EXPLORING THE ROLE OF MICROBIOTA IN THE DEVELOPMENT OF INSULIN-PRODUCING CELLS IN DROSOPHILA MELANOGASTER

by

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Resident microbiotas can influence many aspects of host health and disease. Previous research by the Guillemin lab shows that in zebrafish and mice, gut microbiota promotes the expansion of insulin-producing beta cells in the pancreas through a secreted bacterial protein, beta-cell expansion factor A (BefA). This research investigates the role of microbiota, bacteria, and BefA protein to promote analogous insulin-producing cell (IPC) development in the fruit fly, *Drosophila melanogaster*. In *Drosophila*, 7 insulin-producing cells are present in each lobe of the larval brain. This research first established the effect of germ-free (GF) rearing on IPC numbers in *Drosophila*. The second and third aims tested if feeding flies BefA or if transgenic expression of BefA could restore insulin-producing cell numbers in germ-free flies. We compared the number of insulin-producing cells present in flies that were germ-free, conventionally reared (CV), germ-free and fed BefA protein, and germ-free flies with transgenic expression of BefA. Tissue-specific Dilp3:GAL4/UAS:GFP in all groups made insulin-producing cells visible after dissection and immunohistochemistry. Results showed that germ-free flies have fewer insulin-producing cells per brain lobe than conventional flies, indicating that microbiota is required for normal insulin-producing cell number and development. Further, germ-free larvae fed BefA protein showed a slight but significant increase in insulin-producing

cells per lobe compared to conventional, indicating that BefA has the potential to rescue the effects of germ-free treatment. Transgenic expression of BefA, using the GAL4/UAS system, yielded a trending rescue of insulin-producing cells in germ-free flies, possibly due to lower levels of BefA produced through transgenic expression than via feeding. Results indicate that resident microbiota has a powerful effect on *Drosophila* metabolic pathways and fundamentally affects cell development, including cells in the gut-brain axis. This information can be used to direct research and treatment for diseases like diabetes, helps researchers better understand growth and development, and has implications for the microbiota's effect on the brain. Future experiments include a developmental assay aimed at further investigating the properties of BefA and other similar bacterial proteins, including testing the hypothesis that BefA's membrane permeabilizing properties induce insulin-producing cell expansion.

Acknowledgments

I thank my thesis advisor, Karen Guillemin, and all members of the Guillemin lab for their support, mentorship, and education throughout my years at the University of Oregon. I especially want to thank Stephanie VanBeuge, Jennifer Davidson, and Patrick Horve for their mentorship, teaching, trust in academic research, and special help with this thesis. Additionally, I thank my family. My parents, Yvette Provosty and Eric Mullen, for their continued support of my educational and personal growth, and my brother, Marcus Mullen for his humor and friendship. I would also like to extend my thanks to Judith Eisen, who originally brought me to this research in the Guillemin lab, to my advisor, Daphne Gallagher, for her insight about future endeavors, to Maisie Topping, my fellow scientist, grant writer, and thesis partner. Thank you to all my funding sources, and to all of those who supported my personal, academic, and career growth throughout my time as an undergraduate researcher.

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Introduction

All animals are complex systems of interacting host and microbial cells.[1] This complex ecosystem of microbial interactions begins at birth and come to cover our skin, line our gastrointestinal tracts, and outnumber our own cells. Microbiota, however, are not passive riders. They protect us from the outside environment, help us digest food, make vitamins, and bolster our immune system.[2] These commensal bacteria, also known as the microbes that exist on a host without causing harm, are critically important for health outcomes. Most historical research, however, has focused on pathogenic microorganisms.[3] More recently, researchers are attempting to understand the nuances of the host - microbial relationship and its implications for health and fitness.[3,4] Accumulating evidence shows that the microbiota is involved in everything from host metabolism, immune function, endocrine regulation, brain function and development, to health status such as obesity, inflammation, chronic diseases, and cancer risk.[3,5]

The research described in this thesis focuses on the effect of microbiota on critical systems of animal metabolism and brain development. Research using the model organism *Drosophila melanogaster* provides a powerful model for exploring new molecules and regulatory processes involved in insulin signaling and the gut-brain axis. Although the *Drosophila* microbial community is not identical to the human microbiome, many of the bacterial functions and families are conserved, making *Drosophila* a valuable model organism to study the effect of microbiota on health and behavior.[5] This research aims to better understand the role of the microbiota in normal metabolic function and the mechanism through which bacterial proteins affect metabolism. Findings from this research could inform treatments of metabolic diseases such as Type 1 and Type 2 diabetes.

Background

What is the Microbiota?

