Headless Planarian Phenotype Induced by 8-OH-DPAT Exposure Persists in Subsequent Unperturbed Amputation

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Summary

Previous studies have found that exposing regenerating planaria to serotonergic agonist 8-OH-DPAT results in a headless phenotype in approximately 20% of cases. This study investigates the epigenetic effect of 8-OH-DPAT on planarian morphology across two rounds of amputation. 150 planaria were amputated and half were placed in 10 μM 8-OH-DPAT solution for 24 hours. All regenerated planaria were then amputated again with no 8-OH-DPAT exposure. We found that 11.2% of the planaria exposed to 8-OH-DPAT in the first round exhibited a headless morphology after regeneration, and that 17.6% of these headless planaria regenerated to be headless in the unperturbed second round. The persistence of this altered morphology into the second regeneration suggests that 8-OH-DPAT induces an epigenetic effect that inhibits head formation.

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1. Introduction

Researchers have long sought to understand the mechanism by which planaria, small flatworms found in freshwater and saltwater, are able to regenerate their body parts -- even their brains -- after injury. Even more impressively, they have the ability to detect when their body has been sufficiently repaired and respond by ceasing the regeneration process. Their regenerative ability is widely believed to be a potential model for tissue regeneration in humans. The ability to regenerate tissue *in vivo* would enable more effective treatment of heart disease, various neurodegenerative disorders, and cancer, among other conditions. Furthermore, understanding tissue regeneration would transform the field of artificial organ engineering. However, while significant progress has been made in the past decade, the mechanisms by which planaria heal injured body parts is far from being fully understood. Specifically, the global decision-making that allows planaria to restore the proper size, scale and orientation of their bodies remains largely unknown. This experiment aims to shed light on these decision-making mechanisms at the cellular level by investigating the effects of serotonergic agonist 8-OH-DPAT on two generations of planarian regeneration.

Blastema Formation

At the time of amputation, a reactive oxygen species is released that initiates the process of regeneration. Within 30 minutes, signs of wound healing begin to appear at the location of injury (Durant et al., 2016). This wound healing is possible only because of the abundance of somatic stem cells, known as neoblasts, in planaria. Neoblasts are the only mitotic cells in adult planaria. In the first step of regeneration, neoblasts surround the wound to form a blastema, or a collection of morphologically undifferentiated cells (Tasaki et al., 2011). Simultaneously, apoptosis -- programmed cell death -- occurs at the site of the wound. Furthermore, an injured planarian will experience two mitotic peaks during blastema formation (Durant et al., 2016). The first applies throughout the entire body while the second is specific to the region surrounding the injury and corresponds with an influx of neoblasts to the wound site. The correlation between this second mitotic peak and signaling that triggers neoblast migration is not known. The neoblasts that migrate to the wound cluster together to form a blastema. Once the blastema is formed, the neoblasts need location-specific instructions in order to restore proportionality within and between its structures (Durant et al., 2016).

Polarity and Global Patterning

A combination of several patterning genes, known as position control genes (PCGs), create what is thought of as a positional coordinate system to guide pattern formation during regeneration. These genes, which are continuously expressed in subepidermal muscle cells, provide a frame of reference for blastema at the onset of differentiation (Owlarn, 2016). PCGs provide a partial explanation for the phenomenon of polarity in planaria: an anterior wound leads to regeneration of a head while a posterior wound causes regeneration of a tail. In fact, planaria exhibit polarity along their anterior-posterior, dorsoventral, and mediolateral axes (Pellettieri, 2018). In addition to PCGs, there is another mechanism by which polarity is achieved. Several signaling pathways have been shown to be involved: Wingless/Integrated (Wnt), fibroblast growth factor (FGF), bone morphogenetic proteins (BMP), and the intercellular signaling molecule Hedgehog (*Hh*) (Pellettieri, 2018). In 2008, a team of researchers found that when a homolog of *β-catenin*, a signal-transducing protein that is a key element of Wnt signaling, is silenced, planaria will reconstitute a head regardless of the location of the wound (Iglesias et al., 2008). This discovery further implicated the Wnt signaling pathway in planaria pattern formation, especially in the posterior (see Figure 1). Wnt regulators create a gradient, with Wnt agonists such as *wnt1* concentrated in the posterior and Wnt antagonists such as *notum* in the anterior. This gradient, combined with enhanced transcription of Wnt target genes, helps determine polarity and cell fate (Owlarn, 2016).

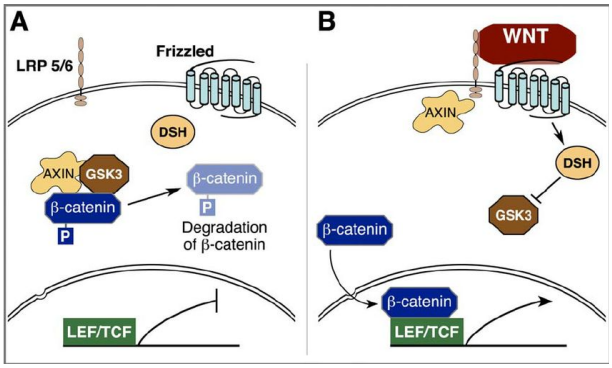

Figure 1 illustrates the pathway for canonical Wnt signal transduction. (Eisenberg, 2007). Before a Wnt protein binds to the Frizzled transmembrane protein (1A), *β-catenin* remains within a cluster of proteins including Axin and glycogen synthase kinase 3 (GSK3). GSK3 phosphorylates *β-catenin* in order to degrade it. When Wnt binds to the Frizzled and LRP5/6 receptors (1B), the Dishevelled (Dsh) protein inactivates GSK3. Consequently, *β-catenin* translocates into the cell's nucleus. Along with LEF/TCF DNA binding proteins, *β-catenin* enhances transcription in order to upregulate Wnt target genes (Eisenberg, 2007). In planaria, there are dozens of Wnt target genes, including tsh, sp5, abdBa (Reuter et. al, 2015).

