J Exp Biol Advance Online Articles. First posted online on 2 July 2013 as doi:10.1242/jeb.087809
Access the most recent version at http://jeb.biologists.org/lookup/doi/10.1242/jeb.087809

TITLE:

An Automated Training Paradigm Reveals Long-term Memory in Planaria and Its Persistence Through Head Regeneration

AUTHORS:

Tal Shomrat, and Michael Levin

Biology Department and Tufts Center for Regenerative and Developmental Biology,
Tufts University,
200 Boston Ave., Suite 4600,
Medford, MA 02155, USA.

CORRESPONDING AUTHOR

Dr. Michael Levin
Biology Department and
Tufts Center for Regenerative and Developmental Biology
Tufts University
200 Boston Ave., Suite 4600,
Medford, MA 02155-4243
Tel. (617) 627-6161
Fax: (617) 627-6121
email: michael.levin@tufts.edu

RUNING TITLE: Memory in Regenerating Planaria

KEYWORDS: flatworms, training, conditioning, learning, planaria, regeneration, behavior

LIST OF ABBREVIATIONS: Automated Training Apparatus (ATA).

SUMMARY

Planarian flatworms are a popular system for research into the molecular mechanisms that enable these complex organisms to regenerate their entire body, including the brain. Classical data suggest that they may also be capable of long-term memory. Thus, the planarian system may offer the unique opportunity to study brain regeneration and memory in the same animal. To establish a system for the investigation of the dynamics of memory in a regenerating brain, we developed a computerized training and testing paradigm that avoided the many issues that confounded previous, manual attempts to train planaria. We then used this new system to train flatworms in an environmental familiarization protocol. We show that worms exhibit environmental familiarization, and that this memory persists for at least 14 days – long enough for the brain to regenerate. We further show that trained, decapitated planaria exhibit evidence of memory retrieval in a savings paradigm after regenerating a new head. Our work establishes a foundation for objective, high-throughput assays in this molecularly-tractable model system that will shed light on the fundamental interface between body patterning and stored memories. We propose planaria as a key emerging model species for mechanistic investigations of the encoding of specific memories in biological tissues. Moreover, this system is likely to have important implications for the biomedicine of stem cell-derived treatments of degenerative brain disorders in human adults.

INTRODUCTION

One of the most interesting capabilities of living systems is processing information. Biological information in multicellular organisms comes in at least 2 flavors: spatial information, needed to create and maintain specific anatomical structures during embryogenesis and regeneration, and temporal information abstracted and stored from environmental stimuli over time by the central nervous system. The intersection of these two fundamental processes has implications for basic neurobiology and engineering of the brain:body interface (Pfeifer and Gomez, 2009; Sampaio et al., 2001), for the synthetic bioengineering of cybernetic systems (Macia et al., 2012; Sole et al., 2007), and for the biomedicine of degenerative brain disease (Murre et al., 2001; Perry and Hodges, 1996). For example, what happens to the personality and mental content of an adult patient with decades of stored memories when his brain is repopulated by the descendants of implanted stem cells (Martino et al., 2011; van Velthoven et al., 2009)? Answering questions about the storage of information in dynamically-remodeling biological tissues, and specifically about the dynamics of memory during brain regeneration,

requires a tractable model system with both – a robust CNS repair mechanism and the ability to learn and remember.

Free-living, planarian flatworms represent the “first” class of organism to have a centralized brain with true synaptic transmission (Sarnat and Netsky, 1985), and shares the majority of neurotransmitters that occur in vertebrate brains (Buttarelli et al., 2008). Planaria have primitive eyes and other sensory capabilities including sensitivity to chemical gradients (Mason, 1975; Miyamoto and Shimozawa, 1985), vibration (Fulgheri and Messeri, 1973), electric fields (Brown and Ogden, 1968), and magnetic fields (Brown and Chow, 1975; Brown, 1966). Their sensory reception mechanisms are integrated by the worm’s nervous system into a rich and complex set of behaviors as they navigate their environment.

Adult stem cell populations (neoblasts) underlie their remarkable regenerative abilities (Reddien and Sanchez Alvarado, 2004; Wagner et al., 2011), and whole worms can regenerate from only a small proportion of the adult worm: a cut off (or damaged) head is rebuilt perfectly within few days (Inoue et al., 2004; Umesono et al., 2011). Recently, planaria have become a popular molecular-genetic system for the investigation of the pathways that allow complex structures such as the head to be regenerated after damage (Aboobaker, 2011; Gentile et al., 2011; Lobo et al., 2012; Newmark and Sánchez Alvarado, 2002; Salo et al., 2009; Sánchez Alvarado, 2006). Thus, planaria are an ideal system in which to probe the dynamics of information stored in the CNS during massive remodeling and repair. While studies have identified several insect organisms in which memories survive the drastic reorganization of metamorphosis (Alloway, 1972; Blackiston et al., 2008; Hepper and Waldman, 1992; Ray, 1999; Sheiman and Tiras, 1996; Tully et al., 1994), planaria are a uniquely tractable system for molecular-biological analyses of large-scale regeneration of adult brains. But can they learn?

Nearly 55 years ago it was demonstrated that planarians could be trained to learn a task, and following amputation of the head, the animals regenerating from the original tail sections remembered the original training (Best, 1963; Corning and John, 1961; McConnell, 1965; McConnell et al., 1959). This stunning finding, suggesting that some memory may be stored outside of the head and imprinted on the new brain during regeneration, led to a myriad of subsequent associative learning studies (Cherkashin et al., 1966; Corning, 1966; Corning, 1967; McConnell, 1965; Morange, 2006; Sheiman and Tiras, 1996). The most common procedure was a classical conditioning protocol based on planarians’ well-known photosensitivity (Dasheiff and Dasheiff, 2002; Inoue et al., 2004; Prados et al., 2013; Stephen, 1963). Acquired memories that could survive the process of head regeneration were demonstrated by measuring a direct

display of a conditioned response or a faster learning rate ("savings") among worm fragments generated from head and tail pieces of previously trained planarians (McConnell et al., 1959).

While learning induced by classical conditioning could be attributed to sensory adaptation rather than consolidation and retrieval of "real, encoded" memory (Halas et al., 1962; Halas et al., 1961), other studies showed that memories formed in more complex discrimination tasks, e.g., eliciting movement in a specific direction in a two-choice maze (Best, 1963; Corning and John, 1961; Corning, 1966; Corning, 1967; Corning et al., 1967; Humphries, 1961; McConnell, 1965; Roe, 1963) or learning to associate odorant cues (Wisenden and Millard, 2001), likewise survived regeneration of the head (Corning, 1966; Ernhart and Sherrick, 1959). The reports of persistent memory in an animal that had to regenerate its entire head (Corning, 1967) suggests approaches for investigating how information can be stored outside of the brain and imprinted on a newly-regenerating brain – a truly fascinating possibility.

These remarkable discoveries have not had sufficient impact on the field and were largely abandoned due to practical difficulties inherent in manual experiments. While the basic findings were validated in some cases, they failed to be reproduced in others (Corning and Riccio, 1970; McConnell, 1966), and the whole line of research became abandoned (Rilling, 1996). While modern discoveries such as epigenetic modification (Arshavsky, 2006; Day and Sweatt, 2010; Ginsburg and Jablonka, 2009; Levenson and Sweatt, 2005) and RNAi (Smalheiser et al., 2001) now offer mechanistic explanations of some of the original results, the primary barrier to molecular-level investigations into the dynamics of memory during CNS regeneration has remained: the difficulty of developing a robust learning assay in planaria. Manual behavior experiments involve limited sample sizes, difficulties in precise reproduction of protocols, and lack of quantitative analysis (Corning and Riccio, 1970; Hartry et al., 1964; Morange, 2006; Travis, 1981). As a result of these difficulties, even, planarians' capacity for long term memory has been questioned (Abbott and Wong, 2008; Takeda et al., 2009; Travis, 1981).

As part of our investigations into information processing by dynamically-organizing tissues, we have begun to develop automated approaches for eliciting learning and recall in planaria to overcome the problems inherent in manual methods (Nicolas et al., 2008; Oviedo et al., 2008b). We thus developed two platforms that allow automated, parallelized, quantitative, and fully objective training and testing of planaria in a wide range of feedback paradigms (Blackiston et al., 2010; Hicks et al., 2006). The aim of this study was to find a learning paradigm that overcomes a number of problems in previous attempts and establishes a modern platform for the use of regenerative planaria for the study of learning and memory.

Best and Rubinstein (Best, 1963; Best and Rubinstein, 1962a; Koopowitz, 1970) showed that planarians which had been fed in a familiar environment will start to eat more quickly than naïve worms which never been exposed to the feeding arena before. As in prior studies, their manually-performed experiments contained small sample sizes and limited controls (Davenport and Best, 1962; Dufort, 1962), and it appears that there have been no later attempts to use or improve this non-punishing paradigm. Here, we modified this environmental familiarization approach, adapting it to the use with a textured substrate (to provide clear haptic cues to the animals) and an automated behavior analysis system (Blackiston et al., 2010). Our protocol minimizes bias caused by manual procedures, allows an unprecedented level of quantitative rigor in behavioral analysis, and applies the procedure to a large sample size in a relatively short time frame. Additionally, in contrast to Best and Rubinstein's protocol, our procedure checks for long-term memory, several days after the training ended. Our results support the findings of Best and Rubenstein, and show a statistically-significant shorter feeding delay for the familiarized worms compare to unfamiliarized worms. Most importantly, the memory survives long enough to allow for regeneration after amputation, and indeed we show that memory traces survive entire brain regeneration in a "saving" paradigm. This simple and promising approach opens great opportunities for the use of planaria as a model organism to understand how specific memories survive large-scale regeneration of neural tissues.

MATERIALS AND METHODS

Experiment apparatus

For training and testing we used a custom-made fully automated training apparatus (ATA) (Blackiston et al., 2010; Blackiston and Levin, 2012) (Figs.1A,2L,M), which minimized bias caused by manual procedures and facilitated the training and testing of large numbers of control and experimental worms, simultaneously within the same conditions including time of the day, temperature, and type of arena. However, we settled on a paradigm that requires path tracking of the animals (Fig. 1B) but no complex training algorithm with instantaneous feedback (light or shock) to each animal. Therefore, this protocol could be done with any of the off-the-shelf system capable of multiple video tracking (Marechal et al., 2004; Noldus et al., 2001).

