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BMP and Delta/Notch signaling control the development of amphioxus epidermal sensory neurons: insights into the evolution of the peripheral sensory system

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SUMMARY

The evolution of the nervous system has been a topic of great interest. To gain more insight into the evolution of the peripheral sensory system, we used the cephalochordate amphioxus. Amphioxus is a basal chordate that has a dorsal central nervous system (CNS) and a peripheral nervous system (PNS) comprising several types of epidermal sensory neurons (ESNs). Here, we show that a proneural basic helix-loop-helix gene (*Ash*) is co-expressed with the *Delta* ligand in ESN progenitor cells. Using pharmacological treatments, we demonstrate that Delta/Notch signaling is likely to be involved in the specification of amphioxus ESNs from their neighboring epidermal cells. We also show that BMP signaling functions upstream of Delta/Notch signaling to induce a ventral neurogenic domain. This patterning mechanism is highly similar to that of the peripheral sensory neurons in the protostome and vertebrate model animals, suggesting that they might share the same ancestry. Interestingly, when BMP signaling is globally elevated in amphioxus embryos, the distribution of ESNs expands to the entire epidermal ectoderm. These results suggest that by manipulating BMP signaling levels, a conserved neurogenesis circuit can be initiated at various locations in the epidermal ectoderm to generate peripheral sensory neurons in amphioxus embryos. We hypothesize that during chordate evolution, PNS progenitors might have been polarized to different positions in various chordate lineages owing to differential regulation of BMP signaling in the ectoderm.

KEY WORDS: Peripheral nervous system, Delta/Notch signaling, BMP, *Achaete-scute*, Cephalochordate

INTRODUCTION

The evolution of the nervous system in metazoan animals has been a topic of great interest. It is thought that the first nerve cell (neuron) evolved in a common ancestor of cnidarians and bilaterians, because neuronal cell types are absent in their sister group, the sponges (Galliot et al., 2009). Although putative sensory cells do exist in the sponge, these cells do not form synapses and do not possess dendrites and axons (Richards et al., 2008). Although cnidarians typically have a diffuse nerve net, some regionalization patterns of neurons and their neurites have been identified in several types of cnidarians (Galliot et al., 2009; Watanabe et al., 2009). It is possible that these cnidarian nerve rings might represent a primitive organized nervous system. After the divergence of cnidarians and bilaterians, a centralized nervous system evolved in the bilaterian lineage (Arendt et al., 2008; Holland, 2003). The evolutionary origin of the central nervous system (CNS) in bilaterian animals has been highly controversial. Traditionally, it was widely accepted that the CNSs of protostomes and deterostomes (or specifically, chordates) evolved independently because they are structurally different and are located in opposite positions along the dorsal-ventral axis (reviewed by Arendt and Nubler-Jung, 1999). However, during the last two decades, many discoveries have been made that challenge this view. Between the protostome and chordates, there are remarkable similarities in gene expression and conserved signaling pathways in the anterior/posterior and medial/lateral patterning of the CNS (reviewed in De Robertis, 2008). This finding has led to the hypothesis that a single CNS evolved in the basal bilaterians, and a dorsal-ventral axis inversion occurred in the chordate lineage.

Conversely, the presence of an ectodermal nerve net and the lack of an apparent CNS in the hemichordates (a group of deuterostome animals) reinforce the argument for independent origins of the CNS in protostomes and chordates (Arendt et al., 2008; Lowe et al., 2003). This controversy is complicated further by the fact that the relationship between the CNS and the peripheral nervous system (PNS) is not always well defined. In addition to the CNS, both protostomes and chordates have a PNS with ectodermal sensory neurons. In the fruit fly *Drosophila*, inhibition of Dpp/BMP signaling by its antagonist Sog (also known as Chordin) during early embryogenesis induces the formation of a ventral neurogenic region that gives rise to the ventral nerve cord and some ventral PNS neurons; subsequently, high levels of BMP signaling in the dorsal ectoderm promote the formation of the dorsal and lateral PNS neurons (Rusten et al., 2002). Similarly, in vertebrate embryos, the CNS is derived from the dorsal neural ectoderm, where antagonists are present to inhibit the BMP signaling. By contrast, most of the neurons of the vertebrate PNS are derived from two ectodermal tissues, the neural crest and the neurogenic placodes, which originate from the boundary between the CNS and the epidermal ectoderm (Baker and Bronner-Fraser, 2001; Graham and Begbie, 2000; Le Douarin and Kalcheim, 1999; Le Douarin and Dupin, 2003; Schlosser, 2006). However, the neural crest and the neurogenic placodes are usually considered to be vertebrate innovations (Shimeld and Holland, 2000), and their evolutionary origins remain largely unresolved. In hemichordates, it is highly debated whether the dorsal or ventral tracts of the axons along with

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the collard cord correspond to the CNS, and whether the ectodermal nerve net corresponds to the CNS or PNS (Holland, 2009; Nomaksteinsky et al., 2009). These uncertainties have hindered our understanding of the evolution of the nervous system. Therefore, it is important to determine the correct homologous relationships of both the CNS and PNS among the key metazoan animal groups by comparative studies.

To gain more insight into the evolution of the nervous system, we focused on the mechanisms of neurogenesis in the cephalochordate amphioxus. Amphioxus is an invertebrate chordate that shares key chordate characteristics (a dorsal CNS, notochord, segmented somites, and pharyngeal gill slits) with vertebrate animals. Among the three major chordate groups (amphioxus, tunicates and vertebrates), the adult body plan and embryology of the amphioxus are highly similar to those of the vertebrates (Whittaker, 1997), whereas most tunicate larvae undergo metamorphosis and lose many chordate characteristics as they become sessile adults. Therefore, it was traditionally thought that amphioxus is the sister group of the vertebrates and that tunicates compose the basal chordate group; however, recent phylogenomic analyses have reverted their positions and placed amphioxus as the basal chordate group and tunicates as the sister group of the vertebrates (Blair and Hedges, 2005; Bourlat et al., 2006; Delsuc et al., 2006; Philippe et al., 2005; Putnam et al., 2008). In addition to the dorsal CNS, amphioxus also have a PNS comprising several types of sensory neurons (Wicht and Lacalli, 2005). Among these amphioxus sensory neurons, the solitary type I receptor cells are the most abundant population of the epidermal sensory neurons (ESNs) and are scattered along the bodies of developing amphioxus larvae and adults (Holland and Yu, 2002; Lacalli and Hou, 1999) (Fig. 1A,B). The cell body of the receptor is located within the epidermis, and a long axon from the base of the cell body extends into the CNS (Fig. 1C,D). Several axons originated from local ESNs merge into parallel bundles, and these axon bundles run along the myotome boundaries on the flank and connect to the CNS (Fig. 1E-I). During the early neurula stage, these ESNs differentiate from the ventral ectoderm and are first characterized by the expression of a pan-neural marker *Hu/Elav* (Fig. 1J-M) (Benito-Gutierrez et al., 2005a; Satoh et al., 2001); subsequently, at the mid- to late-neurula stage, many of these differentiating ESNs appear to delaminate from the ectoderm and invaginate into the sub-epidermal space to migrate towards the dorsal side of the neurula (Fig. 1N-Q) (Benito-Gutierrez et al., 2005b; Kaltenbach et al., 2009). At the late neurula to early larval stage, the ESNs re-insert into the epidermal ectoderm and send out axons to innervate the CNS (Kaltenbach et al., 2009), and many of these ESNs are immunoreactive to antibodies against the neurotransmitter γ-aminobutyric acid (GABA) (Fig. 1R-W) (Anadon et al., 1998). Using embryonic ontogeny and expression data for several homologs of placode marker genes, it has been speculated that these amphioxus ESNs are homologs of vertebrate placode-derived neurons (Benito-Gutierrez et al., 2005a; Benito-Gutierrez et al., 2005b; Holland, 2005; Holland and Yu, 2002; Kaltenbach et al., 2009; Kozmik et al., 2007; Mazet et al., 2004; Meulemans and Bronner-Fraser, 2007; Rasmussen et al., 2007). However, the molecular mechanism controlling the specification of amphioxus ESNs remains unclear, and this situation has impeded our understanding of the relationship of these ESNs to the peripheral sensory neurons in other animals. To address this limitation, we set out to determine the developmental mechanisms underlying the formation of the ESNs in amphioxus.