Tsh is a transcription factor gene that is primarily differentially expressed in the posterior region, and its function is to suppress anteriorization. Thus, the increased transcription of *tsh* as a result of the Wnt signaling cascade ensures that the posterior does not regenerate a head. If *tsh* is suppressed, a planarian's tail will slowly become a head (Reuter et. al, 2015). *Sp5* and *abdBa* are also transcription factor genes that are highly concentrated in the tail in a *β-catenin*-dependent manner, but they facilitate posteriorization rather than inhibit anteriorization. Thus, the upregulation of Wnt target genes triggers a complex web of positive and negative feedback to ensure that cell-fate determination properly restores polarity (Reuter et. al, 2015).

Additionally, gap junctional communication (GJC) is also involved in planaria anterior-posterior patterning (Oviedo et al., 2010). Gap junctions are small channels comprised of plasma membrane connecting two cells, thus facilitating direct cell-to-cell communication. By allowing stem cells to communicate with their immediate environment once they have migrated to the wound site, GJC modulates embryonic development and tissue function in addition to planaria regeneration. These connections allow for both electric and molecular cell-to-cell communication (Yoshimura, 2017). A 2010 study found that when planaria were exposed to GJC inhibitors heptanol or octanol, their anterior-posterior polarity was altered during regeneration (Oviedo et al., 2010). It is believed that GJC blockade interferes with communication in such a way that the target morphology is altered rather than merely improperly executed. Researchers have also shown that serotonin not only regulates GJC by enhancing chemical synaptic transmission, but is itself permeable to gap junctions. Although it is not well-understood, serotonin's GJ permeability suggests that it could be involved in complex feedback loops with downstream consequences (Durant et al., 2016).

Blastema Differentiation

For a long time, the transition between blastema formation (involving neoblast proliferation and migration) and differentiation remained a mystery; few even speculated the mechanism by which

generic neoblasts began to differentiate to form specialized body parts. In 2008, the insights into pattern formation discussed in the previous section gave researchers an idea of how neoblasts "knew" whether to become heads, tails, or other body parts once they migrated to the injury site. A team of researchers in 2011 identified the factor that switches neoblasts from their proliferative to their differentiation state: extracellular signal-related kinase (ERK) activity. In their study, they found that a mitogen-activated protein kinase (MAPK) phosphatase-related gene, which they named *DjmkpA*, is a reliable indicator of blastema differentiation (Tasaki et al., 2011). *DjmkpA* expression was found to be approximately tenfold greater in regenerating tissue than tissue that is not regenerating. By exposing the planaria to various concentrations of an ERK inhibitor, they found that *DjmkpA* expression was highest in planaria with the strongest ERK activity. Another key finding of this study was that *DjmkpA* and ERK form a negative feedback loop to modulate blastema differentiation (Tasaki et al., 2011). In fact, because *DjmkpA* was expressed as part of the initial wounding response and because it is known to tightly regulate ERK, the study speculated the *DjmkpA* itself activates ERK. Lastly, the researchers found that when ERK was inhibited, neoblasts continued proliferating but failed to differentiate, which reinforces their conclusion that ERK triggers blastema differentiation (Tasaki et al., 2011).

Planarian Morphology

As mentioned earlier in this review, various studies have produced planaria with alternate morphologies. When researchers silenced *β-catenin-1* during regeneration, the resulting planaria exhibited a two-head phenotype in which both their anterior and posterior regions grew back heads (Iglesias, 2008). In fact, other planaria in the same experiment demonstrated various other polarity defects, the most extreme of which -- known as radial-like hyper-cephalization -- involves eyes growing all around the periphery of the body. The researchers observed that the severity of the defects was dependent on the inhibitor dosage (Iglesias, 2008). In another study discussed earlier, exposure to GJC inhibitors caused regenerating planaria to exhibit a double-headed morphology (Oviedo et al., 2010). Because neoblasts cannot form gap junctions until they have migrated to the site at which they will differentiate, this finding suggests that patterning decisions do not merely guide cells during migration but during differentiation as well. In another study, researchers found that exposing free-living planaria to praziquantel, an anti-parasitic drug, caused some planaria to die and others to grow two heads (Chan et al., 2014). Praziquantel is known to induce Ca^{2+} uptake in planaria, so the authors believed that the dual effect of the drug was due to varying initial Ca^{2+} concentrations. The researchers also hypothesized that Ca^{2+} entry modulates downstream signaling that contributes to stem cell differentiation. This speculation is consistent with the earlier discovery that Ca^{2+} activates protein kinases and initiates DNA and RNA synthesis (Moraczewski et al., 1986).