The ATA "familiarized" chamber environment contained a Petri dish with rough-textured floor surrounded by the ATA electrode walls (Fig. 2). Rough-textured petri dishes have been made from commercially available polystyrene 15x60mm petri dishes (Fisher Scientific, 0875713A), altered by laser etching (universal laser systems versaLASER VL-300). The laser cuts the circles to a depth of 0.2mm below the level of the dish's floor, but the displaced melted

polystyrene also builds up around each circle to a height of about 0.05mm above the floor of the dish. The pattern (Fig. 2N) is made up of circles drawn at 1.4mm in diameter and spaced 2.15mm at their centers. As cut, the outer diameter of each circle ends up being closer to 1.5mm and 1.2mm inner diameter (the trough that the laser cuts for each circle is about 0.3mm wide).

Worm colonies' maintenance

All planaria used in the current study were *Dugesia japonica*. After examining three planarian species: *Dugesia japonica, Dugesia dorotocephala, and Schmidtea mediterranea*, we found *Dugesia japonica* to be the most suitable for this project. It has remarkable regenerating capabilities, high tolerance for training and dissection procedures, and is very active. Before experiments, planarian colonies were stored in rectangular plastic containers, filled with Poland Springs natural spring water (Oviedo et al., 2008a). *Dugesia japonica* has a high tendency to spontaneously fission. In order to prevent spontaneous fission and allow worms to reach a suitable size for the experiment (1-1.5 cm), containers were stored in an incubator at 10°C in continuous darkness (Morita and Best, 1984) and fed once or twice a week with organic beef liver.

Handling and maintenance during the experiment

In addition to suppressing fissioning, keeping the worms in darkness has been reported to enhanced negative phototaxis (McConnell, 1965)(an important feature for the testing procedure). Worms were kept in continuous darkness during the entire experimental period except for brief periods during water changes and transfers between the experimental environment and their resting petri dish/wells plate. Planarians are more active and display longer exploration phase when kept in 18°C (as compared to 10°C). The experiment room temperature was also kept at 18°C. Therefore, during the experimental period the worms were held in incubator at 18°C. The tails' regeneration rate is also higher in 18°C compared to 10°C, allowing testing the headless fragments worms after only 10 days from decapitation (Fig. 4). Culturing the worms at high density was also found to be effective in suppressing spontaneous fission (Best et al., 1969). Thus, the worms were held in groups, in high density (~12 worms / 2ml water). This high density required water to be changed every day.

Every morning, during the training phase, the experimental apparatus was cleaned and the water was changed. The worms were taken out of the ATA and placed in petri dishes with fresh water in the dark for the cleaning period. The familiarized groups were placed in a dish

with a rough textured floor and the unfamiliarized groups were placed into standard Petri dishes. Rough-textured and standard Petri dishes were reused during the training after being thoroughly cleaned with Kimwipes soaked with ethanol 70% and positionally randomized between trials. The ATA electrodes, used as walls for the "familiar" environment, were also cleaned with Kimwipes soaked with ethanol 70%. At the end of the cleaning procedure the worms were placed back into their experimental environments. In order to suppress fission, the experimental environment was filled with low water levels (~12 worms / 2 ml water) to maintain high density of animals. During the testing sessions the experimental apparatus (ATA-electrodes and dishes) were cleaned between every testing trial. For all worms' handling, we used a plastic transfer pipette with the tip cut off to make a slightly larger opening. During the training, separate pipettes were used for the familiarized and unfamiliarized groups.

Training procedure

Groups of 20-40 experimental worms were placed in an individual ATA chamber (while testing was done on individual animals, familiarization proceeded in groups). The ATA chamber environment contained a Petri dish with rough-textured floor surrounded by the ATA electrode walls (Fig. 2A). The training period last 10-11 consecutive days. The chambers were filled with water (~12 worms / 2ml water) and the lids were closed for darkness. Unfamiliarized (control) worms went through the same procedure, simultaneously with the familiarized (experimental) group but were placed in the ATA in non-textured standard Petri dish (Fig. 2B). Every morning during the training phase, the worms were taken out of the ATA for water change and cleaning. Before being inserted back into the chambers, the worms were inspected and tail fragments caused by spontaneous fissions were extracted. After a 10 day familiarization period, the worms were taken out from the ATA and divided into smaller groups and were kept in 12 multiwell plates (Greiner Bio-One: part number 665102, hydrophobic surface (no treatment)) till the testing (12 worms in a well filled with 2 ml water Fig. 2E). The water in the wells was changed every day. Worms for regeneration experiments were kept in a Petri-dish for a 24-hour rest phase before dissection and division into smaller groups in small wells (to allow all eaten food to be digested before dissection).

Feeding during the training period

Worms were fed throughout the training period, in order to suppress fissioning, and eliminate the possibility of differential starvation levels among worms. The worms were fed in the ATA for one hour, with 1-2 small drops of liver (less than what they are capable of

consuming, Fig. 2C,D). Feeding took place in the morning after every third days of familiarization training (days 1, 4, 7, 10). Just before feeding, chambers were filled with additional ~10ml of water. On the last morning of familiarization training (day 10), the worms were fed intensively with 1-2 drops of liver every 20 minutes, until satiety (revealed by the last drop of liver remaining intact). This procedure "synchronizes" the hunger level of the worms which were tested 4 days later, and suppresses fissioning of the worms during a longer resting phase before testing. In addition, this feeding protocol is designed to create a positive association with the experimental environment. Worms that were tested 11-15 days after the end of training were fed again 1-2 times before the test.

Testing procedure

The ATA contains 12 identical chambers (Fig. 1A). During each testing trial, 6 familiarized and 6 unfamiliarized worms were tested simultaneously, each worm in its own individual chamber. All chambers contained a rough textured floor (a separate set of dishes from those used for the training), surrounded by the ATA electrode walls (Fig. 2J,K). A very small amount of liver was spread with a fine paintbrush on small area of the roughened dishes (Fig. 2J,K,O), and allowed to dry for about 5 minutes before being placed in the ATA and filled with 11 ml of water. In the absence of food, worms prefer to stay on the edge of the dish. Therefore, the liver was applied away from the arena wall (Fig. 2J) so that familiarized worms would be more willing to leave the edge and move toward the center of the dish (Fig. 2P). The worms were inserted to the ATA chambers with a plastic transfer pipette, in alternating order, starting with the unfamiliarized. The worms were placed in the chambers, opposite the liver spot. Worm transfer for all chambers averaged <1min. After all the 12 worms were inside the chamber, the lids were closed and the tracking was initiated.

To identify feeding, we capitalized upon the planarians' strong negative phototaxis (Inoue et al., 2004). Since the worms generally avoid illuminated areas, the quadrant with the spot of liver was illuminated with a strong blue LED light (Azuma et al., 1994; Brown et al., 1968) (Fig. 2L) thus, no worm would stay in this quadrant unless its desire for the liver, overcame their natural light aversion (Fig. 2P). As an indication of feeding, we measured how long it took the worms to reach the criterion of 3 consecutive minutes in the illuminated quadrant, containing the liver spot. Any worms that didn't reach criterion within 60 minutes (e.g., never attempted to eat the liver), as well as worms that showed evidence of any health issue like injuries caused by the transfer pipette, or worms that were in the process of fissioning, were not included in the results.

At the end of each testing trial, the worms were inspected individually, under a dissection microscope, for general health, injuries caused by the transfer pipette, fission, lesions, or incomplete head regeneration in the case of the headless fragment worms. In order to avoid possible interference from moving worms for testing in sequential groups, in the evening before, the testing worms were divided into two groups of 6 familiarized and 6 unfamiliarized worms and each group was placed in a separate well of 12-well plates, filled with 1ml of water (Fig. 2I). As in the experimental period, plates were placed in dark at 18°C till the beginning of the test at the next day.

Producing Headless Fragments

Worms were decapitated 24 hours after the final feeding which occurred at the end of the familiarization session. So that no brain remained, the worms were decapitated at the point between the auricles and the anterior side of the pharynx (Figs. 2F,4). Headless fragments were kept in groups of 12 worms in one well of 12 multiwell plates, in 2ml of water (Fig. 2E), in a dark incubator at 18°C. As with the intact worms, water was changed every day. After 7 days of regenerating at 18°C, the headless fragments were capable of eating (Fig. 4). Seven to nine days after decapitation, the regenerated worms were fed to satiety. Three to four days after feeding the worms were tested for recall. The worms were fed a second time, in cases when the duration between the first feeding to the recall test was longer than 3-4 days. For example, worms that tested at days 13 after decapitation were fed at days 7 and then again at day 9 or 10 from decapitation.

Savings Paradigm

In contrast to the headless fragments' regular protocol, where the feeding took place in the worms' home wells, in the saving protocol, the worms were fed in the familiarization arena. Seven to nine days after decapitation, groups, of both, familiarized and unfamiliarized regenerated worms were inserted in to the ATA's chambers with the surrounding electrode surfaces and the rough floor (the familiarization arena, Fig. 2H). After 30 minutes of exploration phase, drops of liver were placed in the chamber and the worms were allowed to eat until satiety. At the end of the session, the worms were placed back in the multiwall plate (~12 worms in well/2 ml water; Fig. 2E). At the evening, 3 days after the savings session, the worms were divided into groups of 6 familiarized and 6 unfamiliarized (Fig. 2I) and placed back in the dark at 18°C until the beginning of the test at the next day, 4 days after saving session.

Data analysis

The ATA's tracking log files were converted to excel file for data analysis. Because the delay values were not normally distributed (Kolmogorov-Smirnov test), we used the nonparametric Mann-Whitney U test to evaluate statistical significance (Bevins et al., 2001). Fisher's exact test was applied to determine statistical significance of the total number of worms that reach criterion in less than 8 minutes. Tests were one tailed since the direction was predicted in advance based on the previous work of Best & Rubinstein (1962a). To check for any mobility-impairment that might be responsible for behavior differences between the familiarized and unfamiliarized worms, the average movement rate (Pixels/Second) was calculated for the first minute, when the majority of worms were still engaged in exploration behavior.

