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Hemichordate neurulation and the origin of the neural tube

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The origin of the body plan of our own phylum, Chordata, is one of the most fascinating questions in evolutionary biology. Yet, after more than a century of debate, the evolutionary origins of the neural tube and notochord remain unclear. Here we examine the development of the collar nerve cord in the hemichordate *Balanoglossus simodensis* and find shared gene expression patterns between hemichordate and chordate neurulation. Moreover, we show that the dorsal endoderm of the buccal tube and the stomochord expresses Hedgehog RNA, and it seems likely that collar cord cells can receive the signal. Our data suggest that the endoderm functions as an organizer to pattern the overlying collar cord, similar to the relationship between the notochord and neural tube in chordates. We propose that the origin of the core genetic mechanisms for the development of the notochord and the neural tube date back to the last common deuterostome ancestor.

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The evolution of chordates has been investigated for more than a century; however, the origin of the chordate body plan remains controversial^{1–8}. Establishment of the chordate body plan is achieved via several dramatic events, probably including dorsoventral inversion. The dorsoventral inversion was originally proposed as an inversion between protostomes and chordates by Geoffroy–St Hilaire (reviewed by Gee²), and recent molecular studies have offered strong support that the inversion indeed occurred between non-chordates and chordates⁹. Establishment of the chordate body plan is also accompanied by the acquisition of novel organs, such as the neural tube and notochord. Hemichordates, together with echinoderms, and possibly Xenacoelomorpha are the sister group of chordates¹⁰. Compared with the highly specialized radial form of the body plan of extant echinoderms, hemichordates may retain more of the ancestral form of deuterostomes and are consequently more suitable for comparison as an outgroup taxon⁴. Recent molecular developmental biology studies have shown startling similarities between the molecular architectures of the nervous systems between chordates and non-chordate invertebrates^{11–13}. Hemichordates and vertebrates share the genetic mechanisms for anteroposterior patterning of the neuroectoderm^{11,13}. In addition, dorsoventral patterning within the central nervous system via BMP signalling is highly conserved between the annelid *Platynereis* and vertebrates; and thus, the origin of the patterning mechanism predates the last common bilaterian ancestor¹². In hemichordates, BMP signalling is also involved in the patterning of the dorsoventral axis of body regions⁹, but there is no evidence that the nervous system of hemichordates has distinct dorsoventral patterning similar to protostomes and chordates. Rather it is only recently that the hemichordates were revealed to possess a centralized nervous system¹⁴. The presence of a central nervous system in protostomes and hemichordates indicates that the centralization of the nervous system occurred before the divergence of protostomes and deuterostomes¹⁴. However, there is a critical difference in the nervous system of bilaterians. Only chordates have a tubular central nervous system along their anteroposterior body axis. That is, the neural tube and its patterning are chordate novelties. Thus, the origin of the tubular central nervous system is a critical issue for elucidating the evolution of the chordate body plan.

Hemichordates have nerve cords in the dorsal midline and ventral midline. The dorsal nerve cord is divided into the proboscis stalk region, the collar cord and the dorsal nerve cord in the trunk region¹⁴. The ventral nerve cord exists only in the trunk region and is connected to the dorsal nerve cord via the prebranchial nerve ring. Among them, only the collar cord has tubular organization and thus proposed to be a homologous organ to the neural tube^{4,8,15–17}. However, this homology is still controversial^{14,16}, because the enteropneust collar cord, unlike the much more extensive neural tubes of chordates, runs along only a relatively short stretch of the anteroposterior axis¹⁸. In addition, the dorsal position of the collar cord is not consistent with the dorsoventral axis inversion in the chordate lineage^{9,18}. To investigate the potential homology between the collar cord and neural tube, we here examine the development of the collar cord in the enteropneust *Balanoglossus simodensis*, focusing on the expression patterns of genes known to be critical for the early patterning and formation of the chordate neural tube. We find conserved gene expression patterns between neurulations of hemichordates and chordates. The present results suggest that the origin of genetic mechanisms to form and pattern a tubular nervous system predates the diversification of hemichordates and chordates.

Results

Hemichordate neurulation. In *B. simodensis* development, the neural plate is visualized by the expression of the neural marker gene *BsimElav* at the dorsal midline of the prospective collar region at the pre-metamorphic stage (~6 weeks post fertilization) (Fig. 1a–e)¹⁴. The neural plate consisted of several layers of *elav*-positive cells. Two days after settlement, the neural plate invaginated and the collar cord formed in a similar manner as observed during chordate neurulation (Fig. 1f–o). One week after settlement, the collar cord was separated from the epidermis and neurulation was completed (Fig. 1p–t). Note that although some enteropneust species lack an obvious tubular collar cord because the central lumen of the cord is filled with cells, their collar cord still forms via invagination of the neural plate¹⁶.

