Convergent evolution of bilaterian nerve cords

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It has been hypothesized that a condensed nervous system with a medial ventral nerve cord is an ancestral character of Bilateria. The presence of similar dorsoventral molecular patterns along the nerve cords of vertebrates, flies, and an annelid has been interpreted as support for this scenario. Whether these similarities are generally found across the diversity of bilaterian neuroanatomies is unclear, and thus the evolutionary history of the nervous system is still contentious. Here we study representatives of Xenacoelomorpha, Rotifera, Nemertea, Brachiopoda, and Annelida to assess the conservation of the dorsoventral nerve cord patterning. None of the studied species show a conserved dorsoventral molecular regionalization of their nerve cords, not even the annelid *Owenia fusiformis*, whose trunk neuroanatomy parallels that of vertebrates and flies. Our findings restrict the use of molecular patterns to explain nervous system evolution, and suggest that the similarities in dorsoventral patterning and trunk neuroanatomies evolved independently in Bilateria.

The nervous systems of Bilateria, in particular their trunk neuroanatomies, are morphologically diverse1 (Fig. 1a). Groups such as arthropods, annelids, and chordates exhibit a medially condensed nerve cord, which is ventral in arthropods and annelids, and dorsal in chordates. By contrast, other lineages have multiple paired longitudinal nerve cords distributed at different dorsoventral levels. There are even bilaterians with only weakly condensed basiepidermal nerve nets, similar to those in cnidarians (Fig. 1a), which supports the idea that this net-like neural arrangement predates the Cnidaria–Bilatera split2,3 (Fig. 1a). However, the earliest configuration of the bilaterian central nervous system (CNS) is still debated2,4–7 (Fig. 1a), and thus it is unclear when and how often nerve cords evolved in Bilateria.

The conserved deployment of signalling molecules and transcription factors along the bilaterian anteroposterior and dorsoventral axes grounds most scenarios for the evolution of the CNS2,4,7–12. In particular, the similar expression of the transcription factors *nkx2.1/nkx2.2, nkx6, pax6, pax3/7*, and *msx* in the ventral neuroectoderm of the fly *Drosophila melanogaster* and the annelid *Platynereis dumerilii*, and the dorsal neural plate of vertebrates (Fig. 1b), is a core argument for proposing an ancestral CNS comprising a medial ventral nerve cord (VNC) in Bilateria2,4,7,12. In *P. dumerilii* and vertebrates, and to some extent in *Drosophila*, the expression of these genes correlates with the spatial location of neuronal cell types along their trunks4,9,12. Serotonergic neurons form in the ventromedial *nkx2.2/nkx6* region, cholinergic motor neurons develop in the *nkx6*/pax6* area, and *dlbx* interneurons and lateral sensory trunk neurons differentiate in the more dorsolateral *pax6*/pax3/7* and *pax3/7*/msx* domains, respectively (Fig. 1b). The dorsoventral arrangement of these transcription factors and neuronal cell types is absent in hemichordates10,11,14, nematodes15,16, and planarians17, consistent with the idea that the most recent ancestor of Bilateria had a dorsoventrally patterned, medially condensed VNC that has been repeatedly lost in these and perhaps other groups12. However, there is an alternative explanation: that a CNS with a single nerve cord and the similar dorsoventral patterning is the trait that repeatedly evolved, and thus was absent in the most recent common bilaterian ancestor2,4,8,10,11.

**Neuroectodermal patterning in Xenacoelomorpha**

To explore the conservation of neuroectodermal patterning systems in Bilateria, we first studied Xenacoelomorpha (Extended Data Fig. 1), which is the sister group to all remaining bilaterian lineages18,19 (that is, Nephrozoa). We focused our analyses on *Xenoturbella bocki*, the nemertodermatids *Meara stichopi* and *Nemertoderma westblandi*, and the aceloide *Isodiametra pulchra*. As in the aceloide *Hofstenia miamia*20 and most other bilaterians7,10, these xenacoelomorphs differentially express anteroposterior marker genes along their primary body axis21,22 (Extended Data Figs 2a, c and 3). The bone morphogenetic protein (BMP) pathway, which has an ancestral dorsoventral patterning role20,23 and an anti-neural role in *Drosophila* and vertebrates, is also similarly deployed in all studied xenacoelomorphs20, with bmp ligands expressed dorsally and antagonists located more ventrolaterally (Fig. 2a, d and Extended Data Figs 2d and 4). However, the dorsoventral transcription factors that we found in our genomic resources (Supplementary Table 1) did not show a clear staggered expression (Fig. 2b, e). Therefore, Xenacoelomorpha only exhibits the anteroposterior and BMP ectodermal patterning systems, which is reminiscent of the cnidian condition24.