RESULTS

Worms remember a familiar environment

Worms were familiarized to the automated behavior analysis platform (ATA) chambers as described in Methods, and then tracked by the ATA (Fig. 1). The retrieval test for familiar environment took place 4 - 15 days after the ending of the 10 days familiarization period, during which the familiarized worms were kept and fed in ATA chambers in Petri dishes with a rough bottom surface (Fig. 2C). The "unfamiliarized" group were also kept and fed in the ATA but in a standard, smooth-bottom Petri dish (Fig. 2D). During each test session, 6 familiarized worms and 6 "unfamiliarized" control worms were placed individually in the ATA chambers with a rough floor (the familiar environment). A small area of the dish was covered with liver (Fig. 2J,O) and a strong blue light illuminated the quadrant with the liver stain (Fig. 2L). As indication of feeding, we measured how long it took for the worms to reach the criterion of 3 consecutive minutes spent in the illuminated quadrant near the liver. The testing trials lasted 60 minutes. To rule out general physical condition differences between the worms, we checked their movement rate during the first minute, a time period while most of the worms were still during their exploration phase before settled down on the liver area. No significant differences were found between the two groups' motility (Table 1).

We tested for recall of a familiar environment 4 days after the familiarization period. Familiarized worms displayed significantly shorter time to reach criterion compared to the "unfamiliarized" worms (one tailed U-test, $P < 0.001$, Fig. 3B&Table 2). Similarly, testing for the number of worms to reach criteria in less than 8 minutes revealed significant differences

between the trained and control worms (Fisher's exact test, P=0.005, one tailed, Fig. 3Aa&Table 2).

Different groups of worms were tested 12-15 days following training. The familiarized worms displayed significantly shorter time to reach criterion compared to the unfamiliarized control worms (one tailed U-test, P < 0.001, Fig. 3Aa and Table 2). Testing for the number of worms to reach criterion in less than 8 minutes revealed significant difference between the trained and control worms (P=0.014; one tailed, Fisher's exact test, Fig. 3Ab and Table 2). We conclude that worms can remember a familiar environment for at least 14 days.

Worms with regenerated heads also retain some memory in a savings paradigm

The finding that this memory persists for at least 14 days – long enough for the brain to regenerate (Fig. 4), allowed us to check the possibility that this memory can survive brain regeneration. Headless fragments regenerated from familiarized worms displayed slightly shorter feeding latency compared to headless fragments from unfamiliarized worms when tested 10-14 days after decapitation (Fig. 3B&Table 2). However, the effect was not statistically significant. We then checked for the phenomenon of savings (See methods for detailed protocol), as McConnell found in his classical conditioning procedures (McConnell, 1965), where memory was revealed by a significantly faster training in a specific task in groups that had been trained on this task prior to decapitation. Worms that regenerated from headless fragments from original familiarized worms (Fig. 4) displayed significantly shorter feeding latency in the testing assay compared to regenerated worms that had not been familiarized to the environment prior to decapitation (One tailed U-test, P = 0.027; Table 2&Fig. 3B). Testing for the number of worms to reach criterion in less than 8 minutes revealed significant difference between the original familiarized worms and control worms (P=0.013; one tailed, Fisher's exact test, Fig. 3Ac &Table 2). We conclude that some memory of the place familiarization survives decapitation and brain regeneration.

DISCUSSION

During the last decade, planaria have become an important model organism in the field of developmental and regenerative biology; because of their extensive regenerative capacity (driven by an adult stem cell population) and complex CNS, significant efforts are underway to understand the molecular mechanisms behind neural repair and patterning (Aoki et al., 2009; Gentile et al., 2011; Newmark and Sánchez Alvarado, 2002; Nishimura et al., 2011; Salo et al., 2009; Sánchez Alvarado, 2006; Tanaka and Reddien, 2011; Umesono and Agata, 2009).

However, due to their rich behavioral repertoire and ability to learn (Corning, 1967; Oviedo and Levin, 2008), this model system also has the potential to offer unique opportunities for understanding the dynamics of memory during brain regeneration. This question has not only obvious clinical implications for stem cell therapies of adult neurological disorders but also bears on the fundamental issues of mechanisms of memory encoding and storage in the physical processes of the brain.

While planaria are now being used for studies of drug addiction and withdrawal (Pagan et al., 2012; Raffa et al., 2008; Raffa and Valdez, 2001; Ramoz et al., 2012; Rawls et al., 2011; Rawls et al., 2010; Sacavage et al., 2008), the usage of planaria as a model for learning and memory is still very limited (Nicolas et al., 2008; Nishimura et al., 2010; Oviedo and Levin, 2008). Although extensive work on planarians' learning and memory have long suggested that memories can survive brain regeneration (McConnell, 1966), the limitations of previous manual experiments have lead to these important questions being largely neglected by recent workers; these limitations included small sample sizes, difficulties in precise reproduction of protocols, and lack of quantitative analysis (Corning and Riccio, 1970; Travis, 1981). The aim of this work was to find a reliable, state-of-the-art approach that moves beyond past controversies to identify quantitative, objective, high-throughput protocols for eliciting and characterizing planarian long term memory capabilities. By demonstrating evidence for the acquisition of relatively complex, explicit-like memories, the planarian system becomes even more central in modern research into learning and memory.

Environmental familiarity is a well-accepted paradigm for the study of explicit memory mechanism in vertebrates (Heyser and Chemero, 2012; Heyser and Ferris, 2013; Teyke, 1989). Although some invertebrates such as bees and ants are capable of spatial memory and environmental recognition (Collett et al., 2003; Horridge, 2005), environmental familiarity has not been frequently used in learning and memory research with invertebrates. Best & Rubinstein (Best and Rubinstein, 1962a) showed that worms display a shorter feeding delay, when being fed in familiar environment 90 minutes after single, 25 minutes, familiarization session. Here we modified their environmental familiarization protocol and adapted it to the use with an automated behavior analysis system (Blackiston et al., 2010). This system minimizes bias caused by manual procedures, allows an unprecedented level of quantitative, objective rigor in behavioral analysis and data reporting, and applies the procedure to a large sample size in a relatively short time frame. In addition to more rigorous controls (Davenport and Best, 1962; Dufort, 1962), our protocol allows retrieval after at least 14 days from the end of the training.

Since this protocol measures feeding behavior, the worms' performance in the retrieval test is dependent on their baseline appetite level. We examined different starvation periods between 1-30 days (unpublished data) and found differences in the results' significance and variance as a function of the worms' starvation period, as did Best and Rubinstein (Best, 1963; Best and Rubinstein, 1962a). We observed that the best results, in our procedure, were obtained when the worms were fed 3-4 days before being tested. Future users of this procedure must establish the correct hunger level in the worms to observe the best results in this assay. Because hunger level is a pivotal parameter in this approach and could be affected by many variables as manipulation intensity, maintenance temperature, size of the worms, the species of worms, and type of food, we offer an additional heuristic to other workers reproducing this protocol. As a heuristic, the proper hunger level seems to be achieved when not more than a third of the worms initiate feeding in less than 1 minute from the start of the testing trial and stay there until criterion is reached. Also, as seen from the results (fig.3B), although the general protocol was similar between the different groups, there were still differences in the general latency of feeding, between the different categories. Even so, in any of the experiments, both control and experimental groups from each category were from the same colony, trained and tested in the same time and went through identical conditions of feeding and maintenance temperature. As a result, the changes in latency of feeding in each of the categories are both in the experimental and control groups, indicate the importance of rigor with respect to identical parameters and conditions for the experimental and control worms.

Importantly, in contrast to the most commonly-used procedures (classical conditioning protocols), this environmental familiarity protocol cannot be attributed to pseudoconditioning or sensitization effects (Halas et al., 1962; Halas et al., 1961) rather than consolidation and retrieval of "real, encoded" memory and behavior controlled by the brain. Planarians' feeding is a true complex behavior. Although composed of a series of stereotypic actions, it is coordinated and initiated by the central nervous system (Pearl, 1903; Sheiman et al., 2002). The feeding behavior is dependent on sensory integration (Pearl, 1903) , as in our paradigm, of tactile/ mechanical stimulation (Best and Rubinstein, 1962b) , chemotactic (Ash et al., 1973; Pearl, 1903) and optical sensations (Inoue et al., 2004).

Previous studies have shown that when food is placed in direct contact with the opening of the folded pharynx, it can activate the reflexes of extending the pharynx and swallowing, even in decapitated worms (Pearl, 1903; Wulzen, 1917). However, activation of these reflexes in decapitated worm is exceptional (Bardeen, 1901; Pearl, 1903) and the worms need to be

starved (Bardeen, 1901; Wulzen, 1917) and tested directly after decapitation (Bardeen, 1901; Sheiman et al., 2002; Wulzen, 1917).

We never observed such behavior in our worms (*Dugesia japonica,* which fasted for less than a week) and consistent with others' observations (Pearl, 1903; Sheiman et al., 2002), our headless fragments with an intact pharynx did not demonstrate any interest in food until head regeneration (5-7 days after decapitation), even when the tail fragment passed immediately adjacent to the food. Moreover, we observed that extrusion of the pharynx happened just after the head made a first contact with the food, sometime with a kind of stereotypic, drilling-like, movements into the liver. We cannot completely rule out the possibility that the modifications in the peripheral nervous system contribute to change in feeding latency. However, it is well-accepted that the recognition of food and moving directly to it, as in our case, with decision making and a cautious approach, against their natural preference (under the strong light above and away from the edge of the dish, Fig. 2P, Movie S1), are behaviors that are controlled by the CNS (Bardeen, 1901; Pearl, 1903; Sheiman et al., 2002). Finally, our results that show that in contrast to intact worms tested two weeks after training, regenerated worms, with an intact pharynx required "retraining" to demonstrate retrieval (Fig.3, Table 2), suggest that the difference found in latency of feeding is due to modification in the CNS and not/or not just a reflex or peripheral nerve system modification. Thus, our data show the survival of a true complex, brain-regulated behavior program through the process of head regeneration.

The procedure is ideally suited for automated apparatus with minimal handling and does not required manual analysis, as was required for example in studies of conditioned response intensity in classical conditioning procedures (Corning, 1967; Prados et al., 2013; Wells, 1967). Our paradigm requires path tracking of the animals but no complex training algorithm with instantaneous feedback (light or shock) to each animal. Therefore, this protocol could also be done with any of the off-the-shelf systems capable of multiple video tracking (Marechal et al., 2004; Noldus et al., 2001). The protocol avoids operator fatigue and ensures that no scoring biases are introduced into the data by subjective analysis of animal behavior.