Here, we show that the ventral ectoderm of the amphioxus embryo is a neurogenic domain for ESNs. We demonstrate that Delta/Notch signaling is involved in the specification of individual ESNs from neighboring epidermal cells in this domain and that BMP signaling levels regulate the formation of this putative neurogenic domain within the ventral ectoderm. Additionally, we show that the inductions of the dorsal CNS and the ventral ESN progenitor domain are separate events that require opposing BMP signaling levels during amphioxus embryogenesis. Our results demonstrate that conserved Delta/Notch and BMP signaling pathways govern the specification and distribution of peripheral sensory neurons in amphioxus, respectively. These results suggest that a deep homology exists in the PNS sensory neuron formation in metazoan animals.

MATERIALS AND METHODS

Animals, embryos and drug treatments

Amphioxus (*Branchiostoma floridae*) adults were collected in Tampa Bay, Florida, USA, during the summer breeding season. The gametes were obtained by electric stimulation. Fertilization and subsequent culturing of the embryos were carried out as previously described (Yu and Holland, 2009). Amphioxus embryos were staged according to Hirakow and Kajita (Hirakow and Kajita, 1991; Hirakow and Kajita, 1994). For embryos in the neurula stage, we further defined N0 stage as the onset of neurulation, which is characterized by the dissociation of the dorsal epidermal ectoderm from the neural plate before formation of somites. We made a clear distinction between the N1 (early neurula, 1-3 somites), N2 (hatching neurula, 4-8 somites) and N3 (late neurula, 9-12 somites) stages based on the number of somites.

Notch signaling was inhibited by DAPT (Geling et al., 2002) treatment. DAPT (Calbiochem) was dissolved in DMSO to prepare a 10 mM stock solution. The DAPT stock solution was diluted in filtered seawater to a final concentration of 50 μM and applied to amphioxus embryos from the G5 stage (late gastrula). The control embryos were treated with filtered seawater containing an equal amount of DMSO. Manipulation of BMP signaling was achieved by treatment with 250 ng/ml of recombinant zebrafish Bmp4 protein (zBMP4, R&D Systems), as previously described (Yu et al., 2007), or by the small molecular inhibitor dorsomorphin (Sigma). Dorsomorphin was dissolved in DMSO to prepare a 10 mM stock solution, and the experimental embryos were treated with dorsomorphin at 8 or 16 μM from the G5 stage. The control embryos were treated with an equal amount of DMSO in filtered seawater.

Cloning of an *Achaete-scute* homolog in amphioxus

According to the draft genome sequence from the Joint Genome Institute (JGI) and the annotated protein ID 233300 for amphioxus *Achaete-scute* homolog gene product (Satou et al., 2008), a pair of *Achaete-scute* homolog specific primers were designed, and *Bam*HI and *Eco*RI restriction enzyme sites were added to their 5′ ends, respectively (AS1F-*Bam*HI, 5′-CGGG-ATCCCGCAAGCGGAGGATAAACTTC-3′ and AS1R-*Eco*RI, 5′-CGGAATTCCGAACCACGATGTGAAGTCG-3′). The 5′ and 3′ parts of the *Achaete-scute* homolog were amplified from a cDNA library (Langeland et al., 1998) constructed using the pBluescript vector. Vector-specific primer pBluescript-*Not*I (5′-CTTGCGGCCGCTTCACTATAGGGCGAATTGG-GTACC-3′) and AS1R-*Eco*RI were used to amplify the 5′ part of the cDNA, and primers pBluescript-*Not*I and AS1F-*Bam*HI were used to amplify the 3′ part. The amplicon of the 5′ and 3′ parts of the *Achaete-scute* homolog were cloned into the pBluescript vector and sequenced to confirm identity.

In situ hybridization and immunostaining

The plasmid containing the 3′ domain of the *Achaete-scute* homolog was linearized by *Bam*HI digestion and used to synthesize a digoxigenin (DIG)-labeled antisense riboprobe with T3 RNA polymerase. Full-length cDNAs of *AmphiElav/Hu*, *AmphiDelta* and *AmphiSoxB1a* were isolated from an EST library, and the PCR products of the insert cDNA were used as templates for synthesizing DIG-labeled antisense riboprobes with T7 RNA polymerase (Yu et al., 2008a; Yu et al., 2007). For the synthesis of the *AmphiTlx* antisense riboprobe, the primer pDONR222F-SP6 (5′-

