Migratory neuronal progenitors arise from the neural plate borders in tunicates

Alberto Stolfi1, Kerrianne Ryan2, Ian A. Meinertzhagen2 & Lionel Christiaen1

The neural crest is an evolutionary novelty that fostered the emergence of vertebrate anatomical innovations such as the cranium and jaws1. During embryonic development, multipotent neural crest cells are specified at the lateral borders of the neural plate before delaminating, migrating and differentiating into various cell types. In invertebrate chordates (cephalochordates and tunicates), neural plate border cells express conserved factors such as Msx, Snail and Pax3/7 and generate melanin-containing pigment cells2–4, a derivative of the neural crest in vertebrates. However, invertebrate neural plate border cells have not been shown to generate homologues of other neural crest derivatives. Thus, proposed models of neural crest evolution postulate vertebrate-specific elaborations on an ancestral neural plate border program, through acquisition of migratory capabilities and the potential to generate several cell types5–7. Here we show that a particular neuronal cell type in the tadpole larva of the tunicate Ciona intestinalis, the bipolar tail neuron, shares a set of features with neural-crest-derived spinal ganglia neurons in vertebrates. Bipolar tail neuron precursors derive from caudal neural plate border cells, delaminate and migrate along the paraxial mesoderm on either side of the neural tube, eventually differentiating into afferent neurons that form synaptic contacts with both epidermal sensory cells and motor neurons. We propose that the neural plate borders of the chordate ancestor already produced migratory peripheral neurons and pigment cells, and that the neural crest evolved through the acquisition of a multipotent progenitor regulatory state upstream of multiple, pre-existing neural plate border cell differentiation programs.

Progenitor cells that fulfil all the criteria defining the neural crest have not been observed outside vertebrates. These criteria include an embryonic origin at the lateral borders of the neural plate, epithelium-to-mesenchyme transition (EMT), migratory behaviour and the potential to differentiate into diverse cell types such as neurons, bone, cartilage and pigment cells.

In cephalochordates (amphioxus) and the tunicates Halocynthia and Ciona, a subset of neural plate border cells deploy a conserved melanocyte-specific gene network but do not migrate away from the neural tube2–4. Instead, they contribute locally to pigmented photoreceptor organs. In Ciona, the pigment cell precursors undergo an epithelial-to-mesenchymal transition and remain inside the neural tube lumen, but can be induced to exit the neural tube through targeted mis-expression of the mesenchyme-specific transcription factor Twist-related1. Migratory pigment cell precursors have also been reported in larvae of the tunicate Ecteinascidia turbinata8.

In contrast, invertebrate homologues of neural-crest-derived neurons have so far proved elusive. In tunicates, various neurons arise from the neural plate borders, but these remain in the dorsal neural tube or in the epidermis9–11, instead of delaminating and migrating as would be expected for homologues of vertebrate neural-crest-derived neurons. Migratory sensory neurons have been described in cephalochordate embryos, but these arise from ventral epidermis, not the neural plate borders, and reinsert into the epidermis after migrating11–13.

The recently identified bipolar tail neurons (BTNs)12 of Ciona larvae form axon fascicles that extend along the length of the tail on either side of the neural tube (Fig. 1a). These neurons express the proneural basic helix–loop–helix transcription factor Neurogenin (Neurog, Fig. 1b) and the LIM-homeodomain factor Islet (Fig. 1a). Vertebrate Neurogenin and Islet orthologues are involved in specifying various neuronal subtypes including neural-crest-derived dorsal root ganglia neurons (DRGNs), which also have a bipolar or pseudo-unipolar morphology and transmit peripheral mechanosensory inputs to the central nervous system13. Ciona BTNs also express Asic, the orthologue of acid-sensing ion channels (ASICs)14 that modulate touch sensitivity in vertebrate DRGNs. These parallels prompted us to investigate the embryological origins of the BTNs.

Figure 1 | Bipolar tail neurons come from the borders of the neural plate.

a, Larva with a BTN labelled by Islet BTN>unc-76:eGFP (green). Bottom, enlarged view of BTN above. Scale bars, 75 µm (top); 25 µm (bottom).
b, Migrating BTN precursors (arrowheads) labelled by the b-line-specific Neurog b-line>unc-76::Venus reporter construct (green). Scale bar, 25 µm.
c, In situ hybridization for Neurog (magenta) in an embryo electroporated with Mxs>nls::lacZ plasmid (immunolabelling of β-galactosidase in green). White arrowhead, Mxs’/Neurog’ BTN progenitor. Dashed arrowhead, transient Neurog expression in BTN progenitor’s sister cell (epidermal progenitor). Dashed line, midline. Scale bar, 25 µm. d, In situ hybridization for Neurog (red) and Snail (green). Scale bar, 25 µm. Inset is enlarged box showing low levels of Snail expression in BTN progenitor. e, Pax3/7 in situ hybridization (green). Scale bar, 25 µm. Enlarged box inset showing Pax3/7 expression in BTN progenitor. f, Adapted illustration of embryos showing position of pigment cell and BTN progenitors (and their descendants) in the neural plate borders. Lateral views in a, b, dorsal views in c–f. Anterior to the left throughout; st., stage.

