Retinoic acid inhibition impairs planarian eye regeneration.
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Retinoic acid is a known morphogen in regulating animal growth and development. Planaria are a key model system for regeneration and their eyes are a morphological marker of anterior differentiation. We explored the requirement for retinoic acid signaling in the regeneration of body parts in the planaria *S. mediterranea* using an inhibitor of retinoic acid synthesis, diethylaminobenzaldehyde (DEAB). Whole planaria, soaked in DEAB for three days prior to and five days following amputation, produced trunk and tail fragments with defective anterior regeneration. Following regeneration, up to 80% of posterior fragments developed abnormal eyes. The abnormalities included animals without eyes, with only a single eye, with one enlarged eye, or two eyes of different sizes. Eyes were considered to be functional because animals responded to blue laser light with turning behavior. No abnormalities in eye regeneration were observed in side by side vehicle controls. These results suggest that retinoic acid is necessary for normal eye regeneration following injury and supports a previously undocumented signaling role in planaria eye development.

**Abbreviations:** DEAB – diethylaminobenzaldehyde, RA – retinoic acid, ALDH2 - aldehyde dehydrogenase 2

**Keywords:** Amputation; Eyespot; Regeneration; Planaria; Stem Cells; Retinoic Acid; Morphogen

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**Introduction**

Neurodegenerative diseases like Alzheimer’s, Parkinson’s, and Huntington’s are debilitating and currently incurable diseases that cause 16.5% of global deaths (GBD2016 Neurology Collaborators, 2019). These diseases destroy and damage neurons, the principal communicator in the nervous system, which ultimately inhibits movement and mental processing. Research into regenerative therapies can provide critical breakthroughs to repair neurons and provide remedies to these diseases. The low success of regeneration in vertebrate tissues, particularly neurons, has prompted researchers to explore the mechanisms of regeneration in other organisms. One key model organism that has mastered regeneration are freshwater flatworms or planarians.

Planarians are triploblastic organisms that have complex digestive, reproductive, and nervous systems (Nogi et al., 2009). The central nervous system includes a cephalic ganglia with two lobes and two nerve cords that extend along the anterior-posterior axis of the animal. The most notable exterior morphological feature of the nervous system are two anterior eyespots, located in about the same position as the lobes of the cephalic ganglia. Although commonly referred to as eyes, these structures contain only pigment cells and photoreceptors and are primarily involved in the animal’s response to light (Carpenter et al., 1974; Deochand et al., 2016).

Planaria have the remarkable capacity to regrow their entire body in response to food shortage or injury (Morgan, 1898; Sarnat and Netsky, 1985; Reddien and Alvarado, 2004; Accorsi et al., 2016). The large number of somatic stem cells or neoblasts present throughout the planarian mesenchyme enables a small fraction of the flatworm to be able to replace the injured cells and regenerate the entire body, including the central nervous system, in two weeks or less (Gentile et al., 2010). This feat is achieved through the combinatorial use of several cell signaling factors to provide cells with...
positional information (reviewed in Reddien, 2018). For example, along the anterior-posterior axis, high levels of Wnt signaling activity in posterior regions determines tail identities, and manipulations that block this pathway in regenerating trunk fragments result in the transformation of the regenerating tail region into a second head (Reddien, 2018). Similarly, along the dorsal-ventral axis, high levels of bone morphogenic protein activity are required to establish dorsal identities, and in its absence, regenerating fragments develop with a double ventral phenotype (Reddien, 2018). Thus, cell communication is critical for planarian regeneration.

