Analysis of behavior in the planarian model

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Abstract

Planaria are a powerful model species for the study of drug effects and addiction on neural and cognitive function due to their tractability to cell-biological, pharmacological, and molecular-genetic techniques. In order to fully capitalize on the many advantages of this system, it is necessary to be able to analyze behavior and learning in a quantitative manner in worms that have been treated with drug or RNAi reagents. Here, we give a brief overview of behavior and learning analysis in planaria. Classical data demonstrate that planaria can learn, and exhibit many complex behaviors. We present a view of the next generation of work in this field; the development of automated, high-throughput platforms for analysis of planarian behavior will greatly enhance the integration of molecular genetics, nervous system structure, and behavior in the same animal, extending our understanding of drug effects on cognition and opening the way for novel screening approaches to identify new compounds with important nootropic effects.

Introduction

This chapter presents a brief overview of research on learning in planarians, in order to illustrate the types of studies that can be performed in this model species to understand the effects of drugs on behavior and memory. We will provide some background on the history of planarian research and describe examples of planarian learning in the areas of habituation, classical conditioning, and instrumental/operant conditioning. We would like to say at the outset that we believe that when proper procedures are followed, the data conclusively show that planarians can learn. Moreover, we believe that the study of planarian learning should be taken up once again because of the many advantages this animal has for the investigation of the biochemistry of learning and memory.

Planaria represent a critical breakthrough in the evolution of the animal body plan. It is the first organism to have both bilateral symmetry and encephalization, making it capable of detecting environmental stimuli quicker and more efficiently than the lower metazoans, and therefore able to move in a directed fashion. They have developed sensory capabilities for the detection of light [1, 2], chemical gradients [3, 4], vibration [5], electric fields [6], magnetic fields [7, 8], and weak γ radiation [9]. These reception mechanisms are integrated by the worm's nervous system into a rich and complex set of behaviors as they navigate their environment in the search for prey, mates, etc., and represent a fertile background of cognitive processes that may be modulated by drug exposure or withdrawal.

A key component of making use of this system for the study of drug effects and addiction is the ability to perform rigorous analyses of behavior and to establish paradigms in which true memory can be demonstrated. Thus, it is our goal to illustrate the types of analyses that have been done in planaria, and to sketch the outline of the next phase of this field of research, which centers around automated, quantitative characterization of behavior (and learning in particular). As the simplest animal with a bilaterally-symmetrical central nervous system comprised of neurons similar to our own [10], planarians offer an opportunity to study the actions of chemicals in such neurons while working with an easily maintained organism that is highly tractable to pharmacological, surgical, and molecular genetic manipulations [11-14].

These flatworms also allow a researcher to investigate aspects of memory that are impossible to examine in other organisms, due to the planarians' robust ability to regenerate neural structures (see chapter by Oviedo and Levin in this volume). Many human neurotransmitters were first discovered in extracts from planarians [15, 16], and indeed it is clear that neurotransmitters have important functions in determining the structure of neural and non-neural components during planarian regeneration [17]. Over the last decade planarians have been used to model addiction and withdrawal for various psychoactive compounds such as cocaine [18, 19], though the effects such substances have on planarian learning and memory have generally been neglected.

Over the years there have appeared a number of excellent reviews on what has been called the "Planarian Controversy" and the interested reader is

urged to consult these for the type of in-depth analysis that is not possible here [20-22]. Of special interest is a relatively recent review of Russian work [23]. There have also been a number of symposia in which a major focus was planarian learning [24, 25]. For those interested in the pre-1940 planarian research consult [26].

A Brief History of Learning Research in Planaria

One of the more exciting yet often overlooked chapters in the history of the experimental analysis of behavior is the work on learning and memory transfer in planarians [27]. This work captured the imagination of the public during the 1960s because of the possibility of transferring the knowledge of a professor to a student by nothing more than swallowing a pill or for the more macabre, by cannibalism. The scientific literature of the mid 1950s through the early 1970s contained no less than 85 peer-reviewed papers designed to answer the question of whether planarians really learned and whether such learning could be transferred from one animal to another.

Controversies arose among laboratories that failed to replicate some findings, others which suggested alternative explanations and those which firmly held the belief that planarians learn. The interest in planarians by the general public was so great that the "*Worm Runner's Digest*" was created to aid amateur scientists who wanted to conduct their own studies. The Digest contained cartoons, tips, and a few scientific papers. For those interested in more scholarly work on planarians the reader simply turned an issue upside down and found the

"*Journal of Biological Psychology*." The Digest began publication in 1959 and the Journal was added to it in 1967. Both were published together until 1979.

The scientific study of learning in planarians was approached from two interrelated angles. The first was the comparative perspective in which the learning ability of planarians was compared to that of other animals in the expectation of illuminating the similarities and difference in learning across phyla [26]. Unfortunately the comparative analysis of planarian learning was not taken far enough and consisted mostly of a few isolated examples of punishment and maze learning. This lack of attention to training variables and conditioning phenomena would have serious consequences when planarians were subsequently used as a conditioning model.

In addition to the comparative aspect, there was interest in the use of planarians for what they can tell us about the biochemistry of learning and memory. This perspective—which led to what is now known as the simple-system strategy—attempts to uncover the neural circuits associated with a particular behavior [28]. It is ironic that contemporary discussions of the simple systems approach focus on various species of mollusk and insect [29] while neglecting work with planarians. As pointed out by McConnell and Shelby [22] the first experiments on memory transfer were conducted with planarians. However, there is no mention of these experiments (or of McConnell) in contemporary encyclopedic treatments as represented by either the *Encyclopedia of Learning and Memory* [30] or *Comparative Psychology: A Handbook* [31].

The rationale behind the use of planarians as suitable material for simple system research was the work of Donald Hebb [32] and others who argued that learning produced physiological changes at the synapse. In 1953 Richard Thompson suggested to his fellow University of Texas graduate student – James McConnell – that since planarians are the first animal on the phylogenetic tree to possess the type of nervous system required by Hebb for learning, why not use planarians to test Hebb's theory [33]? The first study describing learning in planarians was published in 1955 using a classical conditioning paradigm in which light onset was paired with an electric shock [34]. The results suggested learning but unfortunately, because there was little comparative data, did not contain enough controls to rule out alternative explanations. McConnell followed this study with others that showed that classical conditioning can be transferred either by regeneration or by cannibalism [35, 36]. Following publication of these studies, the "Planarian Controversy" began. The details of experiments that have been done in this field are crucial for those who seek to investigate the effects of drugs on memory and behavior in this species, because they hold many lessons that will enable the field to move forward and avoid the pitfalls that confounded some of the past attempts.