As nothing is known about the molecular mechanism of hemichordate neurulation or dorsoventral patterning of the nerve cord, we examined the expression of genes involved in the formation and patterning of the chordate neural tube. In chordates, BMP signalling performs essential roles in morphogenesis during neurulation and dorsoventral patterning of the neural tube^{19,20}. In the hemichordate *Saccoglossus kowalevskii*, *bmp2/4* is involved in dorsoventral patterning of the body axis⁹, but the function of BMP signalling in the collar cord remains unclear. In *B. simodensis*, *BsimBmp2/4* was expressed in the dorsal surface of the neural plate during neurulation and then was expressed in the dorsal part of the collar cord (Fig. 2a,b). We examined the expression of several genes whose vertebrate homologues mark the neural plate border and pattern the dorsal neural tube, including *dlx*, *pax3/7* and *soxE*²¹. *BsimDlx* was also expressed in the dorsal domain of the neural plate as *BsimBmp2/4* (Fig. 2c,e). After neurulation, *BsimDlx* was expressed in the dorsal domain of the collar cord (Fig. 2d,f). Compared with *BsimBmp2/4* and *BsimDlx*, *BsimPax3/7* and *BsimSoxE* had a different expression pattern at the pre-metamorphic stage. The two genes were expressed at the boundary between the neural plate and epidermis during neurulation (Fig. 2g,i). In the later stage, *BsimPax3/7* and *BsimSoxE* were expressed in the dorsal domain of the collar cord (Fig. 2h,j). The later expression of *BsimSoxE* was expanded more laterally in the collar cord (Fig. 2j–l). We also found that a type A fibrillar collagen gene *BsimColA* was also expressed in the dorsal part of the collar cord (Fig. 2m,n). In chordates, *pax2/5/8* is expressed in differentiating interneurons located laterally in vertebrates and dorsolaterally in amphioxus^{22,23}. In *B. simodensis*, the *pax2/5/8* ortholog *BsimPax2/5/8* is expressed in the whole-collar cord, except for the dorsal region of the neural canal (Fig. 2o,p). In contrast to the conserved expression of these dorsal-patterning genes, we were unable to detect the expression of ventral marker genes, such as *pax6*, in the collar cord (Supplementary Fig. S1). These results suggest that the molecular mechanisms of the hemichordate collar cord and chordate neural tube patterning are only partly conserved. In addition, expression of these genes at the neural plate border indicates that hemichordates possess part of the gene regulatory network necessary for neural crest induction²⁴. We also examined the expression of these collar cord patterning genes at the level of the trunk region, but we did not detect expression in either the dorsal nerve cord or ventral nerve cord.

Dorsal endoderm of the buccal tube and the stomochord. As Hedgehog signalling from the notochord to the neural plate is essential for floor plate induction and for patterning of the neural tube along the dorsoventral axis²⁵, we next examined whether a similar type of cell exists in hemichordates. We found that *BsimHh* was expressed in the stomochord progenitor and the