Retinoic acid (RA) is a signaling factor that is key for vertebrate axonal outgrowth and nerve regeneration (Maden, 2007), whose function in planaria remains largely unknown. In newts and goldfish, both gain- and loss-of-RA function disrupt eye regeneration (Tsonis et al., 2000; Nagashima et al., 2009). In two planaria species, *G. tigrina* and *S. mediterranea*, exposure of trunk and tail fragments to RA caused a long-term delay in head regeneration as measured by the appearance of eye spots, without affecting tail regeneration (Romero and Bueno, 2001; Ermakova et al., 2009). These results suggested that RA suppressed the growth of anterior but not posterior cell population (Ermakova et al., 2009). Importantly, neither study examined whether RA is necessary for head and eye spot regeneration. To analyze the function of RA during planaria head regeneration we took a pharmacological approach to block RA synthesis during eye regeneration events. To eliminate RA, we treated planaria with DEAB, a potent pharmacological inhibitor of aldehyde dehydrogenase 2 (ALDH2), the last enzyme and the rate-limiting step in the RA synthesis pathway (Russo et al., 1988). We used the planaria eyes as markers of anterior specification for several reasons. First, eye photoreceptor neurons and pigment cells are regulated by a single eye stem cell population (Lapan and Reddien, 2011). Second, planarian eye development relies on many of the same genes involved in development in other species (Lapan and Reddien, 2011). Third, timing of eye regeneration is constant and independent of type of injury or metabolic state (Deochand et al, 2016). Finally, eye function can be easily assessed based on stereotypical changes in motor behavior in response to light stimuli (King and Newmark, 2012). We report that RA is required for eye development in planaria, laying a foundation for further exploration of the RA signaling pathway during eye formation in planarian worms.

**Materials and Methods**

**Planarian Care and Maintenance**

*Planaria Dugesia japonica* and *Schmidtea mediterranea* were maintained separately in Ziplock plastic containers of worm water in an incubator at 17 °C. The worm water was prepared with purified water and Instant Ocean sea salts, at an osmolarity between 15-16 mOsM/kg. On occasion, commercially available Poland Spring water was also used. The worm populations were fed pureed and strained organic chicken liver twice weekly followed by container cleaning. Experiments were performed on worms that had been starved for one week prior to amputation to reduce variability in the metabolic state of individual worms. We ensured that worms under maintenance conditions were splitting on their own and the tank had plenty of head and body fragments indicating a healthy population.

**Drug Exposure and Amputations**

Similarly sized worms were selected from the population and transferred to six-well plates with worm water containing 0.1% DMSO (vehicle control) or DEAB (4-Diethylamino-benzaldehyde; 10 µM in 0.1% DMSO; experimental) for three days prior to amputation. All reagents were purchased from Sigma-Aldrich. The rationale for pre-exposing the planaria to the inhibitor three days prior to amputation was to prevent the synthesis of lingering RA. Amputations followed procedures noted in Chan and Marchant (2011). Individual worms were placed on a moist filter paper sitting on top of a Parafilm covering a Petri dish of frozen worm water until the worm became immobile. The setup was then placed under a dissecting microscope and the worm was cut with a scalpel or a single-edge razor blade above and below the pharynx to remove all of the anterior region. Immediately after amputation (day zero), the
trunk and tail fragments were transferred to fresh media containing vehicle control or DEAB for five days. The wells were observed each day for dying or shriveled fragments which were removed to waste. After treatment was complete, surviving worm fragments were transferred to a new multi well plate with fresh worm water to continue regenerating. Fragments were observed at day 8, 11 and again at day 16 when the experiment ended. Because we maintained the worms at 17 °C, full regeneration took 16 days.

Test for Functionality of Eyespots
Following regeneration, S. mediterranea were assessed for the functionality of eye spots based on their phototactic responses on day 16. It has been demonstrated that the eye spots are sensitive to light and planaria will avoid shorter wavelengths of white light and UV light (Paskin et. al, 2014). Planaria show less avoidance behavior to longer wavelengths of green light and will swim directly into red light (Paskin et. al, 2014). The behavioral test compared the responses of the control worms to the RA inhibitor-treated worms.

