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The Time Course and Neuroanatomy of Rhinophore Regeneration in the Nudibranch *Berghia stephanieae*

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**THE TIME COURSE AND NEUROANATOMY OF RHINOPHORE REGENERATION
IN THE NUDIBRANCH, *BERGHIA STEPHANIEAE***

A Master's Thesis Presented

by

ANI MAROYAN

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

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Neuroscience and Behavior Graduate Program

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Approved as to style and content by:

Paul S. Katz, Chair

Karine Fenelon, Member

Ilia Karatsoreos, Member

Kirby Deater-Deckard, Graduate Program Director
Neuroscience and Behavior Program

DEDICATION

I'd like to thank my parents, my younger brother Narek and my friends and mentors who helped shaped me into the scholar I am today. My incredible support system is the reason I am able to push myself to be the best I can be in everything I do. With them by my side I know that I am capable of anything. I'm forever grateful for them.

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To the lab, I'd like to say thank you for being such positive influences in my life. Your eagerness to learn more and the passion for science that each of you possesses is inspiring. You all have shown me what it means to be a successful woman in stem. Thank you.

ABSTRACT

THE TIME COURSE AND NEUROANATOMY OF RHINOPHORE REGENERATION IN THE NUDIBRANCH, *BERGHIA STEPHANIEAE*

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ANI MAROYAN, B.S., UNIVERSITY OF MASSACHUSETTS AMHERST

M.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Paul S. Katz

Within five weeks, the nudibranch, *Berghia stephanieae* (Gastropoda, Mollusca) can regenerate a severed rhinophore, the main olfactory appendage, such that it is indistinguishable from a non-lesioned rhinophore. The rhinophore is a 2 mm long stalk with lateral sides covered in ridges and the distal third covered in fluorescent pigmentation. Its internal morphology is dominated by longitudinal musculature, overlaid by a complex neuronal plexus, which contains neurons that express various neuroactive substances including serotonin and small cardioactive peptide (SCP). Two large nerves originate in the rhinophore ganglion at the base of the rhinophore and run the length of the rhinophore. To quantify the regeneration of these external and internal characteristics, the rhinophore was entirely severed, with animals then sacrificed and dissected at one-day, three-days, five-days, seven-days, 21-days, and 35-days post-lesion.

Immunohistochemical and neuronal tracing techniques were used to study the neuroanatomy of the regenerated structure. At each time point, brains, consisting of the cerebral, pleural, and pedal ganglia with attached rhinophores were quadruple stained with 4',6-diamidino-2-phenylindole (DAPI) and phalloidin to label nuclei and actin respectively, and immunolabeled for SCP and serotonin. The length of regenerated rhinophores was measured and compared with the unlesioned side. There was a period of disorganization in the first five days, followed by rapid increase in size and a gradual slowing of growth from 21 days to 35 days. Ridge reappearance followed a similar trajectory, regrowing from the base of the rhinophore, rapidly after the first five days with gradual slowing and not matching the extent seen on unlesioned appendages. Fluorescent pigmentation on the distal part of the rhinophore was the slowest feature to return, first visible at 21-days post-lesion. These external features were still not equivalent to those seen in unlesioned rhinophores at 35 days. Internally, phalloidin labeling showed that the muscles were disorganized during the first week, but then regrew, matching the return of the external anatomy. Muscle fiber thickness did not vary between regenerated and unlesioned rhinophores. The SCP and serotonin immunoreactivity exhibited different patterns of regrowth. Serotonin fibers were present immediately after lesion at the tip of the rhinophore and increased in density over the rest of the rhinophore with increasing time post-lesion. The SCP plexus, in contrast, was not present until a week after lesion, and at later timepoints there was not a consistent pattern. Lastly, the projections into and from the brain were traced by application of neurobiotin to the cut ends of the rhinophore nerves. There was no obvious difference between regenerated and unlesioned sides five-weeks after the removal of one rhinophore; all major neural tracts were present, including those that traveled from the regenerated side contralaterally through the brain

and up into the contralateral rhinophore ganglion. The neuroanatomy of *Berghia*'s brain and rhinophores thus suggests that after complete severance of one rhinophore, the animal is able to regrow the cells and fiber tracts in five weeks.

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INTRODUCTION

Regeneration is an ability that has contributed to the survival and evolution of many organisms. Gastropods and cephalopods in particular exhibit plasticity in the case of injury (Moffett, 1995). One of the important aspects of regeneration is the regrowth of axons near the site of injury that leads to the regeneration of an appendage over time (Steffensen *et al.*, 1995). Thus, the hallmark of regeneration is to reform axons at the site of lesion and continue to reproduce the features that were once present. These features may not reappear at the same time or follow the same trajectory, which may highlight their importance to the regeneration process and/or the animal's survival.

The time course of regeneration of different organs such as tentacles, siphons, eyes, and mantles vary (Hyman, 1967). When its arm is severed, the cephalopod *Sepia officinalis* takes five days to two weeks to regenerate its appendage depending on the temperature it is kept in (Imperadore and Fiorito, 2018). Similarly, regeneration of the arm in *Octopus vulgaris* was reported to take up to 55 days to fully regenerate (Fossati *et al.*, 2013). Clearly, arms are very important to the prey capture and survival of cephalopods. Other features regenerate more slowly, like the neural fibers in the mantle of *Octopus vulgaris* are not yet organized two weeks after lesion (Imperadore *et al.*, 2017).

The time course of regeneration also varies depending on the type of injury and the features that are regenerating. In the gastropod *Aplysia californica*, about two weeks after nerve crush, sensory innervation began to function again (Dulin *et al.*, 1995). However, in the snail *Melampus bidentatus*, the disrupted synapses and tentacle nerves reconnected to the disrupted nervous system only after 70-days post-cerebral ganglion removal (Moffett and Ridgway, 1988). This is a much longer time course than in *Aplysia* but involved a more extensive manipulation of

removing the entire cerebral ganglion rather than a nerve crush. Even within the same appendage that is regenerating, certain elements of regeneration may take longer to re-appear compared to others. Pigmentation on the skin in the snail *Hydrobia ulvae* forms later in the process of regeneration, after the formation of the tentacle and eye buds (Gorbushin *et al.*, 2001). Exploring patterns of regeneration in different molluscs with different lesions allows for better understanding of how connections and structures re-form during regeneration.

Here, I focus on the regeneration of the sensory organ known as the rhinophore in the nudibranch, *Berghia stephanieae*. Rhinophores are paired olfactory structures on the anterior of the animal's head (*Figure 1A*). They are directly connected to the rhinophore ganglion of the brain, and it is known that the overall outer structure regenerates over several weeks (P.D. Quinlan, unpublished). Previously, *Berghia* was behaviorally tested in a Y-maze before and after rhinophore lesion. It was found that *Berghia* could find prey in a Y maze before lesion but immediately after lesion could only find prey at chance levels (P.D. Quinlan, unpublished). After a five-week regeneration period, they could again find prey indicating that the rhinophores recover behaviorally at that point (P.D. Quinlan, unpublished). However, it is not known how the external and internal neuroanatomical organization regenerates over that same period. I used immunohistochemistry and other staining techniques to explore the neuroanatomical changes that arise following severing of a single rhinophore. Documenting how regeneration occurs for the first time in a nudibranch and determining whether *Berghia* regains all neuroanatomical features will advance knowledge in the field of molluscan regeneration. In the future, it will allow researchers to bridge the gap in understanding causality between regenerated neuroanatomy and behavioral functionality of the appendage.

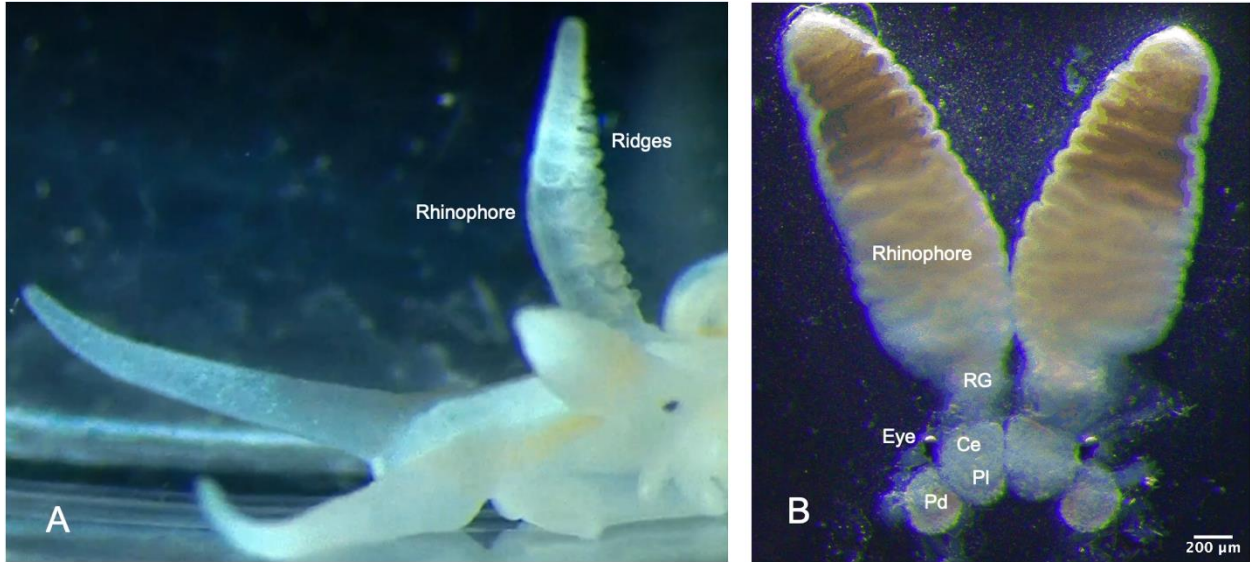


Figure 1 Gross anatomy of the rhinophores. **A.** View of the left side of *Berghia*'s head showing the rhinophores extending dorsally above the eye. Lateral sides of the rhinophores are covered in ridges. **B.** *Berghia*'s bilaterally symmetric rhinophores are situated distally to the rhinophore ganglion (RG). Each RG is connected to the cerebropleural ganglion (Ce, PI) which also has the eyes attached bilaterally. Linked to the CePI are the pair of pedal ganglia (Pd).

