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A novel subset of enteric neurons revealed by *ptf1a*:GFP in the developing zebrafish enteric nervous system

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Abstract

The enteric nervous system, the largest division of the peripheral nervous system, is derived from vagal neural crest cells that invade and populate the entire length of the gut to form diverse neuronal subtypes. Here, we identify a novel population of neurons within the enteric nervous system of zebrafish larvae that express the transgenic marker *ptf1a*:GFP within the midgut. Genetic lineage analysis reveals that enteric *ptf1a*:GFP$^+$ cells are derived from the neural crest and that most *ptf1a*:GFP$^+$ neurons express the neurotransmitter 5HT, demonstrating that they are serotonergic. This transgenic line, Tg(*ptf1a*:GFP), provides a novel neuronal marker for a subpopulation of neurons within the enteric nervous system, and highlights the possibility that Ptf1a may act as an important transcription factor for enteric neuron development.

Keywords

serotonergic neuron; ptf1a; enteric nervous system; zebrafish

Introduction

The enteric nervous system (ENS) is comprised of interconnecting ganglia within the myenteric and submucosal plexuses that run along the gut wall (Bornstein et al., 1984). The ENS regulates gastrointestinal motility and, as the largest portion of the peripheral nervous system, is often referred to as the “second brain” (Furness, 2006). The ENS is largely derived from the “vagal” neural crest that arises from the dorsal neural tube in the post-otic region (LeDouarin and Teillet, 1973; Anderson et al., 2006). Vagal neural crest cells migrate away from the neural tube, moving ventrally toward the anterior foregut. After entering the foregut, they change direction and begin migrating caudally such that they eventually populate the entire gut, a migration process that takes days and is the longest of any embryonic cell migration. Once reaching their final destinations, enteric neural crest (ENC) differentiate into a diverse array of enteric neurons and glial cells along the entire length of the gut (Furness, 2006; Sasselli et al., 2012). Because improper neural crest development gives rise to developmental defects such as Hirschsprung’s disease (colonic aganglionosis) (Bergeron et al., 2012), there has been great interest in understanding the migration, specification and differentiation of enteric neural crest.

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Although recent studies have identified various neurotransmitter markers of terminally differentiated enteric neuron subtypes within the ENS (Uyttebroek et al. 2010; rev. in Sasselli et al., 2012), much less is known about the transcription factors that mediate terminal differentiation towards a particular neuron lineage. Nonetheless, some experiments have shed light on this issue. For example, a null allele of *Ascl1*, a basic helix-loop-helix (bHLH) transcription factor, results in a complete loss of all serotonergic neurons from the ENS, demonstrating its requirement in differentiation of this enteric neuron subtype (Blaugrund et al. 1996). Analogously, inactivation of the bHLH transcription factor *Hand2* in neural crest cells leads to disrupted ganglia patterning and selective loss of VIP^+ neurons, indicating its importance in differentiation of peptidergic enteric neurons in the mouse gut (Hendershot et al. 2007). However, understanding of the transcription factor code responsible for enteric neuron subtype specification and terminal differentiation remains limited. Accordingly, identification of novel factors expressed in the developing ENS will highlight candidate genes that may be involved in these processes.

Pancreas specific transcription factor 1a, *ptf1a*, is expressed in the developing zebrafish pancreas (Lin et al., 2004; Zecchin et al., 2004), retina (Jusuf and Harris, 2009) and cerebellum (Kani et al., 2010) during embryonic development. Ptf1a plays a critical role in zebrafish ventral pancreas specification and exocrine pancreas development (Lin et al., 2004; Zecchin et al., 2004; Dong et al., 2008), as well as functioning during inhibitory neuron subtype differentiation in the retina (Dullin et al., 2007; Fujitani et al., 2006), cerebellum (Hoshino et al., 2005). Given the essential roles of Ptf1a in the subtype specification of various neuron types within the nervous system, we sought to examine if it was also present within the developing ENS. Here, our results using the transgenic fish line Tg(*ptf1a*:GFP) reveal that *ptf1a* is expressed in a subset of neurons in the developing ENS of zebrafish larvae. Thus, these data identify Ptf1a as a novel neuronal marker in the developing ENS.