To follow up on this insight, the same research team exposed planaria to several serotonergic and dopaminergic ligands, amputated the planaria, and observed their regeneration (Chan et al., 2014). Some ligands, such as acetylcholine, did not result in any alternate morphology. Others, such as haloperidol and trifluoperazine, resulted in a double-headed phenotype in a fraction -- from 4% to 64%, depending on the drug -- of planaria. Still other ligands, such as fluoxetine and serotonergic agonist 8-OH-DPAT, caused 20% of planaria to regenerate with no heads (Chan et al., 2014). The mechanism by which serotonin affects planarian pattern formation is not understood. However, as discussed previously, serotonin plays a role in regulating GJC, which in turn modulates planaria polarity (Orellana et al., 2013). In addition, serotonin has long been known to inhibit RNA synthesis in planaria (Franquinet, 1981) and, in cockroaches, serotonergic nerves have been found to innervate muscles (Yoshimura, 2017). Although there is no definite model for the mechanisms of this interaction, it is clear that there is a link between neurotransmitters such as serotonin, GJC, and pattern formation.

Potential Epigenetic Effects

The discovery of altered planarian morphology upon regeneration prompts another question: if the planaria are re-cut in the absence of the original treatment that disrupted pattern formation, will the planaria be able to restore their original morphology? At the heart of this question is whether polarity determination is purely based on the environment or if it can become permanently encoded in a planarian's genes. There is a basis for the idea that bioelectric properties can exhibit epigenetic permanence, as a 2012 study demonstrated in thale cress, nematodes, fruit flies, mice, and yeast (Jablonka, 2012). However, this claim has yet to be tested in planaria. It is important to bear in mind that, for planaria to continue exhibiting alternate morphologies in subsequent unperturbed cuts, the new polarity directions must be reflected in the chromatin of every cell rather than only in the blastema cells that are amputated each round (Durant et al., 2016). To date, one study has tested the permanence of regenerative defects in planaria (Oviedo et al., 2010). The authors amputated planaria while exposing them to GJC inhibitors and found that they grew two heads. After the GJC inhibitors had left the planaria, they re-amputated the same planaria in water and found that they *still* exhibited a double-headed morphology, as in Figure 2 (Oviedo et al., 2010).

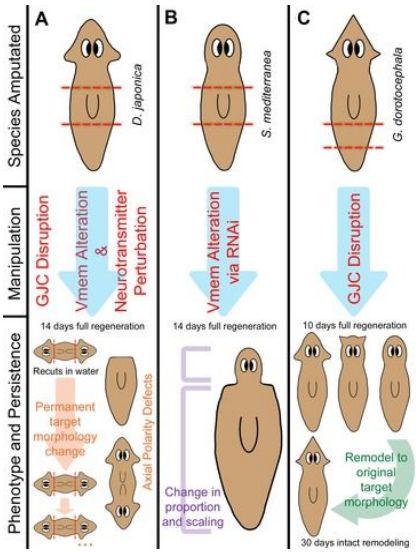

Figure 2 illustrates outcomes of manipulated planarian amputation in different studies. In column A, planaria amputated with GJC and neurotransmitter disruption exhibited a permanent change in their morphology, even after multiple amputation rounds. In column B, hyperpolarization during amputation resulted in a shrunken head phenotype. In column C, GJC blockade initially caused the planaria to grow alternately shaped heads, but after 30 days they remodeled to their target morphology (Durant et al., 2016).

This is a potential precedent for my experiment. Although epigenetic modulation of neoblast differentiation is suspected, its mechanisms are still unknown. Researchers have ruled out DNA methylation as a mechanism; instead, they believe it can be attributed to histone modification or chromatin remodeling (Dattani et al., 2019). As such, the epigenetic role in planarian regeneration is a pressing question worthy of investigation.

The discovery that morphology can be permanently or near-permanently altered in response to GJC inhibition suggests that planarian pattern formation does have epigenetic components.

Questions to Explore

Researchers have been studying planarian regeneration for decades, but our understanding has just scratched the surface. In particular, the possibility of sustained changes in pattern formation raises many questions that could challenge how we think of planarian regeneration. Although long-lasting altered morphology has been demonstrated in response to GJC disruption, it has yet to be shown for other methods of polarity manipulation such as drug exposure. In order to further understand whether planarian pattern formation as a whole can be permanently altered via epigenetics, studies in which planaria are re-cut after exposure to serotonergic and dopaminergic ligands are needed. The following experiment, wherein planaria are exposed to the serotonergic agonist 8-OH-DPAT after amputation and then re-cut in an unperturbed round after regeneration, seeks to investigate this very question. The results of regenerated phenotypes in the second round of regeneration will provide insight into a potential epigenetic model for planarian repatterning.

2. Results

Majority of Planaria Regenerated within Fourteen Days

In order to investigate the morphological effects of 8-OH-DPAT on planarian regeneration over multiple generations, we amputated each planarian into head, trunk and tail fragments and exposed half of them to 8-OH-DPAT solution. Once the fragments completely regenerated, we amputated them a second time without 8-OH-DPAT exposure (see Figure 3). As shown in Table 1, most planaria in both rounds of amputation regenerated fully, whether to a normal morphology or a divergent one. Throughout the experiment, only five planaria -- all of which were Divergent -- failed to regenerate (see Table 2).

