THE ROLE OF THE MICROBIOTA IN PREY CAPTURE BEHAVIOR

by

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A DISSERTATION

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Title: The Microbiota Modulate Prey Capture Behavior by Increasing Inhibition in the Optic Tectum

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DISSERTATION ABSTRACT

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Doctor of Philosophy

Department of Biology

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Title: The Microbiota Modulate Prey Capture Behavior by Increasing Inhibition in the Optic Tectum

There is a growing body of evidence that normal nervous system activity requires signals from resident microbes. We have yet to discover the mechanisms by which the microbiota influence brain function. However, we know that the enteric nervous system (ENS) serves as an important interface between the developing host and its microbiota. In this dissertation I will introduce a novel computer-assisted method for ENS characterization and a novel, incredibly specific mechanism of host-microbe interactions. With new ENS characterization method I developed, it will be possible to better understand the role of the ENS during development, by more rapidly and algorithmically assessing ENS phenotypes. Furthermore, my discovery of a single microbially-sourced protein that influences vertebrate host prey capture behavior and visual system development, will provide a new appreciation for the role resident microbes, both in model organisms and in ourselves. By both establishing a new, less biased, approach to image analysis and describing a surprising new regulatory host-microbe interaction, the work I describe in this dissertation should provide the foundation for an explosion of exciting discoveries in the near future.

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(2) Van Ryswyk L, **Simonson LW**, Eisen J. 2014. Intermediate filament gene *inab* is required for proper axon morphology in zebrafish primary motoneurons. *PlosOne* 9:e886631. PMC3922942.

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CHAPTER I

INTRODUCING HOST-MICROBE INTERACTIONS AND THEIR ROLES IN VERTEBRATE BRAIN DEVELOPMENT

Animals evolved in a microbe rich environment, so it should come as no surprise that microbes can have a profound effect on animal development and function. For example, microbes resident in the vertebrate intestinal tract, the largest concentration of vertebrate host-associated microbes commonly referred to as the microbiota, are known to promote host health, by improving digestion, promoting immune system development, and inhibiting infection[1]. Intriguingly, a number of recent studies provide evidence that these commensal microbes also influence host neural activity and development, promoting social and anxiety-like behaviors[2], although the underlying mechanisms remain unknown. Not only do commensal microbes affect the host nervous system, the host nervous system can also affect the composition of the resident microbial community. Here I provide an overview of interactions between nervous systems of vertebrate hosts and their resident microbiota that lays the groundwork for my dissertation research.

Microbiota affect anxiety-like behavior

It was initially surprising to learn that the microbiota are reported to decrease anxiety-like behavior in mouse models. An important demonstration of the microbiota's influence over host behavior is increased exploration, associated with decreased anxiety-like behavior, in germ free (GF mice) compared to more microbially diverse specific pathogen-free (SPF) mice[3]. As further evidence of the microbiota's role in anxiety regulation, compared to SPF mice, GF mice

are also more inclined to explore the implicitly more dangerous exposed areas of an elevated plus maze[4,3,5,6]. The microbial regulation of these anxiety-like behaviors is remarkably plastic, with microbial inoculation rescuing behaviors in ex-GF mice[7]. Furthermore, in mouse models probiotic supplementation with *Lactobacillus rhamnosus* (JB-1) is sufficient to decrease anxiety-like behavior in a vagal-nerve dependent manner[8]. The vagus nerve synapses with the enteric nervous system (ENS) composed of neurons and glia that reside within the vertebrate gut and provide local innervation, modulating gut activity and host-microbe interactions[9]. These results raise the possibility of ENS signaling as an intermediary in the ability of the microbiota to modulate anxiety behaviors.

Microbiota affect social behavior

Host social behaviors are also modulated by changes in the composition and activity of the resident microbiota[10]. Interestingly, some of these effects appear to occur early in host development, and are no longer influenced by the microbiota in adulthood. As an example, in contrast to their SPF counterparts, GF mice neither seek out other mice nor recognize familiar mice[10]. While the social recognition phenotype cannot be rescued by microbial inoculation, suggesting a developmental defect mediated by microbiota absence, social avoidance is effectively rescued[10]. The ability of the microbiota to modulate host social behavior is not limited to rodents, as recent results in zebrafish supplemented with a probiotic, *Lactobacillus rhamnosus*, showed altered shoaling behavior compared to CV fish[11]. While not conclusively indicating a conserved pathway for microbes to influence social behavior, these data sets establish that both active modulation and developmental critical periods play a role in the behavioral effects of host-microbiota interactions.

Microbiota affect brain function

Not only can the microbiota influence host behavior, they can also influence host brain gene expression[7,4,3,5]. Unlike the anxiety-like behaviors apparent in GF mice, there are several transcriptional defects that are not rescued by microbial inoculation[4]. GF and ex-GF adult mice have lower BDNF and serotonin (5-HT) levels, along with a decrease in several 5-HT receptors in specific brain regions such as the hippocampus and amygdala[7,4,3,5]. These data suggest that there is a critical period for microbial exposure that is necessary for normal transcriptional activity within the early developing brain, however the timing of this exposure remains completely unknown.

Microbiota are linked to complex behavioral disorders

An increasing number of studies suggest that imbalances in the microbiota, often referred to as dysbiosis, can result in both behavioral changes and disease states. For example, there is an interesting correlation between altered microbiota composition and autism spectrum disorder (ASD), a group of social and behavioral disabilities with a wide range of severity[12,13]. Mouse models of ASD harbor a dysbiotic microbiota compared to WT controls[14]. Probiotic *Bacteroides fragilis* supplementation achieved partial restoration of the microbiota in ASD model mice and also rescued several behavioral defects such as anxiety behavior, communication deficits, and stereotyped behavior[14]. An independent study of mice with aspects of ASD also found a correlation between the behavioral changes and shifts in the microbiota[15]. While these studies do not directly implicate the shifting microbiome as a cause of ASD, they do serve as a promising starting point for the study of the role of the microbiota in shaping complex human behavioral disorders.

Microbiota signal to the brain via unknown pathways

The routes by which the microbiota influence brain function are unknown. There are three main candidate pathways: the vagus nerve, the immune system, and via a humoral route. The gnotobiotic zebrafish model will allow me to assess the role of these potential routes in host-microbe interactions during early brain development. The vagal nerve monosynaptically connects the ENS and central nervous system (CNS). Through enteric neural activation of the vagal nerve, there is a possible pathway for molecules secreted by the microbiota[16] to reach the brain either directly or via secondary signaling between neurons. The vagus nerve could also signal to the brain independently of the ENS[17]. The immune system constantly interacts with the microbiota and could potentially conduct signals to the brain. In support of this hypothesis, treatment with anti-inflammatory factors IGF-1 and IL-10 inhibits sickness behavior in mice[18]. There is also the possibility of microbial signaling through secondary messengers that does not fit into either neural or immune pathways. For instance, many microbially-secreted blood-brain barrier (BBB) permeable molecules are neurotransmitter or neuromodulator precursors[19,20]. The microbiota is also capable of manipulating BBB permeability through short chain fatty acid signaling, with GF mice having increased BBB permeability[21]. The use of gnotobiotic zebrafish with mutations in ENS and immune signaling genes combined with bacterial monoassociation studies could allow us to finally pinpoint the signaling mechanism that the microbiota use to influence brain development.

The nervous system influences microbiota composition

Just as the microbiota influence the nervous system, the nervous system can influence the microbiota. The ENS regulates host-microbe interactions by controlling gut motility and secretions, thereby manipulating microbial community dynamics[22]. The ENS is known to directly

interact with the microbiota and also communicates with the CNS via the vagus[16]. Therefore, it follows that the ENS may play an important role in gut-brain axis signaling. Much of the developmental genetics of the ENS remains undescribed, however, leaving this key step in the possible host-microbe interaction pathway unknown.

