The Influence of Toll Receptors on the Developing Peripheral Nervous System of *Gryllus bimaculatus*

Kyla R. Gary
Hadley Horch
Bowdoin College
Neuroscience Department

Abstract

Numerous protein interactions govern the development of an organism's nervous system. The proteins in the Spätzle-Toll signaling system are hypothesized to be neurotrophin-like, where Toll receptors and their Spätzle ligands influence the growth of peripheral neurons toward their targets in the central nervous system. A specific protein, Toll-7, is expressed in the developing limb buds of the Mediterranean field cricket, *Gryllus bimaculatus*. Previous literature suggests that knocking down the expression of Toll-7 will disrupt axonal routing and decrease the volume of the limb buds and pioneer neurons. Our results show an additional process extending away from the central nervous system, implying Toll-7 is important in ensuring proper embryonic peripheral nervous system development in *Gryllus bimaculatus*.

Objectives

The primary objective of this project is to understand how knockdown of Toll-7 impacts the structural development in the peripheral nervous system of the Mediterranean field cricket, *Gryllus bimaculatus.*

Methods

Cricket Husbandry

Gryllus bimaculatus samples originally obtained from Ron Hoy at Cornell University. They were maintained on a 12:12 light/dark cycle at 28°C with 60-80% humidity. They were sustained with water and cat chow *ad libitum*.

Egg Collection

Adult crickets were deprived of damp dirt and water vials for 16 hours. Then, a 35mm petri dish of sand, covered with a damp 18cmx18cm paper towel, was placed over the dish and left in the cricket bins for 2 hours. Two laying dishes were placed in each bin, one dampened with reverse osmosis water and the other with tap water. The eggs collected from the dishes were transferred to an egg well dish created with 1% agarose in RO water. Following injection, the eggs were transferred to a solution of 1% Penicillin-Streptomycin in HEPES-buffered saline (HBS).

Needle Preparation

Microcapillaries were pulled using the DMZ Zeitz-Puller program with the P(A), P10 setting, used for thin-walled glass. A Narishige beveler (Model EG-45) beveled the pulled microcapillaries for 2 minutes at a 20° angle. The needles were visualized using a Leica microscope with LAS Core light visualized software to ensure the diameter of the inner walls adjacent to the opening was 8µL-12µL. Needles with widths outside of this range were discarded.

Egg Injection

A Narishige microinjector was used to inject Toll-7 double stranded RNA or double stranded GFP into the viable eggs. Injection time was set to 0.17 seconds, pressure was set to 10-15 psi, and the machine's balance was set to 0. A mixture of 0.5 µL of rhodamine dye, 0.5 µL of injection buffer, and 4µL of dsRNA (either Sema1a.1 or GFP) was created and 1.5 µL of the liquid was loaded into a needle for injection. Eggs were injected at a point approximately 20-30% of the length of the egg from the posterior end. Following injection, the eggs were stored in their plate in the 28° C incubator at 60-80% RH. They were immersed in the 1% Pen-Streptomycin in HBS solution, which was changed daily. Two days after injection, the eggs were transferred to a 35mm petri dish within three layers of paper towel squares, dampened with 1% Pen-Strep in HBS. These dishes were stored in the incubator. Eggs that were no longer viable removed and substrate changed daily.

Embryo Dissection

Five days after egg injection, the viable embryos were dissected from the eggs in phosphate-buffered saline (PBS) solution. The dissected embryos were transferred to a tube containing 4% paraformaldehyde in PBS for 30 minutes. They were washed with PBSTx (1X PBS with 0.1% TritonX-1000 three times, for five minutes each.

Immunohistochemistry

Alexa Fluor 488-conjugated anti-mouse HRP (1.5mg/ml stock concentration) was diluted 1:400 with PBSTx-HS (10% horse serum in PBSTx). The embryos were rinsed with PBSTx three times, for five minutes each, then blocked in the PBSTx-HS solution for one hour. Embryos were incubated in anti-HRP antibodies on a shaker, overnight at 4°C. Embryos were then washed with PBSTx, 3 x five minutes and mounted in 50% glycerol in HBS.

Visualization and Analysis

Embryos were visualized using Leica SP8 confocal microscope and analyzed with LAS Core Software. Using Imaris software, the volumes and lengths of the pioneer neurons were analyzed.