Results

The transgenic line Tg(ptf1a:GFP) labels a subset of ENS neurons during larval stages of zebrafish development

The transgenic line Tg(*ptf1a*:GFP) previously has been shown to mark cells that express *ptf1a in vivo* (Godinho et al., 2007; Jusuf and Harris, 2009; Kani et al., 2010). To examine if cells that express *ptf1a* are present in the developing ENS, *ptf1a*:GFP^+ larvae were examined from 3 to 6 days post fertilization (dpf), the time during which terminal differentiation commences in the developing zebrafish ENS. Whole mount confocal microscopy revealed that *ptf1a*:GFP^+ cells were first present at 4 dpf in the midgut (Fig. 1A), and persisted at 5 dpf and 6 dpf (Fig. 1B,C), with a few *ptf1a*:GFP^+ cells also seen in the intestinal bulb (foregut) by 6 dpf (Fig. 1F). There was an average of 13 *ptf1a*:GFP^+ cells in the midgut at 4 dpf, 19 cells at 4 dpf and 21 cells at 6 dpf (Fig. 1D). GFP^+ cells were also present in other regions of the embryo, such as the pancreas (Fig. 1F). In order to detect endogenous *ptf1a* transcripts in the developing ENS, we performed in whole mount *in situ* hybridization. Consistent with the distribution of cells observed in the transgenic line, this

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analysis revealed the presence of *ptf1a* transcripts in the developing brain, pancreas and the midgut, but not the hindgut, at 6 dpf (Fig. 1G,H).

To assess if the *ptf1a*:GFP$^+$ cells observed in the gut were neurons, we performed double whole mount immunochemistry against GFP and pan-neuronal markers, Hu or acetylated tubulin. At 4 dpf, confocal images revealed that a minority of *ptf1a*:GFP$^+$ cells co-localized with Hu (Fig. 1A′,A″), or with acetylated tubulin, as seen in both static confocal images and a 3D rendering movie (Fig. 2A′,A″; Supporting Information Movie 1). However, at 5 and 6 dpf, a majority of *ptf1a*:GFP$^+$ cells co-localized with Hu (Fig. 1B′,B″-C′,C″; Supporting Information Movie 2) and acetylated tubulin (Fig. 2B′,B″-C′,C″), revealing their neuronal identity. At 6 dpf, analysis of confocal stack projections along the z-axis revealed that the *ptf1a*:GFP$^+$ cells were located in a concentric pattern along the outer layer of the gut tube, co-localizing with Hu (Fig. 1E). These data indicate that *ptf1a*:GFP$^+$ cells are present within the gut prior to their terminal differentiation into neurons at 4 dpf, and that its expression is maintained in differentiated neurons at 5 and 6 dpf. At 5 dpf, quantification revealed that an average of 28% of Hu$^+$ neurons were *ptf1a*:GFP$^+$ in the midgut, while at 6 dpf 36% were *ptf1a*:GFP$^+$ (Fig. 1I), demonstrating that *ptf1a* is not expressed in all enteric neurons, but rather in a subset of neurons.

Previously, it has been shown that ~30% of midgut neurons contain the neurotransmitter serotonin (5HT) at 5 dpf (Uyttebroek et al., 2010). In order to determine whether *ptf1a*:GFP$^+$ neurons in the midgut were also 5HT$^+$, we performed double immunostaining of *ptf1a*:GFP larvae with antibodies against GFP and 5HT. At 5 dpf, an average of 97% of *ptf1a*:GFP$^+$ cells were 5HT$^+$ (Fig. 3A–A″), indicating that the *ptf1a*:GFP$^+$ neuron population are largely serotoninergic neurons within the larval midgut.

ptf1a:GFP+ cells in the developing ENS are derived from the neural crest

ptf1a is expressed in the developing pancreas, where it plays key roles in regulating pancreas formation (Lin et al., 2004). On the other hand, in the brain and retina, it is expressed in differentiating neuron subtypes (Dullin et al., 2007; Kani et al., 2010) during neurogenesis and retinogenesis, respectively. To date, *ptf1a* expression has not been described in neural crest derived structures. To ascertain whether the *ptf1a*:GFP$^+$ neurons observed in the gut were neural crest derived, we crossed Tg(*ptf1a*:GFP) fish with the Tg(*-4725sox10:Cre;elf1a*:loxp-GFP-loxp-dsRedpA) line, which permanently labels neural crest cells and all of their derivatives by ubiquitous expression of the dsRed fluorescent protein (Rodrigues et al., 2012), allowing for lineage analysis *in vivo*. In live embryos at 5 dpf, 100% of *ptf1a*:GFP$^+$ cells in the gut were also positive for dsRed, indicating that they were indeed derived from the neural crest cells (Fig. 3B–B″).