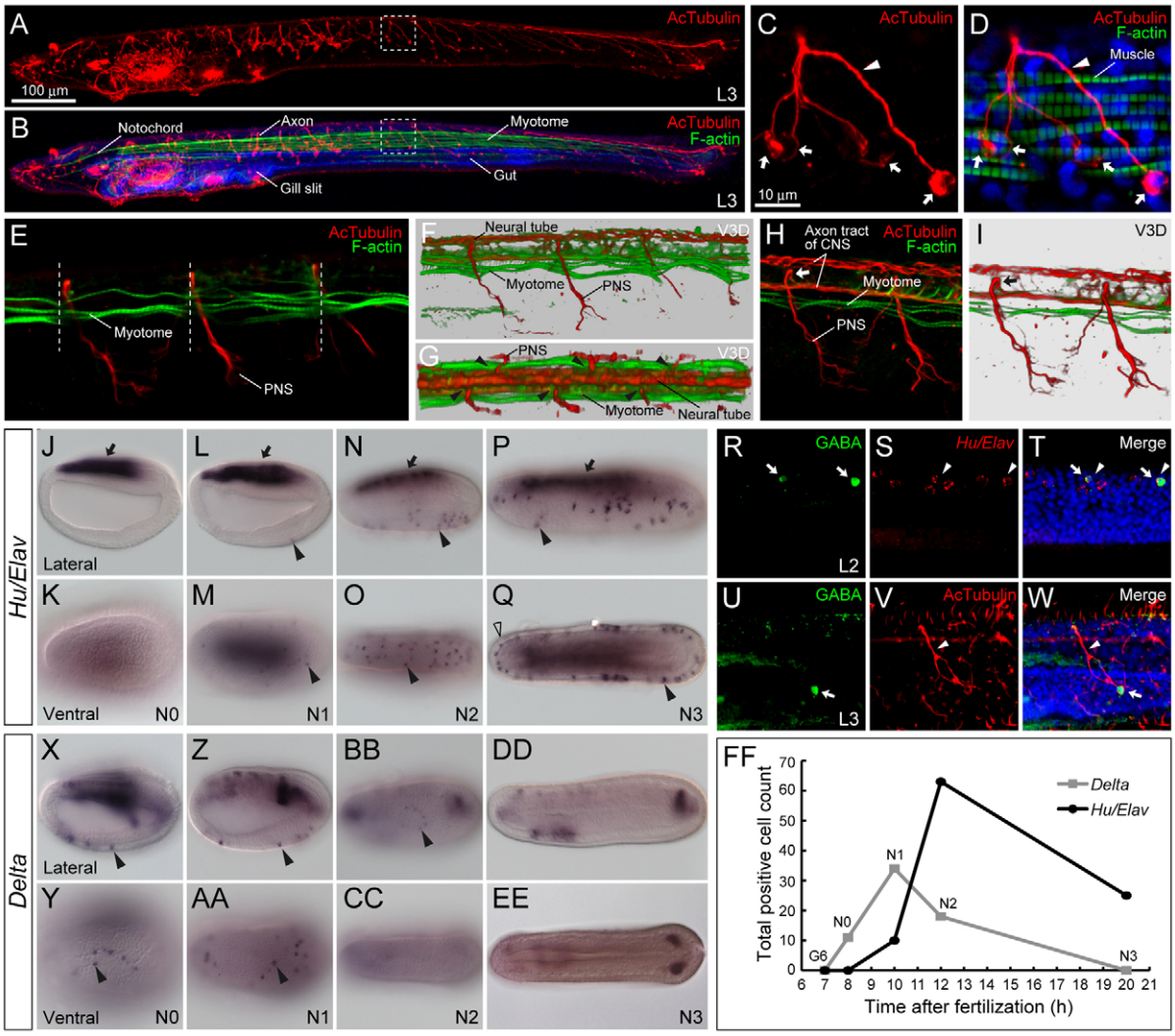

Fig. 1. Distribution of epidermal sensory neurons (ESNs) in amphioxus and expression of ESN markers in early embryogenesis. (**A**,**B**) Confocal images of an L3 stage larva (48 hours post-fertilization). The axonal structure is labeled with anti-acetylated α-tubulin antibody (red). Nuclei are labeled with DAPI (blue). Muscle fibers and myotome organization are visualized by F-actin staining (green). (**C**,**D**) Images show that each individual ESN (white arrows) resides in the peripheral epidermis and projects one axon (arrowhead) towards the CNS. C and D are magnifications of the boxed areas in A and B, respectively. (**E**,**F**) Lateral views of the mid-section of an L3 larva showing the axon bundles (red) running along the myotome boundaries (dashed lines). (**G**) Dorsal view of the same larva showing that the myotomes are asymmetrically positioned, and therefore, the axon bundles (arrowheads) are staggered by a half-segment left to right. (**H**,**I**) Images show the peripheral axon bundles connecting to the CNS (arrows) and traveling along the fiber tracks within the nerve chord. (**J-Q**) Expression of *Hu/elav* during neurula stage embryos. J, L, N and P are lateral views, dorsal to the top and anterior to the left; K, M, O and Q are ventral views. Arrows indicate expression in the CNS (neural tube); arrowheads are examples of expression in the developing ESNs. Open arrowhead in Q indicates the migrating ESN. (**R-T**) Double staining of GABA (green) and *Hu/elav* transcripts (red) in an L2 larva showing colocalization of GABA (arrows) and *Hu/elav* (arrowheads) in ESNs. (**U-W**) One GABA-positive ESN (green, arrow) with its axon visualized by acetylated α-tubulin staining (red, arrowhead). (**X-EE**) Expression of *Delta*. X, Z, BB and DD are lateral views; Y, AA, CC, EE are ventral views. Arrowheads show examples of expression in the developing ESNs. (**FF**) Numbers of observed *Delta*-positive and *Hu/elav*-positive ESN cells during amphioxus embryogenesis. V3D, 3D reconstruction of axonal structures by the visualization-assisted analysis system Vaa3D-Neuron.

ATTTAGGTGACACTATAGAAGACGGCCAGTCTTAAGCTC-3′) and an *AmphiTlx*-specific primer with a 5′ added T7 polymerase binding site (Tlx-R-T7, 5′-TAATACGACTCACTATAGGGAGACCAACGACTTG-GTATGAAGC-3′) were used to amplify a 1276 bp *AmphiTlx* cDNA fragment from the cDNA clone bfne092g20 (Yu et al., 2008b). The procedure for in situ hybridization was performed as previously described (Yu et al., 2008a). Images were taken using a Zeiss Axio Imager A1 microscope with a Zeiss AxioCam MRc CCD camera.

Double fluorescent in situ hybridization and immunostaining of the amphioxus embryos were performed as previously described (Wu et al., 2011). For acetylated α-tubulin and actin filament (F-actin) staining, the embryos were fixed in 4% PFA-MOPS-EGTA and then stored in PBST (PBS with 0.1% Tween 20) at 4°C until needed. For pSmad1/5/8 staining, the embryos were fixed in PFA-MOPS-EGTA and then stored in 70% ethanol at –20°C. The embryos were blocked for 1 hour in blocking solution (3% BSA and 0.1% Triton X-100 in PBS) and incubated in primary antibodies against pSmad1/5/8 (Cell Signaling, 1:150) or acetylated α-tubulin (Sigma, 1:1000) and GABA (Sigma, 1:1000) in blocking solution at 4°C overnight. The immunofluorescent signals were detected after incubation with secondary antibodies (Alexa Fluor 594-conjugated goat anti-mouse antibody for acetylated α-tubulin, Alexa Fluor 488-conjugated goat anti-rabbit antibody for GABA, and Alexa Fluor 594-

conjugated goat anti-rabbit antibody for pSmad1/5/8, Invitrogen, 1:400). DAPI (Invitrogen, 1 μg/ml in PBST) was used for nuclear staining, and BODIPY FL Phallacidin (Invitrogen, 1:40 in 1% BSA/PBST) was used for F-actin staining. Fluorescent images were acquired using a Leica TCS-SP5 confocal microscope.

Measurements and statistical analysis

The embryos subjected to in situ hybridization were mounted with a 100 μm spacer and orientated as ventral side up under a Zeiss Axio Imager A1 microscope. The numbers of solitary or clustered *Hu/elav*- or *Delta*-positive cells were counted in at least ten embryos for both the control and DAPT-treated embryos. Lateral images of the embryos were captured to measure the expression boundary of gene expression. The height of the embryo (H, the vertical distance from the ventral edge to the dorsal edge of the embryo) and the height of the expression boundary (h, the vertical distance from ventral edge to the most dorsal edge of the signal) were measured using the Zeiss AxioVisionLE software. The h/H ratio indicated the relative distance of signal from the base to the dorsal expression boundary. *t*-tests were performed using SigmaStat, and bar histograms were drawn using SigmaPlot.

RESULTS

Delta/Notch signaling controls the specification of ESNs in the ventral ectoderm, and the *Delta* ligand gene is co-expressed with the proneural bHLH gene *Ash*

We confirmed that the earliest differentiating type I receptor cells are individual cells within the ventral ectoderm during the early neurula stage that express the pan-neural marker *Hu/elav* (Fig. 1K,M,O). As development proceeds, these *Hu/elav*-positive cells are observed on the flanks of the embryos (Fig. 1N,P). Interestingly, we noticed that the Notch ligand *Delta* is expressed in a similar pattern in the ventral ectoderm (Rasmussen et al., 2007) and that the expression of *Delta* always precedes *Hu/elav* expression (Fig. 1X-AA,FF). This suggests that Delta/Notch-mediated lateral inhibition (Bray, 2006) might be involved in the specification of these solitary sensory neurons from the neighboring epidermal cells. To test this hypothesis, we used the γ-secretase inhibitor DAPT to block the Notch signaling pathway. The embryos treated with DAPT showed a significant increase of total *Hu/elav*-positive cells, and many of the *Hu/elav*-expressing units were composed of multiple cells (Fig. 2A-C). We also observed a significant increase of *Hu/elav*-expressing units (composed of either solitary or clustered *Hu/elav*-positive cells) in the DAPT-treated embryos (Fig. 2D), which suggests that lateral inhibition might be responsible for limiting the number of these individual sensory neurons. Additionally, when we used other classes of inhibitors that block different functional sites within γ-secretase (Morohashi et al., 2006), we observed similar phenotypes as DAPT treatment (supplementary material Fig. S1). These results indicate that the observed phenotypes are probably caused by blocking Notch signaling. We also observed that in the DAPT-treated embryos, the *Delta*-expressing cells were increased and present in clusters (Fig. 2E-H), suggesting that an auto-regulatory loop might function to restrict the expression of the *Delta* ligand to each solitary neuronal progenitor cell.

