A Conserved Transcriptional Fingerprint Of Multi-Neurotransmitter Neurons Necessary For Social Behavior

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Abstract

Background: An essential determinant of a neuron's functionality is its neurotransmitter phenotype. We previously identified a defined subpopulation of cholinergic forebrain neurons required for social orienting behavior in zebrafish.

Results: We transcriptionally profiled these neurons and discovered that they are capable of synthesizing both acetylcholine and GABA. We also established a constellation of transcription factors and neurotransmitter markers that can be used as a "transcriptomic fingerprint" to recognize a homologous neuronal population in another vertebrate.

Conclusion: Our results suggest that this transcriptomic fingerprint and the cholinergic-GABAergic neuronal subtype that it defines are evolutionarily conserved.

Background

Common convention classifies neuronal subtypes and predicts their functions based on a single neurotransmitter (NT). The limitation of this approach is that it assigns neuronal identity based on the first NT to be detected in a neuron. Often the possibility of other NTs in neurons of interest is not investigated, despite increasing evidence that neurons have the capacity to synthesize and release more than one NT (Burnstock *et al.*, 1976, 2004). The multi-transmitter phenotype has important implications in relation to pharmacological agents that alter the release of NTs. Some classes of drugs are designed based on a single NT released by a specific neuronal type, ignoring other potential NTs they may also release. Some potential outcomes of such a limited approach are a reduced efficacy of pharmacological agents and an increase in unintended consequences (Sevensson *et al.*, 2019).

Neurons can release multiple transmitters via different mechanisms. Co-transmission can be broadly defined as the release of multiple NTs from non-overlapping pools of synaptic vesicles (Tritsch *et al.*, 2012) and co-release happens when the NTs are released from the same pool of synaptic vesicles (Vaaga *et al.*, 2015). Co-transmission of NTs is a common phenomenon in which "classical" small molecule NTs [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). In such cases, multi-transmission enhances neuronal function, as the monamines and purines play a role in accentuating the effect of classical NTs (Ng et al., 2015).

In addition to releasing both classical and neuropeptide NTs, many neuronal subtypes have been shown to release multiple classical NTs (Hnasko and Edwards, 2012). In some cases, the released NTs 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 NTs might have antagonistic effects, such as GABA and ACh release by mouse striatal neurons (Lozovaya *et al.*, 2017). To accurately characterize multi-transmitter neurons, emphasis must be placed on using genetic and protein markers

that are relevant to the synthesis and packaging of specific NTs (Rosenmund *et al*., 2015). Utilizing such an approach provides an accurate identification and characterization of neurons by establishing the presence of the critical machinery required to synthesize, package, or release a specific NT.

In this study, we adopted this approach, and utilized molecular techniques to transcriptionally profile vTel^{y321} neurons, a cluster of forebrain neurons that modulate social behavior. We previously showed that vTel^{y321} neurons are cholinergic (Stednitz et al., 2018), but here we combine transcriptomic, *in situ* hybridization (ISH) and immunohistochemistry (IHC) experiments to reveal that these neurons are also GABAergic. The multi-NT identity is detectable during early embryonic development and identity acquisition is not sequential; rather, machinery for both transmitters is expressed simultaneously. We also discover a combination of LIM Homeobox transcription factor genes that are reliable markers of these neurons in embryos, larvae, and adults. Our analysis provides a “transcriptomic fingerprint” for this cluster of forebrain neurons that we utilize to identify homologous clusters of neurons in rodents. This genetic congruity supports the conclusion that vTel^{y321} neurons are evolutionarily conserved and will be useful to determine whether the transcriptional similarity underlies functional homology.