Importantly, ectodermal patterning systems are deployed independently of the trunk neuroanatomy in Xenacoelomorpha. Similar to cnidarians, xenacoelomorphs have a uniformly distributed, diffuse basiepidermal nerve net5,13–27, *Xenoturbella* species only have this network26. However, nemertodermatids have additional longitudinal basiepidermal nerve cords25, located dorsally in *M. stichopi* (Fig. 2c), and ventrally in *N. westblandi* (Extended Data Fig. 2e). The aceloide *I. pulchra* also has four pairs of subepidermal nerve cords distributed along the dorsoventral axis27 (Fig. 2f). Genes commonly involved in neurogenesis (Extended Data Fig. 5a, d) and neural transmission (Extended Data Figs 2b, f and 5b, c, e) are consistently expressed in the...
sensory structures and neural condensations in these species. However, the dorsoventral transcription factor \( \text{nknx6} \) does not co-localize with the motor neuron marker \( \text{ChAT} \) in the trunk of \( \text{M. stichopi} \) and \( \text{I. pulchra} \), and the relation of \( \text{pax6} \) cells to this and another motor neuron marker \( \text{Hb9} \) is unclear in both species (Fig. 2b, e). Therefore, the diversity of neuroanatomies of Xenacoelomorpha contrasts with the more conserved deployment of ectodermal anteroposterior and BMP patterning systems. This, and the observation that disruption of BMP signalling does not affect CNS development (Extended Data Fig. 6), support the idea that the anti-neural role of the BMP pathway evolved after the Xenacoelomorpha–Nephrozoa split. Likewise, the expression of dorsoventral transcription factors unrelated to the distinct trunk

Figure 1 | CNS evolution and dorsoventral patterning. a, A nerve net is ancestral for Cnidaria and Bilateria. The neuroanatomical diversity hampers the reconstruction of the CNS evolution in Bilateria. b, A central argument for an ancestral medially condensed VNC for Bilateria is the similar deployment of dorsoventral transcription factors in vertebrates, \( \text{Drosophila} \), and the \( \text{P. dumerilii} \) larva. The staggered expression of these genes concurs with specific neuronal populations. D, dorsal; V, ventral; A, anterior; P, posterior; 5-HT, serotonin; ACh, acetylcholine.

Figure 2 | Dorsoventral patterning in Xenacoelomorpha. a, The \( \text{bmp} \) ligands and \( \text{admp} \) are expressed dorsally; \( \text{chd} \) is expressed ventroposteriorly. b, Transcription factors \( \text{nknx2.1} \), \( \text{nknx6} \), and \( \text{msx} \) are expressed ventrally; \( \text{pax6} \) is expressed broadly; \( \text{Hb9} \) and \( \text{ChAT} \) are in the nerve cords. c, \( \text{M. stichopi} \) CNS (green arrowheads indicate the anterior commissures; red arrowheads indicate the nerve cords). Tyr-tubulin, tyrosinated tubulin. d, The \( \text{bmp} \) ligands are expressed dorsally; \( \text{admp-a} \) is expressed ventromedially; \( \text{admp-b} \) is expressed anterolaterally. e, The \( \text{nknx2.1} \) paralogues and \( \text{nknx2.2} \) are expressed ventrally; \( \text{nknx6} \) is expressed laterally; \( \text{pax6} \) throughout the body; \( \text{msx} \) in isolated cells; \( \text{Hb9} \) and \( \text{ChAT} \) are in the brain. f, \( \text{I. pulchra} \) CNS (green arrowheads indicate the brain; red arrowheads indicate the nerve cords). Insets are lateral views. Abbreviations: ac, anterior commissure; bnn, basiepidermal nerve net; dnc, dorsal nerve cord; lnc, lateral nerve cord; np, neuropile; pc, posterior commissure; st, statocyst; vnc, ventral nerve cord. Scale bars, 100 \( \mu \text{m} \).
Dorsoventral patterning in Brachiopoda

To investigate the conservation of the dorsoventral nerve cord pattern in the anteroventral trunk of T. transversa, we identified staggered expression of dorsoventral transcription factors in the anterior ventral midline of the larval trunk. At this stage, nkx2.2 and pax6 are expressed in the apical lobe, albeit pax6 expression projects slightly into the mantle lobe. However, there is a more lateral nkx2.2+/pax6+ domain, a more lateral nkx6+/pax6+/pax3/7+ region, and a broad, dorsolateral msx+ area in the anterior ventral ectoderm of the larval ‘trunk’ (that is, mantle and pedicle lobes) (Fig. 3A, B and Extended Data Fig. 7a). Additionally, a narrow line of cells below the apical–mantle boundary crossing the ventral midline expresses pax3/7 (Fig. 3A, B and Extended Data Fig. 7a). These expression domains disappear in the highly modified adult body (Extended Data Fig. 7a–c). The staggered expression of dorsoventral transcription factors in the ventral anterior ectoderm of the trunk only partly correlates with the larval neuroanatomy, which consists of an anterior condensation and a medial accumulation of serotonergic cells on the ventral side, from which pairs of neurites innervate the chaetae and posterior end (Fig. 3C). The dorsoventral transcription factors do not co-express with most neuronal markers, which are mostly expressed in the anterior region (Fig. 3A, D and Extended Data Fig. 7a, d). Only two tph+ clusters in the medial serotonergic condensation of the larval trunk co-localize with the nkx2.2+/nkx6+ medial domain. Therefore, the brachiopod T. transversa resembles vertebrates, arthropods, and P. dumerilii in the presence of a ventral serotonergic nkx2.2+/nkx6+ area, as well as in the nkx6, pax6, pax3/7, and msx dorsolateral domains, which are, however, not apparently connected to any neural trunk structure.

The staggered ectodermal expression of dorsoventral transcription factors in the anteroventral trunk of T. transversa is largely conserved neuroanatomies suggests that the dorsoventral patterning of the nerve cords also evolved after the Xenacoelomorpha–Nephrozoa split.

