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SUMMARY

Behavioral responses of freshwater planarians have been studied for over a century [1]. In recent decades, behavior has been used as readout to study planarian development and regeneration [2-6], wound healing [7,8], molecular evolution [4,9,10] , neurotoxicology [11-13], and learning and memory [14-17].The planarian nervous system is among the simplest of the bilaterally symmetric animals [18], with an anterior brain attached to two ventral nerve cords interconnected by multiple commissures. We found that, in response to mechanical and near-UV stimulation, head stimulation produces turning, tail stimulation produces contraction, and trunk stimulation produces midbody elongation in the planarian *Dugesia japonica*. When cut into two or three pieces, the anterior end of each headless piece switched its behavior to turning instead of elongation; i.e., it responded as though it were the head. In addition, posterior ends of the head and midbody pieces sometimes produced contraction instead of elongation. Thus, each severed piece acts like an intact animal, with each midbody region having nearly complete behavioral capabilities. These observations show that each midbody region reads the global state of the organism and adapts its response to incoming signals from the remaining tissue. Selective lateral incisions showed that the changes in behavior are not due to nonselective pain responses and that the ventral nerve cords and cross-connectives are responsible for coordinating local behaviors. Our findings highlight a fast functional reorganization of the planarian nervous system that complements the slower repairs provided by regeneration. This reorganization provides needed behavioral responses for survival as regeneration proceeds.

RESULTS AND DISCUSSION

The responses of freshwater planarians seem simple and stereotyped: they primarily glide using cilia on the ventral surface of their exaggeratedly flattened bodies until they sense a physical object, a variation in light levels [19-21], potential food [22], variations in temperature [23], or a toxin [24,25]. They then turn either toward or away from the stimulus, switch to an escape gait [26-28], or stop moving and investigate. However, when the stimulus is localized to a region on their body, different sensitivities and behaviors are observed that depend on the site of the stimulus – similar to behaviors observed in more complex organisms.

Both Mechanical and Optical Stimulation Elicited Regionally Different Responses

Mechanical stimulation with von Frey filaments at different regions of gliding *Dugesia japonica* produced regionally different responses: anterior turning, midbody elongation, and tail contraction (Figure 1A). Weaker stimuli produced lower response probabilities, but we found the same regional distribution of the three responses (Figure S1). Because these regional behaviors were similar to those reported for near-UV light stimulation [29], we also tested near-UV stimulation using a laser pointer at either full intensity (Figure 1B) or restricted by a 0.5 mm wide slit across the width of the animals (Figure 1C). As with mechanical stimulation, light stimuli directed to the anterior end, the middle, or the posterior end, elicited turning, elongation, or contraction, respectively (Video S1). The responses to full beam, slit, and mechanical stimulation were statistically indistinguishable (Loglinear analysis, N = 300 trials, p = 0.998; details of Statistical Analysis in STAR Methods). We therefore used slits for all further experiments because they are more easily applied than mechanical stimuli and better localized than full light beams.

Regional Responses to Near-UV Slits Are Conserved Planarian Behaviors

We stimulated two additional planarian species, *Schmidtea mediterranea* and *Girardia tigrina,* with near-UV slits and observed the same regional behaviors as in *D. japonica*, but the responses were significantly weaker and less robust in *S. mediterranea* (Figure S2). Species differences to near-UV stimulation have been previously reported [30] and may be a result from differences in near-UV sensing. While TRPA1 - a member of the family of transient receptor potential (TRP) channels - was previously reported to mediate the dermal response to near-UV light in *S. mediterranea* [29], knockdown of the *D. japonica* TRPA homolog (DjTRPAa) did not affect the observed behaviors (Figure S3).

Turning Overrides Other Regional Responses

We tested for interactions among the three behaviors in *D. japonica* by presenting two stimuli in close succession at two different body locations. The areas stimulated were those that reliably produced responses: Head (anterior to the eyes), Tail (at the posterior tip), and Mid-body (half-way between Head and Tail) (Figure 1D). By comparing the responses to the second stimulus (black numbers) to the responses to the same stimulus given alone (red numbers), we found that turning overrode the other responses (i.e., stimulating the middle or tail produced a significantly lower response probability when a turn was ongoing). In contrast, stimulating either the middle or the tail neither influenced nor was influenced by responses in any other part of the animal (Fisher's exact tests, N = 60 trials for each; $p < 0.05$ for comparisons with Head first; $p > 0.05$ for all comparisons with Mid-body or Tail first; details in STAR Methods). We conclude that turning is a dominant planarian behavior.

Pieces of Planarians Showed Regional Responses to Stimulation

Attempts to find sharp boundaries between the regions producing the three different behavioral responses produced variable results no matter how narrow the slit, possibly because the light beam was unavoidably scattered. Therefore, we cut the animal into three sections – head, midbody, tail - and stimulated seven points along the length of these sections: three locations in the midbody region but only two locations on the head and tail regions because of their smaller size (Figure 2A). We first tested the pieces with near-UV slits within 1-3 hours after surgery. Stimulating the anterior end of the head piece produced turning and stimulating the posterior end of the tail piece elicited contraction (Figure 2B), similar to the responses in intact worms (Figure 1C; STAR Methods). Surprisingly, the posterior piece produced turning to stimulation of its anterior end, and the middle piece produced the same three behaviors (anterior turning, midbody elongation, posterior contraction) as did intact worms, rather than the single one (elongation) primarily elicited by stimulating these sites in an intact planarian (Video 2). In fact, the distribution of behaviors did not differ between the middle section of trisected animals compared to the whole body of intact animals (Loglinear analysis, N = 180 trials, $p > 0.999$). Moreover, when we cut planarians into two equal pieces (Figure 2C), both halves produced all three behaviors: turning to anterior end stimuli, contraction to posterior end stimuli, and elongation to stimulating the middle of these pieces (Figure 2D). These results from trisected and bisected animals show that stimulating the same midbody location produced different responses depending upon its spatial context, i.e., whether it was in the middle of an intact animal or at the anterior or posterior end of a cut piece.

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The behavior profiles remained the same for trisected animals at 1 and 7 days after surgery (Loglinear analysis, N = 630 trials, p = 0.99), and changed only slightly for bisected animals (due to changes in frequencies of non-responses, and to changes in turning at middles of bisected pieces: Loglinear analysis, N = 599 trials, p = 0.023; Figures 2B, D). Hence, these cutting experiments show capabilities for functional reorganization of behavior that is both rapid (1-3 hours) and long-lasting (7 days). Because regeneration of functioning head and tail pieces takes 3-7 days [31,32], regeneration cannot explain the reorganization present within the first 24 hours.

Unlike the results in intact animals, experiments with dual stimuli on the tail end of freshly cut animals did not show the overriding effect of turning (Table S1; Fisher's exact tests, N = 60 trials, p > 0.05 for all comparisons).

