Characterizing Toll Receptors in the Mediterranean Field Cricket, *Gryllus bimaculatus*

Tabarak N. Al Musawi , Hadley W. Horch
Bowdoin College
Neuroscience Department

Abstract

Neural plasticity describes the change in structure and function of neurons in response to the environment, external stimuli, or injury. It has been found that the Toll pathway, which includes a Spaetzle ligand and a Toll receptor, is involved in development and regenerative processes. To better understand the pathway, we characterized members of the Spz-Toll family by visualizing their expression patterns within cricket brains, ganglia, and embryos through whole mount *in-situ* hybridization experiments. We found that *toll receptor 7* was expressed in the adult brain and in the developing limb buds. We also found that *spz 1* was expressed in the anterior and midline portion of the ganglia whereas *toll 2* was not expressed at all. By characterizing the expression patterns of members of this pathway we hope to better understand Toll function in cricket neuroplasticity.

Project objectives

Neural plasticity and regeneration describe the adaptive changes that lead to new pathways in the nervous system as result of environmental changes, external stimuli, or trauma (*1)*. Injury to the adult nervous system often leads to disruption of neural networks and results in functional loss. Neurotrophins are a family of mammalian growth factor proteins intricately involved in the maintenance of neural circuit connectivity, development, and neural plasticity. Present at the site of injury, neurotrophins supplement and mediate neuronal repair and regeneration (*2)*. Even invertebrates like *Drosophila melanogaster* (fruit fly) express neurotrophin-like proteins. The Spaetzle protein family has been found to be analogous to mammalian neurotrophins due to their characteristic neurotrophin-like cystine-knot domains, which have been evolutionarily conserved among neurotrophin proteins (3). Within invertebrates, members of the Toll receptor superfamily function as receptors for the neurotrophin-like Spaetzle ligands (4). A number of Toll receptors are expressed within the *Drosophila* central nervous system– aiding in neuronal survival and development, indicating that Toll receptors have a role in neuroplasticity (5). While most Toll receptor studies reference *D. melanogaster*, the cricket has recently emerged as a model organism for studying the potential roles of Spz-Toll signaling in neuronal plasticity.

Compensatory plasticity, a form of neural plasticity, describes the structural and physiological rearrangement of the nervous system in response to injury. Two summers ago, I began studying the compensatory capabilities of the Mediterranean Field Cricket *(Gryllus bimaculatus)* with Professor Hadley Horch. The auditory system of the cricket consists of multiple tympanal membranes, or eardrums, located in their forelegs. Upon detecting an external stimulus, the auditory organs relay information through receptor neurons to ascending neurons-1 and -2 (AN-1 & AN-2) in the prothoracic ganglion. Typically, the input received from the right tympanum is conveyed to the right side of the brain and the signal received from the left tympanum is transmitted to the left side of the brain. When a leg is amputated, the ear is lost and the corresponding ANs are disconnected (deafferented). In response to deafferentation, dendrites stemming from the deafferented ANs grow across the midline, a boundary that is generally respected, to connect with the auditory afferents from the intact side, enabling the cricket to regain hearing (6) (figure 1).

The Horch lab recently assembled a transcriptome from the prothoracic ganglia containing transcripts present in both injured and uninjured crickets. After further transcriptional analysis, the lab discovered that members of the Toll signaling pathway, such as *toll 2*, show changes in expression levels 1 and 3 days post deafferentation. These findings in tandem with the potential neuroplasticity-inducing

properties of Toll receptors reveal an exciting research path into the involvement of Toll receptors in compensatory growth mechanisms. Two summers ago, I had the opportunity to explore the evolutionary relationship of Toll receptors among different insect species. Using the constructed cricket transcriptome, we mapped Toll receptor sequences using bioinformatics (NCBI Blast and Conserved Domain Search) and built phylogenetic trees containing Toll receptor sequences from crickets and other insects. We found four distinct Toll protein members in the cricket: Tolls 1, 6, 7 and 8 that have been strongly conserved among insects including fruit flies and termites.

This summer, my goal was to further characterize the Toll receptor family and understand the function of Toll receptor proteins in the compensatory growth of the auditory system in crickets. We approached this inquisition by performing whole mount *in*-situ hybridization experiments to visualize the expression patterns of Toll receptor members in the Mediterranean Field Cricket and interpolated their role as a function of their location within the tissue. This summer, we decided to focus on Toll 2, Toll 7 and Spaetzle 1.

