LETTER

doi:10.1038/nature21679

Ancient evolutionary origin of vertebrate enteric neurons from trunk-derived neural crest

Stephen A. Green[1]*, Benjamin R. Uy[1]* & Marianne E. Bronner[1]

The enteric nervous system of jawed vertebrates arises primarily from vagal neural crest cells that migrate to the foregut and subsequently colonize and innervate the entire gastrointestinal tract. Here we examine development of the enteric nervous system in the basal jawless vertebrate the sea lamprey (*Petromyzon marinus*) to gain insight into its evolutionary origin. Surprisingly, we find no evidence for the existence of a vagally derived enteric neural crest population in the lamprey. Rather, labelling with the lipophilic dye DiI shows that late-migrating cells, originating from the trunk neural tube and associated with nerve fibres, differentiate into neurons within the gut wall and typhlosole. We propose that these trunk-derived neural crest cells may be homologous to Schwann cell precursors, recently shown in mammalian embryos to populate post-embryonic parasympathetic ganglia[1,2], including enteric ganglia[3]. Our results suggest that neural-crest-derived Schwann cell precursors made an important contribution to the ancient enteric nervous system of early jawless vertebrates, a role that was largely subsumed by vagal neural crest cells in early gnathostomes.

The enteric nervous system (ENS) includes thousands of interconnected ganglia embedded within the wall of the gut[4,5], making it the most complex portion of the peripheral nervous system in amniotes. The ENS of jawed vertebrates innervates the entire gastrointestinal tract to regulate muscle contraction, chemosensation, water balance, and gut secretion[6]. Classic transplantation experiments have demonstrated that the neurons and glia of the gut are largely derived from the 'vagal' population of neural crest cells that arise within the post-otic portion of the hindbrain[7,8]. They subsequently migrate to the foregut and embark upon the longest migration of any embryonic cell type, from foregut to hindgut.

The sea lamprey is a jawless (agnathan) vertebrate and an experimentally tractable representative of the cyclostomes. As the sister group to jawed vertebrates (gnathostomes), lampreys are an important model for identifying traits common throughout vertebrates. Lampreys possess migrating neural crest cells that give rise to many cell types, including melanocytes, cartilage, sensory neurons, and glia, but lack other neural-crest-derived structures that are present in gnathostomes such as jaws and sympathetic chain ganglia[9,10]. Given that lamprey embryos lack sympathetic ganglia, we sought to examine other components of the autonomic nervous system, with a focus on the ENS. Adult lamprey have a simple ENS that includes a ganglionated plexus of serotonin (5-HT)-producing cells, as well as a smaller number of catecholamine-containing neurons[11,12]. However, the developmental origin of these enteric neurons is unknown.

As a first step in analysis of lamprey ENS development, we examined the time course of neuronal appearance along the developing embryonic gut by performing *in situ* hybridization for *Phox2b*, which is expressed in enteric neurons in many jawed vertebrates[5,9]. Expression of lamprey *Phox2b* is detectable along the gut from early stages (Tahara stage 25 (T25)[9]), and by stage T28 or embryonic day 20 (E20), *Phox2b*-expressing cells are associated with a depression in the gut that will become the typhlosole, a haematopoietic tissue associated with elements of the ENS (Fig. 1a, b). Using a 5-HT antibody, serotonin-immunoreactive neurons were first observed within the gut wall at ~T28.5 (Fig. 1c, d) in the anterior trunk region. With time, the numbers increased, and 5-HT$^+$ neurons were noted progressively posteriorly, with particularly high cell numbers in the cloacal region, as reported previously[13]. By T30 (E30), there are >100 neurons along the gut in association with the typhlosole and vagus nerve (Fig. 1e and Extended Data Fig. 1). Interestingly, individual serotonergic cells were also often associated with axon bundles emanating ventrally from the dorsal root ganglia (Fig. 2f and Extended Data Fig. 1a). In addition to 5-HT$^+$ neurons, we also noted 5-HT$^+$ columnar cells that may have represented gut enterochromaffin cells (Extended Data Fig. 1a, b).

We next sought to determine the embryological origin of the neurons in the gut by performing lineage labelling with the lipophilic dye DiI[14]. In chicken, vagal neural crest cells that contribute to the ENS arise from the hindbrain neural tube adjacent to somites 1–7 (ref. 7). After exiting the neural tube, they migrate ventrally, invade the foregut, and undergo a collective cell migration along the rostrocaudal extent of the gut. To test whether lamprey possess a homologous cell population, we performed focal injections of DiI into the dorsal portion of the caudal hindbrain (corresponding to the site of origin of vagal neural crest in gnathostomes) of T20 (E6) embryos. Regardless of the exact injection site, dye-labelled cells spread within the hindbrain and emigrated from the neural tube as a stream directly above the pharynx (Extended Data Fig. 2a–c). From this site, they progressed ventrally, then turned

