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Ana Tsulaia April 10, 2024

The Role of *etv1* in specifying the Enteric Nervous System (ENS) Neuronal Subtypes in Zebrafish

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
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Abstract

The Role of *etv1* in Specifying the Enteric Nervous System (ENS) Neuronal Subtypes in Zebrafish

By Ana Tsulaia

The enteric nervous system (ENS) is the largest subdivision of the peripheral nervous system that governs the function of the GI tract and is completely derived from the neural crest. Abnormal migration and differentiation of these neural crest cells cause the human clinical condition, Hirschsprung disease, along with other intestinal neuropathies . Although there have been many studies investigating how the ENS arises, our understanding of the molecular basis of this process is far from complete. Recent single-cell RNA sequence analysis of zebrafish ENS has revealed that the transcription factor ETV1 is specifically expressed in subsets of differentiating ENS neurons, though its precise function remains unclear. The goal of this project is to investigate the role of *etv1* in enteric neuronal specification during the early stages of gut development, using zebrafish as a model organism.

To accomplish this goal, we generated a tg(phox2bb:egfp);*etv1*^{sa14953} mutant zebrafish line, by crossing *etv1*^{sa14953} mutation onto a tg:(phox2bb:egfp) background. Five days-post-fertilization (5dpf) larvae from an in-cross of this line were collected and immuno-stained using three primary antibodies. The first antibody, anti-GFP, was used to label *phox2bb*+ enteric cells. The second, anti-5HT antibody targeted *tph1b*+ serotonin-producing cells, while the third, anti-nNOS, specifically labeled *nos1*+ inhibitory motor neurons. Subsequently, larvae were genotyped, and their guts were dissected for imaging using confocal microscopy. Images were analyzed to determine the number of positive cells for each antibody, in the mid/posterior intestine.

etv1 homozygous mutants displayed a significantly lower number of total *phox2bb*+ enteric cells than their heterozygous and wildtype counterparts, demonstrating that *etv1* affects the total enteric cell count. Mutants also showed a clear aganglionosis at the distal end of their guts, suggesting that ETV1 affects the migration of the neural crest cells along the length of the gut. Furthermore, *etv1* mutants showed a lower percentage of *nos1*+ inhibitory motor neurons as well a higher percentage of *tph1b*+ serotonergic neurons, suggesting the gene's involvement in the differentiation of the enteric neuronal subtypes. Overall, our study provides valuable insights into the molecular and cellular dynamics of enteric neuron differentiation and the impact of the ETV1 transcription factor on this process.

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Acknowledgements

I would like to thank Dr. Iain Shepherd, my principal investigator, who helped me conceive and design the study, as well as my fellow lab members, Anna Han and Anoushka Mukhopadhyay, who assisted with the data collection and analysis.

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Introduction

The enteric nervous system (ENS) is the largest subdivision of the peripheral nervous system (PNS) that makes up the intrinsic nervous system within the gastrointestinal tract (GI). The role of ENS is to regulate multiple aspects of GI function including peristalsis, gastrointestinal absorption and secretion as well as gut immune interactions. It achieves this by acting as an integrated neuronal system encompassing local enteric reflexes and reflexes that communicate between the gut and the central nervous system (CNS).[i] Unlike other parts of the PNS, the ENS can function independently of the CNS when isolated in vitro. This means that the ENS has a fully integrated neuronal network of sensory inter and motor neurons. In most vertebrates, enteric neurons are located in two interconnected gangliated plexi in the gut, the myenteric plexus and the submucosal plexus (Figure 1). The cell bodies of the neurons are located in the ganglia while their axons spread out to make the plexus. These axons also innervate other ganglia and the tissues of the digestive organs including the muscle layers and the mucosa. Most enteric neurons are found in the myenteric plexus which is located between the longitudinal and circular muscle layers of the GI tract. They innervate these muscle layers to control the peristaltic relaxations and contractions of the intestinal wall. In contrast, neurons within the submucosal plexus are located between the circular muscle layer and the mucosa. Nerve fibers in the mucosa regulate the function of entero-endocrine cells while other projections in the intestinal epithelium affect absorption, secretion and vascular circulation within the walls of the gut.[ii]

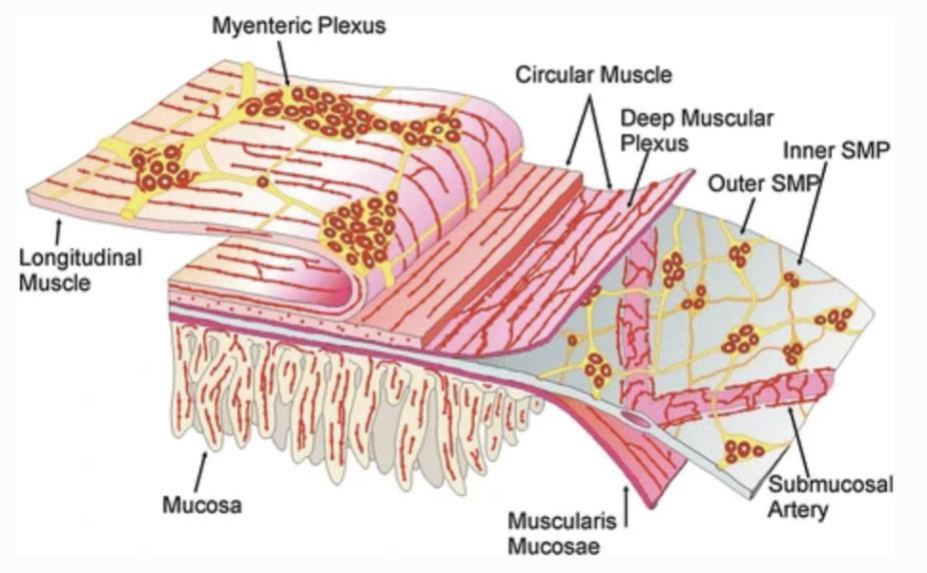

Figure 1. Organization of the ENS of the small intestine of human and medium sized to large mammals. The ENS has two ganglionated plexi, the myenteric plexus between the circular and longitudinal muscle layers and the submucosal plexus between the circular muscle layer and the mucosa. (Furness, John B., et al., 2014)

Given this integrated network in these plexi and the ENS's ability to function independently of the CNS, unsurprisingly, there are number of different types of neurons found in plexi. Previous studies have categorized ENS neurons of the adult guineapigs into 17 different subtypes based on their functions, cell body morphologies, chemistries, key neurotransmitters, and projections to targets.[iii] They are distributed across the different layers of the GI tract based on their function. The 17 subtypes of neurons can be further grouped into three distinct functional classes: primary afferent neurons (IPANs), interneurons, and motor neurons. IPANs detect the physical state of the organs and the chemical features of the luminal content. In order to integrate information about the gut's mechanical and chemical environment, IPANS span across both the myenteric and the submucosal plexi. Motor neurons, on the other hand, have their cell bodies in the myenteric ganglia, where they receive the signals from the interneurons, while their axons project onto the longitudinal and circular muscle layers to control their contractions and

relaxations. Finally, the transfer of signals between motor and sensory neurons is facilitated by the interneurons which span across multiple layers of the gut, synapsing on the other inter or motor neurons.[ii]

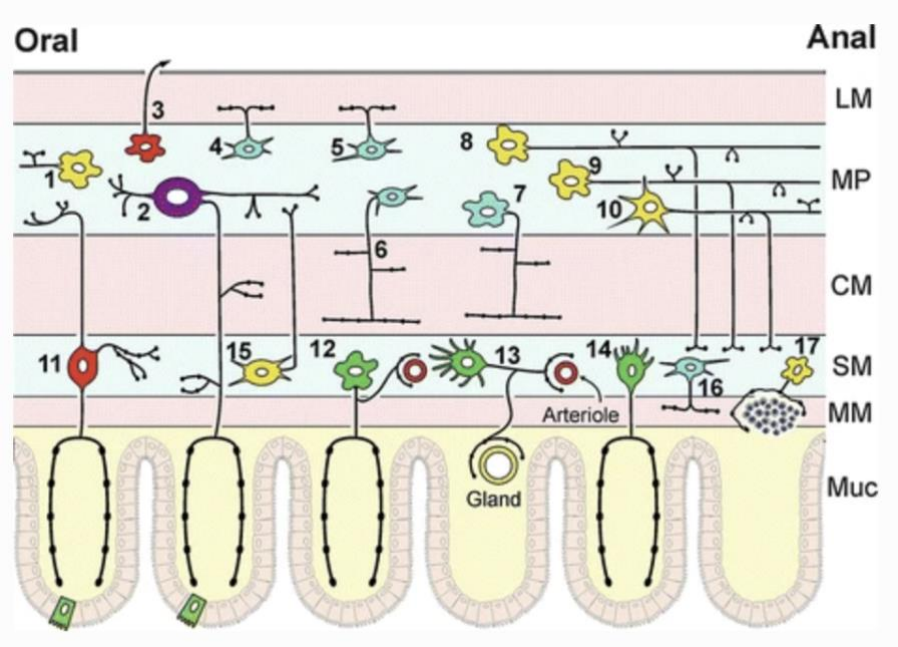

Figure 2. The 17 types of ENS neurons identified in guineapigs.
The neurons span across the different layers of the GI tract based on their function. The layers include the longitudinal muscle (LM), myenteric plexus (MP), circular muscle (CM), submucosal plexus (SM), and the mucosa (Muc). (Furness, John B., et al., 2014)

Other studies have identified a similar complexity of functional neuronal types in other species as well. Zebrafish, a commonly used vertebrate model system, is composed of enteric neurons that are of similar functional, neurochemical, and morphological diversity as those seen in other higher vertebrates.[iv] Zebrafish has the advantage compared to other vertebrate model systems due to its relatively compact size and rapid embryonic and larval development. All zebrafish major organ systems are fully developed within just five days post-fertilization (5dpf).[v]