Learning

Learning has generally been classified into nonassociative and associative learning. Nonassociative learning is considered the more fundamental form, yet it shares many features with associative learning. The prototypical example of

nonassociative learning is habituation. Associative learning is illustrated by such phenomena as classical conditioning, instrumental conditioning and operant conditioning. Information on how to conduct invertebrate learning experiments can be found in [37].

Habituation

Habituation is typically a decrease in some dependent variable (i.e., amplitude, probability) to a monotonously repeated stimulus. Stimuli that no longer transmit significant information tend to be ignored. Before a decrease in responsiveness can be considered a case of habituation several factors must be ruled out including effector fatigue, sensory adaptation, general experience with the training situation, temporal conditioning, and the presence of chemical signals. Effector fatigue and sensory adaptation can often be ruled out by the addition of a distracter stimulus presented when the habituation criteria is met. If the response reappears to the original training stimulus following exposure to the distracter, an interpretation of the response decrease in terms of an inability to sense the training stimulus (sensory adaptation) or an inability to make a response even though the stimulus can still be sensed (effector fatigue) is unwarranted.

The number of planarian experiments on habituation is surprisingly quite small. This is unfortunate for a number of reasons. First, habituation shares many phenomena with associative learning. These include spontaneous recovery, stimulus intensity effects, and generalization [38]. Second, habituation is a fine

comparative tool through which species can easily be compared. Third, habitation can be used as a control for planarian transfer of training experiments that have often been criticized as representing the transfer of general excitatory tendencies rather than a specific behavior. If both excitatory and inhibitory responses can be transferred, the case for physiological and biochemical correlates of the transfer phenomena is greatly enhanced [39, 40].

Although not the primary object of investigation, habituation to light is often assessed prior to using light as a conditioned stimulus in classical conditioning experiments. Several studies have shown that planarians will decrease their responses to light following repeated presentations [41]. Whether such decreased responsiveness is the result of habituation or some other process is not known, because habituation controls, understandably, were not in place. Using habitat rotation and light Walter [42] observed a decrease in responsiveness to repeated stimulation but again controls were not in place.

Perhaps the best studies of habituation in planarians were performed by Westerman [39, 40]. Westerman presented planarians with 3 seconds of light over the course of 25 trials per day for 20 days. The dependent variable was contraction and animals had to meet a criterion of 50 trials with no contraction to light. Animals were not only able to reach criteria but such habituation survived regeneration and could be retained for 7 weeks.

Classical conditioning

To function successfully in a changing environment, planarians must not only learn new behaviors but also call on reflexive responses in new contexts. Classical conditioning is an example of associative learning in which behavior is altered by the pairing of stimuli, one of which is effective in eliciting a biologically important reflex. A common feature of classical conditioning is that the conditioned stimulus (CS) and unconditioned stimulus (US) are presented independently of the organism's actions.

Before it can be concluded that animals have learned to associate a CS and US pseudoconditioning, or "false" learning not based on the explicit pairing of the CS and US, must be ruled out. The easiest way to do this is to use a between group design where a group of animals receiving paired CS-US presentations is compared to another group receiving unpaired CS/US presentations. A statistically significant difference between paired and unpaired groups is evidence for learning. Generally, the intertrial interval for the unpaired group should be half the interval used in the paired group. In this way the time between the CS presentations (the intertrial interval) is approximately equal between experimental and control groups. If the same intertrial interval is used, the time between CS presentations in the unpaired group will be twice as long as in the paired group and any difference between the groups may not be attributed to learning.

In addition to using a group design, a good protocol calls for a within subject design in which two CSs are used – one of which is paired with the US. Evidence for learning is provided by a statistically significant difference between

responses associated with the CS paired with the US and a second CS that is not. Data from both group and within subject designs together strongly supports conditioning.

The vast majority of planarian learning experiments was performed with classical conditioning paradigms using light or vibration as a conditioned stimulus and shock as the unconditioned stimulus. The original Thompson and McConnell [34] experiment served as the model with the technique being refined over the years [21]. Unfortunately an unpaired group receiving the same number of CSs and USs was not run in the original demonstration and this left the investigation open to criticism [43].

Since 1955, a number of studies have employed suitable control groups and have demonstrated classical conditioning. These include backward, delayed, simultaneous conditioning, and various types of pseudoconditioning controls [44, 45]. Using light and vibration as conditioned stimuli, discrimination learning has also been demonstrated in which the planarian is trained to respond to a CS paired with shock and one that is not paired [44, 46-48]. Moreover, conditioning has been demonstrated both in acquisition and in extinction [49, 50]. Of 71 experiments investigating various aspects of classical conditioning in planarians 54 (76%) have produced positive results [21].

Instrumental and operant conditioning

In instrumental and operant conditioning, a contingency is arranged between a motivationally significant stimulus and a specific behavior. The

planarian learns that consequences occur as a result of its actions. Instrumental and operant conditioning are generally considered more complex than classical conditioning although they share many properties.

For instrumental and operant conditioning, controls must be implemented to ensure that the planarian is indeed learning the consequences of its actions. The problem of control is arguably not as great as in classical conditioning because in principle the instrumental and operant response should be a behavior that is not in the repertoire of control animals. For example, if several planarians having been taught to negotiate a maze are placed in a general population containing untrained planarians, it should be an easy matter to determine trained from untrained. In addition, many maze experiments have a built in control by requiring the animal to make a discrimination.

The vast majority of instrumental/operant studies using planarians have employed various types of mazes often using escape from shock or return to the home container as a reward. Several maze configurations have been used including standard T and Y forms. Hexagonal and multi-unit mazes have also been used [21]. Maze performance is often variable with early success giving way to instability and eventually a refusal to run the maze [51]. The most successful maze technique has been developed by Best [52] in which planarians are faced with three arms, any of which can be drained of water. Animals were able to form a light-dark discrimination by entering the correct arm and being reinforced with water. Even here, however, performance can become unstable. Positive results

have been obtained with a hexagonal maze that reduced the intertrial interval and minimized handling [53, 54].

Operant situations have also been used with better success than that reported for mazes. Lee [55] developed a paradigm in which a planarian confined to a cylinder was trained to interrupt a photobeam that briefly terminated a bright light. This work has been replicated [52, 56].