Results

vTel^{y321} transcriptome confirms its transgenic origin

We previously showed that manual and chemogenetic ablations of vTel^{y321} neurons disrupts zebrafish social orienting behavior (Stednitz et al., 2018), prompting us to further understand this cell population. vTel^{y321} neurons are genetically defined by the enhancer trap insertion *Et(rex2-scp1:gal4ff)y321:UAS;GFP)* (Fig. 1a) and lie within the Ventral ventral (Vv) and Ventral dorsal (Vd) nuclei of the zebrafish telencephalon. To learn the molecular identity of this population of neurons, we randomly selected and dissected and dissociated heads of 7-day post-fertilization (dpf) larvae (n = 80) to isolate as much of the forebrain as possible (Fig. 1A). After dissociation, we used fluorescence-activated cell sorting (FACS) to sort, pool, and sequence at least 100,000 sorted GFP positive and negative cells from each sample (Fig. 1A’). 80–90% of dissociated cells were alive (Fig. 1A”) and 3.69% of these cells were GFP positive (Fig. 1A”’). We expected the proportion of GFP positive cells to be small because the vTel^{y321} nucleus is a relatively limited portion of the forebrain, totaling several hundred neurons at this stage (Bruckner *et al*., 2020). We used Ensembl to select protein-encoding genes and compiled lists of genes, ordered by adjusted *P* values, that are differentially expressed (DE) between GFP positive and GFP negative cells. Principal component analysis revealed that one sample from the GFP negative fraction was an outlier (Fig. 1B). This sample was dropped leaving us with 3 GFP positive samples and 2 GFP negative samples. Sorting for genes with an adjusted *P* value ≤ 0.05 generated a list of 2,096 DE genes expressed in GFP positive cells compared to GFP negative cells out of a total of ~ 23,000 genes (9%).

To refine our characterization of vTel^{y321} neurons, we focused on genes documented to be expressed in the forebrain by the Zebrafish Information Network (ZFIN; zfin.org), compiling a list of 454 genes (21.6%

of the 2,096 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 had expected to observe significant enrichment of *lhx8a* in GFP positive neurons since the enhancer trap *Et*^{y321} is located within the *lhx8a* gene locus (Zebrafish Brain Browser, zbbrowser.org) (Additional Table 1). Taken together with the high enrichment of EGFP transcripts in the GFP positive population (Fig. 1D), we conclude that the dataset successfully represents an enriched population of mRNAs from the FACS sorted cells.

Table 1

Neurotransmitter-associated genes expressed in vTel^{y321} neurons

Symbol	Gene name	p-adjusted	Log_2Fold change	Neurotransmitter
slc17a6b	*vglut2*	0.13842785	-0.563783931	Glutamate
slc17a6a	*vglut2.2*	0.97372783	0.094682805	
slc1a2b	*EAAT2 Glutamate transporter*	5.99E-12	1.280660763	
slc1a2a	*EAAT2b*	0.07570601	1.645158684	
slc18a3b	*vachtb*	0.52629943	-1.883485129	Acetylcholine
slc6a7	*Choline transporter*	0.00040338	-8.21997693495	
slc18a3a	*vachta*	0.99634064	-0.015324035	
Chata	*chata*	0.19918698	-0.893354429	
slc18a2	*vmat2*	0.8983514	0.533626413	Dopamine
slc6a3	*DAT*	0.10773861	-0.850246803	
slc6a2	*NE transporter*	0.68157586	0.533626413	Norepinephrine
slc32a1	*vgat*	0.00039023	-1.01476333	GABA
slc6a1b	*gat*	0.00043843	-0.593364614	
gad1b	*gad67b/gad1b*	0.11640804	0.413463156	
gad2	*gad65/gad2*	6.19E-06	-1.01827851	
Gcat	*glycine C-acetyltransferase*	0.58087894	-0.660647133	Glycine
slc6a5	*glycine transporter 2(glyt2)*	0.01674848	-2.009676284	

Highlighted genes are significantly DE with enhanced expression in vTel^{y321} neurons denoted by an adjusted p-value ($p < 0.05$) and negative Log_2Fold change. The letters *slc* denote genes belonging to the solute carrier superfamily of genes.

vTel^{y321} neurons express two classical NTs

The rodent *lhx8a* homolog, *Lhx8*, is required for development of cholinergic neurons in the rat forebrain (Zhao, 2003), and we previously showed that vTel^{y321} neurons are cholinergic (Stednitz et al., 2018). Consistent with our previous observations, ISH confirmed detection of transcripts for cholinergic markers such as the vesicular acetylcholine transporter (*vachtb*, n = 4) (Fig. 2A and 2A') and choline acetyltransferase (*chatb*, n = 4) in vTel^{y321} neurons (Additional Fig. 1). In addition, we also found significant differential expression of *slc5a7*, encoding the choline transporter, further validating the cholinergic identity of these neurons. We compiled a list of NT markers that were expressed, and established which ones were significantly DE (Table 1). We were surprised that, in addition to cholinergic transcripts, vTel^{y321} neurons also express several GABAergic specific transcripts including *slc32a1* (the vesicular GABA transporter, *vgat*) *and* glutamate decarboxylase genes *gad2* and *gad1b* (Table 1), suggesting that vTel^{y321} neurons are both GABAergic and cholinergic.