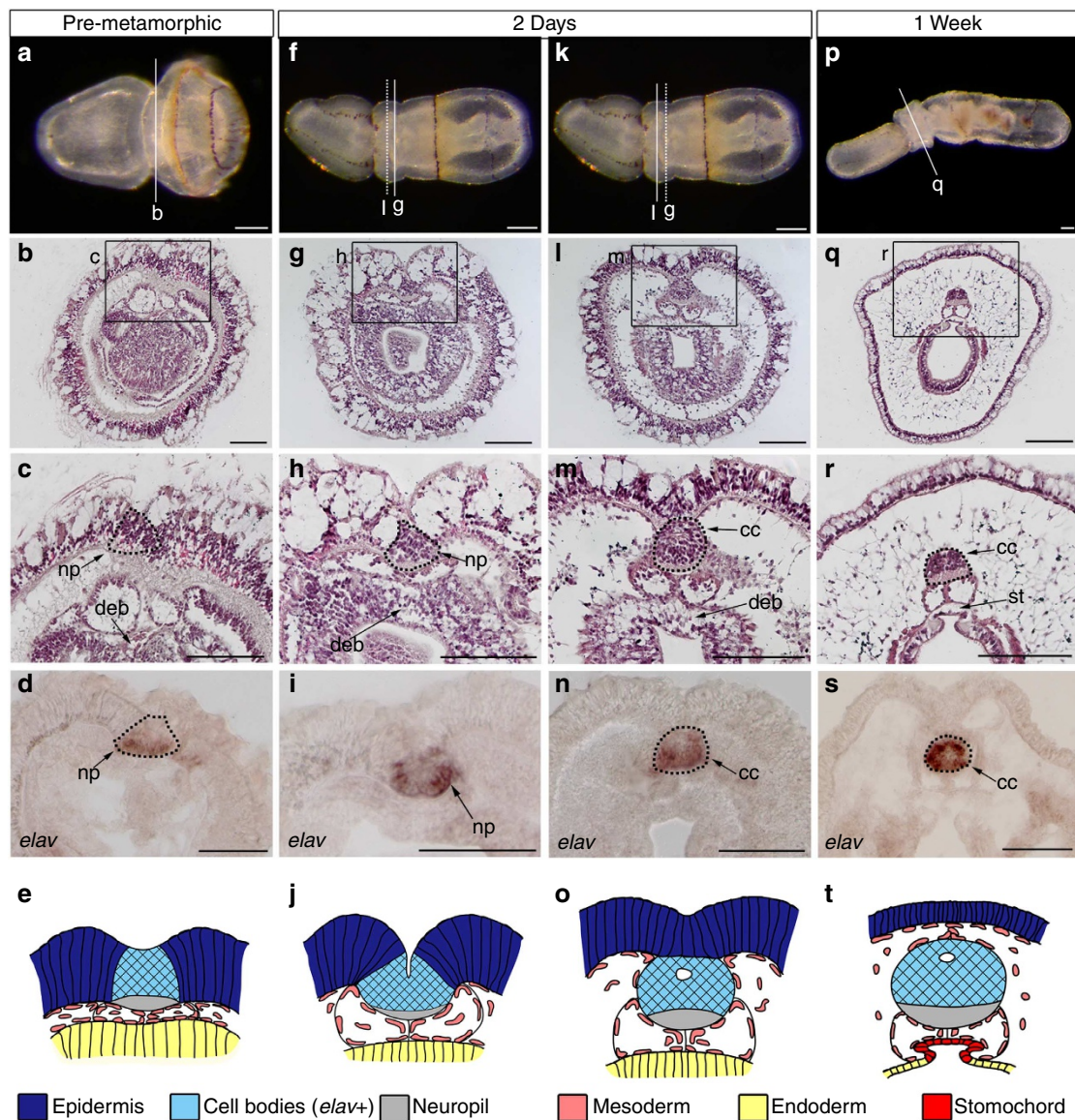


Figure 1 | Neurulation of *B. simodensis*. External morphology, cross sections, *BsimElav* expression and schema of pre-metamorphic larvae (**a–e**), 2-day-old juveniles (**f–o**) and 1-week-old juveniles (**p–t**). (**a**) Dorsal view of a pre-metamorphic larva. Anterior is to the left. (**b**) A cross section of the collar primordium. (**c**) A high-magnification view of the boxed area in **b** showing the neural plate. (**d**) *BsimElav* expression in the neural plate. (**e**) Schematic illustration of the pre-metamorphic stage. (**f**) Dorsal view of a juvenile 2 days after settlement. (**g**) A cross section of the posterior part of the collar where the neural plate was invaginating. (**h**) A high-magnification view of the boxed area in **g**. (**i**) *BsimElav* expression in the invaginating neural plate. (**j**) Schematic illustration of the invaginating neural plate. (**k**) Dorsal view of a juvenile 2 days after settlement. (**l**) A cross section of the middle part of the collar where tubular collar cord formed. (**m**) A high-magnification view of the boxed area in **l**. (**n**) *BsimElav* expression in the collar cord. (**o**) Schematic illustration of the newly formed collar cord. (**p**) Dorsal view of a juvenile 1 week after settlement. (**q**) A cross section of the collar and the stomochord. (**r**) A high-magnification view of boxed area in **q** showing the collar cord and the stomochord. (**s**) *BsimElav* expression in the collar cord. (**t**) Schematic illustration of the dorsal part of the collar. cc, collar cord; deb, dorsal endoderm of the buccal tube; np, neural plate; st, stomochord. Scale bar, 100 μ m.

dorsal endoderm of the buccal tube before settlement (Fig. 3a). Expression was maintained in the stomochord and dorsal endoderm of the buccal tube after settlement (Fig. 3b–d; Supplementary Fig. S2a). Notably, these Hedgehog-positive cells lay beneath the collar cord during metamorphosis. Thus, we asked whether the collar cord has the potential to respond to Hedgehog signalling by examining the expression of patched encoding the receptor for hedgehog. Before settlement, *BsimPtc* was expressed in the mesoderm surrounding the *BsimHh*-expressing endoderm and in the midline of the neural plate (Fig. 3e). *BsimPtc* expression in the collar cord and mesodermal cells was maintained after collar cord formation (Fig. 3f–h;

Supplementary Fig. S2b). These results indicate that the anterior endoderm (stomochord and dorsal endoderm of the buccal tube) functions as the source of Hedgehog molecules, while collar cord and mesoderm cells have the potential to respond to the signal (Fig. 3i–l).

To further test cell type similarity between the notochord of chordates and the anterior endoderm of hemichordates, we examined another aspect of notochord function, which is as a hydrostatic axial skeleton. For the notochord to function as a hydrostatic axial skeleton, prominent vacuoles and a collagenous sheath are critical (Fig. 4a,b)²⁶. We examined the expression of *BsimColA*, the hemichordate homologue of the chordate fibrillar