One of the most successful instrumental training techniques for planarians is known as the "Van Oye Maze" in honor of its developer although in contemporary usage it does not resemble a maze [57]. In this situation food is suspended from a glass rod into a beaker containing several planarians. The rod can be placed to any desired depth with the worms crossing the surface of the water and down the rod to feed. As training progresses the rod is moved to a deeper depth. During the testing phase a clean rod is placed at the final training depth and the results show that significantly more trained planarians are at the end of the rod than untrained planarians. These results have been replicated several times and constitute some of the strongest evidence of the ability of planarians to learn by consequences [20, 57, 58].

Past Drug Experiments

Planarians were one of the first animals examined in biology and studies have been done with them on the effects of drug treatment and withdrawal [59]. Such studies include looking at the effects of caffeine on respiration rate [60] and the development of a dependence on morphine [61]. The most recent drug abuse-

related studies have focused on commonly abused drugs such as cocaine and opioids. To evaluate the effects of drug treatments innate behaviors are typically observed. Evidence for drug impacts has included stereotypically disrupted movements such as “corkscrew” and “head bop” and “tail twist” [19], a loss of the negative phototaxis planaria normally display [62] and hypokinesia [63]. Rarely have these studies considered the impacts of abuse-prone drugs on learning and memory, as did one methamphetamine study [64]. Displays of learning and memory are complex, requiring the correct coordination of central nervous system and peripheral nervous system activities. Therefore, discovering specific disruptions in these processes has the potential to shed considerable new light on the actions drugs have on the nervous system.

Where are we now?

As Corning and Kelly lamented in 1973, interest in the use of planarians for research on learning and memory has declined and such work today is rare. Planarians were the first invertebrate to be used in simple system research and to stimulate interest in the biochemistry of learning and memory. These animals are easy to maintain and easy to manipulate for learning and biochemical experiments. Both cut and uncut worms can be readily exposed to drugs and the increasingly popular use of RNAi in planarian work provides an opportunity to interrupt a particular biochemical pathway as well [65, 66]. Modern molecular studies are beginning to address the cell biology and genetics of neural networks in planaria [67].

Unfortunately, in our view, much time was wasted during the “Golden Age of Planarian Research” on experiments with problematic design investigating whether these animals could learn and whether learning or some non-associative effect was transferred either by regeneration or by cannibalism. Little effort was made toward advancing past basic demonstrations of conditioning phenomena. For example, questions regarding “cognitive” effects such as latent inhibition, US pre-exposure, second-order conditioning, blocking, overshadowing, and within compound associations as well as the effects chemical compounds or RNA-mediated interruptions of endogenous pathways could have on such learning all have remained uninvestigated in planarians.

On the positive side there is little doubt that planarians can learn. Progress has also been made in identifying critical factors that influence such learning. These factors include the presence of slime, housing, diurnal cycles, medium associated with housing, training, and testing, species differences, apparatus, and such training variables as stimulus intensity, intertrial interval, and number of stimulus pairings [21]. Biological research on stem cells and regeneration has created fresh interest in planarians as a model organism, leading to increased knowledge of planarian genetics and neuroanatomy. The species *Schmidtea mediterranea* has a genomic database online at http://smedgd.neuro.utah.edu/ , which should aid researchers working with this species [68, 69]. This type of newly available knowledge of planarian genomics suggests experiments that may combine studies of drug treatments with RNAi targeted at specific neural receptor proteins.

The future of studies in planarian behavior

We believe the time is right to re-invigorate planarian research on learning and memory utilizing the most powerful tools of genetics, cell biology, and biochemistry. It is crucial however, to be able to perform integrative studies that can address the whole path leading from genetics to nervous system structure and ultimately to behavior, and to assess results without subjectivity or experimenter variations. Thus, what is required now is the development of computerized, automated, high-throughput devices that can be used to efficiently and quantitatively characterize behavior of animals that have been manipulated pharmacologically or genetically (e.g., knockouts of specific neurotransmitter receptors). Moreover, functional analyses of the effects of drugs on memory and learning will require a robust assay that can produce a "strong" memory that persists through the course of a specific experiment, and that are easy to measure. This assay also must be reproducible and efficient enough to accommodate large enough sample sizes to ensure high statistical significance of results despite variability among individual animals.

Most available systems for automated behavior analysis focus on rodents [70-76], but a few systems have been developed for small species that are amenable to behavioral screening and large population studies such as crustaceans [41, 55, 77-80], zebrafish [41, 55, 77-80] and *C. elegans* [81]. Unfortunately, none of the existing (commercially-available) solutions meet the necessary criteria; most lacking is the ability to modulate the environment of the animal in

real-time: that is, not only to analyze behavior but to provide feedback (positive and negative reinforcement) individually to each animal so that automated training and testing of recall can occur.

The ideal behavior analysis device would have the following properties. (1) Convenient programming of essential control conditions such as yoked controls, in which control animals receive rewards and punishments based on the behavior of other animals being trained. This controls for the effects of the rewards/punishments *per se*, when these are not linked to the actual behavior of the animals. (2) Full automation to exclude experimenter effects and subjective scoring, enable rich data acquisition, and ensure reproducibility. (3) Programming of individualized combinations of environmental cues and feedback (reward/punishment) in real time to accommodate different rates of memory and learning among animals within a sample group. (4) Flexibility in designing and programming training and testing paradigms, allowing the whole parameter space to be explored for the important variables in a learning trial. (5) Accommodation of large sample sizes for statistical and screening purposes (high-throughput), allowing many different treatments to be efficiently analyzed. (6) Recording of all primary data, for subsequent review, analysis, and transfer to other laboratories. This is essential to allow other investigators to mine the data in different ways, and could be used over the web as a training opportunity for students to interact with such a device remotely. (7) Efficient modular design to enable deployment in other laboratories; this is very important since a considerable degree of discord during the early days of planarian research was

due to the difficulty of reproducing training environments precisely in different labs. A fully-specified automated system that anyone could buy would enable investigators to replicate experimental designs perfectly, and also avoid experimenter effects (providing completely objective scoring). Of course, such devices would also be useful for other powerful model species such as zebrafish.

