

## RESEARCH ARTICLE

# Nodal signaling regulates specification of ascidian peripheral neurons through control of the BMP signal

Yukio Ohtsuka<sup>§</sup>, Jun Matsumoto, You Katsuyama\* and Yasushi Okamura<sup>‡</sup>**ABSTRACT**

The neural crest and neurogenic placodes are thought to be a vertebrate innovation that gives rise to much of the peripheral nervous system (PNS). Despite their importance for understanding chordate evolution and vertebrate origins, little is known about the evolutionary origin of these structures. Here, we investigated the mechanisms underlying the development of ascidian trunk epidermal sensory neurons (ESNs), which are thought to function as mechanosensory neurons in the rostral-dorsal trunk epidermis. We found that trunk ESNs are derived from the anterior and lateral neural plate border, as is the case in the vertebrate PNS. Pharmacological experiments indicated that intermediate levels of bone morphogenetic protein (BMP) signal induce formation of ESNs from anterior ectodermal cells. Gene knockdown experiments demonstrated that HrBMPa (60A-subclass BMP) and HrBMPb (*dpp*-subclass BMP) act to induce trunk ESNs at the tailbud stage and that anterior trunk ESN specification requires Chordin-mediated antagonism of the BMP signal, but posterior trunk ESN specification does not. We also found that Nodal functions as a neural plate border inducer in ascidians. Nodal signaling regulates expression of *HrBMPs* and *HrChordin* in the lateral neural plate, and consequently specifies trunk ESNs. Collectively, these findings show that BMP signaling that is regulated spatiotemporally by Nodal signaling is required for trunk ESN specification, which clearly differs from the BMP gradient model proposed for vertebrate neural induction.

**KEY WORDS:** Epidermal sensory neuron, Nodal, BMP**INTRODUCTION**

The vertebrate peripheral nervous system (PNS) originates from the neural crest and neurogenic placodes, which arise at the boundary between the neural plate and non-neural ectoderm and become a multipotent migratory cell population (LeDouarin and Kalcheim, 1999). Studies of several vertebrates have led to the identification of neural crest specifiers (e.g. Snail family), pre-placodal specifiers (e.g. Six and Eya family) and inductive signaling molecules such as bone morphogenetic protein (BMP), fibroblast growth factor (FGF), Wnt and Notch (Grocott et al., 2012; Meulemans and Bronner-Fraser, 2004). Moreover, comparison of protochordate and

vertebrate gene expression patterns suggests that the neural crest and neurogenic placodes did not arise *de novo* in vertebrates, but originated from the neural plate border in the common ancestor of protochordates and vertebrates (Imai et al., 2004; Mazet et al., 2005; Sauka-Spengler et al., 2007; Yu et al., 2008). To gain more insight into the evolution of the PNS, comparison of the mechanisms underlying the development of the PNS among chordates is essential.

Tunicates are the sister group of vertebrates and are expected to provide information about the transition between invertebrates and vertebrates (Bourlat et al., 2006; Delsuc et al., 2006, 2008). An experimental advantage of the ascidian system is that the larval body plan is relatively simple, and that the cell cleavage pattern is essentially invariant (Nishida, 1987), which enables us to discern precisely the mechanisms governing cell fate specification at the cellular and molecular levels. Ascidian larvae have a dorsal tubular central nervous system (CNS), which is derived from the anterior animal (a4.2), posterior animal (b4.2) and anterior vegetal (A4.1) blastomeres in eight-cell stage embryos (Fig. 1). The a4.2-derived (a-line) cells give rise to the palp and anterior part of the sensory vesicle, whereas the posterior neural tube arises from b4.2-derived (b-line) and A4.1-derived (A-line) cells. Neural induction in a-line and b-line cells is known to occur through direct cell contact with A-line cells, and FGF9/16/20 has been identified as the neural inducer (Bertrand et al., 2003; Miyazaki et al., 2007). In addition, Nodal signaling favors the lateral fate of the neural plate during gastrulation (Hudson and Yasuo, 2005). The ascidian PNS is mainly composed of epidermal sensory neurons (ESNs), which may function as mechanosensory neurons (Torrence and Cloney, 1982). The cell bodies of ESNs lie in the rostral-dorsal epidermis of the trunk and midline epidermis of the tail with long processes extending to the cuticular fin (Ohtsuka et al., 2001b; Takamura, 1998). Previous studies on *Ciona intestinalis* have shown that caudal ESNs are derived from the b4.2 and that caudal ESN formation is controlled by FGF, Nodal, anti-dorsalizing morphogenetic protein (ADMP) and Delta/Notch (Hudson and Lemaire, 2001; Pasini et al., 2006). However, it was not determined when and where these signaling pathways play roles in ESN specification.

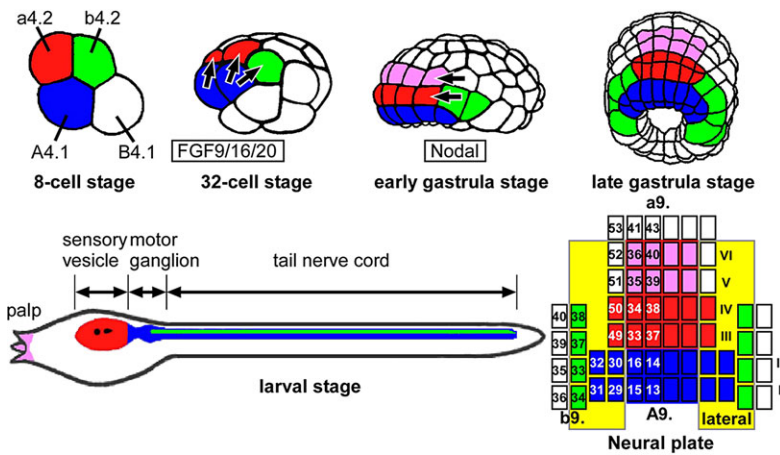
In the present study, we investigated the mechanisms underlying the development of trunk ESNs in *Halocynthia roretzi*, another ascidian species. We previously identified gelsolin as an ESN marker and demonstrated that the interaction between a-line and A-line cells induces trunk ESN specification (Ohtsuka et al., 2001a,b). We now report, based on cell lineage studies using a combination of DiI labeling and immunostaining with anti-gelsolin antibody, that trunk ESNs are derived from the anterior and lateral neural plate border, as is the case for the vertebrate PNS. Gene knockdown experiments showed that moderate levels of BMP activity induce trunk ESNs at the tailbud stage, and that Chordin-mediated antagonism of the BMP signal is only required for anterior trunk ESN specification. We also show that Nodal signaling

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**Fig. 1. The cell lineage of the ascidian larval CNS.** Neural lineages are indicated as follows: a-line cells, red and pink; A-line cells, blue; b-line cells, green. A late-gastrula embryo is shown in neural plate view. In the 8-cell, 32-cell, early-gastrula stage and larval embryos, the anterior is towards the left. A schematic diagram of the neural plate at the late gastrula stage is also shown. Each square represents a neural plate cell (red, pink, blue or green) or adjacent non-neural ectodermal cell (white), and I–VI indicate the row numbers. Blastomere names are indicated on the left of the diagram. The neural fates of a-line and b-line cells are specified by FGF9/16/20 emanating from A-line cells (Bertrand et al., 2003; Miyazaki et al., 2007). During gastrulation, Nodal signaling promotes the lateral fate of the neural plate (Hudson and Yasuo, 2005). Consequently, the a-line cells give rise to the palp and the anterior part of the sensory vesicle, and the posterior neural tube arises from the b-line and A-line cells. The pigment cells, otolith and ocellus are known to be derived from a9.49.

controls the BMP signal required for trunk ESN specification. These results highlight the roles of Nodal and BMP signaling during neural patterning.