Discussion

In this study we report the presence of a novel population of *ptf1a*:GFP$^+$ neurons located within the midgut of the developing ENS of zebrafish larvae, a majority of which are serotonergic. This is the first report describing *ptf1a* expression within the ENS in any organism. This line can be used as an *in vivo* marker of differentiating and terminally differentiated neurons, thus serving as a useful tool for developmental studies of the ENS.

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During zebrafish ENS differentiation, enteric progenitors migrate caudally along the gut tube, within the gut mesenchyme, fully populating the gut by 3 dpf. Subsequently, these enteric progenitors undergo extensive proliferation in order to generate thousands of enteric neurons in the appropriate proportions and in precise locations within the adult zebrafish gut (Uyttebroek et al., 2010). That we did not detect *ptf1a*:GFP$^+$ cells prior to 4 dpf indicates that it is activated after the initial neural crest invasion of the gut, suggesting a possible later role during enteric neuroblast proliferation and/or terminal differentiation. In support of this idea, we found that *ptf1a*:GFP$^+$ cells in the gut co-localized with the neuronal markers Hu and acetylated tubulin, as well as largely co-localizing with the neurotransmitter serotonin, 5HT. During zebrafish ENS development, serotonergic neurons encompass between ~25–30% of total neurons in the larval gut by 5 dpf (Uyttebroek et al., 2010). In adult fish, these proportions are maintained in the proximal gut and midgut, while in the hindgut the proportion reduces sharply to ~10% of total neurons (Uyttebroek et al., 2010). Interestingly, within the developing Xenopus retina, Ptf1a regulates the balance of inner nuclear layer neuron subtypes, specifically controlling the number of GABAergic interneurons born during retinogenesis (Dullin et al., 2007). Ectopic expression of *ptf1a* was sufficient to increase the number of 5HT$^+$ neurons, while Ptf1a loss decreased the number of 5HT$^+$ neurons within the retina (Dullin et al., 2007), suggesting an important role for Ptf1a in the allocation or differentiation of serotonergic neuron subtypes. In conjunction with these previous studies, our discovery of the presence of *ptf1a*:GFP$^+$/5HT$^+$ cells in the gut suggests that Ptf1a may play similar roles. Future studies aimed at further investigating the role of Ptf1a in development of enteric neuron subtypes, as well as its functional regulation within the ENS, will provide novel insight into the mechanisms underlying neurogenesis within the peripheral nervous system.

Materials and Methods

Zebrafish maintenance and fish lines

Zebrafish (*danio rerio*) were maintained at 28.5°C on a 13-hour light/11 hour dark cycle. Animals were treated in accordance with California Institute of Technology IACUC provisions. The Tg(*ptf1a*:GFP) line (Godinho et al., 2007) and/or Tg(*-4725sox10:Cre;elf1a*:loxp-GFP-loxp-dsRedpA) line (Rodrigues et al., 2012) was used for all experiments.

in situ hybridization

Hybridizations were performed as previously described (Uribe and Gross, 2010), with the addition of a 20 minute Collagenase type 1A (Sigma C9891) digestion (1 mg/mL) prior to Proteinase K digestion to facilitate penetration of the probe. To generate *ptf1a* probe, the following primers were used to PCR amplify template containing a T7 polymerase site from pCS2-ptf1a (Zhang et al., 2012), forward 5′ CGACGATGACTTCTTTACGGACC 3′ and reverse 5′ TAATACGACTCACTATAGGTTCCTCGGTGGCAAATGATG 3′, with the T7 site underlined. T7 polymerase was used to generate antisense probe.

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Whole mount immunochemistry

Larval fish at 4 dpf, 5 dpf and 6 dpf were fixed overnight at 4°C in 4% PFA, rinsed three times in 1X PBS, incubated in 100% Methanol at −20°C for 1 hour and then rehydrated step-wise into 1X PBS at room temperature. Larvae were then incubated in 100% acetone at −20°C for 11 minutes, rinsed three times in 1X PBS, then digested at room temperature in 10 μg/mL Proteinase K for 45 min. (4 dpf), 55 min. (5 dpf) or for 65 min. (6 dpf). Larvae were then rinsed three times in 1X PBS, fixed for 10 minutes in 4% PFA at room temperature, rinsed 3 times in 1X PBS and then incubated in 5% Donkey serum block diluted in 1X PBS-tween-20, supplemented with 1% DMSO (PBTD), for 3 hours. Embryos were then incubated in either rabbit anti-GFP 1:500 (Life Technologies, A-11122), goat anti-GFP 1:500 (Abcam, ab6673), mouse anti-HuC/D (Hu) 1:200 (Invitrogen), mouse anti-Acetylated tubulin 1:1000 (Sigma, T6793) or rabbit anti-5HT 1:1000 (Immunostar) overnight at 4°C. Embryos were then washed out of primary antibody in 1X PBS-tween-20, then incubated at room temperature in 1:700 secondary antibodies Invitrogen Alexa Fluor Donkey anti-Rabbit 488, anti-Rabbit 647, anti-Goat 488 or Donkey anti-Mouse 594 for 3 hours at room temperature. Embryos were rinsed in 1X PBST and imaged in 75% glycerol/1X PBS on a Zeiss 710 2-photon confocal microscope (Beckman Imaging Center, Caltech).

