Characterization of FMRFamide Expression in the Enteric Nervous System of *Manduca sexta*

Sabrina Serrano, Jonathan Palacios Alvarez, Dr. Harry Itagaki, Kenyon College
Kenyon Summer Science Scholars, GLCA Neuroscience NSF-REU 2017

Abstract

The Enteric Nervous System (ENS) comprises all of the innervation of the digestive system and, in particular, the gut. Currently, not much is known about the function of the ENS, but it is believed that it could have implications in a multitude of human diseases such as obesity, Diabetes Mellitus, Crohn's Disease, and Irritable Bowel Syndrome (1). A greater understanding of the ENS could lead to new therapies and treatments for these conditions. We are still attempting to gain a sense of how the ENS functions and develops. The enteric microbiome has been shown to play a role in the development of the ENS and CNS (2, 3). Dysbiosis in the enteric microbiome has been linked to Autism Spectrum Disorder, depression, Diabetes Mellitus and Irritable Bowel Syndrome (3, 4, 5).

To study the ENS and the role of diet and the enteric microbiome in its development, we used larval *Manduca sexta. M. sexta* is ideal because of its short 18 day developmental period and the size of its gut in relation to its body mass. In a study on *Lumbricus terrestris*, FMRFamide, a neuropeptide, has been implicated in digestive processes via the neuromuscular regulation by the ENS. We studied FMRFamide expression in 3rd instar *M. sexta* and investigated how two different diets, one artificial and one natural, could affect this expression within the gut. We also looked at the effects that a sterile environment could have on FMRFamide expression and development.

Introduction/Aims

- Studies investigating diseases that affect the gut, like *Diabetes mellitus*, have found loss of inhibitory neuropeptides and an increase in neuropeptides in the GI tract of mice (1)
- Depression, obesity, and autism have been linked to dysbiosis in the enteric microbiome (4, 5, 6).
- Aim 1: We hope to better understand FMRFamide distribution in the *M. sexta* enteric nervous system by characterizing its expression developmentally and in different diet types (artificial vs. tomato leaf)
- Aim 2: To determine the effects that the enteric microbiome has on the enteric nervous system, we will use germ free *M. sexta* reared on both a sterile and non-sterile diets and compare.

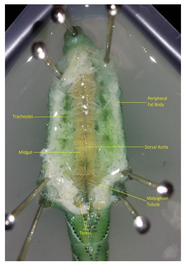
Methods

***Manduca sexta* Preparations**

All *Manduca sexta* were housed in 25°C (16L:8D) chambers and fed on either an artificial diet of wheat germ or a natural diet consisting of tomato plant leaves. *M.sexta* from different instars on both diets were anesthetized on ice before dissection. The guts and central nerve cords were removed and fixed in 4% paraformaldehyde in PBS overnight.

***Manduca sexta* sterile housing and sterile rearing**

M. sexta that underwent sterile rearing were fed an autoclaved wheat germ diet *ad libitum*. All sterile *M. sexta* were housed in sterile petri dishes until reaching the third instar. Prior to hatching all sterile *M. sexta* were also dipped into 70% EtOH. Every other day sterile *M. sexta* were moved into a new sterile petri dish and were handled under a sterile fume hood. Whenever handling these sterile *M. sexta* 70% EtOH washed gloves were used.



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Methods (Cont.)

***Manduca sexta* sterile housing and sterile rearing (continued)**

Each petri dish that contained *M. sexta* was housed in an incubation chamber. Once the sterile *M. sexta* reached the third instar they were sedated with ice and in under 10 minutes were dissected and their midgut and their nerve cord were placed in 4% Paraformaldehyde in PBS.

Immunohistochemistry

After fixation, the dissected tissues were washed in PBT 8x20 min., then blocked in 10% goat serum overnight. Tissues were then incubated with rabbit primary antibody FMRFamide (1:1000, abmcam.com) overnight, washed again, and then incubated in TRITC conjugated goat anti-rabbit 1:400 secondary antibody (Sigma-Aldrich) overnight, then washed and mounted onto slides with 80% glycerol and coverslipped.

Immunofluorescent Microscopy and ImageJ Analysis

Using a Nikon Optiphot 2, the prepared slides were visualized with 4x and 10x objective lenses. Micrographs of the entire structure were taken with these magnifications and will be analyzed later.

Using ImageJ software, the micrographs will be individually analyzed to acquire data on the number of immunoreactive cell bodies present and the diameters of these cells. These data will allow us to determine if differences in FMRFamide expressing cells may be affected by diet.