1Center for Developmental Genetics, Department of Biology, New York University, New York, New York 10003, USA. 2Department of Psychology and Neuroscience, Life Sciences Centre, Dalhousie University, Halifax, Nova Scotia B3H 4R2, Canada.
We detected the earliest expression of Neurog at neurulation, in the caudal-most neural/epidermal boundary cells, which express the conserved neural plate border specification genes Msx, Pax3/7 (ref. 3) and Snail (Fig. 1c–f and Extended Data Fig. 1). During neurulation, these cells drive neural tube closure and their progeny eventually form the neural tube roof plate and dorsal epidermis midline. Together, they continue their migration as a chain of two cells.

Neurog expression distinguishes the BTNs from the caudal epidermal sensory neurons (CESNs), which remain at the dorsal midline and are specified instead by an atonal homologue (Atoh)-dependent regulatory program. We found that the onset of Neurog expression requires MAPK/ERK signalling (Fig. 3a, b). However, later inhibition of MAPK/ERK resulted in the upregulation of Neurog in non-neural cells of the lineage, converting these into supernumerary BTNs (Fig. 3c–e and Extended Data Fig. 4). Overexpression of Neurog also induced ectopic migratory Atonal+ BTN precursors (i) and expression of Protocadherin.c (green) in dorsal epidermis midline but not BTNs (j). Embryos in i, j were electroporated with Neurog b-line >unc-76:mCherry (immunolabelling of mCherry in magenta).

Figure 2 | Bipolar tail neuron precursors delaminate and migrate. a, Embryos electroporated with Neurog b-line >unc-76::eGFP. Top, BTN precursor (arrowhead) extending a lamellipodium. Bottom, BTN precursors (anterior (aBTN) and posterior (pBTN)) delaminating. Scale bar, 25 μm. b, Enlarged view of aBTN in lower panel of a. Top, UNC-76:ecFP, middle, phalloidin; bottom, merged; arrowhead, part of aBTN still in the epithelium. Scale bar, 10 μm. c, Embryo with paraxial mesoderm labelled by Mrf >unc-76::eGFP (green), BTN labelled by Neurog b-line >unc-76::mCherry (magenta) and phalloidin counterstain. Scale bar, 25 μm. Right, cross-sectioned 3D image of same embryo. Only the right side of the embryo was transected. d, 3D slice of embryo showing BTNs (arrowheads) outside neural tube (dotted outline). Only the right side of the embryo was transected. e, Diagram of showing BTNs relative to other tail tissues: neural tube (NT), notochord (NC), myoblasts (M) and endoderm (EN). f, Time series of different embryos co-electroporated with Neurog b-line >unc-76::VenusYFP (green) and Nodal b8.18 >H2B::mCherry (magenta). Right panels are enlarged views of the images on the left. Dashed lines indicate displacement from clonally related epidermal cells (indicated by colour-coded brackets). Scale bars, 25 μm.

Figure 3 | Bipolar tail neuron specification and differentiation. a, Wild-type Neurog b-line >unc-76::VenusYFP expression (green) in embryos treated with DMSO vehicle, counterstained with DAPI (blue). b, Neurog expression was abolished in 43 of 50 embryos treated with 10 μM MEK inhibitor U0126 at 5.5 hours post-fertilization (h.p.f.). c, Supernumerary BTNs were specified in 28 of 50 embryos treated with 10 μM U0126 at 7 h.p.f. d, Two BTN precursors, labelled by Neurog b-line >unc-76::VenusYFP (green) and Neurog b-line >H2B::mCherry (magenta), migrating on one side of a DMSO-treated embryo. e, Expanded chain of four BTNs resulting from treatment with U0126 at 7 h.p.f. f, BTN expression Atonal+c expression of ectopic BTN precursors (i) and expression of Protocadherin.c (green) in dorsal epidermis midline but not BTNs (j). Embryos in i, j were electroporated with Neurog b-line >unc-76::mCherry (immunolabelling of mCherry in magenta). k, Forced overexpression of protocadherin.c in the BTN lineage using the Neurog b-line driver inhibits delamination and migration of BTNs in 7 of 14 embryos. l, Normal BTNs as seen in 9 of 12 control embryos (overexpression of β-galactosidase instead). Embryos in k, l were electroporated with Neurog b-line >unc-76::VenusYFP and Neurog b-line >H2B::VenusYFP (green) and counterstained with phalloidin (magenta). All scale bars 25 μm. Embryos in a–e, i, j fixed at stage 22. Embryos in f–h, k, l at stage 23.
function\(^{21}\). We found that expression of \textit{Cadherin.b}, the predominant cadherin gene expressed in the neural tube of \textit{Ciona} embryos, is absent in BTN precursors (Fig. 3i). Moreover, BTN precursors do not express \textit{Protocadherin.c}, a cadherin superfamily gene expressed in CESNs and epidermis midline (Fig. 3j). Overexpression of \textit{protocadherin.c} protein inhibited delamination and migration of BTN cells (Fig. 3k, l), suggesting that \textit{Ciona} BTN genes and vertebrate neural crest share regulatory strategies for EMT via differential cell–cell adhesion.