The worms were acclimated to the dark from being in the incubator and the experiment was performed with the room lights off. Each worm was individually tested by shining a hand-held blue laser light (a presentation pointer pen, 405 nm) in front of the direction in which they were freely swimming. The blue laser was held perpendicularly, five inches above the well. The light was clicked “on” until a reaction was observed or 3 seconds had passed, which was recorded as no reaction.

Imaging
Representative worms for each regenerative phenotype for the tail and trunk fragments were imaged under white light on day 16 using a Zeiss Stereoscope, MRc5 color camera and AxioVision LE imaging software. Images were imported into Adobe Photoshop, where worm outlines were pasted onto a black background and assembled into figures.

Results
In preliminary experiments, we used the planarian species Dugesia japonica and treated worms with concentrations of DEAB ranging from 10 - 100 µM (n=10 worms per concentration). We used 10 µM as our starting concentration based on our experience using DEAB to inhibit RA production in zebrafish (Lee and Skromne, 2014). As concentrations above 10 µM were determined to be too toxic to use in planaria, we selected 10 µM DEAB as our working concentration. In the D. japonica species, seven worms that survived treatment with 10 µM DEAB showed impaired eye regeneration; 2 worms regenerated without eyes and five regenerated with only a single eye. Because our D. japonica population was too small for a larger study, we switched to using the Schmidtea mediterrenea species. Notably, the results obtained in these two species were consistent in implicating a role for RA in normal eye development in planarians.

Survival of Planarians
Although all S. mediterranea worms survived the 3-day pre-treatment with 10 µM DEAB, there was significant death of worms following amputation and continued treatment with DEAB. Figure 1 plots the survival curves for trunk and tail fragments for the DEAB treated worms; the results are combined from two experiments (131 worms, total). There were noticeable drops in survival within the first 24 hours following amputation and on the fifth day of DEAB treatment following amputation. By day 16, 55% of the trunks and 54% of the tails had survived. Notably, all fragments treated with vehicle control survived until day 16 (21 worms total).

Eye Morphology
Of the surviving S. mediterrenea worm fragments, 100% of the fragments developed one or two areas with less pigmentation reflecting the presence of a regenerated tissue known as a blastema. Trunk regions contained two blastemas and when eyes regenerated, we considered that blastema to be the anterior region. Within the blastema, more than 80% of the trunk fragments regenerated abnormal eye phenotypes which were noticeably observable on day 16 using a
Figure 1. Survival plots for amputated trunks and tails treated with an inhibitor of retinoic acid synthesis. Whole planaria were treated with 10 µM DEAB for 3 days prior to and 5 days post amputation (day of amputation is day zero, indicated with *). Survival plots are averages from two independent experiments; dashed lines indicate days on which observations were not made. Note that following the DEAB treatment (day 5), worms were transferred with a plastic pipette to fresh worm water. For display of the data from day -3 to day 1, the percent survival was offset for the trunks and tails; both had 100% survival during this period. Additional worms were treated in 0.1% DMSO (vehicle control) on the same time schedule and showed 100% survival of trunks and tails (not shown). Worms were maintained at 17°C.

By day 16, several eye phenotypes were observed in trunk and tail fragments treated with DEAB (Figure 2). These phenotypes include eyes absent, cyclops (only a single eye), enlarged (one long eye, horizontally oriented in the body axis), and asymmetric eyes (two eyes of different sizes, one distinctly larger than the other) (Figure 2). Fewer than 20% of the DEAB-treated trunks or tails regenerated two eyes of the same size (normal eyes). In contrast, all of the control trunk and tail fragments exposed to the vehicle DMSO developed normal eyes (Figure 2).

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stereomicroscope. For the absent eye phenotype, we cannot discern anterior and posterior regions. We never saw evidence of eyes on both poles along the anterior-posterior axis for the trunk fragments. Tail regions contain only one blastema and about 65% of these recovered from amputation without developing eyes while others regenerated a distinguishable anterior region but with abnormal eye phenotypes.