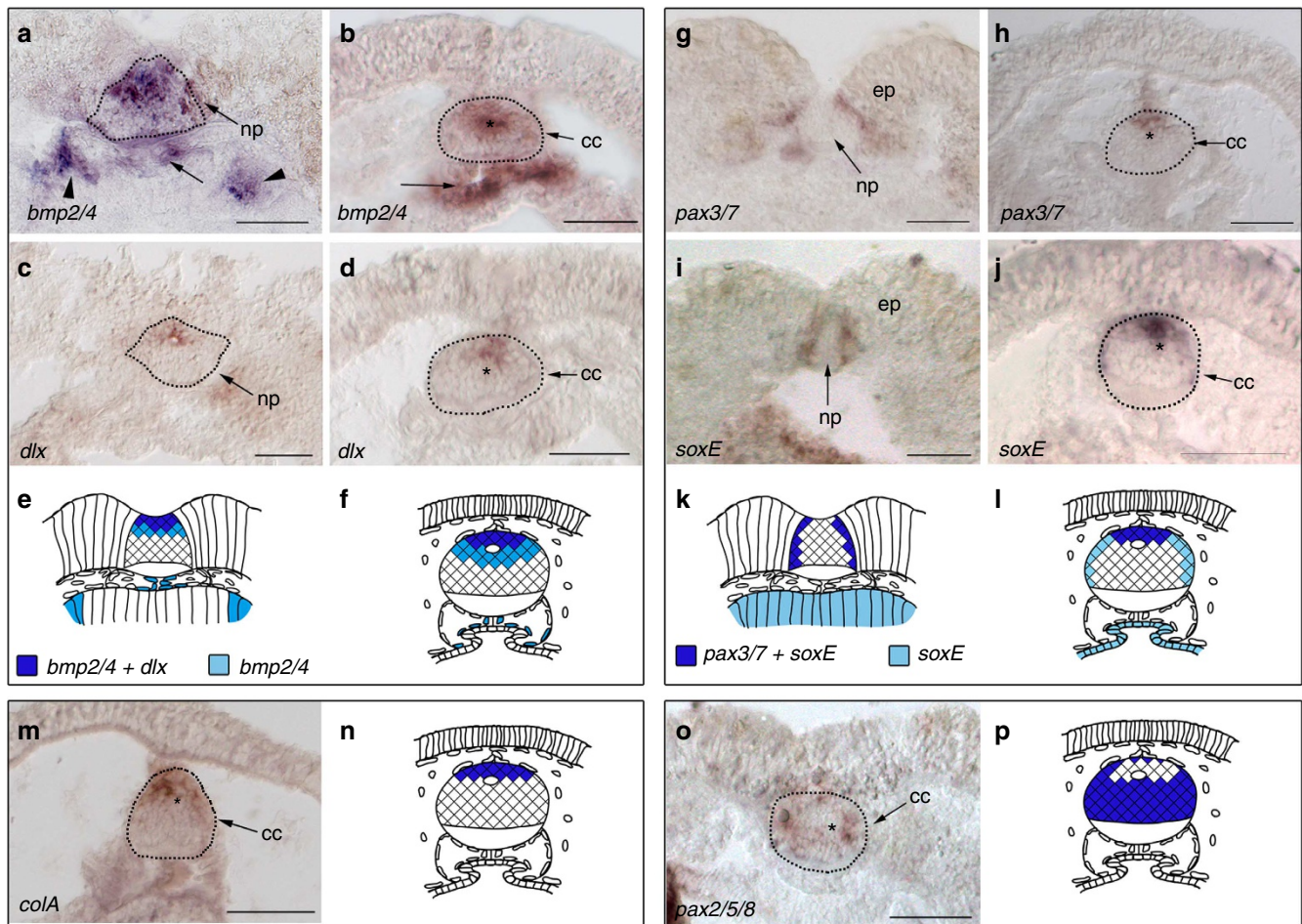


Figure 2 | Gene expression during neurulation in *B. simodensis*. The collar cord is indicated by a dotted line. Asterisks indicate the position of the neural canal. **(a)** *BsimBmp2/4* was expressed on the dorsal surface of the neural plate, mesodermal cells, which gave rise to the blood vascular system and developing gill slits (arrowheads) before neurulation. **(b)** *BsimBmp2/4* was expressed in the dorsal part of the collar cord and mesodermal cells (arrow). **(c)** *BsimDlx* was expressed in the dorsal surface of the neural plate before neurulation. **(d)** *BsimDlx* was expressed in the dorsal part of the collar cord. **(e)** Schematic illustration of *BsimBmp2/4* and *BsimDlx* expressions before neurulation. **(f)** Schematic illustration of *BsimBmp2/4* and *BsimDlx* expressions after neurulation. **(g)** *BsimPax3/7* expression was detected in the border between the neural plate and epidermis before neurulation. **(h)** *BsimPax3/7* was expressed in the dorsal part of the collar cord. **(i)** *BsimSoxE* expression was detected in the border between the neural plate and epidermis before neurulation and the dorsal endoderm of the buccal tube. **(j)** *BsimSoxE* was expressed in the dorsal and lateral parts of the collar cord. **(k)** Schematic illustration of *BsimPax3/7* and *BsimSoxE* expressions before neurulation. **(l)** Schematic illustration of *BsimPax3/7* and *BsimSoxE* expressions after neurulation. **(m)** *BsimColA* was expressed in the dorsal part of the collar cord. **(n)** Schematic illustration of *BsimColA* expression in the collar cord. **(o)** Expression of *BsimPax2/5/8* was detected broadly in the collar cord, except for the dorsal region of the neural canal. **(p)** Schematic illustration of *BsimPax2/5/8* in the collar cord. cc, collar cord; ep, epidermis; np, neural plate. Scale bar, 50 μ m.

collagen genes (clade A)²⁶. *BsimColA* was expressed in the stomochord progenitor of pre-metamorphic larvae and in the stomochord of juvenile worms (Fig. 4c–f). Expression was also detected in the dorsal endoderm of the buccal tube (Supplementary Fig. S3). These expression patterns provide additional information for cell type similarity between the anterior endoderm of hemichordates and the notochord. Hemichordates have a skeletal element called the proboscis skeleton around the stomochord. The proboscis skeleton forms during metamorphosis at the same time as the stomochord (Fig. 4g–m). Thus, the proboscis skeleton is comparable with that of the notochordal sheath. The dorsal endoderm of the buccal tube may also perform a similar role in providing physical strength in the hemichordate collar region.