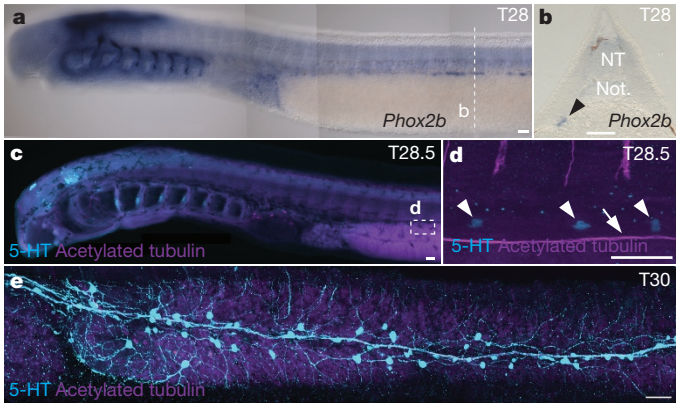


Figure 1 | Early formation of enteric neurons in the lamprey *P. marinus*. **a**, *Phox2b* expression in dorsolateral cells of a T28 embryo, with anterior to left and dorsal up. Letter 'b' marks the site of the cross-section in **b**. **b**, Transverse cross-section of a T28 embryo shows *Phox2b* expression in the depression of the typhlosole (black arrowhead). NT, neural tube; Not., notochord. **c**, 5-HT and acetylated tubulin immunoreactivity in a slightly older T28.5 embryo. **d**, 5-HT is detectable in neurons (white arrowheads) adjacent to the vagus nerve (white arrow). **e**, Serotonergic neurons form small ganglia within the enteric plexus of a T30 embryo. Scale bar, 50 μm.

[1]Division of Biology and Biological Engineering, California Institute of Technology, Pasadena, California 91125, USA.
*These authors contributed equally to this work.

© 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

caudally to populate all of the branchial arches (Extended Data Fig. 2g), similar to previously reported migration patterns of cranial neural crest cells[15,16]. However, despite many focal injections ($n = 46$) into the caudal hindbrain homologous to 'vagal', we failed to find evidence of DiI-labelled cells in the gut. We next looked for expression of the vagal neural crest marker, *Ret*, in lamprey, since it is required for vagal neural crest development in gnathostomes[17–19]. To this end, we cloned a lamprey *Ret* homologue and its co-receptor *Gfra1* and examined their expression patterns by *in situ* hybridization. The results showed lamprey *Ret* and *Gfra1* are expressed in many parts of the embryo, including the typhlosole. *Ret* and *Gfra1* expression were first observed in a region anterior to the gut at T26 (Extended Data Fig. 2i, j) in a manner reminiscent of *Phox2b* expression (Extended Data Fig. 2k). However, we did not observe expression in neural crest cells migrating from caudal hindbrain towards the gut, raising the intriguing possibility that enteric neurons might arise from a different cellular source.

In gnathostomes, migrating trunk neural crest cells fail to invade the immediately underlying gut owing to the presence of repulsive signals such as Slit that block their entry[20]. Recently, however, a secondary contribution to the mammalian ENS has been uncovered that comes not from vagal neural crest but rather from trunk neural-crest-derived Schwann cell precursors (SCPs) associated with extrinsic nerves that contribute postnatally to calretinin-containing neurons of the gut[3]. Using *Dhh-cre* mice to lineage trace immature Schwann cells, it was found that ~20% of colonic neurons in mice were derived from trunk SCPs[3]. Intriguingly, this population persists in *Ret*-null mice[3]. To examine the possibility that a homologous population may exist in lamprey, we asked whether cells emerging from the trunk neural tube might contribute to neurons of the gut. To test this, we performed focal DiI injections into the dorsal trunk neural tube as well as neural tube lumen injections following its cavitation at T22–T23 (~E8), thus labelling the entire neural tube as well as presumptive neural crest cells before their emigration. Both labelling techniques yielded similar results. By T25 (E14), such injections label neural-crest-derived cells in dorsal root ganglia and mesenchyme cells of the fin[9]. Interestingly, at later stages, we observed DiI-labelled cell bodies closely associated with nerve processes, recognized by acetylated tubulin staining above the gut (Fig. 2a–c). These individual DiI-labelled cells were visible as early as T28 (E18–E20) and persisted until the latest stages examined, T30 (E30–E35), by which time the yolk was reduced, facilitating imaging. In the trunk of lamprey, there are two nerve roots per trunk segment: a dorsal root that contains fibres emanating from neural-crest-derived dorsal root ganglia and a ventral root that contains motor-neuron nerve fibres. Interestingly, DiI-labelled cells were associated with these dorsal roots (Fig. 2c). Similarly, serotonergic presumptive neuroblasts were selectively associated with and appeared to migrate only along these dorsal root bundles (Fig. 2f), consistent with a neural crest origin.