Figure 2. Regenerative morphology of _S. mediterranea_ trunk and tail fragments treated with 0.1% DMSO (Controls; No DEAB) or 10 µM DEAB in 0.1% DMSO (DEAB treatment), at day 16 post-amputation. Treatment protocol as described in Figure 1. Images were taken of live worms that were cooled on ice to reduce mobility. This results in contraction of the body and shape changes. Anterior is to the left. Red arrows denote the position of an eyespot. Note that the enlarged eye phenotype appears as a single eyespot. A photograph of DEAB-treated tail fragments that resulted in the asymmetric eyespots was not available (N/A). The bar indicates 1 mm length.
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RA inhibition did not deform the regenerating fragments. The appearance of shape changes in the body of the worms (Figure 2) is largely explained by differences in how the worms contracted their bodies in response to chilling from the plate of ice which was needed to slow worm movement for photography.

The distribution of the eye phenotypes is shown in Figure 3 for trunks and tails treated with DEAB (black and grey bars, respectively) and for trunks and tails treated with DMSO (vehicle control; white and hatched bars, respectively). In both trunk and tail fragments, RA synthesis inhibition reduced the formation of normal eyes to less than 20%. The most common phenotype observed in these conditions was the formation of blastemas without development of eyespots. In trunk fragments, we observed this phenotype in over 35% of the cases. The absence of eyespots increased in regenerating tail fragments to 60%. We never observed the loss of eyespots in control fragments (Figure 3).

Functionality of normal and abnormal eye phenotypes

A randomly selected subset of the regenerated S. mediterranea worms were examined for functionality of the normal and abnormal eyes. In control experiments, all trunk and tail fragments that regenerated following the DMSO treatment (normal eye phenotype) moved their anterior region away from the blue light and re-directed their swimming in a typical avoidance response. This photophobic behavior occurred immediately after clicking the blue light pointer “on” in front of the swimming path for each worm (n = 16 worms tested).

Regenerated worms from DEAB-treated trunks or tails with the eyes absent phenotype failed to respond to the blue light (n = 44 worms tested). For the tail fragments, we aimed the blue light at the amputated end for which a blastema region was sometimes small. We observed less movement with these tail fragments, but also no apparent change of direction, suggesting lack of sensory response to blue light. For trunk fragments with eyes absent, both poles were tested for a response to blue light since an anterior end could not be determined. Neither pole responded to the blue light.

In contrast to the behavior of the eyes absent phenotype, all DEAB-treated trunks or tails with abnormal eye phenotypes (cyclopic, enlarged, asymmetric; n = 39 worms tested) responded to blue light with the same behavior as observed for fragments with normal eyes. These worms with abnormal eyes changed their swimming direction in response to the blue light aimed in their path of movement. This provides evidence that the regenerated abnormal eye morphology confers sensorimotor function, at least in the ability to sense blue light and to integrate this with a change in the direction of movement.

Discussion

RA signaling pathways are implicated in normal animal development, but have rarely been tested in planarian regeneration. Our study examined the requirement for RA by treating worms with DEAB, a drug that inhibits the enzyme ALDH2 which converts retinal to retinoic acid (RA;
DEAB would thus be expected to reduce the activation of RA receptors, which transcriptome analysis indicates to be enriched in epithelial cells (Retinoic Acid Receptor Alpha; Fincher et al., 2018). The impact of RA inhibition by DEAB on the regeneration of planarian eye spots was strong. Several different phenotypes were produced including planaria without eyes or with only a single eye or eyes of different sizes. This suggests that genes regulated by the retinoic acid receptor activation are important in normal eye development. Because the amputated worms were observed to be otherwise normal, we suggest that RA signaling is not needed for wound healing, blastema formation, regeneration of a tail/posterior region without eyes, and healing processes that restore animal movement and eyespots sensitivity to blue light.