Although zebrafish exhibit a complexity of ENS neuronal subtypes similar to that found in higher vertebrates, their anatomy differs from the latter. Zebrafish lack a submucosal plexus; instead, they possess a single layer of myenteric plexus which is non-ganglionated, but rather formed by individual cells.[vi] The sensory and the inter neurons that are found in the submucosal plexus of the higher vertebrates appear to be in the myenteric plexus of the zebrafish. In guinea pigs, however, there is a single layer of submucosal ganglia, mainly consisting of secretory neurons and lacking motor neurons. Furthermore, larger mammals like pigs and humans have submucosal ganglia that form separate, yet interconnected plexi positioned at various levels.[vii]

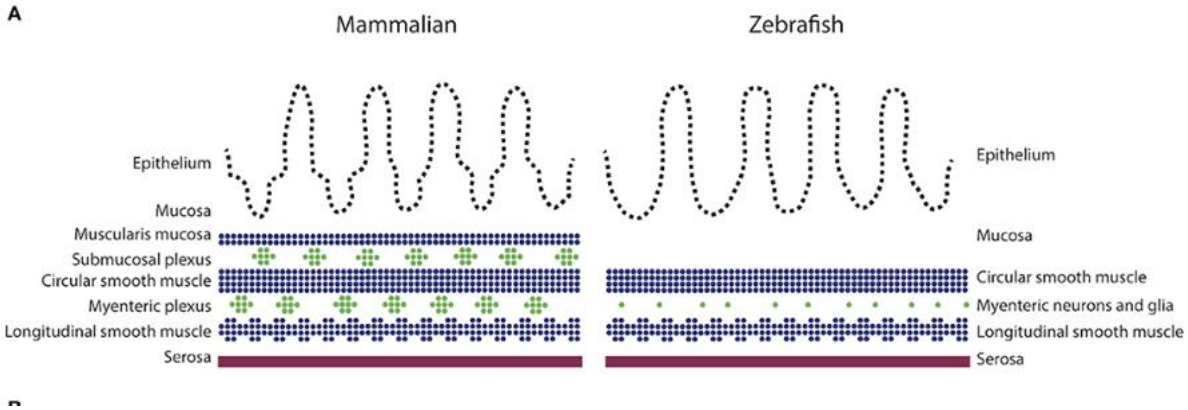

Figure 3. Schematic representation of the GI tract in mammalians and zebrafish.
Zebrafish lack the muscularis mucosa and the submucosal plexus. Moreover, the enteric neurons are not organized in ganglia, but rather as individual cells. (Kuil, Laura E., et al., 2021)

While there have been number of studies aiming to classify the ENS neurons into various subtypes, our understanding of the process by which the ENS cells differentiate into these specific subtypes is still far from complete. Just like all areas of PNS, the enteric nervous system is derived from the neural crest cells. The failure of the neural crest-derived cells to migrate along the length of the gut causes various intestinal neuropathies, so it is of clinical importance to understand the specification, migration, and differentiation of the ENS cells.[viii]

The most studied and comparatively well understood of the intestinal neuropathy is Hirschsprung disease (HSCR), a congenital disorder characterized by an absence of enteric neurons at the terminal end of the GI tract. In this condition, the aganglionic bowel is unable to carry out any propulsive activity which results in bowel obstruction with a high risk of developing more serious conditions such as intestinal inflammation and perforation, leading to potentially fatal infections.[iv] By comparison, other intestinal neuropathies are far less understood.[ix] Key to understanding these conditions as well as gaining further insight in to HSCR and subsequently potentially developing therapeutic strategies to treat these ENS disorders is to gain a greater understanding of the different instructive signals that drive the migration, proliferation and differentiation of ENS cells during the early stages of embryonic development.

To begin to address this problem, more recent studies have undertaken single-cell RNA sequence transcriptomics analysis of ENS neurons and precursors during specific stages of embryonic development.[x] By utilizing this technique, researchers are potentially able to identify the signals that initiate the differentiation of ENS neurons, as it reveals specific genes that are expressed at that stage in differentiated neurons of a specific subtype that are differentially expressed in their other ENS precursor cells or enteric neurons of a different subtype.

This approach has been taken by the Shepherd Lab in collaboration with the Hofstra/Alves Lab at Erasmus University Rotterdam. This single-cell RNA sequence analysis was conducted on the 1369 gut cells isolated from 5 days post fertilization (5dpf) zebrafish embryos. The analysis identified eleven clusters of neurons sharing similar marker genes. Four of these clusters represented distinct subtypes of differentiated neurons (Figure 4). The largest cluster consisted primarily of inhibitory motor neurons expressing the *nos1* gene, but within this cluster there is a

subdivision of serotonergic cells expressing the *tph1* gene. The second major branch/cluster was made up of most intrinsic primary afferent neurons (IPANS) and could be characterized by their expression of the *nmu* gene. The third cluster represented motor neurons while the fourth contained a mix of different subtypes like glutamatergic, GABAergic and IPANS.[xi] Other transcriptomic studies have identified similar subtypes of ENS neurons in other species at comparable stages of development demonstrating an evolutionary conservation of these maker genes.[xii]

Recent single-cell RNA-Seq analysis profiled transcriptomes from the ENS in adult mice identified 21 different clusters of neurons across five major groups including excitatory motor neurons, inhibitory motor neurons expressing the *nos1* gene, sensory motor neurons expressing the *nmu* gene, as well as interneurons, and secretomotor/vasodilator neurons.[xiii]

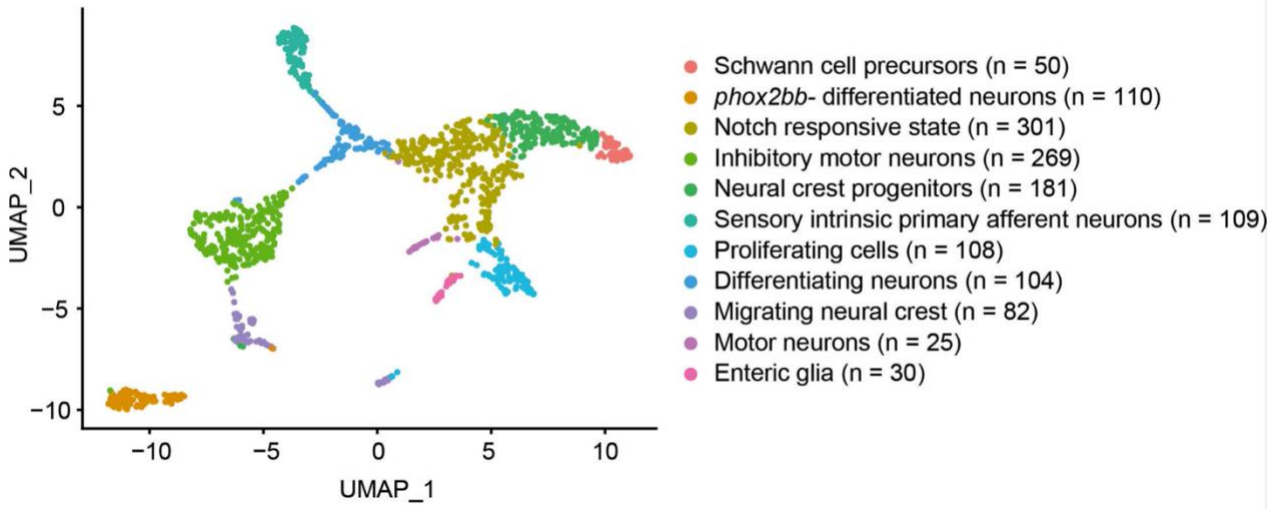

Figure 4. Single cell transcriptomics of 5 dpf zebrafish ENS.
UMAP of 1369 ENS cells, containing eleven different clusters. (Kuil, Laura E., et al., 2023)

Our recent study using the single cell transcriptomics of 5dpf zebrafish ENS has revealed that *etv1* gene might be an important instructive signal in the differentiation of the ENS neurons. A

pseudo-time trajectory plot shows the progression from progenitors to differentiated neuronal clusters (Figure 5). It is evident that during early stages of ENS neuronal differentiation, precursors differentiate into 2 main subtypes: intrinsic primary afferent neurons (IPANS) and inhibitory motor neurons based on their RNA expression. Subsequently, precursors in the inhibitory motoneuron group branch again to give rise to serotonergic neurons.

