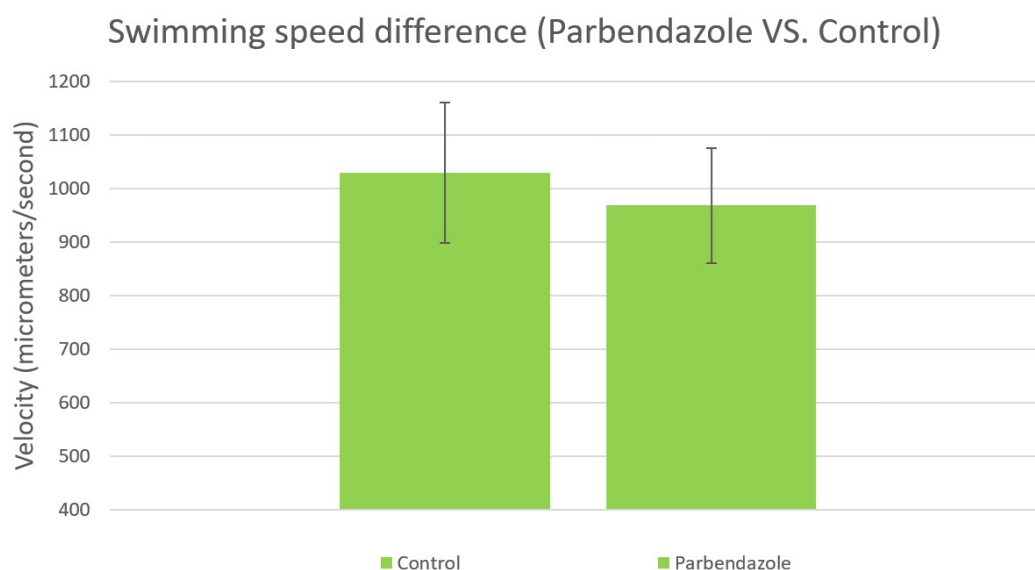
4. Discussion

Implications for information processing and storage in microtubules

In the present study, we showed that the disruption of microtubules does not cause significant impairment in the learning behavior of *P. caudatum*. Therefore, microtubules may not be necessary for memory storage and learning in *P. caudatum*. It is noteworthy to mention that although parbendazole concentration was two orders of

magnitude higher than the IC₅₀ concentration of parbendazole in *P. tetraurelia* and inhibited cellular growth, it did not affect the learning behavior of *P. caudatum*. Additionally, we analyzed the effect of parbendazole on the population density of *Paramecium* in two stationary and logarithmic phases and illustrated its effectiveness on *Paramecium*. Our analysis indicated that parbendazole is effective in the logarithmic phase whether pre-administration population density is compared to post-administration or population density of control is compared to parbendazole groups. However, in the stationary phase, the population density difference was significant only when we compared the control and the parbendazole-treated groups, i.e., comparing the parbendazole group's population density before and after administration did not show significant differences. The differences between the two phases can be explained by the fact that cell division has a higher rate in the logarithmic growth phase, so the disruption of microtubules has more pronounced consequences.

It might be argued that parbendazole only affects dynamic microtubules, and stable microtubules are left untouched. However, it was shown that even stable microtubules reassemble after 6.5 h (Schulze & Kirschner, 1987). Therefore, parbendazole treatment for 24 h should inhibit the reformation of stable microtubules as well. This fact is supported because of the treatment of Vero cells for 21 h with a 20 μ M concentration of parbendazole disassemble microtubules (Havercroft et al., 1981). Since 24-h treatment of cells with a concentration of 100 μ M is well above what was reported in Vero cells, it is reasonable to assume that parbendazole can affect both dynamic and stable microtubules. It is noteworthy that in the (Havercroft et al., 1981) study, centrioles were



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Figure 5. Swimming speed of paramecia without drug treatment (control) and 24 h after parbendazole administration not showing a significant change ()

Independent t-test, $P > 0.05$, $n = 10$; Error bars are $\pm$ SEM.

not harmed by parbendazole at different doses and durations (20 μ M for 21 h or 2 μ M for 45 h).