To establish whether the DiI-labelled cells differentiated into enteric neurons, embryos were sectioned and stained with antibodies to serotonin and acetylated tubulin as a mature neuronal marker. The results, based on examination of transverse sections through 25 representative embryos at T30 (E30–E35) in which we quantitated cells that were positive for acetylated tubulin and serotonin, are summarized in Table 1. We noted numerous DiI-labelled serotonergic neurons (DiI/5-HT/acetylated-tubulin-positive cells) in the anterior gut

Table 1 | Number of DiI-labelled neurons (AcTub$^+$) in 25 representative E30–E35 embryos in which DiI had been injected into the neural tube

Location	DiI$^+$, 5-HT$^+$	DiI$^+$, 5-HT$^-$	Total DiI-labelled neurons
Oesophagus	22	2	24
Intestine (basal typhlosole)	26	0	26
Intestine (typhlosole mesenchyme)	4	5	9
Total	52	7	59

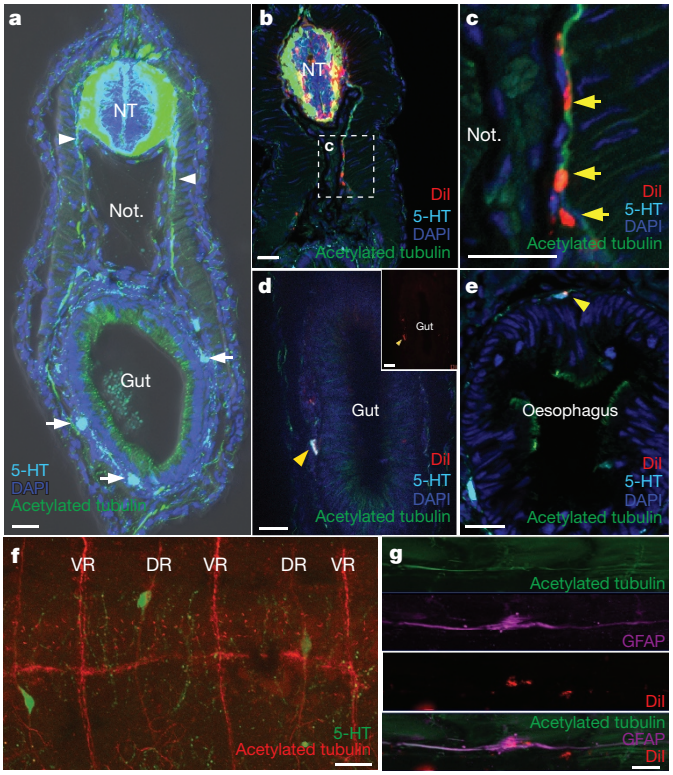


Figure 2 | DiI-labelled cells in the neural tube contribute to enteric ganglia. Immunohistochemistry of T30 lamprey transverse cryosections. **a**, Uninjected control highlighting neuronal projections (white arrowhead) and serotonergic neurons (white arrows) around the gut. Green, acetylated tubulin; blue, DAPI; cyan, 5-HT. **b**–**d**, DiI-injected 'tubefill' embryos. Red, DiI; green, acetylated tubulin; cyan, 5-HT; blue, DAPI. **b**, **c**, DiI (yellow arrows) within the neural tube, dorsal root ganglia, and along axon bundles. **d**, A DiI-labelled enteric neuron (acetylated tubulin$^+$, 5-HT$^+$; yellow arrowhead) within the gut wall typhlosole. Inset shows DiI only. **e**, A DiI-labelled neuron (acetylated tubulin$^+$, 5-HT$^+$) in the oesophagus (yellow arrowhead). See cell counts in Table 1. **f**, 5-HT$^+$ cells along dorsal root (DR) nerve processes but not ventral roots (VR). **g**, A DiI-labelled GFAP$^+$ cell associated with the lateral line. Scale bar, 20 μm.

(Fig. 2d), oesophagus (Fig. 2e), typhlosole, and adjacent tissues, as well as other DiI$^+$ neurons that were serotonin-negative (Extended Data Fig. 1c). These results demonstrate that neural crest cells originating from the trunk neural tube can contribute to enteric neuron populations within the gut wall.

To determine whether markers associated with neural-crest-derived SCPs were present in the lamprey trunk, we stained embryos with an antibody to glial fibrillary acidic protein (GFAP), which labels glial and Schwann cells[21]. We observed prominent GFAP$^+$ cells in lamprey embryos, including some that were DiI-positive along the lateral line (Fig. 2g), consistent with the possibility that neural crest cells give rise to the non-myelinating Schwann cells of lampreys[22].