Furthermore, we followed the morphogenesis of the ESNs in the control and DAPT-treated embryos by *Hu/elav* expression and GABA immunostaining (Fig. 2I-K). During the late neurula stage, individual ESN progenitor cells were observed within the sub-epidermal space during their migration in the control embryos; whereas in the DAPT-treated embryos, we observed many aggregates of *Hu/elav*-positive cells within the sub-epidermal space (Fig. 2I, white arrowheads), suggesting that these ectopic ESN progenitor cells might migrate together as a unit. At the early larval stage when many ESNs become GABA-positive and send out axons to innervate the CNS, we observed a significant increase of clustered GABA-positive cells in the DAPT-treated larvae (Fig. 2J,K). Moreover, the axon bundles connected to each GABA-positive unit appeared to be thicker compared with those in the control (Fig. 2J, white arrows), suggesting that there are more axon tracts in each of the bundles coming from the ectopic ESNs. These data indicate that DAPT treatment not only changes the expression of the neuronal markers during morphogenesis but also affects the actual number of neurons forming at the epidermis.

In the *Drosophila* and vertebrate models, proneural basic helix-loop-helix (bHLH) genes, such as *Achaete-scute* homologs, are required for the correct differentiation of sensory neurons (Quan and Hassan, 2005). Expression of proneural bHLH genes in the neural progenitors activates *Delta* expression, whereas their own expression is inhibited in the adjacent Notch signal-receiving cells. We identified one amphioxus *Achaete-scute* homolog (*Ash*; sequence deposited under GenBank accession number JF779676), the expression of which was colocalized with *Delta* in the individual cells within the ventral ectoderm during the early neurula stage (Fig. 3A,B). DAPT treatment in the amphioxus embryos also increased the number of *Ash*-expressing cells (most of which also expressed *Delta*, as shown in Fig. 3B) and caused clustering of many *Ash*-expressing cells within the ventral ectoderm (Fig. 3B-E). This result is consistent with the known regulatory loop between proneural bHLH genes and Delta/Notch signaling in both *Drosophila* and vertebrates.

High levels of BMP signaling are necessary for generating ESNs from the ectoderm

In amphioxus, BMP signaling is involved in patterning the overall dorsoventral (D/V) axis (Onai et al., 2010; Yu et al., 2007), and manipulation of BMP levels severely affects neural induction and medial-lateral patterning across the dorsal ectoderm (Yu et al., 2008a). To test whether BMP signaling levels control the formation of ESNs from the ventral ectoderm, we used recombinant zebrafish Bmp4 protein (zBMP4) to elevate BMP signal levels globally (Yu et al., 2007). We found that the embryos treated with zBMP4 at the blastula stage were ventralized without a dorsal CNS, whereas the punctate epidermal expression of both *Delta* and *Hu/elav* was expanded throughout the entire embryo (Fig. 4A). We used nuclear phospho-Smad1/5/8 (pSmad1/5/8) staining to visualize BMP signaling levels. In control embryos, we observed strong pSmad1/5/8 staining on the ventral side (Fig. 4B, upper panels, red), which is consistent with the notion that a high BMP level determines ventral structure (Onai et al., 2010; Yu et al., 2007). In the zBMP4-treated embryos, pSmad1/5/8 staining was present globally (Fig. 4B, lower panels, red), and the expression patterns of *Delta* and *Hu/elav* changed corresponding to altered BMP signaling levels across the entire dorsal-ventral axis (Fig. 4B, lower panels, punctate green; supplementary material Fig. S2A,B). To quantify further the effect of manipulation of BMP signaling on the formation of ESNs, we selected one batch of zBMP4-reated embryos that displayed a mild phenotype and a partially formed neural tube, by which we can recognize the D/V polarity of the embryos (Fig. 4C). In the control embryos, the dorsal limits of the *Delta*- and *Hu/elav*-positive cells in the epidermal ectoderm were <40% of the body height at the N1 and N2 stages, respectively, whereas in the zBMP4 treated embryos, the *Delta*- and *Hu/elav*-positive cells were expanded to >80% of the body height at the N1

