The role of semaphorin signaling in the cricket's auditory plasticity

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Abstract

The role of semaphorin signaling between Sema1a.2 and Sema2a in the auditory plasticity of *Gryllus bimaculatus* was examined. Double-stranded RNA (dsRNA) was injected and used to knockdown semaphorin 1a.2 and semaphorin 2 in 7th instar crickets. Physiology recordings quantified responses to auditory stimuli and backfill visualized morphology. Most of the summer focused on troubleshooting the visualization process. Analysis of physiology recordings indicated differences between un-injected and GFP-injected crickets' action potentials and duration of response under specific conditions. Future research will utilize these findings to determine if the process of injection alters the cricket's responsiveness and understand the possible role semaphorin signaling may play in the cricket's auditory plasticity.

Project Objectives

Crickets exhibit an unusual compensatory plasticity in response to injury and are easy subjects to study. When cricket ears are removed via amputation, ascending neurons in the prothoracic ganglion (PTG), part of the central auditory system, sprout across the midline, a boundary they typically respect. The neural pathways reroute and connect to the connections originating in the opposite ear, limiting the disadvantages caused by trauma by re-establishing some ability to localize sound (Scholes, 2020). Proteins involved in this phenomenon have only recently been characterized in cricket. In previous studies, Semaphorins have been shown to guide the development of axons and dendrites which transmit electric signals away from the neuron's cell body and receive electrical signals from other neurons. In developing grasshoppers, a closely related insect, this guidance is achieved by Semaphorin signaling through Plexin receptors. In the cricket, the Horch lab has characterized two distinct Semaphorin1 proteins, known as Sema1a.1 and Sema1a.2 (Horch et al., 2020). Sema1a.2 messenger RNA (mRNA) is expressed in adult thoracic ganglion, but the adult thoracic ganglion lacks appreciable expression of Plexin, the receptor for Sema1 proteins (Horch et al., 2020). In Drosophila melanogaster, Sema-1a is an important regulator of midline crossing, limiting when and where neurons can send axons across the midline. Via reverse Sema signaling, Sema-1a functions can also act as a receptor for secreted Sema2 proteins, causing a surprising type of reverse signaling. This promotes midline attraction and enables axons to cross the midline and form functional midline circuits (Hernandez-Fleming et al., 2017). Such mechanisms could account for the cricket's auditory plasticity. Based on some sequencing completed by the Horch lab, we know that Sema2a is also present in the PTG (Fisher et al., 2018). Furthermore, *G. Bimaculatus*' predicted Sema1a.2 protein sequence was closely related to Drosophila melanogaster Sema1a (Horch et al., 2020). Therefore, it is possible that reverse Sema signaling guides the dendrites post-amputation when they cross the midline to form connections with the opposite side.

Electrophysiology was used to investigate if reverse Sema signaling played an integral role in *Gryllus bimaculatus (G. Bimaculatus)* compensatory plasticity. Downregulation of Semaphorins is associated with promoting structural plasticity in the prothoracic ganglia (Spicer, 2018). Thus, to imitate trauma, double-stranded ribonucleic acid (dsRNA) was introduced into the cricket's hemolymph via injection. Sema2a was determined as a target protein because transcript expression for Sema2a was significantly upregulated suggesting the protein's expression plays a role in midline crossing (Horch et al., 2011). Sem1a.2, also present in the prothoracic ganglion, was considered the other target protein because in situ hybridization localized strong expression of Sema1a.2 in the prothoracic ganglion (Horch et al., 2020). Furthermore, significant downregulation of Sema1a.2 was observed 18 and 30 hours post deafferentation with recovered Sema1a.2 levels 5 days post deafferentation (Chong, 2015). To observe

if reverse Sema signaling played a role in the midline crossing observed post deafferentation, 7th instar crickets were injected with both Sema2a dsRNA and Sema1a.2 dsRNA. Physiological and morphological data was collected when the crickets were 5 to 10 days post molting into adulthood.

Although data for the experimental group was not collected, physiology recordings for GFP-injected crickets and un-injected crickets were compared for delay, number of action potentials, and duration of cricket's response.

Methodology Used

Identity of animals

A colony of inbred male and female Mediterranean field crickets, *Gryllus bimaculatus* (*G. bimaculatus*) were kept in communal cages in a climate of 60-70% humidity and 25°C on a 12:12-hour light:dark cycle. Constant access to food (cat food), water, and shelter (pulped paper egg crates) was ensured. Control group: GFP-injected crickets. Experimental group: Semaphorin-injected crickets.