Figure 3 | Dorsoventral patterning in Brachiopoda. A, Transcription factors nkx2.2 and nkx6 are in the trunk midline (arrowheads), posterior tip (arrows), gut, and apical cells (nkx6); pax3/7 is expressed laterally (arrowheads) and in the apical lobe (arrow); msx is in the mantle and ventral pedicle. B, There is an nkx2.2+/nkx6+ medioventral region, and a more lateral nkx6+/pax6+/pax3/7+ anterior trunk domain. C, T. transversa larval CNS (green arrowheads indicate the neuropile in a, and the trunk serotonergic condensation in b; red arrowheads mark the VNCS). D, Only tph is expressed in the trunk (arrows and arrowheads indicate expression areas). E, Transcription factors nkx2.2 and nkx6 are in the trunk ventral midline (arrowheads), apical lobe (arrows), and gut; pax3/7 is in the mesoderm (arrows), and in two ventrolateral trunk domains (arrowheads); msx is in the trunk, shell epithelium (arrowhead), and mesoderm (arrow). F, N. anomala larval CNS (green arrowheads indicate the neuropile; red arrowheads in a mark the VNCS; red arrowheads in b indicate the innervation of the chaetae). Abbreviations: ao, apical organ; bp, blastopore; ch, chaeta; mo, mouth; np, neuropile; vnc, ventral nerve cord. Scale bars, 50μm.

Figure 4 | Dorsoventral patterning in Nemertea. A, Transcription factors nkx2.1 and nkx2.2 are in the head (arrows), proboscis (nkx2.1), and trunk cells (nkx2.2); nkx6 and pax6 are in the head (arrows) and VNCS (arrowheads). B, In the VNCS, nkx2.2+ cells express tph, but not nkx6; nkx6+ cells express pax3/7 and Hb9, but not VaChT. C, L. ruber CNS (green arrowheads indicate the brain; red arrowheads mark the VNCS and the dorsal neurite in the upper inset). Abbreviations: br, brain; dnc, dorsal nerve cord; mo, mouth; tr, trunk; pb, proboscis; vnc, ventral nerve cord. Scale bar, 100μm.
In metamorphic and definitive juveniles, first detected in the larval imaginal discs (Extended Data Fig. 8a). Lineus ruber staggered expression along the trunk ventral side of the nemertean Similar to brachiopods, some dorsoventral transcription factors show connected to the CNS, suggesting that this system may rather pattern transcription factors in the anteroventral larval trunk is not necessarily Therefore, the conserved staggered expression of the dorsoventral tran- this domain does not co-localize with any serotonergic condensa- also has a medial ventral (arrowheads); and nkx6 is in two ventrolateral larval clusters and midline co-localizes with (ref. 30) and in the brachiopod juvenile head; pax3/7 is in two ventrolateral larval clusters and midline (arrowheads), but in two trunk clusters in juveniles (arrowheads); maxx paralogues are in ventral larval domains and the juvenile VNC (arrowheads). O. fusiformis CNS (green arrowheads indicate the apical larval FMRF-amide cell and juvenile brain; red arrowheads indicate the larval anterior axon and juvenile medial VNC). Abbreviations: ao, apical organ; br, brain; cb, ciliary band; cg, caudal ganglion; ch, chaetae; ln, lateral neurites; mo, mouth; ms, mastax; np, neuropile; vg, vesicle ganglia; vnc, ventral nerve cord. Scale bars, 50 μm.

in the brachiopod Novocrania anomala. In this brachiopod, nkx2.1 (ref. 30) and pax6 (ref. 31) are expressed in the apical lobe, and nkx2.2 and nkx6 are expressed medially in the trunk (Fig. 3E). As in T. transversa, nkx6 extends more laterally at the anterior trunk, where it co-localizes with pax3/7 in the early larva, and maxx is broadly detected in the trunk (Fig. 3E and Extended Data Fig. 7e). Therefore, N. anomala also has a medial ventral nkx2.2/*nkx6* domain; remarkably, however, this domain does not co-localize with any serotonin condensation, which is lacking in the larval CNS of this brachiopod (Fig. 3F). Therefore, the conserved staggered expression of the dorsoventral transcription factors in the anteroventral larval trunk is not necessarily connected to the CNS, suggesting that this system may rather pattern only the ectoderm in Brachiopoda.

Dorsoventral patterning in Nemertea
Similar to brachiopods, some dorsoventral transcription factors show staggered expression along the trunk ventral side of the nemertean Lineus ruber. In this worm, dorsoventral transcription factors are first detected in the larval imaginal discs (Extended Data Fig. 8a). In metamorphic and definitive juveniles, nkx2.1 is expressed in the head and proboscis, and pax3/7 is broadly expressed (Fig. 4a and Extended Data Fig. 8a). However, nkx2.2, nkx6, and pax6 are detected in isolated ventrolateral cells, as well as in cephalic domains (nkx2.2, nkx6, pax6) and isolated trunk cells (nkx2.2) (Fig. 4a and Extended Data Fig. 8a). Remarkably, nkx2.2 and nkx6 do not co-localize, but nkx6 and pax6 do (Fig. 4b). These staggered domains relate to the disposition of the VNCs of L. ruber (Fig. 4c). Furthermore, nkx2.2/* cells co-express the serotonergic marker tph, and nkx6/* cells express the motor neuron marker Hb9, but not VACHT (Fig. 4a, b). Therefore, the staggered expression of the dorsoventral transcription factors nkx2.2, nkx6, and pax6 are linked to the ventral trunk CNS and some neuronal cell type markers in L. ruber, which is similar to the situation described in vertebrates and P. dumerilli9,12,32.