To determine if individual vTel^{y321} neurons are cholinergic-GABAergic neurons (CGNs), we combined GABA immunolabeling and ISH for *vachtb* or *chatb*. We found that vTel^{y321} neurons are both cholinergic and GABAergic (Fig. 2B) based on expression of both *vachtb* (Fig. 2A) and *chatb* (Additional Fig. 1) overlapping with GABA staining. Approximately 92% (1,183/1,287) of GFP positive cells were GABAergic and 85% (1,093/1,287) were cholinergic. At selected anatomical positions (Fig. 2B and Methods) we found that approximately 91 ± 4.9% (670/733) of vTel^{y321} neurons in the rostral telencephalon (RT) are CGNs, expressing both cholinergic and GABAergic properties (Fig. 2C). At the mid-telencephalon position, 84.5 ± 9.31% (181/215) neurons were CGNs. The average proportion of vTel^{y321} neurons that are CGNs in the caudal telencephalon (CT) was 74.15 ± 22.9% (284/383) (Fig. 2C). In the RT and MT, vTel^{y321} neurons are largely located in the Vv and Vd though some are scattered in the dorsal pallium. We conclude that although the proportion of vTel^{y321} neurons that are CGNs is consistently large across the telencephalon, this proportion decreases in the rostral to caudal direction.

The CGN phenotype is constant throughout development

Previous studies suggest that NT fate acquisition could be sequential, in which neurons first develop as single NT neurons and later express an additional NT (Spitzer, 2012, 2017, Li et al., 2020). To learn whether vTel^{y321} neurons develop their cholinergic and GABAergic fates in a particular order, or whether that order differed along the telencephalic axis of these neurons, we examined when they attained their multi-NT phenotype, and whether it occurred sequentially. If the CGN phenotype was attained in a sequential manner, we would expect to see varying proportions of vTel^{y321} neurons that were CGNs. To determine if this was the case, we in-crossed *Et^{y321}* [*Et(rex2-scp1:gal4ff)y321*] fish, raised the resultant F1 embryos to 7 dpf, and performed IHC labeling for cholinergic and GABAergic markers at multiple stages of development. These experiments enabled us to determine that the *y321* transgene begins driving GFP expression in vTel^{y321} neurons at 24 hpf. This was also the earliest timepoint we could detect GABAergic and cholinergic markers (Fig. 3A-A"), and the labeling was consistent across development (Fig. 3A-A",B-B",C-C"). These results suggest that CGNs attain their multi-NT identity very early in development and maintain it throughout life.

To learn whether the number of CGN vTel^{y321} varies from rostral to caudal during early development, we quantified the proportion of CGN vTel^{y321} neurons at 2 and 4 dpf at rostral, medial, and caudal forebrain positions. At least 80% of vTel^{y321} neurons we quantified are CGNs at 2 (81.86±10.08%, n = 4) and 4 dpf (85.99±7.15%, n = 4) (Fig. 3D). Though the general trend between 2 and 4 dpf is an increase in the percentage of CGN vTel^{y321} neurons, the increase is not statistically significant ($T_{(19.8)}$ = -0.59, p = 0.55, n = 8). In addition, there is also a general decrease in the percentage of vTel^{y321} neurons that are CGN multi-transmitter neurons in the rostral to caudal direction. At 2 dpf, 93±3.21% of vTel^{y321} neurons quantified at the RT position are CGNs and the figure declines to 85.45±2.93% at MT and 73.12 ± 9.23% at CT. At 4 dpf, 92 ± 4.73% of vTel^{y321} neurons at the RT position are CGNs, and 87.05 ± 1% at MT are CGNs and 82.31 ± 9.23% at the CT position are CGNs. Overall, the trend we observed in the proportion of vTel^{y321} neurons that are CGNs in adults is replicated at 2 and 4 dpf.