METHODS

Animal Care:

Berghia were housed in the Katz Lab tank room in the Morrill Science Center. *Berghia* and the *Aiptasia* that they fed on were housed in separate tanks in Instant Ocean at pH 7.5-8.8 and temperatures at 20-25°C under 12 hours of light and 12 hours of dark in a day.

Surgical Removal of Rhinophores:

Animals that were subject to surgical removal of rhinophores were first anesthetized using cold 4.5% MgCl₂ for 15 minutes in a dish. The right rhinophore was removed under a dissection microscope immediately using dissection scissors. Animals were then moved to jars to track their regenerative growth. One to three *Berghia* were housed per jar, according to the date of lesion of their rhinophore and were fed one to two times a week.

Quadruple Staining of Regenerated Rhinophores at Specific Timepoints:

Simultaneous DAPI and phalloidin staining, and SCP and serotonin immunolabeling was used to track neuroanatomical changes post-lesion at one-day, three-days, five-days, seven-days, 21-days, and 35-days post-lesion of the right rhinophore (*Figure 2A*). These markers were chosen based on previous work in *Berghia* (unpublished). To stain cell nuclei, I used 4'6-Diamidino-2-Phenylindole, Dihydrochloride, or DAPI (ThermoFisher Scientific). Phalloidin (Rhodamine phalloidin, ThermoFisher Scientific) was chosen to stain for actin filaments because rhinophores are known to have extensive musculature throughout. Out of the multitude of neurotransmitters present, I used antibodies for small cardioactive peptide, or SCP (Developmental Studies Hybridoma Bank, University of Iowa) and serotonin (Immunostar) because of their extremely consistent signal in the rhinophores of *Berghia* specifically. Rather than solely cardioactive properties, SCP is also known as a neuropeptide that plays a role in

synaptic transmission in the gastropod *Aplysia californica* (Lloyd, 1986). In *Berghia*, serotonin is used as a marker for the fiber tracks within the rhinophore, but is also expressed in the central brain, with the particular neurons there sending projections up into the rhinophores.

The process of producing this stain occurred over three days. On the first day, 4.5% MgCl₂ was used to anesthetize the animals for a minimum of 10 minutes prior to dissection. An animal was then pinned in a sylgard dissection plate and its brain and rhinophores were removed intact. After dissection, the brain was taken out of MgCl₂ and placed in 250 microliters of 0.25% protease (from *Streptomyces griseus*; Sigma Aldrich) in seawater in a four-well plate for 15 minutes to permeabilize the sheath the enable antibody penetration. Next, the protease was taken out of the plate and the brain was fixed in 250 microliters of PLP fixative (1.85% lysine monohydrochloride, 0.22% sodium periodate, 2% paraformaldehyde in artificial sea water, modified from Newcomb *et al.* 2006) for two hours at room temperature. After two hours, the fix was taken out and the brain was placed in 250 microliters of a phosphate buffer solution, PBS (ThermoFisher Scientific) wash for 10 minutes. Next, the brain was removed from the PBS wash and was placed in 250 microliters of 5% Normal Goat Serum (ThermoFisher Scientific), 5% Triton-X (Sigma Aldrich) in PBS for one hour to act as a blocking step. After this step, 250 microliters of the primary antibodies were added to the plate with the brain. Rabbit anti-serotonin (1:250) and mouse anti-SCP (1:250) were diluted in 0.5% Triton-X in PBS. These antibodies have been used extensively both in our lab and by other researchers using molluscan species who have done the validations and controls. Incubation occurred overnight in the refrigerator.

On the second day, the primaries were washed from the brain and 250 microliters of PBS were then added for three separate washes each lasting 15 minutes on the rotator. After the third wash, 250 microliters of the secondaries were added to the four-well plate containing the brain

and were then covered in foil to avoid light exposure due to the negative impact of light on the secondaries. The secondaries included goat anti-rabbit Alexa 647 (ThermoFisher Scientific), corresponding to the serotonin primary, and goat-anti mouse Alexa 488 (ThermoFisher Scientific) corresponding to the SCP primary (each 1:250) in 2.5% Normal Goat Serum in 0.5% Triton-X in PBS. This solution also contained the rhodamine phalloidin (3:200, ThermoFisher Scientific) The brain was then incubated in the refrigerator overnight.

On the last day of the immunohistochemistry process, the secondaries were removed and the brain was again washed three times in in 250 microliters of PBS for 15 minutes. After the three washes, 250 microliters of PBS with DAPI (1:1000, ThermoFisher Scientific) was added to the brain for one hour and was again be covered in foil since DAPI is also negatively affected by light exposure. After an hour on the rotator at room temperature, DAPI was removed and the brain was washed in 250 microliters of PBS for 10 minutes. After 10 minutes, the PBS was discarded and 250 microliters of DeepClear (Pende *et al.*, 2020) for clearing was added to the brain for 15 minutes and placed on the rotator. Slides were then prepared for the brain to be mounted. After the 15 minutes, a few drops of DeepClear were placed on the 3D printed slide with a coverslip via a glass pipette and the brain was transferred with forceps to the slide into the DeepClear so as not to dry out. Extraneous debris was removed from the brain with forceps under a microscope before the brain was imaged. Once the extra debris was removed and the brain looked clear, the DeepClear was removed from the slide via the glass pipette and 60 microliters of Vectashield anti-fade mounting medium (Vector laboratories Inc.) was added to the slide containing the brain. Another glass coverslip was carefully placed on top of the brain, ensuring bubbles were not present. The brain was then ready for imaging under the Zeiss

confocal microscope. Images were taken with the 10x air objective, using Zen Black software (Zeiss) for capture, tiling, and stitching of images.

Neurobiotin Fills:

Animals used for backfilling first had their right rhinophore severed and were then given a five-week regeneration period (*Figure 2B*). The axons of the regenerated rhinophore were filled with a neurobiotin tracer (Vector laboratories Inc.) to determine the projections and fibers within the brain while also co-labeling cell nuclei with DAPI to show neural structures post-regeneration

Backfills were a two-day process also consisting of multiple steps. On the first day, 4.5% MgCl₂ was used to anesthetize the animals for a minimum of 10 minutes prior to dissection. A single animal was then pinned in a dissection plate and its brain and two rhinophore nerves emerging from the right rhinophore ganglion remained intact after removal. All other nerves around the brain were cut short to prevent accidental fills if the tracer leaked. After dissection was complete, a Vaseline well was constructed on another sylgard plate. A drop of seawater was placed in the middle of the well and the brain was gently transferred to the droplet using forceps. The rhinophore ganglion as well as the two nerves were gently pulled over the well so that the nerves would not be exposed on the outside of the well. A bit of Vaseline was then placed over the ganglion to ensure that it was covered. The nerves, however, remained exposed over the well. The well was then checked for leaks by adding a drop of seawater to the edges at various sites. The well was properly sealed if the drop did not go through. Next, five microliters of 7% neurobiotin in distilled water was placed on the two exposed nerves, using a method adapted

from octopus (Imperadore *et al.*, 2019). The plate was then placed in a humidity chamber for three hours in the refrigerator.

After three hours, the drop of neurobiotin was removed with a Kim wipe. If the drop had dissipated, neurobiotin had leaked into the well and may not have properly stained the brain and nerves, and these samples were not used in further analyses. After the drop of neurobiotin was removed, the brain was placed in a four well plate with 250 microliters of 0.25% protease in seawater for 15 minutes on the rotator. Next, 250 microliters of the 4% paraformaldehyde (PFA) was added to the well to fix the brain for two hours on a rotator at room temperature. After two hours, 250 microliters of 5% Normal Goat Serum in 5% Triton-X in PBS was added to the well and placed on a rotator in room temperature for one hour. After an hour, the Normal Goat Serum block was removed and 250 microliters of 1:300 streptavidin 649 (Vector laboratories Inc.) in 0.5% Triton-X in PBS was added to the brain. The brain was left in this solution covered in foil overnight in the refrigerator.