Dorsoventral patterning in Rotifera
To explore the conservation of the dorsoventral patterning in Spiralia, we studied the rotifer Epiphanes senta, a member of the sister lineage to all remaining Spiralia38. Different from the brachiopod larvae and the nemertean juvenile, E. senta juveniles lack a staggered expression of dorsoventral transcription factors along their trunks. The three
nkx2.1 paralogs, nkx2.2, and pax6 are all in distinct brain domains of the juvenile rotifer (Fig. 5a). Only the gene nkx6 is detected in two posterior trunk cells (Fig. 5a). As in brachiopods and nemerteans, the trunk CNS comprises two VNCs, and additional paired dorsolateral nerves (Fig. 5b). The trunk expression of nkx6 probably corresponds to the vesicle ganglia1, but it is not related to motor neurons, as inferred by the expression of Hb9 and ChAT (Extended Data Fig. 9a). Therefore, spiralian with paired VNCs deploy the dorsoventral transcription factors without a consistent association with their trunk neuroanatomies.

**Dorsoventral patterning in Annelida**

To investigate the conservation of the dorsoventral patterning in Annelida, the only spiralian lineage with a medially condensed VNC1–3, we studied the annelid O. fusiformis, which belongs to the sister lineage to all remaining annelids

The ancestral nephrozoan neuroanatomy remains unclear (question mark). The dorsoventral (DV) patterning system is not tied to the CNS arrangement in Bilateria (as in Chordata and Annelida). In red, lineages analysed in this study. The green circle with red border indicates that there are annelids with and without the dorsoventral patterning.
the system was independently lost/modified many times. The differences between vertebrates and *Drosophila* in the upstream modulators of dorsoventral transcription factors and in their functional integration should thus be regarded as a case of developmental system drift over large phylogenetic distances. Alternatively, and more parsimoniously, these differences may indicate that the commonalities in dorsoventral nerve cord organization between vertebrates, arthropods, and some annelids evolved convergently (Fig. 6b and Extended Data Fig. 10c).

The similar staggered expression domains of dorsoventral transcription factors in these three lineages, together with those uncovered by our study (Figs 3 and 4), might reflect the existence of ancient ectodermal nerve cord—brain—evolution of the nervous system. *Nat. Rev. Neurosci*. 17, 61–72 (2016).

Hejnol, A. & Pang, K. Xenacoelomorpha’s significance for understanding bilaterian evolution. *Curr. Opin. Genet. Dev*. 39, 48–54 (2016).

Arendt, D., Denes, A. S., Jékely, G. & Tessmar-Raible, K. The evolution of nervous system centralization. *Phil. Trans. R. Soc. B* 365, 1523–1528 (2010).

Hejnol, A. & Lowe, C. J. Embracing the comparative approach: how robust phylogenies and broader developmental sampling impacts the understanding of nervous system evolution. *Phil. Trans. R. Soc. B* 370, 20150045 (2015).

Holland, N. D. Early central nervous system evolution: an era of skin brains? *Nat. Rev. Neurosci*. 4, 617–627 (2003).

Holland, L. Z. et al. Evolution of bilaterian central nervous systems: a single origin? *Evolution* 67, 227 (2013).

Pani, A. M. et al. Ancient deuterostome origins of vertebrate brain signalling centres. *Nature* 483, 289–294 (2012).

Mizutani, C. M. & Bier, E. EvoD/Vo: the origins of BMP signalling in the chordate nervous system. *PLoS Biol*. 15, e2001573 (2017).

Weigert, A. et al. Illuminating the base of the annelid tree using transcriptomics. *Mol. Biol. Evol*. 31, 1391–1401 (2014).

Vellutini, B. C. & Hejnol, A. Expression of segment polarity genes in brachiopods supports a non-segmental ancestral role of engrailed for bilaterians. *Sci. Rep*. 6, 32387 (2016).

Corti, A. M. & Siitonen, T. V. *kna*/*gsh*: conserved regulators of dorsoventral neural patterning? *Curr. Opin. Neurobiol*. 10, 63–71 (2000).

Winterbottom, E. F., Illes, J. C., Faas, L. & Isaacs, H. V. Conserved and novel roles for the Gsh2 transcription factor in primary neurogenesis. *Development* 137, 2623–2631 (2010).

Cheesman, S. E., Layden, M. J., Von Ohlen, T., Doe, C. Q. & Eisen, J. S. Zebrafish and fly *Nkd* proteins have similar CNS expression patterns and regulate motor neuron formation. *Development* 131, 5221–5233 (2004).

Goridis, C. & Rohrer, H. Specification of catecholaminergic and serotonergic neurons. *Nat. Rev. Neurosci*. 3, 531–541 (2002).

Peter, I. S. & Davidson, E. H. Evolution of gene regulatory networks controlling body plan development. *Cell* 144, 970–985 (2011).

