TRANSCRIPTIONAL PROFILING OF MULTITRANSMITER NEURONS IN THE ZEBRAFISH FOREBRAIN

by

DENVER NCUBE

A DISSERTATION

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DISSERTATION ABSTRACT

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Neurotransmitter phenotype is the hallmark of neuronal identity. Neurons are classified based on the neurotransmitters they release. At the inception of the discipline of neuroscience was a fascination with the structure of neurons and morphology of brain tissue. The pioneering work of Santiago Ramon y Cajal (1852-1934) and Henreich Wilhelm Waldeyer (1836-1921) ushered in the famous Neuron Doctrine, which posited that neurons are not only structural units of the nervous system but as trophic, functional, and genetic units. Deciphering the neurotransmitter identity of neurons has been a principal reference point for making interpretations of their function during specific behaviors. In addition, there is a wealth of evidence which shows that neurons can synthesize and release multiple neurotransmitters. However, the development of such neurons and the factors that drive their development has been largely unexplored. Researchers have often narrowed their investigations to establishing the presence of a single neurotransmitter. Mapping these neurons across the vertebrate phyla has not received nearly enough attention though this is conceivably important in understanding common phylogenetic traits. In addition, it is unknown if multi-transmitter neurons attain this identity in a sequential manner or they develop different neurotransmitters at the same time.

To address these questions, I utilized the zebrafish (Danio rerio) as a model system, I characterized a transgenically defined population of multi-transmitter neurons

in the zebrafish forebrain. These neurons which are important in social orienting behavior, synthesize both Acetylcholine and GABA and this study was the first to establish this. My work demonstrates that for this cluster of neurons, the neurotransmitters are co-expressed from the first day of development, and this co-expression persists till adulthood. Further, I identified a constellation of specific marker genes expressed by these neurons, which together enables us to map similar neurons in other vertebrates with great accuracy. I also tested a novel hypothesis on specification of these multi-transmitter neurons by LIM homeobox transcription factor genes. In conclusion, the comprehensive characterization contained this work presents a great platform to tease apart fundamental questions on the impact of different perturbations on the development of neurons.

This dissertation includes previously published and unpublished co-authored material.

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To my wife Nokuthula and my daughter Nandi Nadine Ncube.

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CHAPTER I

INTRODUCTION

The neuron doctrine defines neurons as structural, functional, developmental and trophically independent units (Waldeyer-Hartz, 1891). The view that nerve cells are the building blocks of the nervous system was essentially an application of the Cell Theory to the nervous system (Kuffler and Nicholls, 1976). Neurons principally communicate using neurotransmitters that elicit changes in post-synaptic membranes. For many years it has been thought that neurons release only one neurotransmitter. In the early 1900s the groundbreaking work of Otto Loewi on the autonomic innervation of the frog heart revealed that autonomic nerves released substances that could accelerate or inhibit the heart rate. Later, Henry Dale (1875-1968) helped to identify the released transmitters as noradrenaline and acetylcholine, marking the first time the terms “adrenergic” and “cholinergic” were used in neuroscience. The cumulative of the work of these scientists fundamentally shaped the thinking that neurons release a single neurotransmitter which later turned not to be entirely true.

The “one neuron, one neurotransmitter” doctrine was formalized by John Eccles as “Dales Principle” has been shown to be fundamentally limited depiction of neurotransmitter phenotype. It is important to add that Dale never explicitly dismissed the possibility of neurons releasing more than one neurotransmitter despite this principle being attributed to him. We now know that there are many neurons that can release multiple neurotransmitters across the vertebrate and invertebrate phyla (Avalos and Sprecher, 2021, Broussard J, 2011, Hnasko and Edwards, 2013, Seal and Edwards, 2006). Jan *et a*l.,1982 described multi-transmission in a subset of sympathetic neurons that accompanied the well-established cholinergic transmission. From that time many other studies followed, and this was instrumental in turning the tide on the “one neuron, one neurotransmitter” principle.

To understand the dynamics of neurotransmitter phenotype, we must first consider the synthesis of neurotransmitters. The proteins and enzymes involved in neurotransmitter synthesis brings us a step closer to assigning a definitive identity to specific neurons. Neurotransmitters are classified based on chemical composition/structure, induced postsynaptic responses i.e., excitatory, or inhibitory. Chemical neurotransmitters are synthesized through specific biochemical pathways, packaged into vesicles, and then released into synaptic targets. To a large extent, the synthesis and packaging components are useful in identifying the neurotransmitter phenotype. We can also decipher the mechanism of release of specific based on the presence of specific vesicular packaging molecules (Granger and Sabatini, 2015). Studying the mechanisms of release in multi-transmitter neurons is a daunting task as one must observe specific vesicles releasing neurotransmitters onto their targets and the electrophysiological changes on the post-synaptic membrane (Vaaga *et al.,* 2015).

The realization that there are multi-transmitter neurons in the Central Nervous System (CNS) prompted a debate on how those specific neurotransmitters are a) packaged and b) released. Part of the answer lies in detecting the presence of vesicular packaging proteins or transcripts that code for their biosynthesis in specific groups of neurons. When we consider release of multiple neurotransmitters, there are 2 types of release mechanisms that have been established, namely co-transmission and co-release (Sanders, Granger, Sabatini, 2015). The release of multiple neurotransmitters from a single neuron does not necessarily imply co-release, i.e. Those 2 or more neurotransmitters are packaged into a single population of synaptic vesicles. Co-transmission can be broadly defined as the release of multiple neurotransmitters from non-overlapping pools of synaptic vesicles (Tritsch, Ding and Sabatini, 2012).

In addition, it has been shown that neurotransmitter identity is not static. There is evidence that neurons can change their neurotransmitter phenotype depending on demands placed on the CNS (Spitzer, 2012). Neurotransmitter switching is defined as the gain of one neurotransmitter and loss of another in the same neuron in response to chronic stimulation (Spitzer, 2017). Neurotransmitter receptors on the postsynaptic

membrane change to match the identity of the newly expressed neurotransmitters (Spitzer, 2012). The fundamental point on neurotransmitter switching is that the gained neurotransmitter is not a previously expressed neurotransmitter. Neurotransmitter re-specification can occur during development or in the mature nervous system under the influence of cytokines (Yamamori *et al*., 1989), growth factors (Yang *et al*., 2002; Marek *et al*., 2010; Guemez-Gamboa *et al*., 2014), transcription factors (Demarque and Spitzer, 2010), miRNAs (Dulcis *et al*., 2017), and epigenetic mechanisms (Pritchard *et al*., 2020).

Sensory input can also trigger changes in neurotransmitter profile. In experiments where researchers exposed mice to a 19:5h light: dark cycle stimulated a decrease in the number of immunocytochemically identified dopaminergic neurons in the paraventricular and periventricular nuclei (Dulcis *et al*., 2013). There are postmortem human brain studies drawn from people who would have died in winter or summer specifically comparing differences in the quantity of midbrain dopaminergic neurons. It was observed that the brains of people who died during the winter had a decrease in the number of dopaminergic neurons (Aumann *et al*., 2016). Neurotransmitter switching is believed to confer dynamic and spatiotemporal control over the performance of behavioral tasks. Despite the extensive work that has shown the co-expression of neurotransmitters, the elucidation of developmental mechanisms that drive the specification of such neurons is still missing. There is a gap in our knowledge of the specific genetic programs that drive the developmental trajectory of multi-transmitter neurons. It is also unknown if the multitransmitter phenotype develops in a sequential manner, with neurons starting off as single transmitter neurons and as development ensues assuming additional neurotransmitters. It has also has not been established if the multitransmitter phenotype entails alternate periodic switching.

In this study, I address some of these important questions through transcriptional profiling of a cluster of forebrain neurons which I characterized and determined to be neurotransmitter neurons. Based on the detection of both vesicular transporters of GABA and Acetylcholine, my findings indicate that these neurons are cholinergic-GABAergic neurons, hereinafter referred to as CGNs. The population of multitransmitter neurons in

the zebrafish forebrain in question, had previously shown to be important for social behavior (Stednitz *et al*., 2018). To better understand the dynamics multitransmitter phenotype development, I utilized transcriptional profiling, immunolabelling and in-situ hybridization in larval and adult zebrafish. I established when in development the multi-transmitter phenotype is first detectable in a cluster of forebrain neurons, furthermore I show that the phenotype once established is constant all the way through to adulthood. I also tested a novel hypothesis whether combinatorial expression of three LIM Homeobox transcription factors (LIMTFs) genes is required for the specification of neurons to a multi-transmitter state. In addition, using the combination of LIMTF genes and neurotransmitter markers, we established a transcriptomic fingerprint that allows us to map these neurons in the brains of other vertebrates.

There are many factors that modulate the development and establishment of neurotransmitter phenotype. Microbiota have emerged as potent modulators of neurodevelopment and function. Some clinical studies have revealed altered gut microbiota in patients with neurodevelopmental disorder such as autism (Cryan and Dinan, 2012). Studies have shown that modification of gut microbial communities can influence behavior (Gacias *et al*., 2016). Disruptions in gut microbiota induced by either pathogenic infections or antibiotic treatment have been shown to increase anxiety-like behavior in conventionally raised mice (Bercik *et al*., 2010, 2011; Lyte *et al*., 2006). Bioactive metabolites produced by microbiota have emerged as powerful mediators of gut-brain communication thus revealing that even subtle shifts in gut microbial composition are sufficient to impact brain neurochemistry (Cryn and Dinan, 2012). Understanding interactions between the microbiota and the brain could potentially provide a window into developing therapeutic solutions that can ameliorate neurodevelopmental or behavioral deficits. Considerable strides have been in made in this area with use of fecal transplants from “asymptomatic donors” emerging as part of treatment approaches for autism spectrum disorders and depression (Xiao *et al.*, 2021, Lyu *et al.*, 2021)

Despite a wealth of studies elaborating the extent to which microbiota can modulate brain neurochemistry, there is a gap in our understanding on how microbiota could affect multitransmitter neuron phenotype. The genetic regulation and fate determination of multi-transmitter neurons and how dysbiosis may modulate this is not known. We also identified deficits in social behavior in zebrafish that have been raised germ-free, an observation consistent with similar assays in mice (Desbonnet *et al*., 2014). Given the conservation between fish and mice, it is logical to assume that the same cell type may be mediating social behaviors in humans. Utilized the extensive characterization work we had compiled; I investigated the impact of raising zebrafish in a microbiota deprived condition hereinafter referred to as "germ free"/ GF. We had preliminary data that the GF condition significantly reduced social interaction in zebrafish (Bruckner, 2020) but did not have insight into the exact mechanism that caused this. Through immunolabelling of GABA in forebrain neurons, I show that there is a marked reduction in GABA expression in GF fish. This finding brings us closer to deciphering a possible mechanism through which disruption of gut microbiota impacts brain development and behavior.

This body of work presents data obtained from a cluster of multi-transmitter neurons zebrafish neurons that modulate social orienting behavior. It also explores and leverages the transcriptomic profile of these neurons to construct a reliable mechanism of tracking similar neurons in other vertebrates. In addition, we utilize our knowledge of the unique features of a well characterized cluster of neurons to present data from a study that reveals that raising zebrafish in a GF environment impacts neurotransmitter expression in the forebrain.

CHAPTER II

TRANSCRIPTIONAL PROFILING OF MULTITRANSMITTER NEURONS IN THE ZEBRAFISH FOREBRAIN

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Background

It is a common convention that neuronal subtypes are classified based on a single neurotransmitter they release, and this principle has also been used to predict neuronal function. The limitation of this approach, however, is that it assigns neuronal identity based on the first neurotransmitter to be detected in a neuron. Often the possibility of other neurotransmitters in neurons of interest is not investigated, despite increasing evidence that neurons have the capacity to synthesize and release more than one neurotransmitter (Burnstock *et al*., 2004). The multi-transmitter phenotype has important implications in relation to pharmacological agents that alter the release of neurotransmitters. Some classes of drugs are designed based on a single neurotransmitter released by a group of neurons ignoring other potential neurotransmitters released. Some potential outcomes of such a limited approach are a reduced efficacy of pharmacological agents and an increase in the risk of unwanted effects.

Co-transmission of neurotransmitters is a common phenomenon in which "classical" small molecule neurotransmitters [glutamate, GABA, glycine, acetylcholine (ACh)], purines and monoamines are released with neuropeptides such as somatostatin, neuropeptide Y, substance P and enkephalin (among many others) which slowly alter the properties of target neurons through activation of G-protein coupled receptors (GPCRs). However, many neuronal subtypes have been shown to release multiple classical neurotransmitters (Hnasko and Edwards, 2012). In some cases, the co-released neurotransmitters have similar post-synaptic effects, for example inhibition mediated by GABA and glycine from spinal interneurons (Jonas *et al*., 1998). In other instances, the co-released neurotransmitters might have antagonistic effects, such as GABA and ACh release by mouse striatal neurons (Lozovaya *et al*., 2018). In addition, emphasis must be placed on characterizing neurons using genetic and protein markers that are relevant to the synthesis and packaging of specific neurotransmitters (Rosenmund *et al*., 2015). Using such an approach aids a more accurate identification and characterization of neurons as it establishes the presence of the critical machinery required by neurons to make a specific neurotransmitter.

Deciphering the transcriptional profile of multi-transmitter neurons brings us closer to understanding the genetic mechanisms that regulate their development and, by extension, how their identity influences circuit formation and function. Other researchers in this field have suggested that once neurons have been specified to a particular neurotransmitter identity, they can be re-specified to another identity or express other neurotransmitters in response to electrical activity (Spitzer 2012). Neurotransmitter re-specification can occur during development or in the mature nervous system under the influence of cytokines (Yamamori *et al*., 1989), growth factors (Yang *et al*., 2002; Marek *et al*., 2010; Guemez-Gamboa *et al*., 2010; Guemez-Gamboa *et al*., 2014), transcription factors (Demarque and Spitzer, 2010), miRNAs (Dulcis *et al*., 2017), and epigenetic mechanisms (Pritchard *et al*., 2020). Here we describe a population of multi-transmitter neurons that are specified as such during embryonic development and retain that identity through adulthood.

In this study, we present transcriptional profiling data on a cluster of multi-transmitter neurons which modulate social behavior. We previously showed that these neurons were cholinergic (Stednitz *et al*., 2018), but here we combine transcriptomic, in-situ hybridization and immunohistochemistry data to show that these neurons are also GABAergic. We determined the timepoint that this neurotransmitter identity becomes detectable in the forebrain and that it was not sequential. We also determined a combination of LIM Homeobox transcription factor genes that are reliable markers of these neurons in larval and adult life. Our transcriptomic data is corroborated by in-situ hybridization and provides a "transcriptomic fingerprint" for this cluster of forebrain neurons which we utilize to identify homologous clusters of neurons in rodents. This homology supports the conclusion that this cluster of neurons is evolutionarily conserved and will be useful to determine if the anatomical and transcriptional convergence entails functional similarity.

