

Nervous systems of the sea anemone *Nematostella vectensis* are generated by ectoderm and endoderm and shaped by distinct mechanisms

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SUMMARY

As a sister group to Bilateria, Cnidaria is important for understanding early nervous system evolution. Here we examine neural development in the anthozoan cnidarian *Nematostella vectensis* in order to better understand whether similar developmental mechanisms are utilized to establish the strikingly different overall organization of bilaterian and cnidarian nervous systems. We generated a neuron-specific transgenic *NvElav1* reporter line of *N. vectensis* and used it in combination with immunohistochemistry against neuropeptides, in situ hybridization and confocal microscopy to analyze nervous system formation in this cnidarian model organism in detail. We show that the development of neurons commences in the ectoderm during gastrulation and involves interkinetic nuclear migration. Transplantation experiments reveal that sensory and ganglion cells are autonomously generated by the ectoderm. In contrast to bilaterians, neurons are also generated throughout the endoderm during planula stages. Morpholino-mediated gene knockdown shows that the development of a subset of ectodermal neurons requires *NvElav1*, the ortholog to bilaterian neural *elav1* genes. The orientation of ectodermal neurites changes during planula development from longitudinal (in early-born neurons) to transverse (in late-born neurons), whereas endodermal neurites can grow in both orientations at any stage. Our findings imply that *elav1*-dependent ectodermal neurogenesis evolved prior to the divergence of Cnidaria and Bilateria. Moreover, they suggest that, in contrast to bilaterians, almost the entire ectoderm and endoderm of the body column of *Nematostella* planulae have neurogenic potential and that the establishment of connectivity in its seemingly simple nervous system involves multiple neurite guidance systems.

KEY WORDS: *Nematostella*, Cnidaria, Elav, Evolution, Neural development, Neurogenesis

INTRODUCTION

Cnidarians (sea anemones, corals and jellyfishes) hold a key phylogenetic position for understanding early nervous system evolution: Cnidaria is the likely sister group to the Bilateria and one of only two extant non-bilaterian clades that possess a nervous system (Fig. 1A) (Hejnal et al., 2009; Medina et al., 2001; Putnam et al., 2007; Wallberg et al., 2004; Philippe et al., 2009; Schierwater et al., 2009). Thus, comparing neural development in Cnidaria and Bilateria can help to reconstruct early events in nervous system evolution.

Cnidaria is a diverse clade in which Anthozoa form a sister group to the Medusozoa, consisting of Staurozoa, Scyphozoa, Cubozoa and Hydrozoa (Fig. 1A) (Collins et al., 2006). Cnidarians have two germ layers, the ectoderm and endoderm, separated by extracellular matrix, the mesoglea. Anthozoans, staurozoans and some hydrozoans develop through a swimming planula into a sexually mature polyp (Fig. 1B-D), whereas in many medusozoans sexual medusae develop from asexual polyps.

The nervous system of adult cnidarian polyps consists of epithelial sensory cells, basally located ganglion cells, and neurites from both cell types that constitute basiepithelial networks

alongside the mesoglea (Fig. 1E) (Fautin and Mariscal, 1991; Lesh-Laurie and Suchy, 1991; Thomas and Edwards, 1991). Both sensory and ganglion cells can synapse to epitheliomuscular cells and other neurons. In contrast to most bilaterians, cnidarians often possess both endodermal and ectodermal nervous systems. The overall architecture of the adult nervous system in the hydrozoan polyp *Hydra* resembles a nerve net with regional differences in neuron densities (reviewed by Koizumi et al., 2004), whereas anthozoan polyps often display accumulations of neurons and neurites associated with longitudinal endodermal infoldings termed mesenteries (e.g. Batham et al., 1960). Despite local differences in neuron density and accumulations of neurites, cnidarian polyps lack morphological centralization comparable to the brains of bilaterians.

Whether the strikingly different architecture of bilaterian and cnidarian nervous systems is established by correspondingly different developmental mechanisms, or whether similar mechanisms are used in different ways, is not well understood. In bilaterians, nervous systems are almost invariably generated by the ectoderm [e.g. ecdysozoans (Hartenstein and Campos Ortega, 1984; Scholtz, 1992; Stollewerk et al., 2001), lophotrochozoans (Jacob, 1984; Kadner and Stollewerk, 2004; Meyer and Seaver, 2009) and deuterostomes (Miyamoto et al., 2010; Nakajima et al., 2004; Rao and Jacobson, 2005)]. Initially, the ectoderm becomes divided into epidermal ectoderm and neuroectoderm. Whereas sensory neurons can be generated from both areas (except in vertebrates), interneurons (morphologically equivalent to cnidarian ganglion cells) are generated by the neuroectoderm. In the pseudostratified neuroectodermal epithelium, neural precursor cells (NPCs) develop in the apical layer. NPCs and/or their progeny then

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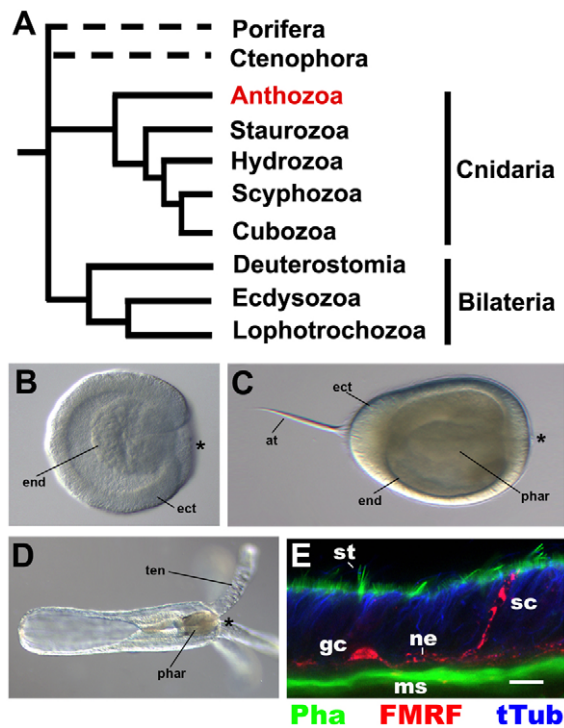


Fig. 1. Phylogenetic position of anthozoan cnidarians and the development and neuron types of *Nematostella vectensis*.

(A) Metazoan phylogeny depicting the sister group relationship between Bilateria and Cnidaria (see Hejnol et al., 2009; Medina et al., 2001; Wallberg et al., 2004; Philippe et al., 2009; Schierwater et al., 2009) and ingroup relationships in Cnidaria (see Collins et al., 2006).

(B-D) Development of *N. vectensis*: gastrula (B), free swimming planula (C) and primary polyp (D). Asterisk, oral pole. Reproduced with permission (Rentsch et al., 2008). (E) Confocal longitudinal sections of a tentacle in adult *N. vectensis*, labeled with phalloidin (green) and antibodies against FMRFamide (red) and tyrosinated tubulin (blue), showing a sensory cell (sc), a ganglion cell (gc) and their basiepithelial neurites (ne) in the ectoderm. at, apical tuft; ect, ectoderm; end, endoderm; gc, ganglion cell; ms, endodermal muscle fibers; ne, neurite; phar, pharynx; sc, sensory cell; st, stereocilia; ten, tentacle. Scale bar: 10 μ m.

move basally/internally, via cell migration and/or cell division oriented parallel to the epithelial surface, to generate mature neurons in the basal epithelium (Harris and Hartenstein, 2008).