Discussion

We present the first evidence that the hemichordate collar cord is subdivided into dorsoventral domains. Furthermore, we suggest

that this patterning may be regulated by Hedgehog signalling from the dorsal endoderm of the buccal tube (Fig. 5). Complex dorsoventral differentiation within the nervous system has been reported in protostomes, such as the polychaete *Platynereis dumerilii*¹². In *Platynereis*, the neuroectoderm is subdivided into dorsoventral domains that match corresponding domains in the vertebrate neural tube¹². Moreover, patterning genes of the neuroectoderm are sensitive to BMP signalling¹². The report indicates that the molecular mechanism responsible for dorsoventral patterning of the neuroectoderm predates the divergence of protostomes and deuterostomes. However, the mechanism patterns the neuroectoderm along the whole-body axis in *Platynereis* (Fig. 5, protostome). In contrast, the mechanism is activated within the neural tube to pattern it dorsoventrally in chordates (Fig. 5, chordate). We show here that the collar cord, the tubular nervous system in hemichordates, has dorsoventral differentiation (Fig. 5, hemichordates). Our present data suggest that the patterning mechanism was already recruited

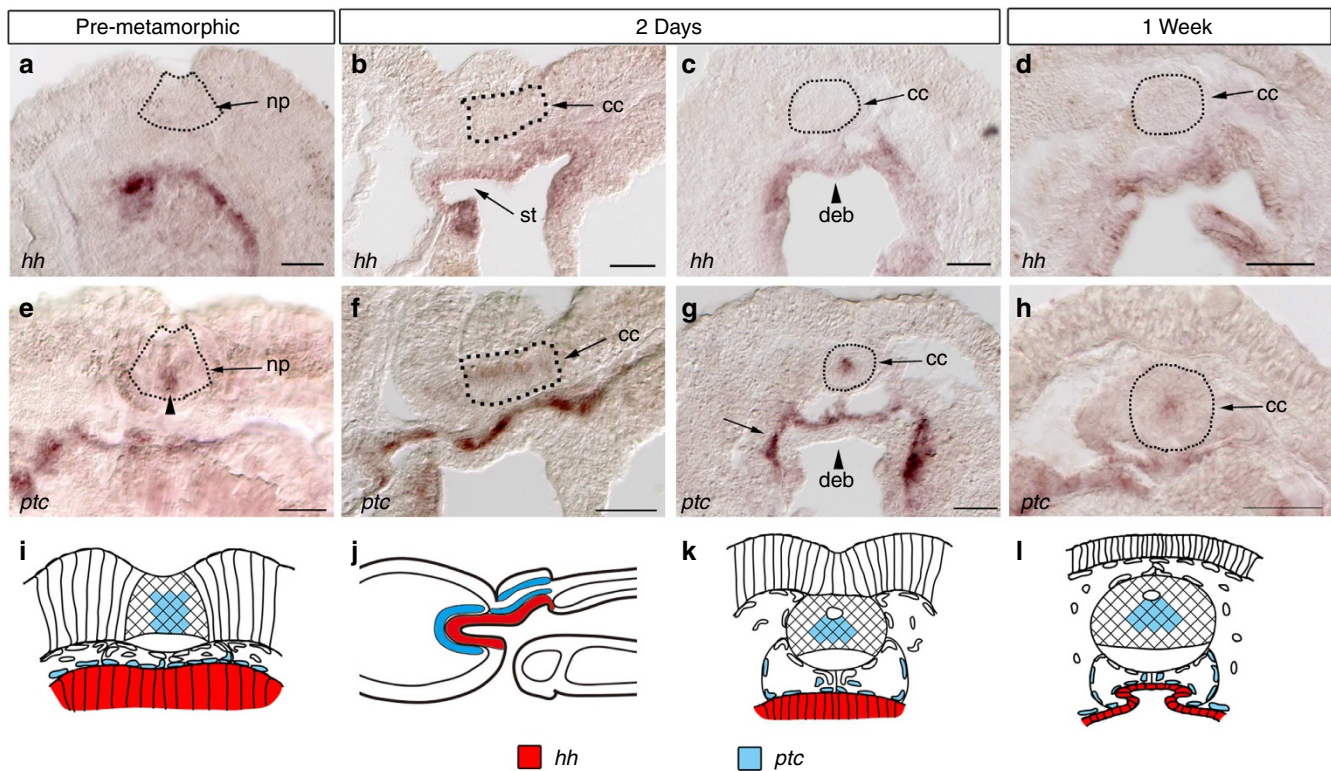


Figure 3 | *Hh* and *Ptc* expression during *B. simodensis* neurulation. (a) *BsimHh* was expressed in the anterior endoderm in pre-metamorphic larvae. (b) A longitudinal section of a juvenile 2 days after settlement shows *BsimHh* expression in the stomochord and the dorsal endoderm of the buccal tube. (c) A cross section of the collar shows *BsimHh* expression in the dorsal endoderm of the buccal tube. (d) A cross section of the collar shows *BsimHh* expression in the dorsal endoderm of the buccal tube. (e) *BsimPtc* was expressed in the mesodermal cells surrounding the endoderm and the midline cells in the neural plate. (f) A longitudinal section of 2 days old juvenile shows *BsimPtc* expression in the mesodermal cells and collar cord cells. (g) A cross section of the collar shows *BsimPtc* expression in the collar cord and mesodermal cells. (h) A cross section of the collar shows *BsimPtc* expression in the collar cord and mesodermal cells. Schematic illustrations of expression patterns of *BsimHh* and *BsimPtc* in pre-metamorphic larva (i), 2-day-old juveniles (j,k) and 1-week-old juvenile (l). cc, collar cord; deb, dorsal endoderm of the buccal tube; np, neural plate; st, stomochord. Scale bar, 50 μ m.