The next day, the brain was washed in PBS for 15 minutes three times on the rotator. After the three washes, the brain was incubated in DAPI (1:1000) for an hour. After an hour, the brain was washed in 250 microliters of PBS for 10 minutes on the rotator and then in 250 microliters of DeepClear for 15 minutes on the rotator. The same protocol was used to prepare slides as in above. After the 15 minutes, a little bit of DeepClear was placed on the 3D printed slide with a coverslip via a glass pipette and the brain was transferred with forceps to the slide into the DeepClear so as not to dry out. Extraneous debris was removed from the brain with forceps under a microscope before the brain was imaged. Once the extra debris was removed and the brain looked clear, the DeepClear was removed from the slide via the glass pipette and 30 microliters of Vectashield was added to the slide containing the brain. Another glass coverslip

was carefully placed on top of the brain, again ensuring bubbles were not present. The brain was then ready for imaging under the confocal microscope, as above.

Solutions:

Solutions such as PBS and PBTx were discarded into appropriate waste bins after a single use. Primaries, secondaries, DAPI, protease, PLP, PFA, goat serum block, and DeepClear were reused for up to two weeks. New solutions were made every two weeks to ensure they would work properly.

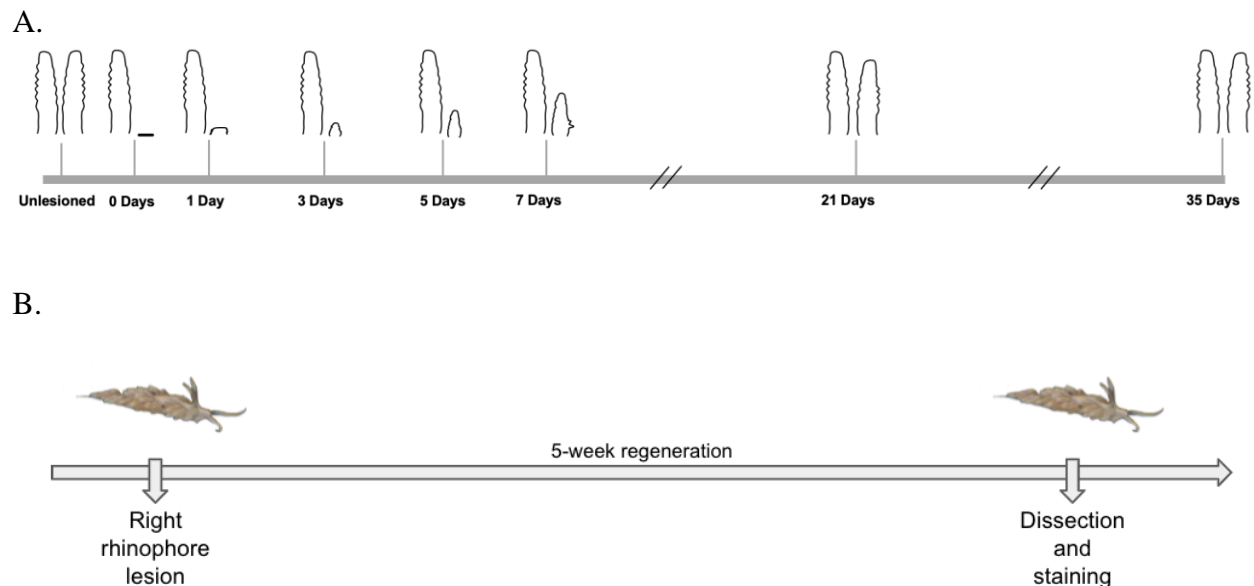


Figure 2 Timelines of quadruple stain and backfill of rhinophore nerves. **A.** All animals first had their right rhinophores lesioned and then were given either a one-day, three-days, seven-days, 21-days, or 35-days regeneration period. Antibodies were used to stain for SCP and serotonin, while cells were also stained for actin with phalloidin. All cells were co-labeled with DAPI. **B.** After animals had their right rhinophores is lesioned, a five-week regeneration period was allowed after which the animal was dissected down to the brain and two rhinophore nerves. Nerves were then filled with neurobiotin tracer and co-labeled with DAPI.

RESULTS

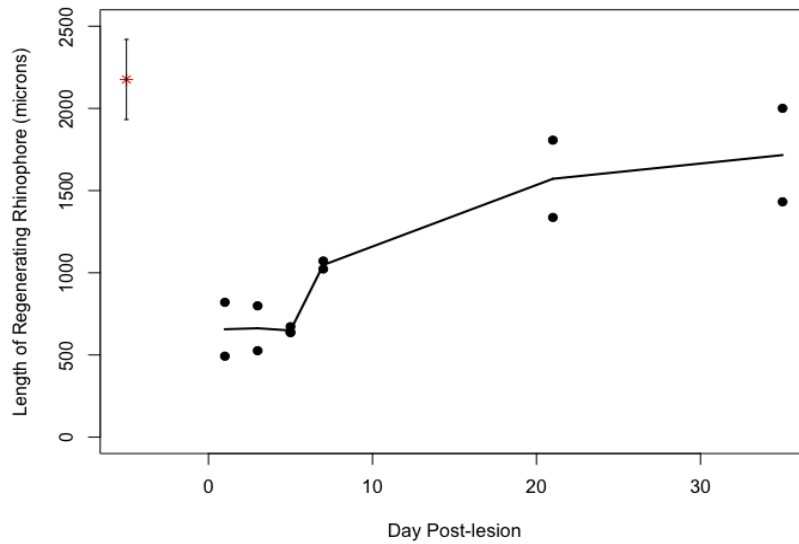
Regeneration of External Features

I. Length

Immediately post-lesion, the rhinophore was a small stump that did not display characteristics that are representative of a rhinophore, such as pigmentation, ridges, or a network of fibers in a plexus. From this short stump, at the measured timepoints, a steep increase in length was noted from one-day to seven-days post-lesion (*Figure 3A*). This accumulation of length plateaued after 21-days post-lesion, as there was no substantial change in length by the 35-day time point (*Figure 3A*). The average of the ratios of the recovered length to the unlesioned rhinophore length at each time point saw a similar pattern of increase until the 21-days post-lesion time point after which the average ratio plateaued at 35-days post-lesion (*Figure 3B*). By 21-days post-lesion the average ratios of the regenerated length showed significant regrowth of the rhinophore. Following the first few days to a week after regeneration, the rhinophore's features began to reappear (*Figure 4*). These will be described in the sections below.

The lengths of the regenerated rhinophores and unlesioned rhinophores were measured to track growth over five weeks. Measurements were taken at the base of the rhinophore in microns where the edge of the rhinophore ganglion met and extended up to the tip of the rhinophore in FIJI (Schindelin *et al.*, 2012). The length was measured under the serotonin stain as serotonin kept up with the regeneration of the rhinophore with staining in the tips. The lengths were graphed using R software.

A.



B.

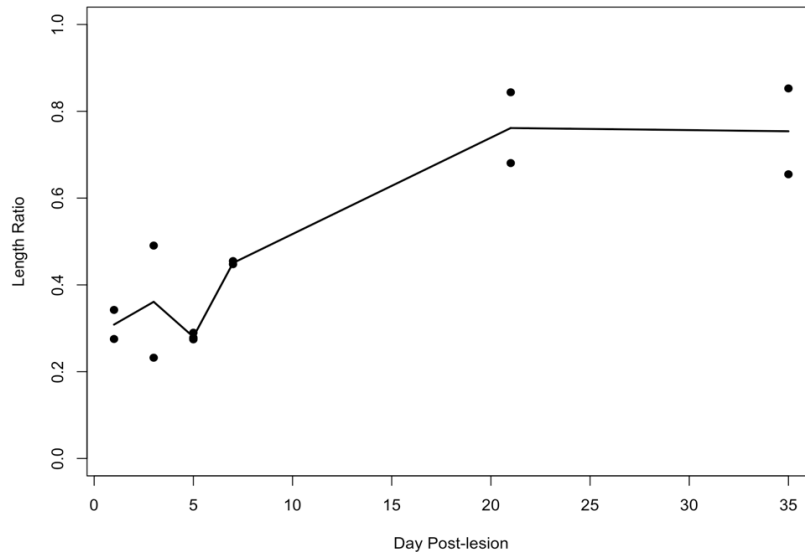


Figure 3 Regenerating rhinophore lengths and ratios at various timepoints post-lesion. **(A)** Rhinophore length increases starting after the five-day post-lesion timepoint and increases more slowly up to five-weeks post-lesion. The black solid line is the average of the individual data (N=2 or N=3) at each timepoint. The lengths of the regenerated rhinophores do not reach the lengths of the unlesioned rhinophores (N=13), with that average shown by a red star and the standard deviation shown by the error bar. **(B)** The average of the ratios of lesioned to unlesioned rhinophore length at each time point increase at one-week post-lesion and continue to increase until three-weeks post-lesion when the ratio plateaus to five-weeks post-lesion. The black solid line is the average of the ratios of each animal's lesioned rhinophore length to unlesioned rhinophore length (N=2 or N=3) at each time point.