## RESULTS

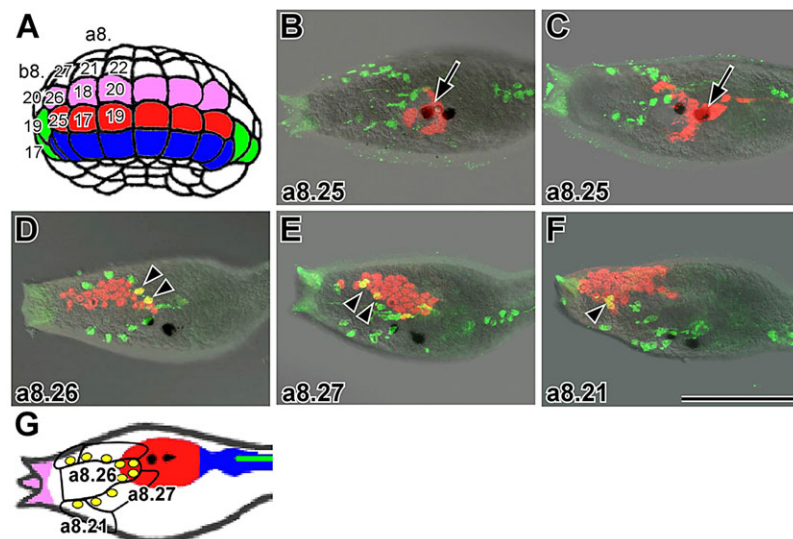
### The origins of trunk ESNs

We initially carried out cell lineage analyses to begin to understand the mechanisms governing trunk ESN specification at the cellular and molecular levels. We individually labeled anterior lateral neural plate cells a8.25 and a8.26 with DiI at the 110-cell stage (Fig. 2A), and then immunostained the resultant larvae using an antibody against gelsolin, an ESN marker. When a8.25 was labeled with DiI, the signals were detected in the sensory vesicle containing pigment cells (otolith and ocellus), as previously reported (Nishida, 1987), but not in trunk ESNs (Fig. 2B,C). In DiI labeling of the a8.26 lineage, the labeled cells were found in dorsal epidermis of the trunk, and gelsolin expression was found in just two posterior cells (Fig. 2D), which reveals that a lateral neural plate cell, a8.26, gives rise to two posterior trunk ESNs (Fig. 2G). Next, to identify the origin of anterior trunk ESNs, we labeled the non-neural ectoderm cells a8.21 and a8.27, which are adjacent to the anterior neural plate (Fig. 2A). In both cases, DiI signals were detected in the anterolateral trunk epidermis (Fig. 2E,F). Immunostaining for gelsolin showed that a8.27 gives rise to two anterior trunk ESNs and that a8.21 gives rise to the anterior-most trunk ESN (Fig. 2E–G).

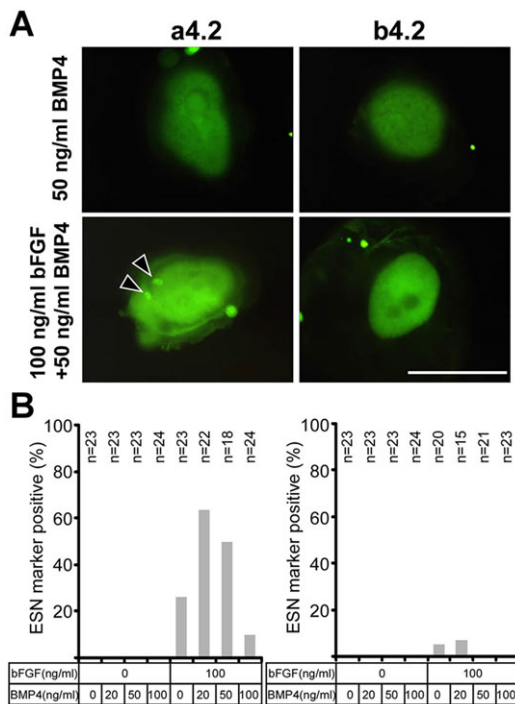
Thus, anterior trunk ESNs are derived from the non-neural ectoderm. In addition, the DiI labeling of the descendants of a8.21, a8.26 and a8.27 showed that the cell fate of trunk ESN was restricted to a9.41, a9.51 and a9.53 at the late gastrula stage (supplementary material Fig. S1). We conclude that trunk ESN precursors border the neural plate anteriorly and laterally, as in the vertebrate PNS.

### Moderate BMP activity is required for ESN specification

In vertebrates, BMP signaling is known to induce neural crest and neurogenic placodes (Grocott et al., 2012; Meulemans and Bronner-Fraser, 2004). To determine whether ESN specification requires BMP ligands, we first investigated the effect of BMP on isolated animal blastomeres. Pairs of animal blastomeres (a4.2 or b4.2) were isolated and continuously treated with recombinant human BMP4 from the 16-cell stage to the larval stage. By itself, BMP4 never induced ESNs in either explant (Fig. 3). Then, we examined the effects of combining BMP4 with bFGF, because bFGF is known to induce neural differentiation (Inazawa et al., 1998; Ohtsuka et al., 2001a). Treatment with BMP4 plus bFGF induced ESNs in a4.2 explants when the BMP4 concentration was 20 ng/ml or 50 ng/ml. A higher BMP4 concentration (100 ng/ml) had no apparent effect on ESN formation, suggesting that moderate BMP activity induces ESNs in bFGF-treated a-line cells. Interestingly, in b-line explants, BMP4 has little effect on ESN specification, even with bFGF



**Fig. 2. Cell lineage of trunk ESNs.** (A) A schematic anterior view of a 110-cell stage embryo in which the neural plate comprises 12 a-line cells (red and pink), eight A-line cells (blue) and four b-line cells (green). (B–F) Merged confocal fluorescence and different interference contrast (DIC) images of DiI-labeled larvae immunostained with AS23. Each blastomere, a8.25 ( $n=23$ , B,C), a8.26 ( $n=28$ , D), a8.27 ( $n=25$ , E) or a8.21 ( $n=16$ , F) was labeled with DiI at the 110-cell stage. Arrows indicate the DiI-labeled otolith (B) and ocellus (C). Arrowheads indicate cells in which DiI and gelsolin were colocalized. Scale bar: 200  $\mu$ m. (G) A fate map of trunk ESNs (yellow circles). Two posterior trunk ESNs arise from a8.26, two anterior trunk ESNs arise from a8.27 and the anterior-most trunk ESN arises from a8.21.



**Fig. 3. Induction of ESNs in a4.2 explants by BMP4.** (A) Expression of gelsolin in a4.2 (left) and b4.2 (right) explants treated from the 16-cell stage with 50 ng/ml BMP4 (top) or a combination of 50 ng/ml BMP4 plus 100 ng/ml bFGF (bottom). Arrowheads indicate gelsolin-positive ESNs. Scale bar: 200  $\mu$ m. (B) Histograms showing the ratios of a4.2 (left) and b4.2 (right) explants with gelsolin-positive ESNs after treatment with BMP4 plus bFGF from the 16-cell stage.

(Fig. 3), suggesting that the competence of b-line cells to respond to BMP/FGF ligands may be different from that of a-line cells.

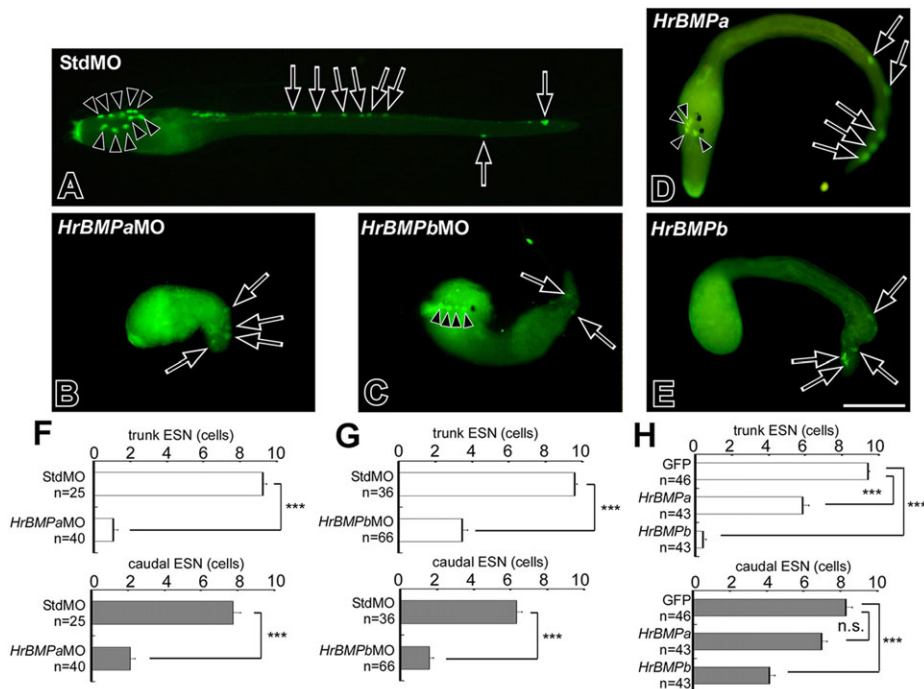
To confirm that ESN specification requires BMP signaling *in vivo*, we carried out knockdown experiments using MOs. Two BMP genes, *HrBMPa* and *HrBMPb*, have been isolated from *Halocynthia* (Miya