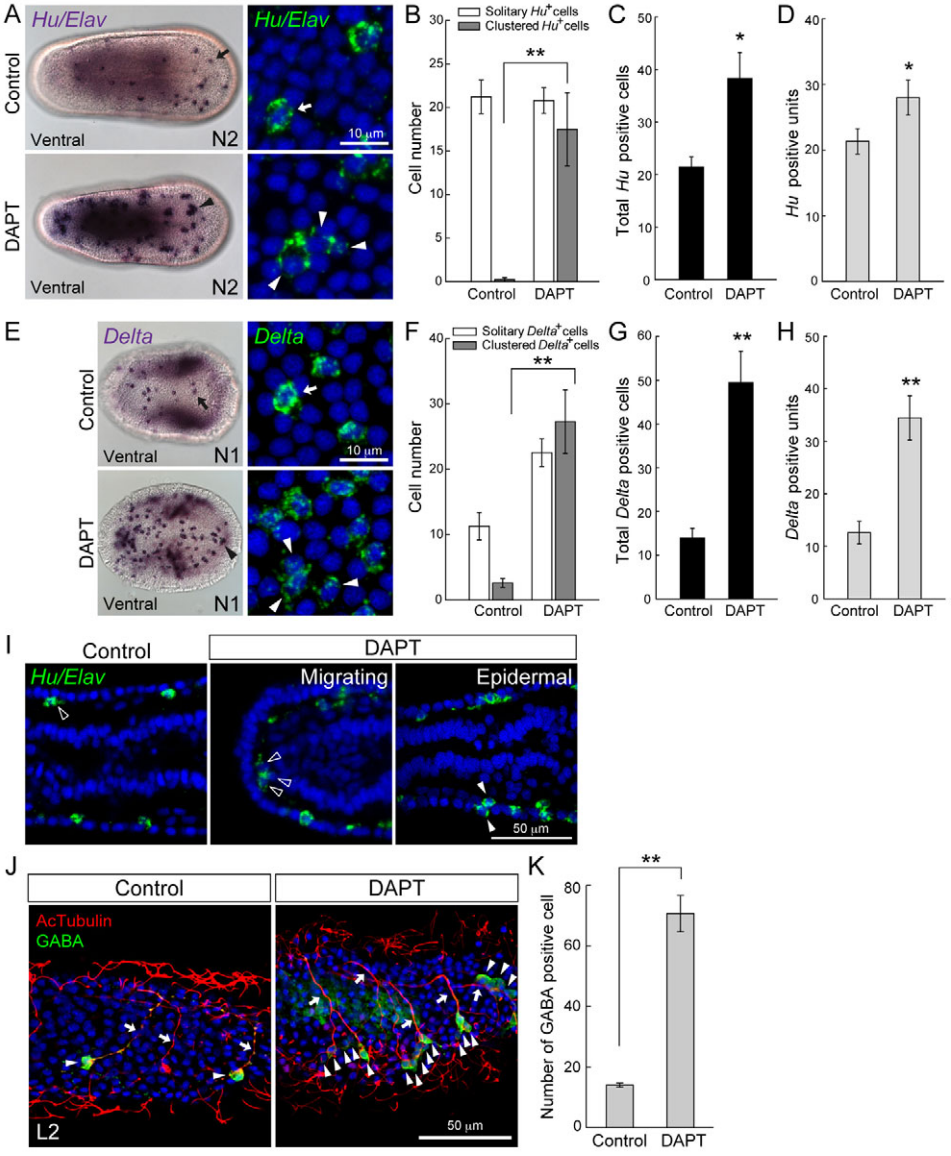

Fig. 2. Specification of amphioxus epidermal sensory neurons is controlled by Notch signaling. (**A**) An N2 stage embryo treated with DAPT at the G5 stage shows clustered *Hu/elav*$^+$ cells in its ventral side. Arrows indicate examples of solitary *Hu/elav*$^+$ cells in the control embryo. Arrowheads indicate the clustered *Hu/elav*$^+$ cells in the DAPT-treated embryo. Fluorescence images show the distribution of *Hu/elav* transcripts (green) and their associated nuclei (blue). (**B-D**) Quantification of DAPT treatment on the distribution and number of ventral *Hu/elav*$^+$ cells. (**E**) N1 stage embryos treated with DAPT at the G5 stage also had more clustered *Delta*$^+$ cells compared with the DMSO-treated control embryos. Arrows indicate the solitary *Delta*$^+$ cells, and arrowheads indicate the clustered *Delta*$^+$ cells. Fluorescence images show the distribution of *Delta* transcripts (green) and their associated nuclei (blue). (**F-H**) Quantification of DAPT treatment on the distribution and numbers of ventral *Delta*$^+$ cells. (**I**) DAPT treatment caused the clustered migration of *Hu/elav*$^+$ ESN progenitor cells in the N3 stage embryo. Open arrowheads indicate the migrating ESNs. Filled arrowheads indicate ESNs that remain in the epidermis. (**J**) DAPT treatment increased GABA$^+$ ESNs in L2 larva. Arrows indicate axon bundles and arrowheads indicate GABA$^+$ ESNs. (**K**) Quantification of DAPT treatment on the number of GABA$^+$ ESNs. **P*<0.05; ***P*<0.001; *t*-test, *n*=10 embryos. Error bars indicate s.e.m.

and N2 stages, respectively (Fig. 4C,D). These results indicate that the level of BMP signaling controls the dorsal-ventral distribution of ESNs.

We also noticed that in our severe zBMP4-treated phenotype, the dorsal neural tube (the CNS, indicated by arrows in Fig. 4A with *Hu/elav* staining) was completely abolished (Fig. 4A); however, the progenitors of the ESNs remained present throughout the ectoderm. This suggests that the patterning of the amphioxus CNS and the ESNs are separate events and require different BMP signaling levels. To test this hypothesis, we applied zBMP4 treatment at a later time point (stage G5) when the CNS was already determined. After this late treatment, the formation of the CNS was mostly unaffected, as shown by the expression of the CNS marker *SoxB1-a* (Fig. 4E). Interestingly, the distribution of punctate *Delta* and *Hu/elav* expression in the ectoderm was still expanded dorsally after this late zBMP4 treatment (Fig. 4E,F). Additionally, combination of DAPT and zBMP4 treatments caused a dramatic increase of clustering *Hu/elav*-positive cells across nearly the entire epidermal ectoderm (supplementary material Fig. S3). These observations suggest that ESN patterning is an independent event that occurs later than CNS induction.

To analyze the role of BMP signaling on ESN patterning further, we used dorsomorphin, a small molecule inhibitor that blocks BMP signaling through the Smad pathway (Yui et al., 2008). Dorsomorphin treatment at the G5 stage caused a dose-dependent reduction of *Hu/elav*-positive ESNs in the neurula stage embryos (Fig. 4G,H). We also observed a decrease of BMP signaling levels as visualized by pSmad1/5/8 staining (Fig. 4I), indicating that BMP signaling is required for the formation of ESNs from the ventral ectoderm. Taken together, these results suggest that high BMP signaling levels might generate a putative ESN progenitor domain in the ventral ectoderm of the amphioxus embryos.

Next, we examined this putative ESN progenitor domain further and investigated the gene that might respond to the high level of BMP signaling. Through a literature search, we identified a homeobox transcription factor gene, *AmphiTlx* (*Tlx*), the expression domain of which is closely similar to the putative ESN progenitor domain (Kaltenbach et al., 2009) (Fig. 5A). At the N0-N1 stage, *Tlx* is expressed in the ventral ectoderm that corresponds to the area from which the dotted *Delta*- and *Hu/elav*-positive cells originate during the early neurula stage (Fig. 1L,M,X-AA; Fig. 5A); subsequently, at the N2-N3 stage, *Tlx* expression is gradually

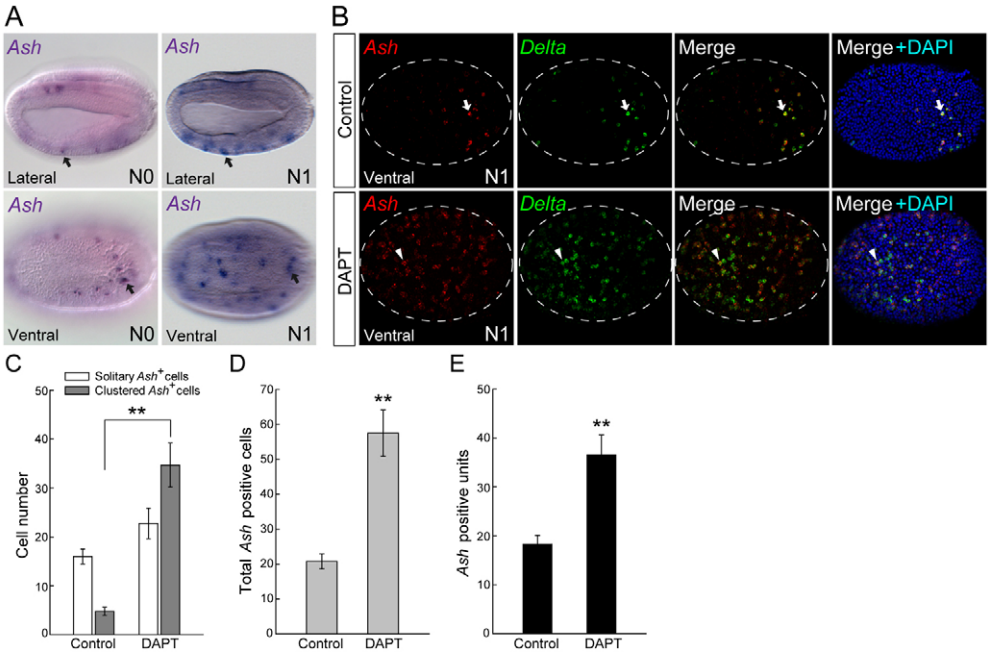

Fig. 3. Amphioxus *Achaete-scute* homolog (*Ash*) is co-expressed with *Delta* in the ventral epidermis of the N1 stage embryos. (**A**) Expression of *Ash* in the N0 and N1 stage embryos. Arrows show examples of spotty *Ash* expression in the ventral ectoderm. (**B**) *Ash* (red) is co-expressed with *Delta* (green) in the ventral epidermis of the N1 stage embryos; an example of a cell co-expressing *Ash* and *Delta* is indicated by an arrow. DAPT treatment also caused a significant increase of clustered *Ash*$^+$ cells (arrowheads). Dashed lines outline embryos. (**C-E**) Quantification of DAPT treatment on the distribution and number of ventral *Ash*$^+$ cells. $^{**}P<0.001$, *t*-test, $n=10$ embryos. Error bars indicate s.e.m.

restricted to colocalize with the *Hu/elav*-positive cells (Fig. 5A,B), suggesting a role for *Tlx* in differentiating ESNs. We found that zBMP4 treatment shifted this ectodermal *Tlx* expression domain dorsally (Fig. 5C,D) and that this dorsal expansion correlated with the change in BMP signaling level as visualized by double fluorescence staining of pSmad1/5/8 and *Tlx* (Fig. 5E; supplementary material Fig. S2C,D). Furthermore, DAPT treatment also increased the number of *Tlx*-expressing cells and caused a clustering of *Tlx*-positive cells in the N2 stage embryos when *Tlx* expression was restricted to the individual developing ESNs (Fig. 5F-I). Taken together, our results suggest that *Tlx* is probably a downstream BMP gene that mediates ESN development in conjunction with *Ash* and Delta/Notch signaling. Further experiments are needed to verify this hypothesis and to link *Tlx* expression to the functions of *Ash* and *Delta* in selected ESN progenitor cells.