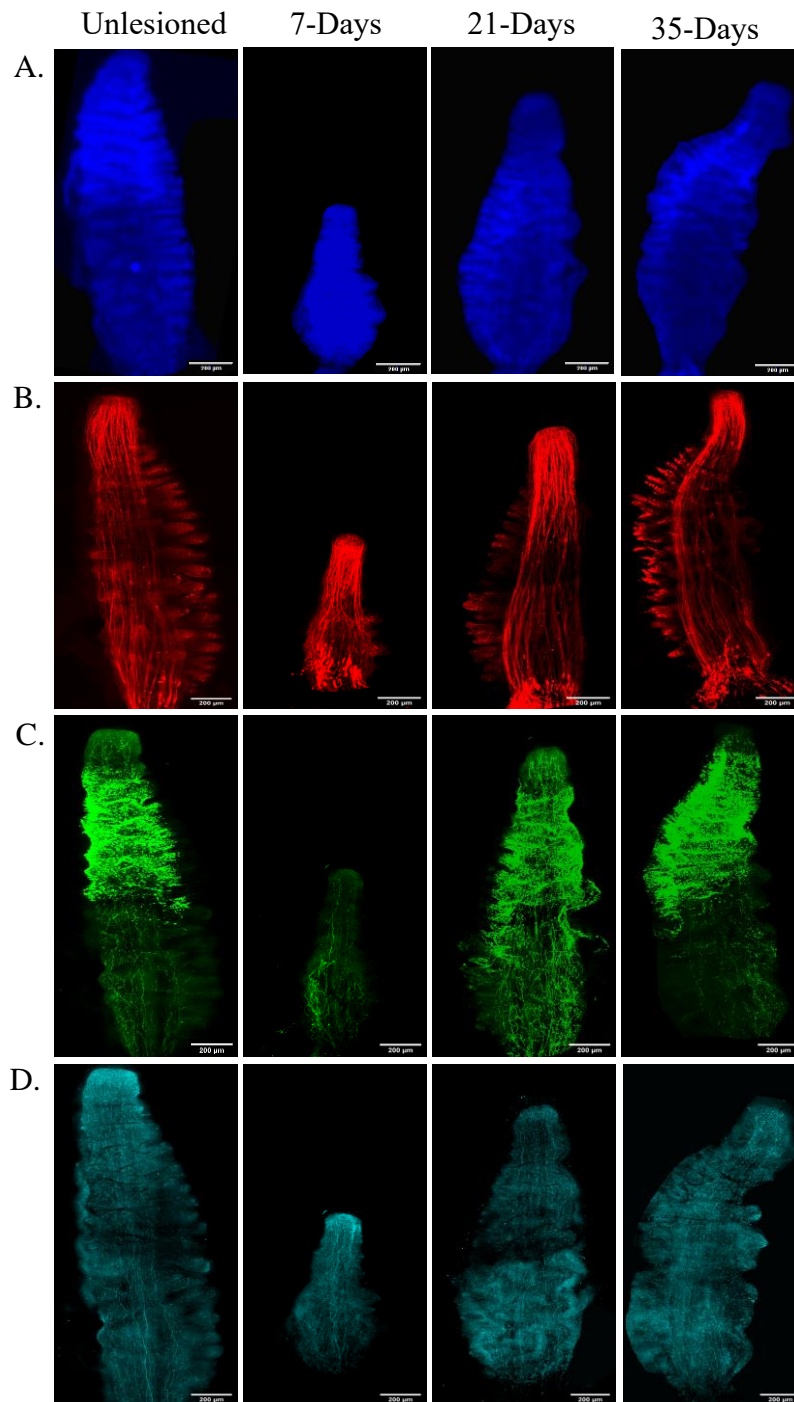


Figure 4 Quadruple staining of rhinophores using DAPI, phalloidin, and immunolabeling for SCP and serotonin. Each column is the same sample. Rhinophores were fixed from an unlesioned side (i), and at a 7-days (ii), 21-days (iii), and 35-days (iv) post-lesion. (A) DAPI shows cell nuclei throughout the rhinophore. The bright area in the distal part is the autofluorescent pigmentation. (B) Phalloidin staining shows actin in muscle fibers extending longitudinally along the rhinophore and actin filaments in the lateral ridges. (C) SCP immunoreactivity shows a plexus at the base of the rhinophore at each timepoint. The bright areas in the distal rhinophore (Ci, Ciii, Civ) are autofluorescent pigmentation. (D) Serotonin immunoreactivity is present throughout the rhinophore at each time point. Scale bar is 200 μm .

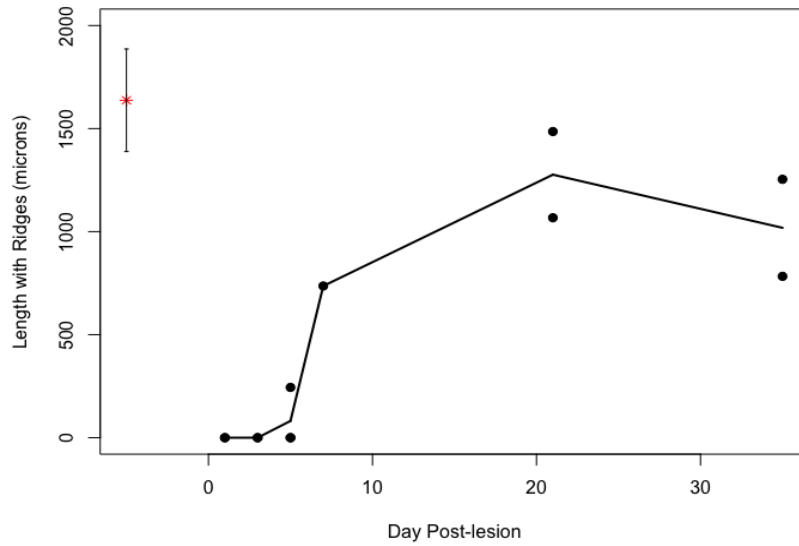
II. Ridges

Phalloidin was used to track the regrowth of the ridges found predominantly on the lateral sides of *Berghia*'s rhinophores. After lesioning the rhinophore, ridges were first noted around five-days to seven-day post-lesion. The one-day, three-days and two of the five-days post-lesions samples had not presented ridges on the regenerated rhinophore, indicating that this feature did not appear until about a week after rhinophore removal. Ridges were first observed on the last sample of the five-days post-lesion rhinophore. By seven-days post-lesion, the length of the ridges was about 2/5 as that of the length of the rhinophore. At this point, the ridges were not yet well defined and were very limited in number (*Figure 4, Bii*). The length of ridges spanning the rhinophores grew significantly at 21-days post-lesion. Here, the ridges looked very similar to ridges of an unlesioned rhinophore, as they were well defined and covered a majority of the length of the rhinophore (*Figure 4, B. and Figure iv, Biii*). The 35-days post-lesion sample did not change significantly from the 21-days post-lesion images, showing a plateau in ridge length growth (*Figure 4, Biv*). At this timepoint, the regenerated rhinophore looked nearly identical to the unlesioned rhinophore. The length of the ridges covering the rhinophore since the 21-day timepoint had not reached the same length covered as the unlesioned rhinophore (*Figure 4, Biii*). Ridges appeared at the same time as muscle fibers became organized, as the length of the regenerated ridges also witnessed a stop in growth at the three-weeks post-lesion time point (*Figure 5A*). The average ratios of the ridged length also increased in a similar manner, indicating that the regenerated ridged length covered almost the same ridged length as the unlesioned rhinophores (*Figure 5B*).

Due to their density, the ridges could not be quantified individually. Instead of counting the ridges, the lengths were taken in FIJI at the point on the rhinophore where ridges are first

observed, up vertically to where the last ridge is seen. Measurements were made in microns. A graph of the ridges was made in R.

A.



B.