Figure 4 contains images of planaria from both rounds of regeneration in order to illustrate the steps of regeneration as well as the distinctions between the ultimate phenotypes. Regeneration begins with blastema formation, which is shown by the blue arrows in Figure 4. Over the next few days, the cells in the blastema begin differentiating into new body parts (purple arrows),

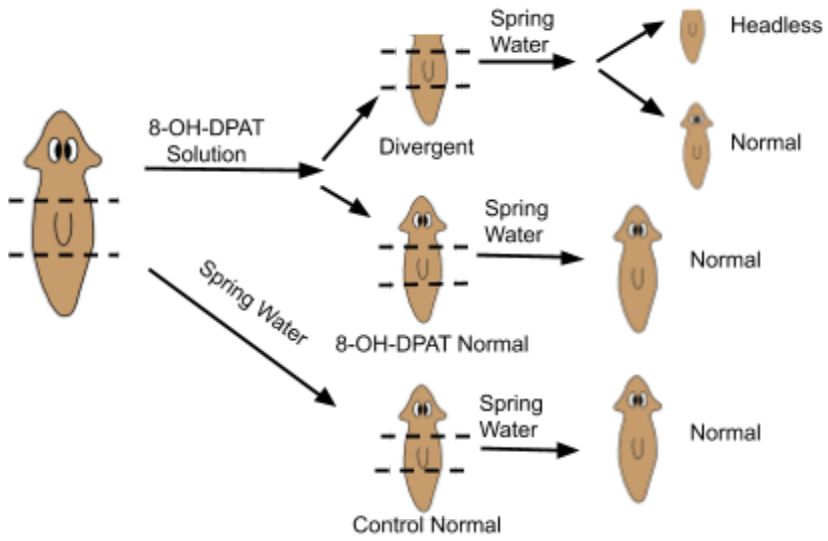

Figure 3 summarizes the experimental process (in chronological order from left to right) as well as the resulting phenotypes of the planaria after each step. The dotted lines indicate the locations of amputation, which are always directly above and below the pharynx. The "Headless" and "Divergent" labels describe the same phenotype, but the separate names are given in order to differentiate planaria that regenerated with this phenotype after the first round of amputations from those with this phenotype after the second round.

which are small and lighter in color than existing fragments. 1C and 1F are photomicrographs of fully regenerated planaria with a typical morphology. The blurriness is due to the quick speed which which fully regenerated planaria swim. 1I depicts a fully regenerated planaria categorized as "No-Head." It qualifies as "No-Head" because its posterior end has a pointy tail, while the anterior region is rounded and lacks the triangular head shape and eyes. Because there were no blastema and this morphology persisted for several days, the possibility that the planaria were still regenerating was ruled out. 1G and 1H are representative of the planaria characterized as "Failed to Regenerate" because they did not grow, replace missing tissue, or gain mobility after amputation. These were the criteria used to score planaria after 14 days of regeneration.

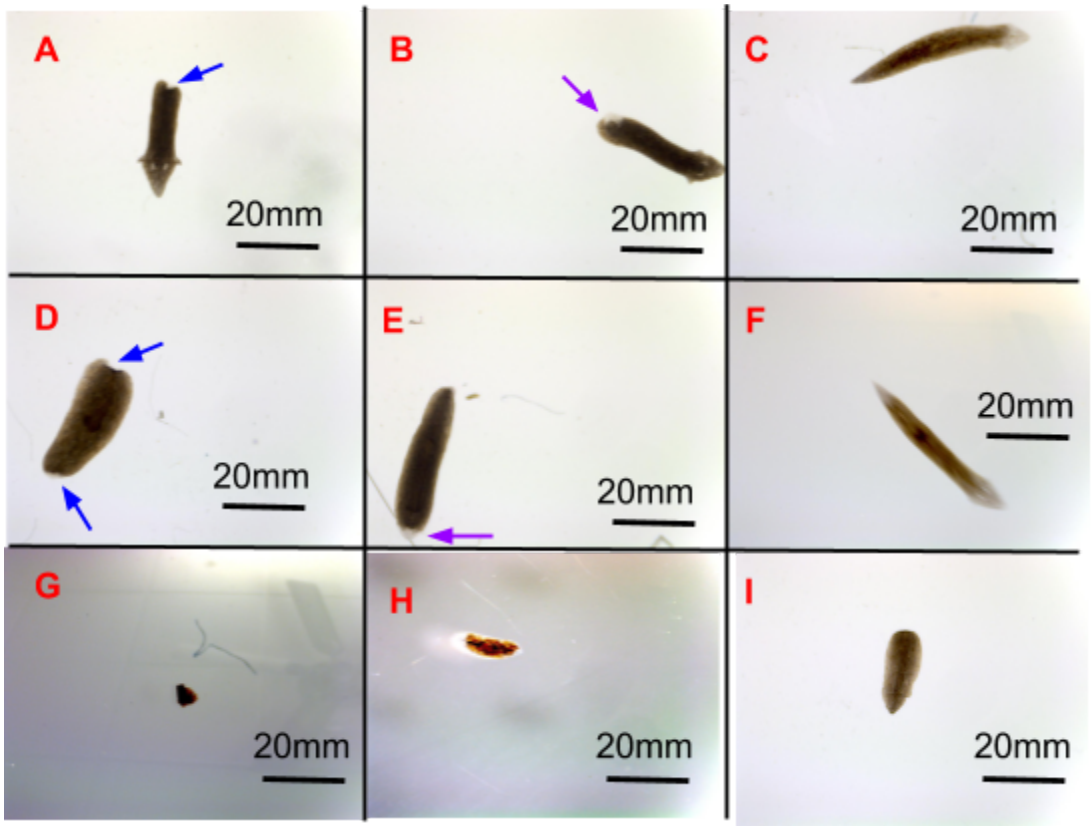

Figure 4 captures a representative sample of planaria to illustrate the process of normal regeneration over time (top two rows) as well as the appearance of planaria that failed to regenerate, as photographed on the Leica Acquire application. (A) A planarian head fragment 24 hours after amputation. (B) A head fragment 6 days post amputation. (C) A fully regenerated planarian with a normal phenotype. (D) A trunk fragment 24 hours post amputation. (E) A tail fragment 6 days post amputation. (F) Another regenerated Normal planarian. (G) A planarian that failed to regenerate after 14 days. (H) Another planarian classified as "Failed to Regenerate" after 14 days due to its lack of growth since amputation. (I) A fully regenerated planarian with a headless phenotype.