et al., 1997, 1996). We injected *HrBMPa*MO, *HrBMPb*MO or a negative control MO (StdMO) into fertilized eggs. StdMO-injected larvae exhibited normal development (Fig. 4A). By contrast, injection of *HrBMPa*MO caused inhibition of tail elongation, loss of trunk ESNs, and a reduction in number of caudal ESNs in the dorsal region (Fig. 4B,F). Injection of *HrBMPb*MO also caused a reduction in trunk and caudal ESNs, but the effect was somewhat milder than that seen with *HrBMPa*MO (Fig. 4C,G). Co-injection of exogenous *HrBMP* RNAs with MOs at the one-cell stage reversed these effects of MOs, suggesting specificity of our MO-mediated knockdown experiments (supplementary material Table S1). The weak recovery in these rescue experiments may be due to a wide range of defects in morphants and a reduction of ESNs that probably comes from injection of an excess amount of *HrBMP* RNAs (see below). These findings indicate that BMP ligands are required for ESN formation in both the trunk and tail. Next, to determine the source of the BMP ligands mediating trunk ESN specification, we injected MOs into a4.2 or A4.1, because formation of trunk ESNs requires interaction between a-line and A-line cells (Ohtsuka et al., 2001a). In both *HrBMPa*MO or *HrBMPb*MO, injection into a4.2 caused a complete loss of trunk ESNs, whereas injection into A4.1 caused no significant defect in trunk ESN formation (supplementary material Fig. S2). Thus, *HrBMPa* and *HrBMPb* originating from a-line cells are required for trunk ESN specification.

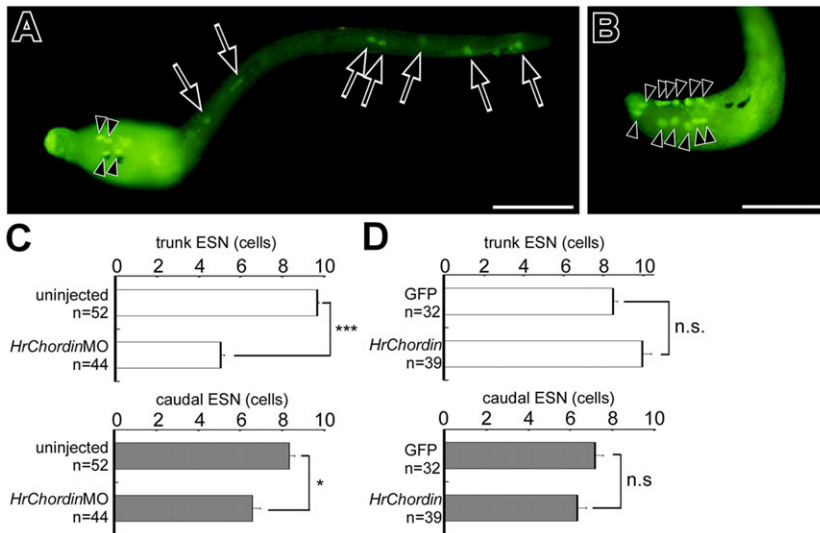
Then, we investigated whether high levels of BMP inhibit ESN formation *in vivo*. Injection of *HrBMPb* RNA markedly suppressed ESN formation in all areas except the tail tip (Fig. 4E,H). Likewise, overexpression of *HrBMPa* diminished ESN formation (Fig. 4D,H). Taken together, these results indicate that moderate levels of BMP activity are required for specification of ESNs.

**Anterior trunk ESN specification depends on expression of *HrChordin* in a-line cells**

To investigate the effect of the BMP antagonist Chordin on ESN specification, we injected *HrChordin*MO into fertilized eggs. In *HrChordin* morphants, ESN formation was diminished in the anterior region of the trunk but not in the tail (Fig. 5A,C), and this



**Fig. 4. Effects of *HrBMP* MOs and exogenous RNAs on ESN formation.** (A-E) Gelsolin expression in larvae after injection of StdMO (A), *HrBMPa*MO (B), *HrBMPb*MO (C), 0.5 g/l *HrBMPa* RNA (D) or 1.0 g/l *HrBMPb* RNA (E) at the one-cell stage. Arrowheads and arrows indicate cell bodies in the trunk and tail, respectively. Scale bar: 200  $\mu$ m. (F-H) Histograms showing the average numbers of gelsolin-positive ESNs in larvae after injection of *HrBMPa*MO (F), *HrBMPb*MO (G) or 1.0 g/l GFP and *HrBMP* RNAs (H) at the one-cell stage. Data are mean  $\pm$  s.e.m. n.s. indicates  $P > 0.05$ ; \*\*\* $P < 0.001$ .



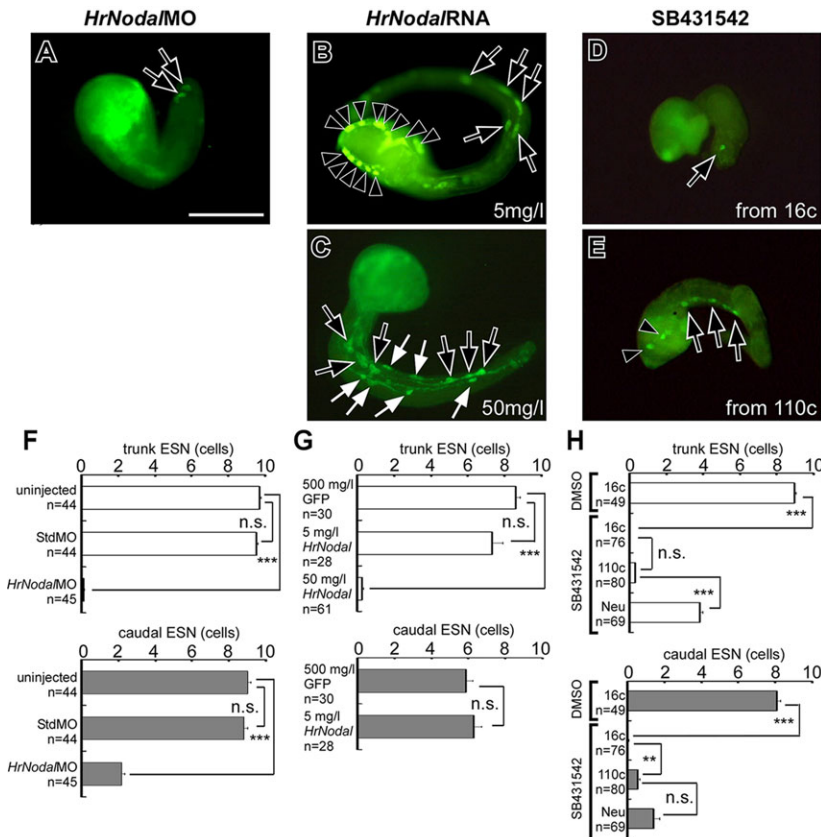
**Fig. 5. Effects of *HrChordin* knockdown and overexpression on ESN formation.** (A,B) Expression of gelsolin in larvae after injection of *HrChordin*MO (A) or *HrChordin* RNA (B). Arrowheads and arrows indicate cell bodies of ESNs in the trunk and tail, respectively. Scale bars: 200  $\mu$ m. (C,D) Histograms showing the average numbers of gelsolin-positive ESNs in larvae after injection of *HrChordin*MO (C) or *HrChordin* RNA (D). Data are mean  $\pm$  s.e.m. n.s. indicates  $P > 0.05$ ; \* $P < 0.05$ ; \*\*\* $P < 0.001$ .

phenotype was reversed by co-injection of exogenous *HrChordin* RNA (supplementary material Table S1). To confirm the cellular source of *HrChordin* affecting trunk ESN specification, we injected *HrChordin*MO into a4.2 or A4.1. Injection into a4.2 resulted in the loss of three anterior trunk ESNs, whereas injection into A4.1 had little effect on trunk ESN formation (supplementary material Fig. S3), demonstrating that expression of *HrChordin* in a-line cells is required for anterior trunk ESN specification. We subsequently assessed whether reducing BMP activity by exogenous Chordin would induce ESN formation. When *HrChordin* RNA was injected into fertilized eggs, ectopic ESNs were found near adhesive papillae, at the anterior-most part of the trunk (43.6%; Fig. 5B,D).