Finally, as an additional means of testing whether enteric neurons originate from within the trunk neural tube, we performed neural tube ablation ($n = 17$), a method classically used to demonstrate the neural crest origin of enteric neurons[8,23], at approximately T24. To accommodate slight natural variation in the number of ENS neurons between embryos, we expressed counts as the ratio of serotonergic cells in the zone adjacent to the ablation versus an identically sized region spaced approximately one somite length to the anterior. Compared with control non-ablated ($n = 6$) or sham-ablated ($n = 4$) embryos (Fig. 3a, c), experimental embryos showed a significant decrease in the ratio of ENS serotonergic cells (Fig. 3b, d). We noted an ~12% decrease of serotonergic neurons in the ablated region compared with sham-ablated embryos and an ~25% decrease relative to stage

© 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

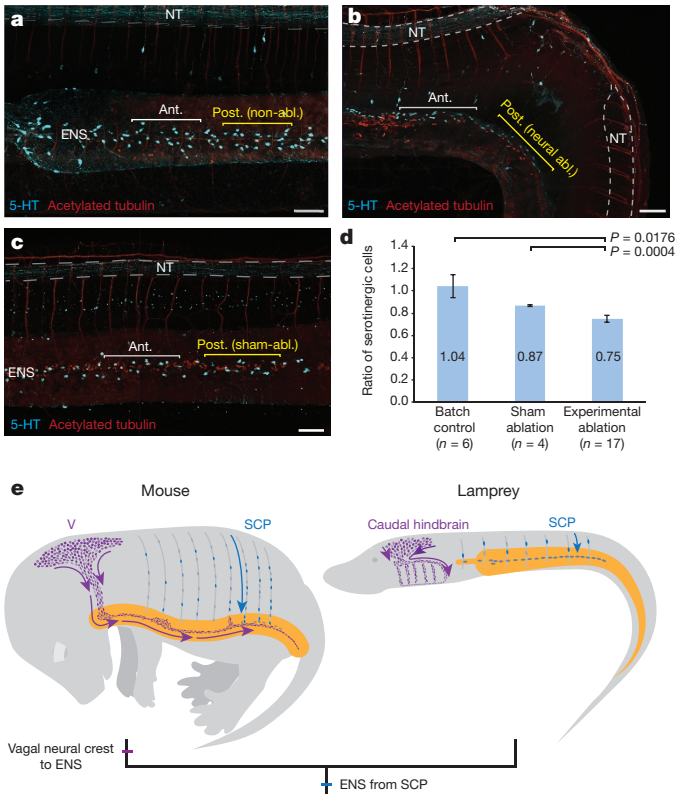


Figure 3 | Neural tube ablation disrupts ENS development. a–c, Whole-mount images of T30 lamprey embryos (red, HuC/D and acetylated tubulin; cyan, 5-HT). Ant., anterior; Post., posterior. **a**, Control non-ablated (non-abl.) embryo. **b**, Ablation of the neural tube (neural abl.) at T24 results in a decrease of serotonergic (5-HT$^+$) cells in the adjacent gut relative to an equivalently sized anterior region. **c**, A sham-ablated (sham-abl.) embryo, in which epidermis and neural tube were cut but not removed, does not show a reduction of cells. **d**, Ablated embryos show a significantly lower ratio of serotonergic cells (number of cells in surgical zone/number of cells in anterior zone) than sham-ablated controls (*t*-test; $P = 0.0004$) and non-ablated batch controls (*t*-test, $P = 0.0176$). Error bars, s.e.m. **e**, Schematic model of neural crest contributions to the ENS in mouse compared with lamprey. V, vagal neural crest (purple); SCP, blue; lamprey caudal hindbrain crest, purple. Scale bar, 100 μm.

controls, consistent with a model of ENS neuronal precursors migrating from trunk neural-crest-derived cells. This decrease was particularly surprising given the remarkable regenerative capacity of the lamprey spinal cord[24,25].

Recent evidence suggests that many neural crest derivatives in post-natal mammals, including skin and peripheral ganglia, arise from SCPs that are closely associated with extrinsic innervation to these structures. For example, SCPs along nerve processes differentiate into melanocytes of the skin and parasympathetic ganglia[1,2,26]. Moreover, SCPs that migrate along trunk spinal nerves contribute to a subpopulation of enteric neurons in mice[3]. These studies prove the existence of neural-crest-derived cells that contribute to the peripheral nervous system and other derivatives at post-embryonic stages. Our results suggest that these trunk neural-crest-derived cell types may represent an ancient and evolutionarily conserved source of cells that contribute to the ENS. Moreover, our data suggest that agnathans might lack a classic 'vagal' neural crest, leading us to speculate that a vagal neural crest population with the ability to form enteric neurons arose[27] in stem gnathostomes (Fig. 3e). We cannot rule out contributions from other sources, but focus here on the positive contribution of trunk neural-crest-derived cells to enteric neurons. Although we cannot formally exclude the possibility that the mechanisms for populating the ENS arose independently in agnathans and gnathostomes, we favour the idea that the contribution of SCPs to the ENS might represent a primitive (plesiomorphic) state retained from early vertebrates, perhaps common to all living vertebrates. With the emergence of jawed vertebrates, new traits, including jaws, sympathetic ganglia, and vagal neural-crest-derived enteric ganglia, appeared under the umbrella of embryonic neural crest derivatives.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 23 February 2016; accepted 31 January 2017.
Published online 20 March 2017.