to the tubular nervous system in the last common deuterostome ancestor. Moreover, no evidence has so far been reported on the involvement of hedgehog signalling in the dorsoventral regulation of the protostome CNS. Our results suggest that recruitment of hedgehog signalling predates chordate ancestors and that the recruitment possibly contributed to the acquisition of a 'tubular' nervous system. However, compared with the highly organized annelid and chordate CNS, differentiation along the dorsoventral axis within the collar cord is limited in hemichordates. The distributions of neurotransmitters and cell types of neurons are not regionalized along the dorsoventral axis in the collar cord^{4,14}. We found that only some dorsal markers show restricted expression in the collar cord. This may be due to the secondary modification in the hemichordate lineage. As enteropneusts burrow in mud, they have probably reduced a part of their locomotion system. Further analyses about the neuromuscular system of enteropneusts should help our understanding on this issue. Some ventral marker genes (*sim* and *chordin*) show expression in the ventral midline of the body of *S. kowalevskii*⁹. These observations suggest the possibility that the hemichordate nervous system extends dorsoventrally along whole body and the collar cord would be comparable only with the dorsal-most part of the neural tube.

Our results lead to the discussion on the homology between the chordate notochord and the hemichordate anterior endoderm, the stomochord plus dorsal endoderm of the buccal tube. The notochord has two functions: as a hydrostatic skeleton and a source of neural tube patterning. The former was supported by

the expression of fibrillar collagen, and as noted by Bateson²⁷, the stomochord is rich in vacuoles. In addition, we show that the dorsal endoderm of the buccal tube is also rich in vacuoles and expresses the fibrillar collagen gene. Thus, the stomochord is quite able to function as a hydrostatic skeleton and indeed functions as a mechanical support to resist pumping of the pericardium¹⁸. The latter was supported from the *hedgehog* expression. In this sense, *ptc* expression in the mesodermal cells is noteworthy. This suggests that the relationship between the anterior endoderm and collar mesoderm is comparable with that between the notochord–paraxial mesoderm in vertebrates.

In contrast, homology between the stomochord and notochord has been challenged mainly from two aspects. The first comes from the differences in gene expressions between the stomochord and notochord; genes that are expressed in the notochord and its precursor. For example, the T-box gene *brachyury* has an essential role in notochord differentiation^{28,29}. A previous report has shown that there is no *brachyury* expression in the stomochord of hemichordates³⁰. Our reexamination of *brachyury* expression in *B. simodensis* did not identify expression in either the stomochord or the dorsal endoderm of the buccal tube, while expression was detected in the posterior end of the trunk (Supplementary Fig. S4).

The second challenge is from the dorsoventral inversion hypothesis. As the notochord develops from the dorsal part of the archenteron, according to the dorsoventral axis inversion hypothesis, the homologous structure should occupy the ventral side of the hemichordate endomesoderm. However, the