**Supplementary Information** is available in the online version of the paper.

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METHODS

No statistical methods were used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

Animal collections and sample fixations. Gravid adults were collected from the coasts near Friday Harbor Laboratories, San Juan Island, Washington, USA (T. transversa), Espeland Marine Biological Station, Norway (M. stichopi and N. anomala), Fanafjorden, Norway (L. ruber), Station Biologique de Roscoff, France (O. fusiformis), and Gullmarsfjord, Sweden (N. westbladi and X. bocki). P. Ladurner (University of Innsbruck) provided a stable culture of I. pulchra, which was maintained as previously described43. A stable laboratory culture of E. senta was maintained in glass bowls with 25 ml of Jaworski's medium in a controlled environment of 20 °C and a 14:10 h light-dark cycle. They were fed ad libitum with the algae Rhodomonas sp., Cryptomonas sp., and Chlamydomonas reinhardtii. Brachiopod, nemertean, and annelid adults were spawned as described elsewhere44–47. Acoelomorph eggs were collected year round (I. pulchra) and in September–October (M. stichopi)44. All samples were fixed in 4% paraformaldehyde in culture medium for 1 h at room temperature. After fixation, samples were washed in 0.1% Tween 20 phosphate buffer saline, dehydrated through a graded series of methanol, and stored at −20 °C in pure methanol. Samples used for immunohistochemistry were stored in Tewen 20 phosphate buffer saline at 4 °C. Before fixation, larval and juvenile stages were relaxed in 7.4% magnesium chloride; E. senta were relaxed in 10% EtOH and 1% bupivacaine. The eggshells of M. stichopi and I. pulchra eggs were permeabilized with 1% sodium thioglycolate and 0.2 mg ml⁻¹ protease for 20 min before fixation.

DMH1 treatments. M. stichopi and I. pulchra embryos were collected at the one- or two-cell stage and cultured with regular water changes in cell culture dishes until the desired developmental stage. Control embryos were treated with 0.1% dimethylsulfoxide and experimental embryos were treated with DMH1 (Sigma) up to 10 μM. Seawater containing the DMH1 was changed every day until fixation. Embryos and hatchinglings were fixed as described above, and stored in Tewen 20 phosphate buffer saline at 4 °C.

Gene identification and expression analyses. RNA sequencing data obtained from mixed developmental stages and juveniles/adults were used for gene identification. Gene orthology was based on reciprocal best BLAST hit. For particular gene families, maximum likelihood phylogenetic analyses were conducted with RAXML version 8.2.6 (ref. 48), after building multiple protein alignments with MAFFT version 7 (ref. 49) and trimming poorly aligned regions with gblocks version 0.91b (ref. 50) (Supplementary Fig. 1). Whole-mount colorimetric in situ hybridization on brachiopod embryos, L. ruber, O. fusiformis, and juvenile E. senta was performed following an already established protocol30,44. Probe concentrations ranged from 0.1 to 1 ng/μl⁻¹, and permeabilization time was 15 min for M. stichopi and post-metamorphic brachiopod juveniles, 5 min for I. pulchra, and 10 min for the other species. Double fluorescent whole-mount in situ hybridization was performed as described elsewhere46.

Immunohistochemistry. Samples were permeabilized in 0.1–0.5% Triton X-100 phosphate buffer saline (PTx), and blocked in 0.1–1% bovine serum albumin in PTx. The antibodies anti-tyrosinated tubulin (Sigma), anti-serotonin (Sigma), and anti-FMRFamide (Immunostar) were diluted in 5% normal goat serum in PTx at a concentration of 1:500, 1:200, and 1:200, respectively. Samples were incubated with the primary antibody solutions for 24–72 h at 4 °C. After several washes in 1% bovine serum albumin in PTx, samples were incubated overnight with Alexa-conjugated secondary antibodies at a 1:250 dilution in 5% normal goat serum in PTx. Before mounting and imaging, samples were washed several times in 1% bovine serum albumin in PTx. Nuclei and actin filaments were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes) and BODIPY FL Phallacidin (Molecular Probes).

Imaging. Representative embryos from colorimetric in situ hybridization experiments were cleared in 70% glycerol and imaged with a Zeiss Axioscope with a Leica SP5 confocal laser-scanning microscope. Images were analysed with Fiji and Photoshop CS6 (Adobe), and figure plates were assembled with Illustrator CS6 (Adobe). Brightness/contrast and colour balance adjustments were applied to the whole image, not parts.

Data availability. All newly determined sequences have been deposited in GenBank under accession numbers KY809717–KY809754, KY709718–KY709823, and MF988103–MF988108. Multiple protein alignments used for orthology assignment are available upon request from the corresponding author. Extended Data Fig. 6c has associated source data.