Whether a similar mode of ectodermal neurogenesis exists in cnidarians is unclear. Hydrozoans contain a particular type of stem cell called interstitial cells (i-cells) that are thought to derive from the endoderm, based on ultrastructural, developmental genetic and embryological experimental evidence (Genikhovich et al., 2006; Martin, 1988; Martin and Archer, 1986; Summers and Haynes, 1969; Thomas et al., 1987). Interstitial cells can give rise to neurons, cnidocytes (stinging cells), gland cells and, in some species, also epithelial cells (Bosch et al., 2010; Bosch and David, 1987; David and Murphy, 1977; Muller et al., 2004; Watanabe et al., 2009). Neurogenesis occurs continuously in adult *Hydra*, and all neurons are derived from interstitial cells (Campbell, 1976), whereas during embryonic development of *Pennaria disticha* and *Clytia gregaria* sensory cells, but not ganglion cells, can be generated in the absence of interstitial cells (Martin and Thomas, 1981; Thomas et al., 1987). In the scyphozoan *Aurelia*, the earliest detectable (FMRFamide-immunoreactive) sensory cells occur in the ectoderm, suggesting an

ectodermal origin (Nakanishi et al., 2008). By contrast, RFamide-immunoreactive NPCs were reported to first occur in the endoderm of late stage gastrulae in the anthozoan *Nematostella vectensis* and later migrate into the ectoderm to differentiate into mature neurons, indicating an endodermal origin (Galliot et al., 2009). However, based on the analysis of gene expression patterns Marlow et al. (Marlow et al., 2009) suggested that early neurogenesis might occur in the ectoderm in *N. vectensis*. Thus, whether ectodermal or endodermal neurogenesis (or both) is the ancestral condition within cnidarians, and whether ectodermal neurogenesis is ancestral for Cnidaria and Bilateria, remain unclear.

After the specification of neurons, the outgrowth of neurites and the formation of synaptic contacts characterize neural differentiation. In bilaterians, growing axons and dendrites are guided to final or intermediate target areas by a combination of target-derived long-range guidance signals and permissive or inhibitory locally acting cues (Burden et al., 2008; Kolodkin and Tessier-Lavigne, 2008). Whether similar guidance mechanisms act during the development of the morphologically less complex cnidarian nervous systems is not known.

Here we investigated nervous system development in the anthozoan *N. vectensis* with the aim of identifying similarities and differences in neural development between this cnidarian model and bilaterians. *Nematostella* has recently emerged as an important model system for cnidarian development that is amenable to the analysis of gene function (Pankow and Bamberger, 2007; Putnam et al., 2007; Rentsch et al., 2008; Technau and Steele, 2011; Wikramanayake et al., 2003). *N. vectensis* gastrulates by invagination before developing into a free-swimming planula with an apical tuft at its aboral pole (Kraus and Technau, 2006; Magie et al., 2007). The planula transforms into a polyp with a tube-shaped body and only one opening, traditionally called the mouth (Hand and Uhlinger, 1992), surrounded by tentacles for prey capture and feeding (Fig. 1B-D). A previous report indicated that it has ectodermal and endodermal nervous systems with presumptive neural structures such as the apical organ in the planula and the oral and pharyngeal 'nerve rings' in the polyp (Marlow et al., 2009) (but see Discussion).

We generated a neuron-specific transgenic reporter line of *N. vectensis* in which the expression of the fluorescent protein mOrange (Renfer et al., 2009; Shaner et al., 2004) is driven by regulatory elements of the *NvElav1* gene, an ortholog of bilaterian *elav1* genes (supplementary material Figs S7, S8) (Marlow et al., 2009). *Elav1* genes encode highly conserved RNA-binding proteins that have been implicated in neural differentiation across Bilateria (Akamatsu et al., 2005; Akamatsu et al., 1999; Campos et al., 1985; Denes et al., 2007; Jimenez and Campos-Ortega, 1987; Meyer and Seaver, 2009; Nomaksteinsky et al., 2009; Satoh et al., 2001). We used this transgenic line together with in situ hybridization, immunohistochemistry, confocal microscopy and embryological experiments to study the formation of the *Nematostella* nervous system in detail. We show that early neurogenesis begins in the ectoderm during gastrulation, followed by endodermal neurogenesis, a change in the orientation of neurite extension of ectodermal neurons during planula development, and tentacular ectodermal neurogenesis during polyp development. Furthermore, we use morpholino-mediated gene knockdown to demonstrate that the development of a subset of ectodermal neurons requires *NvElav1*. Our findings combined with existing comparative data imply that neurogenesis from the ectodermal epithelium evolved prior to the divergence of Cnidaria and Bilateria and support an ancient role for *elav1* genes in neural development. Furthermore,

the *Nematostella* planula appears to have almost uniform neurogenic potential, and its seemingly simple nervous system architecture is established by changing neurite guidance systems.

MATERIALS AND METHODS

Animal culture

Nematostella vectensis were cultured as described previously (Fritzenwanker and Technau, 2002; Hand and Uhlinger, 1992).

RNA extraction, cDNA synthesis and gene cloning

Cloning was performed by standard procedures. For RACE reactions, the SMART RACE cDNA Amplification Kit (BD Biosciences) was used.

Generation of the *NvElav1::mOrange* transgenic line

The *NvElav1::mOrange* transgenic line was generated as described (Renfer et al., 2009). A 2.43 kb region of genomic sequence upstream of the start codon of the *NvElav1* gene (genomic coordinates: scaffold 210, 220684-223104; <http://genome.jgi-psf.org/Nemve1/Nemve1.home.html>) was cloned in front of the open reading frame of mOrange (Shaner et al., 2004) carrying a membrane-tethering CAAX domain at the C-terminus. The *I-SceI*-digested plasmid was injected into fertilized eggs at 20 ng/μl, animals were raised and crossed to wild-type polyps to identify carriers, and two independent lines were established. All animals used in this study are derived from incrosses of F1 heterozygotes. Where indicated in the figure legend, mOrange was visualized with anti-DsRed antibody (Clontech 632496).

Fixation

Late stage larvae (mid-planula II through primary polyp) were anesthetized in 2.43% MgCl₂. Embryos and larvae were fixed as described (Rentzsch et al., 2008), except that PBS was supplemented with 0.5% Triton X-100 (PBSTr).

Immunofluorescence and confocal microscopy

Fixed specimens were rehydrated and washed in PBSTr and blocked with 3% normal goat serum (NGS) in PBSTr. Primary antibodies were incubated with the specimens in PBSTr. We used antibodies against FMRFamide (1:500, Millipore AB15348), GLWamide [1:200 (Schmich et al., 1998)], DsRed (1:100, Clontech 632496), acetylated tubulin (mouse, 1:500, Sigma T6793) and tyrosinated tubulin (mouse, 1:500, Sigma T9028). Following washes in PBSTr and blocking in 3% NGS in PBSTr, specimens were incubated with secondary antibodies (Molecular Probes) conjugated with Alexa Fluor 568 (rabbit, 1:200), Alexa Fluor 488 (mouse, 1:200) and Alexa Fluor 647 (rabbit, 1:200). Nuclei were labeled using DAPI (1:1000, Molecular Probes) or TO-PRO-3 iodide (1:1000, Molecular Probes) and filamentous actin was labeled using Alexa Fluor 488-conjugated phalloidin (1:25, Molecular Probes). Specimens were incubated with fluorescent dyes together with secondary antibodies, washed in PBSTr and mounted in ProLong Gold antifade reagent (Molecular Probes). Images were recorded using a Leica SP5 confocal microscope; confocal stacks were viewed using ImageJ.