1. Espinosa-Medina, I. *et al.* Parasympathetic ganglia derive from Schwann cell precursors. *Science* **345,** 87–90 (2014).
2. Dyachuk, V. *et al.* Parasympathetic neurons originate from nerve-associated peripheral glial progenitors. *Science* **345,** 82–87 (2014).
3. Uesaka, T., Nagashimada, M. & Enomoto, H. Neuronal differentiation in Schwann cell lineage underlies postnatal neurogenesis in the enteric nervous system. *J. Neurosci.* **35,** 9879–9888 (2015).
4. Gibbins, I. in *Comparative Physiology and Evolution of the Autonomic Nervous System* (eds Nilsson, S. & Holmgren, S.) Vol. 4, Ch. 1 (Harwood Academic, 1994).
5. Lake, J. I. & Heuckeroth, R. O. Enteric nervous system development: migration, differentiation, and disease. *Am. J. Physiol. Gastrointest. Liver Physiol.* **305,** G1–G24 (2013).
6. Furness, J. B. & Costa, M. *The Enteric Nervous System* (Blackwell, 2006).
7. Le Douarin, N. M. & Teillet, M. A. The migration of neural crest cells to the wall of the digestive tract in avian embryo. *J. Embryol. Exp. Morphol.* **30,** 31–48 (1973).
8. Yntema, C. L. & Hammond, W. S. The origin of intrinsic ganglia of trunk viscera from vagal neural crest in the chick embryo. *J. Comp. Neurol.* **101,** 515–541 (1954).
9. Häming, D. *et al.* Expression of sympathetic nervous system genes in lamprey suggests their recruitment for specification of a new vertebrate feature. *PLoS ONE* **6,** e26543 (2011).
10. Johnels, A. G. On the development and morphology of the skeleton of the head of *Petromyzon*. *Acta Zool.* **29,** 139–279 (1948).
11. Baumgarten, H. G., Björklund, A., Lachenmayer, L., Nobin, A. & Rosengren, E. Evidence for the existence of serotonin-, dopamine-, and noradrenaline-containing neurons in the gut of *Lampetra fluviatilis*. *Z Zellforsch. mikrosk. Anat.* **141,** 33–54 (1973).
12. Yui, R., Nagata, Y. & Fujita, T. Immunocytochemical studies on the islet and the gut of the arctic lamprey, *Lampetra japonica*. *Arch. Histol. Cytol.* **51,** 109–119 (1988).
13. Nakao, T. & Ishizawa, A. An electron microscopic study of autonomic nerve cells in the cloacal region of the lamprey, *Lampetra japonica*. *J. Neurocytol.* **11,** 517–532 (1982).
14. Serbedzija, G. N., Bronner-Fraser, M. & Fraser, S. E. A vital dye analysis of the timing and pathways of avian trunk neural crest cell migration. *Development* **106,** 809–816 (1989).
15. McCauley, D. W. & Bronner-Fraser, M. Neural crest contributions to the lamprey head. *Development* **130,** 2317–2327 (2003).
16. Horigome, N. *et al.* Development of cephalic neural crest cells in embryos of *Lampetra japonica*, with special reference to the evolution of the jaw. *Dev. Biol.* **207,** 287–308 (1999).
17. Pachnis, V., Mankoo, B. & Costantini, F. Expression of the *c-ret* proto-oncogene during mouse embryogenesis. *Development* **119,** 1005–1017 (1993).
18. Marcos-Gutiérrez, C. V., Wilson, S. W., Holder, N. & Pachnis, V. The zebrafish homologue of the ret receptor and its pattern of expression during embryogenesis. *Oncogene* **14,** 879–889 (1997).
19. Natarajan, D., Marcos-Gutierrez, C., Pachnis, V. & de Graaff, E. Requirement of signalling by receptor tyrosine kinase RET for the directed migration of enteric nervous system progenitor cells during mammalian embryogenesis. *Development* **129,** 5151–5160 (2002).
20. De Bellard, M. E., Rao, Y. & Bronner-Fraser, M. Dual function of Slit2 in repulsion and enhanced migration of trunk, but not vagal, neural crest cells. *J. Cell Biol.* **162,** 269–279 (2003).
21. Wasowicz, M. *et al.* Immunoreactivity to glial fibrillary acid protein (GFAP) in the brain and spinal cord of the lamprey (*Lampetra fluviatilis*). *J. Brain Res.* **35,** 71–78 (1993).
22. Peters, A. The structure of the peripheral nerves of the lamprey (*Lampetra fluviatilis*). *J. Ultrastruct. Res.* **4,** 349–359 (1960).
23. Jones, D. S. The origin of the vagi and the parasympathetic ganglion cells of the viscera of the chick. *Anat. Rec.* **82,** 185–197 (1942).
24. Rovainen, C. M. Regeneration of Müller and Mauthner axons after spinal transection in larval lampreys. *J. Comp. Neurol.* **168,** 545–554 (1976).
25. Selzer, M. E. Mechanisms of functional recovery and regeneration after spinal cord transection in larval sea lamprey. *J. Physiol. (Lond.)* **277,** 395–408 (1978).
26. Adameyko, I. *et al.* Schwann cell precursors from nerve innervation are a cellular origin of melanocytes in skin. *Cell* **139,** 366–379 (2009).
27. Kuo, B. R. & Erickson, C. A. Vagal neural crest cell migratory behavior: a transition between the cranial and trunk crest. *Devel. Dyn.* **240,** 2084–2100 (2011).