The microbiota consists of the indigenous microorganisms that colonize a host. This includes the digestive tract, the skin, and other parts of the organism.[2] While it is difficult to study and remove microbiota from humans, experimental animal models allow scientists to study the effects of a lack of microbiota on the health and fitness of an organism.[3] The growing interest in the role of microbiota in health has increased demand for gnotobiotic animals, or animals with a specific known set of microbes.[3,4,6] Germ-free organisms, or those without any microbes, are a type of gnotobiotic organism. Common model organisms used to study the effects of the microbiota include zebrafish, mice, and the fruit fly, *Drosophila*.[1]

Drosophila as a Microbiome Model Organism

Drosophila has emerged as a powerful model organism to study the effects of the microbiome on the health and fitness of a host. *Drosophila* has ample genomic and genetic resources, relatively simple and tested experimental protocols, is cost effective, and has the capacity for many biological replicates.[3] Using *Drosophila* as a model organism, especially when focusing on such conserved pathways like insulin signaling and brain development, provides exciting opportunities to understand how the microbiome modulates human health.[1]

Microbiota and *Drosophila* Insulin Signaling

Insulin signaling is a conserved pathway that allows animals to use food to make energy. In this pathway, insulin-producing cells secrete the peptide hormone insulin, which allows cells throughout the body to take up sugar from the blood and carry out metabolic processes.[7,2,8]

In humans and other vertebrates, specialized beta cells in the pancreas make insulin. In contrast, in insects such as *Drosophila,* insulin-producing cells (IPCs) are located in the brain. IPCs are the main supply of insulin to the fly, with 7 IPCs located in each lobe of the brain (Luo, Jiangnan et al). These specialized cells produce insulin-like peptides (ILPs) and insulin growth factors (IGFs) that regulate development, growth, reproduction, stress resistance, and lifespan.[7] In *Drosophila*, eight ILPs and the genes that code for these ILPs have been identified (DILP1-8).[2] This study focuses on and utilizes dilp3 specifically for its ease of expression and previous study.[9,10] Both beta cells and IPCs function by sensing the presence of glucose in the extracellular environment and using specific membrane potentials to modulate insulin dynamics and other metabolic chemicals.[11] Studying the effect of microbiota on IPCs in *Drosophila* provides valuable insight into the potential effect of bacteria and bacterial proteins on the development of cells whose function relies specifically on permeability. Further, the location of IPCs in the *Drosophila* brain also allows us to explore the host-microbe relationship's influence on the gut-brain axis.

Disruption to IPCs and Malignant Phenotypes

Researchers found that ablation of IPCs causes developmental delay, growth retardation, elevated carbohydrate levels, and diabetic phenotypes in *Drosophila* that severely impairs normal development.[7] A lack of the cells that produce insulin severely affects normal metabolic processes. When comparing *Drosophila* larvae raised conventionally (CV), or with their normal microbiota, and *Drosophila* larvae raised germ-free (GF), or without their normal microbiota, researchers have found profound effects on insulin pathways. First, disrupting the normal *Drosophila* microbiota adversely effects growth and development, leading to reduced body and

tissue size and delayed phenotypic development.[2,3,9,10,12] These adverse effects seen in GF organisms are phenotypically similar to organisms with insulin and metabolism deficiencies, including diabetic phenotypes. Other research has shown that flies with genetic mutations that mimic a GF phenotype show lower levels of DILPs present in the homogenized fly brain.[9] When researchers supplemented these experimental flies with bacterial secretions, there was a rescue of these DILP levels and some phenotypic markers.[9] Though some phenotypic outcomes for GF or germ-low *Drosophila* are clear and consistent, the mechanism through which the microbiota modulates metabolism and insulin-signaling and what factors contribute to this modulation remains unclear.

Bacterial Proteins, BefA, and a role in Insulin Signaling

Previous research indicates that the absence of microbiota hinders beta cell development in zebrafish.[13] This research has also been replicated in mice.[14–16] Beta cells are analogous to the insulin producing cells (IPCs) present in *Drosophila*. When a specific bacterial protein, called beta cell expansion factor A, or BefA, was reintroduced to both GF zebrafish and mice, it led to the normal proliferation of beta cells in both organisms and rescued diabetic phenotypes in mice.[14,15] This rescue effect suggests that the biochemical mechanism and properties of BefA protein are necessary for the normal development of the metabolic system of both organisms. These data are especially powerful because BefA protein is common in bacteria that colonize the human gut microbiome, suggesting that this relationship between bacterial protein and metabolic development could translate to *Drosophila* and humans.[13] The discovery of BefA and similar proteins offers a new avenue for treating diseases like type 1 and type 2 diabetes, both of which involve a loss of beta cell mass and function. Type 1 diabetes is characterized by the loss of beta

cells due to autoimmune destruction. Type 2 diabetes is primarily caused by increased insulin resistance but can also involve beta cell loss.[17]

Potential Mechanisms of BefA

Because beta cells are intrinsically linked to glucose processing, understanding the mechanism behind beta cell proliferation and the role of proteins similar to BefA in proliferation could potentially offer treatments and a cure for type 1 and type 2 diabetes.[17]

Unpublished research by Patrick Horve and others in the Guillemin lab suggest that BefA facilitated the expansion of IPCs in *Drosophila* and beta cells in zebrafish and mice by acting as an irritant and perturbing agent to membranes. Current data suggests this membrane perturbance then causes an artificial depolarization of the cells, leading to the opening of the voltage dependent Ca^{2+} ion channels. Previous research in zebrafish used a FK506 treatment to block Ca^{2+}- activated calcineurin signaling in the beta cell proliferative pathway and found that FK506 treatment prevented BefA-induced beta cell proliferation. This suggests that BefA is acting through this known pro-proliferative pathway for beta cells and induces proliferation.