Fluorescent in situ hybridization coupled with immunofluorescence

The following protocol was developed by modifying published in situ hybridization protocols (Finnerty et al., 2004; Gates et al., 2002; Nakanishi et al., 2010; Okamoto et al., 2005). Endogenous hydrogen peroxidase activity was quenched by incubating the fixed specimens in 3% hydrogen peroxide in methanol. They were then washed in ethanol and incubated in ethanol:xylene (1:1 v/v). Specimens were then washed in ethanol and methanol, followed by gradual rehydration in water, and were permeabilized in acetone (80%) at -20°C. They were washed in PBSTr, then in 1% triethanolamine in PBS, followed by addition of 0.6% acetic anhydride. Specimens were washed in PBSTr and refixed in 4% formaldehyde, followed by washes in PBSTr. The specimens were then incubated in the hybridization solution (HB: 50% formamide, 5× SSC, 1% SDS, 50 μg/ml heparin, 100 μg/ml salmon sperm DNA, 9.25 mM citric acid, 0.5% Triton-X100) at 60°C. The specimens were incubated with a digoxigenin-labeled riboprobe (MEGAscript Kit, Ambion) at a final probe

concentration of 1 ng/μl for at least 60 hours at 60°C. The specimens underwent a series of washes in HB/2× SSC solutions [75/25, 50/50, 25/75 (v/v)], then in 0.05× SSC, and finally in 0.05× SSC/TNT buffer [1:1 (v/v); TNT: 0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.5% Triton X-100]. After blocking [TNT containing 0.5% blocking reagent (PerkinElmer FP1020)], specimens were incubated with anti-digoxygenin horseradish peroxidase (Boehringer Mannheim) together with other primary antibodies. After washing in TNT buffer, specimens were incubated in fluorophore tyramide amplification reagent (TSA Plus Kit, PerkinElmer). They were then washed in PBSTr and blocked in 3% NGS in PBSTr. Following secondary antibody incubation, specimens were mounted in ProLong Gold antifade reagent and examined using the Leica SP5 confocal microscope.

Transmission electron microscopy (TEM)

A 5-day-old late planula larva was processed and serially sectioned for TEM as described previously (Kraus and Technau, 2006). TEM was carried out at the Molecular Imaging Centre at the University of Bergen (FUGE, Norwegian Research Council).

Morpholinos

Microinjection procedures were conducted as previously described (Rentzsch et al., 2008). Morpholinos were injected at 333 or 666 nM. MO sequences (5' to 3') are:

NvElav MO1, AATTGTCGTCATCATGTCCAACAT (targeting the start codon, underlined);

NvElav1 MO2, ATGAACTGAAATAAGCCGTGAGCGT (targeting the 5'UTR);

NvElav1 MO1mm, AATTCTCGTGCATGATGTGCAAGAT (control MO1); and

Generic control MO, CATGGAGAAATCGGACTTCATATTT (control MO2).

The target sequence of *NvElav1* MO2 is included in the *NvElav1::mOrange* construct, therefore *NvElav1* MO2 could not be used for analysis in the transgenic line. *NvElav1* is a single-exon gene so splice site morpholinos cannot be used.

RESULTS

Generation and characterization of a neuron-specific transgenic *Nematostella vectensis* line

In a search for genes with broad neural expression, we turned to the RNA-binding protein *Elav*, which has been shown to be expressed in a large fraction of neurons in many bilaterians and in scattered ectodermal and endodermal cells during *Nematostella* development (Marlow et al., 2009; Pascale et al., 2008). Moreover, regulatory elements of *elav* genes have been utilized to generate neuron-specific transgenic lines of *Drosophila melanogaster* and the zebrafish *Danio rerio* (Park et al., 2000; Yao and White, 1994). We first used fluorescent in situ hybridization to better characterize *NvElav1*-expressing cells in *Nematostella*. At the early to mid-gastrula stage, which is the earliest stage at which mRNA expression could be detected (Fig. 2A-D) (Marlow et al., 2009), *NvElav1*-expressing cells are columnar/spindle-shaped epithelial cells with middle- to apically positioned nuclei and often with apical cilia (Fig. 2D), exhibiting sensory-cell-like morphology. *NvElav1* expression was not detected in dividing cells (Fig. 2A-C), and none of the *NvElav1*-expressing cells examined incorporated the thymidine nucleoside analog EdU (supplementary material Fig. S1). These observations suggest that early *NvElav1*-expressing cells are largely postmitotic differentiating sensory cells. At later stages of development, *NvElav1* is expressed in a subset of differentiated neurons in the ectoderm and endoderm.

To allow visualization of the nervous system, including neurites, we generated a stable transgenic line in which a 2.4 kb fragment of genomic DNA immediately upstream of the *NvElav1* start codon drives the expression of the fluorescent protein mOrange (Renfer

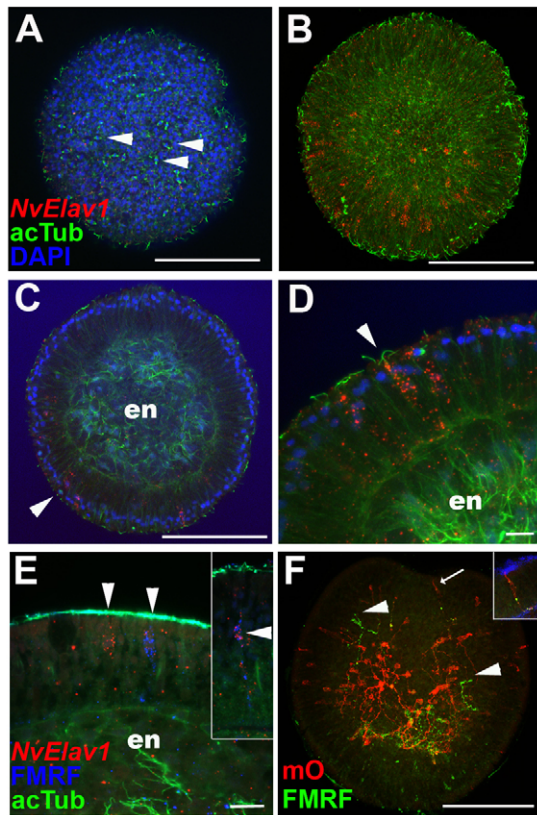


Fig. 2. *NvElav1* is expressed in a subset of early postmitotic neurons. (A–C) Confocal sections of a mid-gastrula *N. vectensis* larva labeled with antisense riboprobe *NvElav1*, antibody against acetylated tubulin (acTub) and the DNA stain DAPI. Oral side is up, aboral side down. Sections are superficial in A, through the ectoderm in B, and medial in C. Note the absence of *NvElav1* expression in mitotic cells in A and C (arrowheads, identified by presence of mitotic spindle and metaphase chromosomes). (D) High-magnification confocal sections of the ectoderm of the same specimen as in A–C, showing a ciliated *NvElav1*-expressing cell with a spindle-shaped sensory-cell-like morphology (arrowhead). (E) Confocal sections of the ectoderm at the mid-planula I stage, labeled with antisense riboprobe against *NvElav1* and antibodies against acetylated tubulin and FMRFamide. Arrowheads indicate individual sensory cells. Note the presence of a FMRFamide-positive *NvElav1*-negative sensory cell (right-hand arrowhead). The inset shows an FMRFamide-immunoreactive neuron that expresses *NvElav1*. (F) Confocal section of the *NvElav1::mOrange* (mO) line at the mid-planula I stage labeled with an antibody against FMRFamide. Arrowheads show FMRFamide-positive neurons that are negative for mOrange expression. Arrow and inset show a sensory cell close to the blastopore. en, endoderm. Scale bars: 100 μ m in A–C,F; 10 μ m in D,E.

et al., 2009; Shaner et al., 2004). Analysis of this line showed that *NvElav1::mOrange* expression is neuron specific, but the transgene is detectable in only 50–70% of all *NvElav1* transcript-positive cells. Co-labeling with antibodies against the FMRFamide and GLWamide neuropeptides revealed that neither *NvElav1* transcripts (Fig. 2E) nor the transgenic line (Fig. 2F) labels all differentiated neurons. Thus, although low-level expression in all neurons cannot be ruled out, *NvElav1* does not appear to be a pan-neural marker in *Nematostella*. We therefore used the *NvElav1::mOrange* transgenic line in combination with in situ hybridization and immunohistochemistry to characterize *Nematostella* nervous system development in detail.