Use of computer controlled training systems would contribute great clarity to this field, by eliminating potential variation between experimenters as well as allowing many animals to be run in parallel. It would also reduce artifacts due to handling, and provide for much greater statistical power. A solid foundation, in terms of modern training techniques and proper controls can help push through the controversies of the past and allow this simple animal to shed light on the fundamental basis for learning and memory. A proposed design, which is currently guiding the efforts in our group, is shown in Fig. 1. A rack-mounted set of drawers contains pull-out trays of 5x5 cells. Each such cell is a Petri dish containing a photoelement (for tracking worm behavior), light-emitting diodes (to establish light and dark quadrants as stimuli), and electrodes (to produce shock for negative reinforcement). This is in effect a set of Skinner chambers, where each animal can be independently trained on an almost limitless number of tasks (e.g., “stay in center”, “follow the lit up quadrant”, “keep moving”, “move when a light flashes”, etc.). A centralized processor controls the environment in each dish based on the behavior of the given worm.

The algorithm for such a system is schematized in Fig. 2A-F. Once the user sets up the training parameters for all of the dishes, there is a repeated

cycle (providing a consistent environment for some number of hours or days). This cycle consists of image processing to detect the position of each worm (Fig. 2C), decisions (determined by the user-specified learning task) as to whether each worm is to be punished or rewarded, and a corresponding change in the light and shock environment of the dish. All of the data (coordinates of the worms' movements plus real-time movies of their activities) are recorded to disk, and may be made instantly available via the Internet. In simpler trials (with no rewards or punishments), the system can simply gather quantitative data on the baseline behavior of control or drug-treated worms and their response to light, shock, and vibration.

Such a paradigm would be a powerful addition to studies on drug effects (Fig. 2G). For example, drugs can be added without learning (to examine effects on behavior), during training (to examine effects on the magnitude and time-course of learning), or during testing (to examine effects on length and quality of recall). Naturally, drugs can be withdrawn from addicted animals to be tested in a similar fashion.

Seeking to develop a prototype for this next step in the field, we produced a small system (suitable for 12 planaria at a time) and illustrated its use in the automated training and testing of control and drug-treated worms [82]. The reduced handling, objective scoring of behavior, and quantitative real-time analysis resulted in a very powerful system that can easily be applied to the study of drug effects; an example application is shown in Fig. 3. The statistical strength of numbers and the reduced tedium (compared to manual training)

opens the way for analyses to be made on the behavioral effects from large numbers of psychoactive chemicals and genetic constructs on behavior, learning and memory. Combinations of chemicals can be observed as can the effectiveness of many different treatments on animals undergoing withdrawal from addictive drugs. With proper controls and careful experimental design, automation that eliminates the possibility of experimenter biases not only offers an opportunity to more clearly illuminate the controversies of the past, but offers a solid foundation for screening potential psychoactive drugs and treatments *in vivo* in a simple lower organism. Moreover, if expanded to tens of thousands of cells in a much larger device, this system offers the possibility of screening drug and RNAi libraries for nootropic compounds (e.g., increases of learning rate), something that is not possible using today's screening platforms that are based on cells in culture or yeast.

As Sarnat and Netsky (1985) noted, the planarian brain is rather unique. The ratio of the brain to body weight is similar to that of the rat. Planarian neurons contain serotonin, acetylcholine, norepinephrine and epinephrine, and there are many morphological and electrophysiological features analogous to the vertebrate brain. As the obstacles that made planarian behavioral research problematic in past decades are overcome, and the work is integrated with the tremendously powerful tools of modern molecular genetics, a new era of planarian research is beginning. Planarians are very simple organisms that utilize an organized central nervous system and human-like neurochemicals; they

clearly have much yet to tell us about the biochemistry of learning and memory, and about the ways in which drug compounds impact cognition.

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References

1. Brown HM, Ito H, Ogden TE. Spectral sensitivity of the planarian ocellus. *Journal of General Physiology.* 1968;51(2):255-260.

2. Brown F, Park Y. A persistent monthly variation in responses of planarians to light and its annual modulation. *International Journal of Chronobiology.* 1975;3(1):57-62.

3. Miyamoto S, Shimozawa A. Chemotaxis in the freshwater planarian Dugesia-japonica-japonica. *Zoological Science (Tokyo).* 1985;2(3):389-396.

4. Mason PR. Chemo-klino-kinesis in planarian food location. *Animal Behaviour.* 1975;23(2):460-469.

5. Fulgheri D, Messeri P. The use of 2 different reinforcements in light darkness discrimination in planaria. *Bollettino - Societa Italiana Biologia Sperimentale.* 1973;49(20):1141-1145.

6. Brown HM, Ogden TE. The electrical response of the planarian ocellus. *Journal of General Physiology.* 1968;51(2):237-253.

7. Brown FA. Effects and after-effects on planarians of reversals of the horizontal magnetic vector. *Nature.* 1966;209(22):533-535.

8. Brown F, Chow C. Differentiation between clockwise and counterclockwise magnetic rotation by the planarian dugesia-dorotocephala. *Physiological Zoology.* 1975;48(2):168-176.

9. Brown F, Park Y. Seasonal variations in sign and strength of gamma-taxis in planarians. *Nature.* 1964;202:469-471.

10. Sarnat HB, and Netsky, M.G. The brain of the planarian as the ancestor of the human brain. *Canadian Journal of Neurological Sciences.* 1985.

11. Eisenstein EM. Selecting a model system for neurobiological studies of learning and memory. *Behavioural Brain Research.* Jan 1997;82(2):121-132.

12. Sanchez Alvarado A, Reddien PW, Bermange A, Oviedo N, Robb SM. Functional Studies of Regeneration in the Planarian Schmidtea mediterranea. *Developmental Biology.* 2003;259:525.

13. Reddien PW, Sanchez Alvarado A. Fundamentals of planarian regeneration. *Annu Rev Cell Dev Biol.* 2004;20:725-757.

14. Newmark PA. Opening a new can of worms: a large-scale RNAi screen in planarians. *Dev Cell.* May 2005;8(5):623-624.

15. Ribeiro P, El-Shehabi F, Patocka N. Classical transmitters and their receptors in flatworms. *Parasitology.* 2005;131 Suppl:S19-40.

16. Welsh JH, Williams LD. Monoamine-containing neurons in planaria. *Journal of Comparative Neurology.* 1970;138(1):103-115.

17. Villar D, Schaeffer DJ. Morphogenetic action of neurotransmitters on regenerating planarians--a review. *Biomedical & Environmental Sciences.* 1993;6(4):327-347.