43. De Mulder, K. et al. Characterization of the stem cell system of the acellos Isodiamictia pulchra. BMC Dev. Biol. 9, 69 (2009).
44. Martín-Durán, J. M., Yellutini, B. C. & Hejnol, A. Evolution and development of the adelphophagic, intracapsular Schmidt's larva of the nemertean Lineus ruber. Evodevo 6, 28 (2015).
45. Freeman, G. Regional specification during embryogenesis in the articulate brachiopod Terebratalia. Dev. Biol. 160, 196–213 (1993).
46. Freeman, G. Regional specification during embryogenesis in the craniiform brachiopod Crania anomala. Dev. Biol. 227, 219–238 (2000).
47. Smart, T. I. & Von Dassow, G. Unusual development of the mitraria larva in the polychaete Owenia collaris. Biol. Bull. 217, 253–268 (2009).
48. Stamatakis, A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30, 1312–1313 (2014).
49. Katoh, K. & Standley, D. M. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30, 772–780 (2013).
50. Talavera, G. & Castresana, J. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. Syst. Biol. 56, 564–577 (2007).
Extended Data Figure 1 | Studied species. a–i, Images of the adult forms of the studied species within a consensus bilaterian phylogeny. Colour boxes highlight major taxonomical clades. Scale bars, 100 μm in a–e, 0.5 cm in g and i, 1 cm in f, h, and i.
Extended Data Figure 2 | Gene expression in *X. bocki* and *N. westbladi*. a, Two six3/6 paralogues are expressed in the anterior head margin in *X. bocki* (arrowheads). b, The neural marker *synaptotagmin* (*syt*) is detected in the circumferential (cf; inset 1) and side (sf; inset 2) sensory furrows in *X. bocki*. c, In *N. westbladi*, the anterior marker *sFRP1/5* (arrowhead) and the posterior genes *gbx* and *wnt1* are asymmetrically expressed along the anteroposterior axis of *N. westbladi*. d, The BMP ligands *bmp2/4-a* and *bmp2/4-c* are expressed dorsally, whereas the BMP antagonist *admp* is expressed dorsolaterally. e, The CNS of *N. westbladi* comprises an anterior ring-like commissure (green arrowheads) and a main pair of ventral condensations (red arrowheads). f, The neuronal marker *syt* is highly expressed in the anterior part (inset 1), and in the nerve cords (inset 2). In the different panels, dotted rectangles indicate magnified areas. In all panels, the anterior pole is to the left. The schematic drawing in e is not to scale. Scale bar, 100 μm in e.
Extended Data Figure 3 | Anteroposterior patterning in Xenacoelomorpha. a, b, Expression of anteroposterior markers in adult specimens of M. stichopi and I. pulchra. In both species, sFRP1/5, vax, six3/6, and BarH are expressed in anterior territories (black arrowheads). In M. stichopi, Rx is also expressed anteriorly, but broadly along the animal body in I. pulchra. In this acoel, emx is detected in the anterior part of the animal (background staining close to the gonads). In the nemertodermatid, the anterior neural markers otx, otp, pax2/5/8, and fezf are expressed along the entire anteroposterior axis, in association with the dorsal nerve cords (black dotted lines in otp). In I. pulchra, otx, pax2/5/8-a, and pax2/5/8-b are broadly expressed. In M. stichopi, an irx orthologue is detected in the posterior tip, whereas it is detected in the anterior tip and around the mouth and copulatory apparatus in the acoel (arrowheads). The gbx orthologue of M. stichopi is expressed posteriorly, and the trunk-related Hox genes are expressed in two lateral rows (anteriposterior Hox) and anteriorly to the mouth and in the posterior tip (posterior Hox). In the nemertodermatid and the acoel, Wnt ligand genes are expressed posteriorly (arrowheads). All images are dorsoventral views with anterior to the left. c, Schematic summary of anteroposterior expression in the nemertodermatid M. stichopi and the acoel I. pulchra. Drawings are not to scale and the extent of the expression domains are only approximate. The expression of posterior Hox in I. pulchra is based on ref. 22.
Extended Data Figure 4 | Expression of BMP components in Nemertodermatida and Acoela. a, In the nemertodermatid *M. stichopi*, the BMP pathway antagonists *twisted gastrulation* (*tsg*) and *crossveinless 2* (*cv2*) are expressed dorsally, whereas the antagonist *BAMBI* is broadly detected in the ventral side. The gene *tolloid* (*tld*) is expressed both dorsally and ventrally. The BMP receptor *bmpR-I* is expressed dorsolaterally and *bmpR-II* is detected more broadly. The genes *smad1* and *smad4* are expressed broadly and *smad6* is expressed along the dorsal nerve cords. b, The BMP ligand *bmp2/4* is not expressed in neuronal cells (*elav1* cells), but in cells located medially to the nerve cords (tubulin positive). The cells expressing *bmp2/4* also express *tsg*, and *cv2* is expressed dorsally along the nerve cords. c, In the acoel *I. pulchra*, the BMP antagonist *tld* is expressed ventrally, *bmpR-I* is detected in the inner body, and *bmpR-II* is expressed anteriorly and posteriorly around the copulatory organ. The genes *smad1* and *smad4* are expressed generally, while *smad6* is expressed in two bilaterally symmetrical anterior clusters. All main panels are dorsoventral views, and the insets are lateral views.
## Extended Data Figure 5 | Expression of neuronal markers in Nemertodermatida and Acoela

### a

In the nemertodermatid *M. stichopi*, the genes associated with neuronal fate commitment, *elav1*, *soxB2*, *ash1*, *ash2*, *atonal*, and *neuroD*, are detected along the dorsal nerve cords.

### b

Similarly, the neuronal markers *synaptotagmin* (*syt*), *tyrosine hydroxylase* (*tyr*), *vesicular monoamine transporter* (*VMAT*), *choline acetyltransferase* (*ChAT*), *vesicular acetylcholine transporter* (*VAcHT*), and *tryptophan hydroxylase* (*tph*) are mostly expressed dorsally, along the dorsal nerve cords.

### c

Morphology of *I. pulchra* embryos stained against tyrosinated tubulin (Tyr Tub) and serotonin (5-HT), and counterstained with phallacidin (actin bundles) and DAPI (nuclei). The first tubulin-positive cells that resemble neurons appear anteriorly (arrowheads) at 24 h post-fertilization (hpf). By 32 h post-fertilization, the anterior and posterior lobes of the brain, as well as some neurite bundles, are visible. Similarly, the first serotonergic cells are detected at 24 h post-fertilization in the anterior end (arrowheads).

### d

In *I. pulchra*, the pro-neural marker *elav1* is broadly expressed, *soxB* is detected in the head region (arrowhead), and *ash1b* is expressed in the anterior tip (arrowhead).