DISCUSSION

The proneural bHLH transcription factor and the Delta/Notch signaling pathway constitute a conserved genetic circuit for sensory neuron formation

Our results demonstrate that BMP signaling is required for the formation of a putative ESN progenitor domain in the amphioxus ventral ectoderm (Fig. 6A). Within this domain, the proneural bHLH transcription factor *Ash* and *Delta* are co-expressed in the ESN progenitor cells. Treatment with DAPT and other γ-secretase inhibitors caused a significant increase in ESN formation and in the clustering distribution of these ectopic ESNs. Although we cannot exclude the possibility that these reagents might also affect other pathways that contribute to ESN specification, and further experiments will be necessary to confirm this, our data suggest that Delta/Notch-mediated lateral inhibition is probably involved in the specification of individual sensory neurons in amphioxus (Fig. 6B). Further experiments using gene-specific manipulations, such as *Delta* knockdown and dominant-negative or constitutively active forms of Notch pathway molecules (Pasini et al., 2006), will help to confirm this finding. Despite the described limitations, our current data are consistent with the theory that the *Achaete-scute* subfamily proneural bHLH genes and Delta/Notch signaling constitute a conserved genetic module to generate external sensory neurons in both protostome and deuterostome animals (Bertrand et al., 2002; Quan and Hassan, 2005). Recently, a functional study in the cnidarian sea anemone *Nematostella vectensis* demonstrated that one of its *Achaete-scute* homologs, *NvashA*, is also expressed in the neuronal cells within the ectoderm, and that manipulating *NvashA* function by morpholino knockdown or mRNA over-expression experiments affects the expression of neuronal genes (Layden et al., 2012). Together with data showing that Delta/Notch signaling is involved in the neurogenesis of certain neural cell types in *Nematostella* embryos (Marlow et al., 2012), these observations suggest that proneural bHLH genes and Delta/Notch signaling might represent an ancient genetic module for generating sensory neurons that evolved in the common ancestor of cnidarians and bilaterians. It is worth noting that in the sponge *Amphimedon queenslandica*, homologs of the proneural bHLH gene *AmqbHLH1* and the *Delta* ligand gene are both expressed in a putative sensory cell type (globular cells) (Richards et al., 2008), although this cell type is not considered to be a bona fide neuron. Interestingly, overexpression of *AmqbHLH1* in *Xenopus* and *Drosophila* embryos can induce ectopic formation of sensory neurons in these organisms (Richards et al., 2008), suggesting that proneural bHLH genes and Delta/Notch signaling might constitute an ancient cell-determination genetic module that originated in the metazoan stem group. In the eumetazoan lineage (cnidarians and bilaterians), this genetic module might have been co-opted into the genetic program for sensory neuron differentiation. In cnidarians and bilaterians, the expansion of proneural bHLH genes and the recruitment of other context-specific co-factors into this conserved genetic module might have further facilitated the spatial and temporal diversification of distinct lineages of neuronal cells within each organism during ontogenetic development. Therefore, they might have allowed for the evolutionary modifications of neuronal cell types and sensory systems in the different organisms (Layden et al.,

