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Article

The effects of regeneration on memory in planarians

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SUMMARY

Planarians are an excellent invertebrate model choice to understand regeneration and the memory of stimulus-response behavior. Unlike vertebrate models, planarians have the unique ability to fully regenerate tissues after damage. Studies using planarians produce conflicting results regarding their ability to retain memory after regeneration. Our research aimed to determine the effects of regeneration as a whole as well as the effects of different regions of regeneration on a planarian's memory of a conditioned stimulus. Here we have demonstrated that regeneration has no significant effect on a planarian's memory. On average, we determined that the non-dissected planarians recalled the conditioned stimulus more frequently, but there was no statistically significant effect on memory retention (p-value=0.143, one-tailed student's t-test). Of the regenerated planarians, the original heads recalled the conditioned stimulus more than the regenerated heads, but this difference in memory was not statistically significant (p-value=0.079, one-tailed student's t-test). Explanations for these findings require a deeper look into the mechanics of learning and retention in primitive organisms such as the planarian.

INTRODUCTION

Neuroplasticity is a process that involves adaptive functional and structural changes to the brain (1). Human brains can reorganize and create new connections after injuries such as strokes and TBI's (traumatic brain injuries) (1). However, vertebrate nervous system cells display limited regenerative capabilities (2). Planarians are a beneficial model organism for studying the effects of regeneration on memory because they can completely regenerate all elements of their morphology (3). However, the history of planarian behavioral studies is full of controversy and misinterpretation (3). The "planarian controversy" refers to the studies performed in James McConnell's laboratory in the 1950s and 1960s and the subsequent responses and replications (3). McConnell's original experiments utilized classical conditioning, a process that causes a neutral stimulus to elicit a response after associating it with another stimulus that already produces that response. In McConnell's design, white light (the neutral stimulus because it elicits no response) was paired with electric shocks (the unconditioned stimulus because it naturally produces a motor response) to transform the light into a conditioned stimulus that now elicits the reaction. Turns and contractions were measured in response to the white light, and these data supported the hypothesis that conditioning survived the regeneration process for both the head and tail sections (3). However, critics claimed that the study was not well controlled, and replications of his studies found more minor differences between experimental and control groups (3, 4). A proposed explanation for McConnell's results is that the electric shocks altered the planarian's physiological response to the light stimuli (3). This would risk invalidating McConnell's conclusion.

Memory has been shown to take various forms, such as metabolic differences in cells and tissues, factors that alter the rate of transcription, bioelectrical circuits, and encoding in neurons (6). Phototactic (related to directed movement in response to a light source) conditioned memory is stored in planarians through physical structures and tissues that make up its nervous system. In planarians, the eye spots carefully coordinate responses with the ganglion and ventral nerve cord; these structures also do not develop until five days post dissection (7). As the eye is necessary to detect non-UV wavelengths (such as the red and green lights chosen in this study), we focused on memory being stored and retrieved as the coordination between these neuronal structures.

Our research aimed to determine the effect of regeneration on the planarian's memory of the conditioned stimulus and compare memory persistence in original and regenerated brains. We hypothesized that if planarians are conditioned to avoid red light and then dissected, then the memory of this conditioned response will be negatively affected due to the process of regeneration. We dissected half of the originally conditioned planarians to stimulate the regeneration process. Post dissection, we sorted the planarians into two groups, those which were the original heads that regenerated tails and those that were regenerated heads (original tails), to determine how the conditioned response persisted post regeneration. In addition, we hypothesized that the original head planarians would exhibit the learned aversion more often than the regenerated head planarians. We found no significant difference between the regenerated and control planarians or the regenerated and original head planarians. This aligns with more recent findings suggesting that planarians can store memories throughout their central nervous system.

RESULTS

We conducted this experiment with the purpose of determining the effects of regeneration on memory in planarians. To control for the fact that regeneration takes time, the effect of time on memory was also studied. We conditioned planarians to display a negative phototaxis reaction in response to red light through classical conditioning

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(Figure 1). We then tested the memory for the conditioned stimulus immediately after training by seeing whether or not the planarian turned away from the red light. This established baseline recall (Day 0). Then we retested and compared to the baseline three weeks later (Day 21) **(Figure 2)**. On average, the baseline planarians turned away from red light 78% of the time, and the average memory for the non-dissected group, three weeks later, was 70% **(Figure 2)**. Although the trend suggests there was a negative trend of memory from initial memory to the three-week measurement, this was not significant **(Figure 2**, *p*-value = 0.374 by two tailed-student's t test**)**.