LIMTF genes are expressed in vTel^{y321} neurons throughout development

Two LIMTF genes, *lhx6* and *lhx8a*, are highly expressed in vTel^{y321} neurons (Fig. 1C and Fig. 1E). These genes show the highest DE between GFP positive and GFP negative neurons. ISH with anti-sense ribo-probes for these genes showed that they are both expressed in adult vTel^{y321} neurons (Fig. 4A-A",B-B"). To determine when these transcription factors were first expressed, we in-crossed *Et^{y321}* [*Et(rex2-scp1:gal4ff)y321*] fish and performed ISH and IHC on their offspring to detect forebrain expression of LIMTF genes. The earliest time point at which we detected LIMTFs was approximately 20 hpf (n = 12) (Fig. 4D, E); at this stage, *lhx6* and *lhx8a* forebrain expression 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 expression of 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). Our bulk sequencing data confirmed that *isl1* is highly differentially expressed in vTel^{y321} neurons (Additional Table 1). 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 are 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 embryonic forebrain in similar clusters (Fig. 4D-F) as *lhx6* and *lhx8a.* We also established that this expression is constant throughout development and can be detected in vTel^{y321} neurons in adult forebrain (Fig. 4C-C"). Additionally, labeling at 24 hpf, once GFP expression from the y321 Et driver is visible, confirms that all 3 LIMTF genes are expressed within vTel^{y321} neurons (Fig. 4G-G", H-H",I-I"). Together these observations indicate that the 3 LIMTF genes are reliable in identifying vTel^{y321} neurons and that they are expressed throughout the life of those neurons, beginning by the end of the first day of development.

The vTel^{y321} “transcriptomic fingerprint” is evolutionarily conserved

Multi-NT neurons are a common feature across vertebrates. However, a major challenge is locating homologous neurons in different vertebrate species. Locating homologous neurons across the vertebrate phyla will enable us to better understand the phylogeny of specific behaviors regulated by multi-transmitter neurons. We were thus interested in examining whether vTel^{y321} neurons are conserved in other vertebrates. To do this, we examined expression of the LIMTF genes, *lhx8a*, *lhx6*, and *isl1*, and the NT pathway genes *gad1* and *chat*, in existing cell sequencing databases. First, we compared our bulk RNAseq-data with single cell RNA-sequencing data from 1, 3 and 7 dpf zebrafish, which was compiled into the Atlas of Zebrafish Development (Farnsworth et al., 2020). Based on expression of the transcriptomic fingerprint from our bulk RNAseq experiments described above, vTel^{y321} neurons map to Cluster 25 of the scRNA-seq Atlas (Fig. 5A). Our dataset of enhanced and statistically significant DE genes was shared with 654 genes from Cluster 25, and therefore served as additional validation of our FACS-based transcriptomic analysis. 212 of the 654 shared genes were classified as forebrain specific using the ZFIN classification.

We turned our attention to the key genes in our proposed transcriptomic fingerprint, such as the LIMTF genes, and found that all 3 of these genes are significantly expressed in Cluster 25 (Fig. 5B). Interestingly, the ohnolog for *lhx8a*, *lhx8b* was also highly expressed in the shared cluster. The data also reveal high expression of GABAergic NT markers such as the glutamate decarboxylase genes (*gad1b*, *gad2)* and other GABAergic markers such as vesicular GABA transporter (*vgat)*, and the *GABA Transporter 1* (*gat1*). There was low expression of *chata* across Cluster 25, however, we previously detected its ohnolog *chatb* in vTel^{y321} neurons using ISH. Unfortunately, *chatb* is not annotated in the Atlas gene list due to inconsistencies in genome alignment, so we were therefore unable to assess its expression via transcriptome analysis. Nevertheless, we felt confident utilizing *chata* in our analysis because there were also other cholinergic markers that we obtained through bulk RNA-sequencing.