11.2% of Planaria Exposed to 8-OH-DPAT Regenerated Headless Phenotype

To determine the morphological effects of 8-OH-DPAT on regenerating planaria, I amputated 75 planaria above and below the pharynx and placed the approximately 225 resulting fragments in a glass dish with 250mL 10 μM 8-OH-DPAT solution. For my control group, I amputated 75 more planaria in the same manner and placed the approximately 225 resulting fragments in a separate glass dish with 250mL spring water. After 24 hours, I replaced the 8-OH-DPAT solution with 250mL spring water. I continued general planaria maintenance and photographing while the planaria regenerated. 14 days later, when the regeneration process was complete, I inspected each planarian in both groups under the microscope and categorized its phenotype (Table 1).

Every planarian in the control group exhibited a “normal” phenotype that corresponded to the pre-amputation morphology. By contrast, 11.2% of the 8-OH-DPAT-exposed planaria regenerated to a headless phenotype.

Table 1: Number of Planaria Exhibiting No-Head Phenotype after First Round of Regeneration

	Normal Phenotype	No-Head	Percent No-Head
8-OH-DPAT	**79**	**10**	**11.2**
Control	**120**	**0**	**0**

17.6% of Divergent Planaria Regenerated Headless Phenotype in Second Round

After the first round of regeneration was complete, I separated the planaria into three groups: those that were exposed to 8-OH-DPAT and regenerated with a headless phenotype (“Divergent”), those that were exposed to 8-OH-DPAT and regenerated normally (“8-OH-DPAT Normal”), and those that were not exposed to 8-OH-DPAT, which were all phenotypically normal (“Control Normal”). I amputated the planaria above and below the pharynx once again, placing them in separate dishes by group as listed above. This round of amputation was unperturbed; no 8-OH-DPAT was dissolved in the spring water.

Once more, I maintained the planaria for 14 days during regeneration and scored their phenotypes once their morphologies stopped changing. Both the 8-OH-DPAT Normal and Control Normal groups had a 0% headless phenotype rate; in other words, they all regenerated to the typical target morphology. However, of the 17 surviving Divergent planaria, 3 of them exhibited a headless phenotype in this second round. This is a 17.6% prevalence, which has no statistically significant difference from the first round rate of 11.2%. However, it’s important to note that the 17.6% statistic is only out of the planaria that regenerated divergently in the first round, not all the planaria that were exposed to 8-OH-DPAT. The proportion of planaria that were initially exposed to 8-OH-DPAT and exhibited a headless phenotype after the second amputation is 1.4%.

Table 2: Number of Planaria Exhibiting No-Head Phenotype after Second Round of Regeneration

	Normal Phenotype	No-Head	Failed to Regenerate	Percent No-Head
8-OH-DPAT Normal	**~200**	**0**	**0**	**0**
Control Normal	**~300**	**0**	**0**	**0**
Divergent	**14**	**3**	**5**	**17.6**

Inferior Mobility in Planaria with Headless Phenotype

Following the precedent of a 2017 study that identified Ca^{2+} as a common denominator in planarian repatterning, nucleic acid synthesis, and neuromuscular function (Chan et al., 2017), I performed two mobility assays during the second round of regeneration: one 8 days post-amputation and another 15 days post-amputation. In these assays, I exploited the negative phototaxis of planaria by measuring how quickly planaria in each group swam away from a concentrated flashlight beam. I started with 10 planaria in the beam and recorded the number left in the beam every 30 seconds for 10 minutes.

In the first mobility assay (Figure 5A), the 8-OH-DPAT Normal planaria were the fastest to leave the light, closely followed by the Control Normal planaria. The Divergent planaria were the slowest by far; 7 of the 10 remained in the light after the 5 minute time frame had passed. However, using a Log-Rank test, none of these differences were statistically meaningful ($p = 0.37$). In the second mobility assay (Figure 5B), each group respectively exhibited higher mobility, as measured by the number that left the light within 5 minutes. This time, the Control Normal planaria left slightly faster than the 8-OH-DPAT Normal ones. The Divergent planaria were still the slowest, but only 3 of 10 failed to leave the flashlight beam. Once more, the differences between these three groups were not statistically significant ($p = 0.23$). However, the Divergent group did exhibit a statistically significant increase in mobility from the first assay to the second ($p < 0.01$), suggesting that Divergent planaria may have delayed mobility development, but are perhaps able to be as mobile as planaria with Normal phenotypes once enough time has passed. An interesting follow-up experiment would be to test whether Divergent planaria ever attain the same level of mobility as Normal ones. Furthermore, it is important to bear in mind that not all Divergent planaria were regenerating Headlessly during this time. The Divergent planaria used in the mobility assays were a representative sample including both Headless and Normal planaria. Visually, we observed that Divergent planaria that were regenerating to be Headless left the light far slower than Divergent planaria that were regenerating to be Normal.