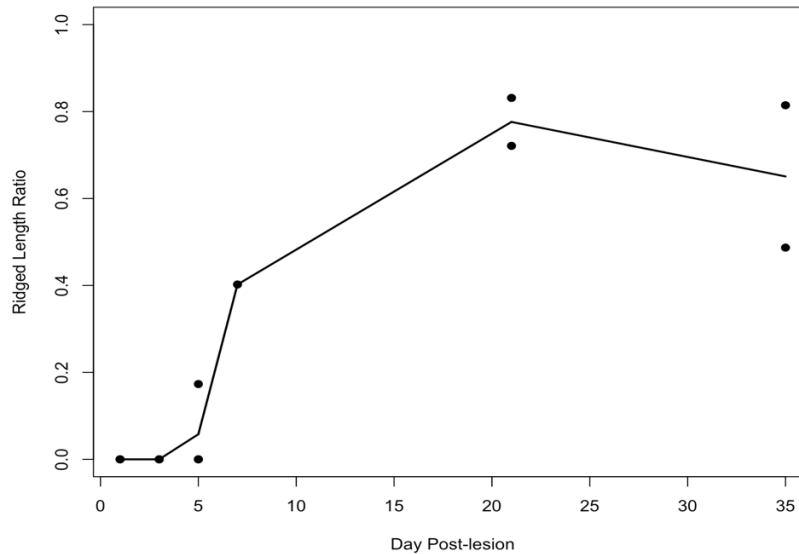


Figure 5 Ridged length and ridged length ratios on the regenerating rhinophores. **(A)** Ridges appear after five days with a maximum at 21-days post-lesion and the average of the data points shown as a black solid line. The mean ridge length of the unlesioned rhinophores (N=13) is shown as a red star with the error bar depicting standard deviation. **(B)** The average of the ratios of ridged length to the length of the entire rhinophore increases sharply from five-days post-lesion to 21-days post-lesion, after which the average ratio drops at 35-days post-lesion. By 21-days post-lesion the regenerated rhinophore has regrown most of its ridges.

III. Pigmentation

Pigmentation on the distal part of the rhinophore re-appeared between seven-days and three-weeks post-lesion. It was visible in the emission spectra used for viewing the DAPI and SCP labelling, which we used to document its presence (*Fig 4A, C*). Pigmentation was not seen until after seven-days post-lesion even though the rhinophore was already increasing in length and ridges were forming after five days (*Figure 4Ciii*). By five-weeks post-lesion, the pigmented region on the regenerated rhinophore was still less than the total area of the unlesioned rhinophore (*Figure 4Ci, Civ, Figure 6A*). The average ratio of the regenerated pigmented area to the unlesioned pigmented area at each time point indicated growth until after three-weeks post-lesion where again the average of the ratios did not substantially change at five-weeks (*Figure 6B*). The average ratio of pigmented area grew to almost the same as that of the unlesioned rhinophore by three-weeks post-lesion (*Figure 6B*).

The area of the pigmented region and the area of the entire rhinophore were calculated for both the regenerated and unlesioned rhinophores in FIJI by drawing regions of interests (ROIs). Measurements were made in microns and the percentages of the area of fluorescence to the area of the entire rhinophore were calculated. A graph of the pigmentation percentages was made in R.

Regeneration of Internal Features

IV. Musculature

Muscle fibers were observed, and at the early stages of regeneration, they appeared disorganized and did not form in specific directions. About five-days to seven-days post-lesion, it was apparent that muscles were elongating and traveling in the right direction as regeneration progressed (*Figure 4Bii*). At 21- and 35-days post-lesion, the muscle fibers continued to elongate with the growth of the rhinophore and maintained the density of the fibers over this period (*Figure 4Biii, Biv*). It was also noted that at the base of the rhinophore, muscle fibers did not undergo changes in thickness throughout regeneration, as they maintained the same breadth over five weeks. Although the thickness of muscles was not affected, the overall appearance and length of the fibers elongated with the growth of the rhinophore from about seven-days post-lesion and continued this pattern throughout the ensuing timepoints.

V. Serotonin immunoreactive plexus

The serotonin antibody labeled fibers traveling up to the tip of the rhinophore across all timepoints, including immediately after lesion (*Figure 4D*). The serotonin plexus was a dense network of fibers across the entire rhinophore. The plexus also formed up to the tips of the rhinophores, expressing serotonin immunoreactivity throughout regeneration (*Figure 7*). Unlike the previously mentioned features, serotonin immunoreactivity was present even at the earliest timepoints post-lesion.

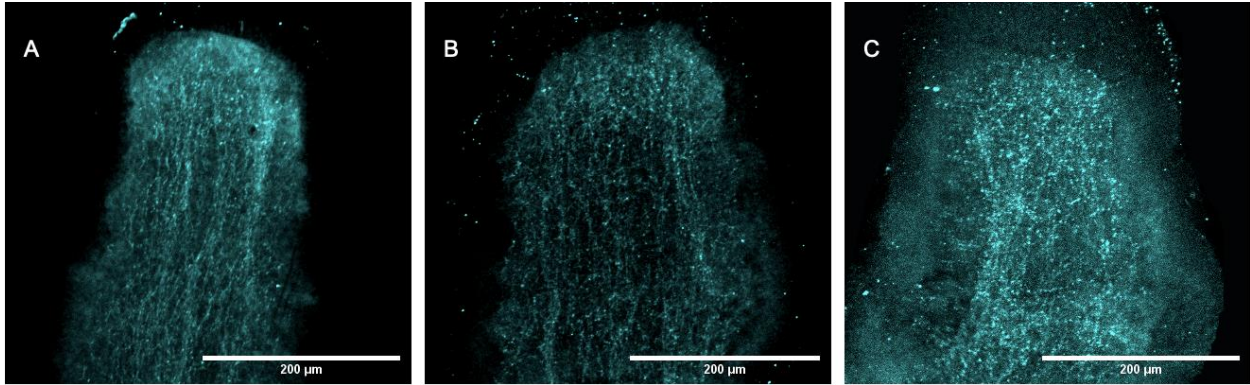


Figure 7 Serotonin plexus at the tip of the regenerated rhinophore. **A.** Seven-days post-lesion. **B.** 21-days post-lesion. **C.** 35-days post-lesion. Scale bar is 200 μm .

VI. SCP immunoreactive plexus

The SCP plexus was first observed much later during regeneration, with the organization of the fiber network visible at seven-days post-lesion, only at the base of the rhinophore. The fibers with SCP were also more intense on the side opposite the ridges, with fibers tapering off towards the tip of the rhinophore (*Figure 8A*). By 21-days post-lesion, the plexus became denser and over-expressed, covering the entire width of the base of the rhinophore (*Figure 8B*). However, at 35-days post-lesion the network seemingly became less dense and once again became localized, this time to the side opposite the ridges and looked similar to the plexus of the unlesioned rhinophore (*Figure 8C*).

The density of the SCP and serotonin fiber plexuses could not be quantified. The development during regeneration is displayed qualitatively as a figure showing their progression over time. Images were collected and generated using FIJI to create z-projections of the standard deviation of each confocal z-stack.

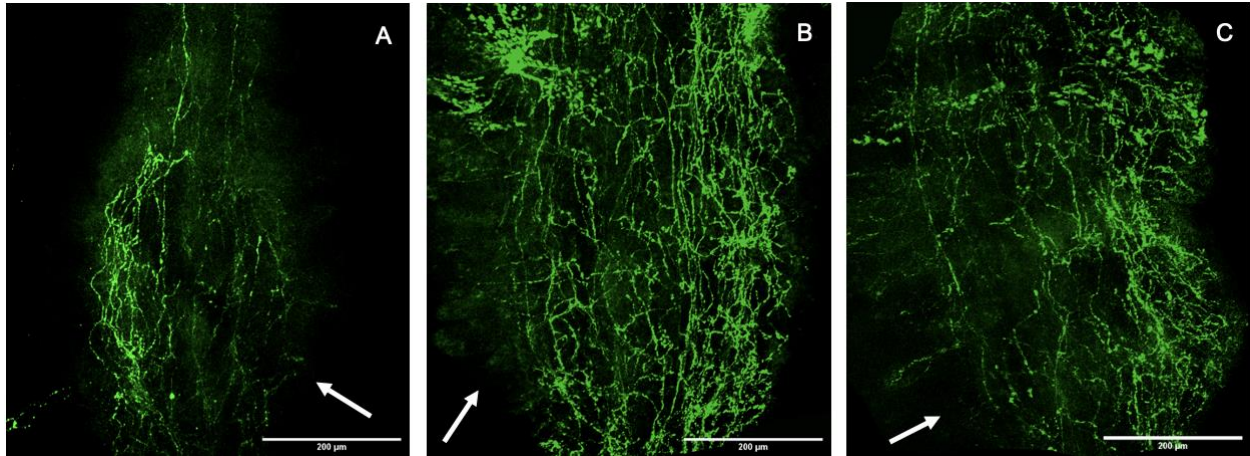


Figure 8 SCP plexus at the base of the rhinophore. **A.** Seven-days post-lesion shows that the fibers were only on the side opposite of the ridges which are indicated by arrows. The network is thin and does not extend up the rhinophore. **B.** 21-days post-lesion, the plexus has become wider, covering the width of the rhinophore. **C.** At 35-days post-lesion, localization of the network looks more prominent on the side opposite the ridges. Scale bar is 200 μm .

VII. Filling of Unlesioned Rhinophore Nerves into the Brain

By filling nerves from the side ipsilateral or contralateral to the regenerated side, it was possible to examine whether projections from the cut rhinophore nerves were similar after five weeks had elapsed. Comparisons were made by filling the rhinophore nerve on the unlesioned side in some preparations (*Figure 9A*, N=7), and in other preparations, filling the rhinophore nerve that has itself regenerated (*Figure 10A*, N=5).