Next, utilizing the three murine LIMTF genes (*Lhx6, Lhx8, Isl1*) and murine NT marker genes (*Gad1* and *Chat)*, we searched for a homologous population of neurons in mice using the Linnarsson Lab scRNA-seq brain atlas (http://mousebrain.org/genesearch.html) .The mouse brain atlas consists of 265 clusters segregated based on expression of tissue specific transcripts. Using the online atlas, we located two similar clusters of neurons in the telencephalon and diencephalon (Fig. 5C) denoted TECHO and DECHO1. These clusters correlate well with our data in which the LIMTF genes are expressed in two clusters, telencephalic and diencephalic (Fig. 4D-F). To test whether our transcriptomic fingerprint was unique to telencephalic and diencephalic clusters, we searched through the mouse brain atlas for other cholinergic clusters and asked whether they bore the same signature. There were six other clusters that were classified as cholinergic neurons, four of these are in the hindbrain (HBCHO1-4), one located in the midbrain (MBCHO4), and one located in the diencephalon (DECHO2) (Fig. 5C). None of these clusters

expressed all the genes in our transcriptomic fingerprint, suggesting that the fingerprint is highly specific for CGN vTel^{y321} neurons.

Discussion

We discovered that a transgenically-defined group of telencephalic neurons required for social orienting behavior in zebrafish co-express ACh and GABA. These neurons, which we refer to as vTel^{y321} neurons, co-express these NTs throughout life from as early as 24 hpf through adulthood. We also discovered that these neurons are marked by the combined expression of three LIMTFs, *isl1*, *lhx8a* and *lhx6* which are expressed starting prior to development of the multi-NT phenotype through adulthood. By combining expression of the three LIMTFs and specific NT markers, we obtained a minimal transcriptomic fingerprint that can be used to track development of these neurons. In addition, we utilized our transcriptomic fingerprint to identify a homologous population of neurons in the murine forebrain. Three broad conclusions can be made from our study: (1) A minimal transcriptomic fingerprint can be applied to identify homologous populations across species. (2) Homologous populations of neurons in the basal forebrain expressing ACh and GABA may perform similar functions in teleosts and mammals, and (3) the high abundance of multi-NT neurons in vertebrate brains requires a concerted effort to understand how dual NT release functions within specific circuits. Here we discuss these conclusions.

In this study, we identified a minimal combination of transcription factors that identify a specific cluster of neurons in the mouse brain. A similar approach showed that combinations of homeobox transcription factors are highly specific in defining neuronal sub-groups (Sugino et al. 2019). Therefore, it will be interesting to determine whether our combined approach can be applied to entire single-cell RNAseq datasets in a high throughput and automated fashion to identify neuronal cell types across species. This will be challenging due to incongruences in gene orthology, particularly for more distantly related species. However, with increasing gene ontology annotation in genome servers, these challenges should soon be remedied. In addition, the minimal fingerprints might also serve as a basis to design intersectional genetic approaches with which to target transgene expression to defined neuronal populations.

The identification of homologous populations of cholinergic and GABAergic neurons in the zebrafish and mouse forebrain raises the question as to whether these neurons also share functional homology. The expression of the three LIMTFs (*lhx6, lhx8, isl1*), together with *nkx2.1*, another homeodomain transcription factor encoding gene enriched in vTel^{y321} neurons (Fig. 1E and Additional Table 1), marks the medial ganglionic eminence (MGE) in the developing rodent brain. Indeed, *Lhx8* and *Lhx6* co-expression controls murine MGE development (Flandin et al., 2011). Zebrafish vTel^{y321} neuron transcriptional profiles map onto the profiles of TECHO and DECHO1 clusters in the mouse forebrain. To our knowledge there have been no functional studies of the TECHO or DECHO1 neuron clusters. However, the DECHO1 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 implicated in various aspects of cognition, memory and social behavior in both rodents and humans. Therefore, it is highly

possible that vTel^{y321} neurons share common functions with TECHO and DECHO1 neurons in modulating aspects of social behavior. Future studies to interrogate the level to which vTel^{y321} neuron functions are mirrored by the homologous cells in rodents will be extremely informative.

We observed that vesicular transporters for both ACh and GABA are expressed in vTel^{y321} neurons, suggesting that synaptic vesicles in these neurons are capable of being filled with these NTs. A next step to understanding the functions of these neurons within their circuits would be to investigate the possible mechanisms of release: co-transmission with mixed vesicle populations, or co-release of both NTs from the same vesicle. Release of both excitatory and inhibitory NTs at the same time, and potentially at the same synapses, presents an interesting problem: how is a consistent signal transmitted to downstream neurons and what is that signal? In some instances, multi-NT neurons might act as pattern generators with alternate release of excitatory and inhibitory NTs; Lozovaya *et al*. (2017) described a population of CGNs in the mouse striatum for which this appears to be the case. This mechanism is perhaps one of the ways that co-transmission and/or co-release of NTs likely serves an important role in the optimization and maintenance of spatio-temporal patterns of neurotransmission critical for the refinement of behavior (Vaaga *et al*., 2015).