Results

vTel^{y321} transcriptome suggests a multi-transmitter neuronal identity

Manual and chemogenetic ablations of vTel^{y321} neurons in a previous study revealed a marked disruption of social orienting behavior in zebrafish (Stednitz *et al*., 2018). vTel^{y321} neurons are transgenically defined by the enhancer trap insertion *Et(rex2-scp1:gal4ff)y321: UAS;GFP)* (Fig1a) and includes the Vv and Vd nuclei of the zebrafish telencephalon. To better understand the molecular identity of this population of neurons, we dissected and dissociated heads of 7-day post-fertilization (dpf) larvae to isolate as much of the forebrain as possible (Fig. 1A). We then used Fluorescence Activated Cell Sorting (FACS) to separate GFP positive and negative cells. After dissociation, FAC Sorting was performed on each sample yielding at least 100000 sorted cells which were then pooled together and sequenced. 80-90% of dissociated cells were live (Fig. 1A) and 3-6% of these cells were GFP positive (Fig. 1A). We expected the proportion of GFP positive cells to be small (3-6%) because the vTel^{y321} cell cluster is relatively a small portion of the forebrain totaling about 200 neurons at this age (Bruckner, 2020).

We selected for protein-encoding genes via Ensemble and compiled gene lists of differentially expressed genes ordered by p-adjusted values. An analysis of the data through principal component analysis determined that one sample from the GFP negative fraction was an outlier (Fig.1C). This sample was dropped leaving us with 3 GFP positive samples and 2 GFP negative samples. Sorting for genes with a p-adjusted value ≤ 0.05, we obtained a list of 2096 genes that were differentially expressed (DE) in the GFP positive neurons relative to GFP negative cells out of a total of ~23000 genes (9%).

To refine our characterization of vTel^{y321} neurons, we focused on genes documented to be expressed in the forebrain (zfin.org), compiling a list of 454 genes (21.6% of the 2096 DE genes). When we reviewed the top 20 differentially expressed genes from the entire dataset and the top 20 forebrain genes (Fig. 1D-E), we discovered that the LIM transcription factor (LIMTF) encoding gene *lhx8a* (Fig. 1C) was highly enriched in the GFP positive population of neurons. We expected to observe significant enrichment of *lhx8a* in GFP positive neurons since the enhancer trap *Et^{y321}* was within the *lhx8a* gene locus Zebrafish Bain Browser, zbbrowser.org). The rodent homologue of this gene, *lhx8*, has been shown be required for development of cholinergic neurons in the rat forebrain (Zhao, 2003). Our in-situ hybridization confirmed detection of gene transcripts for cholinergic markers such as the vesicular acetylcholine transporter (*vachtb*) (Fig. 2B) and choline acetyltransferase (*chatb*) (Fig. S1). In addition, we also found significantly high differential expression of *slc5a7*, encoding the choline transporter, further validating the cholinergic identity for vTel^{y321} neurons. We also compiled a list of neurotransmitter markers that were expressed and established which ones had significant differential expression (Table 1). The basis of the analysis was a comparison of genes that were upregulated in the vTel^{y321} neurons relative to GFP negative neurons. Table 1 shows that other neurotransmitter markers for glutamate and glycine were also significantly highly expressed in the vTel^{y321} neurons. Neurotransmitters that had a negative Log_2Fold change and a p-adj<0.05 were significantly enriched in vTel^{y321} neurons and those that had a positive Log_2Fold change and a p-adj<0.05 were significantly downregulated. It is apparent from Table 1 that there was significant upregulation of some cholinergic and GABAergic markers compared to other neurotransmitter markers. This raised an

interesting scenario where an inhibitory (GABA) and excitatory (ACh) neurotransmitter were co-expressed in vTel^{y321} neurons. There was need to confirm if this was the case.

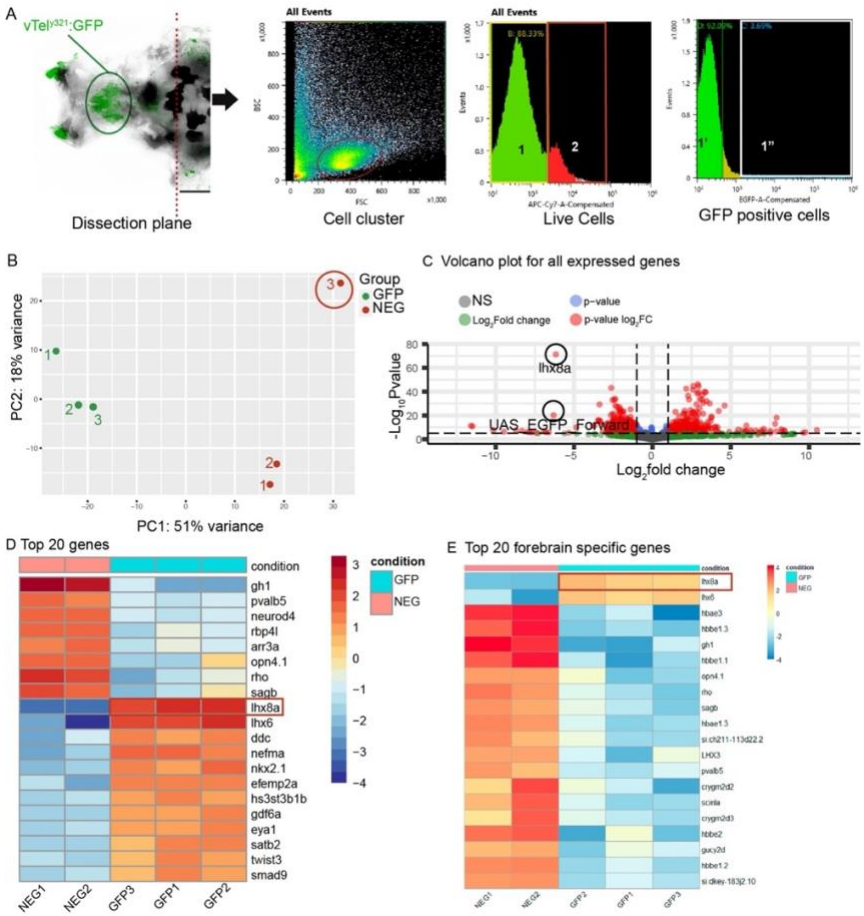


Fig. 1 vTel^{y321} transcriptome suggests a multi-transmitter neuronal identity. **A** Dorsal view of transgenic expression of vTel^{y321} expression in the forebrain and the dissection plane used. Scale bar represents 100µm. The Cell Cluster panel shows flow cytometry isolation of GFP positive neurons (circled) after dissociation. Live Cells panel shows 2 partitions, 1 is the proportion of live cells (88.33%) and 2 is the proportion of dead cells. In 1" (GFP Positive Cells panel), approximately 3.7% of living cells were GFP positive cells and 88% were GFP (1') negative cells. **B** Principal component analysis of GFP positive and negative samples after variance stabilization transformation(vst) of count data. Sample 3 in the GFP negative fraction was dropped from consequent analysis because it was an outlier. **C** Volcano plot comparing the log_2fold change and -log_{10}P of all the individual genes(n=22999) genes. Gray dots – non-signifcantly expressed genes. Red dots- significantly upregulated or downregulated genes depending on where they fall on plot. Red dots in the left partition show singificant padj plus upregulation in GFP positive cells. The blue dots indicate significant p-adj expression but (padj < 0.05**. D** Heatmap of the top 20 genes with the highest differential expression between the GFP positive and GFP negative samples. **E** Heatmap of the top 20 forebrain specific genes with the highest differential expression between the GFP positive and GFP negative sample

Table 1: Neurotransmitter makers expressed in vTel^{y321} neurons

Symbol	p-adj	Gene name	Neurotransmitter
slc17a7a	5.25E-10	*vglut1*	Glutamate
slc17a6b	0.13842785	*vglut2*	
slc17a6a	0.97372783	*vglut2.2*	
slc1a2b	5.99E-12	*EAAT2 Glutamate transporter*	
slc1a2a	0.07570601	*EAAT2b*	
slc18a3b	0.52629943	*vachtb*	Acetylcholine
Slc5a7	0.00040338	*Choline transporter*	
slc18a3a	0.99634064	*vachta*	
chata	0.19918698	*chata*	
slc18a2	0.8983514	*vmat2*	Dopamine
slc6a3	0.10773861	*DAT*	
slc6a2	0.68157586	*NE transporter*	Norepinephrine
slc32a1	0.00039023	*vgat*	GABA
gad1b	0.11640804	*gad67b*	
gad2	6.19E-06	*gad65*	
gcat	0.58087894	*glycine C-acetytransferase*	Glycine
slc6a9	0.00145337	*glycine transporter 1(glyt1)*	
slc6a5	0.01674848	*glycine transporter 2(glyt2)*	

vTel^{y321} neurons are cholinergic and GABAergic neurons (CGNs)

To determine if vTel^{y321} neurons are cholinergic-GABAergic neurons (CGNs), we combined GABA immunostaining and in-situ hybridization for *vachtb*. We found that vTely^{321} neurons are both cholinergic and GABAergic (Fig. 2B) with expression of both

chatb (Supplementary Fig 1) and *vachtb* overlapping with GABA staining (Fig2B). To validate our immunostaining, in-situ hybridization with *gad1b* and *gad2* anti-sense oligonucleotide probes revealed more than 90% overlap with GABA antibody staining (Supplementary Fig 2). We also confirmed the specificity of the antibody by testing it in the spinal cord in wholemount larvae and sections (Supplementary Fig 3). Approximately 92% (1183/1287) of these were GABAergic and 85% (1093/1287) were cholinergic. We then analyzed the number of neurons that are both cholinergic and GABAergic, which we will call CGNs. At the selected anatomical landmarks (see methods) we found that 91.7 ± 4.9 % (670/733) of vTel^{y321} neurons in the RT are CGNs (Fig2d). At the MT position, 84.5 ± 9.31% (181/215) neurons were CGNs. Generally, the total number of vTel^{y321} neurons decreases in the rostro-caudal direction. In the RT and MT, vTel^{y321} neurons are largely located in the Vv and Vd though some are in the dorsal portion/pallium. The average proportion of vTel^{y321} neurons that are CGNs in the caudal telencephalon was 66.7 ± 33.5% (193/339) (Fig2d). We conclude that a large proportion of the vTel^{y321} cells are CGNs, although the population is presumably heterogeneous with the proportion of CGNS decreasing towards the caudal part of the nucleus. There was need to establish if the vTel^{y321} multi-transmitter phenotype was attained in a sequential fashion, where the neurons first develop as single neurotransmitter neurons later expressing an additional neurotransmitter or they are multi-transmitter neurons at the point of specification. It was also important to establish if there was a point during vTel^{y321} neuron development where the neurons would express only one of the GABAergic or cholinergic markers after having expressed both initially.

The CGN phenotype is constant throughout development

To determine the developmental dynamics of the CGN phenotype, we in-crossed 1 month old *Et^{y321}* [*Et(rex2-scp1:gal4ff)y321*] fish and raised the resultant F1 embryos to 7 dpf. We split the embryos into 7 batches, each batch raised to separate ages starting from 1dpf to 7dpf. The 1dpf batch was further split into 3 subgroups, namely pre-19 hpf group for embryos between 16 to 19hpf, pre-22hpf(20-22hpf) and the last one being the 24hpf group. To determine the earliest timepoint when vTel^{y321} neurons became CGNs,

we then set up a series of experiments with 16 hpf to 7 dpf old larvae and performed serial immunohistochemistry assays for cholinergic and GABAergic markers.

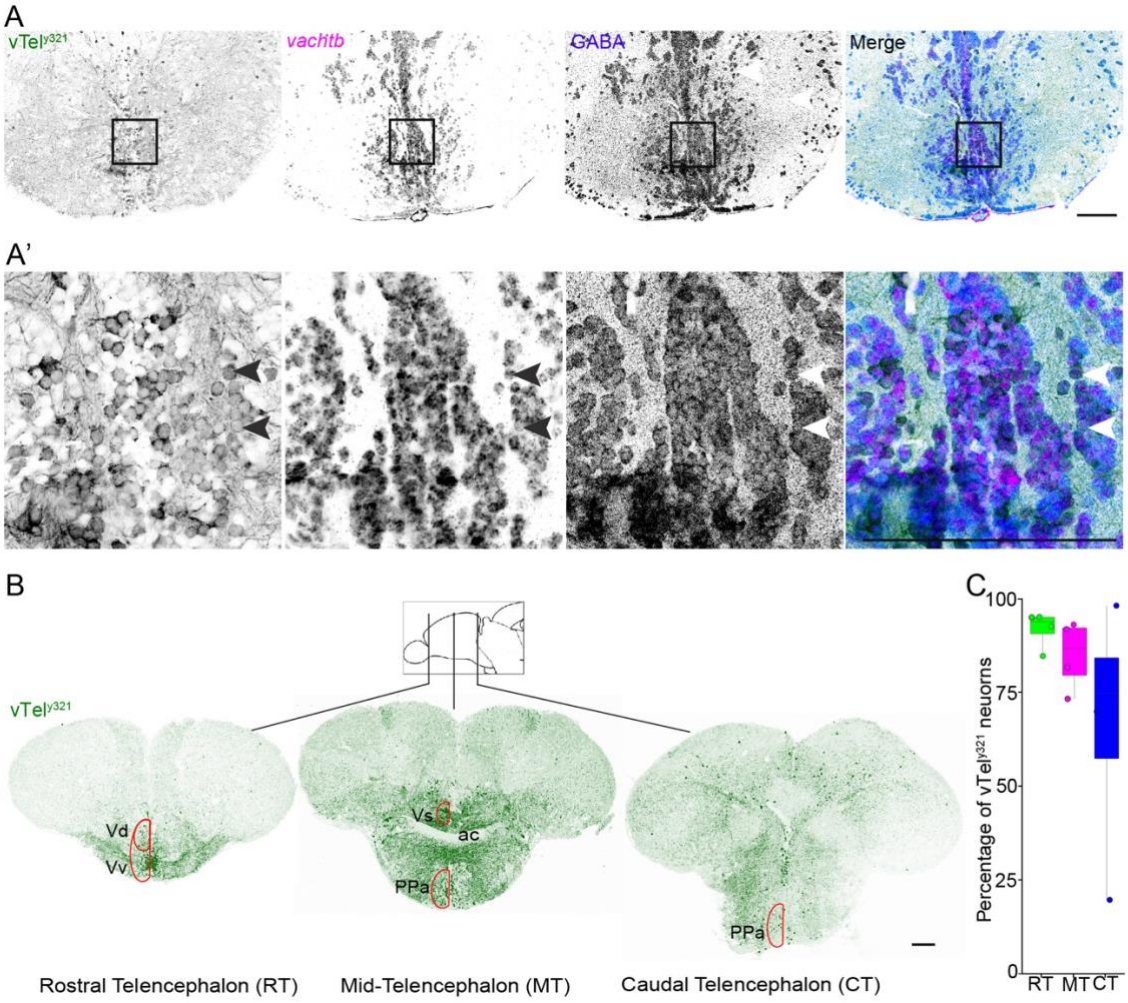


Fig. 2 vTel^{y321} neurons are cholinergic and GABAergic. **A** Coronal cross section of the adult ventral forebrain in the rostral telencephalon showing the expression of the transgene marking out vTel^{y321} and expression of vachtb and GABA. **A'** Row of selected portion of each image from A. Arrows in insert A' point to selected vTel^{y321} neurons that express vachtb and GABA (Scale bar in A and A' represents 100µm). **B** Coronal cross sections of the ventral forebrain depicting known nuclei in the forebrain that include vTel^{y321} neurons and anatomical landmarks used for quantification of vTel^{y321} CGNs (RT-Rostral Telencephalon, MT-Mid-Telencephalon, and CT-Caudal Telencephalon) (scale bar represents 100µm) Vd: ventral dorsal nucleus, Vv: Ventral ventral nucleus, Vs: supracommisural nucleus of ventral telencephalon, Ppa: parvocellular preoptic area, ac: anterior commissure **C** Quantification of the percentage of adult vTel^{y321} neurons that are cholinergic and GABAergic neurons (CGN) at selected anatomical landmarks in the subpallial telencephalon.