My studies contribute to understanding interactions between the host nervous system and the microbiota

In this dissertation, I describe studies using a zebrafish model to investigate both how the ENS might influence the microbiota and how the microbiota influence brain development. I describe a novel method for characterizing the developing zebrafish ENS in Chapter II. In Chapter III, I briefly introduce the study of larval zebrafish behavior and in Chapter IV, I describe a novel role for a microbial protein in regulating the behavior and brain development of larval zebrafish. Chapter V summarizes future directions required to complete the studies described in Chapter IV.

CHAPTER II

CHARACTERIZATION OF ENTERIC NEURONS IN WILD-TYPE AND MUTANT ZEBRAFISH USING SEMI-AUTOMATED CELL COUNTING AND CO-EXPRESSION ANALYSIS

ABSTRACT

To characterize fluorescent enteric neurons labeled for expression of cytoplasmic markers in zebrafish mutants, we developed a new MATLAB-based program that can be trained by user input. We used the program to count enteric neurons and to analyze co-expression of the neuronal marker, Elavl, and the neuronal subtype marker, serotonin, in 3D confocal image stacks of dissected whole-mount zebrafish intestines. We quantified the entire population of enteric neurons and the serotonergic subpopulation in specific regions of the intestines of *gutwrencher* mutant and wild-type sibling larvae. We show a marked decrease in enteric neurons in *gutwrencher* mutants that is more severe at the caudal end of the intestine. We also show that *gutwrencher* mutants have the same number of serotonin-positive enteroendocrine cells in the intestine as wild-types.

INTRODUCTION

We and others have identified several mutant zebrafish lines that exhibit enteric nervous system (ENS) defects and thus may serve as models of genetic diseases that affect ENS function [1-3]. Understanding the roles of the mutant genes requires quantitative expression analysis at several different developmental stages for a number of known cell identity markers, for example, neurotransmitters that distinguish distinct types of enteric neurons [4]. The process of counting enteric neurons in these mutants is very time-consuming, especially if one relies on manual identification of cells in sectioned animals, as we have done in the past [1]. We and many other researchers have resorted to using cumbersome techniques when attempting to quantify

cells in sectioned tissue [for examples and discussion of some of these techniques see [5, 6]]. Without these techniques, fragments of cells in multiple sections would quickly lead to erroneous results. Another approach is to count cells in 3D confocal image stacks from the entire organism or from the specific region of interest, in our case the intestinal tract. Although such whole-mount techniques bypass many of the issues associated with counting cells in sectioned material, determining cell counts from stacks of confocal images poses other problems. Here we describe analysis of the enteric nervous system of *gutwrencher*^{b1088} (*gwr*) mutants using a new method we developed for computer-assisted quantification of cells in whole-mount 3D confocal image stacks of dissected intestines.

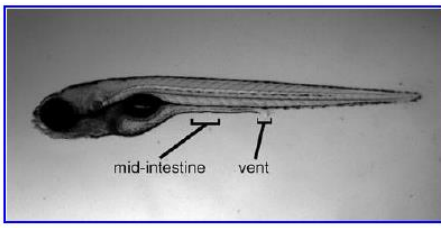

FIG. 1. A representative 5 dpf wild-type larva showing th mid-intestine and vent regions where enteric neurons an enteroendocrine cells were counted.

gutwrencher^{b1088} (*gwr*) is a gene that appears to be pivotal for proper ENS development [1]. Previous counts of enteric neurons in sectioned *gutwrencher* mutant zebrafish larvae revealed a 3.5-fold decrease in enteric neurons overall and a 6-fold decrease in the number of serotonin (5HT) positive enteric neurons compared to wild types [1]. This observed decrease in enteric neurons has also been shown to correlate with dysfunctional gut motility [1]. To better characterize *gwr* and other mutations that affect the enteric nervous system, additional co-expression analyses must be done to show whether all enteric neurons are affected equally, or whether a mutation preferentially affects specific types of enteric neurons.

We were unable to find counting programs that are appropriate for quantifying enteric neurons in whole-mount zebrafish intestines. Many programs used to quantify eukaryotic cell numbers, for example those described by Oberlaender [7] and DeCoster [8] rely on images of nuclear markers, which allow for straightforward image segmentation and watershed analysis algorithms to quickly separate and identify individual cells. However, these programs fail to separate cells with cytoplasmic labeling, such as those we use here. There are also a number of programs [for examples see [9-11]] that are capable of identifying and separating clusters of cells, but only in two-dimensional images.

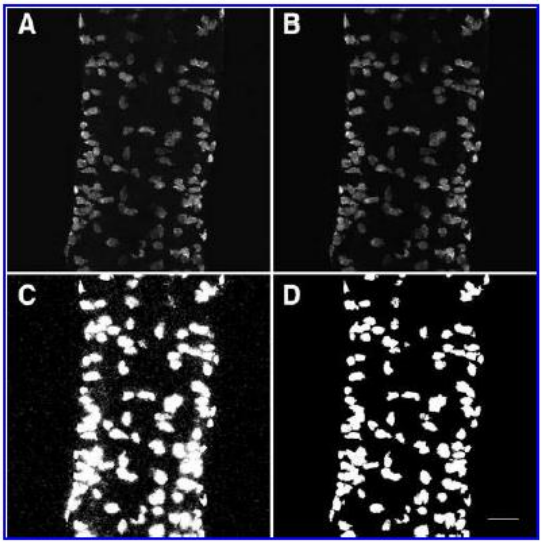

FIG. 2. The algorithm presented here is capable of properly segmenting images of dissected whole-mount zebrafish intestines. **(A)** Maximum intensity projection of dissected, whole-mount, wild-type mid-intestine labeled with anti-Elavl antibody. **(B)** Maximum intensity projection of **A** after deblurring with Wiener filter. **(C)** Maximum intensity projection of output from Otsu's N-thresholding algorithm applied to **B**. **(D)** Maximum intensity projection of **C** after removal of all small pixel clusters. Scale bar = 25 μm.

Whole-mount 3D image stacks of dissected larval zebrafish intestines with neurons fluorescently labeled for cytoplasmic markers are therefore inappropriate for either of these classes of programs. There may be other programs available that would suit our purposes, however, we decided to generate a new program that would be tailored to our specific needs. Here we describe the new program we generated and show that it accurately counts neurons labeled for expression of one or two markers in 3D image stacks of dissected zebrafish intestines. A feature of this program is that it can be trained by the user, and thus could be adapted to count other types of fluorescently labeled cells in the intestine or other regions of whole-mount zebrafish embryos or larvae. Our counts of enteric neurons using this program reveal that even in wild types there are significantly fewer enteric neurons at the caudal end of the intestine than in the

region of the mid-intestine in young larvae, and that this difference is magnified in *gwr* mutants. In addition, we provide counts of serotonergic enteroendocrine cells in the larval zebrafish intestine, and show that their numbers are similar in *gwr* mutant and wild-type larvae.

MATERIALS AND METHODS

Animals Animals were reared at 28.5 °C according to standard zebrafish husbandry [12] and staged by days postfertilization at 28.5°C (dpf).

Immunohistochemistry Antibody staining for Elavl (1:10,000, Molecular Probes Inc., Eugene, OR, catalog number A-21271) and 5HT (1:10,000, Immunostar, Hudson, WI, catalog number 20080) was performed at 5 dpf as previously described (Uyttebroek *et al.*, 2010). Secondary antigens were visualized with standard fluorophore-labeled antibodies for rabbit IgG (1:1,000, Molecular Probes Inc., Eugene, OR, catalog number A-11008) and mouse IgG (1:1,000, Molecular Probes Inc., Eugene, OR, catalog number A-11030). *gwr* mutants were separated from wild-type siblings at 5 dpf according to morphological characteristics [1].