Many secreted bacterial proteins have evolved as inter-bacterial competition factors.[18] Permeabilizing or making the membranes of other bacteria "leaky" is a powerful strategy for microbes as they compete in what researchers have described as a microbial "jungle."[18] The mutualistic evolution of bacteria and bacterial proteins with their host is less well understood.

Drosophila have endogenous pore-forming proteins essential for development.[19] One of these proteins is called torso-like, or Tsl, a known pore-forming protein involved in proper embryogenesis in *Drosophila*.[20] Researchers found that embryos from *tsl* null mutant mothers did not develop correct embryonic structures, including irregular and uncoordinated ventral cell apical constrictions, incomplete furrow formation, and resultant ventral cuticle holes.[20] However,

when the group physically poked holes in the cuticle membrane with needles, there was a partial rescue of the mutant phenotype, meaning that these flies developed normally.[19] This suggests that the mere presence of holes in the membrane, even relatively crude holes formed by a needle, is enough to allow for more normal development of *Drosophila*. In this study, I tested whether Tsl could function like BefA to rescue IPC numbers. The diminished IPC numbers in GF flies led to a real difference in growth and developmental rate between GF, CV, and treatment group flies, which would suggest that the number of IPCs per brain is an important factor in determining normal metabolic function.

Synopsis

This study used GF *Drosophila* to investigate the effect of a lack of microbiota on normal growth and development using specific markers of IPC number per brain lobe and rate of pupariation, or time spent reaching the pupae stage of fruit fly development. I first established that GF *Drosophila* have significantly fewer IPCs per lobe than CV flies. Next, I used feeding assays and transgenic expression of bacterial proteins to test what could rescue the effects of GF treatment. My data suggests that the microbiome and specific bacterial proteins interact with IPC cells in the fruit fly brain and are sufficient for normal growth and development. Feeding assays and transgenic expression of the specific bacterial protein, BefA, was able to rescue these effects. Finally, transgenic expression of an endogenous pore-forming gene, *tsl*, showed rescue effects for GF *Drosophila* that mirrored CV flies, suggesting that a pore-forming mechanism is involved in the normal development of IPCs and metabolic pathways in *Drosophila*. Delayed time to pupariation of GF flies and subsequent rescue with transgenic BefA suggests that the number of IPCs is directly related to normal growth and development.

Thus, our findings show that the microbiome has a profound effect on the development of cells in the brain and may be involved in the metabolic processes of *Drosophila*, providing continued evidence for the importance of the microbiota in gut-brain interaction.

Materials and Methods

Fly Culture and Stocks

The following stocks obtained from the Bloomington Drosophila Stock Center were used in this study: *w/;UAS-(nls?)GFP/(CyO);dilp3Gal4/(TM6B), y[1]w[*]; UAS-BefA/CyO*, . The following stocks were gifts from Johnson/Warr Lab; *UAS-tsl 51C/CyO-GFP, ;; UAS-tsl 51C/CyO-GFP*. Flies were raised on axenic conventional cornmeal-yeast-agar media (Agar 100g, Brewer's Yeast 1000g, Cornmeal 600g, Dextrose 1000g, Propionic Acid 52ml, Tegosept, Prepared (100mg/ml in ETOH)) at 25°C, 60% humidity with 12/hr. light on and off cycles.

Immunostaining

Pre-wandering mid third instar larvae of the relevant genotypes were dissected, fixed, and stained according to standard procedures.[21] Briefly, larvae were dissected in ice-cold PBS, fixed for 20 min in PBS-4% formaldehyde/PFA, permeabilized in PBS-0.1% TritonX-100 (PBS-T) for 30 mins, blocked with 1% BSA in PBS-T (PBS-BT) for 1 hour, incubated with primary antibodies in PBS-TB overnight at 4°C, washed in PBS-BT, incubated with secondary antibodies for 2 hours, rinsed with PBS and incubated in Vectashield (Vectashield, Vector Laboratories Inc.,Burlingame, CA, USA) overnight at 4°C. The samples were mounted using a mounting buffer and analyzed by confocal microscopy. All steps were performed at room temperature unless otherwise noted. Antibodies: Chicken polyclonal anti-GFP (1:500) from the Developmental Studies Hybridoma Bank; Alexa Fluor chicken anti-goat IgG, Invitrogen, Carlsbad, CA (1:1000).