Injection Procedure

Small wing-bud (7th instar) crickets that had no visible deformities were randomly selected. Prior to injection, crickets were anesthetized on ice for one hour. 20uL microloader tips were used to inject 1uL of given dsRNA into each isolate's metathoracic joint. Following injection, crickets were isolated until 5-10 days after their final molt into adulthood to be used for electrophysiology recording.

Dissection for physiology recordings

Crickets were anesthetized on ice for about an hour prior to dissection. The cricket was immobilized ventral side down on Plasticine clay attached to a ball joint platform. Under a microscope, dissection spring scissors and forceps were used to create incisions to remove the rectangular piece of cricket exoskeleton and expose the brain. Fat, trachea, and other tissue were removed from the brain and cold cricket saline prevented drying out tissue.

Physiological recordings and backfilling

A platinum grounding/reference wire was placed in the hemolymph of the cricket's head cavity. A glass electrode was placed in the expected location of the auditory neuropil of the brain which contains ascending neuron 1 (AN1) and ascending neuron 2 (AN2) axonal endings. Audacity was used to play a range of sound frequencies crickets are known to respond to (5kHz, 10kHz, 18kHz, and white noise) at ~70dB for 100 milliseconds each at 250 millisecond intervals to activate the auditory neurons. Next, an audio stimulus emulating predatory bats was played. The tones at 18kHz had a rise and fall period of 5 milliseconds followed by 30 milliseconds of silence. The tone was repeated 5 times to create one 'pulse.' Every pulse of 5X50 milliseconds tones was repeated 5 times with one second between each and was repeated 8 times. Each repetition increased in volume from 50dB to 90dB in 5dB steps. The sound was played from the left speaker and then repeated on the right speaker after 15-20 seconds of silence. Neurobiotin dye was iontophoresed into the brain for 30-60 minutes. Following iontophoresis, crickets were placed in a container with a damp paper towel for 18-24 hours at 4°C.

Tissue preparation

Crickets were pinned ventral side up on a dissection dish and their prothoracic ganglion (PTG) was exposed, removed, and placed in 2% paraformaldehyde (PFA) in phosphate buffer solution (PBS) for one hour. PTGs underwent 4X1-hour washes with 5% triton rinse solution on an orbital shaker and then left in a 1:200 streptavidin 594 in triton rinse solution for 72 hours at 4°C on an orbital shaker. Afterwards, the tissue was rinsed again 4X1-hour washes in 5% triton rinse solution and rinsed in 1X PBS before being placed in VECTASHIELD Mounting Medium for Fluorescence (Vector Laboratories).

Results Obtained

Comparison of physiology recordings showed statistically significant increase between the un-injected crickets and GFP-injected crickets for average number of action potentials in response to the stimuli (Figure 2.) and decrease in duration of response to stimuli (Figure 3.). There were no significant

differences between the two groups of crickets for delay between the start of the stimuli and the beginning of the cricket's response (Figure 1.).

Significance and Interpretation of Results

The presence of significant differences between the un-injected and GFP-injected crickets suggests that further study should be conducted to confirm injection itself does not interfere with the auditory response of the cricket. dsRNA against GFP is injected as the control to compare to crickets injected with the experimental semaphorin dsRNA. However, it is expected that GFP-injected crickets do not differ from un-injected crickets due to the absence of the GFP gene in the cricket DNA. If injection did have an impact, then it would influence data in conjunction with protein knockdown. One factor that could have caused differences would be if a few of the crickets had already experienced the loss of their tympanal leg. *Gryllus bimaculatus* can regrow their frontal leg after loss which looks like their original but loses functional auditory neurons (Bateman & Fleming, 2005). Furthermore, due to time constraints data from the experimental group was unable to be collected.

In addition, issues with visualizing the morphology of the tissue were the focus of the summer. The problem was tackled by isolating possible offenders such as wiring and processing of tissue. It was determined that the reagents used to stain the tissue worked, however it was not fully confirmed that current was being reliably passed through the electrode to drive the dye into the brain.

For future directions, more probing into the process of backfilling will be conducted to determine the root of the issue and restore the Horch lab's ability to visualize the morphology in the prothoracic ganglion. Furthermore, another robust study will be conducted to understand the role semaphorin signaling might play in the auditory plasticity of the cricket. In the presence of more time, more data can be collected from both the control and the experimental groups. This summer has helped me grow as a researcher and gain new skills. I look forward to delving deeper into the role semaphorin proteins play in the cricket's compensatory plasticity and hope to build upon this summer's data.