Manual cell counting After immunohistochemistry, intestines were dissected and mounted in PBS on a cover slip. Z-stacks were acquired on a Zeiss LSM 5 Pascal confocal microscope and subsequently projections were made with the y-axis as turning axis, 180° projections and difference angle 2° using LSM 5 Pascal imaging software (see supplemental movie, available at: http://uoneuro.uoregon.edu/eisen/). Counts of labeled cells were made at the level of the mid-intestine and the level of the vent (Figure 1). In vent images, only the most aboral 200 μm were analyzed. In mid-intestine images we examined a 200 μm region from the top of each image. We counted Elavl positive, Elavl and 5HT double positive, and 5HT positive cells, rotating the

projections to ensure that we counted all cells. Counts are taken from five wild types and five mutants.

Image segmentation and denoising algorithm We identified fluorescence channels within each image as having either relatively high or low levels of background, corresponding in our case to 488nm (5HT; Alexa Fluor 488) and 546nm (Elavl; Alexa Fluor 546) channels respectively. We processed each image channel separately, based on the wavelength being visualized (Figure 2A). To reduce image noise and blurring, a pixelwise adaptive Wiener filter based on statistics estimated from a local 10-pixel neighborhood of each pixel was applied to each 2D matrix (Figure 2B) [13]. These matrices corresponded to a single z-stack channel within a 3D confocal image.

We thresholded each image channel using an automated determination of the threshold level [14, 15] (Figure 2C). Clusters of less than 100 pixels, corresponding to noise or background signal, were deleted from both binary image channels (Figure 2D). To merge punctuate pixel clusters in the 488nm channel, morphological opening and closing operations were performed. We found these morphological operations to be unnecessary in the 546nm channel, because there was relatively low noise and clear labeling of complete cells in the 546nm binary image compared to images from the 488nm channel. Then, in both channels morphological erosion was performed if any pixel clusters exceed six-times the volume of a single stereotyped ENS cell, or if more than 1,000 clusters remained, as these qualities indicate remaining noise or background signal in the binary images. The cells being analyzed have stereotyped sizes, thus, pixel clusters do not need to be separated to the point of containing only one cell, as cluster characteristics can be used to find the number of cells within a pixel cluster.

Cell type identification and coexpression analysis algorithm We identified relevant cell types through analysis of binary images corresponding to individual channels from raw confocal images (Figure 3A). All pixel clusters corresponding to enteric neurons were revealed by the 546nm binary image (Figure 3B). We constructed an image (*C*) consisting of pixels that colocalized in both the 488nm and 546nm binary images such that

$$C = S \wedge E$$

where *S* is the set of all pixels clusters in the 488nm (5HT) binary image, *E* is the set of all pixel clusters in the 546nm (Elavl) binary image, and ∧ represents the operation of identifying all pixels that located at the same coordinates in each image (Figure 3D). To identify clusters that were unique to the 546nm channel, we constructed a binary image (*S'*) such that

$$S' = S - C$$

where all colocalized pixel clusters were removed from the 488nm (5HT) binary image (Figure 3C). Relevant cell types were thereby represented by binary images *E*, *C*, and *S'*, corresponding to enteric neurons, serotonergic neurons, and serotonergic enteroendocrine cells, respectively (Figure 3B-D).

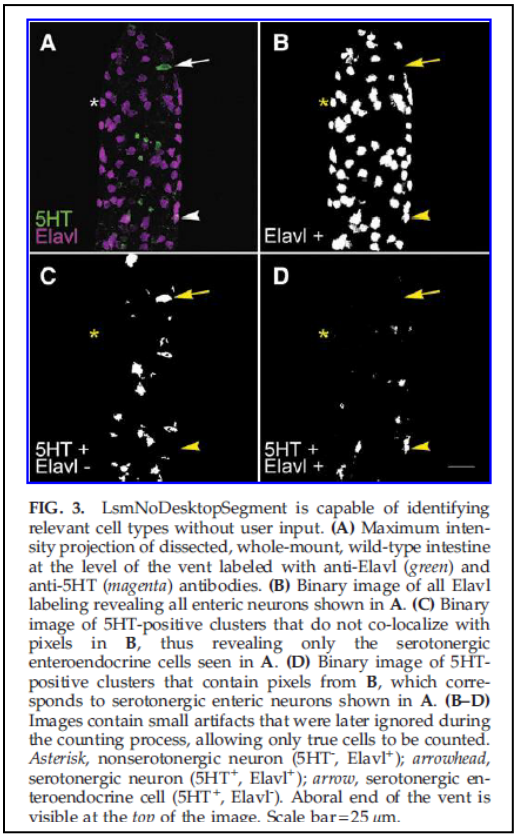

FIG. 3. LsmNoDesktopSegment is capable of identifying relevant cell types without user input. **(A)** Maximum intensity projection of dissected, whole-mount, wild-type intestine at the level of the vent labeled with anti-Elavl (*green*) and anti-5HT (*magenta*) antibodies. **(B)** Binary image of all Elavl labeling revealing all enteric neurons shown in **A**. **(C)** Binary image of 5HT-positive clusters that do not co-localize with pixels in **B**, thus revealing only the serotonergic enteroendocrine cells seen in **A**. **(D)** Binary image of 5HT-positive clusters that contain pixels from **B**, which corresponds to serotonergic enteric neurons shown in **A**. **(B–D)** Images contain small artifacts that were later ignored during the counting process, allowing only true cells to be counted. *Asterisk*, nonserotonergic neuron ($5HT^-$, $Elavl^+$); *arrowhead*, serotonergic neuron ($5HT^+$, $Elavl^+$); *arrow*, serotonergic enteroendocrine cell ($5HT^+$, $Elavl^-$). Aboral end of the vent is visible at the *top* of the image. Scale bar=25 μm.

Cell cluster estimation and counting algorithm We cropped raw and binary images to a region of interest (Figure 4A,B). We then individually examined each pixel cluster using the binary image as a colored mask over the appropriate raw image. We viewed maximum intensity projections of only

the image layers where the cluster of interest appeared. The cluster in question was given a red color while all other visible clusters were colored blue (Figure 4C).

Data from previous analyses greatly informed the cluster size estimation and counting processes, because of the stereotyped size of cells being analyzed. If no previous cluster data was loaded, clusters were initially assumed to be single cells. If we loaded data from a previous analysis, we then approximated the probability of a given cluster being a single cell, or up to four closely joined cells. This estimation was made possible by comparing characteristics for each pixel cluster to characteristics of clusters containing different numbers of cells that had been previously processed. We automatically ignored any pixel clusters that were smaller than 85% of the smallest previously encountered cluster that we had identified as a cell. In addition, we ignored clusters if the raw image intensity in that region was lower than 75% of the least intense

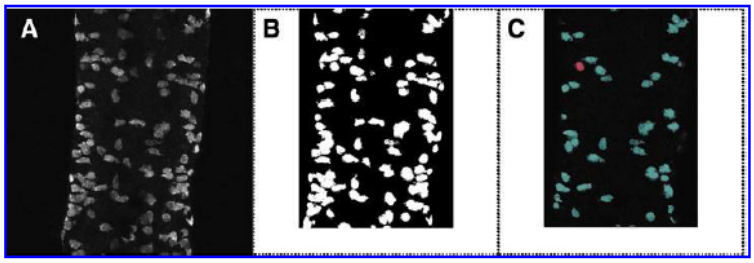

FIG. 4. The algorithm presented here allows for identification of imaged cells within a specified region of interest. **(A)** Maximum intensity projection of dissected, whole-mount, wild-type mid-intestine labeled with anti-Elavl antibody. **(B)** Maximum intensity projection of cropped binary image corresponding to **A**. **(C)** Composite image using colorized **B** masking **A** projected through layers where the *red cluster* of interest occurs.

previously encountered cluster that we previously identified as a cell. We examined each cluster for its volume, maximum cross-sectional area, bounding box volume, and 2D bounding box area, as these four simple criteria accurately stratified clusters into one- through four-cell groups. For each cluster, we performed a z-test for each of these criteria, using data from previous analyses as reference distributions. The z-test probability (z) is given by

$$z = \frac{x - \mu}{\frac{\sigma}{\sqrt{n}}}$$

where x is the sample cluster value for a given characteristic, μ is the mean characteristic value for a given cluster size population, σ is the standard deviation of this population, and n is the population size. We then compared the products (p) of all z-test probabilities for each possible cluster size

$$p_i = \prod_{j=1}^{4} z_j$$

where z_j is the z-test probability with the value of j referring to either cluster volume, maximum cross-sectional area, bounding box volume, or 2D bounding box area, and the value of i referring to the putative number of cells in a cluster.