Unlikely Possibilities for the Functional Reorganization

Some results of the cutting experiments could be explained simply: the turn-eliciting region could extend into the anterior end of the middle piece and the contraction-eliciting region could extend into the posterior end of this same piece. However, this possibility cannot explain why stimulating the anterior end of the tail piece in either bisected or trisected animals produces turning: this site never elicits turning in an intact animal. Likewise, stimulating the anterior end of the middle piece of trisected animals produces turning, but stimulating the same site produces elongation in intact animals.

An alternative explanation is that turning behavior is elicited by nociception and that stimulating any area at or near to the site of the cuts produces nociceptive responses, a phenomenon similar to allodynia (nociceptive response to a stimulus that usually does not elicit such a response) or hyperalgesia (increased response to a nociceptive stimulus); both phenomena are seen in both vertebrates [33] and invertebrates [34]. To test this possibility, we made a variety of cuts that extended from the lateral edge to nearly the midline (Figure 3), then tested the responses of these animals at two sites: just anterior (Site a) and just posterior (Site b) to the cuts. In an intact animal, stimulating this middle region, which includes both sites, produces mostly elongation (Figure 1C), whereas stimulating these sites in bisected animals produced a variety of behaviors (Figure 2D). We tested whether stimulation at Site b, for instance, elicits turning (as in completely bisected pieces) or elongation (as in intact animals). The answer was absolute: for both single (Figure 3A) and offset double (Figure 3B) hemi-sections, stimulation at both Sites a and b produced elongation to every stimulus (n=10 animals, 3 stimuli at each site; Figure 3A, B; Video S3). Thus, despite these major insults to the animals' bodies, they acted like intact animals.

The fact that the behavioral responses in offset hemisected animals are like those in intact animals is likely a property of the ladder-like structure of the planarian nervous system (drawings in each panel of Figure 3). The most likely pathway for coordinating the behaviors is via the two ventral nerve cords and the cross connectives (the "rungs" of the ladder). When both nerve cords were severed at the same location (Figure 3C), stimulating Site a always produced elongation but stimulating Site b always produced turning; i.e., these sites behaved as though they were the back and front ends of completely bisected animals (Figure 2D and Video S4), supporting the idea that information flows via the nervous system and not, for instance, via mechanical coupling between the front and back ends of the body.

An even more extensive surgery, removal of the left or right side of the anterior end of the animal (Figure 3D), gave a mix of turning and elongation responses at both Sites a and b.

Thus, even animals with 25% of their bodies missing responded about half the time like intact animals (i.e., they elongated) and the other half like hemisected animals (i.e., they turned), whether the stimulus was delivered to Site a or Site b. Statistically, frequencies of responses at either site were different from, but intermediate to, head and middle locations of intact animals (Fisher's Exact Test, N = 114 trials; p < 0.05, for 4 comparisons; see STAR Methods). Similarly, frequencies of responses differed from the anterior of bisected tail sections for both Site a and b, and from the middle of bisected tail sections for Site b but not Site a (Fisher's Exact Test, N = 114 trials; p < 0.05, for 3 comparisons. P > 0.05 for 1 comparison; see STAR Methods). These data strongly support the conclusion that stimulation of anterior ends of animal pieces produced turning not from a hypersensitization to the wounds but from the removal of the influence of other body parts.

The Most Likely Explanation for the Observed Functional Reorganization: Interactions among Redundant Circuits

The findings from both moderate mechanical and near-UV stimulation of intact animals (Figure 1) suggest that the neural circuits for three behaviors - turning, elongation, and contraction - reside in different parts of the nervous system: the circuitry for turning is mainly in the head, for elongation is in the middle, and for contraction is in the tail region (solid boxes, Figure 4). Planarians – like most soft-bodied animals - change their body shape by contracting two major muscle types in their body wall: circular (Circ.) and longitudinal (Long.) muscles [35] (ovals, Figure 4). Contraction of circular muscles elongates the worm. Contraction of longitudinal muscles on one side produces a turn to that side whereas simultaneous longitudinal contractions on both sides cause a shortening of the body. Planarians also have oblique muscles; however, the three behaviors we studied can all be explained by activations of longitudinal and circular muscles (green arrows, Figure 4).

The responses of body pieces to stimulation (Figure 2) shows that the circuitry for eliciting turning is present in all body regions (dotted boxes in Figure 4). In addition, the competition experiments (Figure 1D) indicate that the anterior turning system inhibits both the elongation system in the middle and the contraction system in the back end (red arrows to E and C boxes, Figure 4). The fact that this inhibition was observed in both intact animals and pieces suggests that the turning system in each area inhibits the turning systems in more posterior regions (red arrows between T systems in Figure 4). This arrangement guarantees that turning is never activated in a region unless the more anterior regions are silenced or are missing. This concept is similar to a model that was proposed for ensuring proper head regeneration [36], wherein local inhibitory interactions among axial patterning proteins produce the same sort of adaptive local behavior that depends upon the global state of the animal. Importantly, the redundancy of behavioral circuitries means that the production of turning does not depend upon the brain nor is contraction a special property of the tail nervous system because both turning and contraction can be elicited in other body regions. Pieces of an animal can produce all three behaviors immediately—certainly within an hour--which has potentially useful practical consequences. For instance, pieces of a planarian can make directed escape behaviors; having behaviorally competent pieces for the whole duration of regeneration would be a useful survival strategy.

What kinds of sensory neurons (circles, Figure 4) are activated in our experiments? The fact that both mechanical and optical (near-UV) stimuli evoke a similar spatial profile of responses could mean that the dermal mechanoreceptors and photoreceptors feed into the behavioral systems in the same manner. It is also possible that the same receptor neurons - or

even the same receptor channels - are activated by both kinds of stimuli. Previous studies in *S. mediterranea* have shown a role for TRPA1 in dermal near-UV sensing [29], but *Dj* TRPAa knockdown was insufficient to alter the near-UV responses in *D .japonica* (Figure S3), implying the existence of additional near-UV receptors in this species.

Obtaining different responses to the same stimulus at different body locations is a common feature of complex animals. The same light stroking of the back of your hand, the bottoms of your feet, and the surface of your cornea usually elicits scratching, laughing, and violent withdrawal. The neuronal basis of these regionally diverse responses has been studied in more electrophysiologically accessible animals. Stimulating different regions on the shell and legs of turtles produces different classes of scratching movements [37] and putting pieces of acid-soaked filter paper on different skin regions of a frog produces qualitatively different types of wiping responses [38]. Stimulation of three locations along the body of medicinal leeches also produces three different responses (withdrawal, local bending, or crawling) depending upon whether the touch was applied to the front, middle, or back end of the leech [39]. What sets planarians apart from these better-studied behavioral systems is our finding that their regional behavior can adapt in real time to produce the necessary response for survival in severed tissue pieces. It is intriguing that such a relatively simple nervous system as the planarian's can produce such a complex functional reorganization, making it a prime target for a serious effort into understanding the nature of its neuronal processing [40].