These data suggest that all planaria, regardless of serotonergic drug exposure, become increasingly mobile throughout the regeneration process. Furthermore, our findings imply that Divergent morphology in the first round of regeneration is a better indicator of decreased mobility than either 8-OH-DPAT exposure *or* Divergent morphology in the second round. Even Divergent planaria that regenerated normally after the second round of amputations appeared less mobile than 8-OH-DPAT Normal planaria. Consequently, this suggests that the same variable that may cause an 8-OH-DPAT-exposed planarian to regenerate with a divergent morphology also may cause decreased mobility.

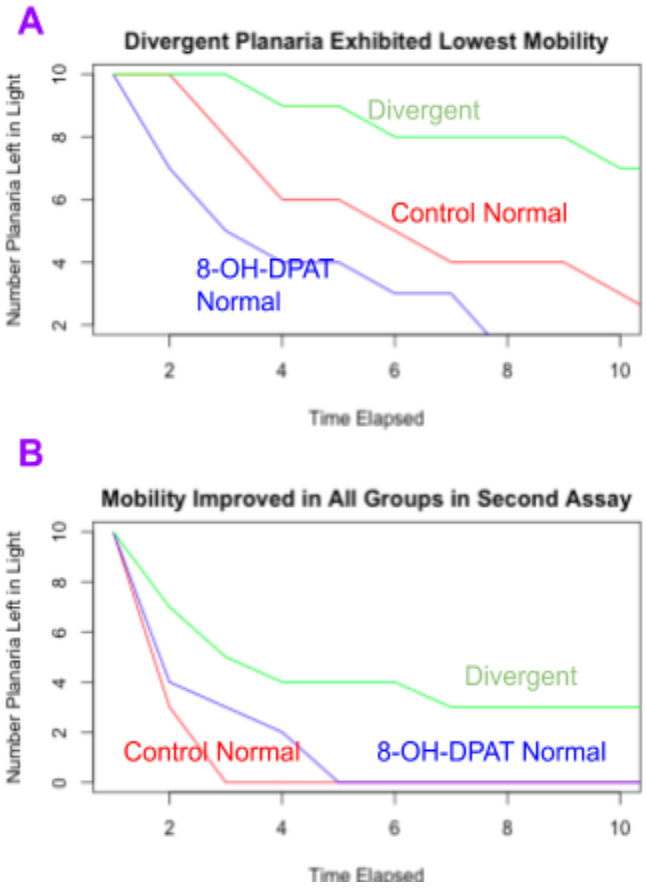

Figure 5 depicts the number of planaria from each group remaining in a narrow beam of light at 30-second intervals; exiting the light beam indicates high mobility. Divergent planaria were less mobile than planaria with Normal phenotypes, regardless of whether they had been exposed to 8-OH-DPAT in the first round, but that all groups became more mobile as regeneration progressed. (A) Results of mobility assay conducted 8 days after the second round of amputations (p=0.37). 8-OH-DPAT Normal planaria were the most mobile, with Control Normal planaria close behind. By contrast, Divergent mobility were far less mobile; by the end of 5 minutes, only 3 had left the light beam. (B) Results of mobility assay conducted 15 days after the second round of amputations (p=0.23). The planaria in each respective group exhibited more mobility than they had one week prior. The difference between the groups was less than it was in the first assay. All 10 of the 8-OH-DPAT Normal and Control Normal planaria exited the light area during the assay, although Control Normal planaria did so faster.

3. Discussion

The objective of this experiment was to verify that 8-OH-DPAT exposure induces a headless phenotype in regenerating planaria and then to investigate whether the headless phenotype persists after a subsequent round of amputation without 8-OH-DPAT exposure. In order to answer these questions, we amputated 150 planaria above and below the pharynx and placed half the fragments in 250mL 10 μM 8-OH-DPAT solution and the other half in 250mL spring water. At the end of regeneration, we grouped the planaria by phenotype (Divergent, 8-OH-DPAT Normal, Control Normal) and performed a second round of amputation without 8-OH-DPAT. At 8 and 15 days after the second round of amputation, we conducted a mobility assay on each of the three groups.

From our findings, we conclude that 8-OH-DPAT exposure after amputation not only induces a headless phenotype in a minority of planaria, but that it creates an epigenetic effect such that this headless phenotype remains present after a subsequent round of amputation in the absence of

8-OH-DPAT. The 11.2% prevalence of headless morphology we observed after the first round had no meaningful statistical difference from the 20% prevalence found in the study we used as our precedent, using a 2 Proportion Z Test ($p = 0.11$) (Chan et al. 2014). Of all the planaria exposed to 8-OH-DPAT, only 1.4% exhibited headless morphologies in the second round, which is a statistically significant decrease, as determined by a 2 Proportion Z Test ($p < 0.01$). We also conclude that the headless phenotype induced by 8-OH-DPAT exposure, but not 8-OH-DPAT exposure itself, leads to decreased planarian mobility.

Using our observations as a starting point, here is the mechanism we propose: 8-OH-DPAT activates extracellular 5-HT (serotonin) receptors on postsynaptic cells, which may activate a downstream signaling cascade that turns off RNA synthesis of head-inducing molecules. Consistent with our observation of continued Headlessness after a second unperturbed cut, we propose that this signaling cascade works to alter gene expression in an epigenetic manner. This hypothesis is supported by our experiment as well as a thorough review of the literature, which has concluded that serotonin inhibits RNA synthesis in regenerating planaria (Franquinet et al., 1981) as well as that histone modification can lead to epigenetic effects in planaria (Dattani et al., 2019). Our proposed mechanism suggests that there is at least one head-inducer whose production is downstream of planarian 5-HT receptors and without which heads cannot form. Although this model is just a hypothesis at this time, if it were to be verified it would provide first-of-its-kind clarity regarding the mechanism of planarian head regeneration and begin bridging the gap between what we currently know and total understanding.