The maximum value of p, corresponding with the most probable identity, became our initial guess (P) such that

$$P = \max p_i$$

where p_i is the z-test probability product for a given cluster size. We then either approved or denied the accuracy of P for each cluster. After all clusters were evaluated, we retrieved cell counts by calculating the sum of each cluster type for each binary image. Cluster data for each analysis is also saved and appended to previous cluster datasets, i to assist with further analyses.

Computer-assisted cell counting Computer-assisted cell counts were taken from the same z-stacks used for manual cell counting. All of the programs described in this paper were written in MATLAB(v2012a). The computer-assisted cell counting programs described in this paper are available for download at: http://uoneuro.uoregon.edu/eisen/

Hardware and software All programs were successfully tested on Windows 7 64-bit and Ubuntu 12.04 LTS laptop computers with Intel core i5-m430 processors and 4GB RAM in MATLAB(v2012a). Images were also processed on Linux supercomputer nodes featuring 12-core CPUs and 72GB RAM, running MATLAB(v2011b).

Different intestinal cell types can be accurately identified and counted by the new program

The LsmNoDesktopSegment program is able to rapidly and properly segment an entire directory of images with no user input necessary (Figure 5). By implementing Otsu's image segmentation algorithm [14, 15] and simple binary image processing techniques, cells with fluorescent cytoplasmic labeling are separated from the background. LsmNoDesktopSegment is also capable of revealing specific cell types by comparing the segmented images for each fluorescent label (Figure 5). All ENS neurons are Elavl positive [3], thus the Elavl and 5HT double positive cells are ENS neurons. The cells positive for only 5HT have previously been shown to be a subset of enteroendocrine cells in the intestinal epithelium [16]. By simply identifying the marker shown in a given image channel, LsmNoDesktopSegment is capable of identifying these relevant cell types. All cell-like clusters of pixels in the Elavl binary image are identified as neurons, and then connected pixel clusters in the 5HT binary image are segregated by the presence or absence of colocalization with Elavl pixels. Ultimately, binary images for all neurons (Figure 3B), enteroendocrine cells (Figure 3C), and 5HT-positive neurons (Figure 3D) are produced. These cell type-specific images are then passed to the LsmCounter program, where pixel clusters are finally identified as either cells, groups of cells, or background signal.

LsmCounter saves descriptive data of each cell and cell cluster that it successfully counts, and these data can then be used to identify cells more efficiently. During the initial operation of

LsmCounter, the false detection rate of cells is high, varying with the complexity of an image. On an initial run of the image presented in Figure 3A, approximately 33% of detections were correct, however analyzing the image with data from only one previous run raised the correct detection rate to 65%. In both cases, the final output counts were the same due to user guidance. During the initial run, the user effectively trains the program by indicating which pixel clusters are not cells. In subsequent rounds of analysis, LsmCounter ignores any pixel clusters of a size that is below a threshold determined by the smallest user-defined cell that was previously encountered. However, LsmCounter is designed to err on the side of false positives rather than false negatives, so that no real cells are missed, and because the user is always easily capable of denying a detection event.

RESULTS

The new program accurately identified and counted cytoplasmically-labeled cells

To be useful, our new program must be able to count cytoplasmically-labeled enteric neurons rapidly and accurately. To learn whether this was the case, we compared manual counts of enteric neurons from 3D confocal image stacks of dissected intestines with counts made by our new program. We found no statistical differences between the numbers of labeled cells detected by either manual or computerized

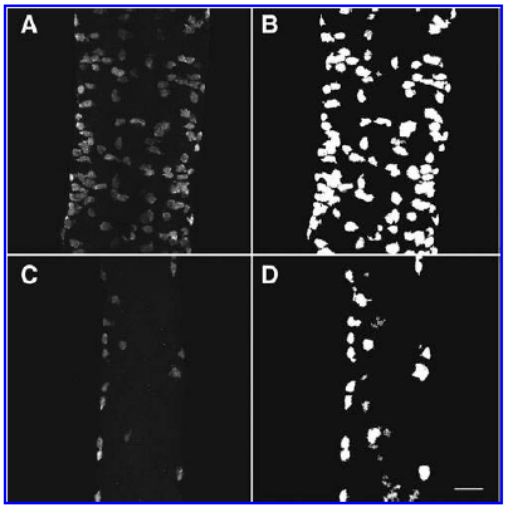

FIG. 5. Segmentation of image stacks reveals isolated cells in dissected, whole-mount, zebrafish intestines. **(A)** Maximum intensity projection of dissected, whole-mount, wild-type mid-intestine labeled with anti-Elavl antibody. **(B)** Maximum intensity projection of the binary image stack produced by Otsu's N-thresholding algorithm applied to **A**. **(C)** Maximum intensity projection of whole-mount *gut-wrencher* mid-intestine labeled with anti-Elavl antibody. **(D)** Maximum intensity projection of the binary image stack produced by Otsu's N-thresholding algorithm applied to **C**. Scale bar = 25 μm.

means (Table 1 and Figure 6). On average, manual and computer-assisted cell counts differed by less than one cell. Relative differences between cell count means from the two different counting methods did not differ significantly (P>>0.05), as determined by two-tailed unpaired student's t-test. We also found that LsmNoDesktopSegment counted cells much faster than they could be counted manually.

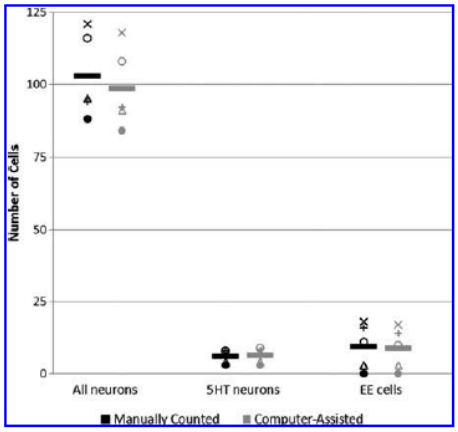

FIG. 6. The data for the wild-type mid-intestine in Table 1 are replotted here to show the high degree of correspondence between manual counts and computer-assisted counts. *Each symbol* represents an individual animal and the *bars* represent the mean. Variability between animals is discussed in the text.

Whereas manual counting takes approximately 5 minutes per dissected intestine for an experienced researcher, LsmNoDesktopSegment can count the dissected intestine in 30 seconds or less when run on a standard laptop computer.

***gutwrencher* mutants have fewer enteric neurons than wild types and this phenotype is more severe in the caudal intestine**

The enteric neuron population of 5 dpf *gutwrencher* mutants is dramatically lower than that of wild-type siblings, with a greater difference in the vent than in the mid-intestine (Table 2). 5 dpf *gutwrencher* mutants exhibit nearly a 10-fold decrease of mid-intestine enteric neurons and a 6.4-fold decrease in 5HT-positive enteric neurons relative to their wild-type siblings. In the vent region, these differences are higher, with an over 50-fold decrease in enteric neurons in *gutwrencher* mutants and a complete absence of 5HT-positive enteric neurons. All of these observed trends are statistically significant, as determined by two-tailed unpaired student's t-test (P<0.05).