18. Raffa RB, Valdez JM. Cocaine withdrawal in Planaria. *European Journal of Pharmacology.* 2001;430(1):143-145.
19. Raffa RB, Desai P. Description and quantification of cocaine withdrawal signs in Planaria. *Brain Res.* Jan 25 2005;1032(1-2):200-202.
20. Corning WC, Riccio D. The planarian controversy. In: Byrne W, ed. *Molecular approaches to learning and memory*. New York: Academic Press; 1970:107-150.
21. Corning WC, Kelly S. Platyhelminthes: the turbellarians. In: Corning WC, Dyal JA, Willows OD, eds. *Invertebrate Learning.* Vol I. New York: Plenum Press; 1973.
22. McConnell JV, Shelby JM. Memory transfer experiments in invertebrates. In: Ungar G, ed. *Molecular mechanisms in memory and learning*. New York: Plenum Press; 1970:71-101.
23. Sheiman IM, Tiras KL. Memory and morphogenesis in planaria and beetle. In: C. I. Abramson ZPS, & Y . M. Burmistrov (Eds.), ed. *Russian contributions to invertebrate behavior*. Westport, CT: Praeger; 1996:43-76.
24. Byrne WL. *Molecular approaches to learning and memory*. New York,: Academic Press; 1970.
25. Corning WC, Ratner SC, American Institute of Biological Sciences. *Chemistry of learning; invertebrate research*. New York,: Plenum Press; 1967.
26. Warden CJ, Jenkins TN, Warner LH. Comparative psychology. Vol 2. New York: Ronald Press; 1940.
27. Rilling M. The mystery of the vanished citations: James McConnell's forgotten 1960s quest for planarian learning, a biochemical engram, and celebrity. *American Psychologist.* Jun 1996;51(6):589-598.
28. Carew TJ, and Sahley, C.L. Invertebrate learning and memory: from behavior to molecules. *Annual review of neuroscience.* 1986;9:435-487.
29. Kandel ER, Schwartz JH, Jessell TM. *Principles of neural science*. 4th ed. New York: McGraw-Hill Health Professions Division; 2000.
30. Byrne JH. *Encyclopedia of learning and memory*. Farmington Hills: Thomson/Gale; 2003.
31. Greenberg G, Haraway MM. *Comparative psychology : a handbook.* New York: Garland Pub.; 1998.
32. Hebb DO. *A textbook of psychology*. Philadelphia,: Saunders; 1958.
33. McConnell JV. The modern search for the engram. In: McConnell JV, ed. *A manual of psychological experimentation on planarians*. 2nd ed. Ann Arbour: Journal of Biological Psychology; 1967.
34. Thompson R, McConnell JV. Classical conditioning in the planarian *Dugesia dorotocephala*. *Journal of comparative and physiological psychology.* 1955;48:65-68.
35. McConnell JV. Memory transfer through cannibalism in planarians. *Journal of Neuropsychiatry.* 1962;3:42-48.
36. McConnell JV, Jacobson AL, Kimble DP. The effects of regeneration upon retention of a conditioned response in the planarian. *Journal of Comparative Physiology and Psychology.* 1959;52:1-5.
37. Abramson CI. *A primer of invertebrate learning : the behavioral perspective*. 1st ed. Washington, DC: American Psychological Association; 1994.

38. Thompson RF, Spencer WA. Habituation - a model phenomenon for study of neuronal substrates of behavior. *Psychological Review.* 1966;73(1):16-&.
39. Westerman RA. Somatic Inheritance of Habituation of Responses to Light in Planarians. *Science.* May 10 1963;140(3567):676-677.
40. Westerman RA. A study of habituation of responses to light in the planarian *Dugesia dorotocephala*. *Worm Runner's Digest.* 1963;5:6-11.
41. Corning WC, Freed S. Planarian behaviour and biochemistry. *Nature.* 1968;219(160):1227-1229.
42. Walter HE. Reactions of planarians to light. *Journal of Experimental Zoology.* 1908;5:35-163.
43. Jensen DD. Paramecia, planaria, and pseudolearning. *Animal Behaviour.* 1965;Supplement 1:9-20.
44. Jacobson A, Horowitz S, Fried C. Classical conditioning, pseudoconditioning, or sensitization in the planarian. *Journal of Comparative & Physiological Psychology.* 1967;64(1):73-79.
45. Vattano FJ, Hullett JH. Learning in planarians as a function of interstimulus interval. *Psychonomic Science.* 1964;1:331-332.
46. Block RA, McConnell JV. Classically conditioned discrimination in the planarian, Dugesia dorotocephala. *Nature.* 1967;215(109):1465-1466.
47. Fantl S, Nevin JA. Classical discrimination in planarians. *Worm Runner's Digest.* 1965;7:32-34.
48. Griffard CD, Peirce JT. Conditioned discrimination in the planarian. *Science.* Jun 19 1964;144:1472-1473.
49. Yaremko RM, Kimmel HD. Two procedures for studying partial reinforcement effects in classical conditioning of the planarian. *Anim Behav.* Feb 1969;17(1):40-42.
50. Crawford T, Livingston P, King F. Distribution of practice in the classical conditioning of planarians. *Psychonomic Science.* 1966;4:29-30.
51. Corning WC. Evidence of right-left discrimination in planarians. *The Journal of Psychology.* 1964;58:131-139.
52. Best JB. Behaviour of planaria in instrumental learning paradigms. *Animal Behaviour Supplement.* 1965;13 (Suppl. 1):69-75.
53. Roe K. In search of the locus of learning in planarians. *Worm Runner's Digest.* 1963;5:16-24.
54. Humpheries B, McConnell JV. Factors affecting maze learning in planarians. *Worm Runner's Digest.* 1964;6:52-59.
55. Lee RM. Conditioning of a free operant response in planaria. *Science.* 1963;139:1048-1049.
56. Crawford FT, Skeen LC. Operant responding in planarian - a replication study. *Psychological Reports.* 1967;20(3P2):1023-&.
57. Wells PH. Training flatworms in a Van Oye maze. In: Ratner WCCSC, ed. *Chemistry of Learning*. New York: Plenum; 1967:251-254.
58. Wells PH, Jennings LB, Davis M. Conditioning planarian worms in a Van Oye type maze. *American Zoologist.* 1966;6(3):295-&.
59. Algeri S, Carolei A, Ferretti P, Gallone C, Palladini G, Venturini G. Effects of dopaminergic agents on monoamine levels and motor behaviour in planaria.

Comparative Biochemistry & Physiology - C: Comparative Pharmacology & Toxicology. 1983;74(1):27-29.

60. Hinrichs MA. A study of the physiological effects of caffeine upon planaria dorotocephala. *Journal of Experimental Zoology.* 1923;40(2):271-300.