While seeking the best complex learning protocol we observed the phenomenon previously called planarian's lethargy (Best, 1963; Best and Rubinstein, 1962b; Corning, 1964; McConnell, 1966; McConnell, 1965). Worms' learning curves during the training phase can suddenly reverse after a steady improvement, while healthy and active worms can begin to refuse to behave at all when inserted into the training apparatuses (Best and Rubinstein, 1962b; McConnell, 1965). Evidence suggests that this phenomenon could be related to familiarization to a dangerous environment, i.e. one in which the animal previously received noxious stimulus

(Shomrat, unpublished data and Best, 1963). The protocol reported here involves natural behavior with minimal handling and without negative reinforcement. This overcomes planarians' lethargy and thus also allows the application to much more sensitive species such as *Schmidtea mediterranea* (Sanchez Alvarado et al., 2002).

No differences were found in general motility between familiarized and unfamiliarized worms (Table 1). Thus, any behavioral differences are not due to simple changes of overall activity level due to the familiarization protocol. The training occurred in complete darkness and the type and amount of water, food, handling and maintenance were identical between the familiarized (experimental) and the unfamiliarized (control) groups. Therefore, the learned difference between the two environments was mainly tactile. In the majority of their exploration phase, the worms were crawling around the edge on the bottom of the chamber. Hence, the experimental worms could feel the roughness of the floor and the dodecagon shape of the chamber walls, which alternated between delrin-plastic and iridium oxide-coated titanium electrode (Fig. 2). Although no shock was delivered and the electrode material does not give off electrolysis products such as metal ions (Blackiston et al., 2010), there is a possibility that additional chemical cues from the electrode metal also facilitated place recognition.

Our results show that planarians can remember previously-encountered habitats for at least 14 days (Fig.3&Table 2). *Dugesia japonica* regenerates a functional head and CNS after 7 days, and in 14 days the worms are fully regenerated (Agata and Umesono, 2008; Inoue et al., 2004), (Fig. 4). Encouraged by the long-term retrieval, we investigated whether trained worms can display retrieval after decapitation and regeneration of a new head (Corning, 1966; Corning, 1967; McConnell et al., 1959). Worms regenerating from decapitated familiarized worms displayed a slightly shorter average, feeding latency compared to regenerated fragments from unfamiliarized worms (Fig. 3 & Table 2), but this effect was not statistically significant. Future work will explore longer training phases and further optimize different starvation periods to determine whether the strength of this effect can be increased.

McConnell's original results revealed a pattern of "savings", where the learning curve of retrained animals is better (faster) relative to that of to naïve animals (McConnell, 1965; McConnell et al., 1959). Therefore, we checked for the presence of savings in the regenerated worms. In our savings protocol, regenerated worms were fed in the testing arena (familiarization environment) in a single 3 hour session, 4 days before the retrieval test. Therefore the feeding session was a previously-encountered environment for the familiarized worms and a first introduction for the unfamiliarized. Worms that had regenerated from headless fragments from original familiarized worms, displayed significant shorter feeding latency compare to

unfamiliarized worms (Fig.3&Table 2), suggesting that memory of the original environment was not located exclusively in the brain, and had become imprinted onto the newly-built brain during regeneration.

In the past, such results have been received with skepticism (Smalheiser et al., 2001; Travis, 1981). The planarian has a centralized brain that guides behavior (Buttarelli et al., 2008; Sarnat and Netsky, 1985), and it is hard to imagine how memory traces (not just reflex arcs mediated by central pattern generators) can be encoded and stored in tissues remaining after complete head removal. However, such results are now made more plausible by modern discoveries such as epigenetic modification that occur in many cell types, not just the central nervous system (Arshavsky, 2006; Day and Sweatt, 2010; Ginsburg and Jablonka, 2009; Levenson and Sweatt, 2005; Zovkic et al., 2013) and RNAi (Smalheiser et al., 2001). It is likely that brain remodeling (plasticity during learning) and regeneration are both regulated via epigenetic pathways that determine patterns of self-organization of neural (Arendt, 2005; Davies, 2012; Kennedy and Dehay, 2012; Saetzler et al., 2011) and non-neural but electrically-communicating cells (Levin, 2012; Mondia et al., 2011; Oviedo et al., 2010; Tseng and Levin, 2013).

It has long been known that regeneration both shapes, and is in turn guided by, activity of the CNS (Geraudie and Singer, 1978; Mondia et al., 2011; Singer, 1952). Thus, it is possible that experiences occurring in the brain alter properties of the somatic neoblasts and are in turn recapitulated back during the construction of the new brain by these adult stem cells. While exciting previous work in insects (Blackiston et al., 2008; Sheiman and Tiras, 1996) suggested the ability of memories to survive significant rearrangements of the brain and CNS (metamorphosis), planaria provide a unique molecularly-tractable model of learned information persisting past complete removal of the brain. Of course, the mechanisms that allow unambiguous mapping (coding and decoding) of environmental sensory facts (e.g., “rough floor”, “metal walls”, etc.) into physico-chemical aspects of genetic material or neural network topologies are poorly understood not only for this case but for the normal relation of conscious memory and its physical substratum in the intact brain.

Our data reveal the presence of memory savings in regenerated tail fragments from trained worms. On the other hand, no significant results were found in experiments that did not include a retraining component after the brain regenerated, indicating the necessity of CNS modification. These results could be due to insufficient training or a sub-optimal protocol. Alternatively, it is possible that only a rough correlate of the memory is present in the neoblasts,

requiring a brief re-exposure to the trigger in order to consolidate into measurable effects on animal behavior (as occurs in the savings paradigm).

We suggest that some trace of memory is stored in locations distributed beyond the brain (since the place conditioning association survives decapitation). A straightforward model implies that information acquired during training must be imprinted on the regenerating (naïve) brain in order to result in the observed subsequent recall behavior. Future work must investigate the properties and mechanisms of such instructive interactions between remaining somatic organs and the regenerating CNS. However, two additional possibilities must be considered.

First is the possibility that the memory is executed entirely by the peripheral nervous system, not involving the brain in learning or recall. Given the similarities between the planarian brain and that of higher animals (in terms of structure, biochemistry, and complex ethology (Nicolas et al., 2008; Oviedo and Levin, 2008; Rawls et al., 2011; Sarnat and Netsky, 1985)), and the fact that worms exhibit no behavior prior to the regrowth of the brain, it is most likely that the planarian brain indeed drives behavior. A pivotal role for the brain is also supported by the need for the Savings portion of the paradigm, and the complexity of the behavior that is very unlikely to be implemented by receptor sensitivity and reflex modifications only (e.g., Fig. 2P and Movie S1). However, if true, this would suggest a remarkable capacity for integration of complex information in the peripheral nervous system of an animal that normally has access to an efficient brain, and thus would suggest a research program into the untapped information-processing abilities of the PNS in other advanced organisms.

Second is the possibility that the new brain is regenerated as a Tabula Rasa and is not imprinted by any traces of the previous memory. Instead, on this model the familiarized worms' PNS (which would have been modified and tuned, e.g., increased/decreased receptor sensitivity to a given stimuli during the training phase) is retraining the new brain: "burning" the association into the new CNS, during the short "Saving" session (which suffices because it is more efficient than in the unfamiliarized worms, due to the modified PNS sensitivity). We believe this scenario is less likely, because of the behavioral complexity of the learned task (Fig. 2P & Movie S1). Experimental and control worms were fed with liver during the entire procedure, and the liver odor would be everywhere in the dish – this means the worms did not have to rely on the rough texture to know that food was somewhere in the vicinity, and both the trained and control groups could have developed positive associations to the smell of the liver. As can be seen in Movie S1, the behavior does not resemble a simple reflex modification but rather the whole environment that makes trained worms initiate feeding sooner. We cannot completely rule out the possibility that the modifications in the peripheral nervous system contribute to change in

feeding latency. However, it should be noted that in order for receptor sensitivity to a *particular* stimulus to change after training, a kind of learning had to take place - the system as a whole (including learning, appropriate modification of PNS, and facilitation of re-training phases) implements an association between the presence of liver and the salient predictor of its presence, the rough surface, out of many other possible sensory modes that could have become more or less sensitized. Thus, this system would provide a novel model in which to examine the interactions between a mature PNS modified by specific experiences and learning in a newly-developed brain (Inoue et al., 2004; Koopowitz and Holman, 1988).

Conclusions:

Our results, obtained using a highly-sensitive, objective, quantitative analysis system, support previous findings of Best and Rubenstein (Best and Rubinstein, 1962a) , that planarians are capable of acquiring a relatively complex, explicit-like memories of environmental familiarity. Moreover, this memory survives long enough to allow full regeneration, after amputation. Remarkably, headless fragments, regenerated from original environment-familiarized worms, display significant environmental familiarity in a saving paradigm. This simple and promising approach opens great opportunities for the use of planaria as a model organism for modern research of learning and memory. Importantly, planarians are the only molecularly-tractable system in which memory and brain regeneration can be studied in the same animal. This is a crucial advantage allows the Investigation of innovative hypothesis as the role of epigenetic and, self-organization mechanisms in memory encoding, brain development, and brain regeneration.

ACKNOWLEDGEMENTS

We thank Punita Koustubhan for general laboratory assistance, Junji Morokuma and Wendy Beane for advice and help with the planarian model system, Douglas Blackiston and Robert Cook for many helpful discussions about behavioral paradigms, Durwood Marshall, Dany S. Adams, and Laura Vandenberg for assistance with statistics, Douglas Blackiston, Michael Romero, and Philip Starks for comments on early versions of the manuscript, and Ethan Golden for fabrication of the rough-textured, petri dishes. This work is dedicated to Paul Van Oye and James V. McConnell, two pioneers of learning and memory in planaria.

FUNDING

This research was funded by the G. Harold and Leila Y. Mathers Charitable Foundation.

REFERENCES

Abbott, S. M. and Wong, G. K. (2008). The conditioning and memory retention of planaria (Dugesia tigrina) for directional preferences. *Bios* **79**, 160-170.

Aboobaker, A. A. (2011). Planarian stem cells: a simple paradigm for regeneration. *Trends in Cell Biology* **21**, 304-11.

Agata, K. and Umesono, Y. (2008). Brain regeneration from pluripotent stem cells in planarian. *Philos Trans R Soc Lond B Biol Sci* **363**, 2071-8.

Alloway, T. M. (1972). Retention of Learning through Metamorphosis in Grain Beetle (Tenebrio-Molitor). *American Zoologist* **12**, 471-472.

Aoki, R., Wake, H., Sasaki, H. and Agata, K. (2009). Recording and spectrum analysis of the planarian electroencephalogram. *Neuroscience* **159**, 908-14.