We observed that each BTN precursor initially migrates anteriorly with a prominent leading edge that becomes the cell’s anterior neurite (or ‘proximal process’), while its Golgi apparatus is located posterior to the cell nucleus. At around 12 h post-fertilization, each BTN precursor undergoes a 180° polarity inversion, with the Golgi repositioning itself anterior to the nucleus immediately before the cell begins to elaborate the posterior segment of its neurite (the ‘distal process’), resulting in a bipolar morphology (Extended Data Fig. 6, Supplementary Video 4 and Supplementary Table 1). These observations suggest that a precisely timed re-orientation of cell polarity underlies the characteristic bipolar morphology of the BTNs.

At hatching, BTN cell bodies are situated in the middle of the tail along the anterior–posterior axis, with their distal processes extending towards the tail tip and proximal processes projecting towards the motor ganglion and brain (Fig. 4a–c)\(^{12}\). Electron microscopy confirmed that the BTN somata lie outside the neural tube and are invariably overlain by epidermal cells (Fig. 4d). BTNs lack junctions with epidermal cells and also lack cilia, thus failing to penetrate the tunic to contact the exterior. These characteristics suggest that while distal BTN neurites may be sensory, their cell bodies lack epidermal sensory receptors found in CESNs\(^{22}\). Along the tail, the BTNs contact overlying CESNs, the short processes of which do not reach the motor ganglion\(^{12}\). At these contacts, synapses form from the CESNs to the BTN (Fig. 4d). Unlike the CESNs, the proximal processes of the BTN form synaptic contacts with the motor neurons that innervate and control the tail muscles (Fig. 4b, c, e). Each BTN establishes many such contacts upon the two most anterior pairs of motor neurons, MN1 and MN2, on both the left and right sides (Fig. 4e and Extended Data Table 1). These synaptic connections are similar to those of mammalian slowly adapting type I DRGNs that, in addition to being mechanosensitive themselves, relay distinct inputs from mechanosensory Merkel cells of the epidermis\(^{33}\). Both tunicate CESNs and vertebrate Merkel cells arise from non-migratory epidermal cells, require Atoh factors for their specification and are glutamatergic in their neurotransmitter phenotype\(^{10,20,24,25}\). These data suggest that BTN may thus be equivalent to vertebrate DRGNs within a homologous ascending sensory pathway (Fig. 4c).

In anamniote vertebrates, evidence for a common progenitor of intramedullary Rohon–Beard neurons (RBNs) and neural crest, in addition to other similarities between RBNs and DRGNs, indicates a deep homology between these cell types\(^{36}\). Fritzsch and Northcutt proposed that a key step in the evolution of neural crest was the elaboration of extramedullary sensory neurons from intramedullary RBN-like neurons\(^{37}\). Following the Fritzsch–Northcutt model, the BTNs may be derived from an ‘intermediate’ extramedullary neuron that evolved in the last common ancestor of Olfactores (vertebrates and tunicates) before the appearance of bona fide neural crest in the vertebrates. The migration of BTN precursors along the paraxial mesoderm, similar to later phases of DRGN migration, suggests that some of the diverse EMT and migratory behaviours displayed by vertebral neural crest cells may pre-date the emergence of vertebrates.

Although the embryological origin (neural plate borders) and molecular signature (\textit{Neurog2}/\textit{Islet}) of the BTNs of \textit{Ciona} also support homology with RBNs, the two do in fact differ in several key aspects. First, BTNs are extramedullary neurons derived from progenitor cells that migrate along paraxial mesoderm lateral to the neural tube. Second, expression of ASICs is shared between BTN and DRGNs, but appears absent from RBNS\(^{38}\). Finally, BTNs are multipolar with extensively branching peripheral neurites that innervate the overlying epidermis\(^{39}\), while we have not observed any peripheral neurites projecting from the bipolar/pseudounipolar BTNs.

We have revealed the developmental history of migratory neuronal progenitors that arise from the neural plate borders of tunicate embryos. Based on their embryological origin, gene expression, cell behaviour, morphology and synaptic connections, we propose that the BTNs are homologous to neural-crest-derived DRGNs. This would imply that the neural plate borders of the olfactorean ancestor gave rise to at least two types of neural crest derivatives: pigment cells and peripheral neurons (Extended Data Fig. 7).

In the invariantly developing \textit{Ciona} embryo, the pigment cell and BTN lineages become separated early in development, but converge at a neural plate border cell identity before parting again towards distinct differentiated fates. This separation between the two lineages may represent the ancestral condition of the neural plate borders before the evolution of the neural crest in vertebrates. This would support models that propose an evolutionary origin for vertebrate neural crest through a heterochronic shift or ‘intercalation’ of a multipotent progenitor state downstream of neural plate border specification but upstream of cell differentiation, based on shared regulatory programs between neural crest and pluripotent cells of the early embryo\(^{30,35}\).

**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 25 March; accepted 30 September 2015.
Published online 28 October; corrected online 18 November 2015
(see full-text HTML version for details).
1. Bronner, M. E. & LeDouarin, N. M. Evolution and development of the neural crest: an overview. Dev. Biol. 366 (2012).

2. Yu, J.-K., Meulemans, D., McKeown, S. J. & Bronner-Fraser, M. Insights from the amphioxus genome on the origin of vertebrate neural crest. Genome Res. 18, 1127–1132 (2008).