Methodology

Tissue Preparation

Embryos were collected from stage 11 or younger eggs (2.5-5.5 days, as described by (8)) and dissected in HEPES Buffer Saline (HBS) to remove egg casing and yolk before fixing in 4% paraformaldehyde in phosphate buffered saline (PFA-PBS) overnight (O/N) at 4°C. Thoracic ganglia and brains were dissected from adult crickets and placed into 4% PFA-PBS (brain: 0.75-1hr, ganglia: 35 min) and then desheathed and stored in PFA-PBS at 4°C O/N. Tissue was rinsed (x3) with 1X PBSTx (PBS with Triton X-100) and dehydrated using a methanol series diluted in PBSTx (25%, 50%, 75% and 100% in consecutive order; 10 min for embryos, 20 min for brain and ganglia). All tissue was frozen in 100% methanol at -20°C for long term storage.

In situ hybridization

Tissue was rehydrated using the aforementioned methanol series (75%, 50% and 25% in consecutive order; 10 min for embryos, 20 min for brain and ganglia) then submerged in 2µg/mL Proteinase K in PBSTx (7 min for embryos and 17 min for brain and ganglia). The tissue then underwent a refixation stage using glutaraldehyde and PFA for 20 mins before O/N hybridization (at 60°C) in probe that was prepared previously. Tissue was treated with 1.5% blocking solution for 1 hr and incubated in anti-DIG AP Fab fragments (1:2500) for 1 hr. Steps were performed by Biolane HTI 16V (Intavis). The color reaction used BCIP & NBT and was left O/N at room temperature.

Visualizing tissue

Embryos were suspended on a slide containing 50% glycerol in PBSTx and visualized using the Zeiss Axioskop 2 Plus microscope with a Leica DFC450 C camera attachment and Leica Application Suite programming (version 4.6.2). Brain and ganglia were suspended in a 1.5% agarose plate containing 50% glycerol in PBST and visualized using a Leica M165 FC microscope with the Leica DFC450 C camera attachment.

Results

To deepen our characterization of toll receptors, we performed *in-situ* hybridization experiments on embryos and adults. We visualized the following Spz-Toll members: s*pz1*, *toll 2*, and *toll 7*. We found that *spz1* was expressed in the anterior and midline portion of the prothoracic ganglia whereas *toll 2* was not expressed at all (figure 2D, E). Further, we found that *toll 7* is expressed in the mushroom bodies of the brain and in the developing embryonic limb buds but not within the

prothoracic ganglia (figure 2A-C). We used a previously studied candidate with a known expression pattern, Sema2a, as a control to verify that our *in-situ* hybridization was indeed working properly.

Significance and Interpretation

The aim of this study was to characterize members of the toll receptor family in the Mediterranean Field Cricket. To do so, we performed whole mount *in-situ* hybridization reactions to visualize the expression patterns of various members of the Spz-Toll family in the prothoracic ganglia, brain, and in embryos. Analyzing the transcript localization can reveal insights regarding the function of the proteins.

Work by others has identified eleven toll members in the cricket genome, some of which are upregulated to promote leg regeneration (9). As this research is largely experimental, we will compare the expression patterns of our Spz-Toll members in the cricket with expression patterns documented in the well-studied *Drosophila melanogaster* (fruit fly) among other insects. We found that *toll 2* was not expressed in the ganglia, however, we remain interested in researching this member of the Toll family as one study found that variants of *toll 2* were found in leg regeneration (9) (fig. 2E). Further, lack of expression may be a result of a technical issue with the probe. We hope to re-make and test the probe in the future.

We found that *toll 7* is expressed in the adult brain and developing limb buds (fig 2A-C). One study focusing on the neurotropic behavior of Tolls 6 and 7 in *D. melanogaster* found that *toll 7* was expressed in the brain and in embryos (10). The expression pattern of *toll 7* is of special interest to us as the expression pattern appears localized to the mushroom bodies of the brain, specifically at the neurogenic tip. This area of the brain has been implicated in plasticity, neurogenesis, and learning (11). Therefore, we hypothesize that *toll 7* plays a role in the neural plasticity within the cricket, which we hope to test in future experiments. We found *spz 1* at the anterior and midline portion of the prothoracic ganglia (fig. 2D). A study done in 2008 concluded that *spz 1* is necessary for neuronal survival within fruit flies and as such it is not a surprise to find it within the prothoracic ganglia, a site of neuronal plasticity (12).

For further research, it would be interesting to continue visualizing the remaining members of the Toll protein family to characterize the overlapping or distinct expression patterns in adult and embryonic crickets. Once we have a baseline for the Toll expression pattern it would be interesting to perform knock-down experiments within the protein family to see how the morphology and physiology of ANs as well as the behavior of the cricket are affected when Tolls are downregulated.