Sensory cells and ganglion cells develop from the ectodermal epithelium during gastrulation and tentacle morphogenesis

The first sign of neurogenesis is already detectable at the early to mid-gastrula stage (see supplementary material Fig. S2 for staging), when transcripts for the neuropeptide precursor *NvRF* (Anctil, 2009; Marlow et al., 2009) are visible in a subset of apically positioned dividing cells (Fig. 3A,B) [here referred to as neural precursor cells (NPCs)] as well as in cells with centrally positioned nuclei within the ectodermal epithelium, presumably differentiating neurons (Fig. 3B). This suggests that at late gastrula stage NPCs differentiate into sensory cells with apical cilia and basal neuronal processes, as well as into ganglion cells (insets in Fig. 3C), albeit fewer in number. At this stage, sensory cells are distributed in the ectoderm except for in the immediate vicinity of the oral and aboral poles (Fig. 3D; supplementary material Fig. S3). However, they become visible in the oral and pharyngeal ectoderm by the mid-planula I stage (Fig. 2F, Fig. 3F). Neurites of these early-born ectodermal neurons lengthen and project in the aboral direction, towards the base of the apical organ (Fig. 3E; see supplementary material Fig. S4 for details of apical organ development). Electron microscopy shows that these neurites contain opaque, dense cored vesicles with evidence of interneuronal synapses (Fig. 3G), but we failed to detect synapses between apical organ cells and underlying neurites (ten separate TEM sections analyzed).

A similar pattern of neurogenesis occurs in developing tentacles during polyp formation (Fig. 3H–J). At the tentacle bud stage, nuclei are separated into superficial and deeper layers in the ectodermal epithelium (Fig. 3I,J); compare with the late planula (supplementary material Fig. S2F). As in NPCs observed during gastrulation, a majority of *NvRF*-expressing cells in tentacle buds contain apically positioned nuclei (Fig. 3I,J); however, mitotic spindles were not observed in these cells. In addition, tentacle bud ectoderm contains *NvRF*-expressing sensory cells and ganglion cells with middle- or basally located nuclei (Fig. 3J), some of which are FMRFamide immunoreactive and extend basal neuronal processes (see Fig. 1E). Thus, putative NPCs occur apically, and their cell bodies appear to shift basally as they differentiate into mature neurons during tentacular neurogenesis, similar to the pattern of ectodermal neurogenesis during gastrulation. We conclude that neurogenesis during gastrulation and tentacle development occurs in the ectoderm.

Sensory cells and ganglion cells are generated autonomously in the ectodermal epithelium

Our observation of early ectodermal neurogenesis during gastrulation and tentacular development indicates that the ectodermal epithelium generates both sensory cells and ganglion cells in *N. vectensis*, in contrast to the previous claim that NPCs are derived from the endoderm (Galliot et al., 2009). In order to distinguish these two alternatives, we first generated endoderm-free larvae via transverse bisection at the early gastrula stage. Oral halves develop into small but complete primary polyps, whereas aboral halves develop into hollow spheres consisting of an ectodermal epithelium without endoderm (Fig. 4A,B) (Fritzenwanker et al., 2007; Lee et al., 2007). Four-day-old endoderm-free larvae developed morphologically normal sensory cells and ganglion cells in the ectoderm (Fig. 4C), showing that the ectodermal epithelium can autonomously (i.e. in the absence of endoderm) generate both types of neurons. Next, we analyzed whether, in the presence of endoderm, sensory and ganglion cells are indeed generated by the ectoderm. At early gastrula stage, we transplanted the invaginating endoderm and some surrounding