Live imaging

Embryos positive for both Tg(*ptf1a*:GFP) and Tg(*-4725sox10:Cre;elf1a*:loxp-GFP-loxp-dsRedpA) were mounted laterally in 1% low melt agarose dissolved in fish water supplemented with 1X PTU and Tricaine anesthetic, in an imaging chamber. 20–80 micron Z-stacks were acquired using a 20x objective on a Zeiss LSM 710 microscope. Z-stacks were compiled and exported using Imaris Image Analysis software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ENS	Enteric Nervous System
ENC	Enteric Neural Crest

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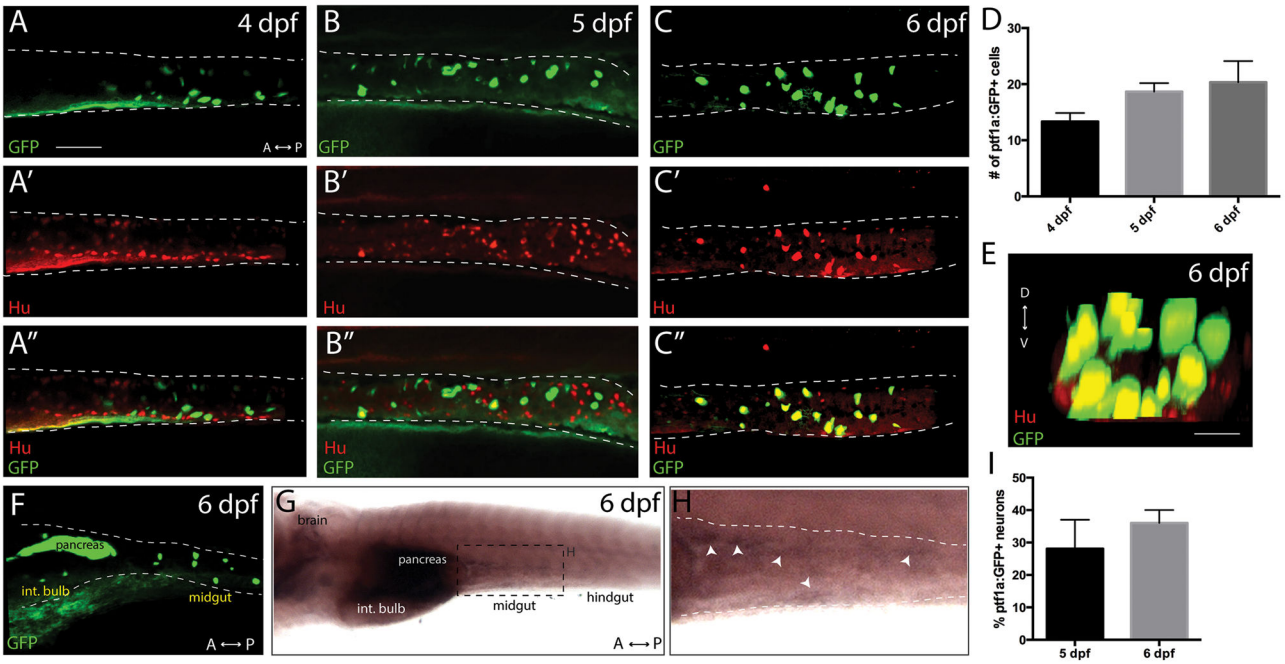

Figure 1. The transgenic line Tg(*ptf1a*:GFP) marks a subset of neurons within the larval ENS