61. Needleman HL. Tolerance and dependence in the planarian after continuous exposure to morphine. *Nature.* 1967;215(102):784-785.

62. Raffa RB, Dasrath CS, Brown DR. Disruption of a drug-induced choice behavior by UV light. *Behav Pharmacol.* Nov 2003;14(7):569-571.

63. Buttarelli FR, Pontieri FE, Margotta V, Palladini G. Acetylcholine/dopamine interaction in planaria. *Comparative Biochemistry & Physiology. Toxicology & Pharmacology: Cbp.* 2000;125(2):225-231.

64. Kusayama T, Watanabe S. Reinforcing effects of methamphetamine in planarians. *Neuroreport.* 2000;11(11):2511-2513.

65. Newmark PA, Alvarado AS. Not your father's planarian: a classic model enters the era of functional genomics. *Nature Reviews Genetics.* 2002;3(3):210-219.

66. Sanchez Alvarado A, Newmark PA, Robb SM, Juste R. The Schmidtea mediterranea database as a molecular resource for studying platyhelminthes, stem cells and regeneration. *Development.* Dec 2002;129(24):5659-5665.

67. Nishimura K, Kitamura Y, Inoue T, et al. Reconstruction of dopaminergic neural network and locomotion function in planarian regenerates. *Dev Neurobiol.* Jul 2007;67(8):1059-1078.

68. Robb SM, Ross E, Alvarado AS. SmedGD: the Schmidtea mediterranea genome database. *Nucleic Acids Res.* Jan 2008;36(Database issue):D599-606.

69. Pagan OR, Rowlands AL, Azam M, et al. Reversal of cocaine-induced planarian behavior by parthenolide and related sesquiterpene lactones. *Pharmacol Biochem Behav.* Dec 15 2007:in press.

70. Nielsen DM, Crnic LS. Automated analysis of foot-shock sensitivity and concurrent freezing behavior in mice. *J Neurosci Methods.* Apr 15 2002;115(2):199-209.

71. Boisvert MJ, Sherry DF. A system for the automated recording of feeding behavior and body weight. *Physiol Behav.* Oct 1-15 2000;71(1-2):147-151.

72. Valentinuzzi VS, Kolker DE, Vitaterna MH, et al. Automated measurement of mouse freezing behavior and its use for quantitative trait locus analysis of contextual fear conditioning in (BALB/cJ x C57BL/6J)F2 mice. *Learn Mem.* Sep-Oct 1998;5(4-5):391-403.

73. Madrid JA, Matas P, Sanchez-Vazquez FJ, Cuenca EM. A contact eatometer for automated continuous recording of feeding behavior in rats. *Physiol Behav.* Jan 1995;57(1):129-134.

74. Hulsey MG, Martin RJ. A system for automated recording and analysis of feeding behavior. *Physiol Behav.* Aug 1991;50(2):403-408.

75. Sanberg PR, Hagenmeyer SH, Henault MA. Automated measurement of multivariate locomotor behavior in rodents. *Neurobehav Toxicol Teratol.* Jan-Feb 1985;7(1):87-94.

76. Torello MW, Czekajewski J, Potter EA, Kober KJ, Fung YK. An automated method for measurement of circling behavior in the mouse. *Pharmacol Biochem Behav.* Jul 1983;19(1):13-17.

77. Fernandez de Miguel F, Cohen J, Zamora L, Arechiga H. An automated system for detection and analysis of locomotor behavior in crustaceans. *Bol Estud Med Biol.* Jul-Dec 1989;37(3-4):71-76.
78. Tiras KP, Aslanidi KB. Device for the Graphic Recording of Planaria Behavior. *Zhurnal Vysshei Nervnoi Deyatelnosti Imeni I P Pavlova.* 1981;31(4):874-877.
79. McConnell JV, Cornwell PR, Clay M. An Apparatus for Conditioning Planaria. *American Journal of Psychology.* 1960;73(4):618-622.
80. Sadauskas KK, Shuranova Zh P. [Method of recording the actograms of small aquatic animals and primary automatic processing of the information]. *Zh Vyssh Nerv Deiat Im I P Pavlova.* Nov-Dec 1982;32(6):1176-1179.
81. Cronin CJ, Mendel JE, Mukhtar S, et al. An automated system for measuring parameters of nematode sinusoidal movement. *BMC Genet.* 2005;6(1):5.
82. Hicks C, Sorocco D, Levin M. Automated analysis of behavior: A computer-controlled system for drug screening and the investigation of learning. *J Neurobiol.* Aug 2006;66(9):977-990.

Figure Legends

Figure 1: Schematic of high-throughput device for analysis of planarian behavior.

(**A**) Rack-mounted system containing pull-out trays of individual dish arrays. Each dish consists of a disposable Petri dish, a photoelement beneath, and a cover that has an array of light-emitting diodes (LEDs) that light up various areas of the dish without introducing heating, and an insert with inert electrodes for providing shock. The whole system is illuminated by a weak light in the far red end of the spectrum which is invisible to the worms but allows the camera to capture their movements.

(**B**) Closeup of one such dish. The LEDs and electrodes are connected to a custom programmable logic array that transduces data and effector commands to and from the software (running on a computer).

Figure 2: Schematic of algorithm for automated analysis of worm behavior

(**A**) The user sets up the trial, indicating the light conditions, what behaviors will be rewarded and punished for each dish, and the parameters of trial length, rest periods, shock strength, light brightness, etc.