### e

In *I. pulchra*, the neuronal marker *syt* is highly expressed in the anterior neuropile. The marker *tyr* is detected in the statocyst and isolated cells. VMAT is detected in isolated dorsal cell clusters in the juvenile that concentrate along the adult brain. *ChAT* and *VAcHT* are expressed in the brain in juveniles and adults (gonadal staining in the adult is background). The gene *tph* is expressed in isolated ventral cells of the adult. All panels are dorsoventral views with anterior to the left. Scale bars, 50 μm in c.
Extended Data Figure 6 | DMH1 treatments in M. stichopi and I. pulchra. a, Schematic overview of dorsomorphin homologue 1 (DMH1) treatments in M. stichopi and percentage of hatching embryos for each experimental condition. b, M. stichopi embryos incubated with DMH1 from 3 to 8 weeks and after hatching show more serotonergic commissures than control animals. c, The differences in the number of commissures are significant in both pre-hatching (asterisk; two-tailed t-test; p<0.0001) and post-hatching (asterisk; two-tailed t-test; p<0.0014) treated embryos. In contrast, the number of serotonin-positive neurite bundles is not significantly increased in any of the treatments. d, Despite the abnormal development of serotonergic axonal tracts, slit and robo genes are expressed similarly. The differences in signal intensity are due to technical variability. e, Schematic overview of DMH1 treatments in I. pulchra and the percentage of hatching embryos for each experimental condition. f, Morphological analyses of DMH1-treated embryos. Treatment in early stages affects normal development, whereas treatments from 4 h onwards do not significantly compromise embryogenesis. g, Embryos treated between 0 and 4 h post-fertilization and fixed at 24 h of development show expanded expression of the ventral marker nkx2.1, reduced expression of the dorsal gene bmp2/4, and unaffected expression of the anterior marker sFRP1/5. The embryo shows a disorganized morphology, as revealed by actin staining. h, The expression of the ventral marker nkx2.1 is expanded in early treated embryos (0–48 h), but unaffected in embryos treated after 4 h of development. In b, d, f–h, the asterisk marks the anterior pole. In b, d, f, panels are dorsoventral views, and in g and h the panels are lateral views.
Extended Data Figure 7 | Gene expression in Brachiopoda. a, Gene expression during early gastrulation and elongation, and in late larvae of *T. transversa*. The gene *nkx2.2* is expressed ventroposteriorly (black arrowhead) and in the pedicle lobe of the larva (arrow). The gene *nkx6* is detected in two bilateral symmetrical ectodermal posterior clusters (arrowheads) and in the archenteron wall. In the larva, *nkx6* is expressed in the pedicle lobe (arrow) and midgut. *pax3/7* is first detected in two ventrolateral domains at the prospective apical-trunk boundary (arrowheads), and in the ventral anterior region of the larva. The gene *msx* is first expressed dorsally, in the future mantle ectoderm (arrowheads), and in the mantle of the larva. The schematic drawing is not to scale, and the blue line represent the commissure. b, In 2-day-old post-metamorphic juveniles, the CNS comprises a main serotonergic anterior commissure (white arrowhead; dorsoventral view) that innervates the developing lophophore. The expression extends posteriorly and concentrates along the midline of the larva (arrow). The gene *nkx2.2* is expressed in the anterior blastoporal lip at the onset of axial elongation, and it is not detected in the late larva. The gene *nkx6* is asymmetrically expressed around the blastopore, in the putative anteroventral neuroectoderm (arrowhead). As the blastopore closes, the expression extends posteriorly and concentrates along the midline of the larva (arrow). The gene *pax3/7* is detected in the posterior mesoderm at the onset of axial elongation (arrow). The gene *msx* is expressed in the prospective mantle lobe ectoderm (arrowheads) and in the dorsal shell-forming epithelium of the late larva. The asterisks indicate the animal/anterior pole and white dashed lines in a and d mark the region of background noise caused by probe trapping in the shell-forming ectoderm. Panel orientations are indicated in the first row/column and apply to the rest of the panels in the same column/row. Scale bar, 100 μm in b.
Extended Data Figure 8 | Gene expression in the nemertean *L. ruber*. a, None of the nerve cord patterning genes is expressed during gastrulation in *L. ruber*. In the intracapsular larva, *nkx2.1* is expressed in the cephalic imaginal discs (arrowheads), *nkx2.2* and *nkx6* in an anterior and a posterior domain of the trunk imaginal discs (arrowheads) respectively, and *pax6* is detected both in the cephalic and in the anterior trunk imaginal discs (arrowheads); *pax3/7* is broadly expressed. With metamorphosis, *nkx2.1* is detected in the head and proboscis, *nkx2.2* is detected in the nerve cords and isolated trunk cells (arrowheads), *nkx6* is expressed in the nerve cords (arrowheads), *pax6* is observed in the head and nerve cords (arrowheads), and *pax3/7* remains broadly expressed. All gastrulae are vegetal views. For larvae and early juveniles, the left column is a dorsoventral view and the right column is a lateral view (anterior to the left). All late juvenile pictures are lateral views, with anterior to the left. b, Lateral views (anterior to the left) of neuronal markers in juveniles. They are all expressed in the VNCs (arrowheads), and not in the dorsal neurite bundle. In all panels, the asterisk indicates the position of the mouth opening. Abbreviations: bp, blastopore; mo, mouth; pb, proboscis.
Extended Data Figure 9 | Molecular patterning and motor neuron markers in Rotifera and Annelida. a, Expression of the motor neuron markers Hb9 and ChAT in juveniles of the rotifer E. senta. The gene Hb9 is detected in neurons of the mastax (arrowheads) and weakly in isolated cells of the brain (arrow). The gene ChAT is detected in the brain (arrow), cells of the corona and mastax (arrowheads). b, Expression of dorsoventral patterning genes in gastrulae and elongating embryos of O. fusiformis. The genes nkx2.2 and nkx6 are expressed in the internalized endomesoderm (arrowheads). The gene pax6 is expressed in two lateral rows during elongation (arrowhead) and pax3/7 in two lateral cells (arrowhead). Of the two paralogues, msx-a is first detected in a posterior ectodermal domain (arrowhead) and in two additional bilaterally symmetrical posterior cells (arrowheads) during elongation. The gene msx-b is only detected during elongation in a posterior domain (arrowhead). c, Ventral view of the expression of nkx2.1 in the juvenile of the annelid O. fusiformis. This gene is detected in the foregut (arrowheads) and hindgut (arrow). d, Expression of the motor neuron markers Hb9 and ChAT in O. fusiformis. Hb9 is first detected in lateral domains of the archenteron/gut during embryogenesis and in the larva, and in isolated cells of the ventral trunk of the juvenile. The gene ChAT is detected in three cells of the apical region of the embryo and larva, and in the neuropile and two lateral ventral cords of the juvenile. Abbreviations: bp, blastopore; mo, mouth; ms, mastax. The asterisk in a marks the position of the mouth.
Extended Data Figure 10 | See next page for caption.
Extended Data Figure 10 | Dorsoventral patterning and the evolution of bilaterian trunk neuroanatomy. a, b, Schematic drawings of trunk neuroanatomy (nerve cords in blue) and expression of patterning genes in spiralian (a) and bilaterian (b) lineages. The overall location of patterning genes expression domains with respect to the dorsoventral axis and nerve cords is indicated by light green. In a, the red dashed squared expression of *pax6* and *pax3/7* in brachiopods indicates that these expression domains are only in the anterior region of the mantle lobe, not all along the trunk. Similarly, the red dashed squared expression of *nkx6* in rotifers highlights that this gene is only expressed posteriorly in the trunk. In b, the red dashed squared expression of *nkx2.1*, *nkx2.2*, and *nkx6* in Cnidaria indicates that these genes are expressed in the pharynx ectoderm. The red dashed squared expression of *nkx6* in *M. stichopus* shows that this gene is only expressed posteriorly. In the acoel *I. pulchra*, the red dashed squared expression of *nkx2.2* specifies that this gene is only expressed between mouth and copulatory organ. Red circles imply that a gene is not expressed in the trunk or is missing. Question marks indicate that there are no available data about the expression of that particular gene. See Supplementary Table 2 and main text for references. Schematic drawings are not to scale and only represent approximate relative expression domains. c, Alternative scenarios for the evolution of the dorsoventral patterning and bilaterian nerve cords. In scenario A, the medially condensed nerve cords of vertebrates, arthropods, and annelids are homologous. Therefore, the dorsoventral patterning was lost multiple times both in lineages with medially condensed nerve cords (for example, the annelid *O. fusiformis*, cephalochordates, and tunicates) and in lineages with multiple nerve cords and diffuse nerve nets. In scenario B, which is supported by this study and is more parsimonious, the similarities in dorsoventral patterning and trunk neuroanatomies of vertebrates, arthropods, and some annelids evolved convergently. The diversity of nerve cord arrangements in nephrozoan lineages hampers reconstruction of the ancestral neuroanatomy for this group (question mark). Animal phylogeny is based on ref. 18.
Experimental design