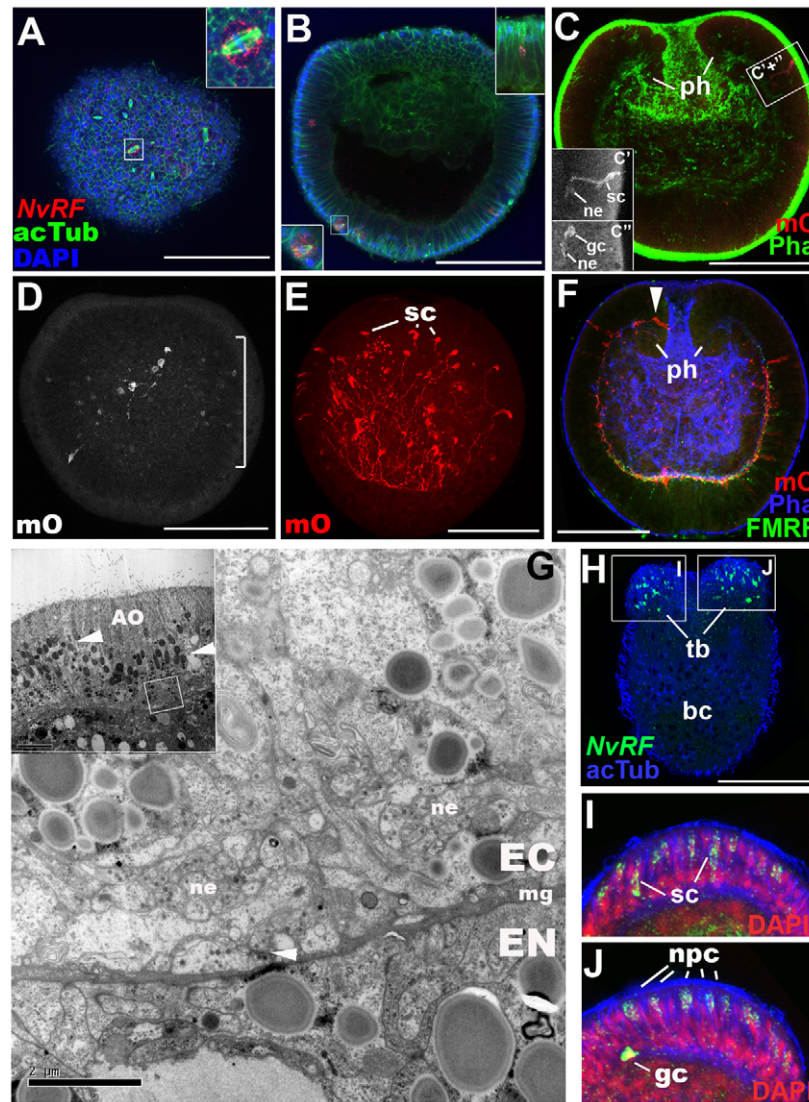


Fig. 3. Ectodermal neurogenesis commences during gastrulation and leads to the formation of a transiently polarized basiepithelial nervous system. (A,B) Confocal sections of an early gastrula *N. vectensis* larva labeled with antisense riboprobe against *NvRF* mRNA (red), an antibody against acetylated tubulin (acTub, green) and DAPI (blue). Oral side is up. Sections are superficial in A and medial in B. The inset in A shows an *NvRF*-expressing mitotic cell, here referred to as a neural precursor cell (NPC). The lower left inset in B shows the apical position of the NPC in the ectodermal epithelium, and the upper right inset shows *NvRF* expression in an elongated epithelial cell with a middle-positioned nucleus. Because nuclei of differentiated sensory cells typically occupy a middle position, we assume that this cell represents a postmitotic, early differentiating sensory cell. (C-D) Confocal images of *NvElav1::mOrange* transgenics at late gastrula stage. Insets at different focal planes in C show the presence of sensory (C') and ganglion (C'') cells. Bracket (D) indicates the distribution of neurons at this stage. (E,F) Confocal sections of mid-planula I larvae of the *NvElav1::mOrange* (mO, red) line, labeled in F with phalloidin (blue) and anti-FMRFamide antibody (green). Oral side is up. Sections are through the entire specimen in E and medial in F. Arrowhead (F) indicates a sensory neuron in the pharynx. (G) An electron micrograph of the base of the apical organ (AO in inset) at late planula stage, showing clusters of neurites (ne) containing opaque and dense cored vesicles. Arrowhead indicates an interneuronal synapse. Note in the inset the characteristic basal position of nuclei of apical organ cells (right-hand arrowhead) relative to that of the neighboring cells (left arrowhead). (H-J) Confocal sections of a tentacle bud stage larva labeled with antisense riboprobe *NvRF* (green), an antibody against acetylated tubulin (acTub, blue) and DAPI (red). Oral side is up. Section is superficial in H; I, J are medial sections of the tentacle buds labeled in H. Note the apically positioned non-dividing putative NPCs, as well as differentiating sensory cells (sc) and ganglion cell (gc) with more basal nuclei. en, endoderm; ph, pharynx; sc, sensory cell; gc, ganglion cell; ec, ectoderm; mg, mesoglea; tb, tentacle bud; bc, body column. Scale bars: 100 μ m in A-F,H; 2 μ m in G.

oral ectoderm tissue of wild-type animals into *NvElav1::mOrange* hosts whose endoderm had been removed (Fig. 4D). mOrange-positive sensory and ganglion cells were readily detectable in the body column ectoderm of the resulting chimeric planulae (Fig. 4E). Thus, during normal development, both neural cell types are generated from the ectoderm.

***NvElav1* is required for the development of ectodermal neurons**

The mode of ectodermal neurogenesis described above is strikingly similar to neurogenesis in bilaterians. To extend these findings to the functional level, we analyzed the role of *NvElav1* in ectodermal neurogenesis. *Elav* is considered a conserved neural marker gene

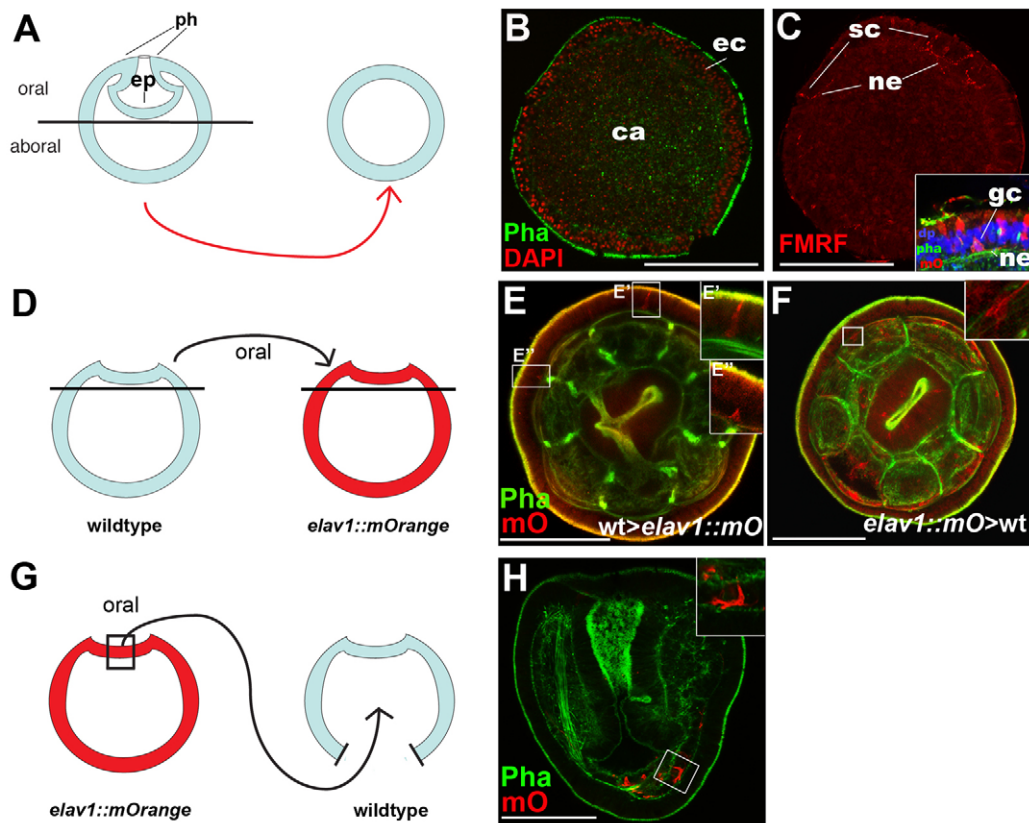


Fig. 4. Neurons develop directly from ectodermal and endodermal epithelia. (A) The bisection experiment. Aboral halves of transversely bisected 20- to 24-hour early gastrulae develop into larvae consisting of ectodermal epithelium without endoderm (right). (B) Medial-level confocal section of a 4-day-old endoderm-free larva derived from the aboral half of a bisected embryo labeled with phalloidin (Pha) and DAPI, showing the absence of the endodermal epithelium. (C) Confocal sections of the larva in B labeled with an antibody against FMRFamide (FMRF). Note the presence of sensory cells (sc) with basal neurites (ne). The inset shows a ganglion cell (gc) with neurites in a 4-day-old endoderm-free larva of the *NvElav1::mOrange* line, labeled with phalloidin, DAPI and an antibody against DsRed, which cross-reacts with mOrange (mO). (D) The transplantation experiments. (E-F) Confocal transverse sections of primary polyps after transplantation of wild-type endoderm plus surrounding ectodermal tissue into *NvElav1::mOrange* hosts (E) or *NvElav1::mOrange* endoderm plus surrounding ectodermal tissue into wild-type hosts (F) at early gastrula stage. mOrange-positive neurons are restricted to the tissue derived from transgenic animals. A sensory cell (E') and a ganglion cell (E'') are shown in the ectoderm. (G) The transplantation of endodermal tissue pieces. Endoderm was excised at early gastrula stage. (H) Confocal sections of mid-late planula showing mOrange-positive sensory cells in the endoderm. Lateral view, oral pole to the top. ao, apical organ; ca, cavity; ep, endodermal plate; gc, ganglion cell; mO, mOrange; ne, neurite; ph, pharynx; wt, wild type. Scale bars: 100 μ m.

in bilaterians and functional data support a role in neural development in vertebrates and *Drosophila melanogaster* (Akamatsu et al., 1999; Campos et al., 1985).

To test the hypothesis that *NvElav1* is required for neuronal development in *Nematostella*, we used two translation-blocking morpholinos (*NvElav1* MO1 and MO2, see Materials and methods) and two control morpholinos (*NvElav1* MO1mm and a generic control MO, referred to as ctr MO1 and MO2). *NvElav1* MO1, but not ctr MO1, efficiently blocked translation of a reporter construct containing the morpholino target site (supplementary material Fig. S4). The number of *NvElav1::mOrange*-positive ectodermal neurons decreased in morpholino-injected 4-day mid-planulae, on average from 102 (uninjected, $n=11$) to 14.8 (*NvElav1* MO1, $n=5$; $P=0.0002$, two-tailed Student's *t*-test; Fig. 5A), whereas injection of control morpholinos had little effect (ctr MO1, 99.4, $n=6$, $P=0.839$; ctr MO2, 93.6, $n=4$, $P=0.720$). Similarly, FMRFamide/GLWamide-immunoreactive neurons were reduced from 92.2 (uninjected, $n=7$) to 27.2 (*NvElav1* MO1, $n=8$; $P<0.001$, two-tailed Student's *t*-test) and 25.2 (*NvElav1* MO2, $n=5$), with

little effect upon injection of control morpholinos (ctr MO1, 93.4, $n=7$, $P=0.958$; ctr MO2, 79.6, $n=5$, $P=0.482$) (Fig. 5B). The morpholino affected the *NvElav1::mOrange*-positive population of neurons more strongly than the FMRFamide-positive population, as the proportion of FMRFamide-positive neurons versus the total number of neurons labeled by FMRFamide and/or mOrange increased, on average, from 0.536 in uninjected controls ($n=5$) to 0.846 in *NvElav1* MO1-injected mid-planula larvae ($n=5$, $P<0.001$), whereas injection of ctr MO1 had no significant effect (0.527, $n=5$, $P=0.854$). This is consistent with the above gene expression data showing that only a subset of FMRFamide-positive neurons express *NvElav1* transcript at high levels. This result, along with the unperturbed development of the ectodermal apical organ and the endodermal longitudinal muscle fibers, support morpholino specificity. We found no evidence for increased numbers of apoptotic cells in *NvElav1* MO-injected animals by TUNEL staining, nor did we observe changes in proliferation by EdU labeling (not shown). Development of the endodermal nervous system was not affected by injection of *NvElav1* MO. We