RA appears only to affect eyespot morphology but not functionally. We did not observe redirection of movement when the blue light was aimed at tissue that did not have eyes. The movement behaviors we observed were only seen in planaria with eyespots, even when number of eyes and their morphology was compromised. It remains possible that a more sensitive test of eyespot function could reveal differences that correlate with eye phenotype.

Other studies have shown that eye regeneration in planaria is also sensitive to increased RA signaling. After treatment with all-trans- and 9-cis-retinoic acids, regenerating fragments of both *G. tigrina* and *S. mediterranea* planarian species experienced a long-term delay in appearance of eye spots (Romero and Bueno, 2001; Ermakova et al., 2009). One of these studies speculated whether this disruption is due to real morphogenetic effects of RA on planarian regeneration or just to toxic effects (Romero and Bueno, 2001). Our loss-of-function results support a role for RA in morphogenesis, as non-regenerating planaria tolerated DEAB treatments very well, while regenerating trunk fragments only developed morphologically abnormalities in anterior tissues. The sensitivity to RA level described here for eye regeneration in planaria has also been described for eye regeneration in other species. In zebrafish, for example, one study found that in the eye, RA agonists promote axonal regeneration (Taha et al., 2010) while another study showed that RA agonists inhibited nerve regeneration (Bremer et al., 2017). Together, these observations suggest a critical role for RA in regeneration of eye structures.

Following amputation and upon transfer of the worms from drug or vehicle to fresh worm water in new multi-well plates, the number of deaths increased (day 1 and day 5, see Figure 1). Both of these are periods of greater trauma for the animals. In addition, tail fragments had lower survival in our experiments, perhaps because they are smaller fragments than the trunks. An improvement to the experiment would have been to use larger planaria. The population used in these experiments was dividing on its own by fission after feedings, which is generally considered to be a sign of a healthy population, but it prevented us from collecting larger worms for the experiment.

**Future directions**

Our studies implicate a requirement for RA in normal eyespot regeneration in planaria. Additional pharmacological studies could prove this requirement. For example, the use of a different RA synthesis inhibitor such as Citral would be expected to mimic the effect of DEAB on eyespot regeneration. Such a result would confirm that the observed effect on eyespot regeneration is specific to the loss of RA synthesis. Upon confirmation, one could examine the ability to rescue the normal eye regeneration by application of exogenous RA to animals pre-treated with DEAB or Citral to downregulate endogenous RA production. This experiment may or may not be informative based on the sensitivity of the tissue to RA levels. Careful titration of exogenous RA would be needed to show eyespot recovery while avoiding tissue toxicity. These results would confirm our conclusion that RA is required for normal eye regeneration in planaria.

In our experience, planarian regeneration was complete within 1 week at 23 °C (room temp), but slowed to 16 days at 17 °C. We selected the cooler temperature for our study because at warmer temperatures, we noticed a higher rate of death of fragments post-amputation, even under conditions in which no drugs were used. Likewise, a regeneration process that is about twice as fast minimizes the
opportunity to observe changes. An experimental variation that may be worth examining at the cooler temperature is the duration of the exposure to DEAB. We used a drug exposure protocol reported by Beane and colleagues (Beane et al., 2013) because even hydrophobic drugs need time to diffuse deep into tissues and cells in order to have the opportunity to act on its target. It might still be useful to test the minimum duration of DEAB exposure that is required to produce the abnormal eye regeneration as this might narrow the time period of cellular mechanisms for normal eye regeneration. Additional future directions include advancing the understanding the role of RA in neurodegenerative disease. RA isomers have protective effects on neurodegeneration of cultured hippocampal neurons (Sahin et al., 2005) and RA is being explored as a neuroprotective agent (Das et al., 2019). A better understanding of the role of RA in the regeneration of neural structures is important for a more complete understanding of its therapeutic potential.

In summary, our study reports the presence of abnormal eye phenotypes for regenerating planaria in which RA synthesis has been blocked. The results are consistent with the hypothesis that RA is required for normal eye development and regeneration.

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