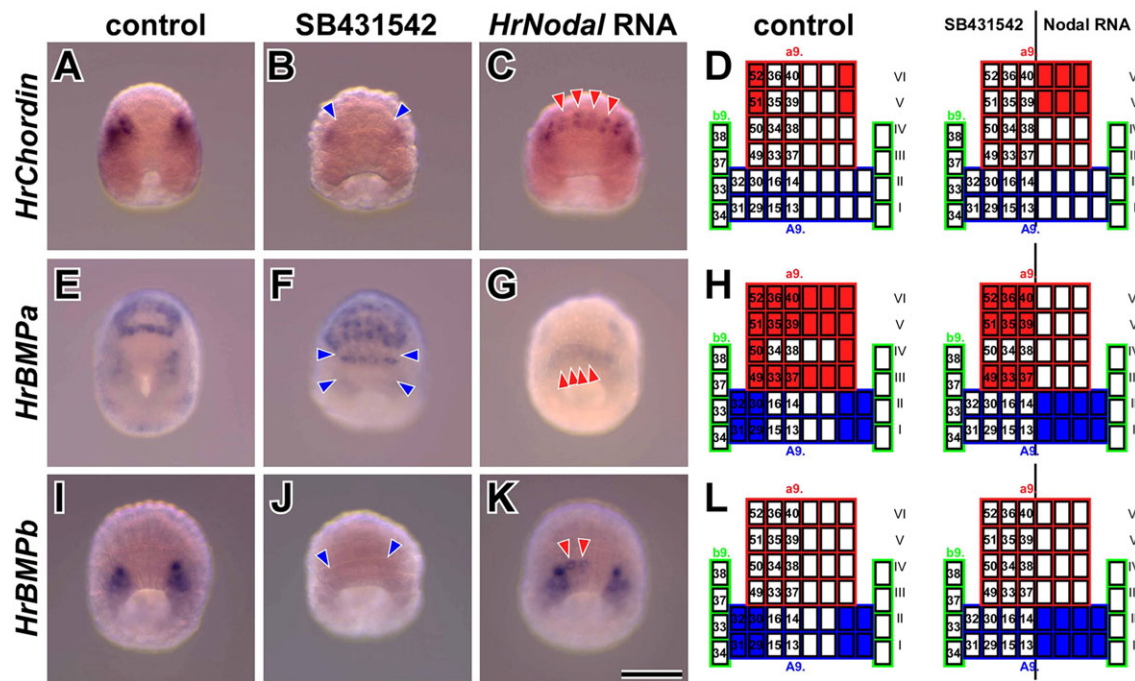
Thus, inhibition of BMP signaling by *HrChordin* contributes to ESN specification in the anterior trunk.

**Nodal signaling is required for ESN specification**

It has been shown that, in ascidians, Nodal regulates gene expression in the lateral border of the neural plate, which contains a posterior trunk ESN precursor, a8.26 (Fig. 1; Hudson and Yasuo, 2005). To determine whether Nodal signaling is required for trunk ESN specification in non-neural ectodermal cells, a8.21 and a8.27, we carried out gene-knockdown experiments. When *HrNodal*MO was injected into fertilized eggs, their larval phenotypes were very similar to those of *HrBMPs* morphants (Fig. 6A). Injection of



**Fig. 6. Effects of perturbing Nodal signaling on ESN formation.** (A-E) Expression of gelsolin in larvae after injection of *HrNodal*MO (A) or 5 mg/l (B) or 50 mg/l (C) *HrNodal* RNA at the one-cell stage, and treatment with 5  $\mu$ M SB431542 from the 16-cell stage (D) or the 110-cell stage (E). Arrowheads and arrows indicate cell bodies of ESNs in the trunk and tail, respectively. White arrows indicate ectopically induced ESNs. Scale bar: 200  $\mu$ m. (F-H) Histograms showing the average numbers of gelsolin-positive ESNs in larvae after injection of MO (F) or RNA (G), and treatment with SB431542 from the 16-cell, 110-cell or neurula stage (H). Data are mean  $\pm$  s.e.m. n.s. indicates  $P > 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .



**Fig. 7. Nodal signaling regulates *HrChordin* and *HrBMPs* expression.** Whole-mount *in situ* hybridization reveals expression of *HrChordin* (A-D), *HrBMPa* (E-H) and *HrBMPb* (I-L) in late gastrula embryos. (A,E,I) Control embryos treated with DMSO. (B,F,J) Embryos treated with 5  $\mu$ M SB431542 from the 16-cell stage. (C,G,K) Embryos injected with 50 mg/l *HrNodal* RNA at the one-cell stage. Arrowheads indicate blastomeres downregulated (blue) or upregulated (red) by SB431542 and *HrNodal* RNA. Scale bar: 200  $\mu$ m. (D,H,L) Schematic diagrams of the neural plate illustrating the results of the expression analyses. Colored squares represent positive cells.

*HrNodal*MO led to a complete loss of ESNs from the trunk and a reduction in ESN number in the tail (Fig. 6A,F), which suggests that Nodal signaling is required for ESN specification in the anterior trunk and tail as well as the posterior trunk. To confirm that the Nodal signal induces ESN formation, we injected exogenous *HrNodal* RNA into fertilized eggs. When a low concentration (5 mg/l) of *HrNodal* RNA was administered, the number of trunk ESNs varied among individuals. Although there was no statistically significant difference in the average number of trunk ESNs between larvae overexpressing *HrNodal* and those expressing GFP (Fig. 6G), more than 10 ESNs were detected in 28.6% of the *HrNodal*-injected larvae (Fig. 6B) but in none of the GFP-injected larvae. Injection of a high concentration (50 mg/l) of *HrNodal* RNA led to the formation of ectopic ESNs in the tail (Fig. 6C). These results indicate that Nodal signaling does induce ESN formation in both the trunk and tail. However, no ESNs were detected in the trunk after injection of a high concentration of *HrNodal* RNA (Fig. 6C,G), indicating that specification of trunk ESNs requires only moderate levels of Nodal signal.