Analysis of the transcriptomic profiles within these different subgroups showed that *etv1* was expressed in sensory intrinsic primary afferent neurons (IPANS) and *nos1* expressing inhibitory motor neurons. However, *etv1* expression was not observed in the serotonin-producing neuron cluster. Instead, these neurons showed a strong expression of the *tph1* gene which encodes the rate-limiting enzyme tryptophan hydroxylase (TPH) that catalyzes the production of Serotonin (5-HT).[xiv] This appears to be an evolutionarily conserved function for *etv1* as other RNA SC RNA seq studies in mouse and human ENS cells also showed *etv1* being specifically expressed in subsets of the differentiating ENS neurons. A recent study aiming to characterize the enteric neurons of the mouse small intestine myenteric plexus found that out of the twelve enteric neuron classes, most differentiated subsets of neurons displayed a strong expression of the *etv1* gene.[xv]

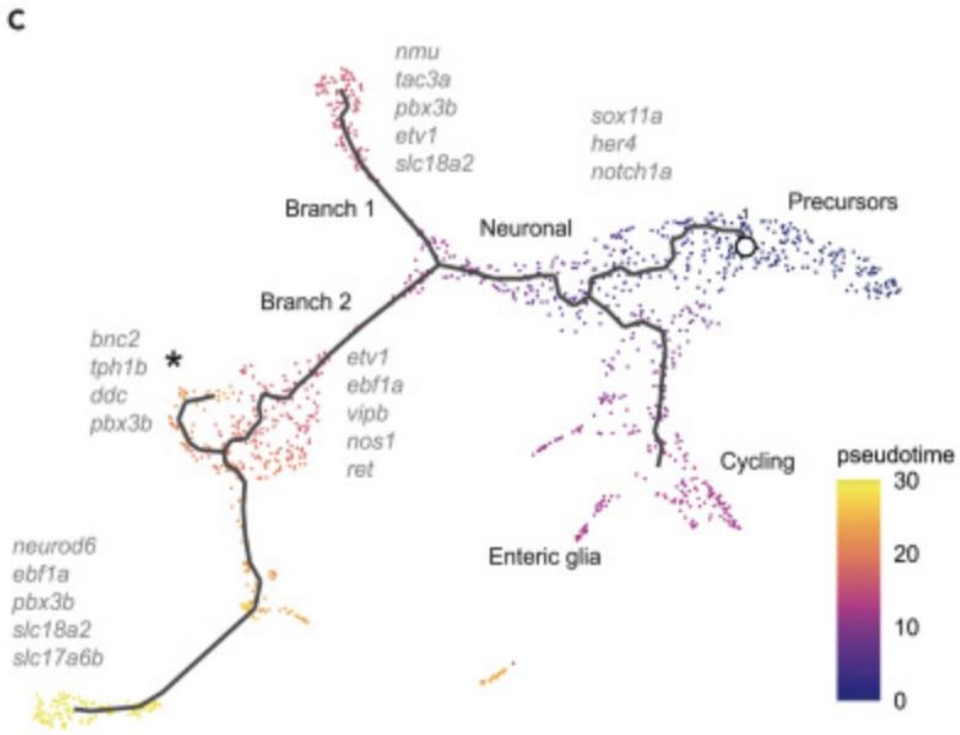

Figure 5: Pseudotime color-coded featureplot showing a bifurcation toward neuronal differentiation (sensory IPAN: branch1 and inhibitory motor neurons: branch2 containing a secondary branch toward serotonergic neurons marked with an asterix)
(Kuil, Laura E., et al., 2023)

ETV1 is a transcription factor who's role in intestinal development and gastrointestinal motility was first associated with development of gastrointestinal stromal tumors (GIST). GIST is the most prevalent human sarcoma, characterized by activating mutations in KIT, a receptor tyrosine kinase that transmit signals from the cell surface into the cell by activating downstream signaling pathways. KIT is highly expressed in interstitial cells of Cajal (ICC), "pacemaker" cells of the GI tract that generate spontaneous flow waves required for peristalsis and mediate the neuronal input from the ENS. Previous studies have shown that GIST arises from subtypes of ICCs which display high levels of *etv1* expression coupled with an activating KIT mutation. Transcriptome profiling and examination of the ETV1-binding sites demonstrate that ETV1 binds to the

enhancer elements in the regulatory regions of the genes controlling the ICC function. Abnormal ICC function and uncontrolled cell proliferation contribute to the development of GIST.[xvi]

Due to its proposed role in the development of GIST and its specific expression in ICC, we cloned the *etv1* gene in zebrafish as a potential ICC marker. However, in situ hybridization analysis using antisense digoxigenin labeled RNA probes revealed that the *etv1* gene was expressed in the gut during the critical stages of ENS development. At 72 hours post-fertilization (hpf), embryos showed a punctate pattern of *etv1* expression along the length of the gut that persisted through 120hpf (Figure 6). Based on this in vivo data and the transcriptomic analysis, it is reasonable to assume that ETV1 is an important determinant in ENS neuron specification during development.

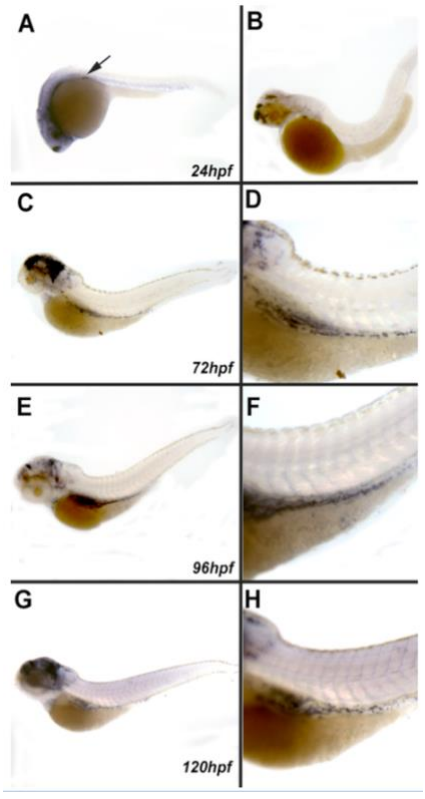

Figure 6: In situ gene expression analysis using antisense digoxigenin labeled RNA probes revealed that the *etv1* gene was expressed in the gut during the critical stages of ENS development. At 72 hours post-fertilization (hpf), embryos have a punctate pattern of etv1

expression along the length of the gut that persists through 120hpf. The pattern of expression looks similar to other enteric neuron-expressed genes such as *phox2bb*.

The aim of this study is to further investigate the role of ETV1 in specifying the different ENS neuronal subtypes in zebrafish model systems. By comparing the total number of enteric neurons as well as the total number and proportion of serotonergic neurons and inhibitory motor neurons between wildtype, heterozygous, and mutant embryos for the *etv1* gene, we determined if the ETV1 transcription factor is an important instructive signal in ENS neuron subtype specification. Our study has found that homozygous mutation of the *etv1* gene causes significant decrease in the total number of *phox2bb*+ cells, leading to aganglionosis at the distal ends of zebrafish embryos' guts. Additionally, our findings demonstrate that the ETV1 transcription factor plays a role in the subtype specification of neural crest cells, influencing their differentiation into serotonergic and inhibitory motor neuron clusters.

Materials and Methods:

Obtaining the ETV1 Mutant Line

The zebrafish *etv1*^{sa14953} mutant line was obtained from ZIRC and crossed onto a *tg:(phox2bb:egfp)* background. The point mutation was generated by introducing a premature stop codon by a G/T base change at chromosome 15. The *phox2bb::egfp* strain contains a stably integrated BAC (Bacterial Artificial Chromosome) clone in which enhanced green fluorescent protein (egfp) had been recombined into the native start site of the *phox2bb* gene. This genetic modification allows for the expression of GFP under the control of the *phox2bb* gene promoter.[xvii] The resulting GFP+ line allows for the identification of all enteric neurons

expressing the *phox2bb* gene. All GFP+ zebrafish embryos that were analyzed in this study were generated from crossing homozygous *tg:(phox2bb:egfp)* line with heterozygote fish carrying the *etv1*^{sa14953} allele. *tg:(phox2bb:egfp); etv1*^{sa14953} adult fish had their fins clipped to extract DNA for genotyping. The zebrafish were sorted into three separate tanks, with each tank corresponding to their *etv1*^{sa14953} genotype: wildtype (+/+), heterozygous (+/-), or homozygous (-/-). To obtain embryos to allow for analysis of ENS neuronal specification and differentiation, three females and two males of each genotype were placed in a breeding tank and were left overnight.

Immunohistochemistry

Embryos from an in-cross of *tg:(phox2bb:egfp)*$^{+/+}$; *etv1*$^{sa14953+/-}$ mutant line were collected and immunocytochemically double-stained at 5 days-post-fertilization (5 dpf). For antibody staining, embryos were first fixed in 4% Formalin/1X fix buffer, washed 3x with PBT (PBS containing Triton 0.5% X-100) for 10 minutes, rinsed 3x with double-distilled water for 1 hour and incubated in the blocking solution with 5% goat serum for 1 hour to reduce nonspecific staining. Subsequently, embryos were incubated overnight in primary antibodies diluted in the blocking solution. After a 3 x 30-minute washing step with PBT, the embryos were immersed for 16-18 hours in the appropriate secondary antibodies diluted in the blocking solution. Subsequently, the embryos were washed with PBT and stored in PBT with 0.01% sodium azide. The primary and secondary antibodies used in this study as well as their dilutions are listed in Table 1.[xviii]

Table 1: Primary and Secondary Antibodies

Primary Antibodies				
Antigen	**Host**	**Dilution**	**Company**	**Catalog No.**
5HT; serotonin coupled to bovine serum albumin with paraformaldehyde	Rabbit	1/10,000–1/20,000	Immunostar, Hudson, WI	20080
Hu; synthetic peptide from human neuronal protein D	Mouse	1/50–1/500	Molecular Probes Inc, Eugene, OR	A-21271
An2-nNOS; synthetic peptide corresponding to Human nNOS	Rabbit	-	Abcam, Cambridge, UK	ab5586

Secondary Antibodies				
Antigen	**Host**	**Conjugate**	**Company**	**Catalog No.**
Rabbit IgG	Goat	Alexa Fluor™ 488	Thermo Fisher Scientific, Waltham, MA	A-11008
Mouse IgG	Goat	Alexa Fluor™ 488	Thermo Fisher Scientific, Waltham, MA	A-11001
Rabbit IgG	Goat	Alexa Fluor™ 568	Thermo Fisher Scientific, Waltham, MA	A-11011
Mouse IgG	Goat	Alexa Fluor™ 568	Thermo Fisher Scientific, Waltham, MA	A-11004
Rabbit IgG	Goat	Alexa Fluor™ 594	Thermo Fisher Scientific, Waltham, MA	A-11012
Mouse IgG	Goat	Alexa Fluor™ 594	Thermo Fisher Scientific, Waltham, MA	A-11005

Genotyping the 5dpf stained embryos

The heads of the stained 5dpf embryos were removed and their DNA was subsequently extracted by lysis. An annealing temperature of 55^{o}C was used in the PCR for the amplification of the ETV1 gene with ETV1 Exon 8 2Forward and Exon 8 2Reverse primers (Table 2). 49

cycles were conducted to amplify the DNA. The PCR product was purified using a QIAquick PCR purification kit and sequenced by Psomagen. PCR sequencing analysis was performed using Sequencher to determine the genotypes of the stained embryos.