Results

To compare typical peripheral nervous system development with that of Toll-7 knockdown subjects, some crickets were injected with GFP dsRNA (Figure 4) to control for the needle poke and the introduction of dsRNA. Since GFP comes from jellyfish and is not naturally found in crickets, the introduction of GFP dsRNA should not impact the typical embryonic development process. Crickets injected with Toll-7 dsRNA show an abnormal process extending from the cell body to the distal tip of the limb bud.

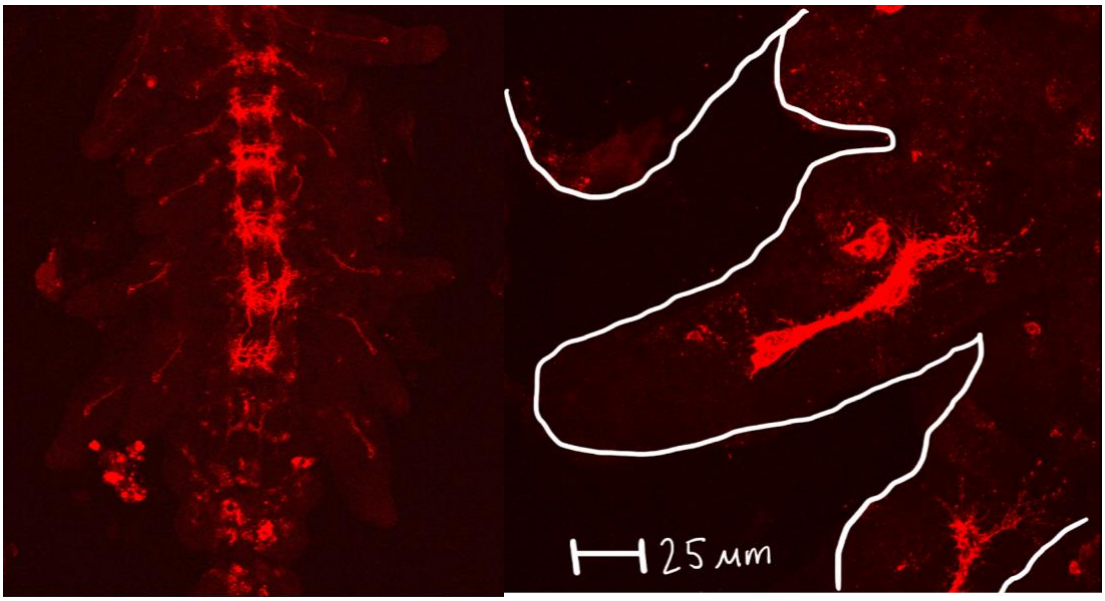

Figure 4. Confocal Images of GFP dsRNA Injected Crickets (Luo, 2023)

Left image (10x) shows the ladder-like central nervous system structure, as well as the developing peripheral nervous system. Right image (40x) shows the prothoracic limb bud.