3. Wada, H., Holland, P. W. H., Sato, S., Yamamoto, H. & Satoh, N. Neural tube is partially dorsIALIZED by overexpression of HrPax-37: the ascidian homologue of Pax-3 and Pax-7. Dev. Biol. 187, 240–252 (1997).

4. Abitua, P. B., Wagner, E., Navarrete, I. A. & Levine, M. Identification of a rudimentary neural crest in a non-vertebrate chordate. Nature 492, 104–107 (2012).

5. Wada, H. Origin and evolution of the neural crest: a hypothetical reconstruction of its evolutionary history. Dev. Growth Differ. 43, 509–520 (2001).

6. Baker, C. V. H. & Bronner-Fraser, M. The origins of the neural crest. Part II: an evolutionary perspective. Mech. Dev. 69, 13–29 (1997).

7. Shimeld, S. M. & Holland, P. W. H. Vertebrate innovations. Proc. Natl Acad. Sci. USA 97, 4449–4452 (2000).

8. Jeffery, W. R., Strickler, A. G. & Yamamoto, Y. Migratory neural crest-like cells form body pigmentation in a urochordate embryo. Nature 431, 696–699 (2004).

9. Mazet, F. et al. Molecular evidence from Ciona intestinalis for the evolutionary origin of vertebrate sensory placodes. Dev. Biol. 282, 494–508 (2005).

10. Pasini, A. et al. Formation of the ascidian epidermal sensory neurons: insights into the origin of the chordate peripheral nervous system. PLoS Biol. 4, e225 (2006).

11. Kaltenbach, S. L., Yu, J.-K. & Holland, N. D. The origin and migration of the earliest-developing sensory neurons in the peripheral nervous system of amphioxus. Evol. Dev. 11, 142–151 (2009).

12. Imai, J. H. & Meinertzhagen, I. A. Neurons of the ascidian larval nervous system in Ciona intestinalis: II. Peripheral nervous system. J. Comp. Neurol. 501, 335–352 (2007).

13. Ma, Q., Fode, C., Guillemot, F. & Anderson, D. J. NEUROGENIN1 and NEUROGENIN2 control two distinct waves of neurogenesis in developing dorsal root ganglia. Genes Dev. 13, 1717–1728 (1999).

14. Coric, T., Passamaneck, Y. J., Zhang, P., Di Gregorio, A. & Canessa, C. M. Simple chordates exhibit a proton-independent function of acid-sensing ion channels. PNAS 105, 1914–1923 (2008).

15. Aniello, F. et al. Identification and developmental expression of Ci-msxbt: a novel homologue of Drosophila msh gene in Ciona intestinalis. Mech. Dev. 88, 123–126 (1999).

16. Wada, S. & Saiga, H. Cloning and embryonic expression of Hsna, a snail family gene of the ascidian Halocynthia roretzi: implication in the origins of mechanisms for mesoderm specification and body axis formation in chordates. Dev. Growth Differ. 41, 9–18 (1999).

17. Nakamura, M. J., Terai, J., Okubo, R., Hotta, K. & Oka, K. Three-dimensional anatomy of the Ciona intestinalis tailbud embryo at single-cell resolution. Dev. Biol. 372, 274–284 (2012).

18. Torrence, S. & Cloney, R. Nervous system of ascidian larvae: caudal primary sensory neurons. Zoomorphology 99, 103–115 (1982).

19. Maksimovic, S. et al. Epidermal Merkel cells are mechanosensory cells that tune mammalian touch receptors. Nature 509, 617–621 (2014).

20. Morisson, K. M., Mise lgases, G. R., Lumpkin, E. A. & Maricich, S. M. Mammalian Merkel cells are descended from the epidermal lineage. Dev. Biol. 336, 76–83 (2009).

21. Theveneau, E. & Mayor, R. Neural crest delamination and migration: from epithelium-to-mesenchyme transition to collective cell migration. Dev. Biol. 366, 34–54 (2012).

22. Artinger, K. B., Chitnis, A. B., Mercara, M. & Driever, W. Zebrafish narrowminded suggests a genetic link between formation of neural crest and primary sensory neurons. Development 126, 3969–3979 (1999).

23. Fritsch-B. & Northcutt, R. G. Cranial and spinal nerve organization in amphioxus and lampreys: evidence for an ancestral craniate pattern. Acta Anat. (Basel) 148, 96–109 (1993).

24. Paukert, M. et al. A family of acid-sensing ion channels from the zebrafish: widespread expression in the central nervous system suggests a conserved role in neuronal communication. J. Biol. Chem. 279, 18783–18791 (2004).

25. O'Brien, G. S. et al. Coordinate development of skin cells and cutaneous sensory axons in zebrafish. J. Comp. Neurol. 520, 816–831 (2012).

26. Paukert, M. et al. A family of acid-sensing ion channels from the zebrafish: widespread expression in the central nervous system suggests a conserved role in neuronal communication. J. Biol. Chem. 279, 18783–18791 (2004).

27. Fritsch-B. & Northcutt, R. G. Cranial and spinal nerve organization in amphioxus and lampreys: evidence for an ancestral craniate pattern. Acta Anat. (Basel) 148, 96–109 (1993).

