**Determining the Roles of Semaphorin 1a Protein in Adult Plasticity in *Gryllus bimaculatus***

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**Abstract**

The role of the protein, semaphorin 1a, in auditory plasticity of *Gryllus bimaculatus* was examined. Control and experimental groups of crickets were used for dsRNA injection and visualization. Double-stranded RNA was used to knockdown semaphorin 1a in 7$^{th}$ instar crickets, and a backfill procedure was then completed in order to visualize the anatomical effects. Much of the summer was centered on trouble-shooting the visualization protocol. Our findings suggest that using lower concentrations of PFA to fix ganglia samples may solve the visualization difficulties the Horch lab has experienced over the years. Future studies within the Horch lab will be able to use these findings to help understand the role semaphorin 1a plays in compensatory plasticity in *Gryllus bimaculatus.*

**Project Objectives**

The auditory system of the cricket, *Gryllus bimaculatus,* has been studied as a prime example of neuronal plasticity after injury. Crickets are unique in their ability to conduct "compensatory plasticity". Amputation of the foreleg, and therefore the ear, from one side of the body greatly impacts the morphology of the neurons within the prothoracic ganglion. In response to the loss of input, the ascending neurons (ANs) on the side of the amputation no longer respect the midline, and instead, their dendrites grow across the midline of the body, forming connections with the auditory system from the opposite side (Figure 1). This "reinnervation" is incredibly precise and recreates the ability of the ANs to fire and distinguish auditory input. Presumably, this greatly increases the ability of the cricket to respond to auditory input, which enhances survival (Horch et al., 2011). As one might imagine, the expression levels of different proteins within the prothoracic ganglion changes drastically in response to damage/injury. This report presents an experimental design for one of these proteins, Semaphorin 1a (Sema 1a), in regards to its role in compensatory plasticity of the cricket auditory system (Chong, 2015).

Semaphorins are a group of proteins that are found across the animal kingdom in both vertebrates, invertebrates, and even viruses (Neufeld et al, 2011). Sema 1a, in particular, is a transmembrane protein found only in invertebrates, and also exhibits large variability in expression levels after differentiation (Chong, 2015). As illustrated by previous research using qPCR, Sema1a expression levels drop significantly 18 hours after deafferentation. Interestingly, beginning at 30-hours through 5-days post deafferentation, expression levels of Sema 1a begin to rise, eventually approaching baseline levels (Chong, 2015). Presumably, this significant downregulation, followed by a substantial upregulation post-deafferentation has an important biological effect, and is at the heart of why we are studying Sema 1a.

This summer, it was our objective to visualize the anatomical effect(s) that downregulating Sema 1a has in the prothoracic ganglia of *Gryllus bimaculatus*. In particular, we wanted to see how knocking down Sema 1a affects the AN desire and ability to cross the midline of the prothoracic ganglia. We hypothesized that when Sema 1a is knocked down, these neurons would no long respect the midline, and would lead to a more haphazard growth. In order to effectively knockdown Sema 1a, dsRNA was synthesized and injected into juvenile crickets. Exactly 2 weeks and 3 days after dsRNA injection, the crickets would then be backfilled to visualize the anatomical effects the dsRNA had on the prothoracic ganglia. Unfortunately, we did not obtain any anatomical data, because much of the summer was focused on trouble-shooting the visualization protocol.

The Horch Lab has spent many years attempting to troubleshoot this part of the procedure, because many of the backfilled ganglia are unable to be visualized. What the lab has coined as a "black

hole", dye seems to penetrate into the ganglia, but stops before in reaches the center of the ganglia (Figure 2). Much of this summer was focused on experimentally manipulating different parts of the visualization protocol in hopes to obtain more successful backfills. With mixed success, we are confident that this summer was incredibly helpful in solving this problem; giving future studies within the Horch Lab potential direction are far as changing the protocol is concerned.

**Methodology Used**

*Specimen Collection*

Male & Female crickets, specifically, *Gryllus bimaculatus*, were used in all experiments, and were raised by Hadley Horch, Ph.D, at Bowdoin College. Crickets were collected as $7^{th}$ instars for injection. Control Group: A foot-chop procedure was performed where the bottom portion of the foreleg was removed. Experimental Group: The foreleg, and therefore ear, of these crickets was amputated.