In both 5 dpf wild types and *gutwrencher* mutants, enteric neuron populations differ significantly along the length of the intestine (Table 3). Wild-types exhibit a 1.5-fold reduction of enteric neurons in the vent region compared to the mid-intestine, and a roughly 3.6-fold decrease in 5HT-positive enteric neurons. In *gutwrencher* mutants, this reduction is exaggerated to a 8.7-fold reduction of enteric neurons and a complete lack of 5HT-positive neurons in the vent region.

Enteroendocrine populations appear constant along the intestine and do not differ between wild types and *gutwrencher* mutants

Enteroendocrine cell numbers do not differ significantly in any of our analyses. When *gutwrencher* mutants were compared to wild-type siblings, we saw no change in mid-intestine enteroendocrine cells and a 1.3 fold change in the vent region that is not statistically significant (Table 2). We also did not observe any significant differences between serotonergic enteroendocrine cell populations in the mid-intestine and vent regions of wild-type or *gutwrencher* mutant larvae (Table 3).

TABLE 1. MANUAL AND COMPUTER-ASSISTED CELL COUNTS ARE NOT STATISTICALLY DIFFERENT

Intestinal region	*Manually counted cells*		*LsmCounter output*		*Relative difference*	p *value*
	Mean	*Std. dev.*	*Mean*	*Std. dev.*		
Wild-type mid-intestine						
All neurons	102.8	14.7	98.6	14.0	−4.2	0.655
5HT neurons	6.0	2.3	6.4	2.7	0.4	0.809
EE cells	9.6	7.9	8.8	7.2	−0.8	0.871
Wild-type vent						
All neurons	65.0	10.0	66.8	10.1	1.8	0.784
5HT neurons	1.6	0.5	1.8	0.8	0.2	0.667
EE cells	10.4	5.3	9.4	4.9	−1.0	0.766

Cell counts were performed on 200 μm lengths of dissected intestine. 5HT-positive, Elavl-negative cells were counted as enteroendocrine cells (EE cells). Relative difference refers to the arithmetic difference between mean cell counts. *P* values were calculated via two-tailed unpaired Student's *t*-test. The overall mean relative difference is −0.6 cells. Abbreviations: Std.Dev., standard deviation.

DISCUSSION

We generated a new MATLAB-based program that enabled us to compare the number of cytoplasmically-labeled fluorescent enteric neurons in different intestinal regions and between wild types and mutants. The LsmNoDesktopSegment program is capable of rapidly processing

TABLE 2. GUTWRENCHER MUTANTS HAVE FEWER ENTERIC NEURONS THAN WILD-TYPE SIBLINGS

		Mean cell counts			*Fold change (p value)*		
Intestinal region	*Genotype*	*All neurons*	*5HT neurons*	*EE cells*	*All neurons*	*5HT neurons*	*EE cells*
Mid-intestine	Wild-type	98.6	6.4	8.8	9.5 ($1.2e^{-6}$)	6.4 (0.006)	1.0 (0.959)
	gutwrencher	10.4	1.0	8.6			
Vent	Wild-type	66.8	1.8	9.4	56 ($5.8e^{-7}$)	undef. (0.001)	1.3 (0.665)
	gutwrencher	1.2	0.0	7.4			

Cell counts were performed on 200 μm lengths of dissected intestine. 5HT-positive, Elavl-negative cells were counted as enteroendocrine cells (EE cells). *P* values were calculated via two-tailed unpaired Student's *t*-test. Undef. represents the undefined value of any value divided by zero.

3D images of whole mount zebrafish intestines and LsmCounter is capable of assisting the user in quantifying the number of cells with a given label. Image processing with LsmNoDesktopSegment requires up to 30 seconds per image file when run on a standard laptop computer, and is easily capable of being run on a distributed computing network for even faster processing. The semi-guided nature of the LsmCounter program allows for oversight over the cell counting process, which means that the runtime is dictated by the researcher, image quality, and the number of pixel clusters in each binary image. The entire computer-assisted counting process typically requires less than 4 minutes per image stack, for a user familiar with the software. The cell counting algorithm presented here is also capable of assessing pixel clusters and estimating the number of cells in an image in approximately 30 seconds per image, though with reduced accuracy, due to the lack of user correction. Also, researchers may be reluctant to adopt fully-automated cell counting software due to a lack of transparency in the counting process, so we choose to maintain user oversight in the counting process, and thereby maintain maximum confidence in the cell counts produced.

In the process of characterizing intestinal 5HT and Elavl expression with LsmCounter, we demonstrated that the program is not limited to counting enteric neurons. Cells in the zebrafish intestine that express 5HT but do not express Elavl have previously been identified as enteroendocrine cells of the intestinal epithelium [16, 17]. Therefore, by simply subtracting the number of 5HT and Elavl co-expressing cells from the total 5HT-positive cells in a given image stack, we quantified serotonergic enteroendocrine cells in the mid-intestine and vent of wild types and *gutwrencher* mutant zebrafish (Figure 3).

TABLE 3. GUTWRENCHER MUTANTS EXHIBIT MORE DRAMATIC REDUCTION OF ENTERIC NEURONS IN VENT VERSUS MID-INTESTINE, COMPARED TO WILD-TYPE SIBLINGS

	Fold change (p value)		
Genotype	*All neurons*	*5HT neurons*	*EE cells*
Wild-type	1.5 (0.003)	3.6 (0.007)	1.1 (0.882)
gutwrencher	8.7 (0.014)	undef. (0.233)	1.2 (0.789)

Mean cell counts are presented in Table 2. *P* values were calculated via two-tailed unpaired Student's *t*-test. Undef. represents the undefined value of any value divided by zero.

The techniques described here are likely to be easily adapted for DIC microscopy images. Because the image segmentation mechanics of LsmNoDesktopSegment simply require regions of high contrast, fluorescent images are unnecessary. LsmCounter tracks and counts objects that are brighter than the background, but this aspect of the program could be changed easily. Alternatively, inverted DIC images could be processed as fluorescent images.

Our results appear to suggest that the phenotype of *gutwrencher* mutants is more dramatic than was initially appreciated. *gutwrencher* mutants were previously described as exhibiting 3.5-fold fewer enteric neurons and 6-fold fewer 5HT-positive enteric neurons [1]. Here we describe a similar 6.4-fold decrease in 5HT-positive enteric neurons, but total enteric neurons appear to be nearly 9.5-fold fewer in the mid-intestine and over 50-fold fewer in the vent of *gutwrencher* mutants (Table 2). Several circumstances may contribute to differences between the fold change of enteric neurons presented here and those previously described. In the current analysis, dissected whole-mount intestines of 5 dpf larvae were examined, whereas

previous cell counts were performed on 4 dpf sectioned larvae, counted in alternating sections to prevent double counting [1]. Enteric neurons are differentiating through this stage of development, thus our results suggest that the *gutwrencher* mutation affects differentiation of enteric neurons in the mid-intestine. Future studies will address whether this results from decreased proliferation of enteric progenitors. Our studies also raise the possibility that *gutwrencher* affects migration of enteric progenitors, because we see significantly fewer neurons at the caudal end of the intestine than in the mid-intestine. However, this could also result from depletion of the progenitor pool, something we can address in future studies.

Our results provide evidence that the serotonergic enteroendocrine population of *gutwrencher* mutants and wild-type siblings do not differ significantly (Table 2). Furthermore, serotonergic enteroendocrine cell numbers appear to remain constant between the mid-intestine and vent region (Table 3). The population of serotonergic enteroendocrine cells in 5 dpf wild types has previously been described as ranging from 10-18 cells in a 3 somite length region of the intestine immediately rostral to the vent [16], which translates to about 3-5 serotonergic enteroendocrine cells per 100 μm of intestinal length. Our numbers are very similar, at 3.7-4.7 serotonergic enteroendocrine cells per 100 μm of intestinal length. These results suggest that *gutwrencher* mutant phenotype does not affect the population of serotonergic enteroendocrine cells, further supporting the idea that *gutwrencher*^{b1088} is an ENS-specific gene. A caveat of this conclusion is that our results do not show whether other subpopulations of enteroendocrine cells are affected, nor do they rule out the possibility that enteroendocrine cell fate is altered. These questions can be addressed in future experiments designed to examine enteroendocrine cells in more detail in both wild types and *gutwrencher* mutants.