© 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

Acknowledgements We thank C. Baker, M. Piacentino, and L. Kerosuo for discussion, and M. Martik, M. Simoes-Costa, and R. Uribe for their comments on this manuscript.

Author Contributions The project was conceived by M.E.B., and analyses were designed by S.A.G. and M.E.B. Descriptive analyses of enteric neurons were performed by S.A.G. Cranial DiI labelling was performed by B.R.U., M.E.B., and S.A.G. Trunk DiI labelling was performed by B.R.U., S.A.G. and M.E.B. Surgeries were performed, imaged and analysed by S.A.G. and B.R.U. Schematics were drawn by S.A.G. The manuscript was written by M.E.B., S.A.G. and B.R.U.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations. Correspondence and requests for materials should be addressed to M.E.B. (mbronner@caltech.edu).

© 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

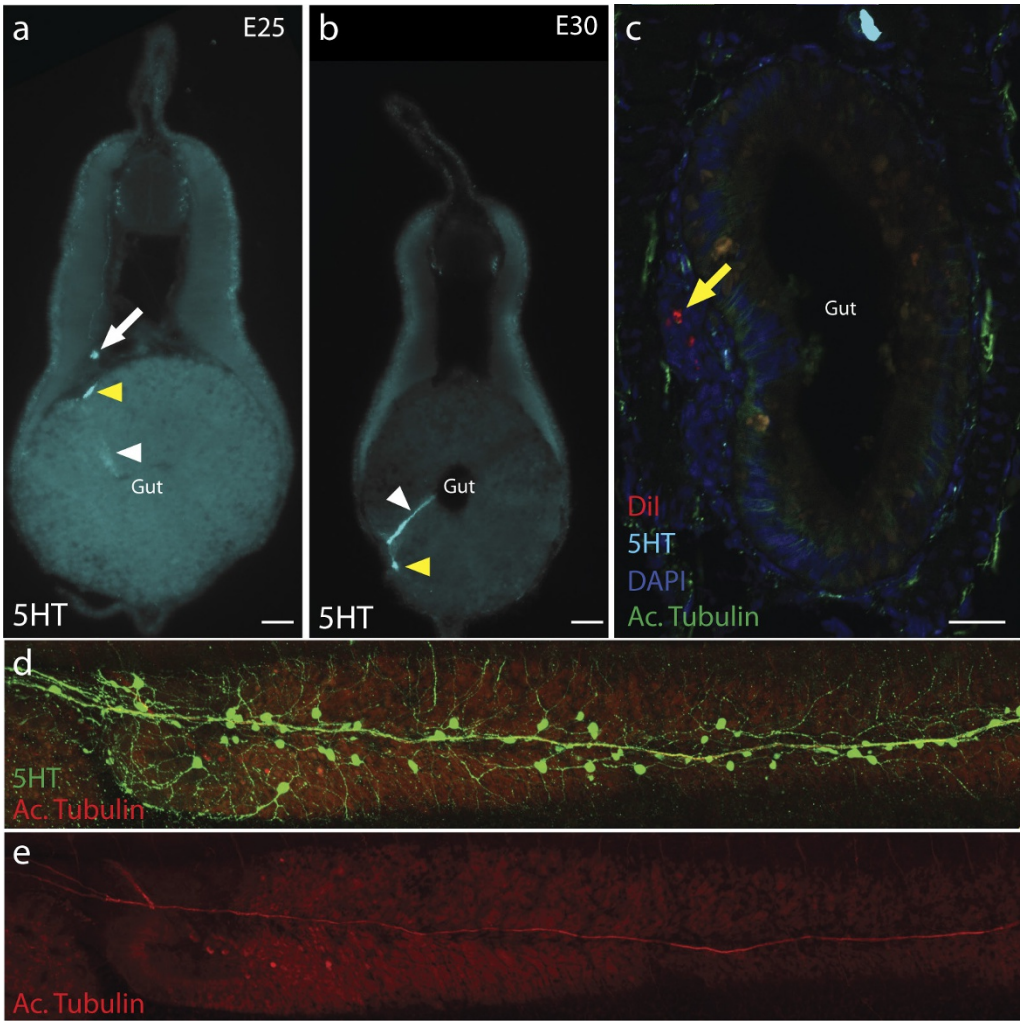
METHODS

No statistical methods were used to predetermine sample size. Adult sea lamprey (*P. marinus*) were supplied by the US Fish and Wildlife Service and Department of the Interior, and cultured according to previous protocols[28] in compliance with California Institute of Technology Institutional Animal Care and Use Committee protocol 1436. Embryo batches with less than 70% survival were excluded from analyses, and all experimental embryos were randomly chosen from appropriately staged cultures. Celltracker CM-DiI (Thermo Scientific) was resuspended as described[9], and surgical ablations were performed using forceps and tungsten needles. For surgeries, only embryos with ablations of two to six somite lengths, adjacent to the anterior intestine and without damage to other tissues, were included in analyses. Serotonergic neurons associated with the typhlosole were counted in a 200–300 μm region adjacent to the ablation site, and an equivalently sized non-ablated region beginning approximately one myotome length anterior to the ablation. Measurements were taken in sibling non-ablated embryos and sham-ablated embryos in which ectoderm and neural tissue was cut, but not removed, at similar axial locations. Investigators were not blinded to group allocations. Probes for *Phox2*, *Gfra1*, and *Ret* were cloned from complementary DNA with primers for Ensembl gene models ENSPMAG00000008433, ENSPMAT00000009324, and ENSPMAT00000008763. *In situ* hybridizations and antibody stainings were performed using previously described protocols[29,30]. Anti-acetylated tubulin (Sigma clone 6-11b-1, T7541; Mouse IgG2b) and anti-5-HT (Immunostar 20080, Rabbit IgG) were used at 1:500. Anti-GFAP (Dako, Z0334; Rabbit IgG) was used at 1:400. Embryos were processed for cryosectioning according to standard protocols, and were sectioned on a Microm HM550 cryostat. Microscopy was performed on a Zeiss AxioImager.M2 equipped with Apotome.2, and fluorescent *z*-stack images were processed and presented as maximum intensity projections. Images were cropped, rotated, and resized using Adobe Photoshop CC, and image panels were constructed using Adobe Illustrator CC. Statistics were analysed in Microsoft Excel.

Data availability. The datasets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request.