Author contributions

Conceptualization, E.-M.S.C. and W.B.K.,Jr.; Methodology, E.-M.S.C., W.B.K.,Jr., and D.L; Investigation, D.L., Z.S., A.C., E.-M.S.C. and W.B.K.,Jr.; Formal Analysis, W.B.K., III; Resources, E.-M.S.C. and W.B.K.,Jr.; Data Curation, W.B.K., III; Writing –Original Draft, E.-M.S.C. and W.B.K.,Jr.; Writing –Review & Editing, D.L., Z.S., A.C., W.B.K.,III., E.-M.S.C., and W.B.K.,Jr.; Funding Acquisition, E.-M.S.C. and W.B.K.,Jr.; Supervision, E.-M.S.C. and W.B.K.,Jr.

Declaration of interests

The authors declare no competing interests.

Figure legends

Figure 1. Mechanical and near-UV stimulation elicit distinct responses in different body regions. A. A 3.0 milligram-force (mgf) was applied to the indicated body locations using von Frey filaments. N = 15 animals, stimulated once at each location. See also Figure S1. **B.** Responses to full beams of a near-UV laser administered at the same five body regions. **C.** Responses of the same five regions to slits of near-UV light. For B and C, N = 10 animals, stimulated once at each site. **D**. Responses to a second near-UV slit stimulus after first stimulating the head, the mid-body, or the tail. Observed responses were turn, elongation, contraction, and no response. Stimuli were presented in pairs, in sequence (e.g., mid-body then head). If the first stimulus did not produce a response within 1 second, the second stimulus was withheld and the response was not counted. Black numbers are the responses to the second stimulus of the pair. Red numbers are the most probable responses obtained from *single* near-UV slit stimuli applied to the head, mid-body, and tail (data from Figure 1C). Each stimulus pair was presented 3X in 10 animals. See also Video S1, Figures S1 and S2.

Figure 2. Responses of body fragments to near-UV slits are similar to whole-body responses. A. Trisected animal, made by cutting across the body at either end of the pharynx, producing Head, Middle, and Tail pieces. **B**. Response probabilities to stimulating the trisected Head and Tail pieces at their anterior (Ant) and posterior (Post) ends, as well as the Middle piece at three locations: Ant, Post and half-way between (Mid). **C**. Bisected animal, made by a single cut across the middle. **D.** Response probabilities to stimulating Head and Tail pieces of bisected animals at three locations—Ant, Mid, and Post. For all experiments, each location was stimulated 3 times in all pieces from 10 animals at 1-3 hours, 1 day, and 7 days. See also Video S2, Figures S2 and S3.

Figure 3. Responses of *D. japonica* after various cuts. In all cases, we elicited responses to small near-UV spots at two locations, Sites a and b. Drawings show the planarian nervous system with representations of the damage caused by the cuts. **A.** A single cut to nearly the midline. **B.** Two offset cuts to nearly the midline on alternating sides. **C.** Two cuts to nearly the midline from both sides at the same longitudinal location. **D.** Removal of one lateral half from the front of the animal. Each graph shows the probability of eliciting each of the three major behaviors (turning, elongation, and contraction) at Sites a and b. In all cases, we delivered 3 stimuli per site each in 10 animals. In B, we cut R-L for 5 animals and L-R for the other 5; for D, we cut 5 animals on the left and 5 on the right. For these experiments, if the animal did not respond to a stimulus, that trial was not counted. Such No Response trials accounted for less than 10% of the total. See also Videos S3 and S4.

Figure 4. **Diagrammatic summary of the major behavioral findings.** Solid lines: Behavioral circuitry, based upon experiments on intact animals (Figure 1). Each region of the animal has longitudinal muscles (Long.) that produce shortening and circular muscles (Circ.) that produce elongation. Each region also has sensory receptors (Sens.) that activate different behaviors in different body regions: turning (T) in the head, elongation (E) in the middle, and contraction (C) in the tail. Green arrows indicate excitation and red arrows indicate inhibition. Dashed lines: Additional circuitry based upon behavioral competition experiments (Figure 1D) and the behavior of body pieces (Figure 2). See also Figure S3.

STAR Methods

Resource availability.
　　See Key Resources Table.

Lead contact
　　Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, William B. Kristan, Jr**.** (wkristan@ucsd.edu)**.**

Materials availability
　　This study did not generate new unique reagents.

Data and code availability
　　Raw data and code are available at https://github.com/Collinslab-swat/Planarian-IdentitySwitch. Standard image and statistical analysis algorithms were used to analyze the data.

Experimental model and subject details
　　Three planarian species--*Dugesia japonica*, *Schmidtea mediterranea*, and *Girardia tigrina*--were used in this study.

Method details

Colony maintenance
　　Dugesia japonica, Schmidtea mediterranea, and Girardia tigrina planarians were maintained in plastic containers in either Instant Ocean salts (0.05 g/L in DI water; Spectrum Brands, Blacksburg, VA, USA) or in 1x Montjuic salts [41] in a dark environment at room temperature (68-72 ºF). For simplicity, we refer to either type of water as planarian water. Planarians were fed 2-3 times weekly using either frozen organic chicken or beef liver from a local butcher or Bellyrubs™ (Amazon) freeze dried beef liver. Planarians were starved for at least 1 week prior to behavioral experiments.

Mechanical stimulation
　　We stimulated the planarians mechanically using "von Frey filaments" produced by heating Tygon tubing (2.5 mm OD, 1 mm ID) above a flame to melt it, then pulling the melted section to short but thin (10 to 125 µm OD) flexible filaments. We measured the force required to bend each filament on a balance (Sartorius BP615). A series of filaments of different forces (ranged from 0.3 to 3.0 mgf) were selected and applied to different regions of freely moving planarians. We report the force as milligram-force (mgf): 1mgf is approximately equal to 9.8 microNewtons.

Optical stimulation
　　Initial studies using flashes of light to the whole body of a planarian at once produced a turning behavior, in which the head moved quickly to the right or left, with the rest of the body following the lead of the anterior end. We noticed that the response to a second light pulse was variable, depending upon the time since the previous pulse. The response to the second pulse became indistinguishable from the response to the first pulse after a delay of 6-10 seconds. To be

sure that we were outside the time-dependent window, we used intervals of at least 10 seconds between stimuli in all experiments. We stimulated localized regions of the planarians optically using Anddicek™ near-UV (406±10 nm) and Green (532±10 nm) laser pointers (Amazon) directed at specific parts of an animal. We constructed a sleeve from the back end of a 10 cc plastic syringe that had been cut across about mid-length and placed over the body of the laser pointer. The unobstructed beam was about 5 mm in diameter. The near-UV beam produced a total power of 1.8 mW (19 x 10^{13} photons/sec/mm^2). An IR filter was placed over the opening to block any stray IR from the laser pointers, a potential problem with standard laser pointers. To further localize the light stimulus, we put an opaque shield over the near-UV light source to admit approximately a 0.5 mm by 5 mm slit of light (0.3 mW total power; 25 x 10^{13} photons/sec/mm^2) or a small spot (0.5 mm diameter; 0.03 mW total power, 31 x 10^{13} photons/sec/mm^2). We activated the beam away from the animal, then brought it across the animal perpendicular to its long (head-tail) axis.