28. Paukert, M. et al. A family of acid-sensing ion channels from the zebrafish: widespread expression in the central nervous system suggests a conserved role in neuronal communication. J. Biol. Chem. 279, 18783–18791 (2004).

29. O'Brien, G. S. et al. Coordinate development of skin cells and cutaneous sensory axons in zebrafish. J. Comp. Neurol. 520, 816–831 (2012).

30. Buitrago-Delgado, E., Nordin, K., Rao, A., Geary, L. & Labonne, C. Shared regulatory programs suggest retention of blastula-stage potential in neural crest cells. Science 348, 1332–1335 (2015).
**METHODS**

Molecular cloning. Reporter constructs were designed based on information of cis-regulatory modules (CRMs) from previously published studies on the following genes: *Isl1*, *Mtx2*, *Neurog3*, *Nodal*, *Asic4*, glutamate decarboxylase (Gad)35, 36, *Slc17a6* (Vglut2)37 and *Fgfbp1*17 (ref. 36). The Neurog b-line CRM (Ciinte.REG.KhC6.1500090-1502346) was cloned using the following primers: Neurog −3,010 forward (5′-GTGTGTCGGCATGATC-3′) and Neurog −773 reverse (5′-CTATACGCGGACCCTCATTG-3′). The Neurog b-line minimal CRM (Ciinte.REG.KhC6.1500090-1500801) was found to be contained within this region and cloned using Neurog −3,010 forward and Neurog −2,599 reverse (5′-GCAAAGCTTCTTGGGATCTTGC-3′) primers. CRMs were cloned upstream of the basal promoter of Neurog (Ciinte.REG.KhC6.1502506-1503107), cloned using the primers Neurog −594 forward (5′-GGTATGCTAGTGTGGTTGTC-3′) and Neurog +9 reverse (5′-ATCCACATTTTGTAGCAAGGC-3′), or the basal promoter of the Zfp gene (also known as friend of GATA, or Fog)42. The full-length MyrCRM (Ciinte.REG.KhC14.4311719-4314636) was cloned using the primers (5′-GAAGATCTCTTGGGATCTTGC-3′) and (5′-CTATACGCGGACCCTCATTG-3′) and Protocadherin (Ciinte.REG.KhC6.39.12.v1.ASL-1). Golgi-targeting sequence was cloned from KH.C14.396.v1.B.DND1-1 cDNA (N-acetylglucosaminyltransferase 7, or Galt7) using the primers Galt7 amino acid 1 forward (5′-ATGAGATTTACAATCACCA-3′) and Galt7 amino acid 1 reverse (5′-GTCTGCGCTGTTCAC-3′). Caenorhabditis elegans unc-76 tags were fused to fluorescent proteins to ensure even labelling of axons49. Probes used for in situ hybridization were transcribed in vitro from templates obtained from previously published gene collection clones43, 44. *Galnt7* was cloned upstream of the basal promoter of Neurog (Ciinte.REG.KhC6.1500090-1500801) and (5′-GGTATGCTAGTGTGGTTGTC-3′) and Neurog +9 reverse (5′-ATCCACATTTTGTAGCAAGGC-3′), or the basal promoter of the Zfp gene (also known as friend of GATA, or Fog)42. The full-length MyrCRM (Ciinte.REG.KhC14.4311719-4314636) was cloned using the primers (5′-GAAGATCTCTTGGGATCTTGC-3′) and (5′-CTATACGCGGACCCTCATTG-3′) and Protocadherin (Ciinte.REG.KhC6.39.12.v1.ASL-1). Golgi-targeting sequence was cloned from KH.C14.396.v1.B.DND1-1 cDNA (N-acetylglucosaminyltransferase 7, or Galt7) using the primers Galt7 amino acid 1 forward (5′-ATGAGATTTACAATCACCA-3′) and Galt7 amino acid 1 reverse (5′-GTCTGCGCTGTTCAC-3′) and Protocadherin (Ciinte.REG.KhC6.39.12.v1.ASL-1).

**Embryo handling, in situ hybridization and immunolabelling.** Images were captured on a Leica inverted TCS SP8 X confocal or DM2500 epifluorescence microscope. For time-lapse image capture, embryos were imaged as they developed in sea water-filled chambers on coverslip-bottom Petri dishes (MatTek). Confocal image stacks were processed in Leica Application Suite or ImageJ. Video annotations were made using Camtasia software (TechSmith). 3D slices and projections were generated using Imaris (Bitplane) or Velocity (PerkinElmer) software. Kaede::nls was photoconverted as previously described42. Neurite lengths and Golgi apparatus positioning were measured using Imaris. Not all cells, neurites and/or Golgi were visible in every embryo. Golgi positioning relative to BDN nuclei was measured using confocal image stacks and analyzed using software (AnalySIS: SIS GmbH, or a Gatan 832 Oris CCD camera using Gatan DigitalMicrograph software to compile multi-panel montages from each section. Comprehensive electron microscopy series identified the cell bodies and axons of BTNs, motor neurons and CENs from their positions and shapes, and these in turn enabled identification of their connections (K.R. and I.A.M., manuscript in preparation).