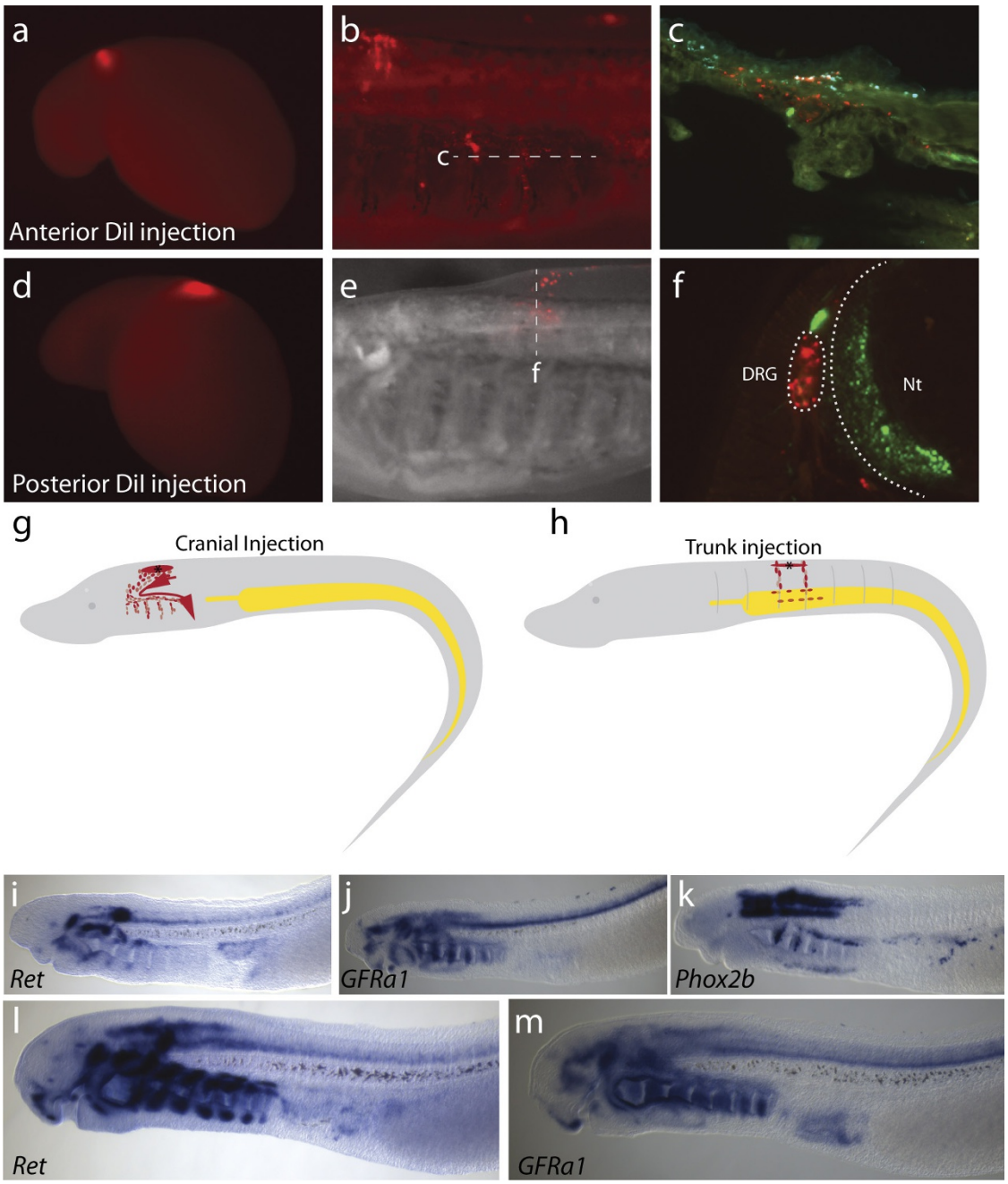
28. Nikitina, N., Bronner-Fraser, M. & Sauka-Spengler, T. Culturing lamprey embryos. *Cold Spring Harb. Protoc.* **2009,** t5122 (2009).
29. Sauka-Spengler, T., Meulemans, D., Jones, M. & Bronner-Fraser, M. Ancient evolutionary origin of the neural crest gene regulatory network. *Dev. Cell* **13,** 405–420 (2007).
30. Nikitina, N., Bronner-Fraser, M. & Sauka-Spengler, T. Immunostaining of whole-mount and sectioned lamprey embryos. *Cold Spring Harb. Protoc.* **2009,** t5126 (2009).

© 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.



Extended Data Figure 1 | Serotonin-immunoreactive cells in the gut, and DiI labelling. a, **b**, 5-HT immunoreactivity in E25 (**a**) and E30 (**b**) embryos. Serotonergic neurons (yellow arrowheads) are positioned within the typhlosole, near the endodermal mucosa. A cell present in the columnar epithelium (white arrowhead) might represent an endocrine (enterochromaffin) cell. Cells positioned dorsal to the typhlosole might be neuroblasts (white arrow). **c**, DiI labelling results in labelled cells (yellow arrow) originating from the neural tube, migrating to the gut and typhlosole. Red, DiI; cyan, 5-HT; blue, DAPI; green, acetylated tubulin. **d**, **e**, Serotonergic (green, 5-HT; red, acetylated tubulin) neurons in a T30 embryo (**d**), and acetylated tubulin staining alone (**e**) shows the position of the vagus nerve.

© 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.



Extended Data Figure 2 | DiI labelling of the caudal hindbrain population shows contributions to the branchial arches. a–**c**, **e**, **f**, Sample time-lapse imaging of two separate DiI-labelled embryos around the hindbrain level. **a**, **d**, Initial injection at E6–E6.5 (T20). **b**, Final DiI localization of embryo in **a**, 10 days after injection (E16). **c**, Frontal cryosection through the branchial basket shows DiI along the branchial arches. Red, DiI; green, neurofilament-M; cyan, collagen type II. **e**, Final DiI localization of embryo in **d** 14 days after injection (E20). **f**, Transverse section through the lamprey branchial basket shows DiI within the dorsal root ganglia (DRG). Nt, neural tube. Red: DiI; green: neurofilament-M. **g**, **h**, Schematic depiction of individual injection sites (*) for cranial (**g**) and trunk (**h**) injections. **i**–**m**, Genes associated with gnathostome enteric neurons, *Ret* (**i**, **l**), *Gfra1* (**j**, **m**), and *Phox2b* (**k**) do not appear to be co-expressed at T26 (**i**–**k**) and T27 (**l**, **m**), before enteric neuron differentiation in lamprey.

© 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.