Table 2: Sequences of the Exon 8 2Forward and Exon 8 2Reverse primers

Primer	Sequence
ETV1 Exon 8 2Forward	5'-TATTGTTCAGATTCCGCCGG-3'
ETV1 Exon 8 2Reverse	5'-AATGTCGTCGTCTCACCTGA-3'

Imaging and Analysis

Guts of the genotyped embryos were dissected and imaged using an Olympus Spinning disc Confocal microscope with SlideBook 6 x64 software. The gut tube was rotated so that it was roughly horizontal and fit within the image frame under 10X magnification. We specifically imaged the mid/postetior end of the gut under both green (GFP) and red channels (RFP) channels. The *phox2bb*+ neurons fluoresced green (due to the secondary antibody which was used to recognize GFP antibody labeled cells was conjugated with Alexa Fluor™ 488) while cells expressing either anti-5HT and anti-nNOS antibodies fluoresced red (as these primary antibodies were recognized by secondary antibodies conjugated with Alexa Fluor™ 568 and 594 conjugates). Projection images of confocal stacks of the different dissected guts just showing the gut expression in the GFP or RFP channels as well as under both channels were saved and exported as a 24-bit TIFF files. For the quantitative analysis, the images were converted into black and white in Adobe Photoshop and processed with Image J, a program that counted the

precise number of positive cells for each antibody in the mid/posterior intestine. In addition, the proportion each neuronal subtype out of the total number of neurons were calculated in Excel. More specifically, the number of total neurons (*phox2bb*+ neurons), the total number and proportion of serotonergic neurons (5HT+ and *phox2bb*+ neurons), and the total number and proportion of inhibitory motor neurons (*nos1*+ and *phox2bb*+ neurons) were calculated in each group. The data were plotted on the stacked dot plot using Excel. Each plot contained 3 groups: Wildtypes (+/+), Heterozygotes (+/-), and Mutants (-/-). Each dot on the plot represented a separate embryo, the position of which showed the number of each type of neuron present in that sample. The plot also showed the total number of embryos in the group as well as the average number of certain type of neuron within each group. To see if there was a statistically significant difference between the average number of neurons as well as the proportion of neurons within each group, the paired T-test with equal variance and a p-value of 0.001 was used. The statistically significant difference between the two groups was denoted by an asterisk while a non-significant difference was denoted as n.s.

Results

etv1 homozygous mutant embryos stained with anti-GFP and anti-5HT antibodies have a significantly lower total number of *phox2bb*+ enteric neurons than heterozygous and wildtype embryos at 5dpf.

We imaged 17 wildtype, 31 heterozygous, and 16 homozygous mutant 5dpf embryos were stained with the combination of the anti-GFP and anti-5HT primary antibodies and complementary secondary antibodies (goat anti-mouse 488 and goat-anti-rabbit 594). Figure 7 displays the gut images of embryos from all three genotypes. Each red “dot” represents an enteric cell expressing serotonin which anti-5HT antibody binds to and fluoresces red. The green “dots”, on the other hand, represent nearly all the differentiated enteric neurons and enteric neural crest cell precursors that express the *phox2bb* gene and produce the Green Fluorescent protein (GFP) that the anti-GFP antibody binds to and fluorescens green. The “lines” extending from each “dot” are the axons projecting from these *phox2bb* expressing enteric neurons. Since *phox2bb* is broadly expressed across nearly all enteric neuronal subtypes and is absent in other cell types within the enteric nervous system (ENS), apart from the undifferentiated enteric neural crest cells, the green “dots” represent nearly all enteric neurons located in these embryos' guts. The anti-5HT antibody binds to all cells producing serotonin, encompassing neurons expressing the *phox2bb* gene and enteroendocrine serotonin cells, which, being non-neuronal, do not express the *phox2bb* gene. In the merged image, the yellow "dots" represent serotonergic neurons expressing both the *phox2bb* gene and the *tph1b* gene. These neurons produce GFP, which binds to the anti-GFP antibody, generating green fluorescence, and serotonin (5HT), produced by the enzyme encoded by the *tph1b* gene, recognized by the anti-5HT antibody, resulting in red

fluorescence. The combination of green and red fluorescence yields a yellow hue when merged. Conversely, cells exclusively fluorescing red are enteroendocrine cells that solely express *tph1b* gene without expressing the *phox2bb* gene, and consequently, not binding to the anti-GFP antibody. It is apparent from the presented embryonic gut images, that there's a small reduction in the overall count of *phox2bb*+ enteric cells in heterozygote compared to wildtype. *etv1* homozygous mutants exhibits a more pronounced decline in *phox2bb*+ cell count relative to both heterozygotes and wildtypes. It is important to note that the mutant exhibits evident aganglionosis, characterized by a complete absence of *phox2bb*+ cells at the distal end of its gut. However, when comparing the total count of 5HT+ cells among mutants, wildtypes, and heterozygotes, no significant disparity in the number of serotonin-producing cells is discernible. In addition to the lack of *phox2bb*+ neurons at the distal end *of etv1* homozygous mutant's gut, we also see a scarcity of serotonergic neurons.

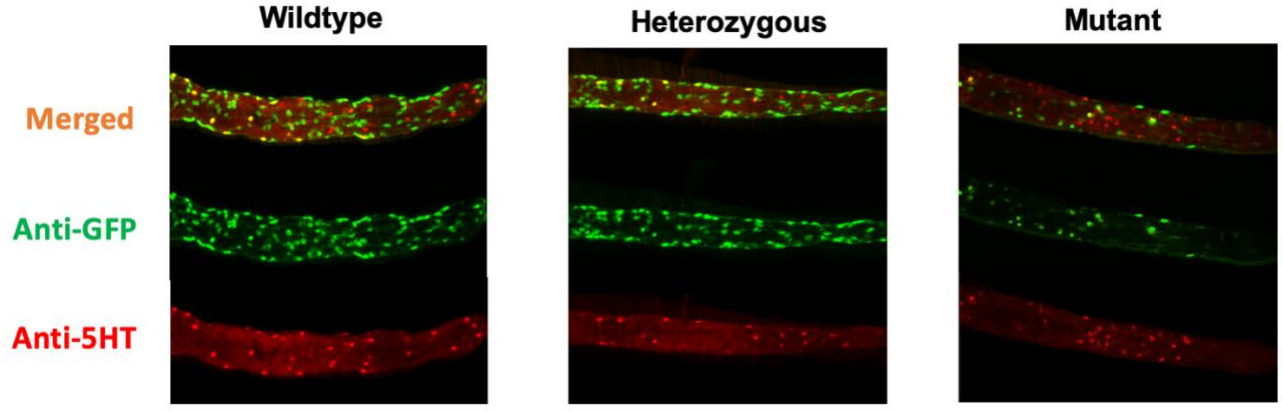

Figure 7: The gut images of the wildtype, heterozygous, mutant 5dpf embryos stained with Anti-GFP and Anti-5HT markers. The guts of *etv1* homozygous mutant embryos display a marked decrease in the number of *phox2bb*+ cells, accompanied by noticeable aganglionosis at the distal end of the gut. There is no substantial difference observed in the number of 5HT+ cells among the three genotypes.

To quantify the difference between the total number of *phox2bb*+ cells, total number of 5HT+ cells, total number of serotonergic neurons, and the percentage of serotonergic neurons between the three genotypes, the data was plotted on the stacked dot plots. Analysis of the data revealed no notable decrease in the total number of *phox2bb*+ cells in heterozygotes compared to wildtypes ($p>0.05$). However, *etv1* homozygous mutants exhibited a significantly lower number of *phox2bb*+ enteric neurons relative to both heterozygotes and wildtypes ($p<0.001$).

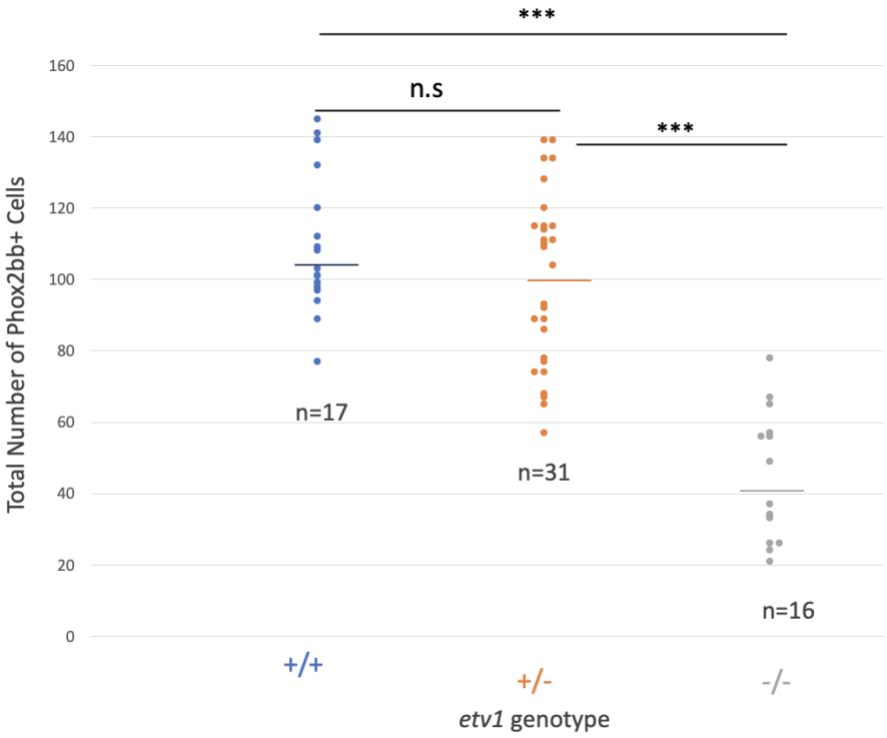

Figure 8: Total number of *phox2bb*+ neurons in 5dpf wildtype, heterozygote, and homozygous mutant embryos for the *etv1* gene stained with anti-GFP and anti-5HT antibodies. There is no significant difference between the total number of neurons between hets and wildtypes ($p>0.05$ denoted by n.s). *etv1 homozygous* mutants display a significantly lower number of *phox2bb*+ cells than hets and wildtypes ($p<0.001$ denoted by ***)

etv1 homozygous mutants have a higher percentage of serotonergic neurons than heterozygous and wildtype embryos at 5dpf.