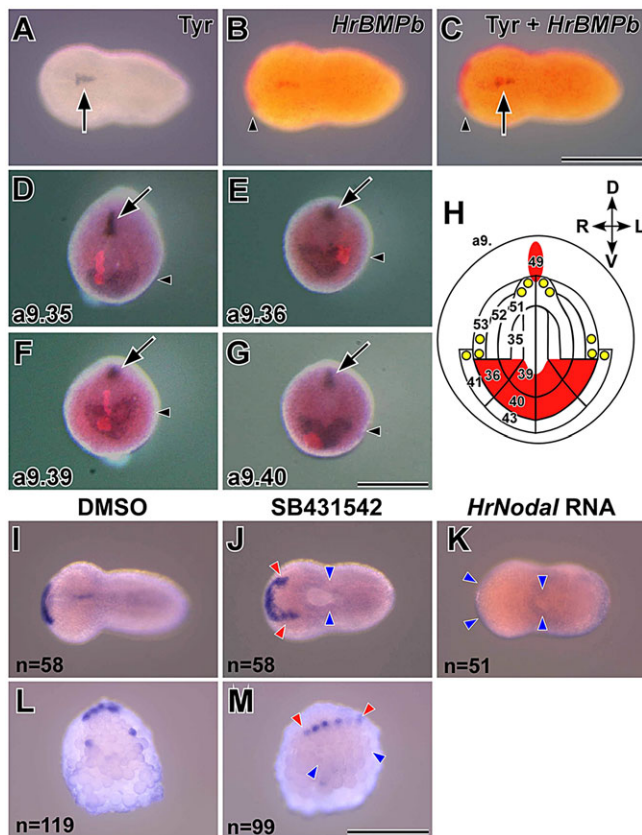
To determine when Nodal signaling is required for ESN formation, a Nodal signal inhibitor, SB431542, was applied at different stages. When embryos were continuously treated with SB431542 from the 16-cell stage to the larval stage, the larval phenotypes were very similar to those of the *HrNodal* morphants (Fig. 6D); i.e. ESNs were completely lost from the trunk and the number was markedly reduced in the tail (Fig. 6D,H). However, SB431542 treatment from the 110-cell stage (initiating stage of gastrulation) or the neurula stage caused only a mild change in morphology (Fig. 6E,H). With treatment from the 110-cell stage, ESNs were occasionally observed in the trunk region, and the number of caudal ESNs was recovered (Fig. 6E,H). With treatment from the neurula stage, the number of ESNs was further increased in the trunk (Fig. 6H). These results suggest that induction of trunk and

caudal ESN formation by Nodal signaling may start at the gastrula stage and continue until the neurula stage.

#### Nodal signaling controls expression of BMPs and Chordin in the lateral neural plate

To determine whether BMP is the downstream mediator of Nodal signaling leading to trunk ESN formation, we assessed the expression patterns of BMP signaling molecules in late gastrula embryos injected with *HrNodal* RNA or treated with SB431542. During normal development, *HrChordin* is expressed in a9.51 and a9.52 in the neural plate (Darras and Nishida, 2001; Fig. 7A,D), but treatment with SB431542 resulted in loss of *HrChordin* expression in the neural plate (Fig. 7B,D). By contrast, injection with *HrNodal* RNA induced ectopic expression of *HrChordin* in rows V and VI (Fig. 7C,D). *HrBMPa* is normally expressed in lateral A-line neural plate cells (A9.29, A9.30, A9.31 and A9.32) and a-line neural plate cells, except for a9.34 and a9.38 (Miya et al., 1996; Fig. 7E,H). In SB431542-treated embryos, however, *HrBMPa* expression was repressed in a9.50 and lateral A-line neural plate cells (Fig. 7F,H). In embryos overexpressing *HrNodal*, *HrBMPa* was ectopically expressed in A-line neural plate cells and was markedly downregulated in a-line cells (Fig. 7G,H). Finally, *HrBMPb* is expressed in lateral A-line neural plate cells of control embryos (Miya et al., 1997; Fig. 7I,L). The inhibition of Nodal signaling resulted in the loss of *HrBMPb* expression, whereas enhancing Nodal signaling caused ectopic expression of *HrBMPb* in rows I and II (Fig. 7J-L). Taken together, these results show that Nodal signaling controls expression of both *HrChordin* and *HrBMPa* in the a-line lateral neural plate.

Trunk ESN formation requires *HrBMPb* expressed in a-line cells; however, at the late gastrula stage, *HrBMPb* is not expressed in a-line cells. Therefore, we examined its expression at the tailbud stage. In control embryos, *HrBMPb* was expressed in epidermal



**Fig. 8. Nodal signaling regulates *HrBMPb* expression in the trunk.**

(A-C) *HrBMPb* expression and tyrosinase activity at the tailbud stage. Arrows indicate tyrosinase-active cells. Expression of *HrBMPb* was detected in tyrosinase-active cells, which were derived from the pigment cell precursor a9.49 (arrows), and in epidermal cells in the anterior-most region (arrowheads). Scale bar: 200  $\mu$ m. (D-G) *HrBMPb* expression in the Dil-labeled embryos. Each blastomere, a9.35 ( $n=17$ , D), a9.36 ( $n=21$ , E), a9.39 ( $n=29$ , F) or a9.40 ( $n=17$ , G) was labeled with Dil at the late gastrula stage. Tailbud embryos are shown in frontal views, with the dorsal at the top. Arrows indicate tyrosinase-active cells; arrowheads indicate epidermal cells in the anterior-most region. Scale bar: 200  $\mu$ m. (H) The frontal epidermis fate map of tailbud embryo, based on the results shown in D-G. *HrBMPb* is expressed in the palp precursors (red). Yellow circles indicate the putative positions of trunk ESN precursors. (I-M) Expression of *HrBMPb* in tailbud embryos treated with DMSO (I,L) or 5  $\mu$ M SB431542 (J,M) from the 16-cell stage, and injected with 50 mg/l *HrNodal* RNA at the one-cell stage (K). (L,M) Cleavage-arrested embryos treated with cytochalasin D from the neural plate stage. Arrowheads indicate downregulation (blue) and upregulation (red) of *HrBMPb* expression by inhibition of Nodal signaling. Scale bar: 200  $\mu$ m.

cells in the anterior-most region and in cells in the anterior part of the CNS (Miya et al., 1997; Fig. 8B,I). Tyrosinase activity showed that *HrBMPb*-expressing cells in the dorsal region correspond to a9.49-derived cells (Fig. 8A-C). In addition, whole-mount *in situ* hybridization with Dil-labeled embryos demonstrated that the *HrBMPb*-expressing cells in the anterior-most region were derived from the palp precursors, a9.36, a9.40, part of a9.35 (possibly a10.70) and part of a9.39 (possibly a10.78) (Fig. 8D-H). When treated with SB431542, *HrBMPb* expression was reduced in pigment cell precursors and was expanded in the anterior-most region (100%; Fig. 8J). To identify *HrBMPb*-expressing cells in SB431542-treated embryos, we carried out cell-lineage analysis using cleavage-arrested embryos. When cell cleavage was arrested from the late gastrula stage to the early tailbud stage, control embryos expressed *HrBMPb* in a9.36, a9.40 and a9.49 (100%;

Fig. 8L), whereas in SB431542-treated embryos, *HrBMPb* expression was lost from a9.49 and ectopically was present in a9.52 (55.6%; Fig. 8M). These results show that Nodal signaling induces *HrBMPb* expression in a9.49-derived cells but inhibits expression in the lateral neural plate. However, injection of *HrNodal* RNA led to a marked reduction in *HrBMPb* expression in both the anterior-most region and in tyrosinase-active cells (100%; Fig. 8K). This suggests that Nodal signaling controls expression of *HrBMPb* dose dependently in a-line cells of tailbud embryos.

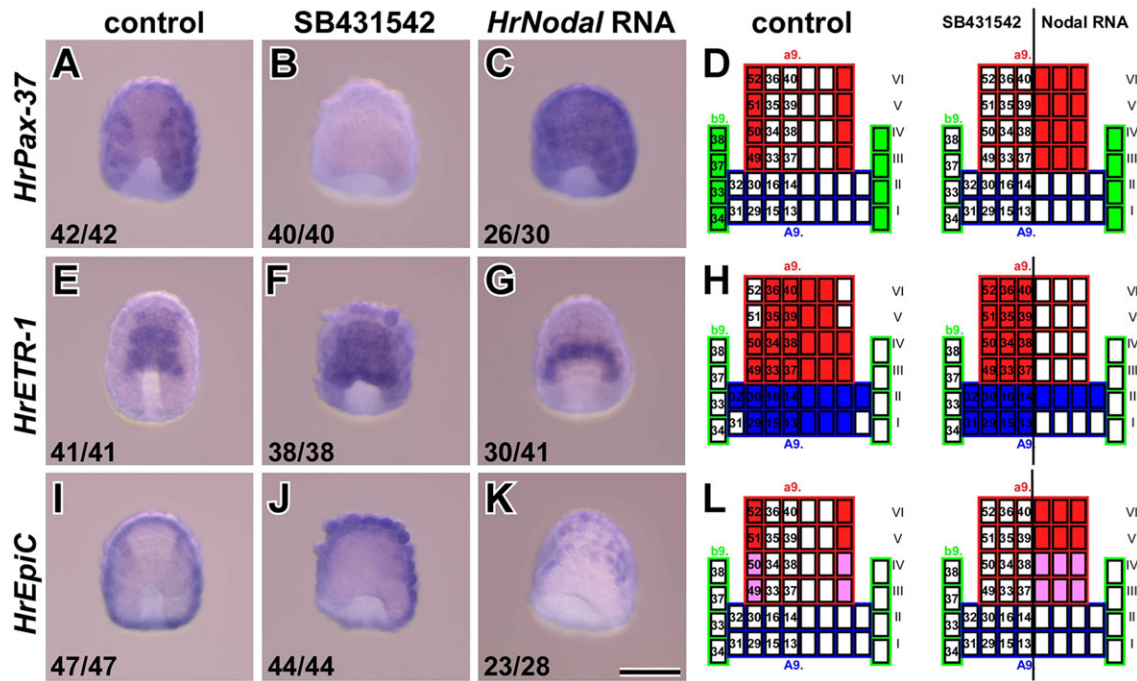
### Nodal signaling specifies the fate of the lateral neural plate