For monitoring behavior, we placed an individual worm in a plastic petri dish (55 mm diameter) filled approximately 2 mm deep with planarian water. After each experiment, we recorded the total laser power using a Newport model 818 photodetector. In initial experiments, we found that the IR filter and the 2 mm of water attenuated the near-UV laser beam by less than 3%. All experiments were performed in a dark room with only red light background illumination, a color that is invisible to planarians [19]. To study interactions between stimuli, we applied optical stimulation with near-UV slits at 2 different locations on the same animal. The interval between stimuli was short but variable because we waited until a response to the first stimulus began before presenting the second stimulus. If the animal did not respond to the first stimulus, we did not give the second stimulus.

In studying the effects of surgical lesions on responses (Figure 3), we used small spots of near-UV light (as indicated in the figure). If the responses were weak or absent, we also used the edge of slits to test for responses, being careful that the edge of the slit did not cross the midline. The qualitative responses were the same for spots and edges of slits, so we pooled these data. Because we were interested only in the nature of the response (turning *vs.* elongation *vs.* contraction), we did not plot "no response" trials for these experiments. Fewer than 10% of the presentation of spots--and none of the presentations of slits—resulted in no response. Most of the responses in Figures 3A and 3B were ipsilateral elongations (i.e., only the side stimulated elongated); both bilateral and ipsilateral responses were tallied as "elongations".

Surgery

Planarians were anesthetized either in saline cooled to less than 5 °C on a custom peltier cooling stage or on saline-drenched filter paper on a cooling block [42]. To section animals into two or three pieces, we used mini-scalpels. We then placed the segments into planarian water at room temperature in petri dishes for recovery for up to a week. Dissected animals remained unfed until all experiments on them were finished.

Response measurement

The responses of the animals clearly depended upon their behavioral state, as noted previously [1]. To establish a consistent behavioral state, we stimulated animals only when they were moving steadily forward in a straight line, a behavior called "gliding" [1,26,43]. After a single stimulation, the animal was aspirated into a plastic transfer pipette and returned to the center of the dish. For most experiments, we tallied just the initial response to the stimulus by visual

observation. For some experiments, we video-taped the responses and measured the rate and direction of movement using ImageJ 1.52u.

Quantification and statistical analysis

The behavioral data are presented as counts of outcomes for planarians subjected to various treatments. The outcomes (turn, elongate, contract, and no response) constitute a categorical variable made up of three levels. The treatments are also categorical, such as stimulus type (mechanical, UV slit, UV beam) and location of the stimulus (head, front middle, middle, back middle, tail). The numbers of times each behavior occurred for each categorical treatment generates a contingency table, with the levels of one variable in the rows, and the levels of another variable in the columns, and tallies how often each row/column combination occurs in the body of the table. With just two variables such data can be analyzed with a Chi-square test of heterogeneity, or (when there are many low counts or zeros in cells, which makes the Chi-square test perform poorly) a Fisher's exact test. In both cases the null hypothesis is that the two variables are independent. All tests used an alpha level of 0.05 as statistical significance.

When there are more than two categorical variables that are being related to one another, instead of contingency table analysis we used loglinear analysis, which models frequencies of outcomes using main effects of and interactions between the variables. Main effects account for different total numbers of observations between the different levels of a single variable, whereas interactions account for the possible dependencies of variables on one another. For instance, a three-way interaction between stimulation type, behavior, and location would test for the possibility that the distribution of the four possible responses across the five stimulation locations are different depending on the type of stimulation that was used. Statistical significance in loglinear analysis is assessed by fitting models with and without the terms to be tested, and then testing for a statistically significant decrease in model support when a term is dropped. Differences in support for loglinear models are tested with a likelihood ratio test.

The complete is available at https://github.com/Collinslab-swat/Planarian-IdentitySwitch and include the code and rationale for all the statistical analyses used in this study. Details of statistical analyses for figures 1-3 in the main text and figures S1 and S2 are provided below.

Figure 1

Figure 1A presents data on mechanical stimulation and compares it with near-UV beam (1B) and near-UV slit (1C) optical stimulation. The data are compiled as frequencies of occurrence of the combinations of stimulus type (mechanical, near-UV slit, near-UV beam), location of stimulation (head, front middle, middle, back middle, and tail), and behavior (turn, elongate, contract). To test the dependency of behavior on location of stimulation and type of stimulation (mechanical, UV beam, UV slit) we used loglinear analysis. The three-way interaction between behavior, location, and stimulus type is the term that matters because to the extent that it is not statistically significant provides evidence that the distribution of behaviors across the stimulus locations do not change with different types of stimulation. We found that the two-way interactions are significant, but the three-way interaction is not; i.e., near-UV slit, near-UV beam, and 3 mgf mechanical stimulation do not produce different patterns of behavior across the five stimulus locations.

Figure 1D shows the rates of each behavior for animals that were stimulated twice. The black numbers in the table are the relative frequencies for the average response obtained when an

animal was stimulated at the second stimulus location. The red numbers are the relative frequency of the typical response for once-stimulated animals at the same location as the second stimulus. Data for UV slit stimulated animals (1C) were used to represent the typical response for once-stimulated animals. We compared these two proportions (aka relative frequencies or probabilities) using a contingency table analysis, using counts of each outcome. To obtain p-values for each comparison, behavior data were coded as either the typical response or "other". Frequencies of typical response or other for twice-stimulated and once-stimulated groups were tabulated and compared with contingency tables. These are all 2x2 tables, many with 0 counts that lead to violations of the assumptions of a Chi-square test, so a Fisher's exact test was used. Based on these comparisons, the only two twice-stimulated patterns that differ from the once-stimulated location are H-M and H-T. In other words, stimulating the head first changes the response of the animal to the second stimulation (and thus makes it different from once-stimulated animals), but stimulating middle or tail first does not alter the response to the second stimulation (thus making them no different from once-stimulated animals).

Figure 2

We tested three body pieces (Figure 2A) with near-UV slits at 3 time points: 1 hour, 1 day, and 7 days after surgery was completed. The middle section of trisected animals was large enough to stimulate at the anterior, middle, and posterior, which allowed us to compare the distribution of behaviors across all three stimulus locations to the head, middle, and tail locations of intact animals. Because this involved a comparison of behaviors among stimulus locations for different types of animals (intact or trisected), we used loglinear analysis. A separate analysis was done for each time point.