When evaluating the total count of 5HT+ cells, encompassing both enteroendocrine cells and serotonergic neurons, across the three genotypes, a notable decrease is observed in the *etv1* homozygous mutants compared to wildtypes and heterozygotes ($p<0.05$). Conversely, there is no significant difference in 5HT+ cell count between heterozygotes and wildtypes ($p>0.05$). It is important to highlight that the reduction in 5HT+ cells observed in *etv1* homozygous mutants is not as pronounced as that observed in their total count of *phox2bb*+ cells (Figure 9). Examining solely the total count of serotonergic neurons, which express both the *phox2bb* and serotonin genes, reveals a substantial decrease in *etv1* homozygous mutants compared to wildtypes and heterozygotes ($p<0.05$) (Figure 10). Nonetheless, this reduction does not correlate proportionally with the decline observed in the total count of *phox2bb*+ cells in *etv1* homozygous mutants, as the decrease in the number of serotonergic neurons is notably smaller ($p<0.05$) than that observed in *phox2bb*+ cells ($p<0.001$).

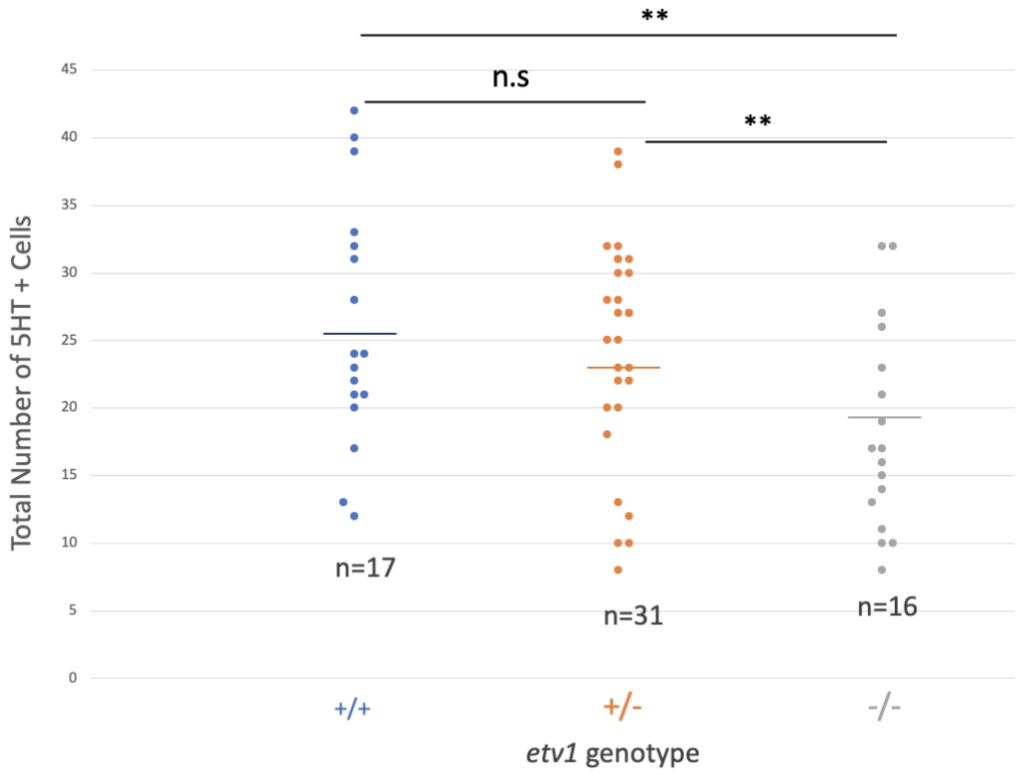

Figure 9: Total number of 5HT+ cells in 5dpf wildtype, heterozygote, and homozygous mutant embryos for the *etv1* gene stained with anti-GFP and anti-5HT antibodies. There is no significant difference between the total number of 5HT+ cells between hets and wildtypes ($p>0.05$ denoted by n.s). *etv1* homozygous mutants display a significantly lower number of 5HT+ cells than hets and wildtypes ($p<0.05$ denoted by **)

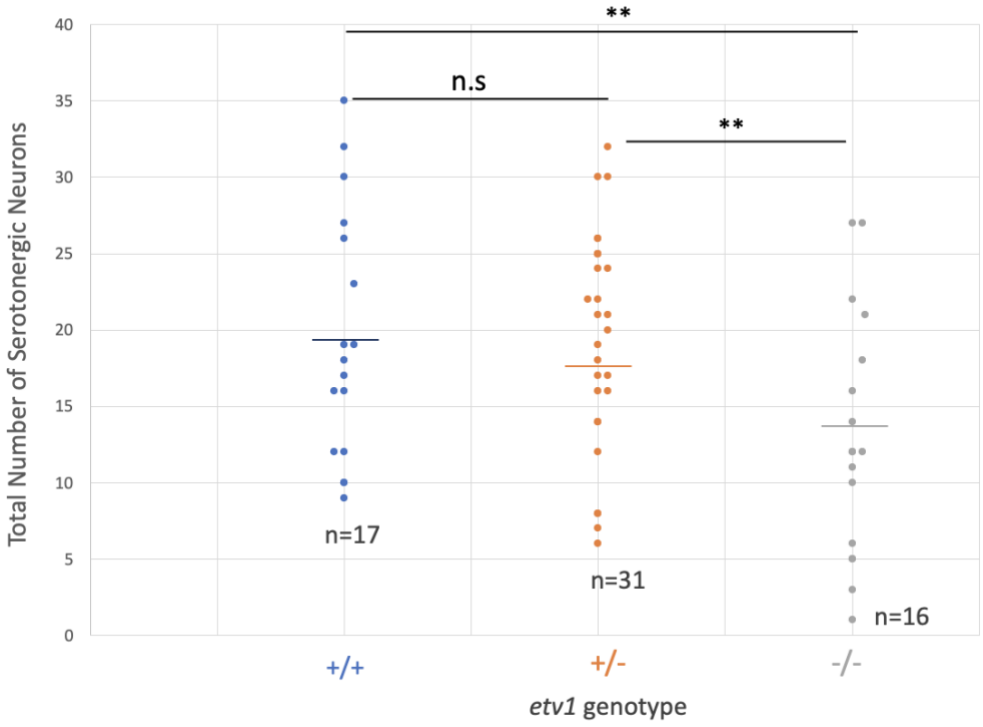

Figure 10: Total number of Serotonergic neurons at 5dpf in wildtype, heterozygote and homozygous mutant embryos for the *etv1* gene stained with anti-GFP and anti-5HT antibodies. There is no significant difference between the total number of serotonergic neurons between hets and wildtypes ($p>0.05$ denoted by n.s). *etv1* homozygous mutants display a significantly lower number of serotonergic neurons than hets and wildtypes ($p<0.05$ denoted by **)

Next, we examined the percentage of serotonergic neurons relative to the total count of *phox2bb*+ cells in embryos of the three genotypes. Analysis of the data revealed that there was a moderate increase in the percentage of serotonergic neurons in *the etv1* homozygous mutants as compared to wildtypes and heterozygous individuals ($p<0.05$) (Figure 11). The difference in the percentage of serotonergic neurons seen in the wildtypes and heterozygous embryos was not significant ($p>0.05$). The relatively high number of serotonergic neurons coupled with the significantly lower total count of enteric neurons resulted in the elevated percentage of serotonergic neurons observed in *etv1* homozygous mutants.