To determine whether Nodal signaling is required for specification of the lateral neural plate, we investigated the expression of a lateral neural plate marker, *HrPax-37*, in late gastrula embryos. *HrPax-37* is normally expressed in a-line and b-line lateral neural plate cells (Wada et al., 1997; Fig. 9A,D). Inhibition of Nodal signaling resulted in the loss of *HrPax-37* expression, whereas enhancement of Nodal signaling caused ectopic expression of *HrPax-37* in the a-line neural plate (Fig. 9B-D), suggesting that Nodal signaling specifies the lateral fate of the neural plate. To further determine the role of Nodal signaling in neural patterning, we examined the expression of a neural marker, *HrETR-1*, and an epidermal marker, *HrEpiC*. In DMSO-treated control embryos, *HrETR-1* was expressed in a-line and A-line neural plate cells, except for a9.51, a9.52 and A9.31 (Fig. 9E,H). In SB431542-treated embryos, however, *HrETR-1* expression was also detected in a9.51, a9.52 and A9.31 (Fig. 9F,H). By contrast, embryos overexpressing *HrNodal* expressed *HrETR-1* only in row II (Fig. 9G,H). *HrEpiC* is normally expressed in entire epidermis and is weakly detected in a-line lateral neural plate cells (Fig. 9I,L). In SB431542-treated embryos, *HrEpiC* expression disappeared from the lateral neural plate (Fig. 9J,L), whereas in embryos injected with *HrNodal* RNA, *HrEpiC* was ectopically expressed in the a-line neural plate, particularly in rows V and VI (Fig. 9K,L). These results suggest that Nodal signaling may negatively regulate the medial (brain/palp) fate in the lateral neural plate.

## DISCUSSION

### Mechanisms for specifying trunk ESNs

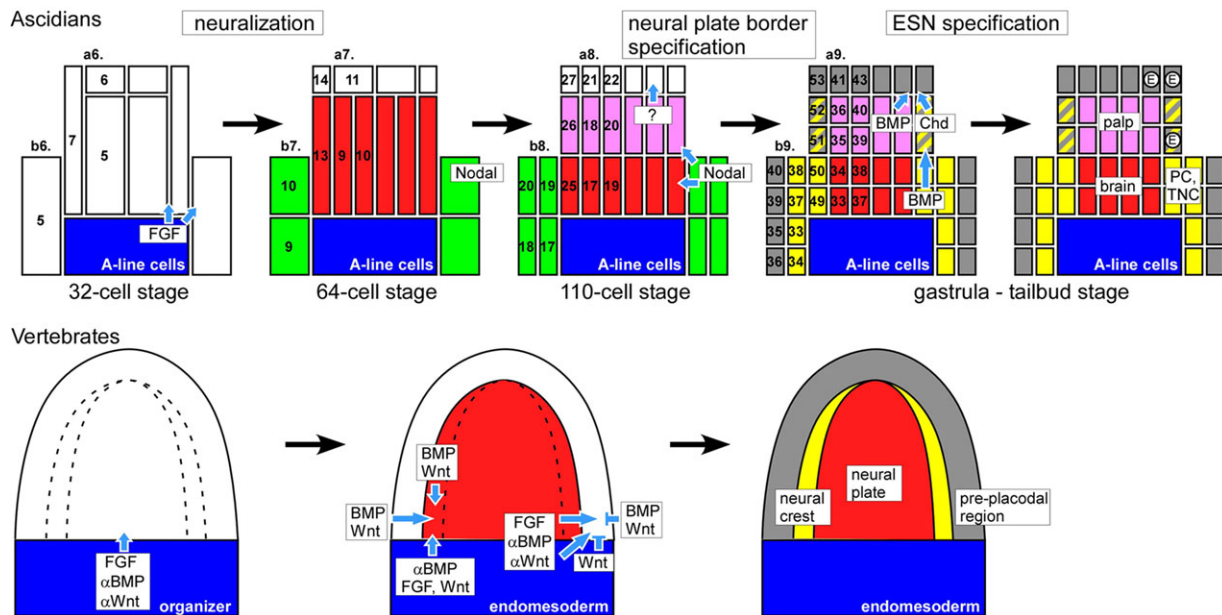
We identified the trunk ESN precursors and ESN-inducing cells in *Halocynthia* embryos, and demonstrated that four signaling molecules, FGF, Nodal, BMP and Chordin, are required for trunk ESN specification. Our findings provide a detailed description of the cell-cell interactions and signal transductions taking place during trunk ESN specification. Trunk ESN specification may occur in at least three phases with distinct signaling requirements (Fig. 10): (1) neuralization of the ectoderm by FGF signal; (2) specification of the neural plate border by Nodal and an unknown signal from the neural plate; and (3) specification of trunk ESNs by BMP signal. First, FGF signaling from A-line cells promotes adoption of a neural fate in a-line cells and induces expression of *Nodal* in b-line cells (Bertrand et al., 2003; Hudson and Yasuo, 2005). Next, Nodal signaling converts from medial neural (brain/palp) fate to lateral neural fate. In the a-line lateral neural plate, Nodal signaling induces the expression of the epidermal marker, *HrEpiC*, as well as the lateral neural plate marker, *HrPax-37*, and it suppresses the expression of the neural marker *HrETR-1* in rows VI and V. We propose here that an unknown signal from the neural plate may specify the anterior neural plate border. This can help to explain why BMP4 by itself has no effect on ESN formation in a4.2 explants. During specification of the neural plate border, Nodal signaling also regulates expression of BMP signaling molecules in the lateral neural plate. In a9.49 descendants, Nodal



**Fig. 9. Nodal signaling is required for specification of the lateral neural plate.** Expression of *HrPax-37* (A-D), *HrETR-1* (E-H) and *HrEpiC* (I-L) in late gastrula embryos. (A,E,I) Control embryos treated with DMSO. (B,F,J) Embryos treated with 5  $\mu$ M SB431542 from the 16-cell stage. (C,G,K) Embryos injected with 50 mg/l *HrNodal* RNA at the one-cell stage. Scale bar: 200  $\mu$ m. (D,H,L) Schematic diagrams of the neural plate illustrating the results of the expression analyses. Colored squares show positive cells. *HrEpiC* expression was frequently undetectable in rows III and IV (pink).

signaling induces *HrBMPb* expression, whereas in a9.51 and a9.52 descendants, it promotes *HrChordin* expression and suppresses *HrBMPb* expression. *HrBMPb* expression in rows VI and V is linked to adoption of the palp fate. Because it was reported that palp

induction requires FGF signaling (Hudson et al., 2003), it is likely that FGF signaling upregulates *HrBMPb* expression in rows VI and V. This speculation is consistent with our result that bFGF by itself induces ESNs in a4.2 explants. Finally, balanced BMP signals