**Electron microscopy**. Adult animals, *Ciona intestinalis* (L.), were collected by P. Darnell from Mahone Bay, Nova Scotia. Two-hour larvae reared at 18°C in the dark were fixed at 4°C for 4 h in 1% OsO4 in 1.25% NaHCO3 adjusted to pH 7.2 with HCl, followed by 2% glutaraldehyde in 0.1 M phosphate buffer. After fixation they were immersed in Epon, and a single larva cross sectioned at 60 nm in the motor ganglion and later at 100 nm down the length of the tail, and the sections post-stained for 5–6 min in freshly prepared aqueous uranyl acetate followed by 2–3 min in lead citrate. Sections were viewed using an FEI Tecnai 12 electron microscope operated at 80 kV and images captured using either a Kodak Megaview II camera using software (AnalySIS: SIS GmbH), or a Gatan 832 Oris CCD camera using Gatan DigitalMicrograph software to compile multi-panel montages from each section. Comprehensive electron microscopy series identified the cell bodies and axons of BTNs, motor neurons and CENs from their positions and shapes, and these in turn enabled identification of their connections (K.R. and I.A.M., manuscript in preparation).

31. Stolfi, T. et al. Early chordate origins of the vertebrate second heart field. Science 329, 565–568 (2010).
32. Russo, M. T. et al. Regulatory elements controlling Cis-mus tissue-specific expression during *Ciona intestinalis* embryonic development. Dev. Biol. 267, 517–528 (2004).
33. Stolfi, A. & Christiaen, L. Genetic and genomic toolbox of the chordate *Ciona intestinalis*. Genetics 192, 55–66 (2012).
34. Khoueiry, P. et al. A cis-regulatory signature in ascidians and flies, independent of transcription factor binding sites. Curr. Biol. 20, 792–802 (2010).
35. Takamura, K., Minamida, N. & Okabe, S. Neural map of the larval central nervous system in the ascidian *Ciona intestinalis*. Zoolog. Sci. 27, 191–203 (2010).
36. lmai, K. S., Stolfi, A., Levine, M. & Satou, Y. Gene regulatory networks underlying the compartmentalization of the *Ciona intestinalis* central nervous system. Development 136, 285–293 (2009).
37. Schlichtherle, L., Zhang, Y., Lamy, C. & Lemaire, P. A combinatorial code of maternal GATA, Ets and β-cat-enin-TCF transcription factors specifies and patterns the early ascidian ectoderm. Development 134, 4023–4032 (2007).
38. Dynes, J. L. & Ngai, J. Pathfinding of olfactory neuron axons to stereotyped glomerular targets revealed by dynamic imaging in living zebrafish embryos. Neuron 20, 1081–1091 (1998).
39. Satou, Y. et al. A CDNAs resource from the basal chordate *Ciona intestinalis*. Genome Res. 13, 153–154 (2003).
40. Roure, A. et al. A multicassette Gateway vector set for high throughput and comparative analyses in *Ciona* and vertebrate embryos. PLoS ONE 2, e916 (2007).
41. Stolfi, A., Wagner, E., Talaiferro, J. M., Chou, S. & Levine, M. Neural tube patterning by Ephrin, FGF and Notch signaling relays. Development 138, 5429–5439 (2011).
42. Davidson, B., Shi, W., Beh, J., Christiaen, L. & Levine, M. FGF signaling delineates the cardiac progenitor field in the simple chordate, *Ciona intestinalis*. Dev. Cell 20, 2728–2738 (2006).
43. Hudson, C. & Yasuo, H. A signalling relay involving Nodal and Delta ligands acts during secondary notochord induction in *Ciona* embryos. Development 133, 2855–2864 (2006).
44. Christiaen, L., Wagner, E., Shi, W. & Levine, M. The sea squater *Ciona intestinalis* as a genetic model of the vertebrate second heart field. Development 126, 267, 271–282 (1997).
45. Ikuta, T. & Saiga, H. Dynamic change in the expression of developmental genes during secondary notochord induction in *Ciona* embryos. Development 133, 2855–2864 (2006).
46. Beh, J., Shi, W., Levine, M., Davidson, B. & Christiaen, L. FGF signaling p, regulates the expression of maternal GATA, Ets and β-cat-enin-TCF transcription factors species and patterns the early ascidian ectoderm. Development 134, 4023–4032 (2007).
47. Ikuta, T. & Saiga, H. A signalling relay involving Nodal and Delta ligands acts during secondary notochord induction in *Ciona* embryos. Development 133, 2855–2864 (2006).
48. Ando, R., Hama, H., Yamamoto-Hino, M., Mizuno, H. & Miyawaki, A. An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein. Proc. Natl Acad. Sci. USA 99, 12651–12656 (2002).
49. Zrinyi-Krajka, F. et al. Collier/OLF/EFB-dependent transcriptional dynamics control pharyngeal muscle specification from primed cardiopharyngeal progenitors. Dev. Cell 29, 263–276 (2014).
50. Nishida, H. Cell division pattern during gastrulation of the ascidian, *Ciona intestinalis*. Dev. Growth Differ 47, 213–222 (2005).
51. Bone, Q. The central nervous system in amphioxus. J. Comp. Neurol. 115, 27–64 (1960).
Extended Data Figure 1 | *In situ* hybridization of neural plate border markers *Snail* and *Msx*. a, Immunolabelling for β-galactosidase (red) and *in situ* hybridization for *Snail* mRNA (green) in stage 12 embryo electroporated with *Msx* > *lacZ*, revealing *Snail* expression in the BTN progenitors (b9.36 cells, arrowheads). Dashed area enlarged in a’. b, Double *in situ* hybridization for *Snail* (green on merged image) and *Msx* (red on merged image) in stage 12 embryos counterstained with DAPI (blue on merged image), showing co-expression in neural plate border cells, including BTN progenitors. Scale bars, 25 μm.
Extended Data Figure 2 | Lineage tracing of b9.36 descendants.