Arendt, T. (2005). Alzheimer's disease as a disorder of dynamic brain self-organization. *Progress in brain research* **147**, 355-78.

Arshavsky, Y. I. (2006). "The seven sins" of the Hebbian synapse: can the hypothesis of synaptic plasticity explain long-term memory consolidation? *Progress In Neurobiology* **80**, 99-113.

Ash, J. F., McClure, W. O. and Hirsch, J. (1973). Chemical studies of a factor which elicits feeding behaviour in Dugesia dorotocephala. *Animal Behaviour* **21**, 796-800.

Azuma, K., Okazaki, Y., Asai, K. and Iwasaki, N. (1994). Electrical responses and K+ activity changes to light in the ocellus of the planarian Dugesia japonica. *Comparative biochemistry and physiology. Part A, Physiology* **109**, 593-9.

Bardeen, C. R. (1901). The Function of the Brain in Planaria Maculata. *American Journal of Physiology* **4**, 175-179.

Best, J. B. (1963). Protopsychology. *Sci Am* **208**, 54-62.

Best, J. B., Goodman, A. B. and Pigon, A. (1969). Fissioning in planarians: control by the brain. *Science* **164**, 565-6.

Best, J. B. and Rubinstein, I. (1962a). Environmental familiarity and feeding in a planarian. *Science* **135**, 916-8.

Best, J. B. and Rubinstein, I. (1962b). Maze learning and associated behavior in planaria. *J Comp Physiol Psychol* **55**, 560-6.

Bevins, R. A., Koznarova, J. and Armiger, T. J. (2001). Environmental familiarization in rats: differential effects of acute and chronic nicotine. *Neurobiology of Learning and Memory* **75**, 63-76.

Blackiston, D., Shomrat, T., Nicolas, C. L., Granata, C. and Levin, M. (2010). A second-generation device for automated training and quantitative behavior analyses of molecularly-tractable model organisms. *PLoS ONE* **5**, e14370.

Blackiston, D. J. and Levin, M. (2012). Aversive training methods in Xenopus laevis: general principles. *Cold Spring Harbor Protocols* **2012**.

Blackiston, D. J., Silva Casey, E. and Weiss, M. R. (2008). Retention of memory through metamorphosis: can a moth remember what it learned as a caterpillar? *PLoS ONE* **3**, e1736.

Brown, F. and Chow, C. (1975). Differentiation between clockwise and counterclockwise magnetic rotation by the planarian dugesia-dorotocephala. *Physiological Zoology* **48**, 168-176.

Brown, F. A., Jr. (1966). Effects and after-effects on planarians of reversals of the horizontal magnetic vector. *Nature* **209**, 533-5.

Brown, H. M., Ito, H. and Ogden, T. E. (1968). Spectral sensitivity of the planarian ocellus. *The Journal of general physiology* **51**, 255-60.

Brown, H. M. and Ogden, T. E. (1968). The electrical response of the planarian ocellus. *Journal of General Physiology* **51**, 237-53.

Buttarelli, F. R., Pellicano, C. and Pontieri, F. E. (2008). Neuropharmacology and behavior in planarians: translations to mammals. *Comparative biochemistry and physiology. Toxicology & pharmacology : CBP* **147**, 399-408.

Cherkashin, A. N., Sheiman, I. M. and Bogorovskaya, G. I. (1966). Uslovniye reflexi y planarii i opiti s regenerazei. *Zhurnal Vysshei Nervnoi Deiatelnosti Imeni I. P. Pavlova* **XVI**, 1110-1115.

Collett, T. S., Graham, P. and Durier, V. (2003). Route learning by insects. *Current Opinion in Neurobiology* **13**, 718-25.

Corning, W. and John, E. (1961). Effect of ribonuclease on retention of conditioned response in regenerated planarians. *Science* **134**, 1363-1365.

Corning, W. C. (1964). Evidence of right-left discrimination in planarians. *The Journal of Psychology* **58**, 131-139.

Corning, W. C. (1966). Retention of a position discrimination after regeneration in planarians. *Psychanomic Science* **5**, 17-18.

Corning, W. C. (1967). Regeneration and retention of acquired information: NASA.

Corning, W. C., Ratner, S. C. and American Institute of Biological Sciences. (1967). Chemistry of learning; invertebrate research. New York,: Plenum Press.

Corning, W. C. and Riccio, D. (1970). The planarian controversy. In *Molecular approaches to learning and memory*, (ed. W. Byrne), pp. 107-150. New York: Academic Press.

Dasheiff, B. D. and Dasheiff, R. M. (2002). Photonegative response in brown planaria (Dugesia tigrina) following regeneration. *Ecotoxicology & Environmental Safety* **53**, 196-9.

Davenport, D. and Best, J. B. (1962). On Planarian Behavior. *Science* **137**, 452-6.

Davies, P. C. W. (2012). The epigenome and top-down causation. *Interface Focus* **2**, 42-48.

Day, J. J. and Sweatt, J. D. (2010). DNA methylation and memory formation. *Nature neuroscience* **13**, 1319-23.

Dufort, R. H. (1962). On Planarian Behavior. *Science* **138**, 400-2.

Ernhart, E. N. and Sherrick, C. (1959). Retention of a maze habit following regeneration in planaria (D. aculatd). In *Paper presented at Midwestern Psychology Association*. St. Louis, Mo.

Fulgheri, D. and Messeri, P. (1973). The use of 2 different reinforcements in light darkness discrimination in planaria. *Bollettino - Societa Italiana Biologia Sperimentale* **49**, 1141-1145.

Gentile, L., Cebria, F. and Bartscherer, K. (2011). The planarian flatworm: an in vivo model for stem cell biology and nervous system regeneration. *Dis Model Mech* **4**, 12-9.

Geraudie, J. and Singer, M. (1978). Nerve dependent macromolecular synthesis in the epidermis and blastema of the adult newt regenerate. *J Exp Zool* **203**, 455-60.

Ginsburg, S. and Jablonka, E. (2009). Epigenetic learning in non-neural organisms. *J Biosci* **34**, 633-46.

Halas, E. S., James, R. L. and Knutson, C. S. (1962). An attempt at classical conditioning in the planarian. *Journal of comparative and physiological psychology* **55**, 969-71.

Halas, E. S., James, R. L. and Stone, L. A. (1961). Types of responses elicited in planaria by light. *J Comp Physiol Psychol* **54**, 302-5.

Hartry, A. L., Morton, W. D. and Keithlee, P. (1964). Planaria - Memory Transfer through Cannibalism Reexamined. *Science* **146**, 274-275.

Hepper, P. G. and Waldman, B. (1992). Embryonic olfactory learning in frogs. *Quarterly Journal of Experimental Psychology. B, Comparative and Physiological Psychology* **44**, 179-97.

Heyser, C. J. and Chemero, A. (2012). Novel object exploration in mice: not all objects are created equal. *Behav Processes* **89**, 232-8.

Heyser, C. J. and Ferris, J. S. (2013). Object exploration in the developing rat: Methodological considerations. *Developmental Psychobiology* **55**, 373-81.

Hicks, C., Sorocco, D. and Levin, M. (2006). Automated analysis of behavior: A computer-controlled system for drug screening and the investigation of learning. *J Neurobiol* **66**, 977-90.

Horridge, G. A. (2005). Recognition of a familiar place by the honeybee (Apis mellifera). *Journal of comparative physiology. A, Neuroethology, sensory, neural, and behavioral physiology* **191**, 301-16.

Humphries, B. (1961). Maze learning in planaria. *Worm Runner's Digest* **3**, 114–115.

Inoue, T., Kumamoto, H., Okamoto, K., Umesono, Y., Sakai, M., Sanchez Alvarado, A. and Agata, K. (2004). Morphological and functional recovery of the planarian photosensing system during head regeneration. *Zoolog Sci* **21**, 275-83.

Kennedy, H. and Dehay, C. (2012). Self-organization and interareal networks in the primate cortex. *Progress in brain research* **195**, 341-60.

Koopowitz, H. (1970). Feeding Behaviour and Role of Brain in Polyclad Flatworm, Planocera-Gilchristi. *Animal Behaviour* **18**, 31-&.

Koopowitz, H. and Holman, M. (1988). Neuronal Repair and Recovery of Function in the Polyclad Flatworm, Notoplana-Acticola. *American Zoologist* **28**, 1065-1075.

Levenson, J. M. and Sweatt, J. D. (2005). Epigenetic mechanisms in memory formation. *Nat Rev Neurosci* **6**, 108-18.

Levin, M. (2012). Molecular bioelectricity in developmental biology: new tools and recent discoveries: control of cell behavior and pattern formation by transmembrane potential gradients. *BioEssays : news and reviews in molecular, cellular and developmental biology* **34**, 205-17.

Lobo, D., Beane, W. S. and Levin, M. (2012). Modeling planarian regeneration: a primer for reverse-engineering the worm. *PLoS Comput Biol* **8**, e1002481.

Macia, J., Posas, F. and Sole, R. V. (2012). Distributed computation: the new wave of synthetic biology devices. *Trends Biotechnol* **30**, 342-9.

Marechal, J. P., Hellio, C., Sebire, M. and Clare, A. S. (2004). Settlement behaviour of marine invertebrate larvae measured by EthoVision 3.0. *Biofouling* **20**, 211-7.

Martino, G., Pluchino, S., Bonfanti, L. and Schwartz, M. (2011). Brain regeneration in physiology and pathology: the immune signature driving therapeutic plasticity of neural stem cells. *Physiol Rev* **91**, 1281-304.

Mason, P. R. (1975). Chemo-klino-kinesis in planarian food location. *Animal Behaviour* **23**, 460-9.

McConnell, J. (1966). Comparative physiology: learning in invertebrates. *Annu Rev Physiol* **28**, 107-36.

McConnell, J. V. (1965). A Manual of psychological experimentation on planarians. Ann Arbor, Mich.

McConnell, J. V., Jacobson, A. L. and Kimble, D. P. (1959). The effects of regeneration upon retention of a conditioned response in the planarian. *Journal of Comparative Physiology and Psychology* **52**, 1-5.

Miyamoto, S. and Shimozawa, A. (1985). Chemotaxis in the freshwater planarian Dugesia-japonica-japonica. *Zoological Science (Tokyo)* **2**, 389-396.

Mondia, J. P., Levin, M., Omenetto, F. G., Orendorff, R. D., Branch, M. R. and Adams, D. S. (2011). Long-distance signals are required for morphogenesis of the regenerating Xenopus tadpole tail, as shown by femtosecond-laser ablation. *PLoS ONE* **6**, e24953.