These serial experiments enabled us to determine that the transgene begins driving GFP expression in vTel^{y321} neurons at 24 hpf. This was also earliest timepoint we could detect the CGN identity (Fig3A-A"). vTel^{y321} neurons maintained their CGN

identity into adulthood. If the CGN phenotype was attained in a sequential manner, we expected to see varying proportions of vTel^{y321} neurons that were CGNs. This would mean that in the early stages of post-fertilization, we would have more single neurotransmitter neurons and then as time went on, an increase in the number of CGNs. In addition, since the vTel^{y321} nucleus runs in the rostro-caudal direction, were there specific positions in the forebrain where there were more single neurotransmitter neurons than double and at what stage? We quantified the proportion of vTel^{y321} neurons at 2 and 4dpf to determine if the proportion of vTel^{y321} neurons that are CGNs changes across development (Fig3C-3D). Interestingly, quantification of CGNs at 2 and 4 dpf recapitulates the trends we observed in the adult forebrain with increased numbers in the rostral region of the telencephalon and a gradual reduction in the caudal region of the telencephalon (Fig 3B). At least 90% vTel^{y321} neurons at the RT position in 2 and 4 dpf fish are CGNs. At the MT position, there is a small increase in the percentage of CGNs at 4dpf. The CT position at 2dpf had the greatest variation. When we examined the number of GFP positive neurons that expressed a single neurotransmitter we found that approximately 1% of vTel^{y321}neurons expressed a single neurotransmitter marker and in both 2dpf(9/700) and 4dpf (12/1157) fish and the single neurotransmitter vTel^{y321} neurons were all ChAT positive.

LIMTF gene expression precedes neurotransmitter marker expression in vTel^{y321} neurons

Two LIMTF genes, *lhx6* and *lhx8a*, are highly expressed in vTel^{y321} neurons (Fig. 1D-E). These genes show the highest differential expression between GFP and non-GFP positive neurons. In-situ hybridization with anti-sense ribo-probes for these genes showed that they are both expressed in adult vTel^{y321} neurons. To determine when these transcription factors were first expressed, we in-crossed 1 month old *Et^{y321}* fish and split the clutch of F1 embryos from this cross into 7 groups that corresponded to specific timepoints in the 7day period that is, 24hpf, 48hpf, 72hpf and so on up to 7dpf. The 24hpf group of embryos was further subdivided into pre- and post-24hpf. The pre-24hpf portion was split into 3 groups which denoted the timepoints that we would take out embryos euthanize and section them for experiments.

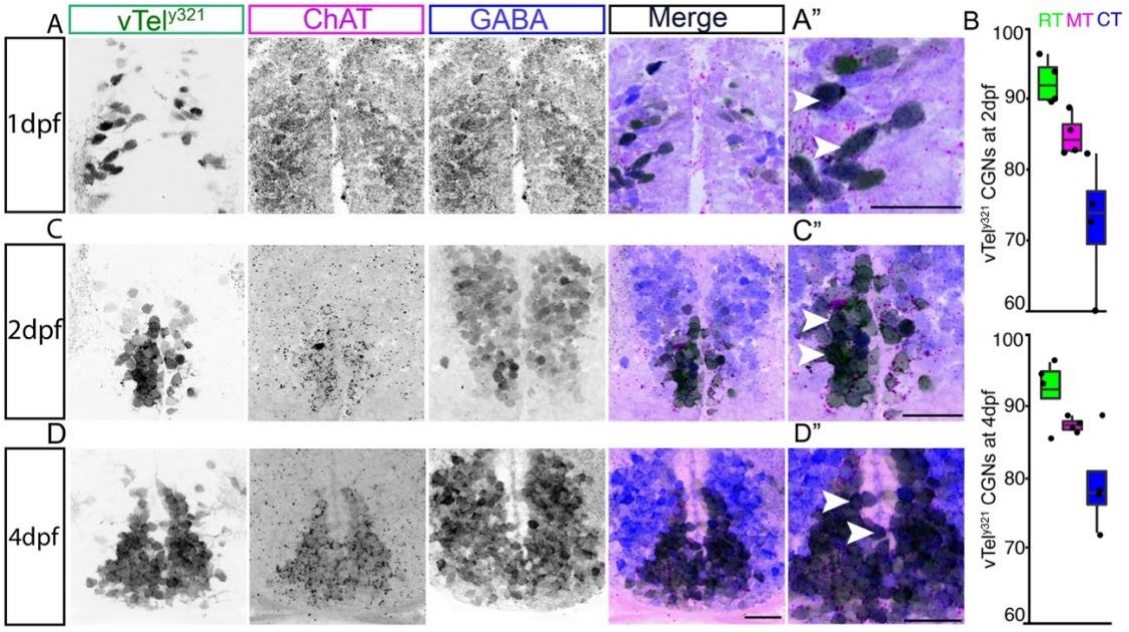


Fig. 3 CGN phenotype is constant throughout development. **A** Transverse section of 1 dpf fish showing expression of cholinergic and GABAergic markers in vTel^{y321} neurons. **A"** Insert of merge between GABA and ChAT expression at 1dpf. Arrows point to selected vTel^{y321} neurons that are CGNs. (Scale bar represents 20µm). **B** Boxplots of the percentage of vTel^{y321} neurons that are CGNs at 2 and 4dpf. **C** Coronal section of the zebrafish subpallial(ventral) forebrain in the rostral telencephalon at 2dpf. **C"** Insert with arrows depicting selected vTel^{y321} neurons that are CGNs. (Scale bar represents 20µm). **D** Forebrain coronal section at 4dpf depicting vTel^{y321} neurons that are CGNs. **D"** Insert from 4dpf forebrain with selected vTel^{y321} neurons that are CGNs shown with arrows.

We then set up a series of experiments with 16-24 hpf old larvae and performed in-situ hybridization and immunohistochemistry to detect expression of LIMTF genes and cholinergic and GABAergic markers in the forebrain. The earliest timepoint at which we were able to detect LIMTFs was approximately 20 hpf (Fig. 4B) and we observe that at this stage, *lhx6* and *lhx8a* expression in the forebrain is restricted to four principal clusters. These clusters have previously been described for *lhx8a* as telencephalic and diencephalic clusters (Thisse *et al.*, 2001). It is likely that these clusters give rise to the population of neurons later marked by the transgene vTel^{y321}. There is evidence that during development, another LIMTF gene, *isl1*, is expressed in the ventral floor plate region of the telencephalon (Wullimann, 2019). In addition, since *isl1* expression is typically used as a marker for cholinergic neurons (Cho *et al*., 2014) and we know that vTel^{y321} neurons were cholinergic, we asked whether *isl1* expression is detectable in vTel^{y321} neurons, and if so, when. *isl1* expression was detected at 20 hpf in the forebrain of *Et^{y321}* fish in the same clusters (Fig4B-4C) as *lhx6* and *lhx8a*. Our experiments

established that the expression of LIMTF genes precedes expression of cholinergic and GABAergic neurotransmitter markers in vTel^{y321}. We also established that this expression is constant throughout development and can also be detected in vTel^{y321} neurons in the adult forebrain (Fig4A). Together these observations indicate that the 3 LIMTF genes and the neurotransmitter markers were reliable in the identification of vTel^{y321} neurons and that they are expressed throughout the life of those neurons beginning at the end of the first day of development.

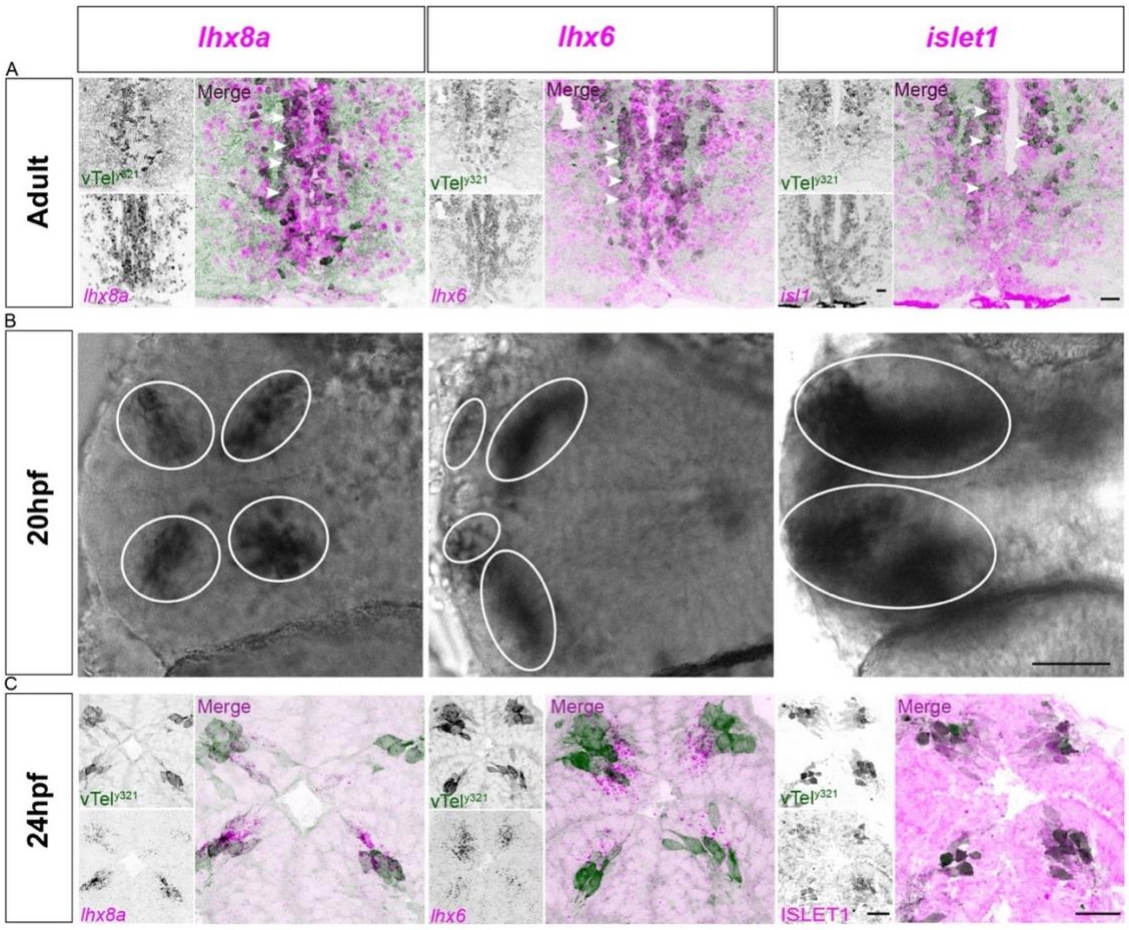
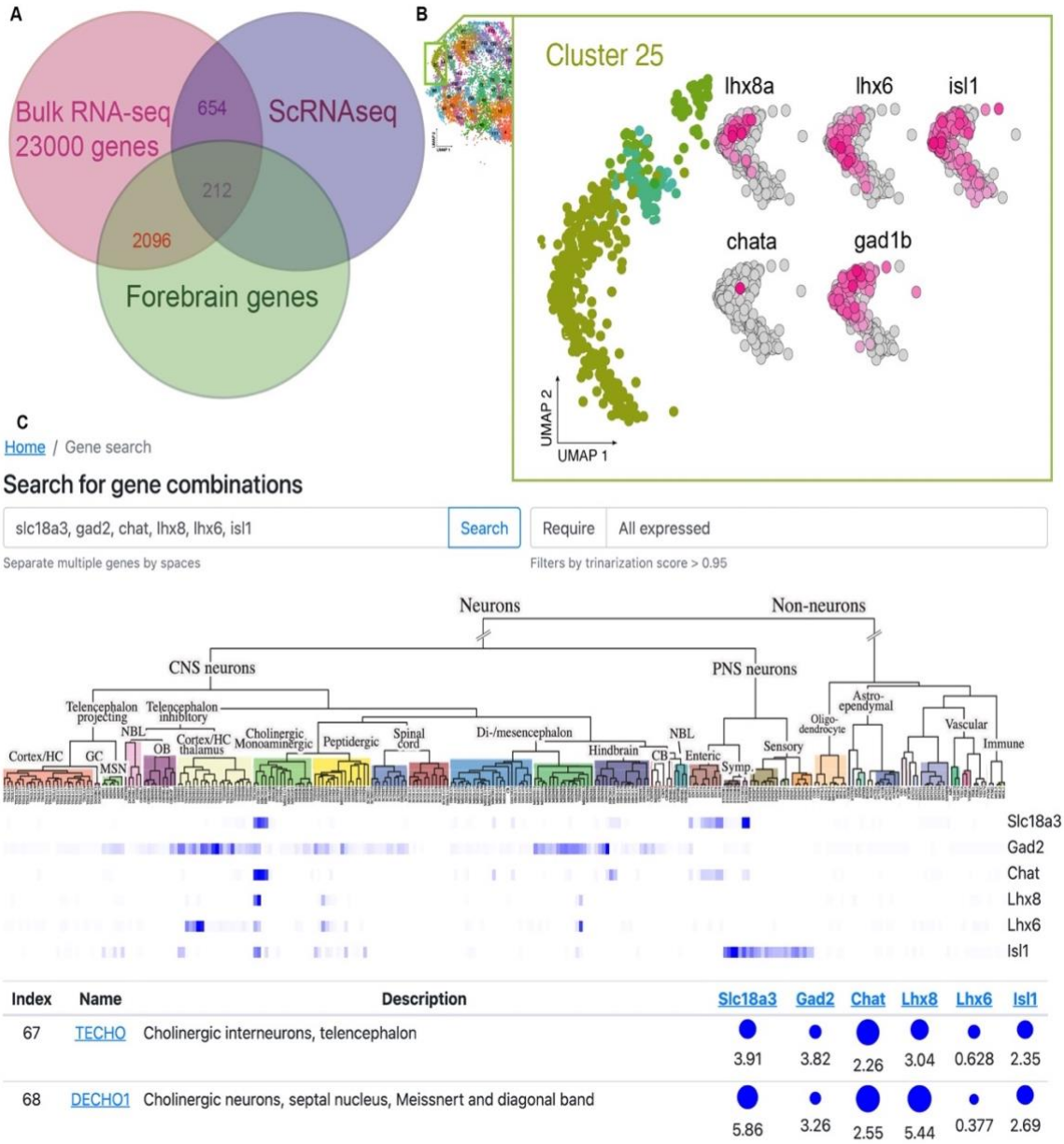


Fig. 4 LIMTF genes are expressed throughout development. **A** Coronal cross section of the ventral forebrain showing, from left to right, expression of lhx8a, lhx6 and isl1in adult vTel^{y321} neurons (select neurons marked by arrows). (Scale bar represents 20µm). **B** Wholemount chromogenic in-situ hybridization in 20 hpf fish. Circles label the principal foci of LIMTF gene expression in the brain. Rostral circles denote diencephalic clusters and caudal circles denote telencephalic clusters (from left to right) respectively at that timepoint (scale bar represents 20µm). Islet1 is broadly expressed as compared to lhx8a and lhx6. **C** 24 hpf larval forebrain transverse sections showing LIMTF gene expression in vTel^{y321} neurons. Detection of *lhx6* and *lhx8a* was done through in-situ probes and an Islet1 antibody was used to detect the homeodomain protein ISELT1 (scale bar represents 20µm).