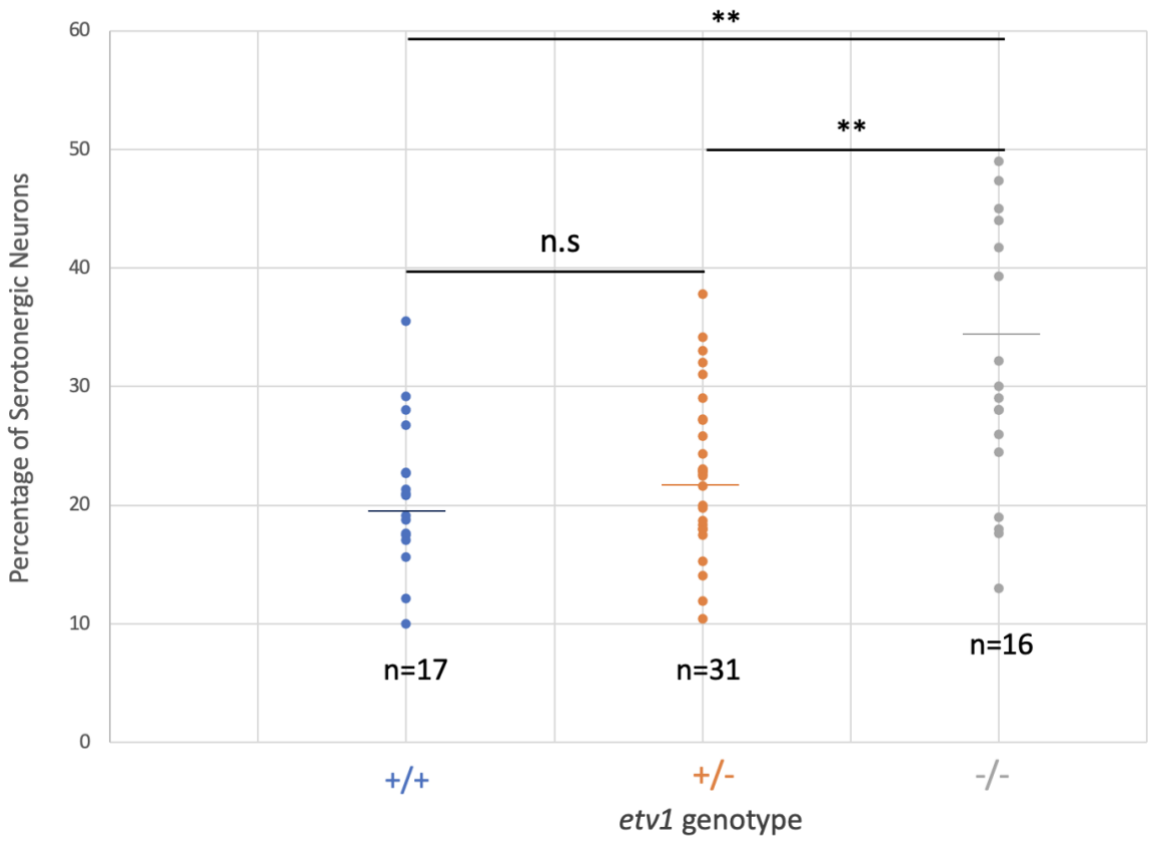

Figure 11: Percentage of Serotonergic neurons in 5dpf wildtype, heterozygote and homozygous mutant embryos for the *etv1* gene stained with anti-GFP and anti-5HT antibodies. There is no significant difference between the percentage of serotonergic neurons between hets and wildtypes ($p>0.05$ denoted by n.s). ETV1 mutants display moderately higher percentage of serotonergic neurons than hets and wildtypes ($p<0.05$ denoted by **)

etv1 homozygous mutants stained with anti-GFP and anti-nNOS antibodies have a significantly lower total number of *phox2bb*+ enteric cells than heterozygous and wildtype embryos at 5dpf

To examine if *etv1* mutation affected the differentiation of nNOS+ inhibitory motor neurons, we stained 14 wildtypes, 17 heterozygous, and 18 mutants with the combination of anti-GFP and anti-nNOS primary antibodies and complementary secondary antibodies (goat anti-mouse 488 and goat-anti-rabbit 594). Figure 12 displays the gut images of embryos from all three genotypes.

Each red “dot” represents an inhibitory motor neuron expressing the *nos1* gene coding for nNOS – neuronal nitric oxide synthase protein that is recognized by the anti-nNOS primary antibody. Green “dots”, on the other hand, represent almost all the enteric cells expressing the *phox2bb* gene and producing the Green Fluorescent protein (GFP) that the anti-GFP antibody binds to and fluoresces green. It is apparent from the gut images that most of the cells that fluoresce red also fluoresce green - this means that most of the enteric cells that produce the neuronal nitric oxide synthase are enteric neurons and thus also express that *phox2bb* gene (Figure 12). The cells that only fluoresce red are not *phox2bb*-expressing cells and therefore are unlikely to be neurons, but just other nonneuronal cells that express the *nos1* gene and produce other forms of the nitric oxide synthase protein that are non-selectively marked by the Anti-nNOS antibody. Comparing the gut images of the three genotypes, it becomes evident that there is a slight decrease in the total number of *phox2bb+* cells in heterozygous individual compared to wildtype. However, a significant decrease is observed in the total count of *phox2bb+* enteric cells in *etv1* homozygous mutant relative to both wildtype and heterozygous individuals (Figure 12). Notably, clear agangliosis is observed at the distal end of the homozygous mutant’s gut. Critically the same observations were made in embryos stained with the anti-5HT antibody. Looking at the number of *nos1+* cells, we see a comparable amount of immune positive cells in wildtype and het, with the cells being distributed evenly across the gut. In *etv1* homozygous mutants, there is a notable decrease in the number of *nos1+* cells, with the majority of the cells being concentrated at the anterior end of the gut. There is a complete absence of *nos1+* cells in the distal end of the homozygous mutant’s gut.

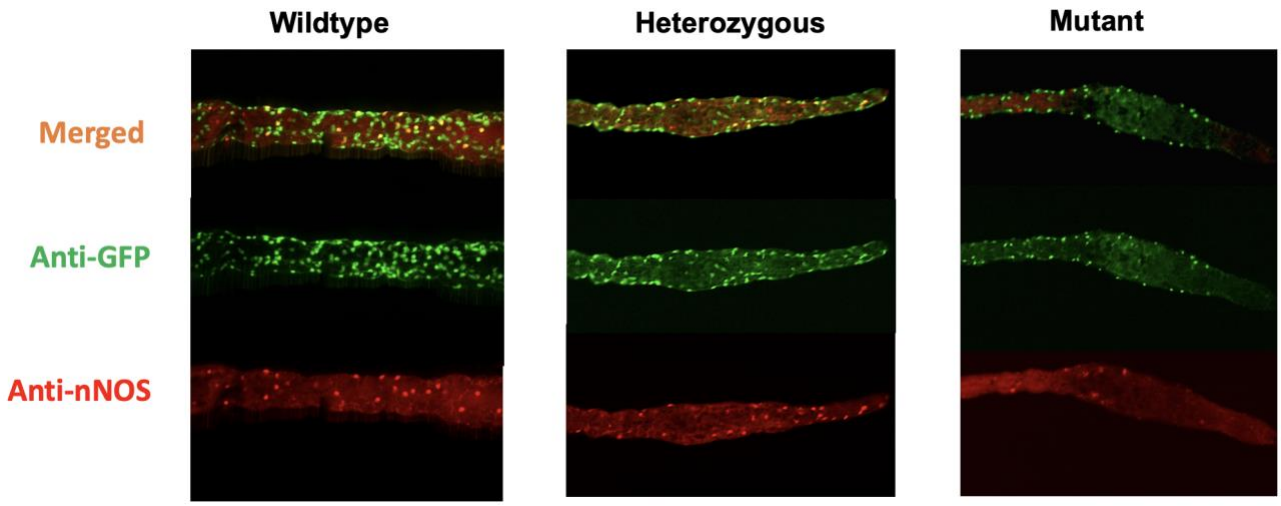

Figure 12: The gut images of the wildtype, heterozygous, mutant 5dpf embryos stained with Anti-GFP and Anti-nNOS antibodies. The gut of *etv1* homozygous mutant embryos display a marked decrease in the number of *phox2bb*+ cells, accompanied by noticeable agangliosis at the distal end of the gut. *etv1* homozygous mutants also have fewer *nos1*+ inhibitory motor neurons than hets and wildtypes, with most of them residing at the anterior part of the imaged gut section.

To quantify the difference between the total number of *phox2bb*+ cells, total number of *nos1*+ cells, and the percentage of double positive nNOS+ *phox2bb*+ cells in the three genotypes, the data were plotted on stacked dot plots. Although there is a slight decrease in the total number of *phox2bb*+ cells seen in the heterozygous embryos as compared to wildtypes, the difference in the numbers seen in the two groups is not statistically significant ($p>0.05$) (Figure 13). *etv1* homozygous mutants exhibited a significantly lower number of *phox2bb*+ cells neurons relative to both heterozygotes and wildtypes ($p<0.001$).

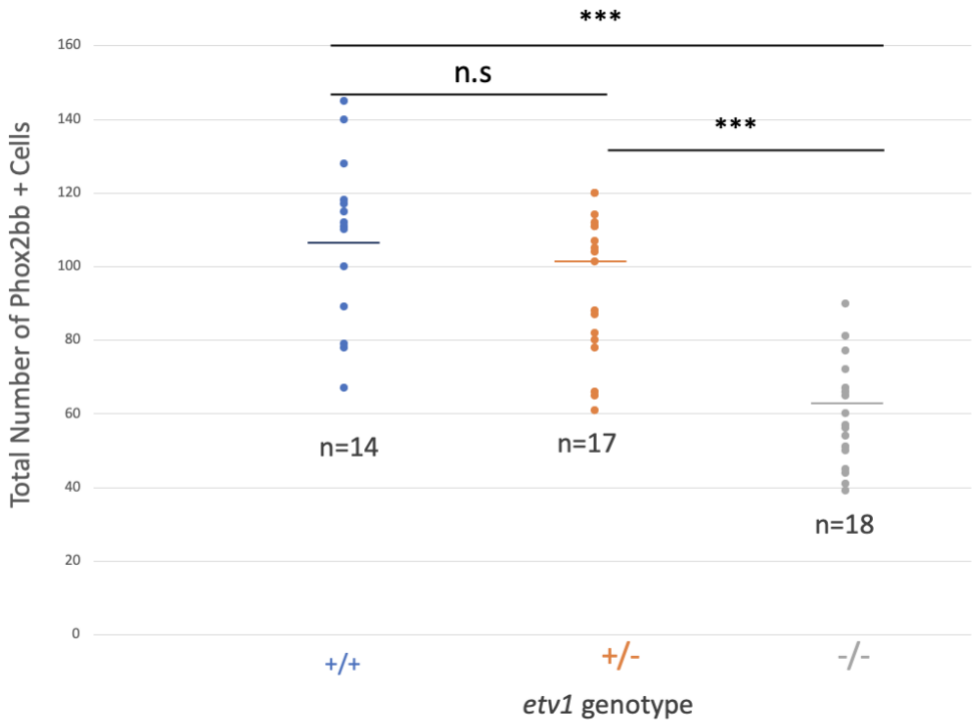

Figure 13: Total Number of *phox2bb* cells at 5dpf in wildtype, heterozygote, and homozygous mutant embryos for *the etv1* gene stained with anti-GFP and anti-nNOS antibodies. There is no significant difference between the total number of *phox2bb*+ cells between hets and wildtypes ($p>0.05$ denoted by n.s). *etv1* homozygous mutants display a significantly lower number of *phox2bb*+ cells than hets and wildtypes ($p<0.001$ denoted by ***)

etv1 homozygous mutants have a lower percentage of nNOS+ neurons than Heterozygous and wildtype embryos at 5dpf.