**Fig. 10. Schematic models for neural patterning in ascidians and vertebrates.** (Top) Summary of *Halocynthia* trunk ESN specification (right side). Neural lineages are indicated as follows: a-line cells, red and pink; A-line cells, blue; b-line cells, green. Each square represents a neural plate cell (red, pink and green) or adjacent non-neural ectodermal cell (white). A blue square represents A-line cells. Blastomere names are indicated on the left half of the drawing. Nodal and an unknown signal from neural plate promote the lateral neural fate (yellow) and neural border fate (gray). The lateral neural plate gives rise to pigment cells (PC) and tail nerve cord (TNC), and the neural plate border gives rise to ESNs (E), which requires BMP signaling. (Bottom) Summary of signaling involved in induction of neural crest (left side) and pre-placodal region (right side) from the 'Binary competence model' (Schlosser, 2006). FGFs, Wnts and BMPs induce neural crest (yellow) at the edge of neural plate (red), while FGFs together BMP and Wnt antagonists induce the pre-placodal region (gray) at the border between neural plate and non-neural ectoderm (white).

regulated by Nodal signaling induce trunk ESNs in the anterior and lateral neural plate border regions. It appears that HrBMPa–HrBMPb heterodimers could act to specify trunk ESNs, because MO-mediated gene knockdown revealed that HrBMPa and HrBMPb play equivalent roles in trunk ESN formation. This is strongly supported by findings in *Drosophila* and zebrafish that BMP heterodimers signal synergistically through heteromeric type I receptor complexes during embryonic patterning, but BMP homodimers do not (Little and Mullins, 2009; Shimmi et al., 2005). We also suggest that the inducing sources of the BMP signal are a9.36 and a9.49 descendants. These descendants express both *HrBMPa* and *HrBMPb*, and are adjacent to trunk ESN precursors (Fig. 8H). We propose here that BMP ligands from a9.36 descendants act on a9.41 and a9.53 descendants to specify anterior trunk ESNs and that BMP ligands from a9.49 descendants induce posterior trunk ESNs in a9.51 descendants. *HrChordin* knockdown studies indicated that anterior trunk ESN specification requires BMP/Chordin antagonism, but posterior trunk ESN specification is Chordin independent, suggesting that trunk ESN specification involves two distinct mechanisms.

Our findings show developmental similarities between ascidian trunk ESNs and vertebrate neural crest and placodes. The ‘Binary competence model’ has recently been proposed as a model for induction of the neural crest and pre-placodal region (for a review, see Grocott et al., 2012; Schlosser, 2006) (Fig. 10). In this model, neural induction initially occurs by signals from the organizer containing FGFs, Wnt antagonists and BMP antagonists. It follows that the dorsal ectodermal region acquires the competence to form the neural plate and neural crest. Induction of the neural crest occurs by activation of FGF and Wnt signaling, and inhibition of BMP signaling from the dorsolateral mesoderm, and the later maintenance requires BMP and Wnt signals from tissues adjacent to the neural crest (LaBonne and Bronner-Fraser, 1998; Steventon et al., 2009). The placodes develop from non-neural ectoderm cells near the border of the neural plate, in which FGFs together BMP and Wnt antagonists from neural plate and/or endomesoderm promote induction of the pre-placodal region, whereas canonical Wnt and BMP signaling prevents pre-placodal gene expression (Ahrens and Schlosser, 2005; Litsiou et al., 2005). Specification of posterior trunk ESNs is similar to that of the neural crest. Posterior trunk ESNs develop from the lateral neural plate cell a9.51, in which BMP signals from the neural plate induce posterior trunk ESNs, as in the vertebrate neural crest. However, specification of anterior trunk ESNs is similar to that of the pre-placodal region. We showed that anterior trunk ESNs develop from non-neural ectodermal cells, a9.41 and a9.53, like vertebrate placodes. In addition, a BMP antagonist Chordin from the lateral neural plate promotes induction of anterior trunk ESNs, whereas high levels of BMP signal inhibit this induction. However, in terms of gene expression, a9.51 is more similar to a placodal cell than neural crest cell. In *Ciona*, a lateral neural plate cell a9.51 expresses the pre-placodal markers, *Six1/2* and *Six3/6*, but not neural crest markers, and neither a9.41 nor a9.53 expresses pre-placodal and neural crest markers (Imai et al., 2004; Mazet et al., 2005). In the meantime, there are also clear differences between trunk ESN precursors and vertebrate neural crest and placodal cells. Our cell lineage analyses showed that trunk ESN precursors may lack migratory abilities. It is known in *Ciona* that trunk ESN precursors express none of downstream genes of BMP signaling involved in vertebrate neural plate border specification, such as *Mxsb*, *Dlx* and *Foxl* (Imai et al., 2004; Mazet et al., 2005). Possibly, trunk ESNs could share a common origin with neural crest and placodes, and genetic changes in cells at the neural plate border led the emergence of vertebrate neural crest and placodes.

### Mechanisms for specifying caudal ESNs

We also elucidated the developmental mechanisms for caudal ESN specification, and showed that they differ from the mechanisms for trunk ESNs and vary widely among regions. In the tail tip, ESN formation is independent of HrNodal, HrBMPa and HrBMPb. Conversely, in the dorsal midline of the tail, FGF/Nodal and BMP signaling are involved in caudal ESN specification, as in trunk ESN specification. Overexpression of *HrNodal*, however, induces caudal ESNs in both the neural and non-neural ectoderm (Fig. 6C), which suggests that adoption of a neural fate by FGF is not necessarily involved in caudal ESN specification. Furthermore, in the dorsal midline, it appears that caudal ESN specification requires another inductive signal in addition to FGF/Nodal and BMP, because treatment of b4.2 explants with BMP4 and bFGF has little effect on ESN formation. Our earlier study showed that ESN induction from b-line cells requires an interaction between b-line cells and B-line cells (Ohtsuka et al., 2001a). This suggests that the inductive signal for specifying caudal ESN formation may originate from B-line cells.

Our findings in *Halocynthia* are clearly different from previous results reported for *Ciona*, where either bFGF or BMP4 applied to b4.2 explants was shown to induce caudal ESN formation, and FGF/Nodal and BMP signals induced caudal ESNs in the dorsal and ventral midline, respectively (Pasini et al., 2006). This difference may result from the distinct expression of BMP ligands between *Halocynthia* and *Ciona*. In *Halocynthia*, *HrBMPa* and *HrBMPb* are expressed in the dorsal and ventral midline of tailbud embryos (Miya et al., 1997, 1996), but their *Ciona* orthologs, *Ci-BMP2/4* and *Ci-BMP5/7-like*, are not (Imai et al., 2004). Here, we suggest that FGF/Nodal and BMP signals coordinate to induce caudal ESN formation in *Halocynthia* but do so independently in *Ciona*. In fact, *Halocynthia* larvae intrinsically lack caudal ESNs in ventral midline, where MAPK activation is not detected (Nishida, 2003). It seems that the competence to respond to a BMP signal is reduced in the b4.2 explant of a *Halocynthia* embryo and that this competence is regulated by an inductive signal from B-line cells before the tailbud stage. Comparison of the *Halocynthia* and *Ciona* gene expression patterns would lead to identification of the competent factors and novel inductive signals involved in caudal ESN formation.

### Evolutionarily conserved roles of Nodal and BMPs in PNS formation

Our results show that Nodal signaling specifies the lateral neural plate fate and regulates dorsal PNS formation via BMP signaling in ascidians. In amphioxus, Nodal/Vg1 signaling regulates expression of *Chordin* and specifies the anterior/dorsal ectoderm (Onai et al., 2010). Similarly, in sea urchin, Nodal signaling induces expression of BMP2/4 and Chordin in the ectoderm, and Chordin-mediated inhibition of BMP2/4 restricts ciliary band fate at the boundary between the dorsal and ventral ectoderm (Bradham et al., 2009; Lapraz et al., 2009; Saudemont et al., 2010). Thus, it seems that Nodal/BMP signaling in ancestor deuterostomes may control medial-lateral (ventral-dorsal) patterning of the nervous system. However, there is relatively little evidence in vertebrates that Nodal signaling governs neural patterning during early development, although it is well known to be essential for specification of primary body axes and for formation of the mesoderm and endoderm (Shen, 2007). Vertebrate *Nodal* is expressed in the organizer, whereas ascidian and sea urchin *Nodal* genes are expressed in the ectoderm and endoderm (Bessodes et al., 2012; Chea et al., 2005; Morokuma et al., 2002). In amphioxus, *Nodal* is also expressed maternally in



addition to the zygotic expression in the dorsal blastopore lip that is considered to be the equivalent of vertebrate organizer (Onai et al., 2010; Yu et al., 2007). Changes in the spatial expression of *Nodal* are likely to have contributed to evolutionary divergence in Nodal function.