Accordingly, previous suggestions about the involvement of tubulins and microtubules in information storage in cells may need further investigation. If microtubules are not involved in information storage, it is hard to argue that they may be involved in information processing. Therefore, It might be reasonable to doubt the role of microtubules as fundamental structures that can support intelligent behavior(s) in an extensive range of species, as suggested by some scholars (Koch & Hepp, 2006; Litt et al., 2006). Since parbendazole does not affect centrioles, our results do not rule out the possibility of centrioles being involved in information processing as suggested by G Albrecht-Buehler (Albrecht-Buehler, 1997; Albrecht-Buehler, 1994, 1995).

Learning in paramecium, phototaxis, effects, and aftereffects of electrical stimulation

Our results show that learning in *P. caudatum* can be impaired upon administering a GABA receptor partial allosteric modulator. However, the same impairment was not observed by disrupting the microtubular dynamics. We cannot explain precisely how this agent disrupts learning in *Paramecium*. It may be explained based on the previously proposed model (Alipour et al., 2017), where learning in *Paramecium* includes three main processes: a) light detection and phototaxis, b) disruption of phototaxis through electrical stimulation, and c) stimu-

lation aftereffects. Some aspects of this model will be discussed in the following.

Light detection and phototaxis in paramecium

It is believed that phototaxis mechanisms in eukaryotes follow a straightforward rule (Jékely Gáspár, 2009). A photosensor molecule codes the light intensity and sends signals to a motor actuator for locomotion. *P. bursaria* is an excellent example of phototaxis. On the one hand, the swimming speed of this ciliate decreases upon exposure to bright light by membrane depolarization (Matsuoka & Nakaoka, 1988). This photosensitivity is independent of the symbiotic alga that exists in *P. bursaria*, as both chlorella-containing and chlorella free forms of *P. bursaria* show photosensitivity (Matsuoka & Nakaoka, 1988). On the other hand, its swimming speed increases upon normal light exposure due to the hyperpolarization of the membrane potential (Matsuoka & Nakaoka, 1988). Interestingly, this membrane hyperpolarization increases the ciliary beat frequency (Machemer, 1974). This effect is mediated through cyclic Adenosine Monophosphate (cAMP) molecules as measured by enzyme immunoassay of cellular cAMP contents (Mitarai & Nakaoka, 2005). Although retinal has been extracted from *P. bursaria* as a possible chromophore molecule (Tokioaka, Matsuoka, Nakaoka, & Kito, 1991), the exact identity of the photosensor molecule in *P. caudatum* is still unknown. It might be reasonable to assume that *Paramecium* uses cAMP as an intermediate messenger