We conclude that the approach we used to obtain a minimal transcriptomic fingerprint for a set of neurons that modulate social orienting in zebrafish is a reliable method that can be translated to other neuronal populations in other vertebrate species. It enabled us to locate phenotypically and transcriptionally overlapping neurons in the mouse brain with great specificity. The convergence of gene expression in our fingerprint between zebrafish and mouse could enable comparative functional studies across different vertebrates. In conclusion, the conservation of NT phenotype, gene expression, and functional overlap between vTel^{y321} neurons and murine neuron clusters constitute a valuable platform to investigate the ontogeny of social behavior.

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*]. All procedures 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 from a random sample of 80 7 dpf larvae expressing GFP from the Et^{y321} enhancer trap line and placed into 250-500uL Neurobasal medium on ice (n = 80). Samples were dissociated using the Worthington Papain Dissociation System (Catalog #: LK003150). Briefly, forebrains were spun at 700g for 5 minutes at RT. Neurobasal medium was removed, Papain + DNase mix was added, and samples were incubated for 30–35 min at RT with constant

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

FACS

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

RNAseq

RNA collected from 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 higher were used for sequencing. RNA libraries were created using the NuGen mRNA Selection Module (Tecan Genomics, Catalog #:0408 – 32) according to the kit protocol. Libraries were analyzed in Next Generation Sequencing (NGS) mode on an Agilent Fragment Analyzer to ensure good quality cDNA for sequencing. Libraries were then run in single-read sequencing on an Illumina HiSeq 4000®.

In situ hybridization

Randomly selected adult *Et*^{y321} zebrafish (2–12 months, n = 4), embryos (16–48 hpf, n = 20) and larvae (2–7 dpf, n = 28 [4 samples per developmental stage]) were randomly selected and anesthetized and euthanized in ice water, decapitated, and placed in 4% PFA for 1-1.5 hours before brains were dissected and fixed overnight in 4% PFA at RT. After fixation, brains were rinsed 3x in PBS, dehydrated in 20% sucrose in PBS for 24 hours, and cryosectioned after mounting in agarose. RNA *in situ* hybridization on 16 mm brain sections was carried out according to the protocol of Yan *et al.* (2011), using digoxigenin labeled probes for *lhx8a, gad2*, *gad1b*, *vachtb* and *chatb.* Sections were incubated overnight at 65ºC with 200ng of probe per section. The following day, sections were washed in a graded series of 5X saline sodium citrate (SSC) with 50% formamide to 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) with Perkin Elmer® block buffer overnight at 4ºC. Sections were washed 8X for 10 min each in TNT and incubated for 5 min in the dark with Perkin Elmer Amplification Diluent, then incubated for 1hr 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 1hr. 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. See Key Resource Table 1 in Additional File 1 for more information on antibodies and Key Resource Table 2 for accession numbers and references for ISH probes.

Immunolabeling

Sections were washed 3X for 5 min per wash in PBS followed by a single wash in 0.1% TritonX in PBS ($PBST_x$) to permeabilize 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. Excess 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. To validate IHC, ISH with *gad1b* and *gad2* ISH probes revealed more than 90% overlap with GABA antibody labeling (Additional Fig. 2). Specificity of the antibody was confirmed by testing on spinal cord preparations of intact larvae and sections (Additional Fig. 3). See Key Resource Table 1 in Additional File 1 for more information on antibodies.