Morange, M. (2006). What history tells us VI. The transfer of behaviours by macromolecules. *Journal of biosciences* **31**, 323-7.

Morita, M. and Best, J. B. (1984). Effects of Photoperiods and Melatonin on Planarian Asexual Reproduction. *Journal of Experimental Zoology* **231**, 273-282.

Murre, J. M., Graham, K. S. and Hodges, J. R. (2001). Semantic dementia: relevance to connectionist models of long-term memory. *Brain* **124**, 647-75.

Newmark, P. and Sánchez Alvarado, A. (2002). Not your father's planarian: a classic model enters the era of functional genomics. *Nat Rev Genet* **3**, 210-9.

Nicolas, C., Abramson, C. and Levin, M. (2008). Analysis of behavior in the planarian model. In *Planaria: A Model for Drug Action and Abuse*, eds. R. Raffa and S. Rawls), pp. 83-94. Austin: RG Landes Co.

Nishimura, K., Inoue, T., Yoshimoto, K., Taniguchi, T., Kitamura, Y. and Agata, K. (2011). Regeneration of dopaminergic neurons after 6-hydroxydopamine-induced lesion in planarian brain. *Journal of Neurochemistry* **119**, 1217-31.

Nishimura, K., Kitamura, Y., Taniguchi, T. and Agata, K. (2010). Analysis of motor function modulated by cholinergic neurons in planarian *Dugesia japonica*. *Neuroscience* **168**, 18-30.

Noldus, L. P., Spink, A. J. and Tegelenbosch, R. A. (2001). EthoVision: a versatile video tracking system for automation of behavioral experiments. *Behav Res Methods Instrum Comput* **33**, 398-414.

Oviedo, N. and Levin, M. (2008). The planarian regeneration model as a context for the study of drug effects and mechanisms. In *Planaria: A Model for Drug Action and Abuse*, eds. R. Raffa and S. Rawls). Austin: RG Landes Co.

Oviedo, N., Morokuma, J., Walentek, P., Kema, I., Gu, M., Ahn, J., Hwang, J., Gojobori, T. and Levin, M. (2010). Long-range neural and gap junction protein-mediated cues control polarity during planarian regeneration. *Dev Biol* **339**, 188-99.

Oviedo, N. J., Nicolas, C. L., Adams, D. S. and Levin, M. (2008a). Establishing and maintaining a colony of planarians. *CSH Protoc* **2008**, pdb prot5053.

Oviedo, N. J., Nicolas, C. L., Adams, D. S. and Levin, M. (2008b). Planarians: a versatile and powerful model system for molecular studies of regeneration, adult stem cell regulation, aging, and behavior. *Cold Spring Harb Protoc* **2008**, pdb.emo101-.

Pagan, O. R., Baker, D., Deats, S., Montgomery, E., Tenaglia, M., Randolph, C., Kotturu, D., Tallarida, C., Bach, D., Wilk, G. et al. (2012). Planarians in pharmacology: parthenolide is a specific behavioral antagonist of cocaine in the planarian Girardia tigrina. *The International journal of developmental biology* **56**, 193-6.

Pearl, R. (1903). The movements and reactions of fresh-water planarians : a study in animal behaviour: London :J. & A. Churchill.

Perry, R. J. and Hodges, J. R. (1996). Spectrum of memory dysfunction in degenerative disease. *Current Opinion in Neurology* **9**, 281-5.

Pfeifer, R. and Gomez, G. (2009). Morphological Computation - Connecting Brain, Body, and Environment. *Creating Brain-Like Intelligence: From Basic Principles to Complex Intelligent Systems* **5436**, 66-83.

Prados, J., Alvarez, B., Howarth, J., Stewart, K., Gibson, C. L., Hutchinson, C. V., Young, A. M. and Davidson, C. (2013). Cue competition effects in the planarian. *Animal cognition* **16**, 177-86.

Raffa, R. B., Stagliano, G. W., Ross, G., Powell, J. A., Phillips, A. G., Ding, Z. and Rawls, S. M. (2008). The kappa-opioid receptor antagonist nor-BNI inhibits cocaine and amphetamine, but not cannabinoid (WIN 52212-2), abstinence-induced withdrawal in planarians: an instance of 'pharmacologic congruence'. *Brain Res* **1193**, 51-6.

Raffa, R. B. and Valdez, J. M. (2001). Cocaine withdrawal in Planaria. *Eur J Pharmacol* **430**, 143-5.

Ramoz, L., Lodi, S., Bhatt, P., Reitz, A. B., Tallarida, C., Tallarida, R. J., Raffa, R. B. and Rawls, S. M. (2012). Mephedrone ("bath salt") pharmacology: insights from invertebrates. *Neuroscience* **208**, 79-84.

Rawls, S. M., Patil, T., Tallarida, C. S., Baron, S., Kim, M., Song, K., Ward, S. and Raffa, R. B. (2011). Nicotine behavioral pharmacology: clues from planarians. *Drug and Alcohol Dependence* **118**, 274-9.

Rawls, S. M., Patil, T., Yuvasheva, E. and Raffa, R. B. (2010). First evidence that drugs of abuse produce behavioral sensitization and cross sensitization in planarians. *Behavioural Pharmacology* **21**, 301-13.

Ray, S. (1999). Survival of olfactory memory through metamorphosis in the fly Musca domestica. *Neuroscience Letters* **259**, 37-40.

Reddien, P. W. and Sanchez Alvarado, A. (2004). Fundamentals of planarian regeneration. *Annu Rev Cell Dev Biol* **20**, 725-57.

Rilling, M. (1996). The mystery of the vanished citations: James McConnell's forgotten 1960s quest for planarian learning, a biochemical engram, and celebrity (vol 51, pg 589, 1996). *American Psychologist* **51**, 1039-1039.

Roe, K. (1963). In search of the locus of learning in planarians. *Worm Runner's Digest* **5**, 16-24.

Sacavage, S., Patel, H., Zielinski, M., Acker, J., Phillips, A. G., Raffa, R. B. and Rawls, S. M. (2008). Withdrawal-like behavior in planarians is dependent on drug exposure duration. *Neuroscience Letters* **439**, 84-8.

Saetzler, K., Sonnenschein, C. and Soto, A. M. (2011). Systems biology beyond networks: generating order from disorder through self-organization. *Seminars in cancer biology* **21**, 165-74.

Salo, E., Abril, J. F., Adell, T., Cebria, F., Eckelt, K., Fernandez-Taboada, E., Handberg-Thorsager, M., Iglesias, M., Molina, M. D. and Rodriguez-Esteban, G. (2009). Planarian regeneration: achievements and future directions after 20 years of research. *Int J Dev Biol* **53**, 1317-27.

Sampaio, E., Maris, S. and Bach-y-Rita, P. (2001). Brain plasticity: 'visual' acuity of blind persons via the tongue. *Brain Res* **908**, 204-7.

Sánchez Alvarado, A. (2006). Planarian regeneration: its end is its beginning. *Cell* **124**, 241-5.

Sanchez Alvarado, A., Newmark, P. A., Robb, S. M. and Juste, R. (2002). The *Schmidtea mediterranea* database as a molecular resource for studying platyhelminthes, stem cells and regeneration. *Development* **129**, 5659-65.

Sarnat, H. B. and Netsky, M. G. (1985). The brain of the planarian as the ancestor of the human brain. *Canadian Journal of Neurological Sciences* **12**, 296-302.

Sheiman, I. M. and Tiras, K. L. (1996). Memory and morphogenesis in planaria and beetle. In *Russian contributions to invertebrate behavior*, eds. C. I. Abramson Z. P. Shuranova and Y. M. Burmistrov), pp. 43-76. Westport, CT: Praeger.

Sheiman, I. M., Zubina, E. V. and Kreshchenko, N. D. (2002). Regulation of the feeding behavior of the planarian Dugesia (Girardia) tigrina. *Journal of Evolutionary Biochemistry and Physiology* **38**, 414-418.

Singer, M. (1952). The influence of the nerve in regeneration of the amphibian extremity. *Q Rev Biol* **27**, 169-200.

Smalheiser, N. R., Manev, H. and Costa, E. (2001). RNAi and brain function: was McConnell on the right track? *Trends Neurosci* **24**, 216-8.

Sole, R. V., Munteanu, A., Rodriguez-Caso, C. and Macia, J. (2007). Synthetic protocell biology: from reproduction to computation. *Philos Trans R Soc Lond B Biol Sci* **362**, 1727-39.

Stephen, W. S. (1963). The influence of varying light intensities on speed of movement in *Planaria Lugubris*. *Worm Runner's Digest* **5**, 40-45.

Takeda, H., Nishimura, K. and Agata, K. (2009). Planarians maintain a constant ratio of different cell types during changes in body size by using the stem cell system. *Zoological science* **26**, 805-13.

Tanaka, E. M. and Reddien, P. W. (2011). The cellular basis for animal regeneration. *Developmental Cell* **21**, 172-85.

Teyke, T. (1989). Learning and Remembering the Environment in the Blind Cave Fish Anoptichthys-Jordani. *Journal of Comparative Physiology a-Sensory Neural and Behavioral Physiology* **164**, 655-662.

Travis, G. D. L. (1981). Replicating Replication - Aspects of the Social Construction of Learning in Planarian Worms. *Social Studies of Science* **11**, 11-32.

Tseng, A. and Levin, M. (2013). Cracking the bioelectric code: Probing endogenous ionic controls of pattern formation. *Communicative & Integrative Biology* **6**, 1-8.

Tully, T., Cambiazo, V. and Kruse, L. (1994). Memory through metamorphosis in normal and mutant Drosophila. *Journal of Neuroscience* **14**, 68-74.

Umesono, Y. and Agata, K. (2009). Evolution and regeneration of the planarian central nervous system. *Dev Growth Differ* **51**, 185-95.

Umesono, Y., Tasaki, J., Nishimura, K., Inoue, T. and Agata, K. (2011). Regeneration in an evolutionarily primitive brain--the planarian Dugesia japonica model. *The European journal of neuroscience* **34**, 863-9.

van Velthoven, C. T., Kavelaars, A., van Bel, F. and Heijnen, C. J. (2009). Regeneration of the ischemic brain by engineered stem cells: fuelling endogenous repair processes. *Brain Res Rev* **61**, 1-13.