1. Sample size
   Describe how sample size was determined.
   We have used between 10 - 100 specimens per experiment which is standard in such experiments. The number was determined so that the expression patterns gave consistent patterns between the specimens. The sample size for one species (Xenoturbella bocci) was between 3-10 because of limited material. The results between the different samples was consistent.

2. Data exclusions
   Describe any data exclusions.
   No data were excluded.

3. Replication
   Describe whether the experimental findings were reliably reproduced.
   In situ hybridizations and antibody staining were repeated twice and led to a reproducible pattern.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   The individual specimens were randomly picked from the culture/environment.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   N.A.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters
   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   n/a
   Confirmed

   ✔️ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)

   ✔️ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly

   ✔️ A statement indicating how many times each experiment was replicated

   The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)

   ✔️ A description of any assumptions or corrections, such as an adjustment for multiple comparisons

   ✔️ The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted

   ✔️ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)

   ✔️ Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.
7. Software

Describe the software used to analyze the data in this study. Fiji, Photoshop CS6, Imaris 7.7 for confocal stacks. RAXML v 8.0, MAFFT for sequence analysis.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

There are no restrictions on availability of unique materials.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

We have used only commercial antibodies (anti-tyrosinated tubulin, anti-FMFamide, anti-serotonin - Sigma Aldrich).

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

N.A.

b. Describe the method of cell line authentication used.

N.A.

c. Report whether the cell lines were tested for mycoplasma contamination.

N.A.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

N.A.

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

In this study we have used whole animal specimens collected from the field. Xenoturbella bocki, Nemertodera westbladi were collected from Kristineberg, Sweden. Novocrania anomala, Meara stichopi, Lineus ruber were collected near Bergen, Norway. Terebratalia transversa was collected near Friday Harbor Station, Seattle, USA. Owenia fusiformis was collected in Roscoff, France. Isodiametra pulchra, Epiphanes senta are from a laboratory culture.

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

N.A.