In this research, planarians were classically conditioned using green light as the unconditioned stimulus and red as the neutral stimulus. We selected these stimuli to avoid possible physiological changes caused by electric shocks, and because planarians exhibit an inverse relationship between the wavelength of light and the intensity of the worm's photophobia (5). This means that planarians react without aversion to lower wavelengths of light like red but turn away from higher wavelengths like green. After conditioning, we measured the planarian's response to the presentation of red light (the conditioned stimulus). Then, we dissected the two experimental groups laterally across to initiate regeneration. After regeneration, we retested the response to the conditioned stimulus in the original heads; the heads regenerated from the original tails, and the non-dissected planarians in the control group.

To determine the effect of regeneration on memory, we tested the memory of both non-dissected and dissected planarians three weeks after conditioning. Dissected planarians were cut in half to initiate regeneration **(Figure 3)**. The memory retention of the non-dissected (control) planarians was compared to all dissected planarians (old and new heads) **(Figure 4)**. The average memory for the control planarians was 70% and the average memory for the dissected planarians was 54.55% **(Figure 4)**. The trend suggests that regeneration has a negative effect on memory retention of the conditioned stimulus, however, it is not statistically significant (p-value=0.143, one-tailed student's t-test).

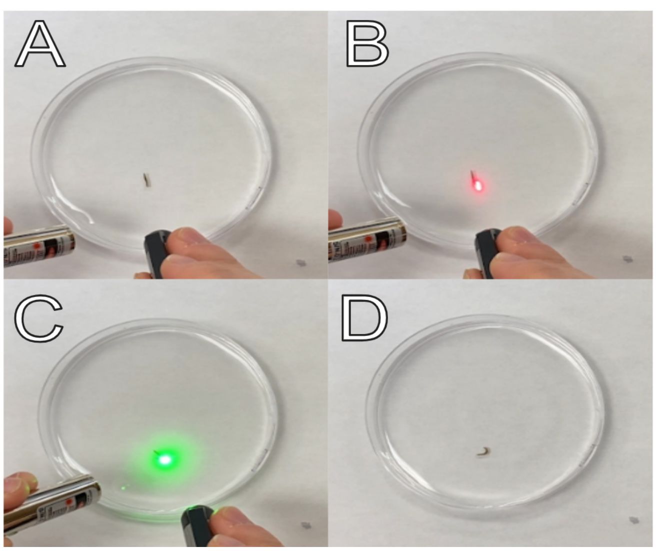

Figure 1: Representation of a conditioning trial. (A) The planarian was placed in the middle of the conditioning dish oriented down. **(B)** The red laser pointer was turned on in front of the head for two seconds. **(C)** The red laser was immediately replaced by the green laser for two seconds. **(D)** The planarian is now oriented to the left and has demonstrated negative phototaxis.

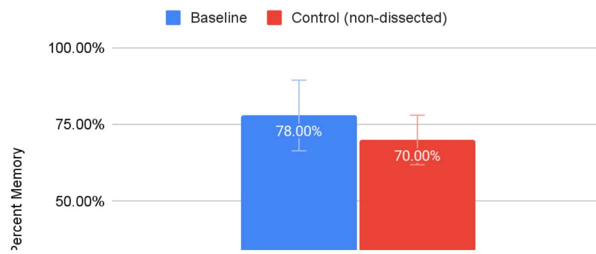

Figure 2: Average initial memory and average retention in the non-dissected planarians after three weeks. Graph represents the percent memory of the baseline (n = 10) and the control after three weeks (n = 4). Data values are represented as the average percentage of planarians showing memory to the conditioned response with blue showing baseline data and red showing after dissection. Error bars represent one standard deviation. Initial memory was tested, then half of the planarians were dissected. Memory was retested three weeks after dissection. Statistical significance was analyzed by a two-tailed students t-test with equal variance with no statistically significant difference after dissection (p = 0.488).

Three weeks after conditioning, we retested and compared the memory in the two experimental groups (old and new heads) **(Figure 5)**. This was done to compare memory persistence in original and regenerated brains. The average memory for the old heads was 63.33%, and the average memory for new heads was 44.00% **(Figure 5)**. The trend suggests the old heads had the highest retention, followed by the new heads **(Figure 5)**. However, we again found the difference was not statistically significant (p-value=0.079, one-tailed student's t-test).