The vTel^{y321} "transcriptomic fingerprint" is evolutionarily conserved

We were interested in understanding whether there is a similar cluster of neurons in other vertebrates and the best method to do this was to define transcripts expressed in vTel^{y321} neurons which conferred them with a unique transcriptomic identity. To this end, we used the expression of the three LIMTF genes and three neurotransmitter marker genes, *gad2*, *vacht (SLC18a3)* and *chat.* First, we compared our bulk RNAseq-data with single cell RNA-sequencing data from Farnsworth *et al*. (2019) drawn from pooled data from 1, 3 and 7 dpf larvae which was later compiled into the UCSC based Atlas of Zebrafish Development. Our dataset shared 654 genes with the single cell sequencing atlas, and this served as additional validity for our data. 212 genes out of the 654 shared genes were classified as forebrain specific genes using the Zebrafish Information Network classification (Fig. 5A). Considering the commonly expressed genes between the two datasets and buttressed by data from our in-situ hybridizations, our population neurons mapped onto Cluster 25 of the scRNA-seq Atlas browser. We turned our attention to some of the key genes in our proposed transcriptomic fingerprint, such as the LIMTF genes, and found that all 3 transcription factor genes are significantly highly expressed in Cluster 25. Interestingly, the ohnologue for *lhx8a, lhx8b* was also highly expressed in the shared cluster. The data also reveals high expression of GABAergic neurotransmitter markers such as glutamate decarboxylase (*gad2),* vesicular GABA transporter (*vgat),* and the *GABA Transporter 1* (*gat1*). Our list of genes did not include the cholinergic specific genes *vachtb* and *chatb* despite having detected them through in-situ hybridization, but there was low expression of *chata* across Cluster 25. Using the expression of the three LIMTF genes (*lhx6, lhx8, islet1*) and neurotransmitter marker genes (*gad2, vacht, chat),* we searched for this population of neurons in mice using the Linnarsson Lab online atlas (http://mousebrain.org/genesearch.html). The Linnarsson Lab online atlas consists of 265 clusters segregated based on expression of tissue specific transcripts. Using the online atlas, we located a similar cluster of neurons in the telencephalon and diencephalon (Fig5C) denoted TECHO and DECHO. This cluster correlates well with the data we obtained from 24 hpf larvae where the LIMTF genes are expressed in two clusters, telencephalic and diencephalic (Fig4C).



Index	Name	Description	Slc18a3	Gad2	Chat	Lhx8	Lhx6	Isl1
67	TECHO	Cholinergic interneurons, telencephalon	3.91	3.82	2.26	3.04	0.628	2.35
68	DECHO1	Cholinergic neurons, septal nucleus, Meissnert and diagonal band	5.86	3.26	2.55	5.44	0.377	2.69

Fig. 5 vTel^{y321} "transcriptomic fingerprint" is evolutionarily conserved. **A** Schematic Venn diagram with the approach we used to obtain our "transcriptomic fingerprint." Comparison of commonly expressed genes between our bulk RNAseq data and single cell RNA-seq data from UCSC Cell Browser Atlas of Zebrafish Development was further analyzed against ZFIN criteria for forebrain specific genes. From the list of 212 genes that met the forebrain expression criteria we further narrowed the search in the Atlas to specific marker genes that constitute the "transcriptomic fingerprint." **B** Single cell RNA-sequencing data from Atlas of Zebrafish Development plotted using Uniform Manifold Approximation and Projection (UMAP) algorithm. "Transcriptomic fingerprint" expressed genes map onto Cluster 25 of the Atlas and their expression is plotted in space .**C** Search result from the Linnarsson Lab online Atlas shows that combined expression of slc18a3 (vacht), gad2, lhx6, isl1, lhx8 and Chat maps onto telencephalic and diencephalic clusters in the mouse brain.

***lhx8a* and *lhx6* mutants express GABAergic and cholinergic markers**

The rodent homologue of zebrafish *lhx8a*, *Lhx8* has also been shown to be required for GABAergic neuron specification when it is co-expressed with another LIM homeobox transcription factor *lhx6*. We confirmed that these neurons express *lhx6* and *islet1(*Fig 4). Data from rodents show that combinatorial expression of *lhx8* and *lhx6* specifies neurons to a GABAergic fate, whereas *lhx8* co-expression with *islet1* specifies neurons to a cholinergic fate (Fig 6A). Given the above we proposed a possible model on how combinatorial expression of LIMTF transcription factor genes could specify vTel^{y321} neurons to a cholinergic and GABAergic fate (Fig 6B). Our model proposes that the expression of all 3 transcription factors would result in a CGN fate. Conversely, by mutating *lhx8a* the expectation would be that vTely321 neurons would not be specified to a CG fate. We expected that knocking out *lhx6* gene would disrupt GABAergic development**.** Since islet1 is an important gene for heart and CNS development, to maintain viability of embryos we chose to utilize *lhx6* and *lhx8a* mutant fish which were viable.

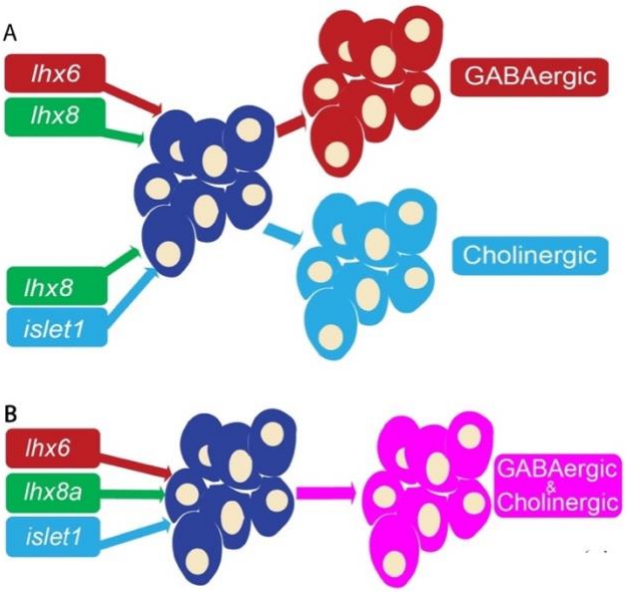


Figure 6 Cholinergic and GABAergic neuron specification through LIMTF genes. **A** Rodent specification of GABAergic and cholinergic neurons (Zhou, 2015). **B** Our hypothetical model proposing that combinatorial expression of LIMTF genes is required for cholinergic-GABAergic neuron specification.

We received double mutants and heterozygotes for *lhx8a* and *lhx6* from Dr Lindsay Barske and genotyped the main stock before outcrossing to AB/Tübingen zebrafish adults to obtain heterozygotes for each allele. F1 heterozygotes for *lhx6* and *lhx8a* were raised to adult stage (1-2 months) and then in-crossed to generate mutants, heterozygotes, and wildtypes for each gene. These F2 animals from the heterozygous in-cross were then euthanized, genotyped and cryosectioned prior to immunostaining for GABA and in-situ hybridization for *chatb* transcripts. From our hypothesis, *lhx6* mutations would result in disruption of

GABAergic specification. However, our data shows that GABA expression was retained in the forebrain even in *lhx6* mutants and heterozygotes. There was no difference between mutants and wildtype animals with respect to GABA expression (Fig7 B). In line with our hypothetical model, mutations to the *lhx8a* gene were expected to disrupt both cholinergic and GABAergic specification. Our results show that GABAergic and cholinergic markers are still expressed in *lhx8a* mutants. (Fig 7A1-A2).

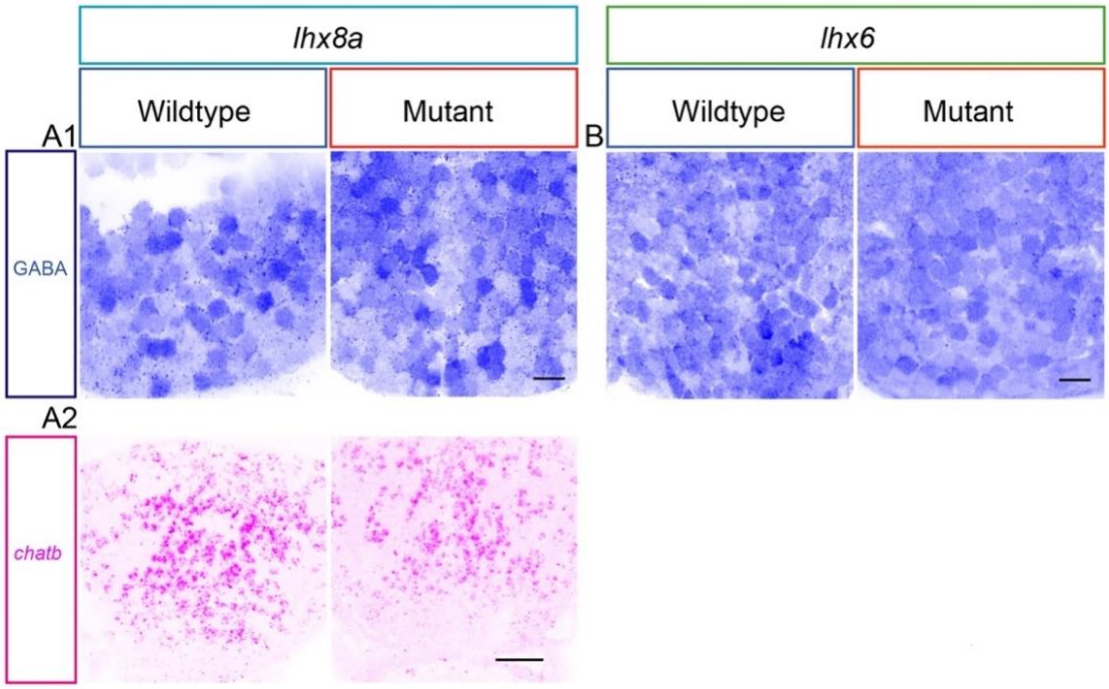


Figure 7 lhx8a and lhx6 mutants retain expression of GABAergic and cholinergic markers. A1-A2 From our hypothesis lhx8a mutations disrupt GABAergic and cholinergic specification, however we still observe GABA expression in mutants at the same levels as wildtype. A2 chatb transcripts detected in the forebrain of both mutants and wildtype lhx8a fish. B. There is no difference in lhx6 mutation in Wildtype and Mutant fish. (Scale bar A1=20µm, A2= 20µm, B = 20µm)

Discussion

We discovered that neurons that define a telencephalic nucleus required for social orienting behavior in zebrafish co-express ACh and GABA. Spitzer *et al.* (2020) suggested that activity dependent neurotransmitter switching may involve turning on transcriptional programs that result in the synthesis of additional neurotransmitters in response to behavioral demands. However, in contrast to Spitzer's studies, we found is that the neurotransmitter fate for the cluster of neurons we defined is constant throughout

development. It is unknown whether this is an indication of the importance of the role of these neurons play in social behavior or other functions.

We determined cholinergic identity using three principal markers, *chatb* which encodes the Choline Acetyltransferase enzyme (ChAT), *vachtb* and the immunostaining for the enzyme ChAT. For GABA we used both *gad2*, and *gad1b* as well and antibody against GABA and the RNA-seq data revealed high expression of the vesicular GABA transporter, *vgat*. Confirmation of the presence of gene transcripts and protein products eliminates the possibility of a situation where the transcripts are not translated. Given the presence of both vesicular transporters in this cell cluster, it might be that the neurotransmitters are packaged separately for eventual release to the same or separate targets, i.e., they are highly likely to be co-transmitted than co-released. Co-transmission of neurotransmitters occurs when neurotransmitters are released from separate vesicular pools either into the same or separate synaptic cleft(s). (Vaaga *et al*., 2015, Granger and Sabatini, 2015). A logical next step would be to investigate the post-synaptic targets that these neurons project to which would reveal the specific roles these neurons play in the circuitry underlying social behavior and how they impact the function of other neuronal populations in the brain. Neurotransmitters act as morphogens during development as well as influencing neuronal plasticity. Determining the post-synaptic targets of this cluster of neurons will be important in studying how multi-transmitter neurons shape circuit formation and plasticity. Lozovaya *et al.* (2018) describes a population of CGNs in the mouse striatum which coincidentally express *lhx8* and *lhx6* and he suggests that similar neurons might act as pattern generators with alternate release of excitatory and inhibitory neurotransmitters. We speculate that the presence of separate vesicular packaging systems for GABA and acetylcholine in vTel^{y321} neurons could support a mechanism by which there is alternate release of acetylcholine and GABA. Co-transmission and corelease of neurotransmitters likely serve an important role in the optimization and maintenance of spatio-temporal patterns of neurotransmission which is critical for refinement of behavior. It would be interesting to establish the dynamics of neurotransmission of vTel^{y321} neurons in relation to their function in social behavior. One of the remarkable features of the nervous system is its plasticity, which the lifelong

capacity to change and adapt in light of intrinsic or extrinsic stimuli (Bertuzzi, Chang and Ampatzis, 2018). It is an open question how the multi-transmitter neuron phenotype influences plasticity and how this is altered in disease states of the brain.

Our in-situ hybridization experiments served as an additional check for RNAseq data. To illustrate this, we consider the absence of vachtb and chatb in both the Bulk and scRNA-seq data. These two genes are not included in the list of 212 overlapping forebrain genes between the 2 datasets. Though the *vachtb* and *chatb* genes were not included in the list of overlapping genes between our bulk RNA-seq data and scRNA-seq data we confirmed their expression through in-situ hybridization. A plausible explanation for this is that these transcripts are also highly expressed outside the vTel^{y321} cluster, thus the statistical comparison of average expression between GFP positive and non-GFP cells will not meet the threshold of statistical significance. *vachtb, vachta* and *chata* are present in the raw data but due to a stringent p-adjusted cutoff threshold ($\leq$0.05) they are not present in the final compilation of the data (Fig1E). The second reason is that we used *vachtb* and *chatb* transcripts which are based on a second cholinergic gene locus only described by Hong *et al*., 2014 thus the genes are yet to be fully annotated. It is also possible that the single cell RNAseq data misses a significant number of cells too.

Expression of the three LIMTFs (*lhx6, lhx8, isl1*) and *nkx2.1* marks the medial ganglionic eminence (MGE) in the developing rodent brain. The MGE is an important proliferative zone that generates neurons which migrate throughout the brain and into surrounding cortical areas. It is still an open question whether vTel^{y321} cluster would correspond in part to the rodent MGE though the similarity in gene expression suggests that to be the case. The convergence between zebrafish adult and larval gene expression data and the mouse brain (mousebrain.org) browser data indicate that this population of neurons is phylogenetically conserved. The next point of enquiry would be to determine if the transcriptional convergence translates to a similarity in function. In the rostral telencephalon, vTel^{y321} neurons are largely located in the Vv and Vd, though some are in the dorsal portion/pallium and this phenomenon applies to the mid-telencephalic region, especially the parvocellular preoptic nucleus and supracommisural nucleus regions. Other

notable nuclei in the telencephalon that are cholinergic and GABAergic is the entopeduncular nucleus dorsal and ventral portions which is believed to be homologous to the hippocampus in mammals. It would be interesting to determine if the mammalian entopeduncular nucleus consists of neurons that share the same neurotransmitter phenotype as vTel^{y321} as it would expand the range of evolutionarily translatable experiments using zebrafish. vTel^{y321} neurons map onto telencephalic and diencephalic clusters in the mouse forebrain based on the mouse brain browser (mousebrain.org). The telencephalic cluster includes dorsal and ventral striatum and the amygdala. The diencephalic cluster includes the medial septal nucleus, diagonal band nucleus and nucleus basalis of Meynert. The diagonal band nucleus and nucleus basalis of Meynert are the second and largest cholinergic nuclei in the basal forebrain respectively, and are implicated in various aspects of cognition, memory and social behavior in both rodents and humans. We are aware that the vTel^{y321} "nucleus" is heterogenous to a certain degree as it is principally defined through transgene expression, and it spans the forebrain in a rostral to caudal fashion. Speculatively, it is worth considering that vTel^{y321} neurons may be part of a phylogenetically older nucleus that modulates several important brain functions which separated into anatomically distinct structures in "higher" vertebrates during evolution.