Cell quantification and analysis

Regions of the telencephalon were selected for quantification using the following landmarks:

(i) 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 (ac). In this position, $vTel^{y321}$ neurons occupy the anterior part of the parvocellular preoptic nucleus and the supracommisural nucleus(Vs) of the ventral telencephalon.
(iii) Caudal telencephalon (CT), at the junction of the forebrain and optic tectum. 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 (n = 4)* and *vachtb(n = 4)* was quantified using ImagePro Plus version 6.0 ®. GFP expressing cells were manually identified in a separate image channel and thresholding performed. Data tagged cell templates identified in the GFP channel were loaded onto a second image channel with either *vachtb* or *chatb* labeling. In this channel (in this case, *vachtb)*, the fluorescence intensity was assessed for the tagged cell regions. This allowed for quantification of data points which were GFP, *vachtb* or *chatb* positive. Updated points from the combination of GFP and *vachtb* points were then transposed to the third channel (GABA) and the fluorescence intensity for that channel was assessed. Each of the channel specific fluorescent intensity data from tagged points was exported into Microsoft Excel. In Excel, the fluorescent intensity for all channels was normalized through subtraction of the sum

of the average background intensity (Aμ) and the standard deviation (Aμ+∂) i.e. (Individual intensity – [Aμ + ∂)]. These data were then used to determine the number of GFP neurons that were GABAergic and/or cholinergic. Plots and graphs for the data were generated in R Studio®. For all developmental stages, a sample size of at least 4 animals were used with quantification of GFP neurons that were cholinergic and GABAergic quantified at the 3 anatomical landmarks (RT, MT, and CT). To avoid confirmation bias, all slides with samples for control and analysis were assigned blindly.

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 run 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 custom construct (EGFP sequence) were 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. Samtools® (version 1.5) was then used to convert aligned .fastq reads to .bam files, then sorted and indexed. 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 Ensemble Biomart and then used for processing in DESeq2 in R Studio® (R version R.3.5.2). From here, data were analyzed in R using the DESeq2 pipeline instructions provided by Love *et al.*(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.

Abbreviations

ACh: acetylcholine; CT: caudal telencephalon; CGN: cholinergic-GABAergic neuron; DE: differentially expressed; dpf: days postfertilization; Et: enhancer trap; FACS: fluorescence activated cell sorting; GABA: gamma-aminobutyric acid; GPCR: G-protein coupled receptor; IHC: immunohistochemistry; ISH: *in situ* hybridization; LIMTF: LIM homeobox transcription factor; MT: medial telencephalon; NT: neurotransmitter; RT: rostral telencephalon; Vd: ventral dorsal; vTel: ventral telencephalon; Vv: Ventral ventral.

Declarations

Ethics Approval

All animal procedures were carried out in accordance with the relevant guidelines and regulations under an approved protocol with the University of Oregon Animal Care and Use Committee. All the experiments were conducted in line with the ARRIVE guidelines for in-vivo experimental scientific research.

Availability of Supporting Data

The data set supporting the results of this article are available in the LabArchives repository (https://mynotebook.labarchives.com/login) with file names: Vtel_y321_Bulk_RNAseq.xlsx and 654_Genes_overlap_with_Vtel_y321_FACS.xlsx, in Folders: Conserved Transcriptional Fingerprint/RNAseq Data.

Competing Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author Contributions

Conceptualization: P.W., J.S.E., D.N.; Methodology: D.N., J.S., A.T., D.R.F., J.B. Investigation: D.N., J.S.; *Software and Formal Analysis*: D.N., J.S., A.T., D.R.F., A.C.M.; *Writing Original Draft*: D.N.; *Review and Editing*: D.N., P.W., J.S.E.; *Funding Acquisition*: P.W., J.S.E.; *Supervision*: P.W.

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Figures

Figure 1

vTel^{y321} transcriptome confirms its transgenic origin. **A** Dorsal view of vTel^{y321} transgene expression in the forebrain and the dissection plane (dotted red line) for dissociation. Scale bar = 100µm. **A'** FACS isolation of cell cluster (circled) after dissociation. **A"** Graph shows cutoff between live (1) (88.33%), and dead (2) cells (11.67%). **A"'** Proportions of GFP positive (3.69%) and negative cells (92.09%) from the live cell fraction. **B** Principal component analysis of GFP positive and negative samples after variance stabilization transformation of gene count data. Sample 3 in the GFP negative fraction was dropped from subsequent analysis as an outlier. **C** Volcano plot of the ~23,000 genes expressed in the samples. Gray:

Not significantly enriched and/or no fold change; Blue: significant differential expression ($p<0.05$) but with fold change below threshold; Green: Up or downregulated (positive or negative fold change) without significant differential expression; Red: Significantly differentially regulated**. D** Heatmap of the 20 genes with the greatest differential expression between GFP positive and GFP negative samples. **E** Heatmap of the 20 forebrain specific genes with the greatest differential expression between GFP positive and GFP negative samples.