A possible limitation for furthering our understanding of enteric mutant phenotypes is that cells cannot easily be counted in the anterior intestine. This is not because our program cannot handle the counting, but rather because the thickness of the tissue prevents sufficient resolution on our confocal microscope. However, other microscopy methods, such as light sheet microscopy [18], should be able to solve this problem.

One of the things we found striking was the variability in the number of specific types of enteric cells, even for animals of the same genotype. This is graphically illustrated in Figure 6. We believe that this variability is real, because we and others have found similar variability in the numbers of enteric neurons [1] , the numbers of serotonin-positive enteric neurons [1] , the numbers of serotonin-positive enteroendocrine cells [16] , and the numbers of goblet cells [16] , whether these cells were counted in whole mount or in sections. This variability calls into question the sensitivity of any counting method for detecting subtle phenotypic differences between wild types and mutants. If mutants with very slight decreases in enteric neurons were present in our initial screen [1], we may have overlooked them, as we screened animals stained for enteric neurons on a stereomicroscope. In any case, as in other situations in which there is variability, counting cells in more animals will provide a more sensitive measure of the ability to discern subtle phenotypes.

ACKNOWLEDGMENTS

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CHAPTER III

SIGNALING FROM GUT TO BRAIN: A BEHAVIORAL WINDOW INTO DEVELOPMENTAL DYNAMICS

With a more refined technique to characterize ENS development, we are in a better position to pursue possible modulators of ENS development and identify possible signals that require ENS mediation such as the resident microbiota. The ENS interacts with and shapes resident microbial communities[1] and is therefore likely pivotal in maintaining the necessary signaling environment during host development. The ENS also controls the secretion of blood-brain barrier permeable neuroactive molecules[2,3] and synapses with the vagus nerve[4], giving the ENS and thereby the microbiota two possible neural routes to influence host nervous system activity and brain development.

One of the easiest ways to identify any possible early neurodevelopmental roles of the ENS and microbiota on brain development and when they might occur is to use a behavioral assay that reflect the underlying activity of particular cells in a specific brain region. Zebrafish larvae are capable of completing complex behavioral tasks within 4-6 days post fertilization[5,6,7,8,9]. Couple this with the fact that zebrafish develop externally in sterile chorions that can be surface sterilized to produce germ-free (GF) larvae[10], and I have the optimal system for assessing the downstream behavioral effects of the microbiota on host neural activity and development.

Many of the most robust behavioral assays in larval zebrafish revolve around visual acuity and gross motor activity[5,6,8,9]. To differentiate between visual defects in the retina versus

the brain, I can use the reflexive optokinetic response (OKR) assay, in which a larva is immobilized, exposed to horizontally moving stimuli, and the rate of saccades is recorded[7], and the prey capture assay in which larvae are placed in individual culture wells, given rotifers to eat, and their capture efficiency compared under different conditions[8,9] such as GF and conventionally reared (CV). In the following chapter, I will demonstrate the use of these assays, among others, in the identification of a novel role for a microbially-sourced protein in host brain development and behavior.

CHAPTER IV

THE MICROBIOTA MODULATE PREY CAPTURE BEHAVIOR BY INCREASING INHIBITION IN THE OPTIC TECTUM

The resident microbiota provide factors that influence host postnatal development[1,2]. Host brain development may be influenced by microbial factors that alter the host's immune signaling, nutritional status, or nervous system activity through direct interaction with the peripheral or central nervous system[3,4,5] The microbiota may also secrete effectors that circulate within the host and interact with developing nervous tissue, altering complex behaviors[1,6]. However, the ability of a specific microbial effector to influence particular neural subtypes within the host brain has not been demonstrated. Here we show that microbially produced chitin binding protein (CBP) influences host visual system development and behavior. We found a striking correlation between the number of GABAergic tectal cells and the efficiency of prey capture in germ free (GF) zebrafish larvae. Monoassociation with a single zebrafish bacterial isolate, *Aeromonas* ZOR1, or exposure to a single *Aeromonas*-produced protein, CBP, was sufficient to restore both tectal cell numbers and prey capture efficiency to wild-type levels. Our results provide a molecular mechanism by which the microbiota affect host brain development by modulating identities of specific neural subtypes. We expect ours to be the first of many descriptions of particular microbial effector molecules influencing development of specific host neural subtypes. For example, complex behaviors, from sociability to anxiety[7,1,8,9,10,11] may be modulated by exposure to microbial factors that affect development of particular brain regions, providing new insights into environmental influences on animal development.

We used the larval prey capture assay[12,13] to assess complex behavioral differences between GF and conventionalized (CV) zebrafish. GF larvae showed decreased prey capture efficiency compared to CV clutchmates (Fig. 1). A trivial reason for differences between GF and CV prey capture could be changes in visual acuity or general activity levels. To rule this out we assessed optokinetic motor response (OKR) (Fig. 2) and spontaneous locomotion (Table 1) and found that GF larvae showed no difference from CV clutchmates. These results indicate that GF larvae are not overtly deficient in visual or locomotor capacities and suggests behavioral differences result from alterations in brain development. We tested this hypothesis first through RNAseq analysis of larval brains. Clustering analysis of differentially expressed genes revealed a dissimilarity between GF and CV brain transcriptomes and a clear in-group similarity within both sets (Fig. 3). We cross-referenced differentially expressed genes with a list of genes expressed within the larval optic tectum (www.zfin.org) and found a pattern of up-regulation among pro-proliferative genes in GF larvae. We also discovered that GF brains had an upregulation of *gad1b* transcripts, which mark superficial tectal cells necessary for prey capture[14]. This transcriptional upregulation correlated with the presence of supernumerary *gad1b*$^{+}$ cells within GF tecta (Fig. 4). We also observed an increase in the number of GABAergic inhibitory synapses in GF tecta (Fig.

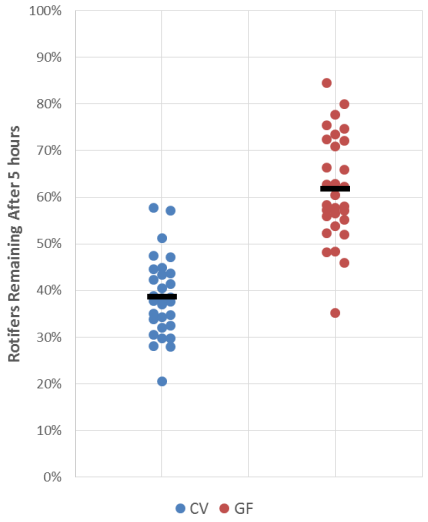

Figure 1 | Germ free larvae are less effective predators. 7dpf GF larvae fail to capture rotifers as efficiently as CV clutchmates. P < 0.001, t-test, 20 fish/condition, 3 biological replicates, bars = mean values.

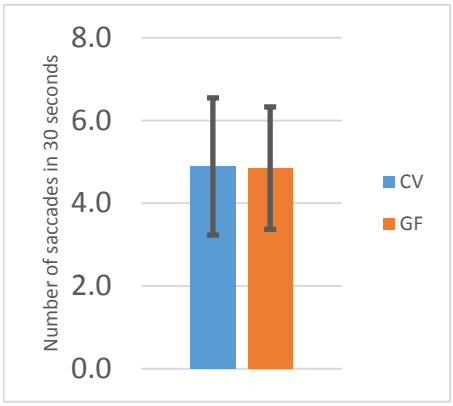

Figure 2 | GF and CV larvae can see. Both GF and CV larvae perform identically in OKR tests at 7dpf. 20 fish / condition, 4 replicates.