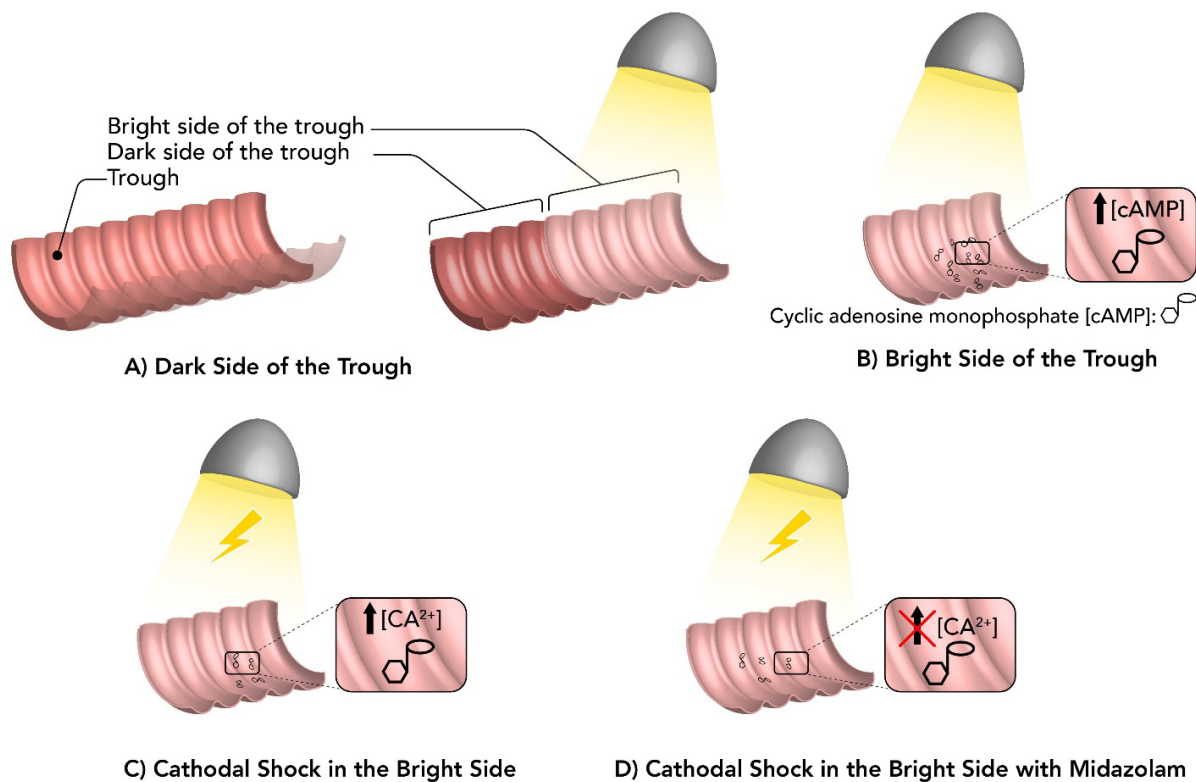


Figure 6. A schematic representation of the proposed model for learning in *Paramecium*

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to coordinate its unknown photosensor and its cilia. A similar molecular pathway might be responsible for phototaxis in *P. caudatum*. Since freely swimming paramecia spend only 39% of their time in the light side of the trough (≈ 100 s out of 270 s; Figure 2), it might be possible that *P. caudatum* avoids light exposure with a similar mechanism as *P. bursaria*. This mechanism probably involves cAMP, membrane potential fluctuations, and an unknown photosensor.

Disruption of phototaxis through electrical stimulation

It is known that the *Paramecium* membrane contains Ca^{2+} channels (Thiele & Schultz, 1981) used in the movement behaviors of the organism (Doughty & Dryl, 1981; Hinrichsen, Saimi, Hennessey, & Kung, 1984; Machemer, 1974) 1974. Interestingly, it was also shown that membrane depolarization could reverse the ciliary beating direction of the organisms by changing the flow of Ca^{2+} ions across the membrane (Nakaoka, Imaji, Hara, & Hashimoto, 2009) the amplitudes of which reach a few mV. When the resting potential fluctuation is positive and large, a spike-like depolarization is frequently elicited that reverses the beating of the cilia associated with directional changes during swimming. We aimed to study how the resting potential fluctuation is amplified. Simultaneous measurements of the rest-

ing potential and intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$). The link between membrane potential fluctuations and Ca^{2+} was established by the observation that adding a calcium chelator (BAPTA) intracellularly or Ba^{2+} ions extracellularly would reduce the membrane fluctuations (Nakaoka et al., 2009). Therefore, since the resting membrane potential of *Paramecium* is around -25 mV (Nakaoka et al., 2009) the amplitudes of which reach a few mV. When the resting potential fluctuation is positive and large, a spike-like depolarization is frequently elicited that reverses the beating of the cilia associated with directional changes during swimming. We aimed to study how the resting potential fluctuation is amplified. Simultaneous measurements of the resting potential and intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$, successive cathodal shocks may cause an outward flow of positive ions (Ca^{2+} in particular) and cancellation of *P. caudatum*'s light aversion behavior by reducing its swimming speed (Figure 6). This hypothesis can be further explored in future studies.