Backfills of the rhinophore nerves into the brain were first viewed with FIJI. A map of filled structures was then constructed on a basic *Berghia* brain schematic using Adobe Illustrator and Microsoft PowerPoint. Images were generated as above, by creating z-projections of the standard deviation of the confocal stacks in FIJI.

Backfilling of cut nerves from *Berghia*'s unlesioned rhinophore consistently stained certain projections and cells throughout the brain. First, representative of projections in the brain of a fill from the unlesioned side, all animals showed presence of medial, central, lateral (1, 2 and 3) and rhino-rhino contralateral (R-RCT) projections (*Figure 9A, B*). These projections were

thick and extended from the rhinophore ganglia ipsilateral to the stain into the ipsilateral cerebropleural (Ce-PI) ganglion (1, 2 and 3) with the R-RCT crossing from the ipsilateral Ce-PI ganglion into the contralateral Ce-PI ganglion. Additionally, small distal cells were found in the rhinophore ganglia, with a stronger presence in the rhinophore ganglion ipsilateral to the fill. Cells ventral to the ipsilateral eye within the cerebral ganglion where the buccal connective was present also stained with neurobiotin (*Figure 9*). *Berghia*'s eyes, when present, provided insight into the consistent structures that filled via the rhinophore nerves. A posterior ipsilateral photoreceptor was stained often (*Figure 9A*). The fill through the buccal connective was typically present in both Ce-PI ganglia which extend dorsally to travel past the eye (*Figure 9D*). The presence of the projections and cells in the fills from the unlesioned rhinophore nerves highlight that *Berghia* was able to regenerate its tracts back to a pre-lesion state.

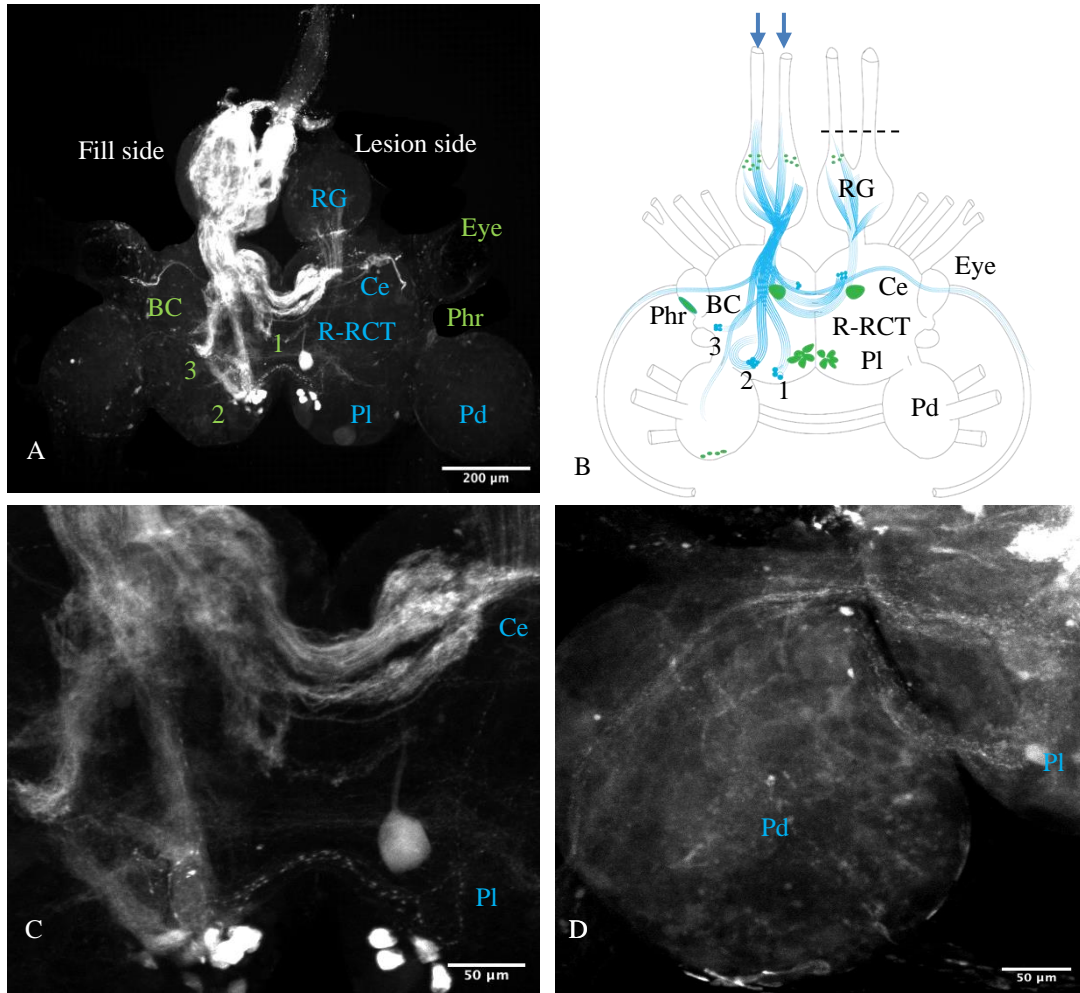


Figure 9 Projections and cells filled in an unlesioned nerve. **A.** Fill of the rhinophore nerves contralateral to the lesion. Fill enters the rhinophore ganglion (RG) from the nerves and travels down to the ipsilateral cerebropleural (Ce-Pl) ganglion while also staining the ipsilateral buccal connective (BC). The Rhino-rhino Contralateral Tract (R-RCT) crosses over to the contralateral rhinophore ganglion while the lateral and medial projections extend into the ipsilateral Ce-Pl ganglion. The photoreceptor (Phr) posterior the contralateral eye is also stained. **B.** Map of an unlesioned nerve fill shows notable cells in green and major fiber tracts in blue. Lesion is indicated with a dotted line and fill is indicated by blue solid arrows. **C.** The lateral, medial and R-RCT projections showing terminal endings with stained cells. A symmetric group of cells in both Ce-Pl ganglia are stained. **D.** The filled axonal projections at the dorsal region of the ipsilateral pedal (Pd) ganglion. Scale bar is 200 µm and 50 µm in C and D.

VIII. Filling of Lesioned Rhinophore Nerves into the Brain

The lesioned side of *Berghia*'s brain mirrored the cells and projections seen in the unlesioned side. The medial, central and R-RCT projections were also present in all of the

lesioned side backfills, however appearing thinner than the projections in the unlesioned side fills (*Figure 10A, C*). The R-RCT also appeared in all animals, showing a connection between the lesioned and unlesioned side rhinophore ganglia (*Figure 10A, B*). The unlesioned side also stained cells in the distal rhinophore ganglion near the site of fill (*Figure 10B*). When the ipsilateral eye was present on the lesioned side backfills, the ipsilateral photoreceptor usually but did not always stain (*Figure 10D*). The buccal connective axons were stained when filling from the lesioned side, which is typically found farthest away in the brain from other filled structures. In the Ce-Pl ganglion ipsilateral to the fill were a burst of staining covering a patch of the ganglion close to the midline (*Figure 10C*). This patch of staining was persistent in the Ce-Pl ganglion ipsilateral to the fill and was not indicative of cells usually filled in that region of *Berghia*'s neuroanatomy.

The pedal ganglion of the lesioned side of the brain displayed less consistent staining of cells. First, the dorsal region of the pedal ganglion ipsilateral to the fill showed a thin projection stemming from the Ce-Pl ganglion while the projection did not reach the outgoing pedal nerves (*Figure 10*). The thin projection leading into the pedal ganglion was less often seen on the lesioned side fills. Additionally, staining of cells in the pedal ganglion ipsilateral to the fill were less consistent (*Figure 10A, 10B*). This region was also carefully examined to rule out possible leakage from the pedal nerves. These results highlight that there may have been effects of regeneration on cell presence in the pedal ganglia.