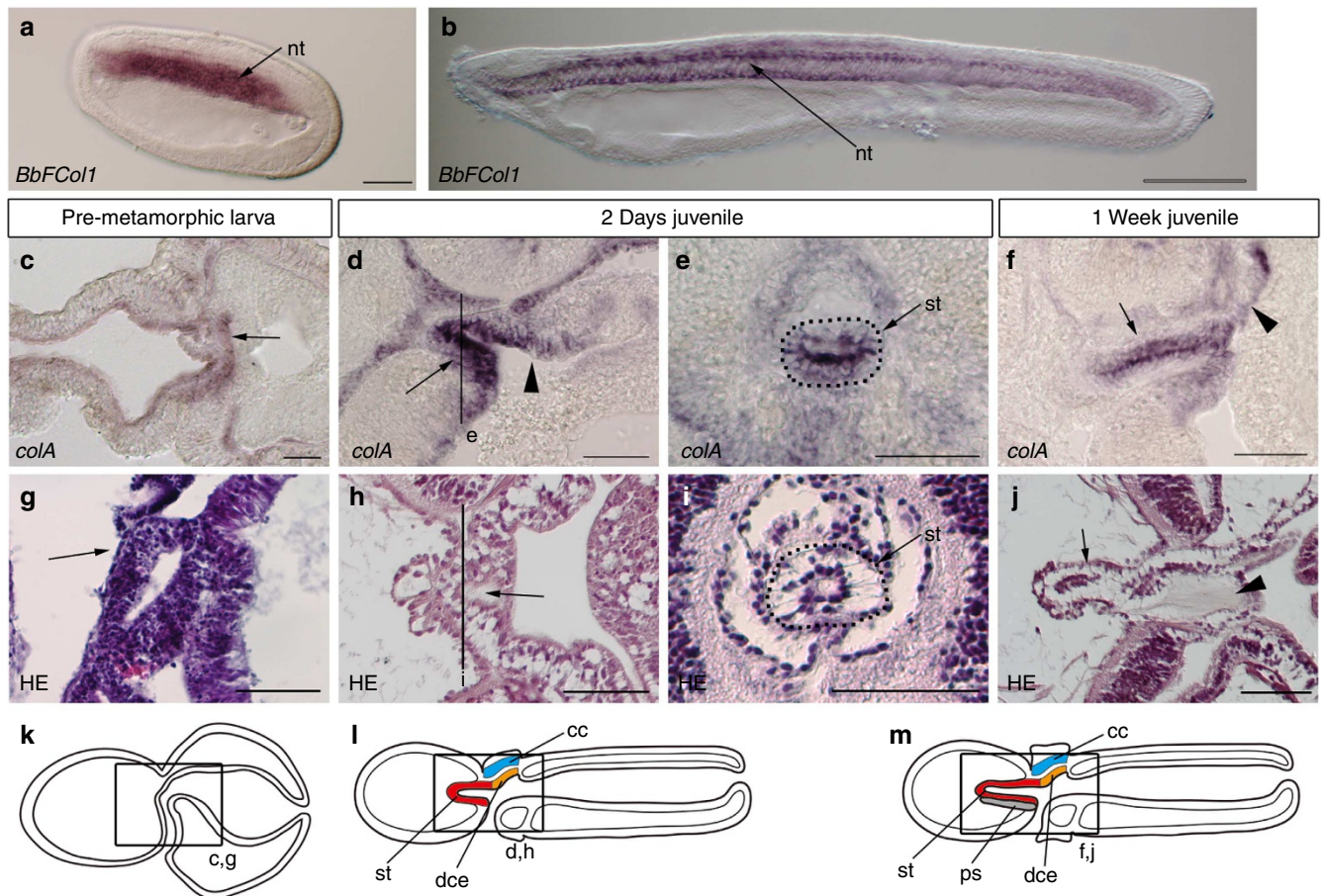


Figure 4 | Morphogenesis and gene expression of the anterior endoderm during metamorphosis of *B. simodensis*. Expression of the amphioxus fibrillar collagen gene *BbFCol1* at 14 h (a) and 22 h (b) post fertilization. (c) *BsimColA* expression was detected in the anterior endoderm in pre-metamorphic larva. (d) A longitudinal section of 2 days juvenile shows *BsimColA* expression in the stomochord (arrow) and the dorsal endoderm of the buccal tube (arrowhead). (e) A cross section shows *BsimColA* expression in the stomochord. (f) A longitudinal section shows *BsimColA* expression in the stomochord (arrow) and the dorsal endoderm of the buccal tube (arrowhead). (g) A longitudinal section of the anterior endoderm in a pre-metamorphic larva. (h) A longitudinal section of the anterior endodermal region shows invagination of the stomochord (arrow). (i) A cross section of the developing stomochord. (j) A longitudinal section of the anterior endodermal region showing the stomochord (arrow) and the proboscis skeleton (arrowhead). Schematic illustrations of longitudinal sections of pre-metamorphic larva (k) and 2-day- (l) and 1-week-old (m) juveniles. Boxed areas in k, l and m indicate positions of c and g, d and h, and f and j, respectively. cc, collar cord; deb, dorsal endoderm of the buccal tube; nt, notochord; p, proboscis; ps, proboscis skeleton; st, stomochord. Scale bar, 50 μ m.

stomochord develops as an anterior protrusion from the dorsal wall of the buccal tube. Similarly, homology between the hemichordate dorsal collar cord and chordate CNS is not consistent with the dorsoventral inversion hypothesis. Rather, the ventral trunk nerve cord and the pygochord, which are vacuolated cells in the posteroventral mesentery, were proposed as homologous structures to the chordate notochord and CNS, respectively^{31,32}.

Based on these viewpoints, we consider two possible evolutionary scenarios. First, in the deuterostome ancestors, hedgehog-positive notochord/stomochord-like organs occupy the endoderm midline both dorsally and ventrally. In hemichordates, hedgehog-positive cells are retained only in the anterodorsal part of the endoderm, where it patterns the dorsally located nerve cord. In contrast, in the chordate lineage, the hedgehog-positive cells are only retained ventrally. The ventral PNS of amphioxus³³ and ascidians³⁴ may be evolutionary remnants, homologous to the dorsal nerve cord of hemichordates.

The second possible scenario is co-option of hedgehog-dependent nerve patterning originally establishing the

anterodorsal part of the endoderm in the last common deuterostome ancestor. This hedgehog-dependent nerve patterning may be activated on the other side: the dorsal side of chordates. In this case, the stomochord and notochord share only the genetic regulatory machinery for the hydrostatic skeleton and patterning of the nervous system. It is notable that the *hedgehog* and *ColA*-positive cells are also found in the anterior endoderm in chordates^{35,36}, which may represent the homologue of the hedgehog-positive endoderm cells of hemichordates. In either scenario, the genetic linkage between the hedgehog secreting cells and *brachyury* expression was established in the chordate ancestors.