Gut Immunofluorescence (Tomato Leaf)

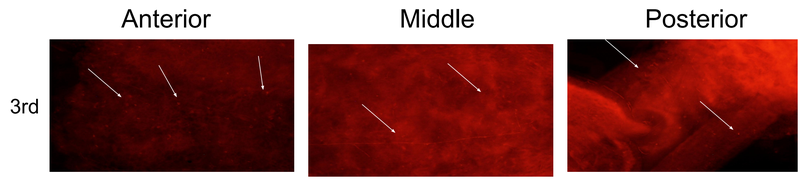

Figure 1. Micrographs displaying, from left to right, the anterior, middle, and posterior midgut of 3rd instars at 10x magnification. FMRFamide-like immunofluorescent cells can be seen (arrows).

Gut Immunofluorescence (Artificial Diet)

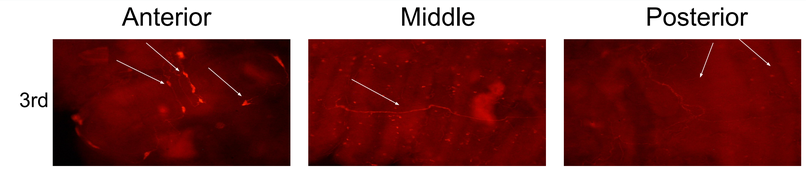

Figure 2. Micrographs displaying from left to right the anterior, middle, and posterior midgut of 3rd, 4th, and 5th instars (by row) at 10x magnification. FMRFamide-like immunofluorescent cells can be seen (arrows).

Gut Immunofluorescence (sterile vs. non-sterile)

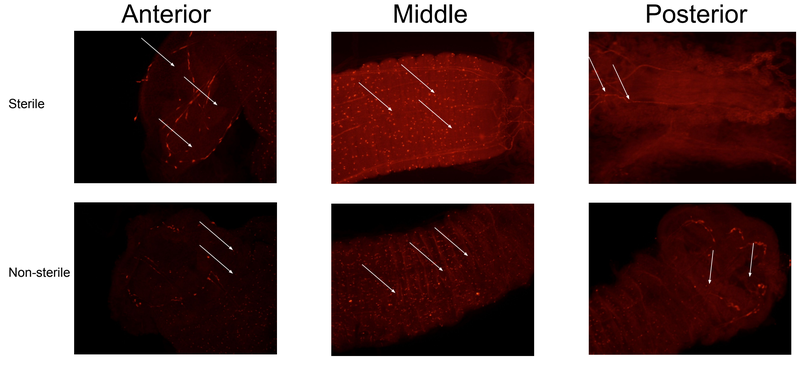

Figure 3. The white arrows point to examples of immunoreactive cells labeled for the neuropeptide FMRFamide in third instar *M. sexta*. The tissue shown in the first row are from a *M. sexta* reared on a sterile diet and the tissues shown on the second row are from a *M. sexta* reared on non-sterile food. The columns go from the anterior of the *M. sexta* to the posterior of the *M. sexta* tissues.

Results (Contd.)

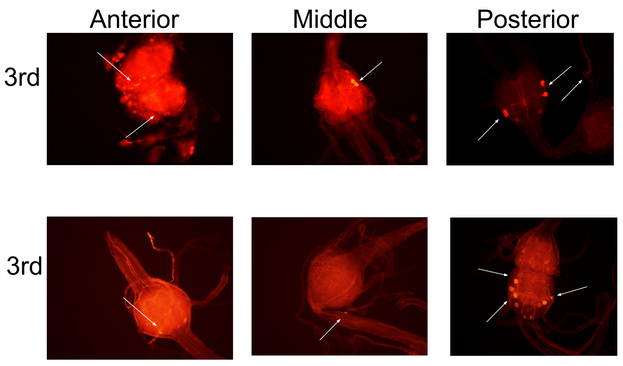

Figure 4. Micrographs depicting anterior, central, and posterior ganglia of the central nerve cords in 3rd instar by tomato leaf diet (top row) and artificial diet (bottom row) at 10x magnification. Bright red colors indicate FMRFamide immunofluorescent cells. We do not see a developmental pattern of FMRFamide responsive cells, but we do see consistency in location, similar to tomato leaf raised diet *M. sexta* CNS.

Conclusions/Future Directions

- Initial data indicate that expression of FMRFamide decreases as development reaches later stages, consistent with the work presented by Witten and Truman (1996).
- There appears to be a slight decrease in FMRFamide expression in the *M.sexta* that have been fed only the natural tomato leaf diet compared to the artificial diet. This suggests that FMRFamide expression in the gut is affected by diet, consistent with the values observed in an investigation of FMRFamide responsive cells and nutritional diets and eating cycles in the *Locusta* gut (Zudaire et al. 1998)
- We are using ImageJ to analyze the micrographs and obtain quantitative data of FMRFamide expressing cell count and size between different diet types and sterile environment types.

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Acknowledgments

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