Stimuli to both locations on the head section produced turning responses at 1 hour at the same rates as the head of intact animals for the anterior location in intact animals (p > 0.05), but at a lower rate for posterior locations (p < 0.05). Turning continued to be observed at the same rate as the head of intact animals at the other two time points (p > 0.05), but no response was elicited at the posterior of the head region at 1 day or 7 days after surgery. Stimulating the posterior end of the tail section elicited contraction, although at a lower rate than for intact animals (p < 0.05) (Figure 2B). Differences in the number of non-responses led to statistically significant differences at each time point, but all the responses elicited were those expected for the equivalent location in intact animals (Figure 1B, C).

With bisected animals it was possible to stimulate at three locations consistently, so comparisons to the head, middle, and tail of intact animals were possible for both bisected sections. This analysis was done separately by time point for each section, starting with the tail section. There were minor differences in the responses in the two pieces of bisected animals. For instance, the more mixed distribution of behaviors in head sections of bisected animals produced differences from the more consistent responses seen in intact animals (p < 0.05). The probability of turning responses in the anterior nds of tail sections were either not significantly different from the heads of intact (p > 0.05) or differed only due to greater frequencies of non-response in the bisected tail sections. All three of the time points show a different pattern between head section posterior locations and intact tails. Tails of intact animals contracted 90% of the time and never elongated, but head sections were more often unresponsive (42%) and elongated frequently (10-29%). The head of intact animals always turned, and the anterior of tail sections either turned or did not respond. Differences between anterior ends of tail sections and heads of intact animals were due only to differences in non-responses, since no other behavior was observed for either condition.

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Figure 3

There are two analyses - a contingency table of behaviors by stimulus location for panel D (quartered animal) and an analysis of differences between the quartered animal and the other three surgeries. There is a slight, statistically significant dependency of behavior of stimulus location, but none of the standardized residuals are greater than 2. Differences in non-responses are a factor. Next we compared panel D to panels A, B, and C one at a time. Because this adds another grouping variable, surgery, we used loglinear models. When comparing D to A or B, we found that the two-way interactions are significant, but the three-way is not. Thus, behavior depends strongly on surgery and less strongly on location. In contrast, for D and C, both the 2-way and 3-way interactions are significant, which means that in this case behavior depends both on surgery and on location.

TRPAa RNAi

RNAi of DjTRPAa was performed as previously described [28]. Briefly, *D. japonica* planarians were injected on four consecutive days with *in vitro* transcribed dsRNA to a final concentration of at least 1 μg/μL using a Leica dissection microscope and Pneumatic PicoPump Model PV 820 (World Precision Instruments, Sarasota, FL, USA). The *C. elegans* gene unc22 was used as a control. After the fourth injection, planarians were fed organic beef liver mixed with *trpaa* dsRNA, cleaned 2 hours after, then 2 days after, and only used for experiments > 5 days post feeding. Successful knockdown was verified by exposure to 100uM AITC; while *(unc22)* RNAi worms scrunched in response to this TRPAa specific chemical inducer [28, 30], *(trpaa)* RNAi worms did not scrunch (Figure S3A).
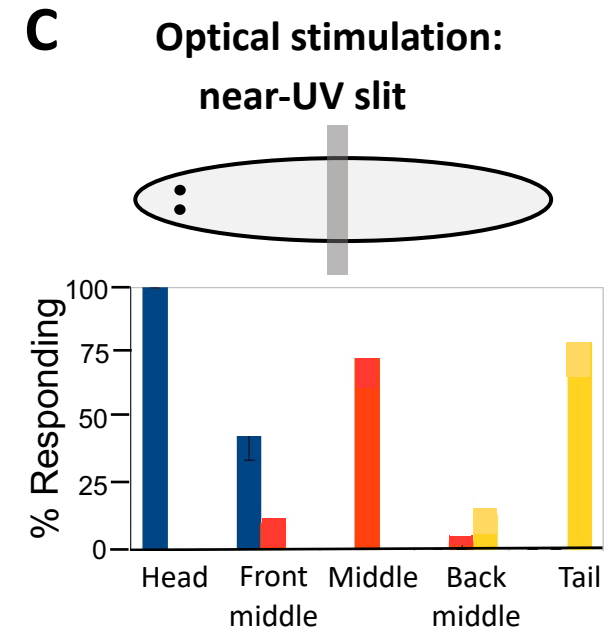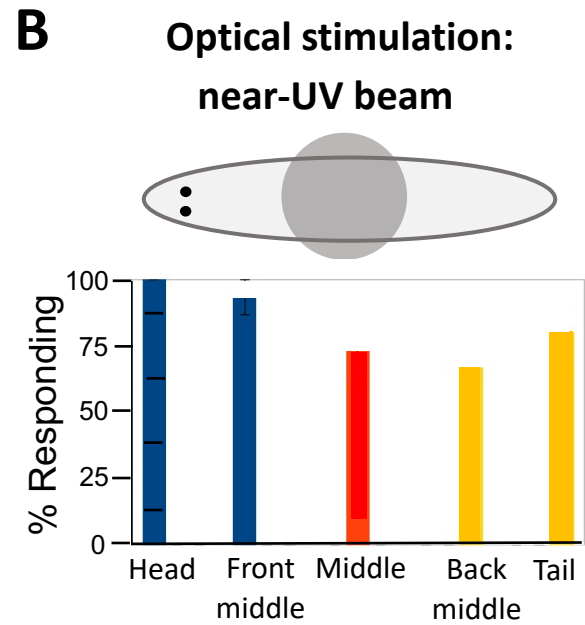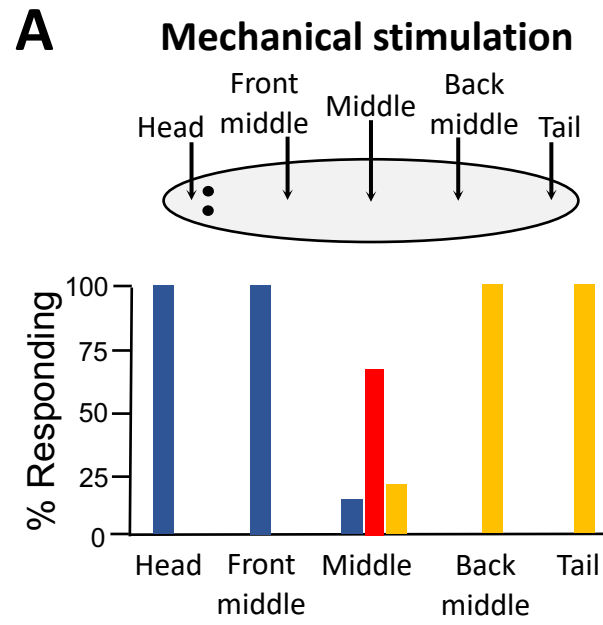
.