We are of the view that our transcriptomic fingerprint is highly precise in identifying specific clusters of neurons in the mouse forebrain that correspond to vTel^{y321} neurons in zebrafish. In Table 1 we show that vTel^{y321} neurons may also be glutamatergic, a finding which is also confirmed when we consider the identity of the mouse brain neuronal clusters. Both the telencephalic (http://mousebrain.org/celltypes/TECHO.html) and diencephalic (http://mousebrain.org/celltypes/DECHO1.html) clusters are also glutamatergic.

Multi-transmitter neurons potentially complicate the construction and interpretation of connectomic wiring diagrams (Granger, Wallace, and Sabatini, 2017). It challenges conventional approaches of constructing models due to the potential for release of neurotransmitters that exert antagonistic effects such as the vTel^{y321} neurons.

We know that the ablation of vTel^{y321} neurons results in marked deficits in social orienting behavior (Stednitz *et al*., 2018). Stednitz and Washbourne, (2020) showed that social behavior is hierarchical and cumulative during early zebrafish development. Considering this evidence, it would be interesting to investigate the specific roles that each of the neurotransmitters released by vTel^{y321} neurons play in refining circuits that govern social behavior across development.

Some researchers (Lozovaya *et al.*,2018) have suggested that there might be a higher number of multi-transmitter neurons in primates. It is still an open question whether the multi-transmitter state is a trait which appeared early in the phylogeny of vertebrates has been retained. A large-scale study on zebrafish spinal cord neurons by Pedroni and Ampatzis (2019) revealed that approximately 15% of spinal cord neurons express multiple transmitters. Such neurotransmitter maps enable us to gain a closer look into the organization of the nervous system as a precursor to understanding the functions and roles that these neurons played. The quantification of multi-transmitter neurons in our study is valuable in that we identify some brain nuclei that house multi-transmitter neurons and regionally map the extent of their distribution. A follow up study would be to conduct a volumetric assessment of the total number of CGNs in the forebrain and link that to a connectomic map of the zebrafish brain such the one constructed by Kunst *et al*., 2019. Results from such a study would enable us to establish the different circuits that vTel^{y321} neurons are involved in and bring into contextualize how ablation or perturbations of these neurons in impacts specific brain functions.

lhx8 and *lhx6* are implicated in the neurodevelopmental disorder Tourette Syndrome (Paschou, 2012). Their possible role in CGN neuron specification could be instructive on revisiting the pathophysiology of the syndrome since both cholinergic and GABAergic neurons are affected. The syndrome is characterized by substantial striatal cholinergic neuron loss, possibly implicating dysfunctions in the *lhx8* gene which plays a role in maintaining cholinergic neuron identity and function through a positive feedback mechanism with nerve growth factor (Tomioka *et al*.,2014). In other cases of the syndrome, mutations in the 3’ UTR of *lhx6* gene are present potentially making it

possible to design a zebrafish model for Tourette Syndrome which could be useful in investigating mechanisms that drive the syndrome and possibly design screens for therapeutic agents.

We tested the hypothesis whether combinatorial expression of LIMTFs was responsible for specifying vTel^{y321} neurons to a CGN phenotype. The data we obtained does not support this hypothesis since GABA expression is clearly retained. We believe this might be down to several factors. The first factor we consider is that the orthologue for one of the key drivers of both cholinergic and GABAergic neuron development in rodents *lhx8a* has a duplicate, *lhx8b*. Genetic compensation in zebrafish is known to be a common situation when the mutation results in a stop codon resulting in a feedback mechanism that leads to upregulation of other genes which perform a similar function(s). When we compared our bulk RNA-seq data with data from the Cell Browser we found that *lhx8b* is also highly expressed in this cluster of neurons (Supplementary Table 1). In fact, *lhx8b* appears to be almost exclusively expressed in the forebrain. It is thus possible that in *lhx8a* mutants, *lhx8b* drives both GABAergic and cholinergic specification. The *lhx6* gene was recently renamed to *lhx6a* on ZFIN (January 2021), with indications that there is a *lhx6b* gene. At the time this manuscript was compiled the *lhx6b* gene had not yet been annotated and as such there is no information on whether it is expressed the zebrafish forebrain. Genetic compensation also occurs through genes that have sequence similarity. LIMTF genes share a lot of sequence similarity (Hobert, 2000) so it is conceivable that compensation could be triggered when there is degradation of mRNA transcripts of related genes. The family of *dlx* transcription factors has also been shown to be involved in the generation of GABAergic neurons. From our data these genes are highly expressed (Supplementary Table 1) and this could. Rodent data shows that DLX1 and DLX2 are necessary and sufficient for *Gad* gene expression (Le *et al.*, 2017) and it could be an indication of why *lhx6* or *lhx8a* mutations alone will not disrupt GABAergic specification.

Conclusion

Combining our previous work on vTel^{y321} neurons modulating social orienting behavior (Stednitz *et al.,* 2018*)*, and our characterization which yielded a "transcriptomic

fingerprint" provides a good platform that can be used by researchers interested in studying neurodevelopment and therapeutics. The impact of developmental perturbations can be assessed by examining changes or alterations in the expression of specific markers in our transcriptomic fingerprint as well as any changes in the multi-transmitter phenotype. We established that vTel^{y321} neurons are specified as multi-transmitter neurons and the evidence at hand suggests that they remain in that state permanently. Such a feature which allows further enquiry into the role played by multi-transmission in shaping the development circuits that govern social behavior.

Our transcriptomic fingerprint allows investigation of similar neurons in the mouse brain and potentially other vertebrates. The set of LIMTFs genes we utilize have been implicated in the specification and function of neurons that express them. Inasmuch as mutations of these genes did not affect the multi-transmitter phenotype, these genes might have a significant role in the modulation of social behavior by vTel^{y321} neurons

Methods

Zebrafish Husbandry

All zebrafish embryos, larvae, and adults were raised and maintained at 28.5°C according to standard protocols (Westerfield, 2000). Lines used were AB/Tübingen and *Et^{y321}* [*Et(rex2-scp1:gal4ff)y321*]. We also utilized *lhx6*^{e1617} and *lhx8a*^{el640} lines courtesy of Dr Lindsay Barske from Gage Crump's lab at University of Southern California. All procedures carried out in this study were approved by the University of Oregon Institutional Animal Care and Use Committee.

Tissue Dissection

Forebrains (cut right behind the eyes, see Fig. 1A) were collected using a fine blade from zebrafish larvae expressing GFP from the Et^{y321} enhancer trap at 7 days post-fertilization (dpf), and placed into 250-500uL of Neurobasal medium on ice. The number of forebrains in each sample was n=80. Samples were dissociated using the Worthington Papain Dissociation System (Catalog #: LK003150). Briefly, forebrains were spun down at 700g for 5 minutes at RT. Neurobasal medium was removed, Papain + DNase mix

was added, and samples were incubated for 30-35min at RT with constant agitation. Cells were then dissociated further via pipetting until the mixture was homogeneous after incubation. Cells were then spun down at 700g for 5min at RT. Supernatant was removed, cells were resuspended in stop solution, and incubated with constant agitation for 5min. Cells were then washed 2 times in wash solution (containing glucose, 1M hepes, FBS, and DPBS then 0.22μM filtered), filtered through a Falcon tube with 35μm strainer cap (Catalog #:352235), and placed on ice. To each of these tubes, 1uL of Ghost Red 780 Viability Dye (Tonbo Biosciences, 13-0865-T100) was added to 1mL of wash solution used to resuspend the cells. All cells were sorted within 4hr of starting the dissection - longer than this resulted in less cell viability.

FACS

Fluorescent Activated Cell Sorting (FACS) was performed on a Sony® SH800. Forward scatter (FSC) was set at 16, the back scatter (BSC) was set to 34-36%, and a 120μm chip was used for sorting all cells for this experiment. Negative controls (GFP (-) cells) were used to set thresholds to control for GFP autofluorescence prior to sorting. Positive controls for dead cells (cells that had been treated with ethanol prior to sorting) were used to set thresholds for the cell viability dye prior to sorting. Once the thresholds for controls were set, 100,000 cells per sample were sorted directly into 3mL of Trizol-LS, always ensuring the ratio of cells and sheath fluid to Trizol-LS was 1:3. In this experiment, three samples of GFP (-) cells (n=3) and three samples of GFP (+) cells (n=3) were collected for downstream RNAseq.

RNAseq

The RNA collected from the sorted cells was first quantified and checked for quality on an Agilent Fragment Analyzer System. Only samples that had a RNA Quality Score (RQN) value of 8 or more were used for sequencing. RNA was then used to create libraries using the NuGen mRNA Selection Module (Tecan Genomics, Catalog #:0408-32) according to the kit protocol. The prepared libraries were then analyzed in Next Generation Sequencing (NGS) mode on the Agilent Fragment Analyzer to ensure good

quality cDNA for sequencing. The libraries were then run in single-read sequencing on Illumina HiSeq 4000® machine.

In situ hybridization

Adult *Et*^{y321} zebrafish (age 2-12 months), embryos (16-48 hpf) and larvae (48hpf-7dpf) were anesthetized and euthanized in ice water, then decapitated and placed in 4% PFA for 1-1.5 hours before brains were dissected and fixed overnight in 4% PFA at room temperature. After fixation, brains were rinsed 3x in PBS and dehydrated in 20% sucrose in PBS for 24 hours, followed by cryosection after mounting in agarose. RNA in situ hybridization on 16 mm brain sections was carried out according to the protocol by Yan, Talbot and BreMiller 2011, using digoxigenin labeled probes for *lhx8a, gad65&67b, vachtb* and *chatb.* Sections were incubated overnight at 65ºC with 200ng of probe per section. The following day, sections were washed in a graded concentration series of 5X saline sodium citrate (SSC) with 50% formamide:2X SSC ending in an incubation step with anti-digoxygenin Fab fragments (Roche) suspended in 1M Tris HCl pH 7.5, 5M NaCl and 0.25% Tween 20 (TNT) and Perkin Elmer® block buffer overnight at 4ºC. Sections were washed 8X for 10 minutes each wash in TNT and incubated for 5 minutes in the dark with Perkin Elmer Amplification Diluent, then incubated for 1 hour at room temperature in the dark with Cy3 for subsequent visualization. Endogenous peroxidase activity was quenched through incubation in 2% hydrogen peroxide in TNT for 1 hour. Sections were first incubated in primary antibodies (chicken anti-GFP, 1:500, Aves Laboratories) and then secondary antibody (goat anti-chicken IgY-488, 1:500, Molecular Probes) overnight in 0.25% PBS Tween. Sections were imaged on a Leica DMI8-CS confocal fluorescence microscope using a 40x objective.

Immunohistochemistry

Sections were washed 3X for 5 minutes per wash in 1XPBS followed by a single wash in 0.1% PBS TritonX ($PBST_x$) to permeabilize the tissue. The sections were then incubated in block buffer (0.1%$PBST_x$ 2% Bovine Serum Albumen and 5% Normal Goat Serum) for a minimum of two hours and then incubated in appropriate primary antibodies overnight at 4ºC. Primary antibodies were washed off using 0.1%$PBST_x$ buffer before

incubation with secondary antibodies in block buffer overnight at 4ºC. Secondary antibodies were washed off using 0.1%PBST$_x$ before applying DAPI Fluoromount-G® (Southern Biotech) and coverslips overnight in the dark at room temperature.

Cell quantification landmarks

We quantified neurons at 3 anatomical positions in larval and adult fish

1. the rostral telencephalon, at the junction of the ventral telencephalon and olfactory bulb (RT). In this position, vTel^{y321} neurons occupy the ventral and medial portions of the telencephalon including Vv and Vd nuclei.
2. mid-telencephalon, at the anterior commissure (MT). In this position, vTel^{y321} neurons occupy the anterior part of the parvocellular preoptic nucleus and the supracommisural nucleus of the ventral telencephalon.
3. the caudal telencephalon at the junction of the forebrain and optic tectum (CT). In this position vTel^{y321} neurons occupy the ventral and medial portions of the forebrain, forming a strip of cells apposed that sweeps dorsally and laterally in the dorsal portion of the telencephalon.

Co-expression of GFP, *chatb* and *vachtb* was quantified using ImagePro Plus version 6.0 ®. GFP expressing cells were manually identified in a separate image channel and thresholding performed. GFP fluorescent intensity data from the GFP channel were loaded onto a second image channel with any one of *vachtb, chatb, gad65/67b.* In this channel, the background for the second channel (in this case, *vachtb)* was set with fluorescent intensity for points from the GFP channel updated. This allowed for quantification of data points which were also *vachtb* positive. The updated points from the combination of GFP and vachtb points were then transposed to the third channel (GABA) and fluorescence intensity updated. With each of the channel updated fluorescent intensity the data from individual points we exported into Microsoft Excel. In Excel, the fluorescent intensity for all channels was normalized through subtraction of the sum of the average intensity(Aμ) minus the standard deviation(Aμ+∂) i.e. (Individual intensity – [Aμ + ∂)]. This data was then used to determine the number of GFP neurons

were GABAergic and Cholinergic. Plots and graphs for the data were generated in R Studio®.

Statistics

RNAseq Data Analysis

Raw data files were unzipped and checked for quality using FastQC (Babraham Bioinformatics, version 0.11.8). After ensuring all reads were good quality, cutadapt (v1.18) was used to trim off adapters with the following parameters: -n 3 -O 1 -m 30. Another FastQC was fun on these files to ensure the adapter sequences were gone. Trimmomatic (USA Del Lab, version 0.36) was then used to trim off any poor-quality reads in single-end mode. The Zebrafish genome index was uploaded (Danio_rerio.GRCz11.dna.primary_assembly.fa.gz) and a custom construct (gal4 sequence) was added into the genome index using gmap_build from GMAP-GSNAP package (version 2018-03-25). GSNAP was then used to align the reads from samples to the Zebrafish reference genome (+ gal4 sequence). Samtools® (version 1.5) was then used to convert aligned. fastq reads to .bam files, then sorted and indexed them. To generate the counts matrix, htseq-counts (Python version 3.7.0) was used with the following parameters: -f bam --stranded=yes -m intersection-strict. From the counts matrix, protein coding genes were selected out using Ensembl Biomart and then used for processing in DESeq2 in R Studio® (R version R.3.5.2). From here, data was analyzed in R using the DESeq2 pipeline instructions provided by Michael I. Love, Simon Anders, and Wolfgang Huber (2014). After analysis, genes with an adjusted p-value of "NA" and/or above 0.05 were removed so that only significant differences were included in the results.

***lhx6* and *lhx8a* mutant allele information**

We received double mutants and heterozygous for *lhx8a* and *lhx6* from Dr Lindsay Barske (Gage Crump's lab at University of Southern California) and genotyped the main stock before outcrossing to ABTU zebrafish adults to obtain heterozygotes which were later in crossed to generate mutants, heterozygotes, and wildtypes for the respective alleles.