a, Photoconversion of Kaede::nls driven by the Msx driver was used to follow the cell divisions of the BTN progenitors from the late gastrula stage to the early tailbud stage. Both b10.71 and b10.72 divide once. b11.141 will give rise to a definitive anterior BTN (see Extended Data Fig. 4). Numbers in each panel represent time in minutes elapsed from the initial photoconversion event. Scale bar, 50 μm. b, Lineage tree showing specification of aBTNs in relation to other cells of the posterior neural plate borders. For simplicity, only one side of the embryo is depicted. c, Lateral view of a 110-cell-stage embryo showing the positions of blastomeres in b. Red lines connect sibling cells. d, Dorsal view of a neurula-stage embryo showing zippering of posterior neural-plate-border-derived capstone cells as neural tube closure is initiated. Panels b and d are courtesy of H. Hashimoto and F. Robin (University of Chicago) and N. Takatori (Tokyo Metropolitan University), and partially modelled after ref. 17. Panel c modelled after ref. 49.
Extended Data Figure 3 | Neurog cis-regulatory sequences. a, Schematic diagram representing Neurog locus and 5′ cis-regulatory sequences including b-line and b-line minimal cis-regulatory modules. Peaks represent nucleotide sequence conservation with Ciona savignyi genome. b, Late gastrula embryo (stage 13) electroporated with full-length Neurog (blue) and Nodal b-line (red) reporter constructs. Reporter co-expression is seen in b9.36 descendants on either side of the neural plate. Neurog expression also marks tail-tip lineages of uncertain provenance, previously reported to be descended from b8.21 (ref. 10). Scale bar, 25 μm. c, Neurog b-line reporter. d, Neurog b-line minimal reporter. Scale bars in c, d, 50 μm.
Spatiotemporal restriction of Neurog expression.

a, Lateral view of in situ hybridization (ISH) for Neurog (green) in embryo electroporated with Neurog b-line > H2B::mCherry (red) shows that Neurog expression is selectively maintained in only a subset of initially Neurog-expressing neural plate border cells. a', In the b9.36 lineage, the anterior-most cell (b11.141, solid arrowhead) is always the sole one to express Neurog at this stage, and will go on to become the anterior BTN. Dashed arrowhead indicates b11.142, the sister cell of b11.141, which has downregulated Neurog relative to its sibling. b, b'. The identities of the cells in the tail tip (presumed b8.21-derived) lineages are unclear, but Neurog is similarly restricted (arrowheads) to a single cell on either side of the midline, which we interpret as the definitive posterior BTNs. c, Control embryo treated with DMSO vehicle, showing wild-type pattern of Neurog expression only in b11.141. d, Neurog is expanded to b11.142 upon treatment with the MEK inhibitor U0126 at 7 h.p.f. This condition also results in specification of supernumerary BTNs, presumably due to expanded Neurog expression (see text for details). Thus, downregulation of Neurog in b11.142 also requires MEK/ERK signalling. e, Diagram of the aBTN lineage, descended from the b8.18 blastomere. Scale bars in a, b, 25 μm. Scale bars in c, d, 10 μm.
Extended Data Figure 5 | Perturbation of Notch signalling does not alter Neurogenin expression or bipolar tail neuron specification and differentiation. **a**, Top, lateral view of a stage 23 embryo electroporated with Msx>H2B::mCherry (magenta nuclei), Neurog b-line>unc-76::eGFP (green) and Msx>nls::lacZ, serving as the wild-type control condition. Bottom, embryo electroporated with same reporters as upper panel, plus Msx>Su(H)-DBM, which encodes a DNA-binding mutant form of the Notch co-activator Rbpj. No discernable difference in Neurog activation or BTN specification was observed between control and Su(H)-DBM conditions (1 of 32 versus 2 of 42 embryos showing ectopic Neurog+ BTNs, respectively). **b**, Late overexpression of Su(H)-DBM using the Neurog b-line driver similarly did not alter BTN specification/differentiation, as monitored by Asic>unc-76::eGFP reporter expression (0 of 50 control versus 0 of 50 Su(H)-DBM embryos showed ectopic Asic+ BTNs). Scale bars, 50 μm.
Extended Data Figure 6 | Cell polarity and morphogenesis of bipolar tail neurons. a, Embryo at 11.5 h.p.f. (18°C) with BTNs displaced from clonally related epidermal cells (epid.) labelled by UNC-76::VenusYFP (red), Galnt7ΔC::CFP (green), and H2B::mCherry (blue) driven by Neurog b-line cis-regulatory module. Targeted localization of CFP by the Galnt7 N-terminal signal sequence reveals polarized subcellular distribution of Golgi apparatus on posterior side of BTN nuclei as migration and proximal process extend in an anterior direction. This is distinct from the apical (dorsal) location of the Golgi apparatus in epidermal cells. b, Embryo at 12.5 h.p.f. (18°C) showing 180° inversion of Golgi apparatus localization to the anterior side of the nucleus, immediately preceding distal process extension. Scale bars in a, b, 50 μm. c, Still frames from a confocal image stack time lapse movie (Supplementary Video 4) showing inversion of Golgi complex (Galnt7ΔC::VenusYFP, green) relative to nuclei (H2B::mCherry, red) in migrating BTNs. Time lapse imaging initiated at 11.5 h.p.f. (18°C). Time in minutes elapsed from start shown at bottom right of each panel. Anterior BTN (aBTN) indicated by magenta arrowhead, posterior BTN (pBTN) indicated by white arrowhead. Scale bar, 25 μm. d, Diagram showing correlation of average length of proximal (left) and distal (right) processes and angle of Golgi apparatus location relative to cell nucleus along the anterior–posterior axis in BTNs at different time points. Locations of Golgi apparatus represented by rose plots of bins of 20° spanning anterior (0°) and posterior (180°) endpoints around dorsal edge of BTN nucleus. Bin diameters indicate number of cells. Embryos analysed belong to the same pool as embryos in a and b. See Supplementary Table 1 for source data.
Extended Data Figure 7 | Proposed evolution of neural crest through the acquisition of multipotency by neural plate border cells. 