References

1   Pearl, R. (1903). The movements and reactions of fresh-water planarians: A study in animal behaviour. Q. J. Microsc. Sci. *46*, 509-548.
2   Umesono, Y., Tasaki, J., Nishimura, K., Inoue, T. and Agata, K. (2011) Regeneration in an evolutionarily primitive brain--the planarian *Dugesia japonica* model. *The* Eur. J. Neurosci. *34*, 863-869, doi:10.1111/j.1460-9568.2011.07819.x..
3   Ivankovic, M, Haneckova, R., Thommen, A., Grohme, M. A., Vila-Farré, M., Werner, S., and Rink J.C. (2019). Model systems for regeneration: planarians. Development *146*, doi:10.1242/dev.167684.
4   Umesono, Y., and Agata, K. (2009). Evolution and regeneration of the planarian central nervous system. Dev. Growth Differ. *51*, 185-195.
5   Elliott, S. A., and Alvarado, A. S. (2018). Planarians and the history of animal regeneration: Paradigm shifts and key concepts in biology. Methods Mol. Biol. *1774*, 207-239.

6	Pietak, A. A.-O., Bischof, J. A.-O. X., LaPalme, J., Morokuma, J. A.-O., and Levin, M. A.-O. (2019). Neural control of body-plan axis in regenerating planaria. PLOS Comput. Biol. *15*. doi:https://doi.org/10.1371/journal.pcbi.1006904.

7	Wenemoser, D., Lapan, S. W., Wilkinson, A. W., Bell, G. W., and Reddien, P. W. (2012) A molecular wound response program associated with regeneration initiation in planarians. Gene Dev. *26*. 988-1002, doi:10.1101/gad.187377.112.

8	Oderberg, I. M., Li, D. J., Scimone, M. L., Gaviño, M. A., and Reddien, P. W. (2017). Landmarks in existing tissue at wounds are utilized to generate pattern in regenerating tissue. Curr. Biol. *27*, 733–742.

9	Riutort, M., Álvarez-Presas M Fau - Lázaro, E., Lázaro E Fau - Solà, E., Solà E., Fau - Paps, J., and Paps, J. (2012). Evolutionary history of the Tricladida and the Platyhelminthes: an up-to-date phylogenetic and systematic account. Int. J. Dev. Biol. *56,* 5-17.

10	An, Y., Kawaguchi, A., Zhao, A., Toyoda, A., Sharifi-Zarchi, A., Mousavi, S. A., Bagherzadeh, R., Inoue, T., Ogino, H., Fujiyama, A., *et al.* (2018). Draft genome of *Dugesia japonica* provides insights into conserved regulatory elements of the brain restriction gene *nou-darake* in planarians. Zool. Lett. *4*, 24. doi:10.1186/s40851-018-0102-2.

11	Hagstrom, D., Cochet-Escartin, O., Zhang, S., Khuu, C., and Collins, E.-M. S. (2015). Freshwater planarians as an alternative animal model for neurotoxicology. Toxicol. Sci. *147*, 270-285. doi:10.1093/toxsci/kfv129.

12	Zhang, S., Hagstrom, D., Hayes, P., Graham, A., and Collins, E.-M. S. (2019). Multi-behavioral endpoint testing of an 87-chemical compound library in freshwater planarians. Toxicol. Sci. *167*, 26-44.

13	Zhang, S., Hagstrom, D., Siper, N., Behl, M., and Collins, E.-M. S. (2019). Screening for neurotoxic potential of 15 flame retardants using freshwater planarians. Neurotoxicol. Teratol. *73*, 54-66, doi:https://doi.org/10.1101/538280.

14	Turel, Z., Prados, J., and Urcelay, G. (2020). Heat shock disrupts expression of excitatory and extinction memories in planaria: Interaction with amount of exposure. Behav. Process. *179*, 104197. doi:10.1016/j.beproc.2020.104197.

15	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*, 977-990. doi:10.1002/neu.20290.

16	Blackiston, D., Shomrat, T., and Levin, M. (2015). The stability of memories during brain remodeling: A perspective. Commun. Integr. Biol. *8,* e1073424. doi:10.1080/19420889.2015.1073424

17	Shomrat, T., and Levin, M. (2013). An automated training paradigm reveals long-term memory in planarians and its persistence through head regeneration. J. Exp. Biol. *216,* 3799-3810. doi:10.1242/jeb.087809.

18	Bullock, T., and Horridge, G. A. (1965). Structure and Function in the Nervous Systems of Invertebrates (Freeman Press).

19	Paskin, T. R., Jellies, J., Bacher, J., and Beane, W. S. (2014). Planarian phototactic assay reveals differential behavioral responses based on wavelength. PloS One *9*, e114708-e114708. doi:10.1371/journal.pone.0114708.

20	Lambrus, B. G., Cochet-Escartin, O., Gao, J., Newmark, P. A., Collins, E. M., and Collins, J. J. 3rd. (2015). Tryptophan hydroxylase is required for eye melanogenesis in the

planarian *Schmidtea mediterranea*. PloS One *10*, e0127074. doi:10.1371/journal.pone.0127074.

21   Reynierse, J. H. (1967). Reactions to light in four species of planaria. J. Comp. Physiol. Psychol. **63**, 366–368.

22   Mori, M., Narahashi, M., Hayashi, T., Ishida, M., Kumagai, N., Sato, Y., Bagherzadeh, R., Agata, K., and Inoue, T. (2019). Calcium ions in the aquatic environment drive planarians to food. Zool. Lett. *5,*31-46*.* doi.org/10.1186/s40851-019-0147-x

23   Inoue, T., Yamashita, T., and Agata, K. (2014). Thermosensory signaling by TRPM is processed by brain serotonergic neurons to produce planarian thermotaxis. J. Neurosci. *34,* 15701-15714, doi:10.1523/JNEUROSCI.5379-13.2014.

24   Hagstrom, D., Cochet-Escartin, O., and Collins, E.-M. S. (2016). Planarian brain regeneration as a model system for developmental neurotoxicology. Regeneration *3*, 65-77, doi:10.1002/reg2.52.

25   Grebe, E., and Schaeffer, D. J. (1991). Planarians in toxicology, standardization of a rapid neurobehavioral toxicity test using phenol in a crossover study. B. Environ. Contam.Toxicol. *46*, 866–870.

26   Cochet-Escartin, O., Mickolajczk, K. J., and Collins, E.-M. S. (2015). Scrunching: a novel escape gait in planarians. Phys. Biol. *12*, 055001. doi:10.1088/1478-3975/12/5/056010.

27   Cochet-Escartin, O., Carter, J. A., Chakraverti-Wuerthwein, M., Sinha, J., and Collins, E. M. S. (2016). Slo1 regulates ethanol-induced scrunching in freshwater planarians. Phys. Biol. *13*, 1-12. doi:10.1088/1478-3975/13/5/055001.

28   Sabry, Z., Ho, A., Ireland, D., Rabeler, C., Cochet-Escartin, O., and Collins, E. M. S (2019). Pharmacological or genetic targeting of Transient Receptor Potential (TRP) channels can disrupt the planarian escape response. PLoS One, 753244-753244, doi:10.1101/753244.