*dsRNA Injections*

$7^{th}$ instar crickets were isolated the day of injection and placed on ice for ~1 hour before manipulation. The crickets were positioned and temporarily immobilized using wax. Using a 5µL Hamilton syringe, crickets were injected into one side of the body in the soft piece of tissue at the base of the large hind leg. The syringe was inserted along the side of the body such that it did not injury any major structures, and was close to the prothoracic ganglia. A volume of 0.5µL of 20µM dsRNA was injected. The needle was removed slowly to avoid any leakage from the injection site.

*Backfills & Fixation*

Animals ready to be backfilled were placed on ice for roughly 1 hour, and then pinned to a dissecting dish. The cricket was placed ventral-side up, and each of the four hind-legs were removed. The cuticle was removed in order to expose nerve 5, the prothoracic ganglia, and the 2 parallel neck-connectives. Nerve 5 was filled with Biocytin 594 (conjugated), while AN-2 was filled with 4% biocytin (unconjugated) (Lillis, 2017).

The next morning, the prothoracic ganglia was removed from the body of the cricket and submerged in 4% PFA for 1.5-2 hours. The 4% PFA was diluted in 1x PB solution. The ganglia were then washed 3x with PB. The next step included 4 x 1 hour 0.5% Triton Rinses, replacing the triton after each wash and placing the samples on an orbital shaker. After the fourth hour, 399µL of 0.5% Triton rinse solution was added, along with 1µL of streptavidin (1:400). The samples were then placed in the 4C cold room for three nights. Once removed, the 4 x 1-hour rinse protocol was repeated. The ganglia were then rinsed 3x with PB and a series of 6 x 10 minute ETOH washes were conducted. Finally, the ganglia were cleared in methyl salicylate and placed on a slide for visualization.

**Results Obtained**

Much of our summer was focused on trouble-shooting the visualization protocol of the procedure. We executed multiple ideas with mixed success. First, instead of using PBS (Phosphate buffered saline), we used PB (Phosphate buffer) to dilute our PFA, and for all washes. We didn't notice any significant difference between using one verses the other. We then tried fixing the ganglia in 4% PFA for overnight instead of for 1.5-2 hours. The ganglia appeared very dehydrated and were unable to be visualized under confocal microscopy- not recommended for future experiments. Next, we changed our 0.5% Triton rinses to be 8 x 30 minute washes instead of 4 x 1 hour. While we did not see any significant effects between the two, we felt that fresh triton could only help the process. Additionally, after the 8 x 30 minute washes, we left the ganglia in 0.5% Triton solution overnight, which still left the ganglia dehydrated - not recommended for future experiments. As far as dye concentration is concerned, we switched to a 1:100 streptavidin ratio. While this did help with the visualization, nearly everything inside the ganglia appeared florescent. This was only a temporary fix because in order to produce publication quality photographs, the lab needs to produce very specific and specialized photos. Finally, we tried fixing our ganglia in 0.5%, 1%, and 2% PFA instead of 4% PFA. While the 0.5% PFA did not work well, both the 1% and 2% PFA-treated ganglia looked fantastic.

**Significance and Interpretation of Results**

As a whole, I feel as though this summer's research was incredibly valuable to current and future members of the Horch Lab. While we did not accomplish everything we set out to, but we did complete some very important work. The issue with visualization has been a problem that has plagued the Horch Lab for many years (Figure 2), but I feel as though we made great progress in spearheading this issue. While many of the things we tried either made our ganglia harder to visualize or did not help at all, our final troubleshooting idea of the summer proved to be quite successful.