Wagner, D. E., Wang, I. E. and Reddien, P. W. (2011). Clonogenic neoblasts are pluripotent adult stem cells that underlie planarian regeneration. *Science* **332**, 811-6.

Wells, P. H. (1967). Training flatworms in a Van Oye maze. In *Chemistry of Learning*, eds. W. C. Corning and S. C. Ratner), pp. 251-254. New York: Plenum.

Wisenden, B. D. and Millard, M. C. (2001). Aquatic flatworms use chemical cues from injured conspecifics to assess predation risk and to associate risk with novel cues. *Animal Behaviour* **62**, 761-766.

Wulzen, R. (1917). Some chemotropic and feeding reactions of Planaria maculata. *Biol Bull* **33**, 67-69.

Zovkic, I. B., Guzman-Karlsson, M. C. and Sweatt, J. D. (2013). Epigenetic regulation of memory formation and maintenance. *Learn Mem* **20**, 61-74.

FIGURE LEGENDS

Fig. 1. The Automated Training Apparatus (ATA).

(A) A picture of the 12 channel fully automated device we used. The device contained 4 blocks of 3 isolated chambers. Each chamber contained 1 worm in a petri dish, allowing the simultaneous tracking and training of 12 individual worms (Blackiston et al., 2010). All coordinate data are processed, allowing an objective and quantitative analysis of each animal's behavior during testing trials.

(B) The basic workflow loop of the device. Continuously and independently, cameras in each cell determine the position of each worm and record it. The device also has provisions for providing changes of light or electric shock in response to specific worm positions. Such negative reinforcement was not used in these experiments, but the ability to provide real-time feedback to each individual animal allows very sophisticated training and testing paradigms to be employed.

Fig. 2. Experimental protocol

Training phase: Groups of worms were placed in the ATA's chambers for 10 consecutive days. (A) The "familiarized" group was in Petri dishes with a rough textured bottom, while the "unfamiliarized" (control) group was placed in standard Petri dishes with smooth bottoms (B). (C&D) In the morning days 1, 4, 7, 10, the worms were fed in the ATA with 1-2 small drops of liver (white arrows). On the morning of the last day the worms were fed extensively by being given more liver than they could consume. Every day, the experiment arenas (dishes + electrodes) were cleaned and water was changed. During the cleaning procedure the familiarized worms were placed in a dish with a rough textured floor and the unfamiliarized worms were placed into standard Petri dishes, in the dark.

Resting phase: (E) After 10 familiarization days, the worms were kept in 12 multiwell plates in the dark until testing. The wells' water was changed every day. (F) Illustration of a worm before and after decapitation. To ensure that no brain tissue remained, the worms were decapitated at the point between the auricles and the anterior side of the pharynx (White arrow). Worms were fed in the 12 multiwell plates 4 days before retrieval test (G). Saving session: (H) regenerated worm were fed in the ATA chambers with a rough floor (the familiar environment), 4 days before retrieval test. (I) In the evening before the testing day, the worms were divided into two groups of 6 familiarized and 6 unfamiliarized worms and placed in separate wells of a 12-well plate.

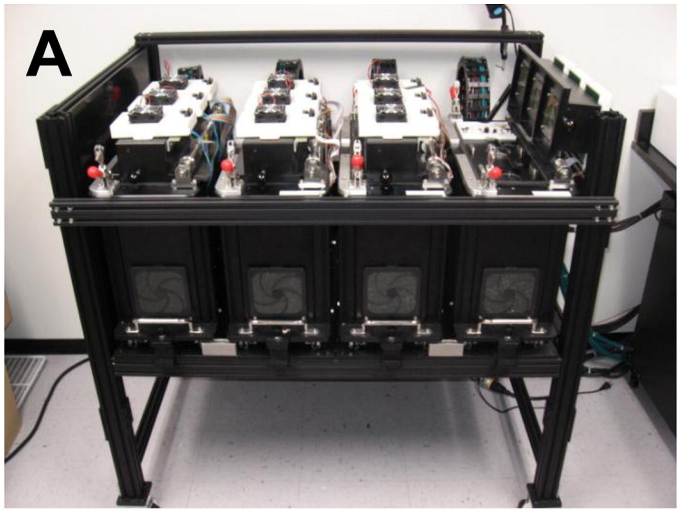
Testing phase: After the resting period, the retrieval test took place. To test recall, 6 familiarized worms and 6 unfamiliarized worms were placed individually in the ATA chambers with a rough floor (the familiar environment). (J&K) A small area of the dish was covered with liver (red arrow point on the liver stain) and (L) a strong blue light was illuminating, from above the quadrant with the liver stain (opened lid of the ATA with the light setting during the test). The device measured how long it took each animal to begin feeding. Panel (M) shows the worm as seen from below by the tracking camera, Red arrow indicates the worm's pharynx. (N) Enlargement of the rough textured bottom of the experimental environment with worm for comparison. (O) Enlargement of the testing dish floor with the small stain of liver (inside the dashed red circle). The black stain in the middle is made on the outer side of the dish by a black marker to label the area where liver is. This enabled to place the dish in the right position with the liver under the illuminated quadrant. (P) Typical exploration/foraging trail during the test. At the start (red arrow) the worms are mainly moving around the edge of the chamber, avoiding the illuminated quadrant (Blue area) containing the liver stain (dashed red circle). In some cases, as in this example, the worm will make more than one, short, enters to the illuminated quadrant with the liver, before making a sharp turn toward the liver stain and initiating feeding.

Fig. 3. Worms in a familiar environment display significantly shorter exploration phase before initiating feeding:

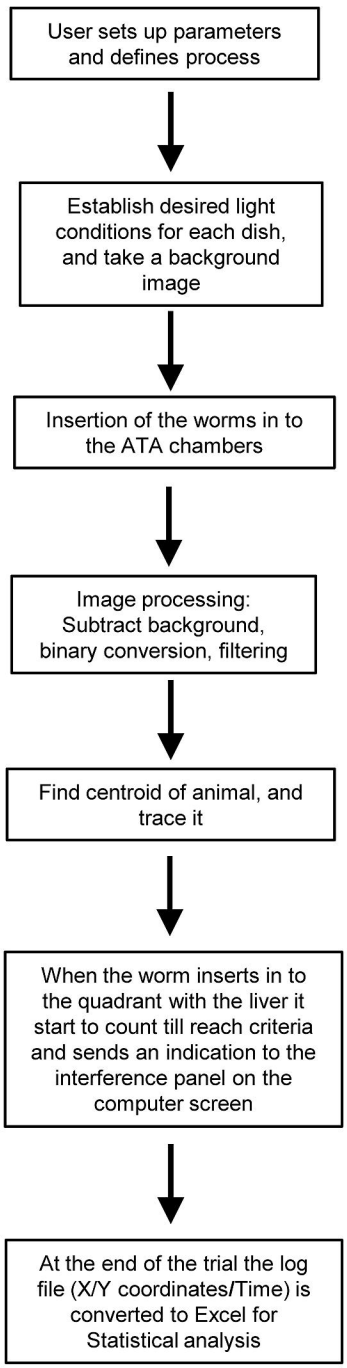
A. Percentage of worms to reach criterion in less than 8 minutes. (a) Intact-4-days: 60.4% of familiarized worms (n=225, red column) and 48% of the unfamiliarized worms (n=229, black column), which have been tested 4 days after training, reach criterion in less than 8 minutes (<8 minutes, p=0.005; one-tailed, Fisher's exact test). (b) Intact-14-days: 84.2% of familiarized worms (n=70, red column) and 67.1% of the unfamiliarized worms (n=70, black column), which have been tested 12-15 days after training, reach criterion in less than 8 minutes (<8 minutes, p=0.014; one-tailed, Fisher's exact test). (c) Saving paradigm: 79.5% of familiarized worms (n=106, red column) and 64.5% of the unfamiliarized worms (n=104, black column), which have been tested, 11-13 days after decapitating, with the Saving paradigm, reach criterion in less than 8 minutes (<8 minutes, p=0.013; one-tailed, Fisher's exact test). **B.** Median delay of feeding (time in minutes). The same groups as in A, including the category of Headless Fragments, Regular Protocol which are worms regenerated from tail fragments and have been tested, 10-14 days after decapitating, (Familiarized n=164, Unfamiliarized n=171).The right points are form the familiarized groups, (Trained), and the left points are from the Unfamiliarized, (Control) groups. Red line: Intact-4-days (Familiarized 6.641±0.47;

960 Unfamiliarized 8.341±0.48, P<0.001; one-tailed, U-test). Black line: Intact-14-days (Familiarized
961 5.012±0.49; Unfamiliarized 6.991±0.41, P<0.001; one-tailed, U- test). Green line: Headless
962 fragments, Regular Protocol (Familiarized 10.15±0.7; Unfamiliarized 10.325±0.69, No statistical
963 significance). Blue-line, Saving paradigm (Familiarized 7.166±0.58; Unfamiliarized 8.304±0.55,
964 P=0.027; one-tailed, U-test).
965 Error bars show SEM.
966 # Criterion was 3 consecutive minutes in the illuminated quadrant, containing the liver spot.
967
968
969 Fig. 4. Decapitation and regeneration
970 Illustration of worm regeneration sequence in our protocol conditions of 12 worms / 2ml
971 water in 18°C and constant darkness (not the same worm in each of the panels)**.** Worms were
972 decapitated at the point between the auricles and the anterior side of the pharynx (red arrows).
973