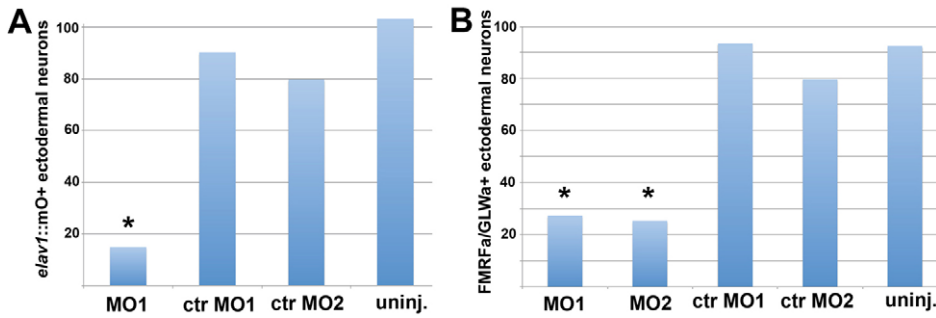


Fig. 5. *NvElav1* is required for the development of ectodermal neurons. The effects of *NvElav1* translation-blocking morpholinos on the number of (A) *NvElav1::mOrange*-positive and (B) FMRFa/GLWamide-positive neurons. *, $P < 0.05$, versus ctr MO1. Note that *NvElav1* MO2 could not be used to analyze the *NvElav1::mOrange* line because its target sequence is included in the transgene.

assume that this is due to decreased efficiency of the *NvElav1* MO at the later developmental stage of endodermal neurogenesis. However, it might also reflect a differential requirement for *NvElav1* in ectodermal versus endodermal neurogenesis.

In summary, these observations show that *NvElav1* is required for normal development of a subset of ectodermal neurons.

The orientation of neurite extension changes during planula development

The outgrowth of ectodermal neurites before the mid-planula stage occurred with unexpected polarity towards the aboral region, the site at which the apical sense organ develops (supplementary material Fig. S5). This polarized outgrowth is difficult to reconcile with the formation of what is often assumed to be a diffusely organized nerve net of cnidarian polyps. However, from the mid-planula II stage, we observed that newly born ectodermal neurons develop transversely oriented neurites (Fig. 6B). The average angle of the neurites from *NvElav1::mOrange*-positive ectodermal sensory cells changed from

30° ('longitudinal') in the mid-planula I stage to 80° ('transverse') in the tentacle bud stage ($P = 1.28 \times 10^{-11}$, two-tailed Student's *t*-test; Fig. 6A-C) and the number of ectodermal neurons in the body column (including the aboral region) increased on average from 30 to 100 ($P < 0.01$, two-tailed Student's *t*-test).

This change was also observed in GLWamide-immunoreactive ectodermal neurons, the majority of which were *NvElav1::mOrange* negative (mid-planula I, 26.4°; tentacle bud, 72°; $P = 9.0 \times 10^{-6}$; Fig. 6D,E). Thus, the development of the ectodermal nervous system consists of two phases with different preferential orientation of neurite outgrowth.

Endodermal neurons develop in the endodermal epithelium and form a complex basiepithelial network

The presence of endodermal nervous systems has been described in cnidarian polyps and medusae (e.g. Anctil, 2000; Davis, 1974; Grimmelikhuijzen, 1983; Singla, 1978), but information about the

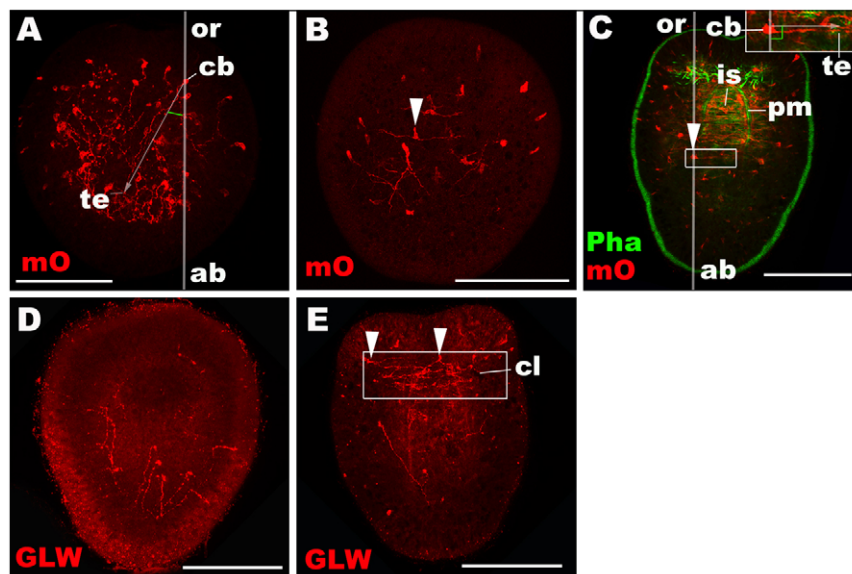


Fig. 6. The orientation of ectodermal neurite extension changes from longitudinal to transverse during planula development.

(A-E) Confocal sections through the ectodermal epithelium showing cell bodies and neurites of ectodermal neurons; in C, the endoderm [e.g. parietal muscle fibers (pm) and isolated neurons (is)] is partially exposed. Oral side is up. (A,D) Mid-planula I stage. (B) Mid-planula II stage. (C,E) Tentacle bud stage. (A-C) *NvElav1::mOrange*-positive neurons; in C, anti-DsRed is used to label the *NvElav1::mOrange*-positive neurons. (D,E) GLWamide-immunoreactive neurons. For measurements of neurite angles, the beginning of neurite extension, typically at the base of the cell body (cb), and neurite termini (te) were connected in a straight line (arrows in A and C), and its angle with respect to the oral-aboral axis was measured (e.g. green lines in A and C). Arrowheads (B,C,E) indicate neurons extending transverse processes. The inset in C shows a magnified view of the boxed region. The boxed region in E shows a cluster of newly born GLWamide-immunoreactive neurons (cl), which are absent in D. ab, aboral; cb, cell body; is, isolated neuron; or, oral; pm, parietal muscle; te, neurite termini. Scale bars: 100 μ m.

development of these endodermal nervous systems is scarce. We observed that, in *Nematostella*, endodermal *NvElav1*-expressing sensory cells become evident at the early planula stage (Fig. 7A) and differentiate during planula stages throughout the endodermal epithelium (Fig. 7B,C) (Marlow et al., 2009). After transplantation of wild-type endoderm plus surrounding ectoderm into *NvElav1::mOrange* host ectoderm we could not detect any mOrange-positive cells in the endoderm (Fig. 4E). Reciprocal transplantations (*NvElav1::mOrange* endoderm plus surrounding ectoderm into wild-type ectoderm) demonstrated that the