29   Birkholz, T. R., and Beane, W. S. (2017). The planarian TRPA1 homolog mediates extraocular behavioral responses to near-ultraviolet light. J. Exp. Biol. *220*, 2616-2625. doi:10.1242/jeb.152298 .

30   Sabry, Z., Rabeler, C., Ireland, D., Bayingana, K., and Collins, E.-M. S. (2020). Planarian scrunching as a quantitative behavioral readout for noxious stimuli sensing. JoVE, (161) doi:10.3791/61549.

31   Shettigar, N., Joshi, A., Dalmeida, R., Gopalkrishna, R., Chakravarthy, A., Patnaik, S., Mathew, M., Palakodeti, D., and Gulyani, A. (2017). Hierarchies in light sensing and dynamic interactions between ocular and extraocular sensory networks in a flatworm. Sci. Adv. *3*, e1603025) 1-14. doi:10.1126/sciadv.1603025.

32   Cebrià, F. (2007). Regenerating the central nervous system: how easy for planarians! Dev. Genes Evol. *217*, 733-748. doi:10.1007/s00427-007-0188-6.

33   Jensen, T. S., Finnerup, N. B. (2014). Allodynia and hyperalgesia in neuropathic pain: clinical manifestations and mechanisms. Lancet Neurol. *13,* 924-935. doi:10.1016/S1474-4422(14)70102-4.

34   Walters, E. (2018). Nociceptive biology of molluscs and arthropods: Evolutionary clues about functions and mechanisms potentially related to pain. Front. Physiol. *9,* (1049) 1-22. doi:10.3389/fphys.2018.01049.

35   Kristan, W. B. Jr., Skalak R., Wilson, R. J. A., Skierczynski, B. A., Murray, J. A., Eisenhart, F. J., and Cacciatore, T. W. (2000) Biomechanics of hydroskeletons: Lessons
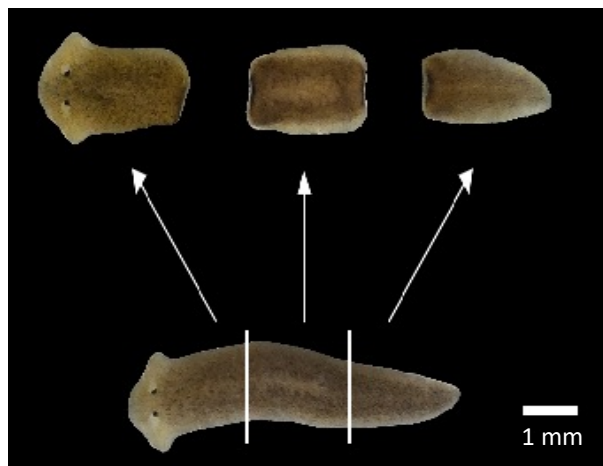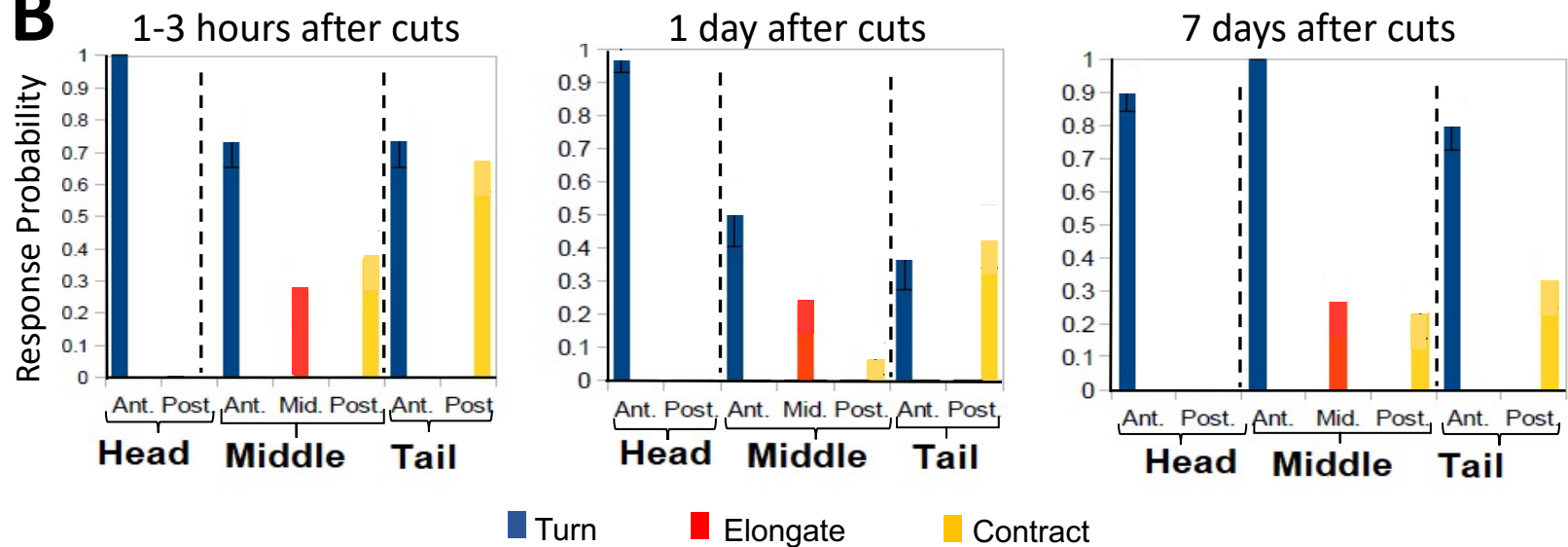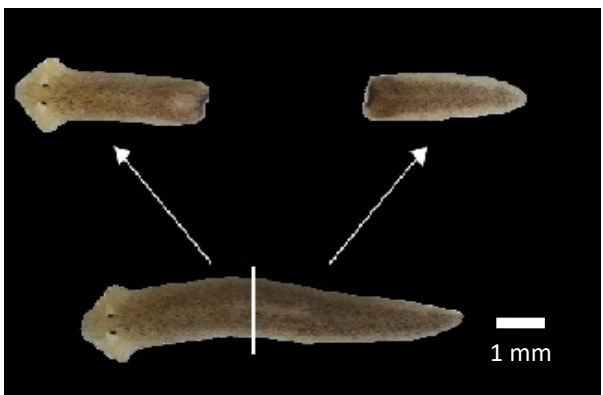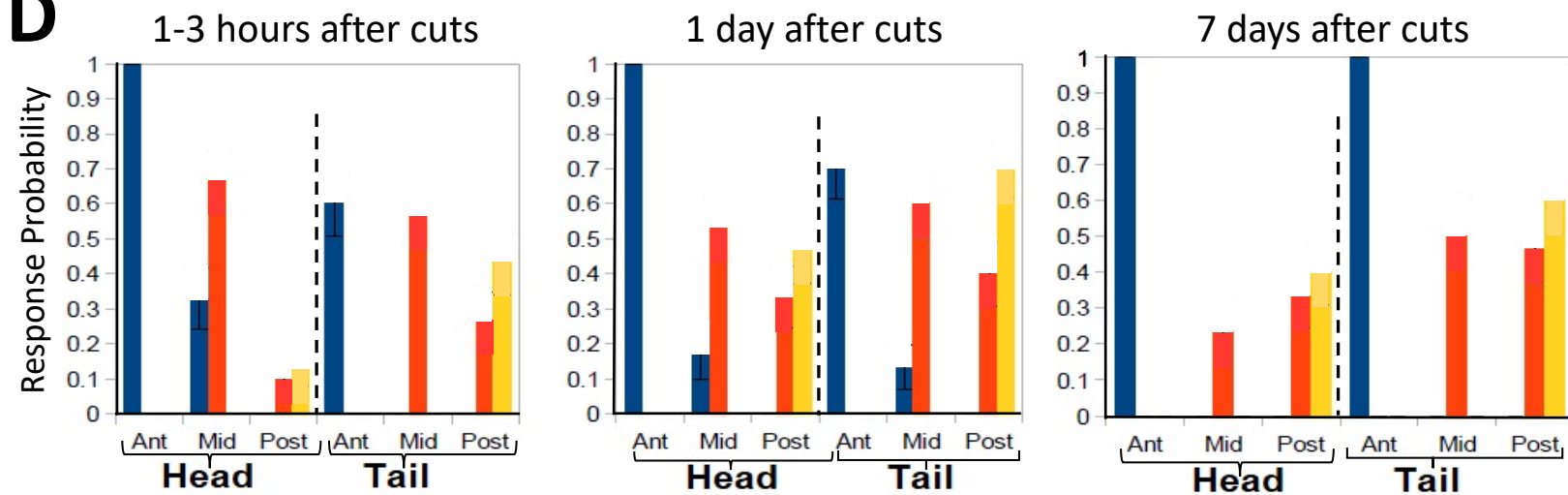
learned from studies of crawling in the medicinal leech. In: Biomechanics and Neural Control of Movement, J. Winters and P. Crago, eds. (Springer-Verlag), pp. 206-220.