Several issues remain to be resolved to bridge the deep gap between the body plan of non-chordates and that of chordates. For example, we do not understand how metameric musculature was acquired or how coordinated development was established between metameric muscle blocks and the CNS in chordate ancestors. Further examination of hemichordate developmental biology will provide greater insight into the dramatic events in the evolution of the chordate body plan.

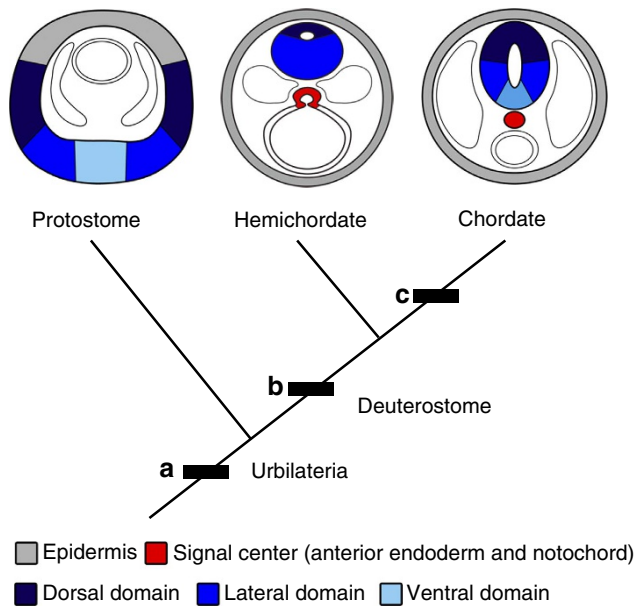


Figure 5 | Schematic illustration of bilaterian nervous systems. A complex dorsoventral patterning in the neuroectoderm was present in the common ancestor of protostomes and deuterostomes (a). In the deuterostome lineage, a tubular neural structure was acquired and the dorsoventral patterning was recruited to the tube. The tube was induced and patterned by the endodermal signals in the ancestor (b). The endodermal signal was activated (co-opted) to the newly evolved dorsal midline mesoderm, the notochord, and the signals induced the neural tube (c).

Methods

Animal collection. Adult acorn worms (*B. simodensis*) were collected along the rocky seashore of the city of Shimoda. Induction of spawning, culture of larvae and induction of metamorphosis were achieved in the laboratory³⁷.

Molecular cloning and phylogenetic analyses. Fragments of *bmp2/4*, *colA*, *elav*, *soxE*, *pax2/5/8*, *pax3/7*, *pax6*, *dlx*, *hedgehog*, *patched* and *brachyury* were amplified using degenerate primers (primer sequences and accession numbers are in Supplementary Table S1). Gene orthologies were inferred using ML analyses (Supplementary Fig. S5) and multiple alignments (Supplementary Fig. S6). Amino acid alignments were made with ClustalX ver. 2.0 (ref. 38). Amino acid evolutionary models were selected using Modelgenerator³⁹. Maximum likelihood analyses were performed with PhyML ver. 3.0 (ref. 40).

in situ hybridization. For section *in situ* hybridization, juvenile worms were treated with 2% HCl in filtered sea water (FSW) to remove mucus at 4 °C, 5 min. Then, specimens were fixed with 4% paraformaldehyde (PFA) in Mops buffer (0.1 M Mops, 0.05 M NaCl) at 4 °C, over night. Frozen sections were air dried and washed with PBST and fixed with 4% PFA/PBS for 10 min at room temperature (RT). Then, the slides were washed with PBS and digested with 1 μg ml⁻¹ proteinase K/PBS for 10 min at RT. After a brief wash with PBS, the samples were postfixed in 4% PFA/PBS for 10 min at RT. The slides were washed with PBS three times and acetylated in 0.1 M triethanolamine with 0.25% acetic anhydride for 15 min at RT and washed with PBS three times. The slides were prehybridized at least 1 h in hybridization solution (50% formamide, 5 × SSC, 5 × Denhardt's, 200 μg ml⁻¹ yeast RNA) at 60 °C and hybridized with a DIG-labeled RNA probe at 60 °C at least 16 h. The slides were washed in 50% formamide/2 × SSC for 60 min, 2 × SSC for 30 min twice, 0.2 × SSC for 30 min twice at 60 °C. Then, they were rinsed twice with maleic acid buffer (MAB), blocked 2% blocking reagent (Roche, Indianapolis, IN, USA) in MAB for 60 min at RT and incubated over night at 4 °C with a 1:1,500 dilution of anti-DIG-AP antibody (Roche) in blocking buffer. They were washed with MAB for 30 min four times and transferred into AP buffer (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM MgCl₂, 2% polyvinylalcohol) and were developed by incubating the slides in NBT/BCIP (Roche) in AP buffer until a signal was visible. The reaction was stopped in PBS, postfixed in 4% PFA/PBS over night, washed with PBS and mounted with 80% glycerol. Then, they were observed under a light microscope.

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Author contributions

N.M. and H.W. contributed to the design of experiments. N.M. performed sample collections and experiments. N.M. and H.W. discussed the results and wrote the manuscript.

Additional information

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