Stimulation aftereffects

Our assumptions to this point may explain why paramecia in the training group are relatively attracted to the light side. However, they do not explain the mechanisms of memory retention in *Paramecium*. We believe that memory retention in this organism is related to the stimu-

lation aftereffects. Light exposure produces a substantial amount of cAMP in the cytosol, while the reduction of Ca^{2+} in the cell—due to electrical shocks—may oppose 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 light or dark side of the trough. This finding is in line with the experimental finding that paramecia in the experimental group spend an almost equal amount of time in both halves of the trough (Figure 2). Therefore, we suggest that memory in *P. caudatum* in this task might be encoded in the concentration of cAMP molecules. However, future studies should be performed to measure cAMP concentrations during the test period to validate this hypothesis.

Pharmacological manipulations of learning in *P. caudatum*

Our results indicate that a benzodiazepine drug (a GABA receptor partial allosteric modulator) can disrupt the learning behavior of *P. caudatum*. As shown in the previous reports, a GABA_B agonist (baclofen) can cancel the reversal movement of *P. primaurelia*, and its effect can be blocked by a Ca^{2+} channel blocker (verapamil) (Bucci et al., 2005). Midazolam, as a GABA PAM, can potentially oppose the effect of electrical shocks and help *Paramecium* to exert its normal photophobic behavior towards the light (Figure 6).

Study limitations

One of the main limitations of the current study is the lack of behavioral data for multiple doses of parbendazole or midazolam. Since our primary goal in the present study was to identify (and not quantify) agents involved in *Paramecium* learning, we only studied single non-toxic high concentrations of parbendazole and midazolam. This condition gave each of these pharmacological agents their best chance to show their effects. However, lower dosages of the same pharmacologic agents might have different effects. Additionally, we used population density as a proxy for parbendazole effectiveness, while a more direct measure (such as immunocytochemistry) might be the appropriate technique to illustrate the effects of parbendazole on the microtubular structure.

A) When *Paramecium* is swimming in the dark side of the trough, there is a baseline cAMP concentration that maintains a normal swimming speed. B) When *Paramecium* enters the light side of the trough, light exposure causes an increase in cAMP levels, which consequently increases the swimming speed. C) When *Paramecium*

enters the light side of the trough and receives successive electrical shocks, electrical shocks will cause subtle and temporary backward movements, leading to a normal swimming speed almost equal to its swimming speed on the dark side. This condition causes the accumulation of cAMP molecules in the cytosol, which eventually cancels the photophobic behavior of *Paramecium* during test trials. This issue seems to be a possible mechanism for learning in *Paramecium*. D) Administration of midazolam as a GABA PAM can cancel the backward movements, as mentioned in the text. This process can eliminate the effect of electrical shocks and the subsequent accumulation of cAMP molecules in *Paramecium*. Therefore, midazolam might disrupt the learning behavior of *Paramecium* in this manner.

5. Conclusion

In the present study, we have shown that microtubular networks in the cytosol do not seem to be significant players in the learning process of *P. caudatum*. Instead, ionic flow disruption by a GABA receptor allosteric modulator can impair memory in *P. caudatum*. We disrupted microtubular dynamics in *Paramecium* using parbendazole to see its effect on *Paramecium*'s learning process. We observed that a partial allosteric modulator of GABA (midazolam) could disrupt the learning process in *Paramecium*, but parbendazole could not. Our results suggest that microtubules are probably not involved in information storage in *P. caudatum*, and there might be other mechanisms for this process. Our findings may have significant consequences for theories that consider a significant role for microtubules in information storage or processing in a wide range of animals, including *Paramecium*.

Further studies on the molecular cascade involving cyclic monophosphate and calcium ions could explain the learning behavior of *P. caudatum*. We suggested a molecular pathway to explain the learning behavior in *P. caudatum* based on the effect of a GABA PAM on this phenomenon, which can be further evaluated through future experiments. To this end, one experimental test can be using calcium channel blockers. If reversal beating direction (due to depolarization and calcium influx) is the primary mechanism responsible for *P. caudatum*'s learning, Ca^{2+} channel blockers could neutralize the learning behavior of *P. caudatum*.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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