Although our mobility assay was mostly inconclusive, we are confident in our observation that Headless planaria were less mobile than non-Headless planaria, regardless of 8-OH-DPAT exposure or Divergence in the first round of regeneration. Our proposed mechanism, as supported by the dual effect of Ca^{2+} as an establisher of axial polarity as well as a key agent at the neuromuscular junction (Chan et al., 2017), is that planarian with altered morphologies may have a Ca^{2+} imbalance during regeneration that inhibits signaling in planarian musculature. The potential connection between 8-OH-DPAT and Ca^{2+} has yet to be explored outside of their shared importance in repatterning.

A primary limitation of our study was the lack of information it provided regarding the mechanisms of remodeling and, by extension, how 8-OH-DPAT alters them. We can draw speculative conclusions based on our findings, but further study will be needed for confirmation. Another setback was the small sample size of Divergent planaria; only 14 of the initial 30 survived the second round of regeneration. In order to draw more conclusive results, an even larger number of planaria should be exposed to 8-OH-DPAT in the first round. Additionally, we were limited in the precision with which we could create a 10 μM 8-OH-DPAT solution. Due to the small bottle in which the powder arrived and its low solubility, it is likely that some of it was

lost during the transfer. A final limitation of this experiment was our inability to track each planaria fragment individually due to the large number of planaria. Consequently, we could not determine whether Divergent or Headless planaria regenerated from head, trunk or tail fragments as well as whether there were subtle phenotypic differences between Divergent planaria that remained headless after the second round and those that went back to a normal phenotype.

The most pressing question that should be investigated by future experiments is the mechanisms of this phenomenon. These studies should perform Western Blots or other whole-mount immunostaining techniques to detect the presence of head and tail markers, such as *wnt1* and *notum*, before and after regeneration in the presence of 8-OH-DPAT. It would be interesting to learn whether head markers are present in headless planaria. These assays must be performed across multiple rounds of regeneration in order to detect potential changes in these markers, which will give insight into the epigenetic model. Moreover, we hope that future experiments will separate regenerating planaria into heads, trunks and tails to answer one final question: can head fragments exposed to 8-OH-DPAT regenerate into headless planaria? If this technique of separating fragments is used in tandem with essays against head and tail markers, the findings should reveal crucial information about how 8-OH-DPAT interferes with repatterning. Lastly, cutting-edge forms of epigenetic sequencing such as chromatin immunoprecipitation (ChIP) sequencing, which have recently been deployed in planaria studies (Dattani et al., 2019), should be used to gather genotypic observations to corroborate phenotypic studies such as this one. Whether these experiments verify the proposed model set forth in this paper or discover a different mechanism, the insight into the complex process of planarian regeneration will have weighty implications in the fields of tissue engineering and regenerative medicine.

Lastly, in addition to molecular mechanisms, the duration of the 8-OH-DPAT effect was not conclusively determined by my experiment. Does the headless phenotype propagate indefinitely through countless generations, or does it stop after two rounds of regeneration? Does the proportion of headless planaria decrease linearly over time? The answers to these questions may also provide a key for unlocking crucial information about planarian epigenetics.

In summary, we found that 24 hours of 8-OH-DPAT exposure induces a headless phenotype during the first two rounds of planarian regeneration. It appears that 8-OH-DPAT has an epigenetic effect on planaria.

4. Materials and Methods

General Maintenance

Brown *Dugesia Dorotocephala* planaria were obtained from Carolina Biologicals and kept in 250mL Arrowhead spring water in glass Pyrex dishes. The pie dish had diameter 12 cm, the circular dish had radius 10 cm, and the square dish had side length 15 cm. Before amputation, planaria were fed twice per week with a pea-sized chunk of hard-boiled egg yolk for 1 hour before removal of the yolk. Their water was changed twice a week (24 hours after the feeding period ended) by pipetting the planaria into a plastic jar, replacing the spring water in the dish, and pipetting the planaria back. Beginning one week before amputation and continuing for the duration of the experiment, the planaria were no longer fed. Their water was then changed every 24 hours for 4 days after amputation, then every second day. Due to the large number of planaria after amputation, their water was simply decanted and replaced while they remained in their dish. Throughout the experiment, the water was kept at room temperature: 68°C. Plastic wrap with generous holes poked was stretched over the surface of the containers to prevent evaporation.

Amputation Technique

A thin layer of spring water was added to a fresh Petri dish and chilled on ice for 30 minutes. One planarian was pipetted into the Petri dish. Using a scalpel with a curved blade, a firm and swift guillotine-like motion was used to amputate the planarian immediately above its pharynx. No sawing motion was used. The blade was promptly lifted straight upwards and then pressed down once more in a guillotine-like action directly below the pharynx. The blade was removed and carefully wiped dry with a paper towel. All three fragments of the amputated planarian were then pipetted into a glass Pyrex dish containing 250mL freshly-changed spring water. No microscope was used.