The fact of the matter is that none of our future experiments can take place until our visualization protocol has been fixed. It is not sustainable for the lab to continue spending time and resources on dsRNA, injections, crickets, etc. if we cannot reliably visualize the effects our experimental conditions have on the cricket auditory system. Professor Horch has attempted to trouble shoot nearly every step of the protocol, with some success; however, this problem always seems to eventually reoccur. With the help of Lisa Ledwidge, lab technician at the Horch Lab, we came up with the idea to change the concentration of PFA we used to fix our ganglia with (Zukor, 2010). By testing 0.5%, 1%, 2% PFA, and comparing it to our usual, 4% PFA results, we may have cracked the code in figuring out why our ganglia are so difficult to visualize. Many of our samples appeared very dehydrated under confocal microscopy, which made it nearly impossible for our streptavidin dye to enter inside the ganglia. It was thought that the 4% PFA was too high of a concentration, and that a lower concentration would still fix the ganglia, but still allow the streptavidin to penetrate. Our first round of results looked very promising, with both the 1% and 2% PFA being able to be visualized, and with no sign of dehydration. Granted, these results were only with a total of 4 samples (2 in each PFA condition). Thus, the following week, we attempted to recreate our results. The backfills completed were not great to begin with, but the ganglia did not appear dehydrated or have other hallmarks of the previous issues we faced with the 4% PFA. We did not gather any photographs of great quality, but was due to an unrelated issue with backfilling the ganglia, and not with the concentration of PFA. Going forward, we would recommend that the lab fixes samples in 2% PFA for 1.5-2 hours.

For future directions of research within the Horch Lab, we will now be able to inject crickets with Sema1a dsRNA and visualize its effects. Additionally, there is a new technique which describes the ability to backfill neurons via a suction electrode placed directly on the outer surface of the brain in crickets (Isaacson & Hedwig, 2017). It is possible that this could be a future technique used in the Horch Lab to study both the physiology and morphology of a single cricket. As a whole, this summer will certainly be considered a success and there were some incredibly important experiments conducted. It is my hope and intuition that we provided current and future Horch Lab members with important information regarding the visualization of cricket ganglia samples. I can hardly wait to continue this research in the semesters to come.

## Figures/Charts

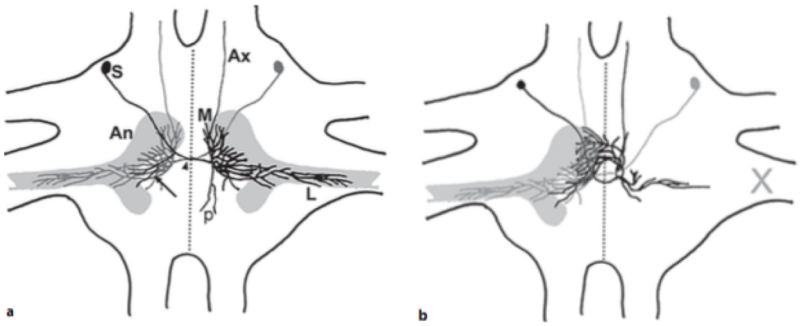

Figure 1. a) The prothoracic ganglion of an uninjured cricket. AN-2 dendrites respect the midline and do not innervate nerves receiving signals from the contralateral ear. b) Amputation on the right results in profound degeneration of the auditory nerve. In addition, AN-2 dendrites do not respect the midline and undergo a profound reorganization. (Adapted from Horch, et al., 2011)

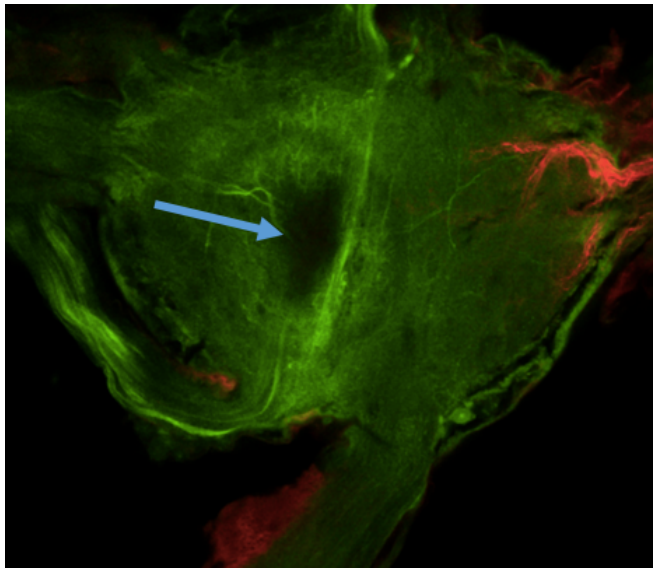

Figure 2. Confocal microscopy image of *Gryllus bimaculatus* prothoracic ganglia. Blue arrow indicates area of the ganglia where the dye did not fully penetrate- the area deemed as the "black hole".

**Acknowledgements and References**
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