***lhx6*^{e1617}**

This allele is a 5 bp deletion that causes a frameshift and early stop codon after five incorrect amino acids. The line was generated using TALENs and had not yet been published when this manuscript was compiled. In green are the L and R TALEN target sites.

ref TCTCTTGCAGTCACAGTCTGACGGGGAATCTCATAGATCTAATATGGAGAAAGACGA
el617 TCTCTTGCAGTCACAGTCTGACGGGGAA--------TAGATCTAATATGGAGAAAGACGA

lhx8a^{el640}

This allele is a 5 bp deletion that causes a frameshift and early stop codon after 21 incorrect amino acids. The line was generated using CRISPR/Cas9 and had not yet been published at the time when this manuscript was compiled. Shaded in green is the CRISPR target site.

ref GACTGCATGTTGGACAACCTGAAGCGTGCCATGGAGAATG
el640 GACTGCATGTTGGACAACCTGAAGCGT----------GGAGAATG
Both alleles had not yet been published when this manuscript was compiled.

Key Resource Tables
Antibodies

Target	Manufacturer	Cat#	Dilution	Species	Immunogen	RRID
GFP	Aves Lab	GFP-1020	1:1000	Ck	IgY	**AB_10000240**
ISLET1/2	Developmental Studies Hybridoma Bank	39.4D5	1:1000	Rat	Partial protein	**AB_2314683***
GABA	EMD Millipore	ABN131	1:1000	Rb	GABA mouse, rat IgG	**AB_2278931**

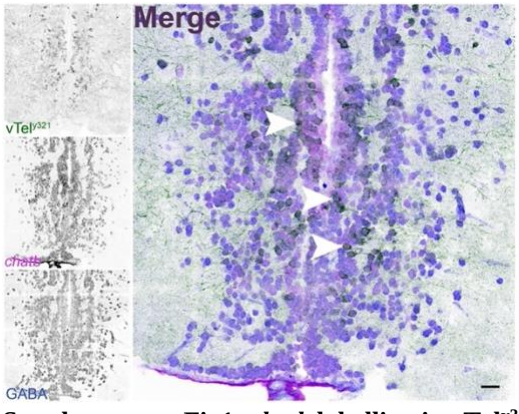
Antibodies continued

ChAT	Aves Lab	CAT	1:500	Ck	Sequence shared between the mouse (Q03059) and human (P28329) gene products (IgY)	**AB_2313537**
GFP	Invitrogen	A11120	1:1000	Ms	IgG2a	**AB_221568**
GFP	Abcam	AB290	1:1000	Rb	IgG	**AB_303395**
Goat anti-mouse	Invitrogen	A21131	1:500	Gy	IgG2a	**AB_2535771**
Goat anti-Chicken (FITC)	Aves Lab	F1005	1:1000	Gt	IgY	**AB_2313516**
Goat anti-Chicken IgY 555	Invitrogen	A21437	1:1000	Gt	IgY(H+L)	**AB_2535858**
Goat anti-Rabbit 546	Invitrogen	A11035	1:1000	Gt	IgG (H+L)	**AB_143051**
Goat anti-Rabbit 633	Invitrogen	A21071	1:1000	Gt	IgG	**AB_2535731**

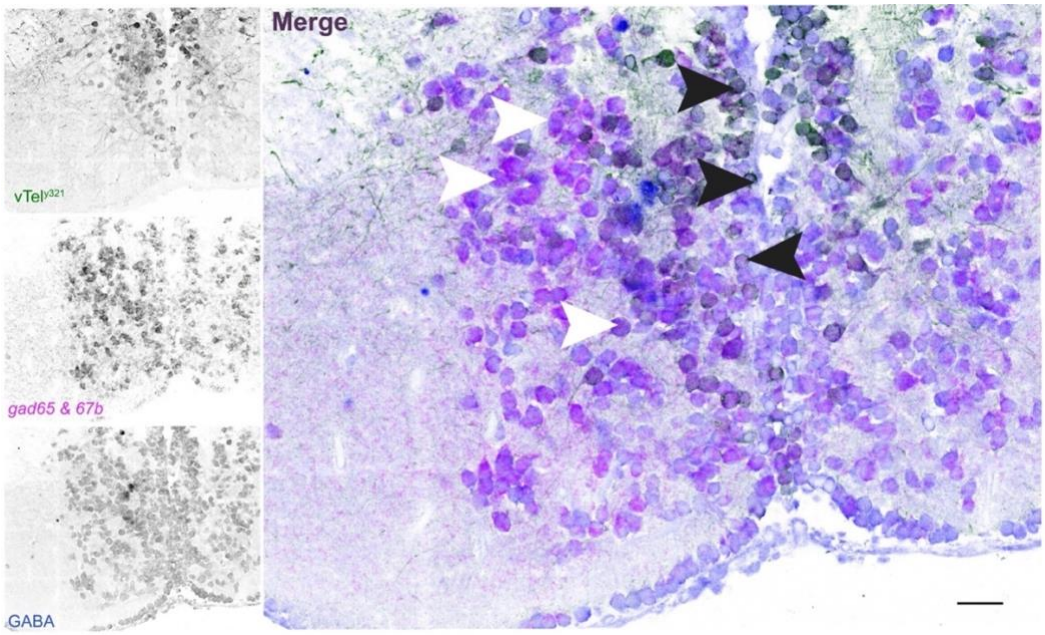
In-situ hybridization probes

Probe	Accession number	Source
vachtb	NM_201107.1	Hong, *et al*.,2013
chatb	NM_001291882.1	Hong, *et al*.,2013
gad65/gad2	NM_001017708	Higashijima, S., Mandel, G. and Fetcho, J, (2004)
gad67b/gad1b	AB183390	Higashijima, S, Mandel, G. and Fetcho, J, (2004)
islet1	NM_130962.1	Hutchinson, S and Eisen, J, (2006)
lhx8a	AY664404	Jackman, *et al.*, (2004).
lhx6	AY664403	Jackman, *et al.*, (2004).
Microscopy		
Leica TCS SP8	Confocal Leica 10X/. Leica N/A 40X/1.10 Water Objective Leica 11506357	https://www.leica-microsystems.com/ products/confocal-microscopes/details/ product/leica-tcs-sp8/

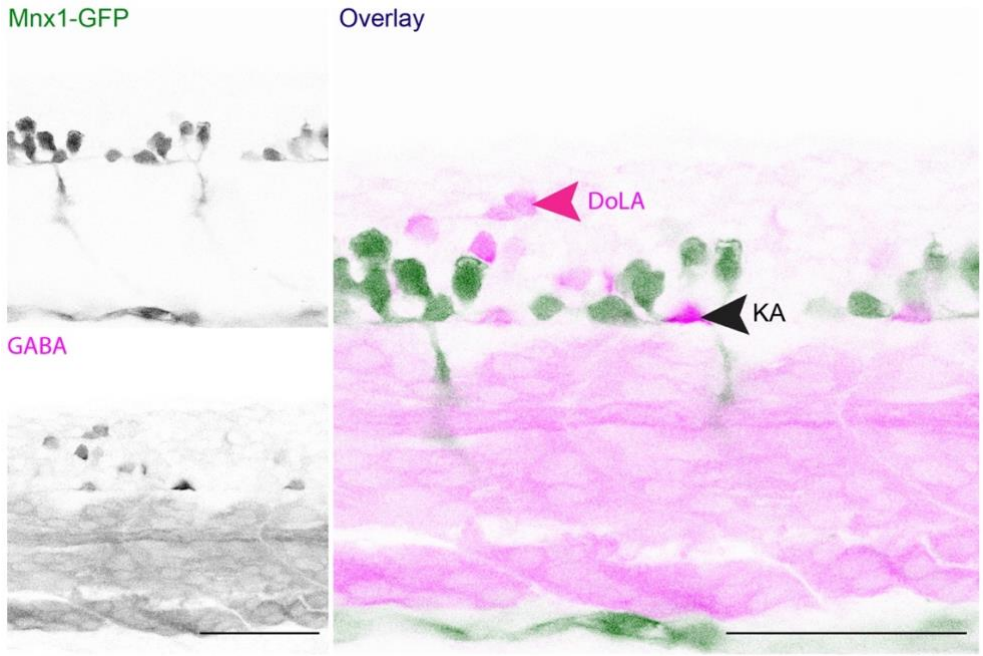
SUPPLEMENTARY FIGURES



Supplementary Fig1: *chatb* labelling in vTel^{y321} neurons. Validation of cholinergic identity of vTel^{y321} neurons with *chatb* antisense probes. Arrows point to selected vTel^{y321} neurons expressing both *chatb* and GABA. (Scale bar represents 20µm)



Supplementary Fig 2 *gad65&67b* labelling validates GABA immunostaining. *gad65* and *67b* transcripts were labelled in vTel^{y321} neurons and validation staining was done with GABA antibody. White arrows denote GABA and *gad65&67b* double staining, Black arrows label vTel^{y321} neurons which are positive for both GABA and *gad65&67b*. GABA antibody labelling overlaps extensively with *gad65&67b* confirming its specificity. (Scale bar represents 20µm)



Supplementary Fig 3: GABA antibody stains GABAergic neurons in the larval spinal cord. To test the specificity of the GABA antibody we utilized 24-48phf larval spinal cords in the transgenic line *tg (GAL4: mnx1; UAS: GFP*) which drives GFP expression in motor neurons. Black arrow denotes KA neurons and white arrow points to DoLA neurons which are known GABAergic neurons in the spinal cord. (Scale bars in the GABA and Overlay image represents 50µm)

Supplementary Table 1
Comparison of commonly expressed genes between scRNAseq data and FACS data

GENE	p_val	avg_logFC	p_val_adj	FACS list	fb genes
dlx5a	0	1.91997372	0	dlx5a	dlx5a
dlx1a	0	1.7966013	0	dlx1a	dlx1a
gad2	0	1.4606234	0	gad2	gad2
slc32a1	0	1.17929098	0	slc32a1	vgat
slc6a1b	0	1.17817005	0	slc6a1b	slc6a1b(gat)
dlx6a	0	1.09868991	0	dlx6a	dlx6a
nrxn3a	0	0.54763587	0	nrxn3a	#N/A
lhx8b	0	0.26782977	0	lhx8b	#N/A
nkx2.1	8.94E-303	0.87809747	2.91E-298	nkx2.1	nkx2.1
elavl3	1.03E-249	1.6746611	3.36E-245	elavl3	elavl3
fez1	3.56E-234	1.11198908	1.16E-229	fez1	fez1
gng3	1.02E-212	1.54877531	3.30E-208	gng3	#N/A
isl1	5.87E-182	0.93586708	1.91E-177	isl1	isl1
lhx6	2.74E-171	0.99715636	8.90E-167	lhx6	lhx6
lhx8a	7.30E-64	0.79136587	2.38E-59	lhx8a	lhx8a

CHAPTER III

MICROBIAL INFLUENCE ON MULTI-TRANSMITTER PHENOTYPE

Microbiota modulate GABAergic expression in vTel^{y321} neurons

Authors

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Specific Author contributions:

Conceptualization: P. Washbourne, J. S. Eisen.; Methodology: D. Ncube, J. Bruckner.;
Investigation: D. Ncube
Software and Formal Analysis: D. Ncube.
Writing Original Draft: D. Ncube.
Review and Editing: D. Ncube, P. Washbourne, J.S. Eisen.
Funding Acquisition: J.S. Eisen & P. Washbourne.; Supervision: P. Washbourne
This work is contained in a manuscript in preparation.

Background

There is increasing evidence that host-associated microbes influence the development and function of multiple host organs and processes. Rapid developmental changes in host organ development that underlie metamorphosis have been correlated with dynamic changes in microbial communities and specific metabolites (Burns *et al*., 2016, Stephens *et al*., 2016). Microbial communities have inhabited the planet for over 3 billion years long before the emergence of multicellular life. It is believed that endosymbiosis was key to the origin of eukaryotes, culminating in the evolution of organelles, enabling cellular structural and functional compartmentalization. (Mereschkowsky, 1995, Marguilis 1967, Timmis, 2004) providing host cells with new metabolic pathways (Pokes and Valenzano, 2019). It is conceivable that eukaryote evolution was achieved in the context of a specific microbial sphere. Mutualistic interactions between microbes and simple or complex eukaryotes are believed to have

evolved over time leading to a profound ecological and physical interdependence between multicellular organisms and microbial communities. There are many examples of host-microbe co-evolution, notable examples being the bobtail squids which house bioluminescent *Aliivibrio fischeri* (McFall-Ngai, 2014) and ruminants which wholly depend on their cellulose-fermenting microbes in their digestive chambers such as the rumen. (Nocek and Russell (1988). Microbiota have established mutually beneficial relationships with their hosts, thus entrenching them in the totality of organ development and function.

The microbiota-gut-brain axis (MGBA) is a concept in biology informed by the ability of microbiota to alter brain development and behavior. (Desbonnet *et al*., 2014, Lee *et al*., 2021). Bioactive metabolites produced by microbiota have emerged as powerful mediators of gut-brain communication thus revealing that even subtle shifts in gut microbial composition are sufficient to impact brain neurochemistry (Cryn and Dinan, 2012). Understanding interactions between the microbiota and the brain could potentially provide a window into developing therapeutic solutions that can ameliorate neurodevelopmental or behavioral deficits. Considerable strides have been in made in this area with use of fecal transplants from "asymptomatic donors" emerging as part of treatment approaches for autism spectrum disorders and depression (Xiao *et al.*, 2021, Lyu *et al.*, 2021). Fecal transplant interventions position microbiota as potent modulators of human behavior whose presence overrides genetic driven deficits in behavior. Experiments in germ-free (GF) model systems demonstrate that the microbiota is required for normal neurodevelopment, including neurogenesis, neurotransmitter expression, and host behavior (Hsiao *et al*., 2013). However, the mechanisms by which the microbiota modulates neurodevelopment and behavior remain poorly understood. We had preliminary data that raising zebrafish in a GF condition reduced social orienting behavior. Since we had determined that vTel^{y321} neurons modulate social orienting behavior (Stednitz *et al*., 2018), there was no information on how raising zebrafish GF could potentially impact the neurotransmitter phenotype of vTel^{y321} neurons, and whether the effect on social behavior could be explained by changes in neurotransmitter phenotype. Strandwitz *et al*., 2018, suggests that the possible mechanism through which

microbiota modulate brain function is likely through alteration of neurotransmitter levels and specifically GABA, norepinephrine, and dopamine. Since we already knew that vTel^{y321} neurons are GABAergic, we tested they hypothesis that the deficits in social behavior in GF were a result of a change in multi-transmitter phenotype in vTel^{y321}.

Our results indicate that there was a significant reduction in percentage of vTel^{y321} neurons that were GABAergic in the mid and caudal telencephalon. Inasmuch as we did not find a statistically significant reduction in GABAergic levels in the rostral telencephalon, there was still a reduction in the percentage of vTel^{y321} neurons that were GABAergic. Our data, coupled with previously published data brings us closer to establishing the mechanisms through which dysbiosis impacts brain function by loss or reduction of specific neurotransmitters in specific neuronal clusters. This study adds to evidence that microbiota can impact behavior through modulating neurotransmitter levels in the brain.