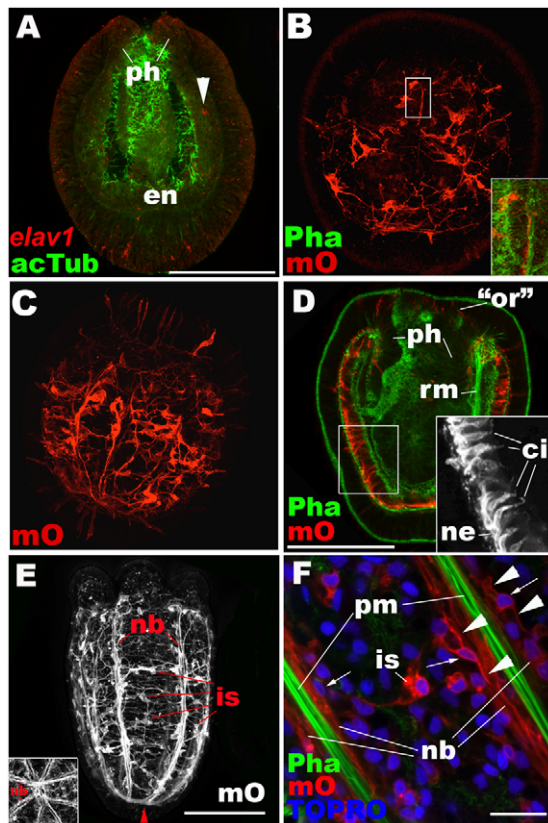


Fig. 7. Endodermal sensory cells develop from the endodermal epithelium and form a complex basiepithelial network containing neuronal bundles. (A) Medial-level confocal sections of an early planula

labeled with antisense riboprobe *NvElav1* and an antibody against acetylated tubulin (acTub). *NvElav1*-expressing cells occur in the endoderm (arrowhead). (B–D) Confocal sections of *NvElav1::mOrange* planulae (B, mid-planula II; C, late planula) showing the developing endodermal nervous system and some ectodermal sensory cells. (D) Medial-level confocal sections of a late planula of the *NvElav1::mOrange* line labeled with phalloidin (Pha) and an antibody against DsRed. The inset in D is a magnified view of the boxed region, showing sensory cells with cilia (ci) and neurites (ne). (E) Projection of confocal sections of a tentacle bud larva of the *NvElav1::mOrange* line, showing the *NvElav1::mOrange*-positive endodermal nervous system. Sections are through the entire larva. Inset shows an aboral view of the convergence of endodermal neurite bundles (nb) at the aboral pole (red arrowhead). (F) Confocal sections of a primary polyp of the *NvElav1::mOrange* line labeled with phalloidin (Pha) and TO-PRO. Arrows indicate cell bodies that are integrated within the neuronal bundles flanking the parietal muscle fibers (pm). Arrowheads indicate individual neurites in the neuronal bundle; note that extension of neurites occurs in oral (up) as well as aboral (down) directions. ph, pharynx; en, endoderm; rm, retractor muscle; or, oral nerve ring; is, isolated neuron. Scale bars: 100 μ m in A,D,E; 10 μ m in F.

transplanted endoderm is able to generate mOrange-positive neurons (Fig. 4F). Similarly, after integration of small pieces of *NvElav1::mOrange* endodermal plate into the endoderm of wild-type hosts, mOrange-positive neurons were detected in the endoderm but not in the ectoderm (Fig. 4G,H). These experiments show that there is little or no migration of *NvElav1::mOrange*-positive neurons or their precursors from the ectoderm into the endoderm and that endodermal neurons are generated directly from the endodermal epithelium.

The endodermal neurons form a basiepithelial network that initially does not display obvious polarity. However, once parietal muscle fibers form at the junction between the mesentery and the body wall, neurites turn either orally or aborally at this site, leading to the formation of prominent tracts of longitudinally oriented neurites along each side of these muscle fiber bundles (Fig. 7B,C,E,F). These neurite bundles converge at the base of the aboral pole, forming a mesh-like structure (Fig. 7E, inset), but a concentration of neuronal cell bodies was not observed at the point of convergence. Irregularly spaced small clusters of *NvElav1::mOrange*-labeled neurons can be found along the longitudinal neurite bundles (Fig. 7F). A set of neurons occurs between neighboring parietal muscle bundles and sends basiepithelial neurites that connect with each other and with parietal muscle-associated neurons (Fig. 7E,F). These endodermal neurites do not follow the circumferential musculature (supplementary material Fig. S9A). Although the number of endodermal neurons increases during further development of the polyp, the overall organization of the endodermal nervous system is maintained (Fig. S9B; see supplementary material Fig. S6 for details on the primary polyp nervous system).

The endodermal neurons that we observed initially display the elongated morphology of sensory cells and extend a cilium into the gastric cavity (Fig. 7D). However, as thinning of the endodermal epithelium occurs during planula development (supplementary material Fig. S2C–F), the cell bodies of the endodermal sensory cells appear shortened (superficially resembling ganglion cells) along the apical-basal axis at later stages (e.g. polyps).

Taken together, endodermal neurons are generated from the endodermal epithelium and the establishment of the endodermal nervous system architecture appears to be controlled by guidance systems that differ from those present in the ectoderm.

DISCUSSION

Elav1-dependent ectodermal neurogenesis is ancestral for Cnidaria and Bilateria

We have shown via transplantation experiments that the ectodermal epithelium generates both sensory cells and ganglion cells in *N. vectensis*. During gastrulation, cell bodies of dividing NPCs occur apically in the ectodermal epithelium, whereas differentiated neurons have centrally or basally located cell bodies, suggesting basally oriented translocation of postmitotic neural cell bodies. Although basal translocation of postmitotic cell bodies or nuclei of epithelial cells might not be restricted to neural cells (Meyer et al., 2011), this migration is strikingly similar to interkinetic nuclear migration described in vertebrate neuroepithelia (Taverna and Huttner, 2010) and to the basally oriented migration of differentiating neural cells/cell bodies in a broad range of bilaterians (e.g. Denes et al., 2007; Jacob, 1984; Kadner and Stollewerk, 2004; Meyer and Seaver, 2009; Stollewerk et al., 2001). In addition, the function of *NvElav1* appears to be similar to that of its orthologs in Bilateria: postmitotic expression is required for development of ectodermal neurons in *N. vectensis*,

vertebrates and *Drosophila melanogaster*. These parallels imply that, in the last common ancestor of Cnidaria and Bilateria, NPCs in the ectodermal epithelium divided apically and some of their daughter cells expressed *elav* postmitotically to differentiate into neurons during basal migration of the cells/cell bodies (Fig. 8A), probably including sensory and ganglion cells. Thus, our findings together with evidence for ectodermal neurogenesis in the hydrozoans *C. gregaria* (Thomas et al., 1987) and *P. disticha* (Martin and Thomas, 1981), as well as in the scyphozoan *Aurelia* (Nakanishi et al., 2008), suggest that neurogenesis from ectodermal epithelial cells was present in the last common ancestor of cnidarians and bilaterians (Fig. 8B).

This hypothesis explains why bilaterian neurogenesis is invariably derived from the ectoderm. Adult bilaterian central nervous systems are internal organs, but they are generated by the outermost layer of the embryo, the ectoderm, and need to be subsequently internalized. This is unusual because other internal organs (e.g. muscle, kidneys, gonads) are generated by the middle layer of the embryo, the mesoderm, and it has not been clear why the central nervous system derives from the ectoderm. We propose that this is simply due to the ancestry of ectodermal neurogenesis; that is, ectodermal neurogenesis predates the origin of bilaterian mesoderm.