5). To learn whether altered tectal inhibition could affect behavior, we measured prey capture efficiency of individual GF and CV larvae and then counted the *gad1b*$^{+}$ tectal cells in the same individuals. We found that both CV and GF larvae showed a direct correlation between the number of *gad1b*$^{+}$ tectal cells and prey capture behavior (Fig. 6). In both the GF and CV cohorts, larvae with fewer *gad1b*$^{+}$ tectal cells performed better in the prey capture assay, with an overlap in both performance and cell number in the worst performing CV and best performing GF larvae. This continuum of phenotypes between GF and CV fish highlights the dynamic nature of host-microbe interactions during development.

Table 1 | Locomotor activity of GF and CV larvae is not statistically different

Gnotobiotic Status	Time spent in motion (%)	Std. dev.	*P* value
CV	31.1	28.3	0.808
GF	31.8	20.1	

Time spent in motion was automatically calculated by tracking software. Images were taken at 1Hz. Std. dev. = standard deviation. 24 fish / condition.

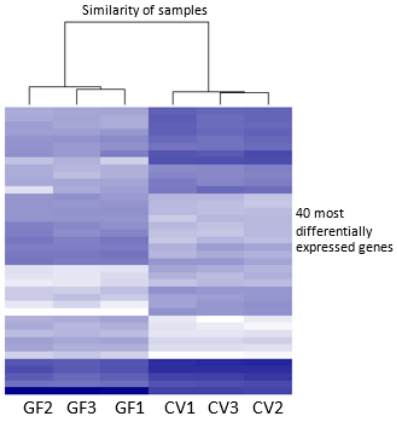

Figure 3 | Blind clustering analysis of RNAseq reveals consistent CV/GF transcriptional changes. Quantseq RNAseq analysis on 7dpf larval larval zebrafish brains. n = 10/replicate.

To identify a microbial signal or signals responsible for modulating tectal development and prey capture, we monoassociated GF larvae with bacteria known to colonize the larval zebrafish gut. Fish monoassociated with *Aeromonas* ZOR1, a primary constituent of the zebrafish microbiota[15,16] isolated from zebrafish guts,

performed like CV clutchmates in the prey capture assay (Fig. 7). Likewise, larvae monoassociated with the closely related and genetically tractable *A. veronii* isolated from leech[17] exhibit CV-like *gad1b*$^{+}$ tectal cell numbers and prey capture efficiency (Fig. 8).

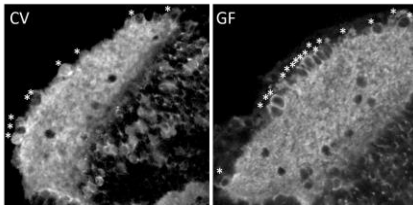

Figure 4 | GF zebrafish have an increase in the number of optic tectum gad1b cells. Gad1b antibody labeling of 7dpf conventionalized (CV) and germ free (GF) clutchmates reveals a nearly contiguous layer of supernumerary superficial inhibitory tectal cells in GF individuals, and sparse cells in CV. The void between cells in the superficial layer represent unlabeled cells, not an absence of cells.

Many host-microbe interactions are the result of secreted factors[18]. In gram negative bacteria such as *Aeromonas*, the type II secretion system serves as one of the main conduits of toxin secretion[19]. To determine whether *A. veronii* provide a secreted effector that modulates host development and behavior, we monoassociated fish with a leech isolate of *A. veronii* that had a mutation in the type 2 secretion system ($T2SS^-$)[17] and found that neither prey capture behavior nor $gad1b^+$ cell number were restored (Fig. 9). To learn the molecular nature of the *A. veronii* secreted factor that restored prey capture behavior and $gad1b^+$ cell number, we isolated cell-free supernatant (CFS) from WT and ΔT2SS *A. veronii*. Mass spectrometry revealed a short list of possible secreted effectors produced by WT *Aeromonas*. We performed ammonium sulfate precipitation on *A. veronii* CFS and found that exposure to high molecular weight extract was sufficient to rescue prey capture in GF larvae (Fig. 10). We successfully cloned each identified *A. veronii* effector into *Escherichia coli* vectors, a non-constituent component of the zebrafish microbiota. After identifying the transgenic protein in vector CFS via SDS-PAGE gel electrophoresis, we exposed larvae to the transgenic *E. coli* CFS. Exposure to *E. coli* CFS containing *A. veronii* CBP, the most abundant high molecular weight secreted effector, was sufficient to rescue prey capture and $gad1b^+$ tectal cell numbers in GF larvae (Fig 11).

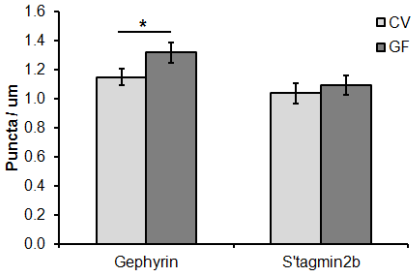

Figure 5 | Antibody labeling reveals supernumerary inhibitory synaptic puncta in GF larval tecta. The number of inhibitory synapses (as defined by the postsynaptic marker gephyrin) is increased by ~15% (n >= 32 images for each condition, p = 0.033). The synapse-type-independent marker synaptotagmin 2b (znp1) shows no change within the same images. S'tagmin2b = synaptotagmin 2b.

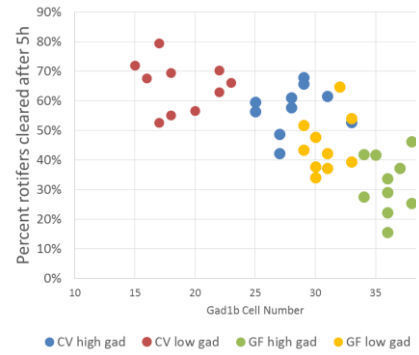

Figure 6 |The number of superficial gad1b cells correlates with prey capture efficiency. Gad1b antibody labeling of 7dpf conventionalized (CV) and germ free (GF) clutchmates reveals supernumerary gad1b cells and prey capture deficiencies in GF larvae. The severity of these phenotypes correlate with a linear $R^2 = 0.54$.

We provide the first evidence of a single secreted microbial product modulating early postnatal development of a specific neural population within the vertebrate brain and the behavioral consequences of that modulation. These findings have important implications for the role of shifting microbial populations among developing humans and other vertebrates[20]. There are likely countless

other such individual bacterial proteins required for normal host development and behavior. Not only must we now consider the composition, but the genomic content, of an individual's microbiome when attempting to assess disease states. Furthermore, we should consider the pivotal role resident microbiota have likely had in shaping the behaviors of vertebrates throughout evolutionary history.

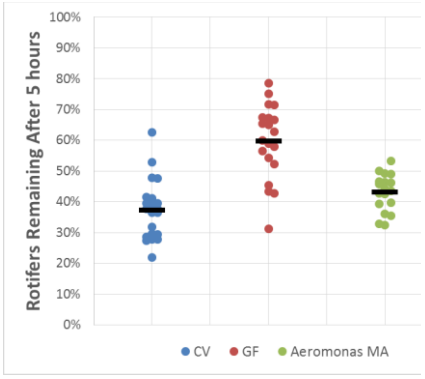

Figure 7 | Zebrafish isolate *Aeromonas* monoassociation rescues larval prey capture. GF 7dpf larvae monoassociated with *Aeromonas* ZOR1, isolated from zebrafish, are more efficient at capturing rotifers than GF clutchmates. P < 0.001, t-test, n = 40/condition.