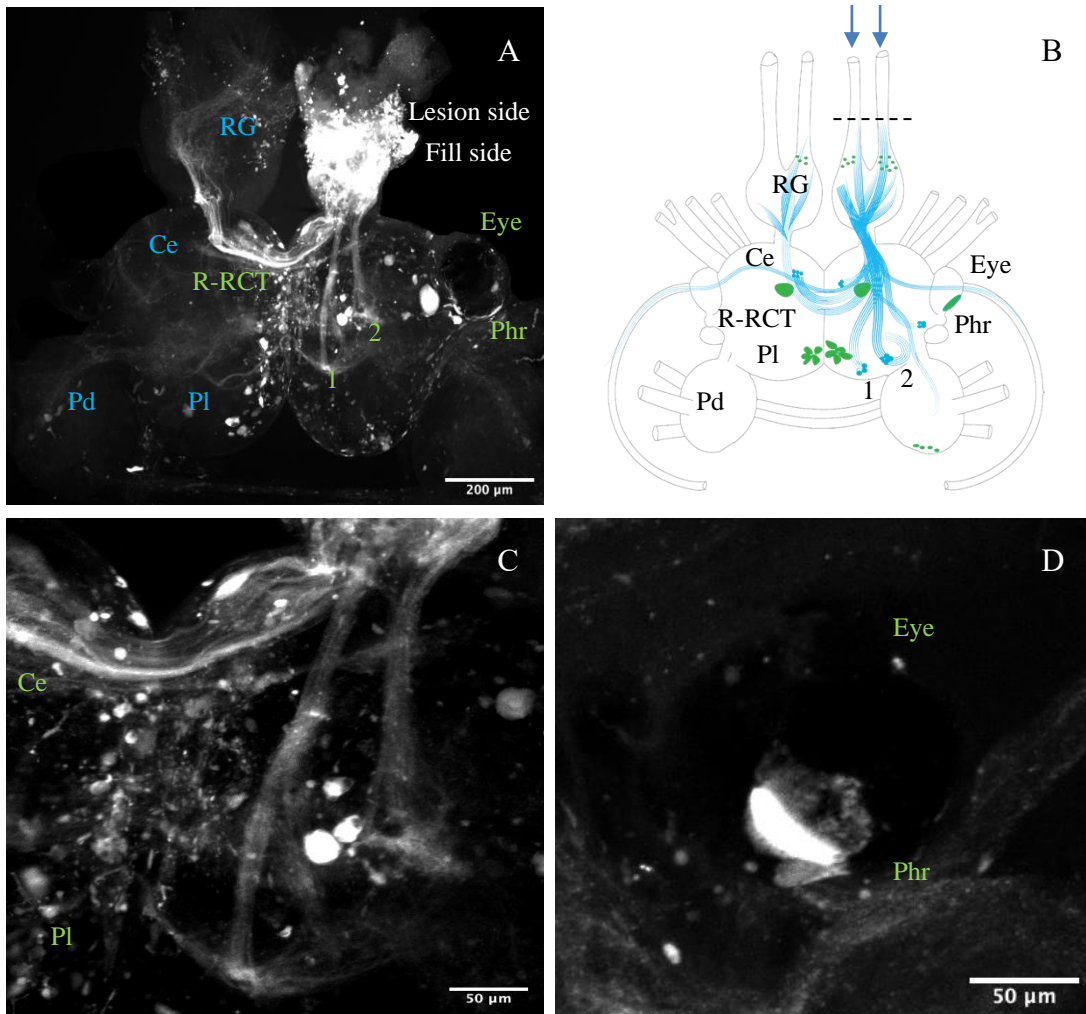


Figure 10 Projections and cells filled in a lesioned nerve. **A.** Fill of the rhinophore nerves ipsilateral to the lesion. Fill enters the Rhinophore ganglion (RG) from the nerves and travels down to the ipsilateral cerebropleural (Ce-Pl) ganglion while also staining the ipsilateral buccal connective (BC). The R-RCT crosses over to the side contralateral to the lesion and fill as the lateral and medial projections travel farther down the ipsilateral Ce-Pl ganglion, as seen in the unlesioned side fills. The photoreceptor (Phr) is stained on the ipsilateral posterior eye. **B.** Map of a lesioned nerve fill shows notable cells in green and major fiber tracts in blue. Lesion is indicated with a dotted line and fill is indicated by blue solid arrows. **C.** The lateral, medial and R-RCT projections that are in the same location as the projections in the fills of the unlesioned. A burst of staining lining the division between the two Ce-Pl ganglia is also shown. **D.** The Phr stained in a different brain of the lesioned nerve fill, showing that characteristics of the eye are back at five weeks post-lesion. Scale bar is 200 μm and 50 μm in C and D.

DISCUSSION

External features of the rhinophore during regeneration

The lesioned rhinophore grew back but did not reach the same length

After a five-week regeneration period, *Berghia*'s rhinophore grew back in length. Additionally, although the rhinophore grew in length, it did not grow back to the same height as the unlesioned rhinophore. This may be due to the unlesioned rhinophore growing as a compensatory effect of the missing rhinophore, though data was not collected to track the length of the unlesioned rhinophore during contralateral rhinophore regeneration. The regenerating rhinophore's inability to reach the same length also may be due to the rhinophore not being given enough time to regenerate to the same length as the unlesioned side. Studies on other molluscan species have indicated a wide range of time to regenerate an appendage. For example, the arm of *Sepia officinalis* took from five-days to two weeks to fully regenerate (Imperadore and Fiorito, 2018), while in *Octopus vulgaris* the arm did not reach full regeneration until 55 days post-lesion (Fossati *et al.*, 2013). These findings highlight that different species of molluscs may require different lengths of time to fully regenerate appendages, suggesting that *Berghia* may also need a longer regeneration period for its regenerating rhinophore to reach the same height as the unlesioned rhinophore. The findings from this study showed that although the regenerated rhinophore seemed to grow back during regeneration, it did not reach the same length as the unlesioned rhinophore within the allotted five-week time period.

Rhinophore ridge regrowth occurred later in regeneration

The ridges that were observed on the lateral sides of *Berghia*'s rhinophores showed a delayed response in reappearing on the regenerated side. The presence of ridges on the exterior

of *Berghia*'s rhinophore were not presented until about a week post-lesion. The ridges grew along the rhinophore up until the five-week period, but much like the length of the rhinophore, they did not regenerate to cover the same length as the ridges on the unlesioned side.

While the ridges looked grossly normal, it could be that their innervation had not been fully established by the five-week timepoint. In similar view, when the axons of motorneurons in the wings of mollusc *Clione limacina* were crushed, synapsing onto both correct and incorrect muscles occurred about one to two weeks post-lesion (Panchin *et al.*, 1997). It was not until after the two-week timepoint that the neurites began innervating the correct muscle fibers (Panchin *et al.*, 1997). It may be that it first took a week for muscle fibers to move into the rhinophore, and then proceeded to take several weeks for them to correctly innervate and form the ridges.

Likewise, one of the main nerves that innervates the tail of *Aplysia californica* was severed and allowed to regenerate (Steffensen, 1995). The regeneration of sensory fibers, which included the innervation of cilia and microvilli endings, took one and a half to two weeks to return (Steffensen, 1995). This time course closely parallels that of the regeneration of ridges on *Berghia*'s rhinophores, suggesting that the one to two-week period is when concentrated growth of structures such as the ridges and cilia occurs. It may take more time after this burst of regeneration to grow the remaining structures back to what they were before the lesion. The continued growth in ridge coverage up till the end of the regeneration period may suggest that with more time, the ridges could continue to develop. Although the ridges reform after a delay, they appear to regrow by the five-week period while still not having reached the same coverage of length as the unlesioned side.

Pigmentation returns more slowly than other features

The pigmentation of *Berghia*'s rhinophores nearly re-developed at the five-week post-lesion timepoint but did not grow back to the same area of pigmentation as the unlesioned rhinophores. Pigmentation was also displayed in a DAPI stain of the cells in rhinophores to prove that it was not autofluorescence as a product of SCP immunoreactivity. Pigmentation was not apparent until 21-days post-lesion. The late appearance of pigmentation could be an indication of its lesser importance to the functioning of *Berghia*, since it was one of the last features to re-appear in the rhinophore. The snail *Hydrobia ulvae* also did not regrow its pigmentation until after it had formed its tentacle and eye buds (Gorbushin *et al.*, 2001), highlighting the idea that pigmentation was not imperative in functionality and therefore did not have to regenerate immediately after a lesion. When the pigmented areas of regenerated rhinophores in *Berghia* were compared to the pigmented areas of the unlesioned side, pigmentation had not yet fully developed at the five-week timepoint. Pigmentation arose last of all features previously discussed and had a similar delay in the other findings.

Internal features of the rhinophore during regeneration

Muscle fibers organized after a short period of disorganization

The interior of the regenerating rhinophore exhibited a delayed response in the re-arrangement of muscle fibers. During the early stages of development at one-day, three-days and five-days post-lesion, muscle fibers were present in a disorganized manner within the rhinophore. The fibers did not look like that of an unlesioned rhinophore, where fibers usually follow patterns of vertical and horizontal projections, representing longitudinal and transverse musculature. It was not until seven-days post-lesion that the fibers began to rearrange in these

patterns. Comparatively, a study on the gastropod *Aplysia californica* reported that reconnection of fibers and ensuing organization after nerve crush did not occur until about two weeks after the crush, displaying a longer regeneration period for the inner fibers of the appendage (Dulin *et al.*, 1995). Similarly, Imperadore *et al.* (2017) discovered that *Octopus vulgaris* did not organize the neural fibers in its mantle until about two weeks after lesion. These findings suggest that similar to muscle fiber organization in *Berghia*, other molluscs also experience delays in internal reorganization. In *Berghia*, such delays could be due to the rhinophore being too small to be able to fix the fibers within until it could grow to a length where there was enough room within to reorganize.