Results

Percentage of vTel^{y321} neurons that are GABAergic is reduced in Germ Free zebrafish

In order to establish if the GF state had an impact on vTel^{y321} neurotransmitter phenotype, we utilized the transgenic zebrafish line *Et^{y321}* [*Et(rex2scp1:gal4ff)y321*] and AB/Tübingen. We outcrossed *Et^{y321}* fish with AB/Tübingen and split the resultant F1 progeny at fertilization into GF and Conventionalized (CVZ) groups. The F1 were raised to 7dpf and euthanized and sectioned for immunostaining. We used the same anatomical landmarks that we employed in our previous study on transcriptional profiling, that is RT, MT, and CT:

(i) the rostral telencephalon (RT), at the junction of the ventral telencephalon and olfactory bulb. In this position, vTel^{y321} neurons occupy the ventral and medial portions of the telencephalon including Vv and Vd nuclei.

(ii) mid-telencephalon (MT), at the anterior commissure. In this position, vTel^{y321} neurons occupy the anterior part of the parvocellular preoptic nucleus and the supracommisural nucleus (SC) of the ventral telencephalon.

(iii) the caudal telencephalon at the junction of the forebrain and optic tectum (CT). In this position vTel^{y321} neurons occupy the ventral and medial portions of the forebrain, forming a strip of cells apposed that sweeps dorsally and laterally in the dorsal portion of the telencephalon.

Table 3 presents the descriptive statistics from the analysis of a total of 40 fish used to investigate the effect of raising zebrafish Germ Free on GABAergic levels in vTel^{y321} neurons.

Table 3: vTel^{y321} neuron statistics by region and condition

Region	**Average number of vTely321 neurons per condition (µ±Std. error)**		**% GABAergic vTel^{y321} neurons (µ±Std. error)**		**Effect size**
	CVZ	**GF**	**CVZ**	**GF**	
RTEL	139.65±8.11	141± 6.03	92.07±2.34	82.85±4.74	0.54
MTEL	75.8±7.34	70±5.69	94.4±0.96	80.75±6.08	0.69
CTEL	46.8±2.45	44.5±2.68	85.6 ±5.03	57.7±8.75	0.86

We first quantified the number of vTel^{y321} neurons as indicated by GFP expression and at the RT position, there were on average slightly more vTel^{y321} neurons in GF (141± 6.03) fish as compared to CVZ (139.65±8.11). 92.07±2.34% of vTel^{y321} neurons in CVZ fish were GABAergic and 82.85±4.74 % in GF. An independent sample T-test revealed that there was an insignificant difference between the percentage of vTel^{y321} neurons that were GABAergic in the forebrain of GF and CVZ fish [t_{40} (27.79) =1.74, p = 0.093)]. An analysis of the effect size at the RT position using Hedges' g (Hedges, 1981) was 0.54, which meant that the GF condition had a moderate effect on GABAergic levels at this specific position. Quantification of the number of vTel^{y321} neurons at the MT landmark revealed that there were almost equal numbers of neurons between CVZ (75.8±7.34) and GF (70±5.69) fish. CVZ fish had an average of 94.4±0.96 %, while GF fish averaged 80.7±6.08 %. The difference in GABAergic levels in the MT was statistically significant t_{40}(19.94) =2.22, p= 0.038). The effect size at this position

was 0.69, which falls in between the large and moderate effect criteria. At the CT, the average number of vTel^{y321} neurons between the two conditions was similar, with 46.8±2.45 for CVZ and 44.5±2.68 for GF. At this position, there was a statistically significant difference in the percentage of GABAergic vTel^{y321} neurons between GF and CVZ conditions (t_{40}(30.29) = 2.77, p=0.01). The GF condition had a large effect on GABAergic levels at the CT position (g= 0.86). The trend we observed shows that the differences in GABAergic levels between GF and CVZ groups increases in the rostro-caudal direction.

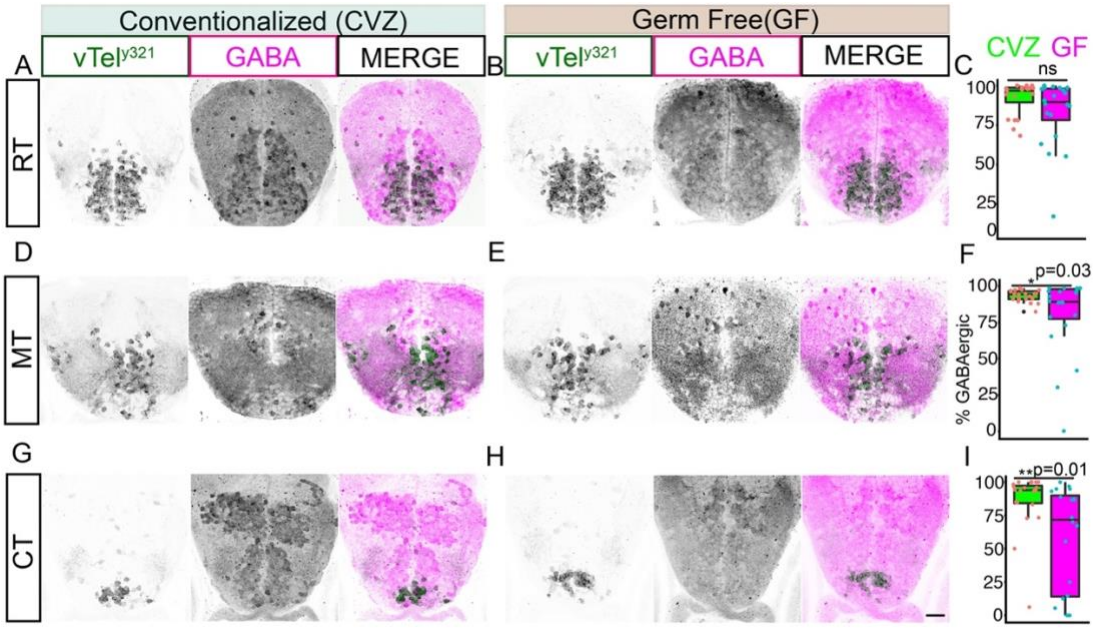

Figure 10. GF condition reduces the percentage of GABAergic vTel^{y321} neurons A-B Coronal cross sections of the forebrain in GF and CVZ fish Conventionalized (CVZ) express almost similar levels of GABA with Germ Free fish. **C** Statistical analysis of the percentage of GABAergic neurons revealed no significant difference in GABAergic levels between GF and CVZ conditions [(t_{40} (27.79) =1.74, p = 0.093)] in the rostral telencephalon. **D-E** Comparison of percentages of vTel^{y321} neurons at the anterior commissure in GF and CVZ fish. **F** Statistical analysis of the percentage of GABAergic neurons revealed a significant difference in GABAergic levels between GF and CVZ conditions [(t_{40}(19.94) =2.22, p= 0.038)]. **G-H** Coronal section of the caudal telencephalon. There were similar numbers of vTel^{y321} neurons between the 2 conditions. **I** Statistical analysis of the difference between the percentage of GABAergic vTel^{y321} neurons between GF and CVZ revealed a statistically significant difference. [(t_{40}(30.29) = 2.77, p=0.01)]. (Scale bar = 100µm)

GF condition reduction in percentage of GABAergic vTely321 neurons is uniform across the telencephalon

To assess if the region and the condition (GF or CVZ) of the animal affects the

percentage of vTel^{y321} neurons, we analyzed the data with a two-way ANOVA test. The rationale behind this analysis was that it was possible that the percentage of GABAergic neurons were affected by the GF condition in a region-specific manner. We had observed that the GF condition led to a reduction in GABAergic levels, we wanted to establish if this effect was specific to region, or it was an overall effect. From our analysis (Fig 9), we conclude that the GF condition significantly reduced the GABA levels overall, with no significant differences between the regions as shown in the plot below. Our analysis showed that the region ($p = 0.00379$) and condition ($p = 8.06e^{-05}$) of the animal had a significant effect separately on the percentage of vTel^{y321} neurons that were GABAergic but there was no interaction effect between the region and the condition ($p=0.27$)

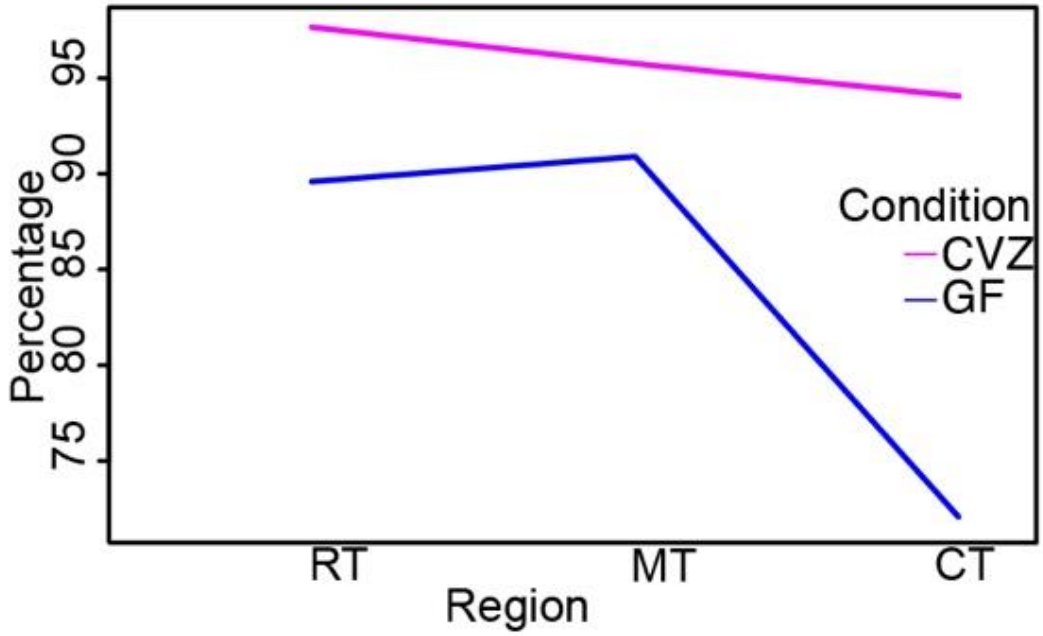

Figure 11 Interaction analysis between condition, region, and percentage. An Interaction plot is a useful way to determine if there is a difference in means between independent groups that are split on two factors. In this plot we treat the three regions are independent of each other and use a two-way ANOVA to establish if the 2 specific factors (region and condition) affect the percentage of vTel^{y321} neurons that are GABAergic. The absence of intersection between the 2 lines is an indication that the reduction of GABAergic levels is uniform across regions and that there is no interaction between region and condition.

Discussion

Our data indicates that the GF condition results in a reduction of GABAergic levels in vTel^{y321} neurons. Our samples were similar in terms of the number of vTe^{ly321} neurons, but GABAergic levels were consistently lower in GF as compared to CVZ. The

difference between GABAergic levels in GF and CVZ conditions rises to the point of statistical significance in the MT and RT positions. It is important to note that though the difference in GABAergic levels in the RT is not necessarily statistically significant, the changes in social behavior are pronounced. This brings into view that even subtle differences in neurotransmitter levels arising from the dysbiosis are significant enough to cause a marked change in specific behaviors. The trends in our data also revealed a stepwise increase in the magnitude of the effect size in a rostral and caudal direction (RT, g=0.54, MT, g=0.69, CT, g =0.86). It is an open question whether the rostro-caudal increase in effect size and increase in statistical significance is a result of differential sensitivity to deprivation of microbiota. An analysis of possible interactions between the region and GABA levels would be useful to determine the specific weight that each forebrain region plays. It is possible that statistical power might be a limitation in our study. We utilized 20 animals for each group and quantified the number of GABA positive vTel^{y321} neurons at only 3 positions per animal. It could be possible that increasing the number of positions in the rostral region would recapitulate the differences in GABAergic neuron numbers we observe in the MT and CT. In addition, perhaps increasing the number of animals for each condition could raise statistical power.

Another approach to gain a closer understanding on the impact of the GF condition on GABA expression would be spatial in-situ transcriptomics approaches such multiplexed fluorescence *in situ* hybridization (FISH) or *in situ* sequencing (Levsky *et al*., 2002, Ke *et al*., 2013, Chen *et al*., 2015, Shah *et al* 2016, Lein, Borm and Linnarsson, 2017, Meng *et al*., 2018) Spatial transcriptomics preserve the structural integrity of the brain whilst providing sufficient detail on the variations in expression patterns of selected transcripts across brain regions.

An additional approach would be to assess the levels of expression of LIMTF genes in our “transcriptomic fingerprint” between the different conditions. It is known that *lhx8a* plays a role in the maintenance of cholinergic identity, perhaps the other transcription factors also play a role. It is critical to keep in view that the changes in neurotransmitter phenotype are not restricted solely to vTel^{y321} neurons but also other

GABAergic neurons in the telencephalon. The anatomical positions we use in this study give somewhat precise locations in the brain where we can examine the effect of reduction in GABAergic neurons in specific brain nuclei whose function(s) have been established.

It would be interesting to determine if adding increasing GABA levels using GABA agonists in GF fish would rescue the reduced social interaction. At this point, we do not know the exact mechanism through which microbiota modulate GABA levels and specifically which microbial species are responsible for this. Preliminary sequencing data at hand indicates that there is elevation of *gad2* transcripts in GF animals despite a reduction in GABA expression. This could mean that whatever metabolites or secreted substance from microbiota is important for parts of GABA synthesis or translation of enough gad2 transcripts into protein. It would be a logical step to first establish the specific molecular substrates secreted by microbiota and determine the exact steps in neurotransmitter synthesis that they modulate.

It is important to note that the reduction in GABA expression in vTely321 neurons in GF animals is happening when the neurons have already been specified. It is an open question if the loss in GABA expression is driven by de-differentiation or reduced expression levels of genes like *gad1a&b* or *gad2*. There is a need to determine the exact strains of microbes that mediate the maintenance of GABA levels in neurons. Determining the exact gut microbe metabolites that mediate the maintenance of normal neurotransmitter levels will be useful in establishing the mechanism by which GABA levels are modulated. Since vTel^{y321} neurons are not only GABAergic and cholinergic, but possibly glutamatergic and glycinergic too, it would be important to investigate if these other neurotransmitters are affected in a similar manner in GF zebrafish.

Conclusion

The reduction in GABAergic levels in vTel^{y321} neurons adds to evidence that indicates that microbiota modulate expression of neurotransmitters. A normal gut microbiome is essential for the maintenance of GABA expression in the zebrafish

forebrain. This important result could entail that recolonization of the gut with microbes could restore normal neurotransmitter expression. We established that the reduction in GABAergic levels worsens in a rostro-caudal fashion though our interaction analysis revealed that there was no interaction between the condition and region. Our statistical analysis revealed an increase in effect size possibly indicating that there could be some differential sensitivity to dysbiosis based on anatomical location of specific neurons. Our results are an example of how we could utilize the unique features of vTel^{y321} neurons to assess the impact of different perturbations on neurodevelopment.

Methods

Zebrafish Husbandry

All zebrafish embryos, larvae, and adults were raised and maintained at 28.5°C according to standard protocols (Westerfield 2000). Lines used were AB/Tübingen and *Et*^{y321} [*Et(rex2scp1:gal4ff)y321*]. All procedures carried out in this study were approved by the University of Oregon Institutional Animal Care and Use Committee.