Maximum projection confocal stacks at (A–A″) 4 dpf, (B–B″) 5 dpf and (C–C″) 6 dpf depicting *ptf1a*:GFP$^+$ and Hu$^+$ cells within the midgut (dashed lines), scale bar: 70 microns, for A–C. (D) Bar graph illustrating total number of *ptf1a*:GFP$^+$ cells in the gut from 4 dpf to 6 dpf, error bars represent s.e.m., n=6. (E) Maximum intensity confocal projection along the z-axis reveals that *ptf1a*:GFP$^+$/Hu$^+$ cells co-localize in a concentric pattern along the gut tube at 6 dpf, scale bar: 100 microns. (F) Lateral view of a 6 dpf *ptf1a*:GFP$^+$ larval fish. (G,H) Ventrolateral view following whole mount *in situ* hybridization against *ptf1a* reveals that *ptf1a* localizes to the brain, pancreas and midgut of 6 dpf larvae. (I) Bar graph illustrating the percentage of total Hu$^+$ neurons that are *ptf1a*:GFP$^+$ in the midgut at 5 dpf and 6 dpf, error bars represent s.e.m., n= 6. Int. bulb-intestinal bulb.

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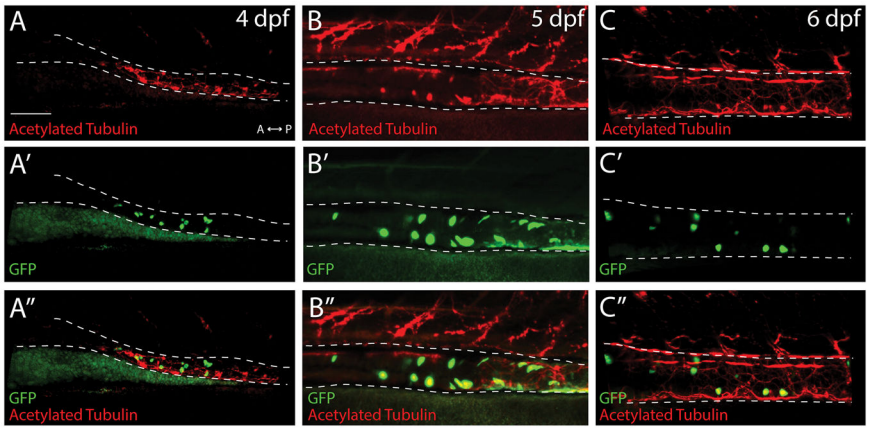

Figure 2. *ptf1a*:GFP^{+} cells co-localize with the neuronal marker acetylated tubulin in the gut

Maximum projection confocal stacks at (A–A″) 4 dpf, (B–B″) 5 dpf and (C–C″) 6 dpf depicting *ptf1a*:GFP^{+} and Acetylated tubulin^{+} cells within the midgut (dashed lines), scale bar: 70 microns.

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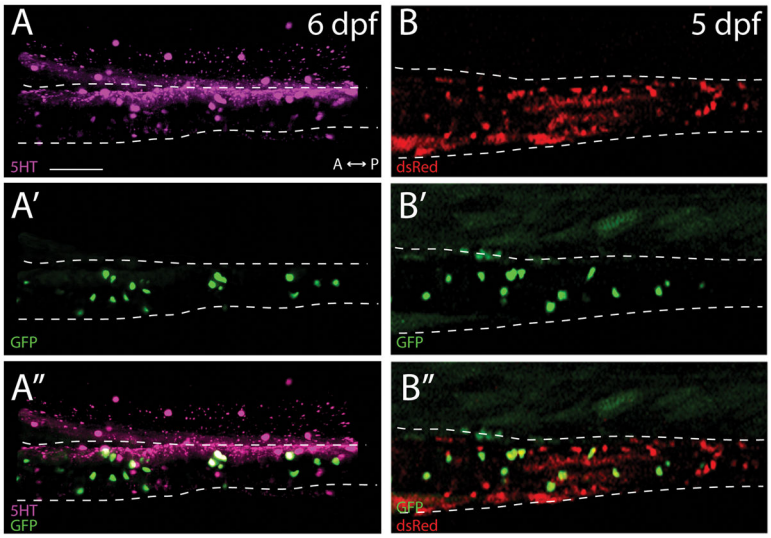

Figure 3. *ptf1a*:GFP cells are largely serotonergic enteric neurons that are derived from the neural crest

Maximum projection confocal stack of the midgut (A–A″) reveal that *ptf1a*:GFP^{+} cells co-localize with the neurotransmitter 5HT at 6 dpf. (B–B″) Images of live triple transgenic larval fish, *ptf1a*:GFP;*-4725sox10:Cre;elf1a*:loxp-GFP-loxp-dsRedpA, shows that *ptf1a*:GFP^{+} cells are neural crest derived. Scale bar: 70 microns.