8-OH-DPAT Exposure

25mg 8-OH-DPAT arrived from Sigma in powder form stored in a bottle. In order to make it into a stock solution, the bottle was inverted into a 200mL tube and tapped to release as much powder as possible. Then, the remaining powder was suspended in 200μL distilled water, pipetted up and down to mix, and then pipetted into the 200mL tube. This was repeated 4 additional times to ensure that the entire 25mg of 8-OH-DPAT was in the 200mL tube with 1mL distilled water. However, the powder was still not dissolved, so 9 additional mL distilled water were added to

the solution for a total of 10mL. At this point, the tube was covered in tinfoil and used as a stock solution at room temperature.

In order to obtain the desired concentration of 10 μM 8-OH-DPAT in 250mL spring water for the planaria, 328μL of the 8-OH-DPAT solution was pipetted into 250mL spring water in a glass dish. Upon amputation, planaria fragments that were designated to be exposed to 8-OH-DPAT were added directly into this dish. 24 hours later, the 8-OH-DPAT solution was replaced with 250mL spring water during the regular water-change.

Photographing Planaria

To obtain photographs of regenerating planaria under a microscope, planaria were placed in a Petri dish of chilled spring water and observed under a Leica Microsystems dissection microscope. The Leica Acquire application was connected to the camera in the Leica dissection microscope. At regular intervals after amputation, representative fragments were photographed using Leica Acquire. These photographs were then printed in black-and-white for documentation.

Mobility Assay

3 Petri dishes were filled with a thin layer of room-temperature spring water. 10 planaria per group (Divergent, 8-OH-DPAT Normal, Control Normal) were pipetted into respective Petri dishes, which were brought to a dark room and placed on a table covered in black paper. The wristband of flashlight was wound around a ring stand on the table such that the flashlight hung from the stand and its beam created a circle of light with a smaller radius than the Petri dish. One Petri dish was placed under the beam of light and all of its planaria were moved into the light. Then, a timer was started and the number of planaria remaining in the light circle was recorded every 30 seconds for 5 minutes. Finally, this was repeated with the other Petri dishes.

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References

Beane, W.S., Morokuma, J., and Adams, D.S. (2012). A Chemical Genetics Approach Reveals H,K-ATPase-Mediated Membrane Voltage is Required for Planarian Head Regeneration. Chem Biol. *18*, 77-89.

Chan, J.D., Agbedanu, P.N., and Zamanian, M. (2014). 'Death and Axes': Unexpected Ca2+ Entry Phenologs Predict New Anti-schistosomal Agents. PLoS Pathog. *10 2*, 10.1371/journal.ppat.1003942.

Dattani, A., Sridhar, D., and Aboobaker, A.A. (2019). Planarian flatworms as a new model system for understanding the epigenetic regulation of stem cell pluripotency and differentiation. Seminars in Cell & Dev. Bio. *87*, 79-94.

Durant, F., Lobo, D., and Hammelman, J. (2016). Physiological controls of large‐scale patterning in planarian regeneration: a molecular and computational perspective on growth and form. Dev. Bio. *6*, 78-102.

Eisenberg, L. and Eisenberg, S. (2007). Evaluating the Role of Wnt Signal Transduction in Promoting the Development of the Heart. Scientific World *7*, 161-176.

Franquinet, R. and Martelly, I. (1981). Effects of serotonin and catecholamines on RNA synthesis in planarians; in vitro and in vivo studies. Cell Differ. *10 4*, 201-209.

Gentile, L., Cebria, F., and Bartscherer, K. (2011). The planarian flatworm: an in vivo model for stem cell biology and nervous system regeneration. Dis. Model Mech. *4*, 12-19.

Iglesias, M., Gomez-Skarmeta J.L., and Salo, E. (2008). Silencing of *Smed*-β*catenin1* generates radial-like hypercephalized planarians. Development *135*, 1215-1221.

Jablonka, E. (2012). Epigenetic Variations in Heredity and Evolution. Clinical Pharmacology & Therapeutics *92 6*, 683-688.

Moraczewski, J., Martelly, I., and Franquinet, R. (1986). Protein phosphorylation and the role of Ca2+ in planarian turbellarian regeneration. Hydrobiologia *132 1*, 223-227.

Orellana, J.A., Martinez, A.D., and Retamal, M.A. (2013). Gap junction channels and hemichannels in the CNS: regulation by signaling molecules. Neuropharmacology *75*, 567-582.

Oviedo, N.J., Morokuma, J., and Walentek, P. (2010). Long-range neural and gap junction protein-mediated cues control polarity during planarian regeneration. Dev. Bio. *339*, 188-189.

Owlarn, S. and Bartscherer, K. (2016). Go ahead, grow a head! A planarian's guide to anterior regeneration. Regeneration *3 3*, 139-155.

Pellettieri, J. (2018). Regenerative tissue remodeling in planarians – The mysteries of morphallaxis. Seminars in Cell & Developmental Biology, https://doi.org/10.1016/j.semcdb.2018.04.004.

Reuter, H., Marz, M., and Vogg, M. (2015). β-Catenin-Dependent Control of Positional Information along the AP Body Axis in Planarians Involves a Teashirt Family Member. Cell Reports *10 2*, 253-265.

Tasaki, J., Shibata, N., and Nishimura, O. (2011). ERK signaling controls blastema cell differentiation during planarian regeneration. Development *138*, 2417-2427.

Yoshimura, R., Suetsugu, T., and Endo, Y. (2017). Serotonergic transmission and gap junctional coupling in proventricular muscle cells in the American cockroach, *Periplaneta americana*. Journal of Insect Phys. *99*, 122-129.