**a**, Cartoon diagram depicting a hypothetical path for neural plate border and neural crest evolution, starting with the reconstructed last common olfactorean ancestor, which could have had neural plate borders lined with committed progenitor cells giving rise to several pigmented ocelli and BTN-like peripheral neurons, a condition that may be conserved in extant cephalochordates. These cells would have been reduced in the highly miniaturized embryos of extant tunicates, while vertebrates are proposed to have co-opted a mesenchymal, multipotency program to bestow these cells with the potential to give rise to pigment cells, peripheral neurons or other derivatives, after a prolonged period of EMT and migration.

**b**, Diagram representing idealized cell lineages in the neural plate borders of tunicate and hypothetical urolfactorean ancestor, in which segregated lineages at the neural plate borders give rise to committed pigment cell or peripheral neuronal progenitors.

**c**, Diagram of simplified neural crest cell lineage deploying a multipotency program downstream of neural plate border specification and upstream of cell differentiation. Thus, neural crest cells could have evolved through redeployment of a multipotency program (intercalation hypothesis), or through its maintenance from earlier embryonic stages (heterochrony hypothesis).
### Extended Data Table 1 | Synaptic input from bipolar tail neurons to motor neurons, identified by electron microscopy

| Postsynaptic motor neuron identity | Synapse partnership | Number of synapses | Total number of sections with synaptic profile |
|-----------------------------------|---------------------|--------------------|---------------------------------------------|
| MN1 Left (A11.118)                | BTN1→MN1L           | 27                 | 134                                        |
|                                   | BTN3→MN1L           | 21                 | 88                                         |
| **Total**                         |                     | **48**             | **222**                                    |
| MN1 Right (A11.118)               | BTN1→MN1R           | 3                  | 14                                         |
|                                   | BTN2→MN1R           | 22                 | 94                                         |
|                                   | BTN3→MN1R           | 1                  | 4                                          |
|                                   | BTN4→MN1R           | 11                 | 55                                         |
| **Total**                         |                     | **37**             | **167**                                    |
| MN2 Left (A10.57)                 | BTN1→MN2L           | 10                 | 51                                         |
|                                   | BTN3→MN2L           | 6                  | 30                                         |
| **Total**                         |                     | **16**             | **81**                                     |
| MN2 Right (A10.57)                | BTN2→MN2R           | 17                 | 90                                         |
|                                   | BTN4→MN2R           | 10                 | 73                                         |
| **Total**                         |                     | **27**             | **163**                                    |
| MN3 Left                          | BTN1→MN3L           | 1                  | 2                                          |
| **Total**                         |                     | **1**              | **2**                                      |
| MN4 Left                          | BTN1→MN4L           | 2                  | 9                                          |
| **Total**                         |                     | **2**              | **9**                                      |
| MN4 Right                         | BTN2→MN4R           | 2                  | 5                                          |
|                                   | BTN4→MN4R           | 1                  | 2                                          |
| **Total**                         |                     | **3**              | **7**                                      |
| MN5 Left                          | BTN1→MN5L           | 1                  | 3                                          |
| **Total**                         |                     | **1**              | **3**                                      |
| MN5 Right                         | BTN4→MN5R           | 1                  | 3                                          |
| **Total**                         |                     | **1**              | **3**                                      |

BTN, bipolar tail neuron. MN, motor neuron. Axons of BTN1 and BTN3 lie on the left hand side of the embryo, and BTN2 and BTN4 on the right. The axons are not traced to their somata to indicate which would be anterior and posterior.