DISCUSSION

The original purpose of our experiment was to determine effects of regeneration on planarians' memory. Our study found no significant difference in memory between regenerated and non-regenerated planarians. There was also no significant difference between regenerated and non-regenerated heads. The trends we observed showed that in general, dissection had a negative impact on memory retention and that new planarian heads recalled the stimulus less than new planarian tails.

We speculate that there were several possible factors that contributed to our findings. Regeneration is a stressful process for the organism since energy and resources must be diverted towards healing and regrowing the body rather than typical daily functions (2). In planarians, injury that results in the loss of the anterior (front portion) triggers the pluripotent neoblasts to proliferate for additional cell turnover and also requires specific transcription factors to be expressed for differentiation of these cells (8). We believe that these additional demands on the metabolism of the planarian due to regeneration may weaken memory retention. In addition,

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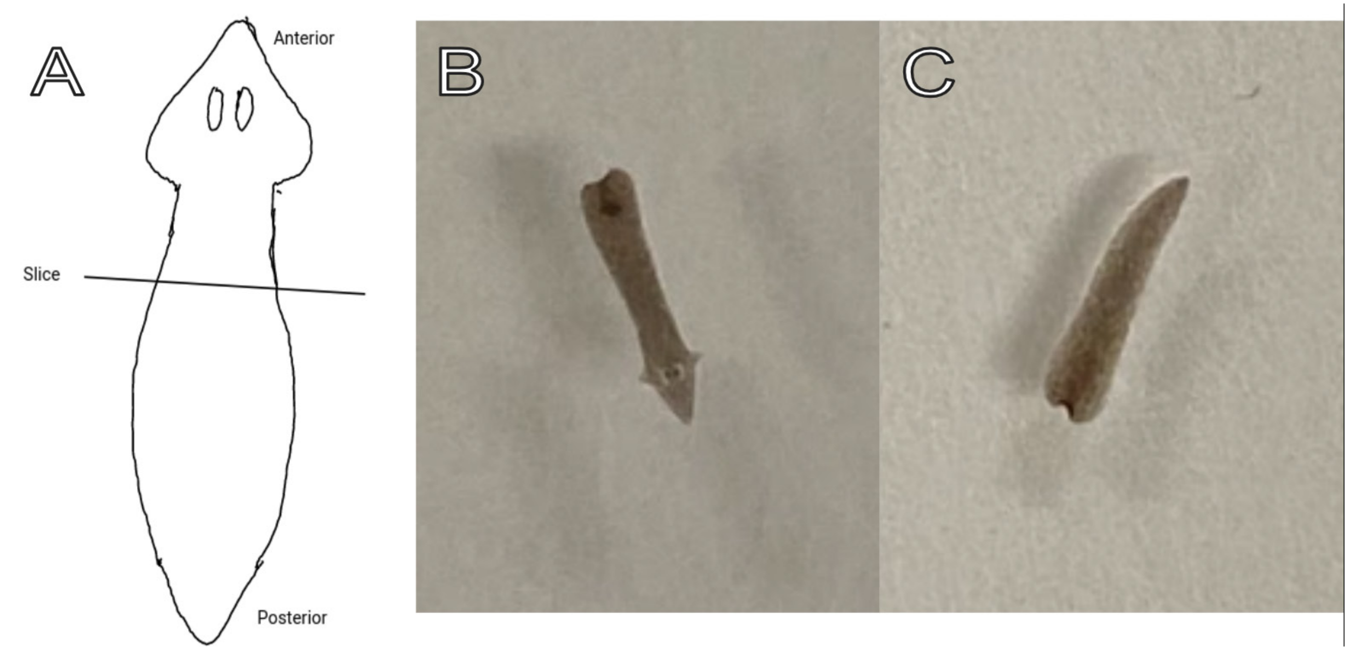

Figure 3: Representative image of dissected planarian. (A) Planarian dissection procedure: a drop of water was placed on a petri dish lid. The planarian was placed in the drop. Once stretched, the planarian was dissected with a scalpel laterally. **(B)** An image of the anterior end (anterior end is facing towards the bottom of the image). **(C)** An image of the posterior end (posterior end is towards the top of the image).