GF Derivations

GF derivation followed standard procedure for gnotobiotic animals.[6] In short, embryos were collected from apple caps with yeast deposit and bathed in 10% bleach for 5 mins, rinsed with EtOH and water, and deposited onto sterile media. GF tests were conducted with homogenized embryos on MRS-Agar plates and viewed after 48 hours of incubation 30°C.

Feeding Assays

Early third instar larvae were fed liquid fly food consisting of purified protein, 500 ng/mL, 5% sucrose and 0.05% bromophenol blue dye over a 24hr period before dissection. Dye ensured larvae consumed food.

Time to Pupariation Assay

Time to pupariation assay followed previous methods for developmental time assays.[12] In short, embryos were collected in a 5–6-hour time window and underwent GF treatment and testing. Pupariation was measured twice daily until 100% of population had reached pupariation. Because larval growth is affected by crowding and variable numbers of larvae survive GF treatment, only vials with 10-45 individuals were included in analysis.[6]

Data Collection

Confocal image stacks were acquired with 10x and 40x oil lenses using the Leica SPE Laser Scanning Confocal and Widefield microscope. Images are taken at 1024 transparency with Alexa488 and DAPI lasers. Images were processed in Fiji. Cells exhibiting both GFP and DAPI stain were quantified per brain lobe. When adjustments to brightness and contrast were needed, they were applied to the entire image uniformly. Mosaic images to show different focal planes were assembled in Fiji.

Statistical analysis

Statistical significance is denoted by letters, with $p<0.05$. All statistical Student's t-tests and one way ANOVA with Bonferroni post-hoc test were performed using GraphPad 6 Prism software. The results are stated as mean ± s.d., showing all points unless otherwise noted.

Results

GF larvae have fewer insulin-producing cells per brain lobe than CV larvae

To investigate whether the microbiome is necessary for normal IPC number and development in fruit fly brains, GF larvae were generated using previously established methods.[6] GF larvae were homogenized and plated on nutrient rich MRS-Agar plates, which were then incubated at 30° C for 48 hours to ensure larvae are GF. Both GF and CV flies were raised on yeast-based medium at 25° C until reaching the 3rd instar larvae phase. Third instar GF and CV larvae were then dissected, stained, mounted, and imaged. Tissue specific *dilp3:GAL4* driving expression of *UAS:GFP* allowed for visualization of IPCs for all groups. Larvae brains were stained with antibody against GFP to illuminate dilp positive cells, and with DAPI, which stains for DNA within the nucleus of the cell. Only double positive cells were quantified. Results showed that a lack of microbiota has a profound diminishing effect on the number of IPCs per brain lobe (Fig. 2). CV larvae, from the quantification of 149 brain lobes, had a consistent and average IPC per lobe of 6.973 +/- 0.04145. In contrast, GF larvae, quantified from 105 brain lobes, had fewer IPCs with an average of 5.876 +/- 0.1113 per lobe (Fig 3.) We also found that there was less overall GFP staining in GF brains compared to CV brains. Qualitative observations saw smaller and slower development of GF larvae compared to CV, which is consistent with previously published findings.[12] These data were collected from 8 independent experiments.

Feeding of BefA protein expands insulin-producing cells in GF larvae

BefA is a bacterial protein shown to rescue IPCs in young GF zebrafish and mice when administered orally. We investigated whether feeding of BefA to GF *Drosophila* larvae was sufficient to increase IPC numbers. In this assay, all fly larvae at the 2nd instar phase were fed a liquid diet of sucrose, water, and bromophenol blue dye. Because larvae are translucent, the blue dye was visible in their intestinal tracts serving as a check to ensure the larvae consumed their specific liquid food. Only larvae with blue dye in their intestinal tracts were removed, dissected, and stained for imaging. The experimental group of GF flies was fed the same liquid fly food, with the addition of BefA protein in a 500 ng/mL ratio, purified through standard procedures.[16] Results from the feeding assay show that feeding larvae BefA protein was successful in rescuing some but not all the effects of the GF treatment. The average IPC number for GF is 4.872 +/- 0.2299 from 39 lobes. The average IPC number for GF+BefA is 5.611 +/- 0.1794, n = 36, which was significantly higher than the GF average for these specific feeding assay experiments.