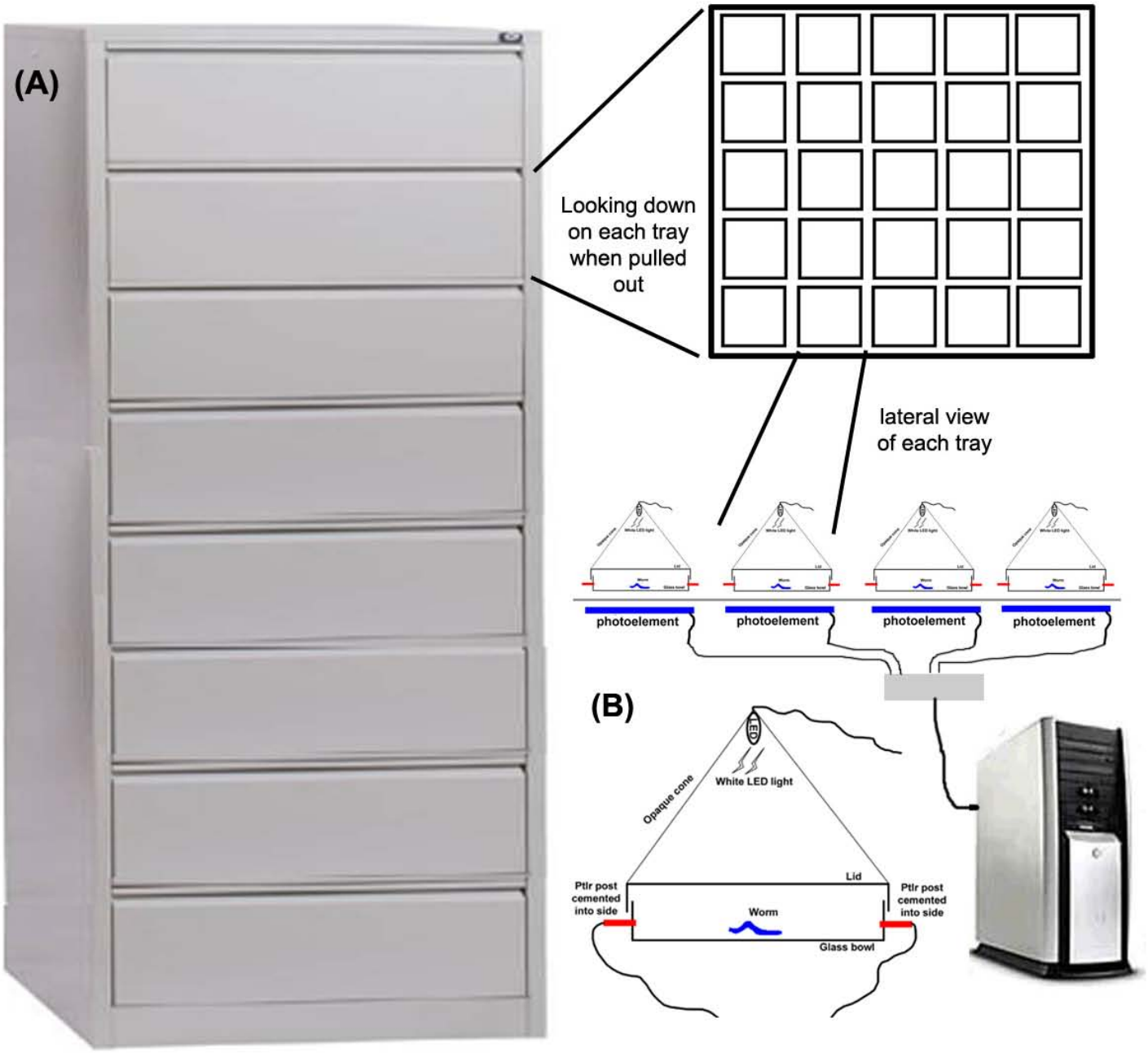
Throughout the trial, the device will repeat a cycle consisting of (**B**) changing the lights if needed and grabbing images of each dish, (**C**) low-level image processing, (**D**) detecting the centroid of the worm's position (actual such image shown in C', centroid indicated by red asterisk), (**E**) sending commands to shock those worms that are to be punished, and recording all the data to files on disk. The cycle can be performed at any rate (e.g., 5 complete cycles per second), and (**F**) statistical analysis is performed when it is completed.

(**G**) Schematics of how this could be used to investigate drug effects or withdrawal. The device will be used to characterize the behavior (collecting data on rate of movement, preferences for the edge vs. bottom of dish, aversion to light, sensitivity to shock, etc.) of worms exposed to various pharmacological reagents or removed from exposure to drugs to which they are addicted (vs. controls). This can be done in the absence of learning (for basic effects on behavior), during training (to characterize effects on learning), and during recall (to study effects on duration or quality of recall).

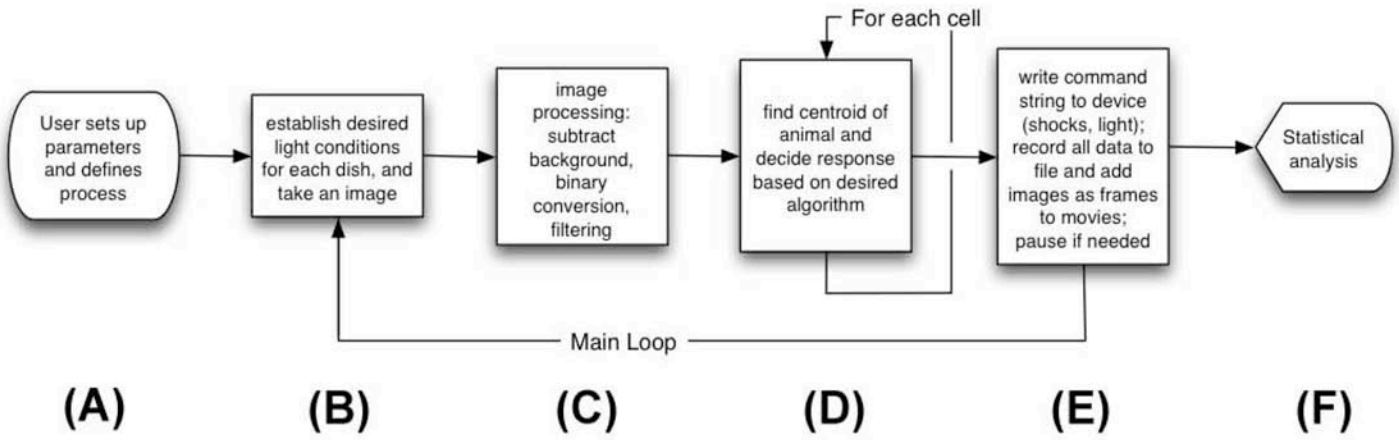
Figure 3: Sample data from prototype behavior analysis device

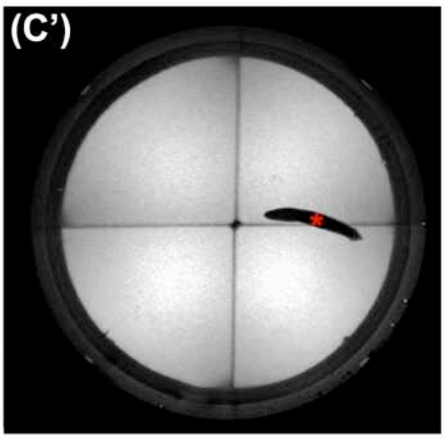
(**A**) One of the output streams is shown here for a typical experiment, where worms are trained against preference (punished by a weak electric shock for avoiding a brightly lit region in the dish). Ignoring the many metrics that are also gathered by this system (average velocity, % of time at the edge vs. center of dish, etc.), this panel focuses on the progress of a learning trial. Yoked controls (worms that are punished according to the behavior of the trained worms, and thus have no opportunity to discern a cause-effect relationship), show no improvement over time. In contrast, the trained group shows a considerable decrease in their presence in the normally-preferred dark portion of the dish. Effects of drug exposure and withdrawal can easily be detected by such metrics when applied during training or testing phases.