In vertebrates, neural patterning depends on BMP activity: high levels of BMP activity induce epidermis, intermediate levels of activity induce neural crest and low levels of activity adapt neural plate (Faure et al., 2002; Nguyen et al., 1998; Tribulo et al., 2003). The gradient of BMP activity is generated by the secretion of BMP antagonists such as Chordin from the organizer (Ben-Zvi et al., 2008). Similarly, in amphioxus and fruit fly, BMP signaling is reportedly involved in neural-epidermal segregation and specification of dorsal nervous system containing PNS (Dorfman and Shilo, 2001; Lu et al., 2012; Rusten et al., 2002; Yu et al., 2008, 2007). In ascidians, however, it is likely that BMP signaling is required for PNS formation but not neural-epidermal segregation. A previous study showed that overexpression of *HrBMPb* and *HrChordin* affects expression of *HrEpiC* and specification of pigment cells, but not specification of the brain lineage (Darras and Nishida, 2001). This finding, together with our present study, suggests that expression of BMP ligands in the neural plate border region is necessary to promote PNS formation. Presumably, BMP activity may be maintained at a low level throughout the ectoderm during gastrulation and may be induced in the neural plate by FGF and Nodal signaling. In ascidian embryos with a very small cell number, inductive events appear to occur by direct cell contact. Therefore, it is plausible that ascidians might have evolved to use the spatiotemporal regulation of the BMP signal by FGF/Nodal signaling to specify sensory neurons. Such a loss of neural-epidermal segregation has been reported in hemichordates and annelids. Within those organisms, neuroectodermal patterning is positively regulated by BMP signaling. Administration of BMP4 to these embryos results in expanded expression of sensory neuron markers and dorsal midline markers into entire ectoderm (Denes et al., 2007; Lowe et al., 2006). Taken together, these findings suggest that the role of BMP signaling in PNS formation is conserved among bilaterians.

## MATERIALS AND METHODS

### Animals and embryos

Adult ascidians, *Halocynthia roretzi*, were purchased from fishermen in Sanriku and Asamushi, Japan. Spawed eggs were fertilized with non-self sperm and allowed to develop in Millipore-filtered seawater or artificial seawater (Jamarin-U; Jamarin Laboratory) at 7°C or 10°C. Cleavage was inhibited using 0.5 µg/ml cytochalasin D (Sigma-Aldrich), after which the cleavage-arrested embryos were cultured until the tailbud stage. The isolated blastomeres were cultured in 0.5% agarose wells filled with 0.1% BSA in Jamarin-U-containing recombinant bovine bFGF (Progen) and/or recombinant human BMP4 (R&D Systems) until unoperated sister embryos developed into swimming larvae. To investigate the role of Nodal signaling, embryos were treated with 5 µM SB431542 (Sigma-Aldrich), an inhibitor of ALK4/5/7, until they were fixed, as described previously for *Ciona* embryos (Hudson and Yasuo, 2005). As a control, embryos were cultured in 0.1% DMSO in filtered seawater.

### Histochemistry, immunohistochemistry and *in situ* hybridization

Whole-mount immunohistochemistry and *in situ* hybridization were performed as described previously (Ohtsuka et al., 2001a; Wada et al., 1995). Immunostaining for gelsolin, an ESN marker, was carried out using the AS23 monoclonal antibody. DIG-labeled RNA probes for gelsolin (Ohtsuka et al., 2001a), *HrBMPa* (Miya et al., 1996), *HrBMPb* (Miya et al., 1997), *HrChordin* (Darras and Nishida, 2001), *HrPax-37* (Wada et al., 1997), *HrEpiC* (Ueki et al., 1991) and *HrETR-1* (Yagi and Makabe, 2001) were synthesized using a DIG RNA-labeling Kit (Roche). The signal was detected by staining with NBT/BCIP or Fast Red (Roche). To detect tyrosinase enzymatic activity,

embryos were fixed overnight at 4°C in 4% paraformaldehyde in buffer containing 0.5 M NaCl and 0.1 M MOPS (pH 7.5). They were then washed with 16 mM sodium phosphate buffer (pH 7.4) before incubation in the same buffer containing 4 mM 3,4-dihydroxy-L-phenylalanine (DOPA) at 37°C. After the tyrosinase reaction, the embryos were dehydrated in methanol and subjected to whole-mount *in situ* hybridization.

### Lineage analysis and microinjection

Microinjection was carried out as previously described (Okada et al., 2002). For lineage tracing, the blastomere was labeled with DiI [DiI18(3), Molecular Probes] at the gastrula stage as previously described (Nakatani et al., 1999). To determine the trunk ESN lineage, the DiI-labeled larvae were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature and then incubated in 1 mg/ml digitonin in PBS overnight at 4°C. After washing with PBS, the larval tunic was manually removed from each larva using needles and the larvae were immunostained with AS23. The specimens were examined using laser scanning confocal microscopy (Zeiss LSM5 PASCAL). Serial optimal sections were taken at 10 µm intervals, and a three-dimensional image was reconstructed using the software supplied with the LSM5. To identify lineages of *HrBMPb*-expressing cells, the DiI-labeled tailbud embryos were fixed for whole-mount *in situ* hybridization. After DOPA staining and treatment with 1 mg/ml digitonin in PBS for 10 min, the embryos were subjected to whole-mount *in situ* hybridization without using detergents. Overlays of pictures were created using Adobe Photoshop.

### Morpholino antisense oligos (MOs)

MOs for *HrNodal* (Tokuoka et al., 2007), *HrBMPa*, *HrBMPb* and *HrChordin* were purchased from Gene Tools. Their sequences were as follows: *HrNodal*MO, 5'-TAGCAGGTAGGTAGAATAGAAGAAT-3'; *HrBMPa*MO, 5'-TCTTCTCCTCCACTCATCTTGTC-3'; *HrBMPb*MO, 5'-CATGTCCCTCAACGTGTGATCTCC-3'; *HrChordin*MO, 5'-ACTTCGCTAATCGACGAGATCCCAT-3'.

MOs other than *HrNodal*MO were injected at 0.5 mM; *HrNodal*MO was injected at 0.25 mM. Standard control morpholino-oligo (StdMO; Gene Tools) served as a negative control.

### Synthetic RNA

Plasmids for *in vitro* transcription of *HrNodal* (Morokuma et al., 2002) and *HrChordin* (Darras and Nishida, 2001) were kindly provided by Dr H. Nishida (Osaka University). To generate expression plasmids for *HrBMPa* and *HrBMPb*, the full open reading frames were amplified using the following primers: for *HrBMPa*, 5'-TTTAAGCGGCCGCGA-TGAGTGGAG-3' and 5'-TTTGAGCTCGAGGGATGCGC-3'; and for *HrBMPb*, 5'-TCACACGCGGCCGCGACATGTTTAC-3' and 5'-TGCCTCTCGAGACTACCGAC-3'.

The PCR products were cloned into the pSD64TR vector (kindly provided by Dr T. Snutch, University of British Columbia). Capped RNAs were synthesized using a MEGAscript SP6 Kit (Ambion), after which poly (A) tails were added using a Poly(A) tailing Kit (Ambion).

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### Competing interests

The authors declare no competing financial interests.

### Author contributions

Y. Ohtsuka performed the majority of the experiments. J.M. and Y.K. constructed the plasmids. Y. Ohtsuka and Y. Okamura designed the experiments. Y. Ohtsuka, Y.K. and Y. Okamura wrote the manuscript.

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## Supplementary material

Supplementary material available online at  
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