Transgenic expression of BefA protein expands insulin-producing cells in GF larvae

We next tested whether BefA could be administered to GF flies via transgenic expression in the IPCs and whether this would be sufficient to increase IPC numbers. To test if transgenic expression of BefA showed a similar rescue effect as orally delivered BefA, we constructed a fly line using virgin *dilp3:GFP* flies crossed with virgin *UAS:BefA* flies (Fig. 5). The progeny of this cross expressed both GFP and BefA in dilp3 expression cells. GFP allows for the visualization of IPCs that express dilp3 and are supplemented with BefA protein synthesized from the *Drosophila* genome. Vials were kept post-virgining to ensure flies were properly selected and in fact virgins. Results from the transgenic expression assay showed a significant rescue of IPCs in GF larvae. Both groups, GF and GF + transgenic BefA, showed statistical differences, but not a

full rescue of IPCs with the transgenic BefA treatment. The GF larvae had an average of is 5.983 +/- 0.1335 IPCs per lobe from 49 brains. The average for GF+BefA transgenic is 6.273 +/- 0.1809 IPCs per lobe from 33 lobes. The effect of transgenic BefA expression was not as robust as with the feeding assay (Fig. 4). This could be due to the possibility that transgenic expression of BefA results in less protein than when it is administered via feeding. The results from the transgenic expression and feeding assay suggest that the effect of BefA protein may be dose dependent.

Transgenic expression of pore-forming tsl also expands insulin-producing cells in GF larvae

The Guillemin lab is actively investigating the mechanism through which BefA supports the proliferation of IPC cells in animals. The Guillemin lab has shown BefA acts as a membrane perturbing protein.[16] These membrane perturbing abilities, while likely originally functioning as a bacterial attack system against other microbes, may have functions within the host that developed because of coevolution.[16]

We hypothesize that it is the membrane perturbing abilities of BefA that affect the highly membrane dependent, neuron-like IPC cells in the fly brain. With this hypothesis, we tested the effect of transgenically expressing the membrane-permeabilizing Torso-like (Tsl) protein on IPC development. Tsl is a known *Drosophila* pore-forming protein.[20] Previous studies document that the pore-forming properties of Tsl are involved in the normal development of *Drosophila* larvae.[19,20] The eggs laid by homozygous *tsl* null mutant mothers are mispatterned and lack normal cuticle formation. The *tsl* gene is also required zygotically for proper development from the cellular immune system.[20,22]

The known properties of *tsl* prompted us to explore and compare the effect of Tsl and BefA expression on the development of IPC cells. Specifically, we assayed the potential of Tsl to

rescue the diminished IPC counts in GF larvae as seen above. We used tissue specific GAL4:UAS system in a manner analogous to the transgenic expression of BefA. We generated fly lines that expressed *tsl* and GFP under regulation of *dilp3:GAL4*. The same fly lines without the *tsl* insert were used in the conventional control. The larvae underwent the same dissection, staining, mounting, and imaging process as previously described.

I found that transgenically expressed *tsl* had a profound rescue effect on the GF treatment. GF larvae had an average of 5.455 +/- 0.2223, IPCs per lobe, which was consistent with previous IPC data. GF larvae with transgenically expressed *tsl* in the IPCs had an average of 6.950 +/- 0.08811 IPCs per brain lobe (n=20) (Fig. 6). The transgenic expression of *tsl* restored IPC numbers in GF larvae to levels comparable to those measured in CV animals. These data suggest that a pore-forming mechanism may be involved in the normal development of IPC cells and metabolism.

GF flies have delayed time to pupariation

We next asked whether the diminished number of IPCs correlated with slower growth and development in *Drosophila*. Previous research demonstrated the lack of microbiota slowed larvae development.[12] We worked to replicate this result by measuring time to pupariation, where the percentage of larvae reaching the pupal phase was measured and recorded twice daily. GF and CV larvae were compared in three biological replicates. The data showed that GF larvae develop an average of 1-2 days more slowly than CV larvae. This assay supports qualitative observations that GF larvae develop more slowly and are generally smaller in size compared to CV larvae. This suggests further that the diminished number of IPC cells, as well as the general lack of microbiota, contributes to reduced insulin pathway signaling and an observable decrease in normal growth and development.

Discussion

Microbiota and bacterial proteins are influential in many aspects of host physiology, growth and development, and behavior.[3,5,23] A growing body of research indicates that the microbiota performs a key role in modulating metabolic systems, growth and development, and the gut-brain axis.[24] It is still unclear the exact mechanism through which the microbiota influences metabolic function and development of neurons and insulin-producing cells specifically. This research is the first to our knowledge to suggest that the microbiota and specific bacterial proteins can bolster the specific number of IPC cells in *Drosophila*.