Neural development occurs throughout ectoderm and endoderm

In vertebrates NPCs originate from a restricted area of the ectoderm (the neuroectoderm) and can first be detected after gastrulation (Harris and Hartenstein, 2008). By contrast, in *Nematostella* we observe NPCs already at early gastrulation stages and neurons develop throughout the ectoderm during gastrulation and planula stages, with the exception of a small area at the aboral pole from which the apical organ develops. The broadly distributed

ectodermal neurogenesis also includes ganglion cells/interneurons, which in most bilaterians originate only from the neuroectoderm. Together with the development of sensory and ganglion cells in endoderm-free planulae these findings suggest that vertical induction from the endoderm is not required for ectodermal neurogenesis and that the ectoderm does not become subdivided into territories of differential neurogenic potential.

The transplantation experiments show that endodermal neurons of *Nematostella* develop from the endodermal epithelium. This indicates that both the ectoderm and endoderm of *Nematostella* planulae are neurogenic. Since the widespread occurrence of endodermal nervous systems appears to be unique to cnidarians, it will be interesting to understand the genetic basis of this phenomenon. *NvElav1* is expressed in both ectodermal and endodermal nervous systems during development, suggesting the possibility of deployment of pre-existing genetic mechanism(s) of ectodermal neurogenesis to neurogenesis from the endodermal epithelium. This suggests that the genetic mechanisms that control neural specification and differentiation might be similar in *Nematostella* and bilaterians, although it remains to be determined whether the signaling pathways that control the segregation of neural and non-neural tissues and territories in vertebrates have equivalent functions in *Nematostella*.

Additional genetic data comparing ectodermal and endodermal nervous system development in *N. vectensis*, and across distantly related cnidarian taxa, should shed further light on how this potential novelty was achieved in Cnidaria.

Polarity and accumulations of neurites in the *Nematostella* nervous system

At mid-planula I stage, the neurites of most ectodermal neurons project aborally towards the base of the apical organ. Since the apical organ itself is typically considered a sensory structure, it could be that sensory information from the ectoderm becomes integrated around the aboral pole. However, there is no accumulation of neural cell bodies or a ganglion-like structure as is often associated with apical organs in bilaterians. Similarly, from mid-planula II stage, prominent bundles of endodermal neurites flank the developing mesenteries and form a mesh at the aboral pole area, but again no accumulation of neural cells at the aboral pole is detectable. We do observe clusters of endodermal neurons along the mesenteries, but we could not detect any regularity in their positions along the oral-aboral axis or in their distribution among the eight mesenteries. Although a cluster of sensory cells develops around the mouth opening by the tentacle bud stage (Fig. 7D), we did not observe the formation of oral or pharyngeal nerve rings.

Thus, despite clear accumulations of neurites and the occurrence of small clusters of neural cell bodies, we cannot detect any morphological structure that would be comparable to the nervous system centralization observed in bilaterians.

Possible mechanisms of the change in the neurite guidance system during planula development

A drastic change in the overall ectodermal neurite orientation occurred in the body column during planula development, from longitudinal in early-born to transverse in later-born neurons. The initial aboral neurite orientation might reflect putative global attractive or repulsive signals originating from the aboral or oral pole, respectively, and could therefore be related to the patterning of the oral-aboral body axis. Alternatively, endodermal processes that penetrate the mesoglea may form synapses with ectodermal neurites and might be the source of attractive guidance cues. These

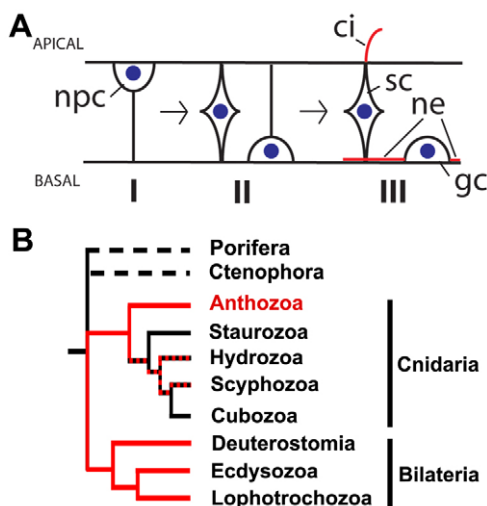


Fig. 8. Summary of ectodermal neurogenesis in *Nematostella* and its evolutionary implications. (A) Proposed model for ectodermal neurogenesis in *N. vectensis*. Neural precursor cells (npc) occur apically (I). NPC cell bodies migrate basally (II) and differentiate into sensory cells (sc) and ganglion cells (gc) (III). Blue circles represent nuclei. ci, cilium; ne, neurite. (B) Evolutionary history reconstruction of ectodermal neurogenesis in Metazoa. Red lines indicate clear evidence for neurogenesis from the ectodermal epithelium, whereas red-black dashed lines indicate indirect support for ectodermal neurogenesis. Parsimony assumption favors the scenario in which ectodermal neurogenesis is ancestral in Cnidaria, as well as in Cnidaria and Bilateria.

processes from the endoderm are initially concentrated in the aboral region and later occur throughout the body column, correlating with the change in neurite orientation (supplementary material Fig. S10).

We were unable to identify morphologically distinguishable landmarks that transverse ectodermal neurites would project to. Moreover, endodermal neurites developing at the same time do not display preferential orientation, suggesting that the architectures of the endodermal and ectodermal nervous systems are shaped by separate guidance mechanisms. We thus recognize three different neurite guidance mechanisms (two in the ectoderm and one in the endoderm) during *Nematostella* development. Although we cannot exclude the possibility that additional subpopulations of neurons display other patterns of neurite orientation, our results suggest that guidance mechanisms acting more globally and probably over long distances (mainly in the early stage ectoderm) and others acting more locally (mainly in the endoderm and late stage ectoderm) contribute to the formation of the nervous system in *Nematostella*. These mechanisms might differ from each other in the type of the signaling molecules that are used and/or in the spatiotemporal expression patterns of the same guidance molecules.

Evolution of cnidarian nervous system development

Our results suggest that in *Nematostella* neurogenesis commences in the ectoderm and that neurons originate from epithelial cells. This is in contrast to the situation in hydrozoans, where most neurons originate from interstitial stem cells. The similarity between neurogenesis in *Nematostella* and that in bilaterians indicates that the condition in hydrozoans might be derived.

It remains to be determined to what extent other findings of our study can be generalized for cnidarians. For example, strictly aboral orientation of early ectodermal neurites in the hydrozoan *Podocoryne* is restricted to neurons in the oral region (Groger and Schmid, 2001; Momose and Schmid, 2006), whereas RFamide-positive ectodermal neurites in the scyphozoan *Aurelia* (Nakanishi et al., 2008) and the anthozoan *Acropora millepora* (Hayward et al., 2001) develop oral and aboral projections. Similarly, the extensive endodermal nervous system development in planulae observed in *N. vectensis* has not been described in other cnidarians (de Jong et al., 2006; Groger and Schmid, 2001; Martin, 2000; Nakanishi et al., 2008; Piraino et al., 2011). Additional studies of nervous system formation at high temporal and spatial resolution in these and other cnidarians are needed for a detailed reconstruction of the evolutionary histories of cnidarian neural development.

In conclusion, our data suggest that neurogenesis from ectodermal epithelial cells is ancestral for cnidarians and bilaterians and that post-transcriptional regulation of gene activity by *elav1* genes is an ancient feature of neural development. We show that, in contrast to bilaterians, almost the entire tissue of the *Nematostella* planula is neurogenic and that, similar to the situation in bilaterians, multiple neurite guidance mechanisms are involved in the formation of the only superficially simple nervous system of *Nematostella*.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.071902/-/DC1>

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