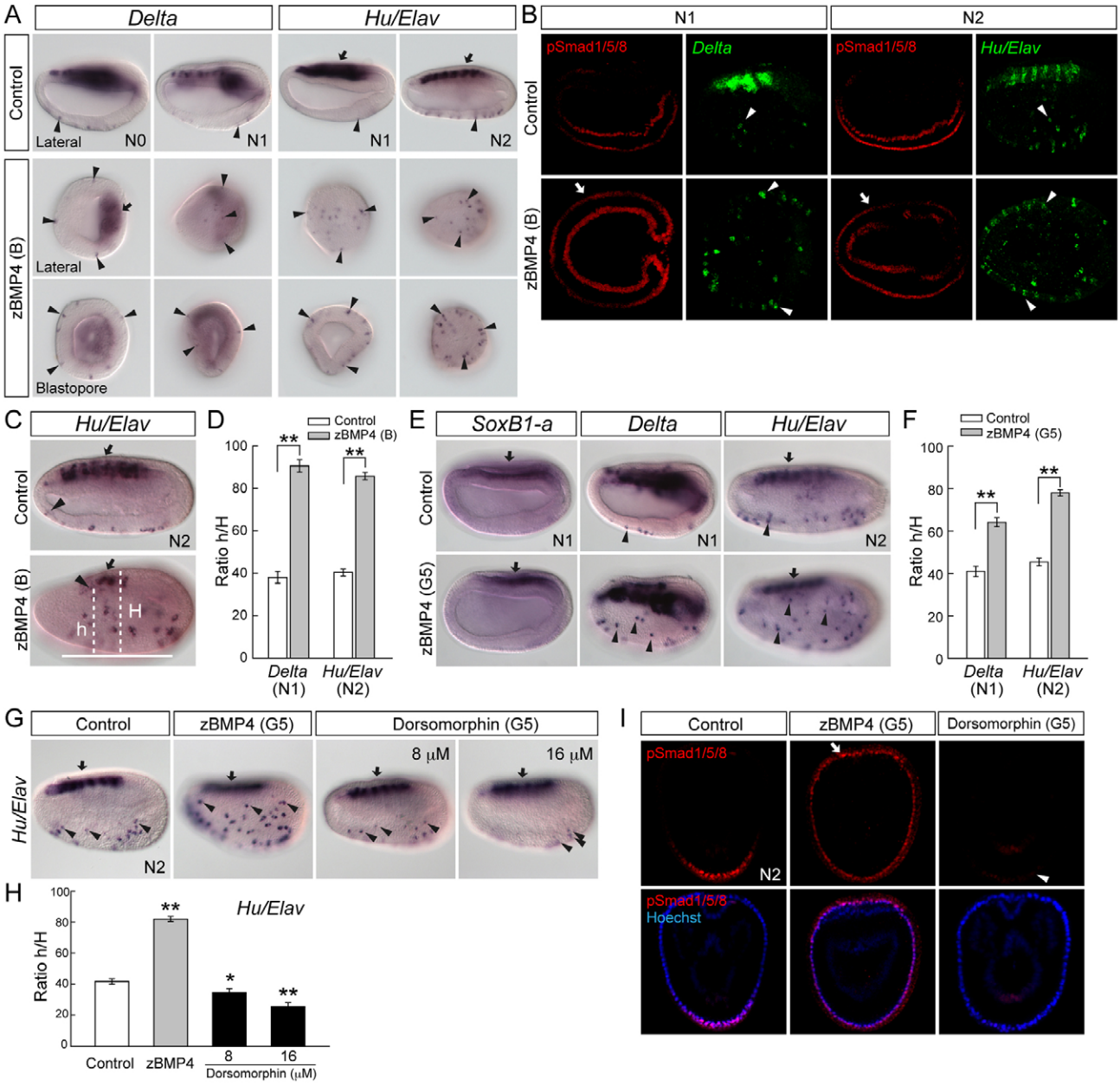

Fig. 4. BMP signaling levels control the distribution of putative ESN progenitor cells in amphioxus embryos. (**A**) The punctate *Delta* and *Hu/elav* expression were expanded throughout the epidermis in embryos treated with zBMP4 at the blastula stage. Arrowheads indicate examples of scattered ESN progenitor cells. The CNS (arrow) is indicated in the control embryos. Blastopore views of zBMP4-treated embryos are also shown (bottom panels). (**B**) Double staining of pSmad1/5/8 (red) with *Delta* or *Hu/elav* transcripts (green) in the control (upper panels) and zBMP4-treated (lower panels) embryos. Images show a lateral view of the embryo with the anterior towards the left and the dorsal at the top. Arrows indicate the dorsal expansion of pSmad1/5/8 signals. Arrowheads indicate the punctate expression of *Delta* or *Hu/elav*. (**C**) Distribution of ESNs in an embryo treated with zBMP4 at the blastula stage that displays a milder phenotype. Arrows indicate the CNS in both the control embryo and the zBMP4-treated embryo. Arrowheads indicate the dorsal limits of the ESN distribution (h). The body height (H) of the embryo is also depicted. (**D**) Quantification of the effect of mild zBMP4 treatment on the distributions of *Delta*$^+$ and *Hu/elav*$^+$ ESN progenitor cells. $^{**}P<0.001$; *t*-test, $n=10$ embryos. Error bars indicate s.e.m. (**E**) Embryos treated with zBMP4 at the G5 stage had a mostly intact CNS (indicated by arrows in embryos stained with *SoxB1-a* or *Hu/Elav*); however, their ESNs, as shown by the punctate expression of *Delta* and *Hu/elav* (arrowheads) at N1 and N2 stage, respectively, were distributed more dorsally compared with the control embryos. (**F**) Quantification of the effect of late zBMP4 treatment (G5 stage) on the distributions of *Delta*$^+$ and *Hu/elav*$^+$ cells. $^{**}P<0.001$; *t*-test, $n=10$ embryos. Error bars indicate s.e.m. (**G**) In contrast to the zBMP4 treatment, dorsomorphin treatment at the G5 stage reduced punctate *Hu/elav* expression in the ventral ectoderm (arrowheads). The CNS in each image is indicated by arrows. (**H**) Quantification of the effect of manipulating BMP signaling (G5 stage) on the distribution of *Hu/elav*$^+$ cells. $^*P<0.05$; $^{**}P<0.001$; *t*-test, $n=10$ embryos. Error bars indicate s.e.m. (**I**) Frontal views of pSmad1/5/8 staining (red) in control, zBMP4-treated and dorsomorphin-treated embryos with dorsal to the top and ventral to the bottom of the images. Nuclei are labeled with DAPI (blue). Arrow indicates the dorsal expansion of pSmad1/5/8 signals in the zBMP4-treated embryo; arrowhead indicates the contraction of pSmad1/5/8 signals in the dorsomorphin-treated embryo.

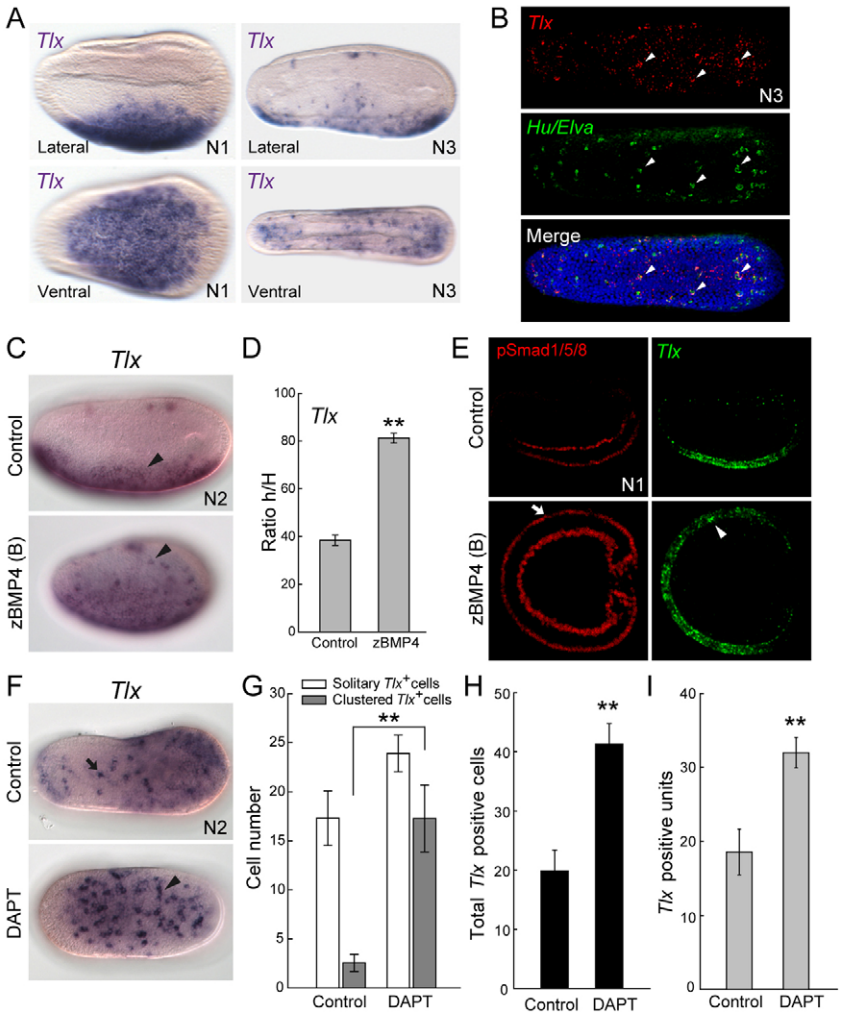

Fig. 5. *AmphiTlx* expression marks a ventral epidermal domain corresponding to the putative ESN progenitor field, and its expression is correlated with high BMP signaling levels. (**A**) *Tlx* expression in the normal neurula stage (N1 and N3 are shown here) embryos. Upper panels are lateral views, dorsal to the top and anterior to the left; lower panels are ventral views. (**B**) *Tlx* (red) is co-expressed with *Hu/elav* (green) in the ventral epidermis of the N3 stage embryos; examples of cells co-expressing *Tlx* and *Hu/elav* are indicated by arrowheads. (**C**) Lateral view of *Tlx* expression in the control and zBMP4-treated embryos. Arrowheads indicate the dorsal limits of *Tlx* expression in the epidermis. (**D**) Quantification of the effect of zBMP4 treatment on the ectodermal expression domain of *Tlx*. $^{**}P<0.001$; *t*-test, $n=10$ embryos. Error bars indicate s.e.m. (**E**) Double staining of pSmad1/5/8 protein and *Tlx* transcripts in the control and zBMP4-treated embryos (lateral views). Arrow and arrowhead indicate the expansion of dorsal pSmad1/5/8 and *Tlx* signals, respectively. (**F**) *Tlx* expression in the control and DAPT-treated embryos (late N2 stage) viewed from the ventral side. The arrow shows example of solitary *Tlx*-expressing cell in the control embryo, and the arrowhead indicates an example of clustered *Tlx*-expressing cells in the DAPT-treated embryo. (**G-I**) Quantification of DAPT treatment on the distribution and number of ventral *Tlx*-expressing cells. ** $P<0.001$, *t*-test, $n=10$ embryos. Error bars indicate s.e.m.

2012; Quan and Hassan, 2005). Thus, comparing how this conserved genetic module is activated and utilized for neurogenesis in different organisms should provide important information for understanding the evolution of the nervous system.

A conserved function for BMP signaling in promoting sensory neuron formation across bilaterians and its implications for deuterostome PNS evolution

Inhibition of BMP signaling by BMP antagonists in the dorsal ectoderm is required for amphioxus CNS formation (Onai et al., 2010; Yu et al., 2008a; Yu et al., 2007). Our work demonstrates that high BMP signaling positively regulates the developmental program of the peripheral ESNs in the ventral ectoderm and that manipulating BMP signaling levels affects the distribution of ESNs during amphioxus embryogenesis. Our data are consistent with findings from *Drosophila* and *Platynereis* which conclude that the motor neurons and interneurons in the CNS develop from the non-BMP side of the body, whereas the formation of the peripheral sensory neurons are positively regulated by BMP signaling (Denes et al., 2007; Rusten et al., 2002). This finding suggests that the function of biphasic BMP signaling in CNS and PNS development exists in the bilaterian common ancestor.