This research demonstrated that microbiota are necessary for normal IPC numbers in *Drosophila*. Further, BefA protein alone, when delivered through a feeding assay, is sufficient to rescue IPC numbers in GF flies, making them phenotypically closer to conventionally raised flies. BefA delivered transgenically showed some, but less robust rescue of IPC number, suggesting dose dependance. Finally, transgenic expression of the pore-forming, endogenous *tsl* gene showed the most robust rescue of the IPC cells in GF flies. Though CV flies were used as controls in all experiments, we did not include CV flies in the analysis when investigating whether BefA or *tsl* rescued GF phenotypes. This was because CV raised flies developed more quickly than GF flies and reach pupariation sooner making the timing of the feeding assay in particular challenging.[11,12] The data showing that the fly microbiota promotes normal growth and development is consistent with previous research showing the delayed growth patterns in GF animals.[12]

The finding that BefA and *tsl* supplementation show varied levels of IPC rescue after GF treatment raises further questions. First, it is still unclear to what extent the BefA and Tsl proteins are disrupting the delicate IPC membranes found in the fruit fly brain. IPC cells and

neurons are both cells whose function is highly dependent on the specific permeability of the membrane at different times.[11] The perturbing properties of BefA and the pore-forming properties of Tsl, when expressed in the brain, both bolster the development of IPC cells in *Drosophila* lacking a normal microbiota. They do not, however bring IPC levels higher than CV flies or cause abnormal development of these cells in other areas, to the best of our knowledge. It remains unclear what level of membrane perturbance yields ideal IPC growth, as it is likely not a linear relationship between membrane perturbation and IPC growth. Additionally, it is unclear, to what level BefA is perturbing membranes at different doses. Further investigation into the mechanisms through with BefA bolsters IPC growth and beta cell growth is required.

The finding that the microbiota specifically effects the development of neuron-like cells continues to support the importance of the microbiota in the gut-brain axis, the bidirectional pathway between the central nervous system and the gastrointestinal tract. Though the brain is a highly conserved organ, these data shows that the removal and addition of bacteria proteins has a profound and measurable impact on the number of IPCs in the brain. Bacteria and their secreted proteins likely have conserved roles in the development of neurons and neuronal pathways. Previous research shows that bacteria can produce chemicals and proteins, specifically amines, that aid the production of the neurotransmitter serotonin, which is synthesized in the gut.[25–27] Other studies have shown that the microbiomes of individuals with psychological disorders are significantly different in their microbial membership and chemical compositions than microbiomes of individuals without disorders.[27,28] The mechanism through which microbiota specifically effects the gut-brain axis is still a subject of active investigation. Studies using *Drosophila* specifically have shown that the gut microbiome modulates some behaviors, like aggression, but has no specific effect on locomotion or sleep.[27,29] The effect of the microbiota on

some behaviors and not others suggests that the impact of bacterial proteins on the brain is highly specific. Because of the demonstrated physiological difference in the IPC development in the *Drosophila* brain, insulin and insulin signaling pathways remain a promising area of research for the role of bacterial proteins in host-microbial relationships.

These data support the hypothesis that the membrane perturbing abilities of BefA function to rescue IPCs in not only *Drosophila*, but also other model organisms. The robust rescue suggests that the development of membrane-voltage dependent cells, like neurons, IPCs, and beta cells, may be intricately connected with the membrane perturbing qualities of many bacterial proteins.

This research demonstrates that host microbiota has a powerful effect on metabolic pathways and fundamentally affecting development of the cells in the brain. Understanding the role of the microbiota in metabolic pathways will aid in our treatment and understanding of diseases like type 1 and type 2 diabetes which both involve a loss of beta cell mass and function.[14,15,30] This research further contributes our knowledge of the growth and development of cells, particularly membrane-voltage dependent cells.

Figures

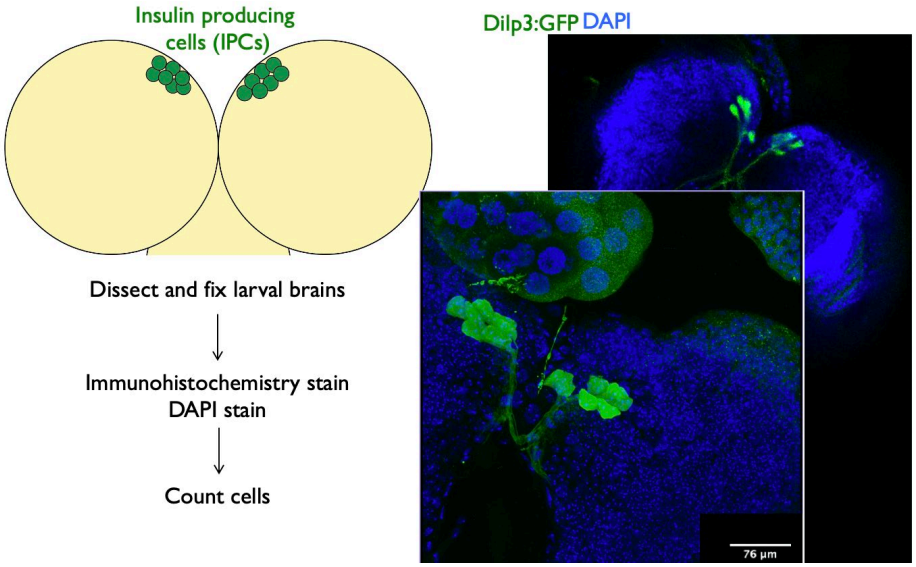

Figure 1. Graphical Methods of IPC viewing and data generation

Drosophila embryos are grown on apple caps before undergoing GF derivation as previously described. Once at third-instar phase, Drosophila larvae are dissected, fixed with PFA, and undergo immunohistochemistry staining and DAPI staining with primary and secondary antibodies for GFP and DNA nucleotides. Brains are separated from the carcass and mounted before viewing on Leica microscope. IPCs are quantified per brain lobe by double counting cells positive for both GFP and DAPI stains, indicating both DNA and dilp3 presence.