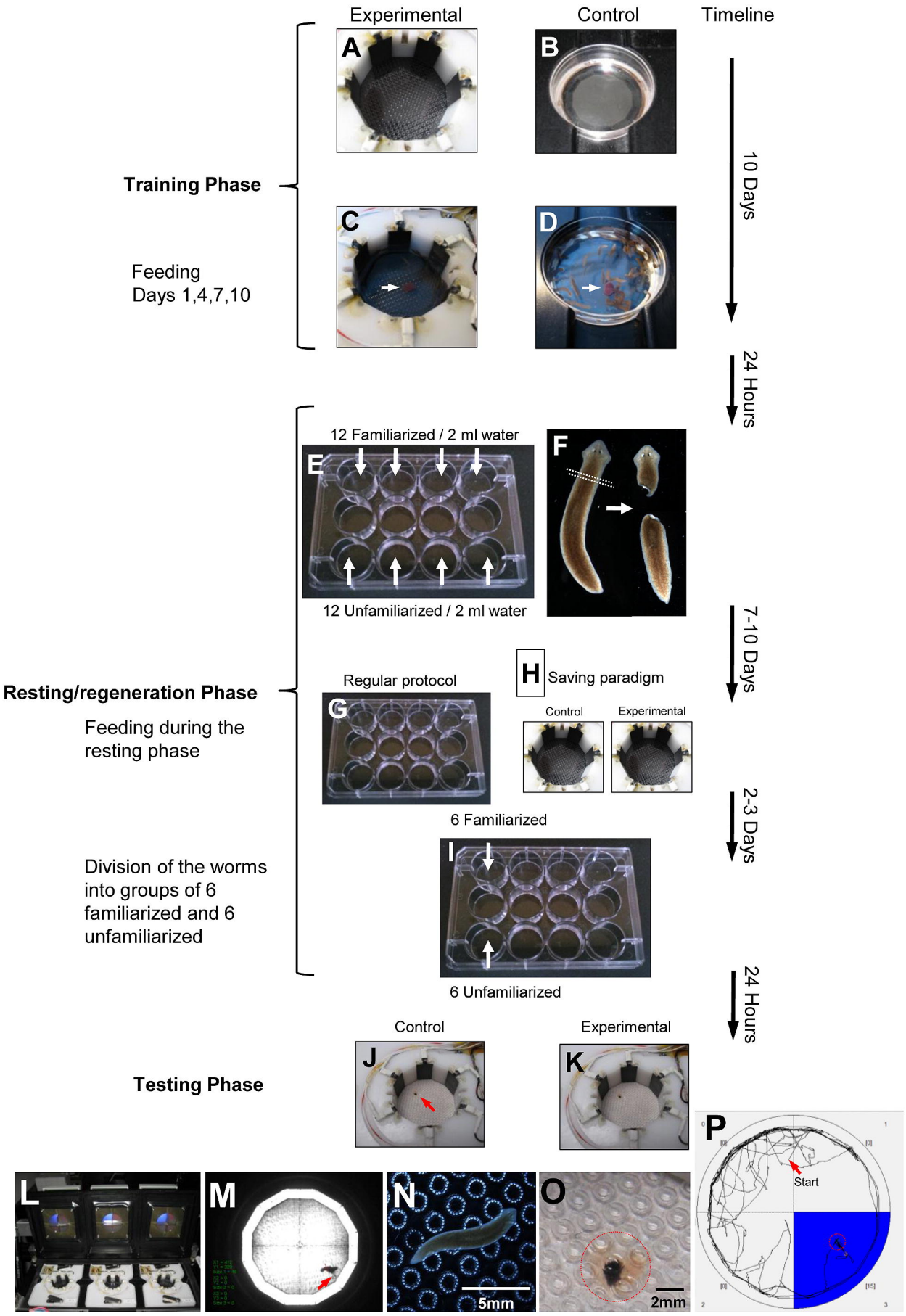
A



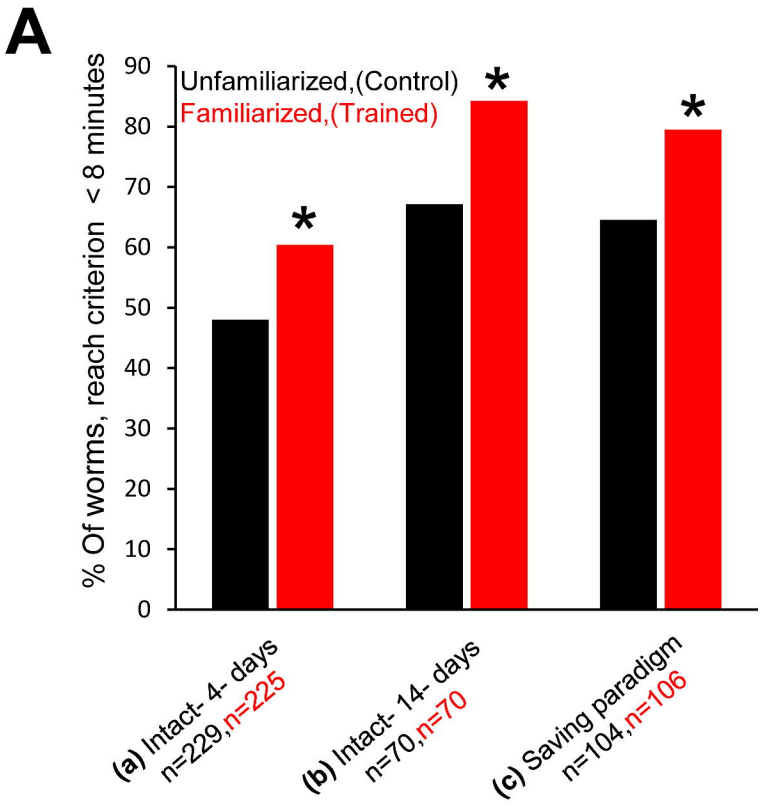
B

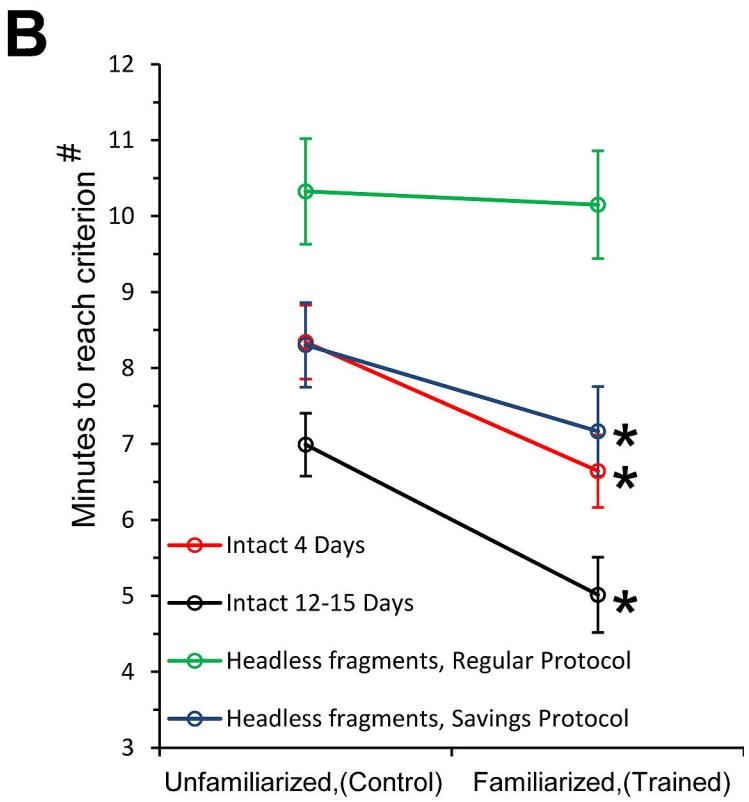


Shomrat and Levin, Fig.1

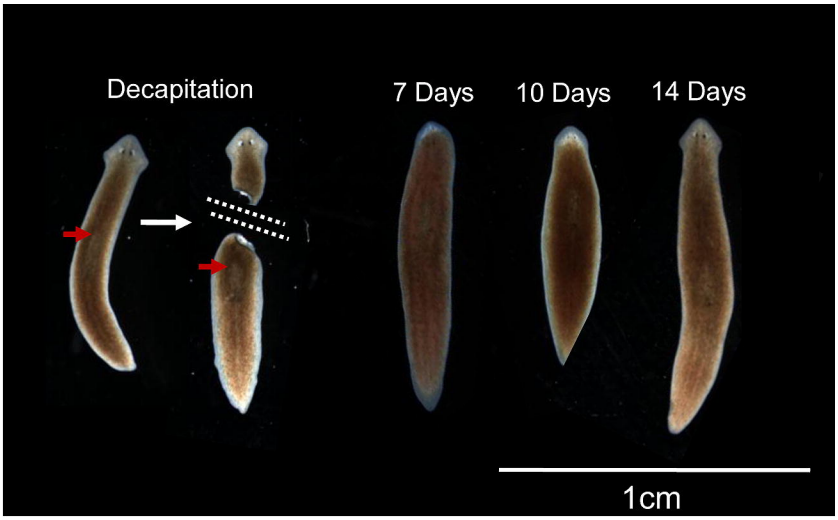


Shomrat and Levin, Fig.2





Shomrat and Levin, Fig.3



Shomrat and Levin, Fig.4

Table 1: Motility During the Testing Session

	Movement Rate Average Pixels/Second (± s.e.m)	
Protocol	Familiarized:	Unfamiliarized:
Intact: tested 4 days after end of training	8.775±0.2	8.818±0.2
Intact: tested 12-15 days after end of training	8.102±0.33	8.859±0.27
Headless fragments (saving paradigm): tested 11-13 days after decapitating	7.34±0.24	7.858±0.25

Table 2: Latency of Feeding During the Testing Session

	N	**Average Latency** Minutes to reach criteria (± s.e.m)		**Median Latency** Minutes to reach criteria (± s.e.m)		**Statistical Significance**	
Protocol	**Reach Criteria / Tested**	**F**	**C**	**F**	**C**	**U-test** (One tailed)	**Fisher's exact test (n<8min)** (One tailed)
Intact: tested 4 days after end of training	Familiarized : 225/233 Unfamiliarized: 229/238	8.817 ±0.47	10.339 ±0.48	6.641 ±0.47	8.341 ±0.48	P < 0.001	P=0.005
Intact: tested 12-15 days after end of training	Familiarized: 70/72 Unfamiliarized: 70/72	5.932 ±0.49	7.326 ±0.41	5.012 ±0.49	6.991 ±0.41	P < 0.001	P=0.014
*** Regular Protocol Headless fragments** tested 10-14 days after decapitating	Familiarized: 171/201 Unfamiliarized: 164/199	12.93 4±0.7	12.603 ±0.69	10.15 ±0.7	10.325 ±0.69	No statistical significance	No statistical significance
****Savings Protocol Headless fragments** tested 11-13 days after decapitating	Familiarized: 106/117 Unfamiliarized: 104/115	8.532 ±0.58	9.545 ±0.55	7.166 ±0.58	8.304± 0.55	P = 0.027	P=0.013

Legend: F = familiarized; C = controls (unfamiliarized)

*Regular Protocol: The feeding session before the test was taken place in the worm multi plate wells (Fig. 2G)

**Saving Protocol: The feeding session before the test was taken place in the in the familiarization arena (ATA chamber with the electrode insert and the rough floor (fig. 2H).