we believe that regeneration of specific structures may have impacted the ability to remember the stimulus aversion. The planarians that regenerated heads had to regenerate the cells of the eye spots as well as the basal ganglia (planarian brain), which have been associated with storing memory (6). Without the original connections between the neuronal components that learned the conditioned response, a system built from new stem cells would not have had that stimulus-response pathway created. Future studies could focus on the amount of additional resources needed by the planarians during regeneration to support this idea. While we believe that the memory was stored in the nervous system cells of the planarian ganglia, the mechanisms of information storage, encoding, and retrieval in planarians that are regenerated is not well known (6). Further studies into how this information is stored would improve our understanding on why our results showed no change in the planarians before and after regeneration.

We encountered some limitations in our experimental design. The first problem relates to the frequency and spacing of training sessions. We were only able to train the planarians five times a day for five days (25 times per planarian total) in the first replicate. None of the twelve worms in this initial replicate were able to demonstrate negative phototaxis towards the neutral stimulus, and we terminated the trial before moving forward with dissection. This replicate demonstrated that more conditioning trials were necessary to establish memory. Because of this and the influence of the study by James McConnell, the conditioning procedure was modified to 25 trials each day for five days (9). This modified procedure is what led to our analysis of the results in this study as the conditioning could still be demonstrated most of the time (70%) three weeks after the last day of conditioning.

The second limitation we ran into is the length of time required for regeneration. Planarian regeneration typically takes two to three weeks and undergoes fourteen main stages of regeneration (9, 10). This poses a challenge to the study, because retesting the memory of planarians post-dissection must occur weeks after the end of training, and not all of these stages are easy to visually discern. The decision to retest the planarians' memory three weeks after conditioning and dissection instead of two was made because the regenerated heads are still transparent after two weeks. Our study required retesting memory after complete regeneration. It is

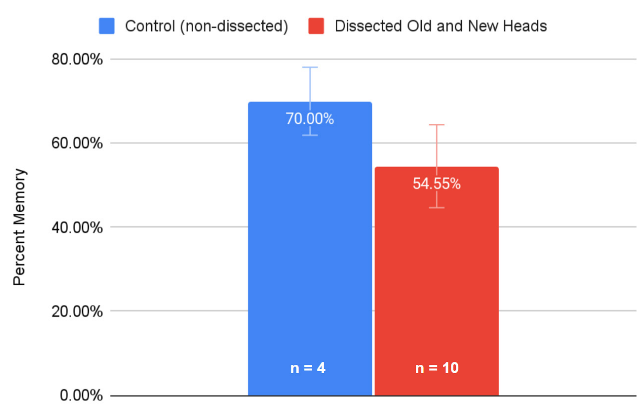

Figure 4: Average memory retention in dissected and non-dissected planarians after three weeks. Graph represents the percent memory of the control (n=4) and the dissected old and new heads (n=10). Data values are represented as the average percentage of planarians showing memory of the conditioned response with blue showing non-dissected planarians and red showing both types of dissected planarians. Error bars represent one standard deviation. Planarians were conditioned to display a negative phototaxis reaction to red light. Half of the planarians were dissected, then memory was tested three weeks later. Statistical significance was analyzed by a one-tailed student's t-test with equal variance. p= 0.143 is not statistically significant.

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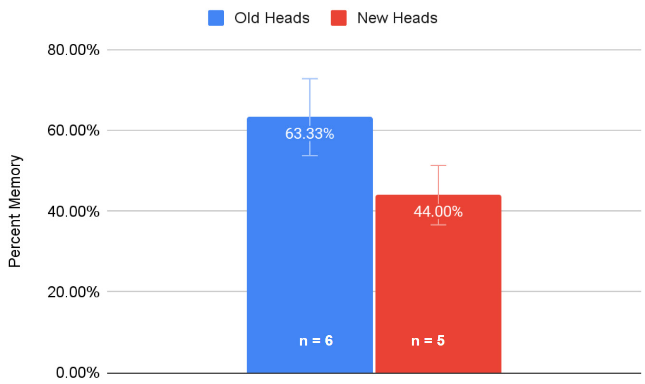

Figure 5: Average memory retention based on regenerated section. Graph represents the percent memory of the old heads (n=6) and the new heads (n=5). Data values are represented as the average percentage of planarians showing memory of the conditioned response with blue showing old planarian heads and red showing new planarian heads. Error bars represent one standard deviation. Planarians were conditioned to display a negative phototaxis reaction to red light. Half of the planarians were dissected, then memory was tested three weeks later. Statistical significance was analyzed by a one-tailed student's t-test with equal variance. p = 0.079 is not statistically significant.

possible that regeneration was still not fully completed at the three-week point. This would have a negative impact on the new head's ability to see the red laser because the ocelli (eye spots) would have been one of the last parts to regenerate (2).