36   Owlarn, S., Klenner, F., Schmidt, D., Rabert, F., Tomasso, A., Reuter, H., Mulaw, M. A., Morita, S., Gentile, L., Weidinger, G., *et al.* (2017). Generic wound signals initiate regeneration in missing-tissue contexts. Nat. Commun. *8,* 1-13. doi:doi:10.1038/s41467-017-02338-x.

37   Stein, P. S. G. (2018). Central pattern generators in the turtle spinal cord: selection among the forms of motor behaviors. J. Neurophysiol. *119*, 422-440. doi:10.1152/jn.00602.2017.

38   Giszter, S., McIntyre, J., and Bizzi, E. (1989). Kinematic strategies and sensorimotor transformations in the wiping movements of frogs. J. Neurophysiol. *62*, 750-767. doi:10.1152/jn.1989.62.3.750.

39   Palmer, C., Barnett, M., Copado, S., Gardezy, F., and Kristan, W. (2014). Multiplexed modulation of behavioral choice. J. Exp. Biol. *217*, 2963-2973. doi:10.1242/jeb.098749.

40   Yartsev, M. (2017). The emperor's new wardrobe: Rebalancing diversity of animal models in neuroscience research. Science *358,* 466-469. doi:10.1126/science.aan8865.

41   Cebrià, F., and Newmark, P. A. (2005). Planarian homologs of netrin and netrin receptor are required for proper regeneration of the central nervous system and the maintenance of nervous system architecture. Development *132*, 3691-3703. doi:10.1242/dev.01941.

42   Morton, J., Saad, M., and Beane, W. (2017). Surgical ablation assay for studying eye regeneration in planarians. JoVE. doi:10.3791/55594

43   Rompolas, P., Patel-King, R. S., and King, S. M. (2010). An outer arm dynein conformational switch is required for metachronal synchrony of motile cilia in planaria. Mol. Biol. Cell *21*, 3617-3759. doi:10.1091/mbc.E10-04-0373.
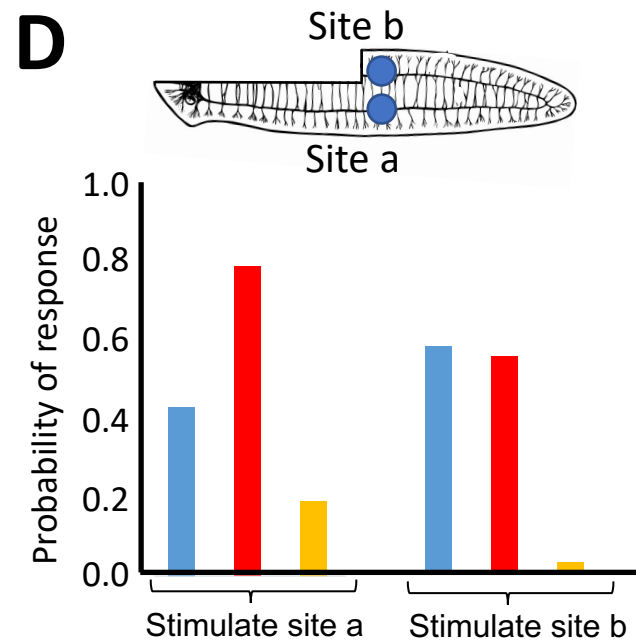
A Mechanical stimulation

B Optical stimulation: near-UV beam

C Optical stimulation: near-UV slit

■ Turn   ■ Elongate   ■ Contract

D. Interactions between responses elicited by near-UV slits at two body locations.

Stimulus pair	Turn	Elongation	Contraction	No response
M-H	0.87 (1.0)	0	0	0.13
T-H	0.87 (1.0)	0.07	0	0.07
H-M	0	0.40 (0.72)	0	0.60
T-M	0	0.80 (0.72)	0	0.20
H-T	0	0.60	0.10 (0.80)	0.30
M-T	0	0	0.73 (0.80)	0.27

A

B

1-3 hours after cuts

1 day after cuts

7 days after cuts

Response Probability

Head   Middle   Tail

Turn   Elongate   Contract

C

D

1-3 hours after cuts

1 day after cuts

7 days after cuts

Response Probability

Head   Tail

A

Site a Site b

Probability of response

1.0
0.8
0.6
0.4
0.2
0.0

Stimulate site a   Stimulate site b

B

Site b

Site a

Probability of response

1.0
0.8
0.6
0.4
0.2
0.0

Stimulate site a   Stimulate site b

Turn
Elongate
Contract

C

Site b

Site a

Probability of response

1.0
0.8
0.6
0.4
0.2
0.0

Stimulate site a   Stimulate site b

D

Site b

Site a

Probability of response

1.0
0.8
0.6
0.4
0.2
0.0

Stimulate site a   Stimulate site b

HEAD

MIDDLE

TAIL

Turn

Elongate

Contract

T

T

T
T
E
E
C
T
T
C