Figures/Charts

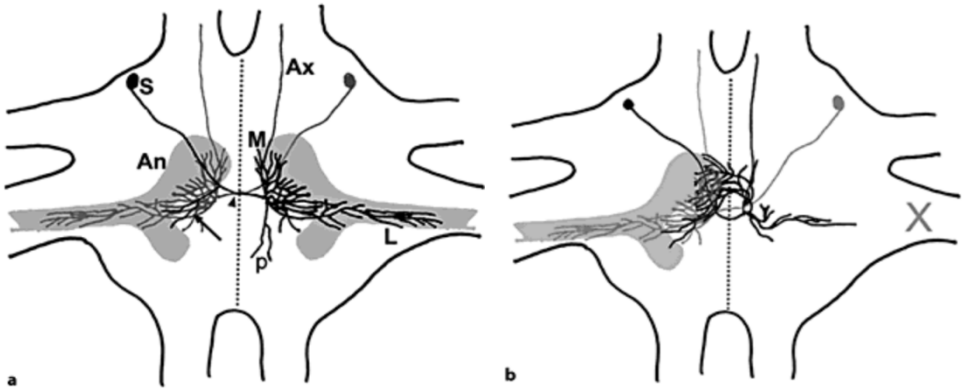

Figure 1: Crickets exhibit compensatory growth in their auditory system following deafferentation. A) Dendrites (black) stemming from ascending neurons (AN) receive input from auditory afferents (gray), and do not transgress the midline (dotted line) in the prothoracic ganglia. B) Injury on the right (X) results in unilateral loss of the auditory afferents followed by significant rearrangement of ipsilateral AN dendrites that cross the midline to form connections with the uninjured side. Figure adapted from (7).

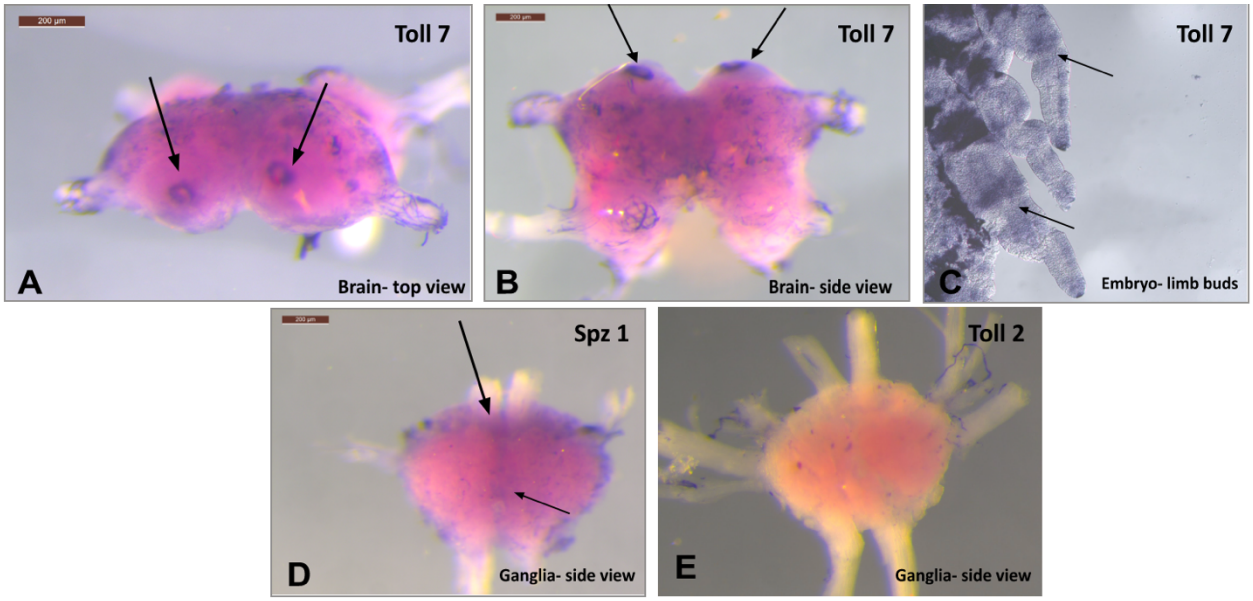

Figure 2: *Toll 7* is expressed at the neurogenic tip of the adult brain and in the developing limb buds whereas Spaetzle 1 is found in the anterior and midline portion of the prothoracic ganglia. A) Top view of adult cricket brain expressing *toll 7* at the tip of the mushroom bodies (black arrows). B) Side view of the adult brain expressing *toll 7*. C) Developing limb buds expressing *toll 7*. D) Prothoracic ganglia expressing *spz 1* in the anterior and midline portion. E) Ganglia exposed to *toll 2* showed no patterning indicating lack of presence.

Funded by: The Maine Space Grant Consortium

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