In addition, the temperature variable was not fully controlled in the experiment. The planarians were housed in a cabinet drawer in the science classroom. Planarians do best at room temperature. However, we collected the data in the winter months. This often caused a low temperature in the classroom and the drawer. The temperature fluctuated daily but was also inconsistent in different areas of the drawer. The dishes of each experimental group were rotated daily to counteract the effect of varying temperatures on different groups of planarians. Despite this, colder temperatures experienced by some groups may have negatively affected their regeneration and put their bodies under stress. Lastly, the length of planarian memory may not have been long enough. We waited 21 days for the planarians to fully regenerate, but most studies that study memory show that 14 days is the longest time interval that memory has been recorded (11,12).

A fourth limitation was in our sample size. The old heads (regenerated tails) had a sample size of six (the initial and those that regenerated) and the old tails (regenerated heads) had a sample size of five (the initial and those that regenerated). It is difficult to determine statistical significance with such small samples. With smaller sample sizes, there is a higher risk of the small sample being unusual just by chance and not a reflection of the actual population studied. In addition, there were two unusual worms. One of the new heads died during the regeneration period, so no post-regeneration data could be collected; however, this was the only instance out of all the worms studied. Also, one of the original heads was one-third the size of the rest of the planarians and very light in color after the regeneration period. It is possible that this planarian had not fully completed the regeneration process. Both of these unusual worms lowered the averages of an already small sample size and could explain why the regenerated fraction had a 40% average memory when the average for original head memory was 63.33%. Another issue was the high standard deviations of both proportions. The original heads had a standard deviation of 15.06% and the regenerated heads had a standard deviation of 16.73%. This is attributed to the low number of test trials. With higher sample sizes and a higher number of training sessions, it may have been possible to see statistical significance between the groups analyzed.

The final limitation was the lack of matched pairs. It would have been ideal to directly compare the old and new head from the same original planarian. However, this was not feasible due to the lack of space and time for care during a class period.

Our findings can be extended in larger-scale studies with higher sample sizes to support these relationships further. In addition, future research should be done into the physiological effects of green light (532 nm). Part of the planarian controversy was attributed to possible physiological changes caused by the neutral shock stimulus. Our experiment's neutral stimulus (red light) should also be investigated. A further concern of the Planarian Controversy was possible pseudo-conditioning (when a previously neutral stimulus elicits a response). Research should be done to establish a baseline of the planarian's response to random presentations of red light at non-regular intervals for different periods of time and see if this would have an effect. UV light is not detected by the eye but through extraocular/whole body sensing in planarians (7). It would be interesting to see if this detection pathway shows similar results to our work with regeneration post dissection.

This work is important because classical conditioning is a powerful method for studying basic learning and memory in animals (14). This "basic memory" in classical conditioning refers to implicit or unconscious memory (15). Implicit memory is associated with the basal ganglia, neocortex, and cerebellum in humans; not all of these structures are found in planarians (15). However, in the planarian, the cerebral ganglia, which is a group of nerve cells forming a nerve center, leads in behavior. The cerebral ganglia then send impulses down the two central nerve strands and throughout the body (9). Because the regenerated heads and original heads do not have significantly different percent memories, the cerebral ganglia may be necessary for learning but not retention of the conditioned stimulus **(Figure 5)**. It can be inferred that structural changes must occur throughout the nervous system so that planarians with regenerated heads can recall the conditioned response. This requires a deeper look into the mechanisms of learning and retention in primitive organisms such as the planarian.

Finally, this work is vital because it has implications for research into human stem cells. Planarians are a beneficial model organism for humans, but their regenerative capabilities far surpass those of humans. Humans are incapable of regenerating brain cells the way that planarians can (2). In this study, we have demonstrated that regeneration has no significant effect on the memory of a planarian. It is possible that structural changes throughout the nervous system of a planarian occur during learning. More research should be done into the mechanisms involved in human memory. In the future, information gained from planarian regeneration could be applied toward this end.