Gnotobiology

Zebrafish embryos were raised GF, or CVZ as previously described by Bates *et al*., 2006 and Melancon *et al*.,2017. Briefly, embryos were treated from 0-6 hours post-fertilization (hpf) in embryo medium (EM) containing 100 μg/ mL ampicillin, 250 ng/mL amphotericin B, 10 μg/mL gentamycin, 1 μg/mL tetracycline, and 1 μg/mL chloramphenicol. In a class II A2 biological safety cabinet, embryos were briefly surface-sterilized with 0.1% PVP-I and 0.003% sodium hypochlorite, washed with sterile EM, and transferred to 50 mL tissue culture flasks at a density of 1 fish/1 mL sterile EM. CVZ flasks were inoculated with 200 μl water from the parental tank. Sterility was assessed by direct visualization of microbial contaminants with phase optics on an inverted microscope at 40x magnification once per day and by culturing media on LB agar at 28° C for two days following terminal sampling. CVZ siblings were inoculated with system water at 7 dpf and fed rotifers three times daily.

Fixation and Cryosection

7dpf larvae were euthanized in ice water and then placed in 4% paraformaldehyde (PFA) for a minimum of 16hrs or overnight at Room Temperature. After fixation the larvae were washed 3X in 1X PBS to remove PFA and placed in 20% sucrose in PBS overnight in preparation for cryosectioning. The sections were sectioned at 16µm thickness and placed on glass slides.

Immunohistochemistry

Sections were washed 3X for 5 minutes per wash in 1XPBS followed by a single wash in 0.1% PBS TritonX ($PBST_x$) to permeabilize the tissue. The sections were then incubated in block buffer (0.1%$PBST_x$ 2% Bovine Serum Albumen and 5% Normal Goat Serum) for a minimum of two hours and then incubated in appropriate primary antibodies overnight at 4ºC. Primary antibodies were washed off using 0.1%$PBST_x$ buffer before incubation with secondary antibodies in block buffer overnight at 4ºC. Secondary antibodies were washed off using 0.1%$PBST_x$ before applying DAPI Fluoromount-G® (Southern Biotech) and coverslips overnight in the dark at room temperature.

Key Resources Table

Target	Manufacturer	Catalog#, clone	Dilution	Species	Immunogen	RRID
GABA	EMD Millipore	ABN131	1:1000	Rb	GABA mouse, rat IgG	**AB_2278931**
GFP	Invitrogen	A11120	1:1000	Ms	IgG2a	**AB_221568**
Goat anti-Ms 488	Invitrogen	A21131	1:500	Gt	IgG2a	**AB_2535771**
Goat anti-Rb633	Invitrogen	A21071	1:1000	Gt	IgG	**AB_2535731**

Cell quantification

Co-expression of GFP and GABA was quantified using ImagePro Plus version 6.0 ®. GFP expressing cells were manually identified in a separate image channel and thresholding performed. GFP fluorescent intensity data from the GFP channel were

loaded onto the GABA channel. In this channel, the background for the second channel was set with fluorescent intensity from the GFP recorded points updated. The updated fluorescent intensity for the GFP points would allow determination of which GFP positive neurons are also GABAergic. The fluorescent intensity data was then exported into and Excel sheet for analysis in R Studio®.

CHAPTER IV

CONCLUSION

Multi-transmitter neurons are present in both vertebrate and invertebrate animals, raising several questions on the evolution and function of neurotransmission. One such questions is to consider if the multi-transmitter state is a phylogenetically older and preserved state compared to the single transmitter phenotype? Also, are there any general rules or principles that neurons employ in their selection of specific combinations of neurotransmitters? If there are, how are they rules genetically determined?

It would be interesting if there is a is a format of specific neurotransmitter combinations in circuits involved in social behavior across vertebrates. This would enable us to interrogate the phylogeny of social behavior and the common elements across different species.

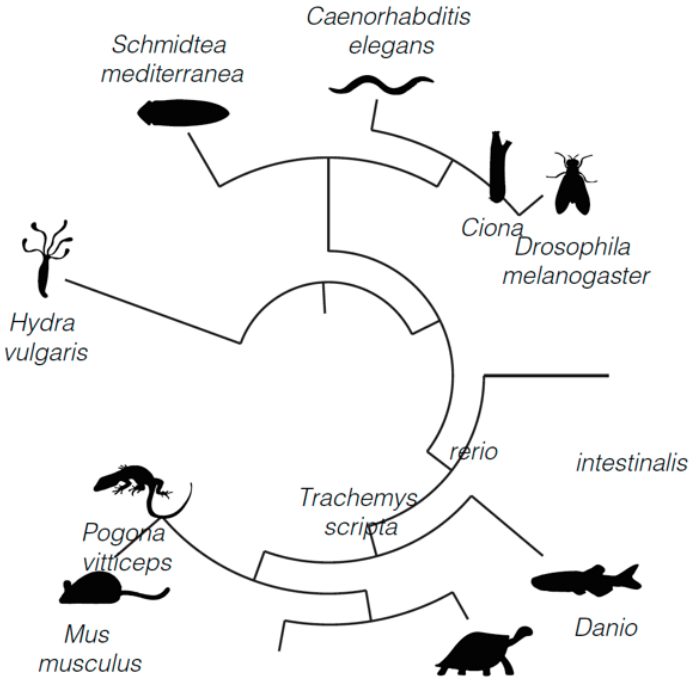

Figure 12. **Conserved dual and multi-transmitter neurons across metazoans.** Circular phylogenetic tree displaying the different species analyzed to study neuronal composition of the brain, or neuronal networks, in terms of their neurotransmitter phenotype. Figure from Avalos and Sprecher(2021)

The "transcriptomic fingerprint" approach presented in this work is one approach that researchers could locate similar neurons across the vertebrate phyla. There is evidence that short homeobox transcription factors offer a reliable way to locate unique sets of neurons in the CNS. They exhibit low transcriptional noise, and their unique combinations allow for isolation and identification of distinct neuronal clusters (Sugino *et al.*, 2017). The approach we used enabled the isolation of *only* 2 out of a total of 265

clusters from the mouse brain browser. These 2 clusters of neurons have the same neurotransmitter phenotype and location as vTel^{y321} neurons. It would be interesting to investigate if the convergence of gene expression between vTel^{y321} neurons and telencephalic and diencephalic neurons in the mouse brain entail a convergence of function. We envisage that the same approach can be utilized to isolate the same neurons in humans other primates.

Interestingly, the LIMTF homeodomain proteins are also phylogenetically well conserved across vertebrates and invertebrates. (Hobert, 2000). Specification of cholinergic neurons in the mouse brain is controlled by the expression of the gene *lhx8* (Zhou, 2004) which is also important in the maintenance of cholinergic neuron function. We observe that this gene is expressed in forebrain neurons from about 4hpf all the way through to adult stages. The phylogenetic conservation in expression of LIMTF genes and the resultant homeodomain proteins might be indicative of the critical roles they play in multitransmitter neuron function. We tested the hypothesis whether combinatorial expression of LIMTF genes is required for multi-transmitter neuron specification. Inasmuch as there are several plausible explanations why the specific mutations did not affect the multi-transmitter phenotype, it is possible that the LIMTF genes play roles in the maintenance of vTel^{y321} neuron identity and function. Since the specific alleles are not lethal, it would be critical to assess if LIMTF gene mutations impact social behavior. This could be practically achieved by raising the mutant zebrafish to 14dpf and testing them through the social orienting behavior experimental setup and determine if the mutations have an impact. It is an open question whether the reduction in GABA levels in GF fish would in any way be influenced by changes in expression of LIMTF genes. Preliminary sequencing data from GF zebrafish reveals that lhx8a expression is upregulated in vTel^{y321} neurons. The impact of developmental perturbations can be assessed by examining changes or alterations in the expression of specific markers in our transcriptomic fingerprint as well as any changes in the multi-transmitter phenotype. We established that vTel^{y321} neurons are specified as multi-transmitter neurons and that they remain in that state throughout development. Such a feature allows further enquiry into the role played by multi-transmitter neurons in shaping circuits.

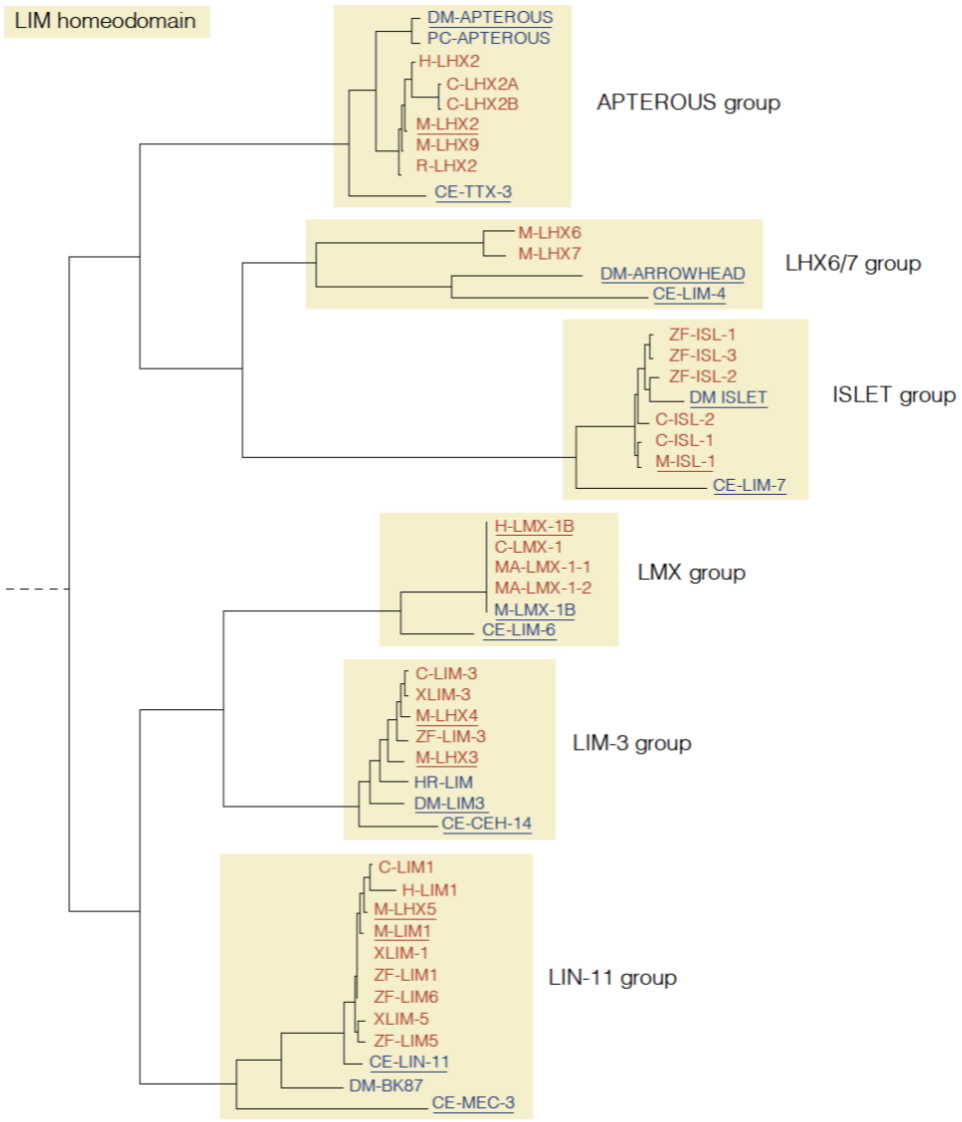

Figure 13 Phylogenetic classification of LIM-HD proteins. The phylogenetic tree of the homeodomains of the LIM-HD proteins was constructed using the neighbor-joining method. Invertebrate members are labeled in blue, vertebrate members in red. LIM-HD group names were chosen based on the first identified member of each group. Genes for which mutant phenotypes have been characterized (see text) are underlined. Other major classes of HD proteins are schematically depicted to illustrate that members from distinct HD classes are not merely defined by the presence of additional domains, but by distinctive sequence features within their homeodomains that make them cluster in a separate branch of a phylogenetic tree of homeodomain proteins. A detailed phylogenetic analysis of other homeodomain classes can be found elsewhere. Species designations are: C, chicken; CE, *Caenorhabditis elegans*; DM, *Drosophila melanogaster*; H, human; HR, *Halocynthia roretzi*; M, mouse; MA,hamster; PC, butterfly; R, rat; X, Xenopus; ZF, Zebrafish. Alternative gene names can be found at: http://www.informatics.jax.org/. (Hobert, 2000)

Our transcriptomic fingerprint is highly precise as it maps onto only 2 clusters out of the 265 clusters compiled by the Linnarsson Lab on the mousebrain.org Browser. This precision allows investigation of similar neurons in the mouse brain and potentially, other

vertebrates. The set of LIMTFs genes have been implicated in the specification and function of neurons that express them. Inasmuch as mutations of these genes did not affect the multi-transmitter phenotype, these genes might have a significant role vTel^{y321} neuron function. The transcriptomic fingerprint we establish in this study, allows investigators to consider the relevance of multi-transmitter neurons in the phylogeny of social behavior. Stednitz and Washbourne, (2020), have shown that social behavior is hierarchical and progressive, with the animals becoming more adept and skilled at performing specific behaviors as they age. We do not know whether these neurons are part of circuits that modulate social behavior in the rodent brain. The fact that vTel^{y321} neurons are cholinergic and GABAergic before the orienting behavior is first observed raises the question whether the progression in the development of social orienting behavior is driven by a commensurate change in firing patterns and or projections to other parts of the brain. Such data would be valuable in helping us understand the underlying features of the circuits that vTel^{y321} neurons are part of. The basal forebrain cholinergic nuclei in rodents are known for their importance in the performance of many behaviors. Are vTel^{y321} neurons part of a homologous network of neurons in mice and other vertebrates? From the Linnarsson lab browser the telencephalic cluster of neurons that is transcriptionally identical to some extent, to vTel^{y321} neurons maps to the ventral and dorsal striatum and amygdala. The ventral striatum in both mice and humans plays a role in the computations that take place during social behavior (Baez-Mendoza and Schultz, 2013). Utilizing the transcriptomic fingerprint, vTel^{y321} neurons also map onto medial septal nucleus, diagonal band nucleus and the nucleus basalis of Meynert. The last 2 nuclei are the largest cholinergic nuclei of the basal forebrain are instrumental in many behavioral and cognitive processes including attention, learning, memory, arousal, and sleep (Arendt *et a*l., 1985, Semba, 2004) and have been the focus for many studies of neurodegenerative disorders such as Alzheimer's disease (Mesulam, 2013).

It has been established that the gut microbiome co-evolved and, in some cases, directly influenced the evolution of multi-cellularity in vertebrates. It is unknown if the phylogeny of multi-transmission fate was in any way, influenced by gut microbes. Several bacteria have been shown to produce and/or consume a wide range of

mammalian neurotransmitters, including dopamine, norepinephrine, serotonin, or gamma-aminobutyric acid (GABA) (Strandwitz, 2018). It is interesting to note that rodents raised germ free also experience deficits in social behavior. Our mapping of vTel^{y321} neurons onto specific telencephalic and diencephalic nuclei which are crucial for the execution of social behavior and cognition could be a portal into understanding the role of the gut microbiome in modulating neurotransmitter phenotype in the vertebrate basal forebrain.

Given the convergence of transcriptional data and function, there is evidence to conclude that vTel^{y321} neurons could represent a phylogenetically older cluster of neurons that later diverged in "higher" vertebrates into distinct telencephalic and diencephalic nuclei which retained the multi-transmitter phenotype. More work on the parcellation of this cluster of neurons into distinct subgroups could enable us to go back and forth in comparing common and divergent features across species and thus get closer to understanding the evolution of social brain in vertebrates.

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