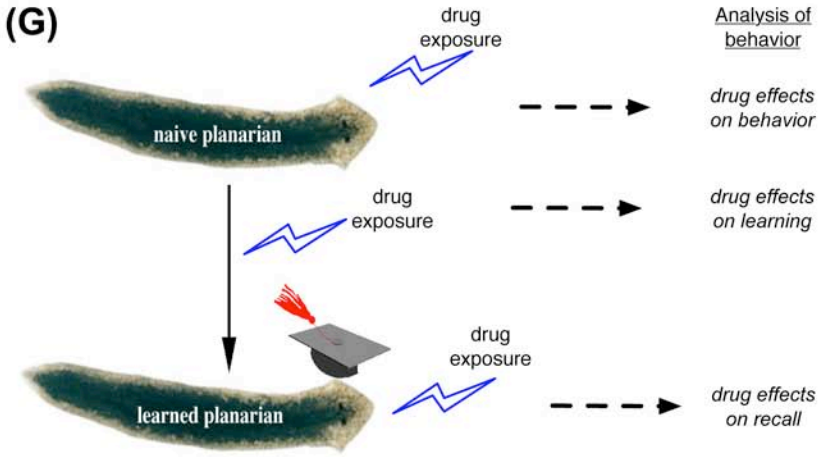
Effects of drugs on motor behavior can also be studied via a “curiosity plot”, where the device pseudocolors the area of a dish based on how often the worm visited that region. This allows one to determine, at a glance (and without recourse to the timelapse movies), how much of a dish was explored by a given animal. (**B**) A control animal that explored the dish and settled in the upper-right-hand corner. (**C**) A worm treated with PCPA, which exhibited the expected frantic activity of continually circling around the edge of the dish. (**D**) A worm treated with reserpine (targeting serotonin levels), which exhibited the expected lethargy and never moved from its spot. These data and relevant methods/controls are given in Hicks et al., 2006.



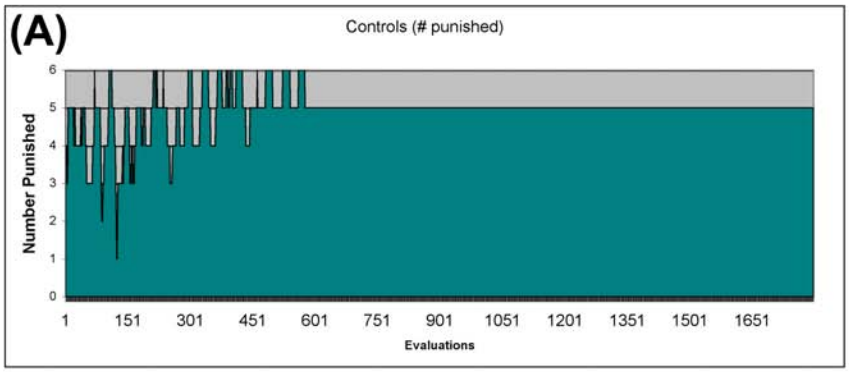
Nicolas et al., Fig. 1

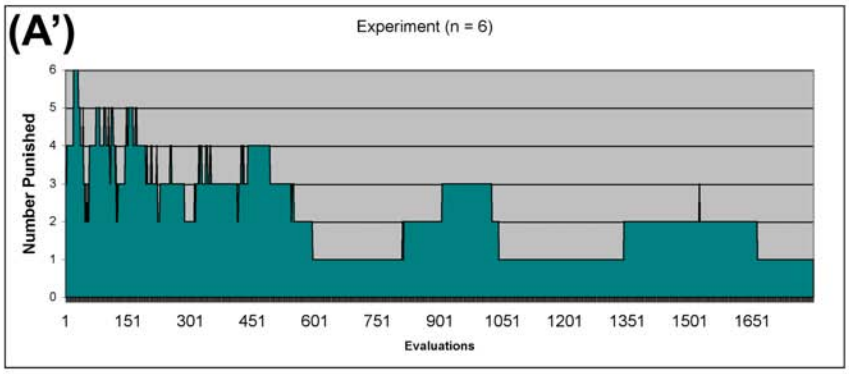


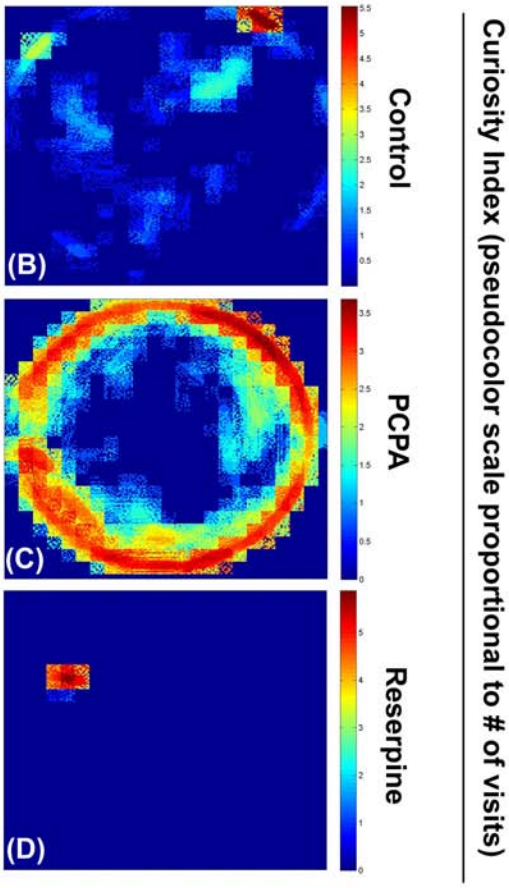




Nicolas et al., Fig. 2







Nicolas et al., Fig. 3