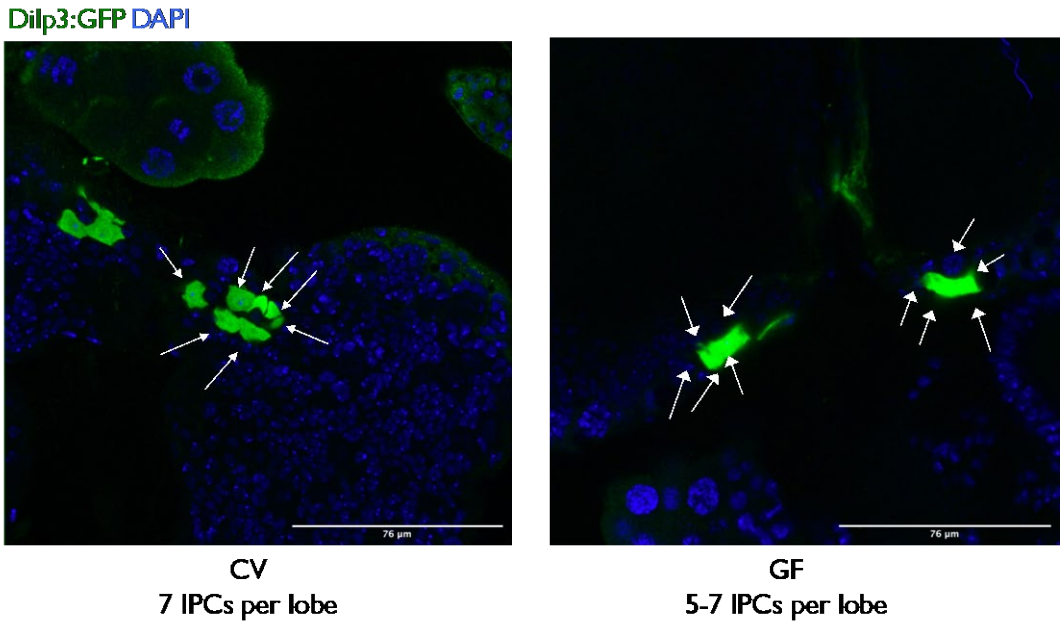

Figure 2. GF flies exhibit a significant reduction in IPCs per lobe compared to CV flies

IPCs in the Drosophila brain. IPCs are labeled for GFP and DNA for DAPI. Both GF and CV brains exhibit IPCs, but GF flies show a significant qualitative reduction in mass and fluorescence.

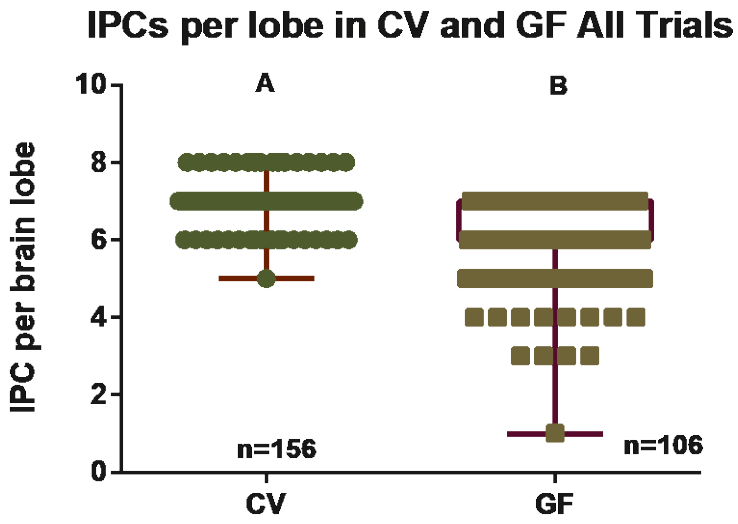

Figure 3: GF flies exhibit a significant reduction in IPCs per lobe compared to CV flies

Letters indicate $p<0.05$, students t-test. Data are presented as min/max, showing all points from eight biological replicates. Mean for CV is 6.973 +/- 0.04145, n = 149. Mean for GF is 5.876 +/- 0.1113, n = 105.