Figure 2

vTel^{y321} neurons are both cholinergic and GABAergic. **A** Coronal section of the adult ventral forebrain in the rostral telencephalon showing expression of the Et y321 transgene-dependent GFP, mRNA expression of vachtb and GABA immunolabeling. **A'** Inserts of selected portion of sections in **A**. Arrows point to selected vTel^{y321} neurons that express vachtb and GABA (Scale bars: A = 100μm; A' = 50μm). **B** Coronal sections of ventral forebrain depicting known nuclei in the forebrain that include vTel^{y321} neurons and anatomical positions used for quantification of vTel^{y321} CGNs (RT, rostral telencephalon; MT, mid-telencephalon; and CT, caudal telencephalon; Vv, Ventral ventral nucleus of the telencephalon; Vd, ventral dorsal nucleus of the telencephalon; Vs, supracommisural nucleus; PPa, Parvocellular pre-optic nucleus; ac, anterior commissure) (scale bar: B = 100μm). **C** Boxplots showing percentage of adult vTel^{y321} neurons that are cholinergic and GABAergic at selected anatomical forebrain landmarks.

Figure 3

CGN phenotype is constant throughout development. Expression of cholinergic and GABAergic markers between 1-5 dpf. **A** Transverse section at 1 dpf showing expression of cholinergic(ChAT) and GABAergic(GABA) immunolabeling. **A',B'** and **C'** Merged images showing vTel^{y321} neurons labeled that are cholinergic and GABAergic from 1dpf to 4dpf**. A", B" and C"** inserts from areas defined by in **A',B'** and **C'**. Arrows in insert A", B" and C" show selected vTel^{y321} neurons that are cholinergic and GABAergic. **B**, **C** Coronal sections of ventral telencephalon showing cholinergic and GABAergic markers at 2 and 4 dpf. (Scale bar for all images = 20μm). **D** Boxplots showing the percentage of vTel^{y321} neurons that are CGNs at 2 and 4 dpf.

Figure 4

LIMTF genes are expressed throughout development. **A-C** Coronal sections of adult ventral forebrain showing (left to right) expression of lhx8a, lhx6 and isl1 in adult vTel^{y321} neurons. Selected neurons that express the representative transcripts are denoted by white arrows. **D-F** Wholemount chromogenic ISH in

20 hpf embryo showing expression of lhx8a, lhx6 and isl1 (left to right). Arrows label the principal foci of LIMTF gene expression in the brain. Magenta arrows denote diencephalic clusters and white arrows denote telencephalic clusters respectively at that timepoint. islet1 is more broadly expressed compared to lhx8a and lhx6. **G-I** 24 hpf larval forebrain transverse sections showing LIMTF gene expression in vTel^{y321} neurons. **G** lhx8a mRNA expression in 24hpf in vTel^{y321} neurons. **H** lhx6 mRNA expression in in vTel^{y321} neurons. **I** islet1 protein expression in in vTel^{y321} neurons. (Scale bar in A, A', A" to C, C', C" and G, G', G" to I, I', I" = 30μm; D-F = 50μm).

Figure 5

vTel^{y321} "transcriptomic fingerprint" is evolutionarily conserved. **A** Single cell RNA-sequencing data from Atlas of Zebrafish Development plotted using Uniform Manifold Approximation and Projection (UMAP) algorithm. **B** "Transcriptomic fingerprint" expressed genes map onto Cluster 25 of the Atlas and their expression is plotted in UMAP space. vgat expression in Cluster 25 included as a corroboratory marker. **C** Search result from the mousebrain.org online sc RNAseq Atlas show that combined expression of Lhx6, Isl1, Lhx8, Gad1 and Chat maps onto telencephalic (**TECHO)** and diencephalic (**DECHO1**) clusters in the mouse brain. Other cholinergic clusters HBCHO1-4, MBCHO4, and DECHO2 do not express the full transcriptomic fingerprint.

Supplementary Files

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