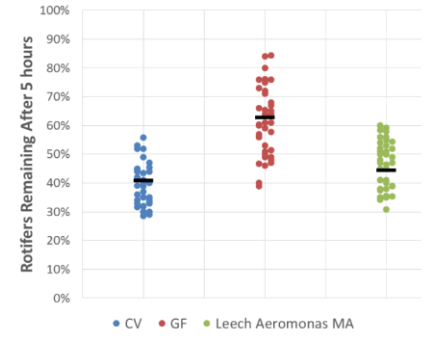

Figure 8 | Leech isolate *Aeromonas* monoassociation also rescues larval prey capture. GF 7dpf larvae monoassociated with *Aeromonas veronii* are more efficient at capturing rotifers than GF clutchmates. P <

Methods

Animals were reared at 28.5°C according to standard zebrafish husbandry[21] and staged by days postfertilization at 28.5°C (dpf).

All experiments and analyses were performed in a blinded fashion.

For prey capture assays, individual 7 dpf larvae were presented with approximately 100 live rotifers and imaged for 5 hours. Larval zebrafish were individually placed into tissue culture wells. Between 0.2 and 2.0 ml of rotifer culture was added to each tissue culture well. Embryo medium was added, if needed, to maintain an even water level across each culture tray. Fish were imaged from 0 to 5h as previously described[6].

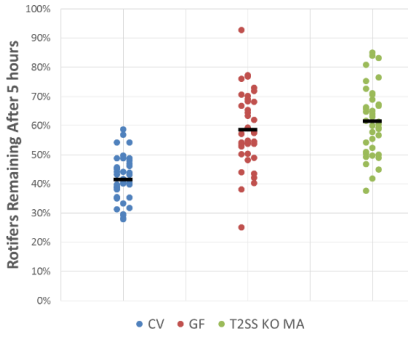

Figure 9 | T2SS mutant leech *Aeromonas* monoassociation fails to rescue larval prey capture. GF 7dpf larvae monoassociated with *Aeromonas veronii* ΔT2SS are as efficient at capturing rotifers as GF clutchmates. n = 40/condition.

Locomotor assays were performed with individual 7 dpf larvae in individual tissue culture wells. Image analysis was performed live with sampling at 1Hz[22,23].

OKRs were measured in 7 dpf larvae immobilized in methylcellulose and presented with a drum of alternating black and white vertical stripes rotating at 0.125Hz. Each larva was presented with a total of 12 alternating clockwise and counterclockwise rotation trials[24].

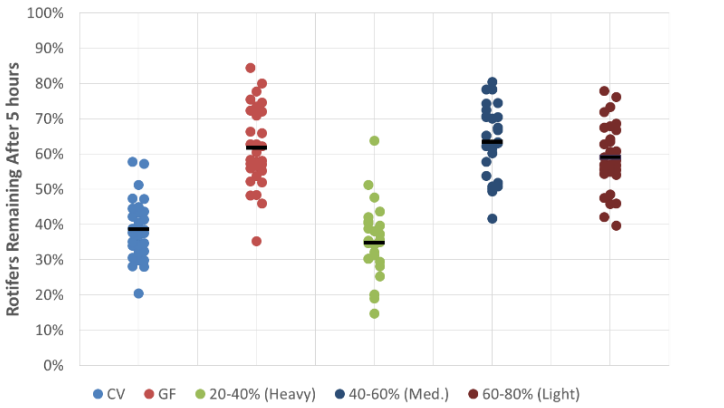

Figure 10 | *Aeromonas veronii* heavy CFS fraction rescues larval prey capture. GF 7dpf larvae exposed to *Aeromonas veronii* more efficient at capturing rotifers than GF clutchmates. n = 30/condition, 3 replicates.

Antibody staining for Elavl (1:10,000, Molecular Probes Inc., Eugene, OR, catalog number A-21271) and Gad1b (1:1,000, GeneTex, Irvine, CA GTX124460) was performed at 7 dpf as previously described[25]. Secondary antigens were visualized with standard fluorophore-labeled antibodies for rabbit IgG (1:1,000, Molecular Probes Inc., catalog number A-11008) and mouse IgG (1:1,000, Molecular Probes Inc., catalog number A-11030).

Figure 11 | Chitin binding protein is sufficient to rescue larval prey capture. GF 7dpf larvae exposed to CBP$^+$ *E. coli* CFS (CBP MA) are more efficient at capturing rotifers than GF clutchmates. $P < 0.001$, t-test, n = 40/condition.

GF embryos were derived and maintained as previously described[26]. Monoassociations, CFS preparations and protein expression construct preparations were performed as previously described[27,28].

Brains were dissected at 7 dpf, homogenized and RNA extracted with TriReagent (catalog number TR 118). Stranded sequencing of single transcripts was performed using the Quantseq method[29]. Genomic alignment to zebrafish genome assembly Zv9 was performed with BowTie2 and differential expression was assessed via DeSeq[30].

CHAPTER V

FUTURE DISSECTION OF THE MECHANISMS BY WHICH MICROBIAL PRODUCTS AFFECT BRAIN DEVELOPMENT

My work demonstrates that a single molecule, CBP, secreted by *Aeromonas* is sufficient and necessary to modulate larval zebrafish tectum development and behavior. However, several questions remain unanswered, such as how information from this signal is conducted to the brain and when the key signaling moment in development occurs. There are three main candidate pathways for gut-brain signaling: the vagus nerve, the immune system, and a humoral route. My future goal is to use mutant zebrafish lines and precise temporal introduction of CBP to learn when CBP is required and the route by which it signalsto influence developing host brains.

The larval zebrafish immune system relies solely on innate immune signaling[1], therefore if the immune system is necessary for CBP-brain signaling, it must be via a component of the innate immune system[1]. By both exploiting the lack of a mature adaptive immune system at 7 dpf and utilizing a mutation in *myd88*, a gene encoding an adapter protein necessary for innate immune signaling[1], I will be able to test whether immune signaling is required to conduct information from CBP to the brain to establish a normal tectal phenotype. As the immune cells of the gut continuously interact with the microbiota and their products, along with the host ENS[1,2], this remains a promising line of inquiry.

Along the same line, the microbiota also interact with a direct route to the CNS via the vagus nerve[3], either through monosynaptic connections between the ENS and CNS or via the vagus nerve directly. Through ENS activation of the vagal nerve, there may be a route for

molecules secreted by the microbiota[3] to reach the CNS. By examining the behavior and tectal phenotype of *sox10* mutantlarvae[4], which lack enteric neurons, I can assess whether ENS signaling is required for CBP-brain signaling. However, this experiment does not rule-out the possibility of ENS-independent vagal activity. This could be tested genetically by killing vagal neurons, running the risk of confounding results by ablating non-target cells, or by ablating vagal neurons physically, which may prove to be unreasonably difficult.

There is also the possibility of signaling through secondary messengers that does not fit into either neural or immune pathways. If the above experiments prove inconclusive, the CBP-brain signaling mechanism may involve a blood-brain barrier (BBB) permeable molecule, possibly a secondary messenger from a CBP-reactive cell[5,6]. If this is the case, a much more detailed analysis of the dispersion of CBP signaling and reactivity would be necessary to identify the signaling cell type.

Finally, the role of CBP exposure timing is completely unknown. The larval tectum may receive a CBP mediated signal later than normal inoculation would occur in the wild, between 3-5 days post-fertilization, and reach day 7 with an apparently conventional tectum and behavior. This possible plasticity would suggest that the CBP signal-receptive cells within the tectum remain as such past their normal timing in development. I plan to learn when CBP signaling is required by exposing larvae to CBP at varying timepoints and assessing their tectal phenotype and behavior.

Throughout this dissertation I have introduced and discussed novel methods for ENS characterization and a novel mechanism of host-microbe interaction with previously unheard-of specificity. By more rapidly and algorithmically assessing ENS phenotypes, we can better understand the role of the ENS during development. And with the evidence of CBP influencing

host behavior and brain development, we now have a new level of appreciation for the role our resident microbes played during our own development. I look forward with anticipation to the responses and feedback from these studies.

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