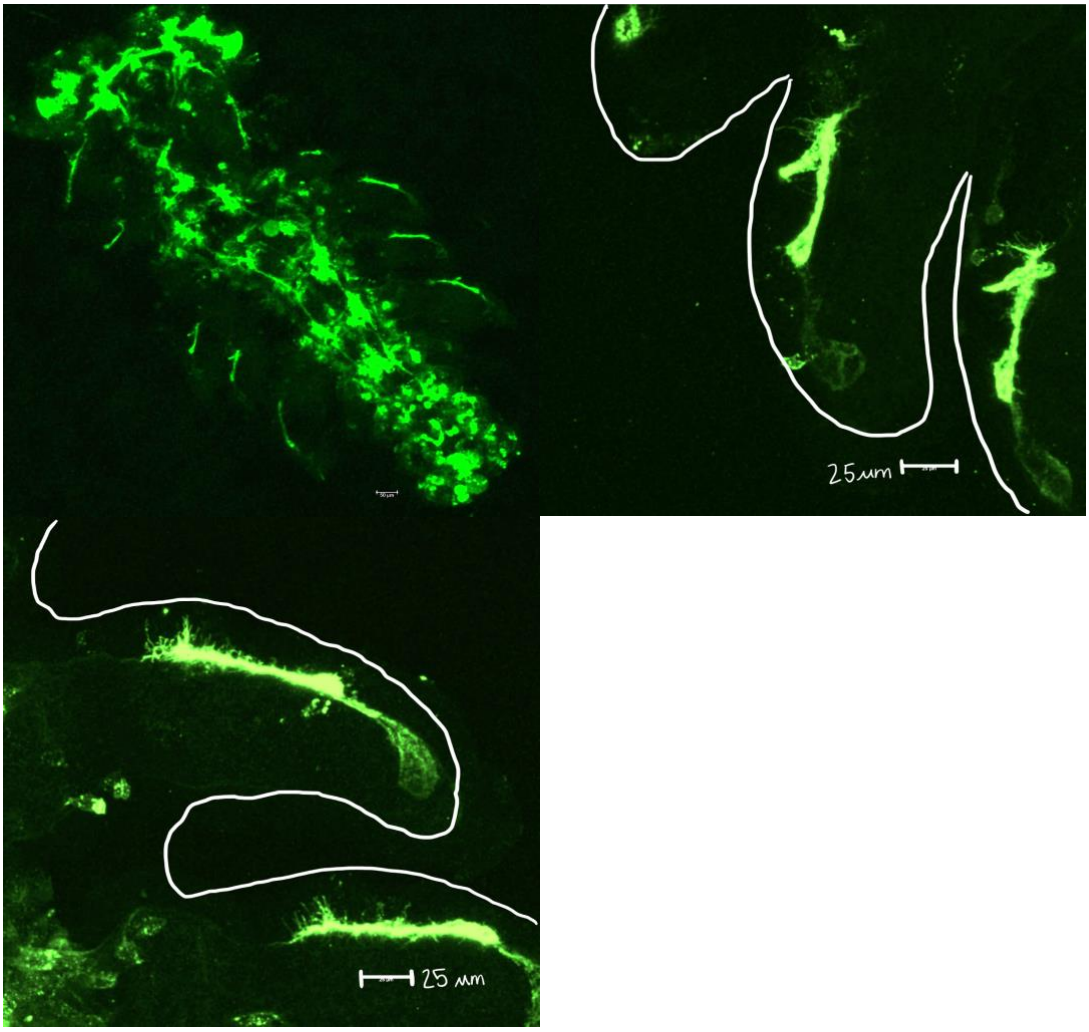

Figure 5. Confocal Images of Toll-7 dsRNA Injected Crickets

Top left image (10x) shows the ladder-like central nervous system structure, as well as the developing peripheral nervous system. Top right image (40x) shows the prothoracic limb bud, with a process extending towards the CNS as well as the distal tip of the limb bud. There is also a protrusion extending towards the anterior side of the limb bud, away from the central nervous system. Bottom left image (40x) shows the prothoracic limb bud, also with processes extending from the cell body to the central nervous system, distal tip, and anterior side of the limb bud.

Discussion

In the Toll-7 dsRNA injected crickets, there appears to be a process extending from the cell body towards the distal end of the limb bud that is not present in the GFP injected crickets. There are a few plausible explanations for this observed phenotypic abnormality. Toll-7 has been implicated with cell survival in *Drosophila melanogaster*, where animals with mutant forms of Toll-7 experienced higher levels of programmed cell death than their wild-type counterparts (Li et al., 2020). Therefore, knocking down the expression of Toll-7 could cause increased death of guidepost cells or even pioneer neurons. This loss of axonal guidance may cause the processes to extend towards unintended targets.

Additionally, Bentley and Keshishian (1982) characterized limb bud development in grasshoppers, while Luo (2023) confirmed this progression in *Gryllus bimaculatus.* Specifically, around Stage 9.0 of development, tarsal neurons appear at the distal end of limb buds. Perhaps, these

processes may be the tarsal neurons appearing too early and beginning to extend towards the central nervous system.

More experiments are needed to confirm the consistent appearance of the extra process extending towards the distal tip of the limb bud. It is beneficial to recognize that even while the crickets are mounted on their dorsal sides, the angles in which their legs sit on the slides may be inconsistent. Using Imaris software for data analysis may be able to control for some of this variability, but it should remain a consideration for future trials and conclusions. Further, we will use Imaris to measure filopodia quantity and density to determine if Toll-7 knockdown causes a decrease in either of these parameters.

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