Figures/Charts

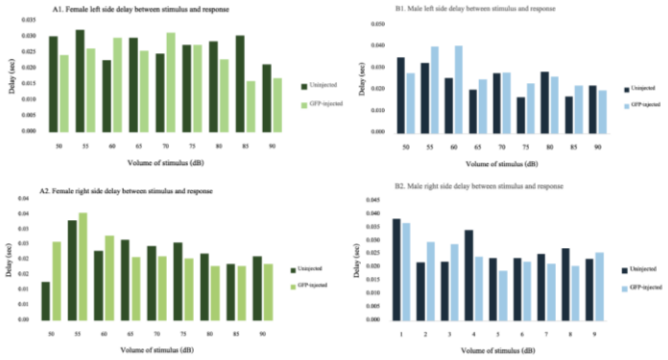

Figure 1. No significant difference in delay between the beginning of the stimulus and beginning of response from the cricket's auditory system for Uninjected versus GFP-injected crickets; Uninjected female (n = 3), GFP-injected female (n = 3), Uninjected male (n = 2), GFP-injected male (n = 3). An unpaired, two-tailed T-test determined lack of statistical significance (A1. p = 0.184, A2. p = 0.97; B1. p = 0.373, B2. p = 0.65).

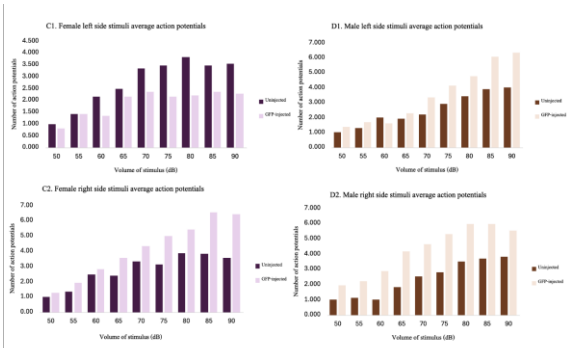

Figure 2. Significant increase between the average number of action potentials for female left stimuli and male right stimuli with Uninjected female (n = 3), GFP-injected female (n = 3), Uninjected male (n = 2), GFP-injected male (n = 3). An unpaired, two-tailed T-test determined statistically significant increase between Uninjected and GFP-injected female crickets' number of action potentials in response to left stimuli (C1. p = 0.045) and between Uninjected and GFP-injected male crickets' number of action potentials in response to right stimuli (D2. p = 0.010). There was no statistically significant difference for the female right stimuli (C2. p = 0.075) or the male left stimuli (D1. p = 0.199).

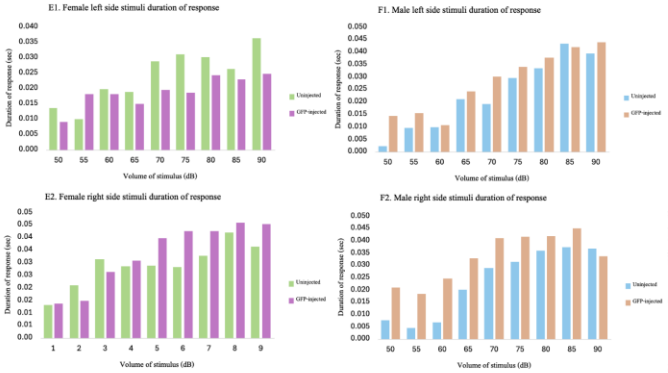

Figure 3. Significant decrease in duration of response between female Uninjected and GFP-injected crickets in response to right stimuli with Uninjected female (n = 3), GFP-injected female (n = 3), Uninjected male (n = 2), GFP-injected male (n = 3). An unpaired, two-tailed T-test determined a statistically significant decrease in length of response duration in seconds (E2. p = -0.397). There was no statical significance in duration of response for the female left stimuli (E1. p = 0.162) and both male left and right stimuli (F1. p = 0.45, F2. p = 0.095).

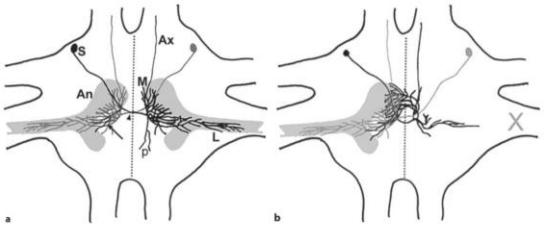

Figure 4. a) uninjured cricket prothoracic ganglion. AN-2 dendrites respect the midline and do not innervate nerves receiving signals from the contralateral ear. b) injured cricket prothoracic ganglion where trauma causes degeneration of the auditory nerve. AN-2 dendrites do not respect the midline and innervate nerves receiving signals from contralateral ear to compensate for loss of auditory input (adapted from Horch et al., 2011).

Acknowledgments and References
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