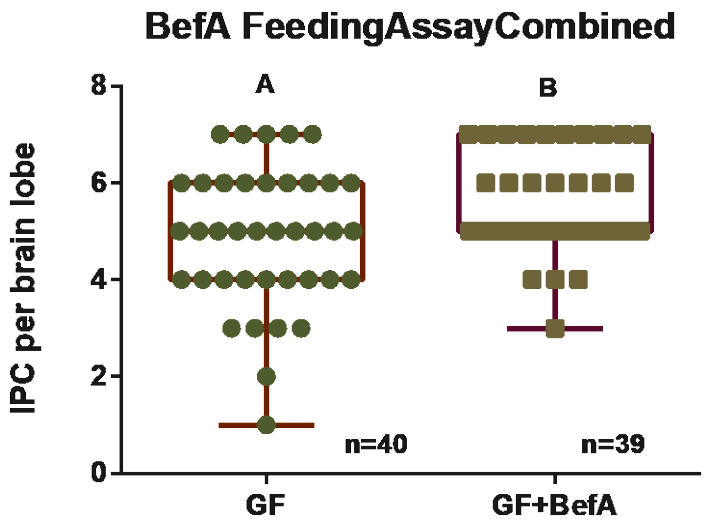

Figure 4: BefA delivered through a feeding assay showed a significant difference in IPC per lobe between GF+BefA and GF groups.

Letters indicate p<0.05, students t-test. Mean for GF is 4.872 +/- 0.2299, n = 39. Mean for GF+BefA is 5.611 +/- 0.1794, n = 36. Data are presented as min/max, showing all points from three biological replicates.

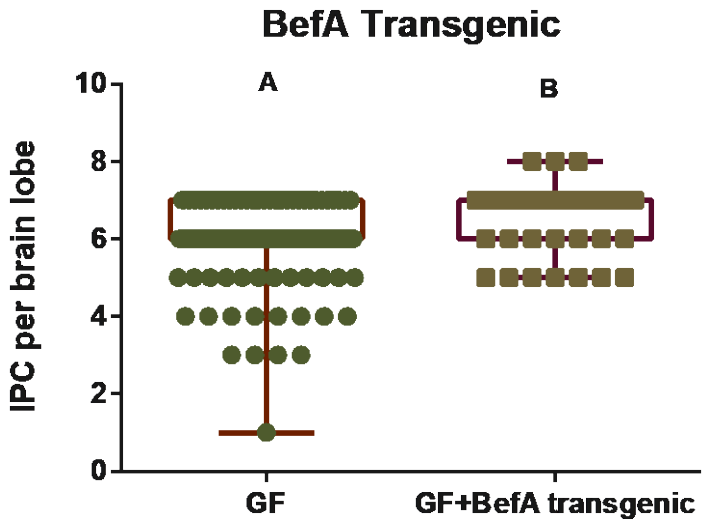

Figure 5: BefA delivered transgenically shows a significant difference in IPC per lobe between GF and GF+BefA.

BefA alone showed full rescue effects of GF treatment, bringing IPC counts to CV levels. Letters indicate p<0.05, students t-test. Mean for GF is 5.876 +/- 0.1113, n = 105. Mean for GF+BefA transgenic is 6.273 +/- 0.1809, n = 33. Data are presented as min/max, showing all points from three biological replicates.

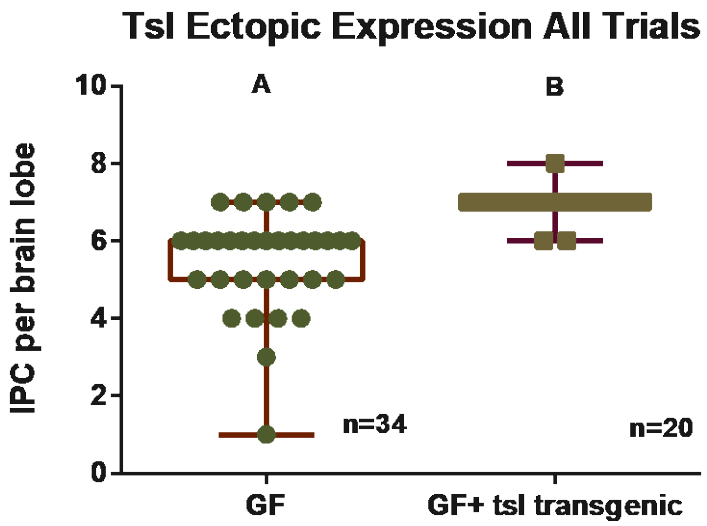

Figure 6: Tsl delivered transgenically showed a significant difference in IPC per lobe between CV, GF, and GF+BefA

Tsl alone showed full rescue effects of GF treatment, bringing IPC counts to CV levels. Letters indicate $p<0.05$, students t-test. Mean for GF is 5.455 +/- 0.2223, n = 33. Mean for GF+ tsl transgenic is 6.950 +/- 0.08811, n = 20. Data are presented as min/max, showing all points from three biological replicates.

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