Within the chordate lineage, the patterning process of amphioxus ESNs is highly similar to the formation of the ascidian larval epidermal sensory neurons derived from the ventral epidermal midline (Pasini et al., 2006), suggesting that they might be homologous. However, whether these amphioxus ESNs are homologous to the vertebrate sensory neurons derived from the neural crest and neurogenic placodes remains a contentious issue. In vertebrates, neural crest and placode development is intimately associated with neural induction, and the level of BMP signaling plays a crucial role in establishing the medial-lateral boundaries along the dorsal ectoderm (Meulemans and Bronner-Fraser, 2004; Schlosser, 2008). It has been determined that BMP signaling is active at the neural plate border in the chick embryo (Faure et al., 2002). Moreover, BMP signaling is involved in sensory neurogenesis in epibranchial placodes (Holzschuh et al., 2005; Kriebitz et al., 2009). The amphioxus sensory cells show some similarity to the vertebrate placode-derived neurons in the following ways: (1) they both originate from the epidermis and are induced by relatively high BMP signaling levels; (2) they express the proneural bHLH homolog of *Achaete-scute* and differentiate into neuronal cells by Delta/Notch-mediated lateral inhibition; and (3) they can delaminate and migrate (Benito-Gutierrez et al., 2005b; Kaltenbach et al., 2009). However, there are differences between the amphioxus ESNs and the vertebrate placode-derived neurons. In vertebrates, the pre-placodal field is closely adjacent to the dorsal neural plate (Schlosser, 2008), whereas the presumptive ESN domain in amphioxus is not. Thus, it can be hypothesized that a conserved developmental module for PNS neurogenesis is activated at different locations of the body

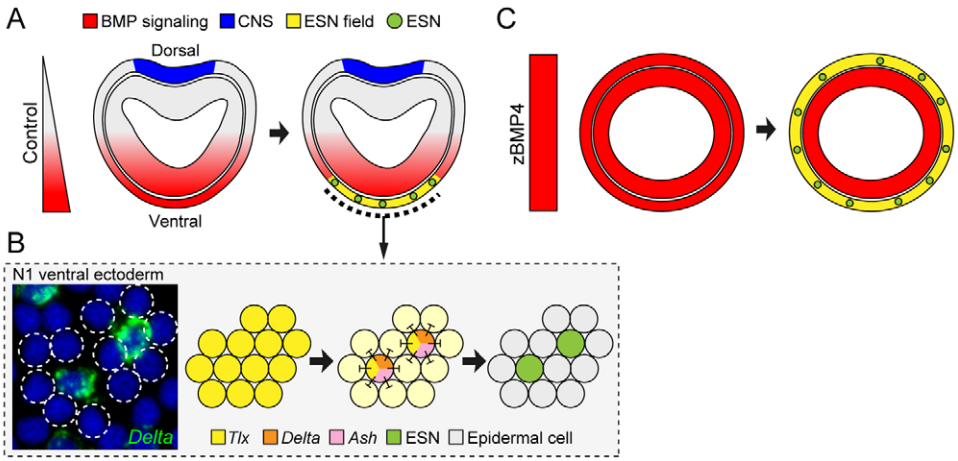

Fig. 6. Schematic models for the development of amphioxus ESNs. (**A**) During embryogenesis, inhibition of BMP signaling by BMP antagonists induces the formation of the CNS in the dorsal ectoderm (blue), whereas high levels of BMP signaling induce a putative ESN progenitor field (yellow) in the ventral ectoderm, which is approximately equivalent to the *Tlx* expression domain. (**B**) Within this ESN progenitor field (yellow, expressing *Tlx*), *Ash* and *Delta* are expressed in specific cells to promote the differentiation of individual ESN cells (green) through Delta/Notch-mediated lateral inhibition. Image shows individual ESN progenitor cells expressing *Delta* in the ventral ectoderm; dashed lines encircle their neighboring epidermal cells. (**C**) Exogenous zBMP4 treatment elevates BMP signaling levels globally and converts the entire ectoderm into an ESN progenitor field.

in the different chordate lineages. It is tempting to speculate that BMP signaling might function as a labile plug-in (Davidson and Erwin, 2006) and can be deployed in different spatial contexts to activate conserved neurogenesis modules for generating epidermal sensory neurons. It is possible that during early vertebrate evolution, certain embryonic ectodermal domains for generating the peripheral sensory system might have shifted more dorsally to become the pre-placodal field in vertebrates. Taken together, our results support the hypotheses that the amphioxus ESNs are possible homologs of the vertebrate placode-derived neurons and that the ventral ectoderm of the amphioxus embryo is a neurogenic region that might be homologous to the pre-placodal field of vertebrates (Holland, 2009). Further study of the regulation of BMP signaling in the ectoderm to initiate sensory neuron development in the different chordate lineages is important for determining the evolution of the chordate sensory systems.

There is evidence to suggest that the amphioxus *Tlx* gene is probably downstream of BMP signaling and involved in ESN development. We have observed that the initial *Tlx* expression in the ventral ectoderm roughly corresponds to the area from which the dotted *Hu/elav*-positive cells originate during the early neurula stage (Fig. 5); subsequently, *Tlx* expression is gradually restricted to colocalize with *Hu/elav*-positive cells. In addition, elevated BMP signaling level (visualized by pSmad1/5/8 staining) leads to an expansion of ectodermal *Tlx* expression in accordance with the ectopic ESN formation in the ectoderm (Fig. 5E). It has been shown that vertebrate *Tlx* homologs (*Tlx1* and *Tlx-3*) are expressed in certain developing neurons of the CNS as well as in the cranial sensory neurons and that *Tlx-3* can control the expression of some proneural bHLH genes to promote neuronal differentiation (Kondo et al., 2008; Langenau et al., 2002). We are currently carrying out gene-specific knockout experiments to examine the necessity of amphioxus *Tlx* function for *Ash* and *Delta* activation and the differentiation of ESNs. Additionally, it will be interesting to investigate the epistatic relationships of *Tlx* with other transcription factors that are also expressed in the developing ESNs, such as *Coe* and *SoxB1c* (Mazet et al., 2004; Meulemans and Bronner-Fraser, 2007). We anticipate that comprehensive comparisons such as these will facilitate our understanding of the modification of the PNS during chordate evolution and might reveal the developmental evolution that led to the emergence of complex peripheral sensory organs in vertebrates.

Finally, our data demonstrate that manipulating BMP signaling in different time windows can cause different phenotypes in the amphioxus embryos. Elevating BMP signaling level at the blastula stage abolishes the formation of the CNS and converts the entire ectoderm into the ESN progenitor domain (Fig. 4A). By contrast, elevating the BMP signaling level at the late gastrula stage (when neural induction has presumably completed) has little effect on the formation of CNS, but it still expands the distribution of peripheral ESNs to almost the entire ectoderm (Fig. 4E). These phenotypes are similar to the hemichordate ectodermal nerve net and raise the intriguing question of whether this ectodermal nerve net is homologous to the CNS or PNS. Hemichordates have a mostly diffused nervous system with dense aggregates of neurons associated with their dorsal and ventral cord (Lowe et al., 2006; Lowe et al., 2003; Nomaksteinsky et al., 2009). Recent anatomical and molecular analyses have shown that the dorsal cord of hemichordates is internalized in the collar region, which is reminiscent of the neural tube in chordates, suggesting that it might represent the CNS in hemichordates (Kaul and Stach, 2010; Nomaksteinsky et al., 2009). These new findings also suggest that the neurons of the hemichordate ectodermal nerve net might actually represent the PNS. It has been shown that in the direct developing hemichordate *Saccoglossus kowalevskii* embryos, the pan-neural marker *Hu/elav* is expressed as spots in the entire ectoderm but is stronger in the dorsal and ventral midlines. After exogenous zBMP4 protein treatment, *Hu/elav* is expressed at a uniformly strong level throughout the ectoderm (Lowe et al., 2006). This observation suggests that the formation of certain neurons in the *S. kowalevskii* ectoderm is also positively regulated by BMP signaling. We speculate that the *Hu/elav*-expressing cells in the dorsal ectodermal midline of *S. kowalevskii* are probably the ones induced by the positive BMP signaling, because the

Bmp2/4 ligand is expressed in this position, and that they might be equivalent to the ESNs in amphioxus. Further molecular genetic studies on the formation of neuronal cells in hemichordates will help to resolve this issue and shed light on the evolution of the nervous system.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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