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MATERIALS AND METHODS

Maintenance of planarians

Dugesia dorotocephala planarians from Carolina Biological Supply (Item #: 132954) were cultured by first creating a stock solution of 35 grams of Instant Ocean Sea Salts per liter of distilled water. This solution was adjusted with sodium bicarbonate to ensure the pH was within 6.9-8.1 using a pH indicator kit. If the pH was not correct, it was adjusted by adding sodium bicarbonate.

The planarians were kept in petri dishes with 1 mL of water for each 6-8 mm planarian. The lids of the dishes remain closed but not sealed to allow gas exchange to occur.

The planarians were fed hard-boiled egg yolks every other week. For feeding, a pea sized portion of egg yolk was mixed into the planarian water. Then the dish was placed in a dark drawer for 30 minutes. Then the planarians were transferred into a new dish with fresh planarian water using a new bulb pipette. Two days after feeding, the planarians were again transferred into fresh water. Planarians were fed the week before each experiment and then were not fed for four weeks during the experiment.

Conditioning

Each replicate contained four initial planarians. Four planarians of roughly the same size were selected from the culture and moved into a new petri dish. Each planarian was conditioned individually in the separate conditioning petri dish. Conditioning was done by placing the planarian in the center of the dish with its head oriented down. Then a red laser pointer (wavelength 670 nm) was turned on directly in front of the planarian's head for two seconds. We chose red light as a neutral stimulus because planaria do not show negative phototaxis when exposed (5). Using a shorter wavelength of light for the first part of the training would not have worked as the planaria would have already had an innate response to it (5). The red laser pointer was then immediately replaced with the green laser pointer (wavelength 532 nm) for two seconds for the purpose of exposing the planaria to a negative unconditioned stimulus and pairing it with the previous neutral stimulus (red light) **(Figure 1)**. We chose green light, as we could see the negative response from the planaria. After twenty seconds, the planarian was reoriented in the dish as stated above, and another conditioning trial was done. Each planarian in a replicate was trained 25 times a day for 5 consecutive days.

Memory testing

After five days of conditioning, each planarian's memory of the conditioned stimulus (red light) was tested to establish a baseline. One of the four planarians was placed in the conditioning dish. The planarian was placed in the center oriented down, like in training. Then, the red laser pointer was turned on in front of its head for three seconds. We recorded whether the planarian moved away from the light. This was our measurement for a response to a stimulus. This testing was repeated five times each for all planarians.

Three weeks (21 days) after dissection, the planarian's memory of the red light was retested. This testing was done identically to the baseline testing; however, we performed memory testing with six fully grown planarians instead of four. The different sized groups resulted from the two dissected planarians regenerating into four individual planarians. Additionally, the results were separated into categories based on the regenerated section (or lack thereof).

Dissection

After the memories of the four planarians in a replicate were recorded, two of the four planarians were dissected. This was done by placing a large drop of chilled planarian water on a clean petri dish lid. One planarian at a time was transferred into the water drop with a bulb pipette. When the planarian was stretched out, a clean razor blade was pressed into the body of the planarian laterally across the middle between the anterior and posterior ends **(Figure 3)**. After dissection, the dissected sections were separated into different petri dishes **(Figure 3)**. The two heads were placed in one petri dish, the two tails were placed in another, and the non-dissected control planarians were in another.

Statistical Analysis

Statistical analysis was performed by calculating the average percent memory of five groups: baseline, non-dissected, dissected (combines original and regenerated heads), original heads, and regenerated heads.

Creating these groupings, while important to test our multiple hypotheses, did mean that there were fewer planarians per trial. A power analysis at the 80% level based on our baseline data suggested that 5 planarians per trial is enough for statistical analysis. As we generally had four planarians per condition, this could affect the statistical analysis.

The average percent memory per group was calculated as follows

$$Average\ memory = \frac{Number\ of\ planaria\ that\ displayed\ negative\ phototaxis}{Total\ number\ of\ planaria\ tested\ in\ group}$$

Three total tests were performed. Each test was either a one-tailed or two-tailed student's t test of equal variance comparing the average percentage of planarians that demonstrated memory by responding to the conditioned stimulus.

Data were input into a Google Sheet for analysis and then calculated manually with an alpha level of 0.05 used to determine statistical significance.

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