When evaluating the total count of nNOS+ neurons across the three genotypes, a notable decrease is observed in the *etv1* homozygous mutants compared to wildtypes and heterozygotes ($p<0.001$) (Figure 14). It is important to note that the decrease in the number of nNOS+ neurons seen in the *etv1* homozygous mutants is bigger than the decrease in the number *phox2bb*+ cells seen in the same group. There is a slight decrease in the number of nNOS+ neurons in the heterozygotes as compared to the wildtypes, however, the decrease is not statistically significant

(p>0.05). The decrease in the number of nNOS+ neurons seen in heterozygous individuals is proportional to the decrease in the number of *phox2bb*+ cells of the same group.

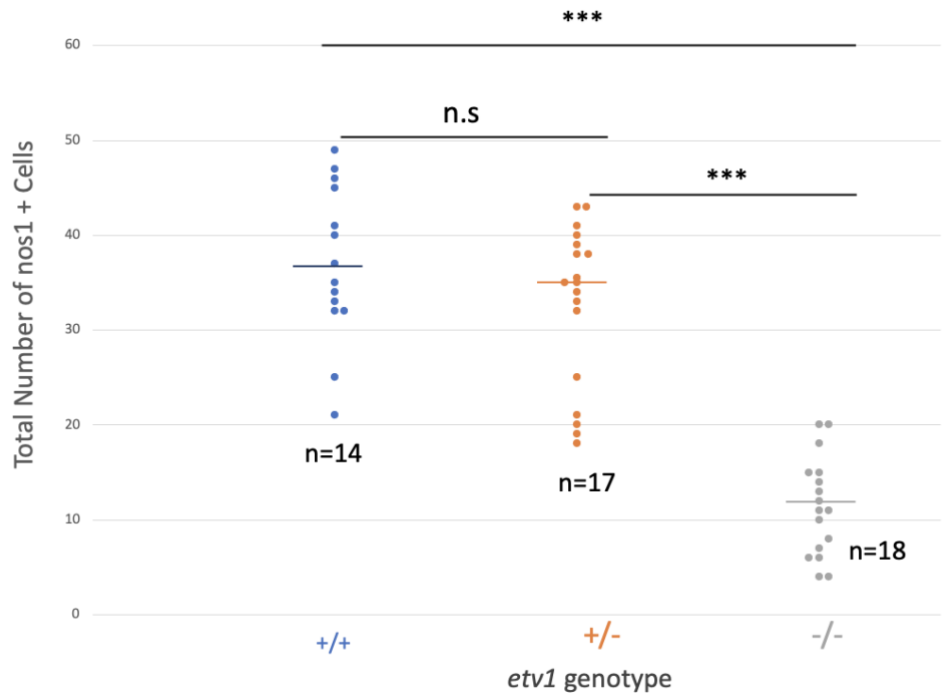

Figure 14: Total Number of nNOS+ cells in 5dpf wildtypes, heterozygotes, and homozygous mutants embryos for the *etv1* gene stained with anti-GFP and anti-nNOS antibodies. There is no significant difference between the total number of nNOS+ neurons between hets and wildtypes (p>0.05 denoted by n.s). *etv1* homozygous mutants display a significantly lower number of total nNOS+ neurons than hets and wildtypes (p<0.001 denoted by ***)

Next, we examined the percentage of nNOS+ enteric neurons relative to the total count of *phox2bb*+ enteric cells in embryos of the three genotypes. Analysis of the data revealed that the difference in the percentage of nNOS+ enteric neurons seen in the wildtypes and heterozygous embryos was not significant (p>0.05). However, there was a moderate decrease in the percentage of nNOS+ enteric neurons in the *etv1* homozygous mutants as compared to wildtypes and heterozygous individuals (p<0.05) (Figure 11). This observed phenomenon can be attributed to

our previous findings - the reduction in the number of nNOS+ enteric neurons was bigger than the one seen in the number of *phox2bb*+ enteric cells in the same group of stained embryos. The comparatively higher number of *phox2bb*+ enteric cells in *etv1* homozygous mutants coupled with the significantly lower total count of nNOS+ enteric neurons resulted in the lowered percentage of nNOS+ enteric neurons observed in *etv1* homozygous mutants.

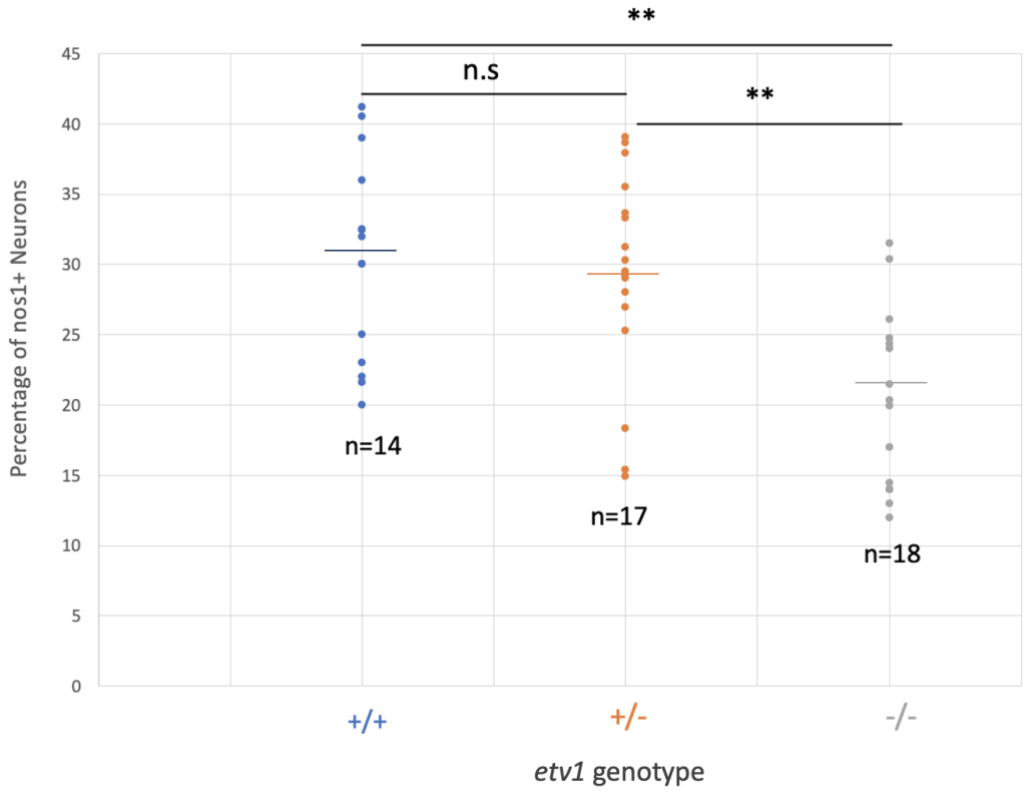

Figure 15: Percentage of nNOS+ enteric neurons at 5dpf in wildtype, heterozygote, and homozygous mutant embryos for the *etv1* gene stained with anti-GFP and anti-nNOS antibodies. There is no significant difference between the percentage of nNOS+ enteric neurons between hets and wildtypes ($p>0.05$ denoted by n.s). *etv1* homozygous mutants display a lower percentage nNOS+ inhibitory enteric neurons than hets and wildtypes ($p<0.05$ denoted by **)

Discussion

The findings from our study shed light on the molecular and cellular mechanisms underlying the development and differentiation of enteric neuron subtypes in zebrafish. Given the evolutionarily conserved expression of *etv1* in the ENS of other vertebrates it is possible that *etv1* will play a similar function during ENS neuronal differentiation as we describe here in zebrafish. Through the use of single-cell RNA sequencing, immunofluorescence staining, and imaging on the 5dpf embryos, we have gained insights into how a mutation in *etv1* affected the differentiation and migration of the serotonergic and inhibitory motor neuron subtypes in the gut.

Our results demonstrated that *etv1* homozygous mutants have a significantly lower total number of *phox2bb*+ enteric cells than their heterozygous and wildtype counterparts. This reduction in *phox2bb*+ enteric cell number is accompanied by a clear agangliosis at the distal end of the homozygous mutants' guts, indicating disrupted enteric nervous system development similar to that previously described for other zebrafish ENS mutants.[xix] The decrease in the *phox2bb*+ cell population is evident in both sets of embryos – those subjected to anti-5HT staining and those subjected to anti-nNOS staining. Notably, the extent of reduction observed in both sets is similar, with both groups showing p-values below 0.001 when compared between homozygous mutants and wildtype/heterozygotes. These findings indicate that *etv1* influences the overall count of *phox2bb*+ cells, encompassing both enteric neurons and enteric neural crest cells.

Furthermore, we observed a scarcity of nNOS+ and 5HT+ enteric neurons at the distal ends of the *etv1* homozygous mutant guts, with the majority of these specific enteric neuronal subtypes localized to the anterior and mid regions of the gut. This observation suggests that the ETV1

transcription factor plays a role in the migration and/or the differentiation of enteric neurons along the length of the gut.