It was also apparent that muscle fibers grew with the length of the rhinophore after this period of organization, suggesting that the rhinophore's interior was growing back to its original state. It could have been that within the week after the rhinophore was lesioned, the fibers near the lesion site contracted and assembled in a manner not indicative of parallel growth with the rhinophore. It may have taken them up to a week to restructure themselves to follow the growth of the rhinophore. Although there was a delay in the establishment of clear muscle fiber pattern, such development occurred nearly a week after the rhinophore was lesioned and continued to organize throughout the rest of the regeneration period.

Serotonin fibers reappeared at the earliest days post-lesion

Serotonin immunoreactive fibers were observed within the entire rhinophore immediately after lesion. Similarly, Koert *et al.* (2001) observed that production of serotonin was not obstructed after the axon of a serotonergic cerebral giant cell of gastropod mollusc *Lymnea stagnalis* was crushed, indicating that serotonin could have contributed to functionality in

regeneration. This was similar to results seen in *Berghia*, with fibers immunoreactive for serotonin present immediately after lesion.

The regrowth of the SCP fiber plexus occurs more slowly than serotonin

The importance of staining against SCP was to provide another means of comparing regenerated rhinophore features to the characteristic features shown in unlesioned rhinophores. One of these features was the SCP fiber plexus, a network of fibers that covers the base of the rhinophore and tapers off towards the tip. This network was visible as early as seven days post-lesion but was localized to one side of the regenerating rhinophore. At the 21-day post-lesion period the plexus looked over-expressed compared to its counterpart on the unlesioned rhinophore. However, the plexus looked similar in size to the unlesioned rhinophore by the 35-day period, insinuating that some sort of increase in expression took place before the plexus returned to what it looked like on the unlesioned side. This could represent the biological result of pruning, or it could be indicative of individual differences in regeneration rate, as each data point was a single animal sacrificed at that time. Similar findings were seen in the sea anemone *Nematostella* where a whole-body lesion led to shorter regenerated animals that grew back nerve nets reduced in size (Havrilak *et al.*, 2019). It is unclear why *Berghia*'s SCP plexus looked denser and larger at 21-days post-lesion before returning to baseline at 35 days, and further studies could use more animals to investigate this.

The formation of the SCP plexus was similar to that of muscle fiber organization and ridge formation. The SCP plexus and muscle fibers form under the same depth of the rhinophore, and therefore could have an interaction. An intertwining of epithelial cells and the nerve net was noted in the cnidarian *Hydra*, depicting how when one moved during cell division, the other

followed (Bode *et al.*, 1988). In *Berghia*, the density of muscle fibers at the base of the rhinophore corresponds with the location of the SCP plexus, indicating that the two may not be mutually exclusive in their re-growth. It is also possible that the plexus had its origin on one side of the rhinophore which then expanded, which could explain why the network appeared localized at seven days post-lesion. The same study on *Hydra* showed that the nerve net on the skin naturally displaced the net due to cell division, moving it from the base of the tentacle to the tip (Bode *et al.*, 1988).

Berghia's SCP plexus could also be moving across the width of the rhinophore as the network expands, since data showed the plexus moved from seven-days to 21 days post-lesion. In the oyster *Crassostrea gigas* and scallop *Mimachlamys nobilis*, SCP is the extracellular messenger that causes fast relaxation of muscle, providing a physiological connection between SCP and muscle fibers (Tsutsui *et al.*, 2007). SCP's activity in muscle relaxation may explain why images of *Berghia* display similarly dense overlapping bundles of SCP fibers and actin filament at each time point. Data on the regeneration of the SCP plexus suggested that this feature was among the middle of the order of characteristic rhinophore features to return to normal.

The backfilling of rhinophore nerves showed some differences in the brain of Berghia after regeneration

Backfilling nerves of animals already at the five-week regeneration point allowed us to consider whether the period was enough time for *Berghia* to regrow its projections. Analyzing the number of tracts that regrew at this timepoint, it was apparent that *Berghia* was not significantly affected at this point in regeneration. Nearly all structures and projections, from the

R-RCT traveling into the contralateral rhinophore ganglion to the ipsilateral posterior photoreceptor that sends an axon into the nerve, had regrown by this time, with the exception of the presence of cells and a projection in the pedal ganglion. The distance from the site of backfill may have been why the pedal ganglion was the only region of the brain that was not consistently stained. However, the lesioned side backfills had the presence of the buccal connective axons at five-weeks post lesion, and thus supported that the regenerated rhinophore was back to normal functioning, as the buccal ganglion neurons that send those axons are farther than the pedal ganglion. In *Nematostella*, animals that were lesioned for regeneration did not grow back to their original lengths, however their nervous systems regrew all of the tracts seen in unlesioned animals in proportion to the size of the regenerated body (Havrilak *et al.*, 2019).

Berghia may have also experienced atrophy before the five-week period and may have had enough time to form new synaptic connections before it was sacrificed. The burst of staining in some of the samples near the cerebral commissure may indicate such atrophy, while the re-formed projections may be the result of successfully synaptic re-formation in the allotted five-week period. These consistent stains supported the view that although animals may have experienced differences in regrowth of projections, they were able to regenerate tracts to their pre-lesion appearances within a five-week regeneration period.

It appeared that projections from the lesioned side of the *Berghia*'s brain looked thinner than the projections stemming from the unlesioned side. This finding may suggest that *Berghia* also regrew all its tracts, perhaps not to the size pre-lesion, but proportionate to the regenerated rhinophore size. Therefore, evidence for the regeneration of the projections that spanned across the rest of the brain suggested that the stained projections had regenerated, but it may be that the fill did not reach far enough down the opposite end of the brain due to experimental error.

Additionally, the effect of tentacle regeneration on the terminal mass in the procerebrum in terrestrial slug *Limax valentianus* showed that the terminal mass was reduced in size two-weeks post-lesion (Matsuo *et al.*, 2010). However, by 58-days post-lesion, the terminal mass had recovered in size and a longer period of regeneration had not affected its growth (Matsuo *et al.*, 2010). Similarly, if *Berghia* had experienced a degeneration of its axons, it could have been that the five-week regeneration process was enough for it to re-grow to its original projections.

An abnormal patch of staining in the Ce-PI ganglion ipsilateral to the fill may suggest a role of axonal degeneration during the regeneration period. Such was seen in larvae *Xenopus* where a transection on the olfactory nerve led to the disassembly and degradation of axons with increased dye accumulation from staining at one-week post-transection (Hawkins *et al.*, 2017). It could be that *Berghia* experienced degeneration of axons in the medial tract which led to the burst of dye on the side ipsilateral to the fill. However, since nearly all projections and cells were at their pre-lesion state by five weeks, it may be that like *Xenopus*, *Berghia* experienced degeneration of axons earlier in its regeneration period and regenerated the structures by the five-week time point. Results from additional studies may suggest functional re-organization after lesion. Shortland and Woolf (1993) showed that with respect to shrinking receptive fields in the dorsal horn of rats, abandoned synapses may have atrophied while new synapses formed, suggesting plasticity within the regenerating spinal cords.

Backfilling from rhinophore nerves from both sides of the brain may show the presence of more projections in *Berghia*'s brain. Although tracer neurobiotin can cross gap junctions, it is the only tracer small enough to travel through the narrow axons, while larger tracers like Dextran cannot navigate through. Thus, using neurobiotin for fills was the most effective way of staining for the maximum number of projections. The effectiveness of neurobiotin as a tracer was also

seen in studies on cats that had used horseradish peroxidase injections following lesions of two different nerves. Using both types of tracers, it was found that horseradish peroxidase showed about half of the projections as neurobiotin, highlighting that neurobiotin was the more successful of the two tracers in showing regenerated projections from the two different sites (Koerber *et al.*, 1995). In *Berghia*, using tracer neurobiotin from two lesioned sites, such as the left and right rhinophore nerves, could lead to similar results of the displaying of more projections than a single-sided fill, although it would be impossible to tell projections apart unless using forms of neurobiotin that were already conjugated with different colored dyes.

CONCLUDING REMARKS

Although further research is necessary to support molecular details of rhinophore regeneration in *Berghia*, these data lay a foundation by defining stages of rhinophore regeneration both internally and externally. The five-day to seven-day post-lesion points were when characteristic features of *Berghia* first re-appeared, such as ridge growth along the rhinophore and the presence of pigmentation. Results supported the view that serotonin expression may have a role in rhinophore regrowth, as it was entirely present throughout all timepoints of regeneration, even the disorganized earliest timepoints. Due to the five-week timepoint of the backfills, it is not known whether the tracts undergo degeneration before they grow back to their original state. Using the backfill technique with more timepoints will provide a better understanding of the pathways that axons may follow during regeneration. Because this study presented how the neuroanatomy of *Berghia* changed with regeneration, pairing this research with behavioral studies in the future would provide a more detailed acknowledgement of how olfactory navigation may be related to rhinophore functionality post-regeneration.

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