Interestingly, the decrease in *phox2bb*+ enteric cell count in *etv1* homozygous mutants was not paralleled by a proportional decrease in the total count of *tph1b*+ serotonergic enteric neurons. While there was a modest decrease in the serotonergic neurons in the mutants, relative to wildtypes and heterozygotes ($p<0.05$), this reduction was not as pronounced as the decline observed in their total count of *phox2bb*+ cells ($p<0.001$). As a result of these findings, we observed a moderate rise in the percentage of serotonergic neurons in *etv1* homozygous mutants relative to both heterozygotes and wildtypes ($p<0.05$). We cannot assert that the increase in the percentage of serotonergic neurons in the mutants was significant due to two main reasons. Firstly, the p-value is greater than $p=0.001$. Secondly, the sample size is small, with a large standard deviation. In future investigations, obtaining a larger sample size would be essential to improve the reliability of the results and reduce the impact of outliers. Nonetheless, the trend seen in the mutants suggests a differential impact of *etv1* mutation on the emergence of the distinct enteric neuron subtypes during gut development.

It is imperative to acknowledge that second set of embryos stained with the combination of anti-GFP and anti-nNOS antibodies displayed cells emitting only red fluorescence, indicating their identity as nos1+, *phox2bb*- cells (Figure 16). This observation seems logically perplexing because, as depicted in the UMAPs of *nos1* and *phox2bb* (Figure 17), all *nos1*-expressing cells are expected to co-express *phox2bb*. This discrepancy can be explained by the possibility of the anti-nNOS antibody cross-reacting with the other cell-type specific forms of NOS enzyme proteins, like eNOS, that are expressed by other nonneuronal cells of the intestine.

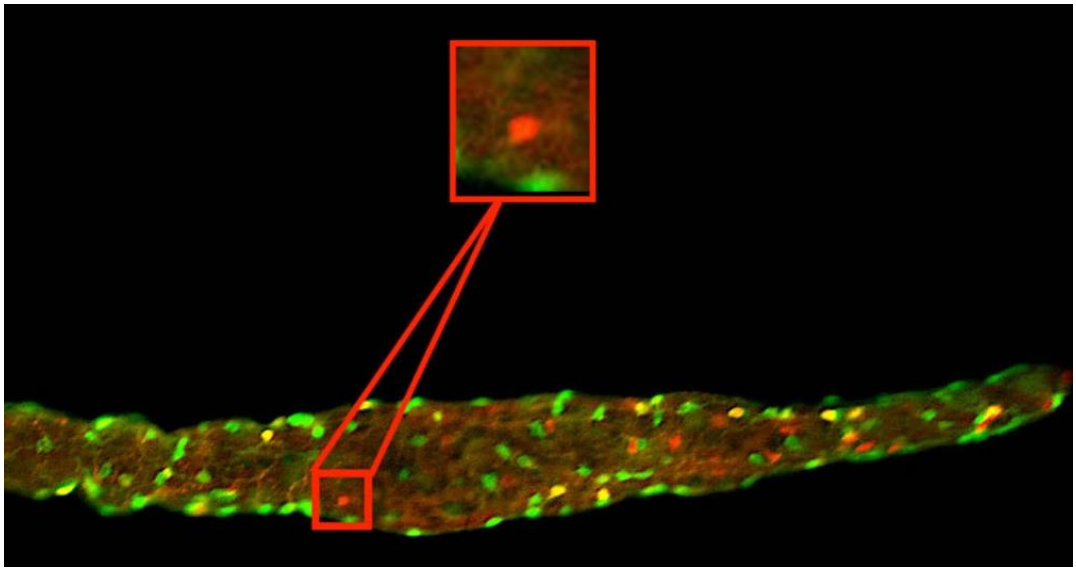

Figure 16: Embryo gut stained with anti-GFP and anti-nNOS antibodies revealing a *phox2bb*- and *nos1*+ cell that only fluoresces red.

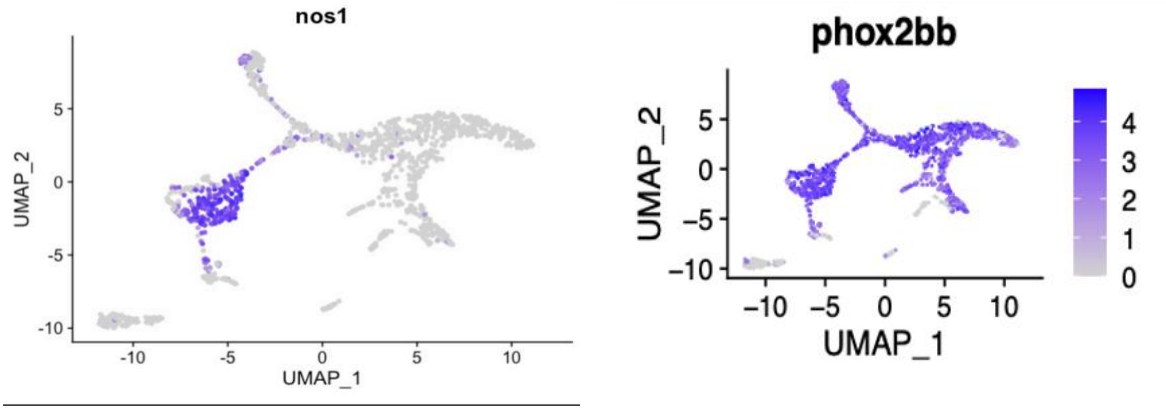

Figure 17: UMAPs of *nos1* and *phox2bb*. All cells expressing the *nos1* gene are enteric neurons also express *phox2bb*. Therefore, we can conclude that the *nos1+*, *phox2bb-* cells are enteric cells that produce other cell type specific NOS enzyme proteins like eNOS that the anti-nNOS antibody cross-reacts with.

etv1 mutants stained with the combination of anti-GFP and anti-nNOS antibodies showed a significant decrease in the total number of *phox2bb*+ cells compared to hets and wildtypes ($p<0.001$). However, they exhibited an even more significant decrease in their nNOS+ neuron

population ($p<0.0001$). These findings explain why we observed a moderate decrease in the percentage of the nNOS+ enteric neurons in the *etv1* homozygous mutants ($p<0.05$).

The moderate increase in the percentage of serotonergic neurons combined with the moderate decrease in the percentage of nNOS+ neurons in the *etv1* homozygous mutants could be explained by examining the UMAPS of the three genes (Figure 18). The *etv1* gene is highly expressed in the inhibitory motor neuron population, so we would expect to see a decrease in this population among *etv1* homozygous mutants. Conversely, the *etv1* gene is not expressed in the serotonergic neurons. The loss of nNOS+ neurons in the mutants might have resulted in the precursor cells that normally give rise to these neurons choosing a different cell fate, potentially becoming serotonergic neurons.[xx] This line of reasoning could explain why we saw a moderate increase in the percentage of serotonergic neurons in the *etv1* mutants.

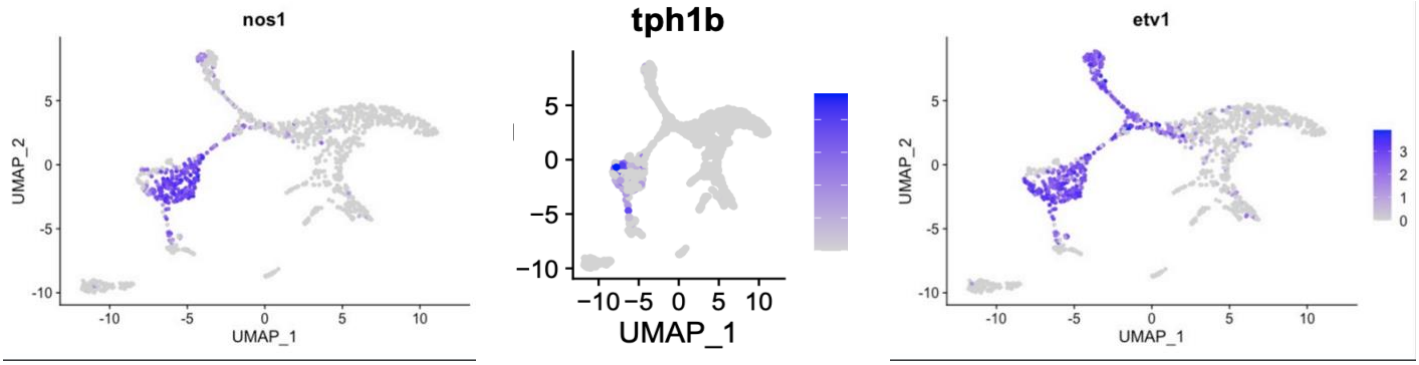

Figure 18: UMAPs of *nos1*, *tph1b*, *etv1*. *etv1* is expressed in the inhibitory motor neurons which also express the *nos1* gene. In *etv1* homozygous mutants we potentially would expect to see a decrease in this population if it was a factor in determining this specific cell's fate. *etv1* is not expressed in the serotonergic neurons which instead express the *tph1b* gene.

Lastly, it's crucial to note that we have identified a smaller than expected number of homozygous *etv1* mutant adult zebrafish. These fish were rare and notably smaller in size compared to their wildtype and heterozygous siblings. Their presence suggests that, despite the compromised ENS,

these zebrafish mutants can survive. This observation isn't uncommon in zebrafish ENS mutants, as other zebrafish with distal gut aganglionosis have also been documented to reach adulthood. For instance, it's been demonstrated that heterozygous *ret* fish, which display a similar distal gut aganglionosis phenotype as the *etv1* homozygous mutants, are capable of surviving to adulthood.[xxi]

Overall, our study provided valuable insights into the molecular and cellular dynamics of enteric neuron differentiation and the impact of ETV1 transcription factor on this process. Future investigations aimed at elucidating the underlying mechanisms driving these observed alterations may offer novel therapeutic targets for gastrointestinal disorders associated with enteric neuron dysfunction.

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