

Anatomy and Development of the Nervous System of *Nematostella vectensis*, an Anthozoan Cnidarian

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ABSTRACT: *Nematostella vectensis*, an anthozoan cnidarian, whose genome has been sequenced and is suitable for developmental and ecological studies, has a complex neural morphology that is modified during development from the larval to adult form. *N. vectensis*' nervous system is a diffuse nerve net with both ectodermal sensory and effector cells and endodermal multipolar ganglion cells. This nerve net consists of several distinct neural territories along the oral–aboral axis including the pharyngeal and oral nerve rings, and the larval apical tuft. These neuralized regions correspond

to expression of conserved bilaterian neural developmental regulatory genes including homeodomain transcription factors and *NCAMs*. Early neurons and stem cell populations identified with *NvMsi*, *NvELAV*, and *NvGCM*, indicate that neural differentiation occurs throughout the animal and initiates prior to the conclusion of gastrulation. Neural specification in *N. vectensis* appears to occur through an independent mechanism from that in the classical cnidarian model *Hydra*. © 2009

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INTRODUCTION

An ability to sense variation in the environment and coordinate appropriate behavioral response is a hallmark of morphologically diverse extant eumetazoan nervous systems. Many questions surrounding early neural evolution remain unanswered. To what extent were neural axes patterned along the major body axes, as in flies, nematodes, and vertebrates, or in the

dispersed nerve nets of earlier branching taxa? How many neural cell types existed, how were they specified, and what was the mode of communication between them? Characteristics of the ancestral eumetazoan nervous system can only be inferred by studies of extant species (structural, developmental, and molecular traits), including lineages, which predate the origin of central nervous systems [Fig. 1(A)]. Investigations into early branching metazoans are particularly useful in this regard. *Nematostella vectensis* is an anthozoan cnidarian and thus, a member of the sister clade to all Bilateria.

Although the molecular basis of the formation and function of bilaterian nervous systems is understood in some detail, comparatively little is known about these processes in the basal metazoans. Patterning of

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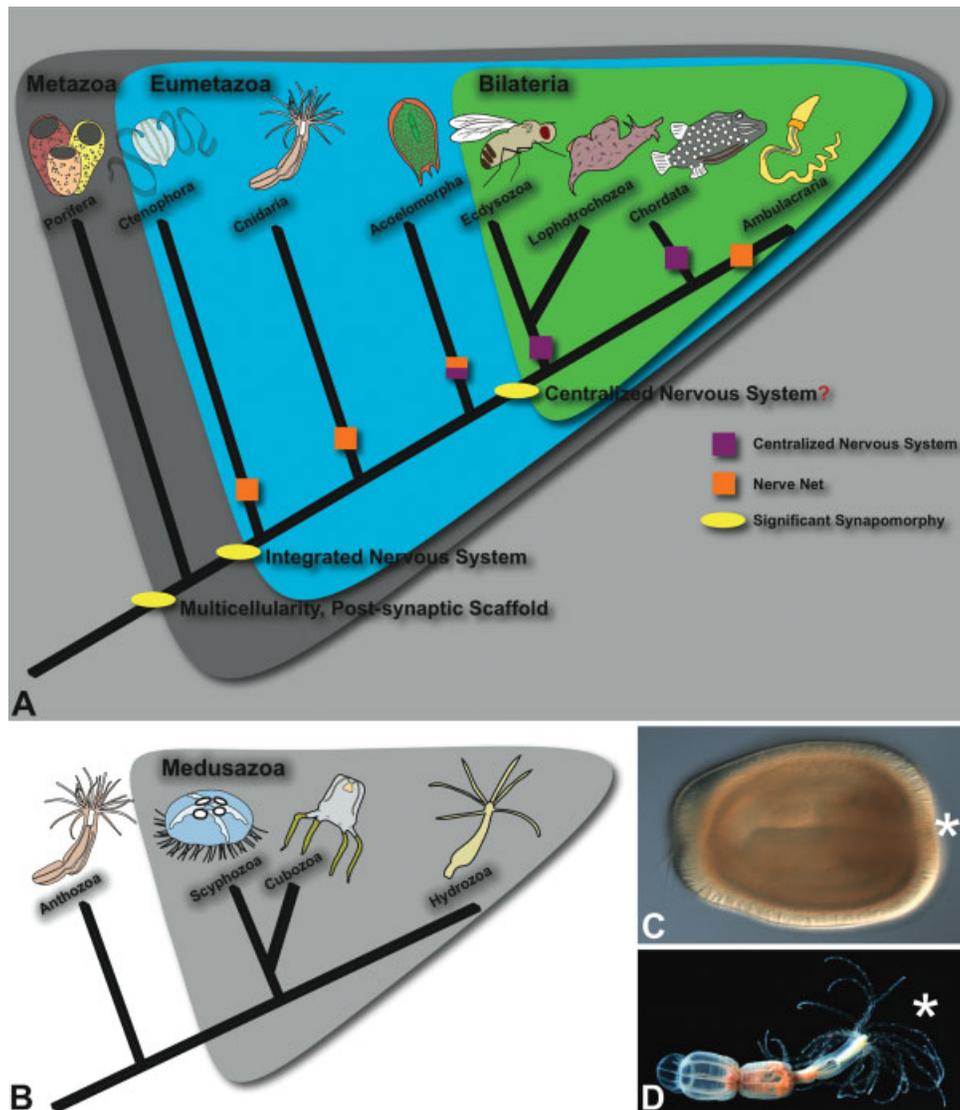


Figure 1 Evolution of neural characters in the metazoa (A) Protostomes (Ecdysozoan and Lophotrochozoan) and Deuterostomes (Chordate and Ambulacrarian) comprise lineages in which at least some taxa possess centralized nervous systems. Basal metazoan taxa display varying degrees of neural organization ranging from a lack of neural elements (Porifera) to nerve nets (Ctenophora and Cnidaria) to anterior ganglia and multiple nerve cords (Acoelomorpha). (B) Cnidarian phylogeny showing phyla level relationships (Collins, 2006 no. 2075). (C, D) Planula (B) and adult polyp (A) oriented with oral pole (asterisks) at right.

distinct neural populations along the anterior-posterior axis is conserved throughout bilaterian phyla (Hirth et al., 2003), notably even in those which retain a nerve net, suggesting that these mechanisms were in place in the urbilaterian ancestor (Lowe et al., 2003). Although it is generally accepted that centralized nervous systems evolved from simple “nerve nets” (Parker, 1919), it is not clear when this transition occurred and if it occurred more than one time in the bilateria. An investigation of neural patterning mechanisms in early branching non-bilaterian taxa,

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which retain such a nerve net (cnidarians and ctenophores), is critical for understanding the evolution of the nervous system.

Cnidarians, as an outgroup to Bilaterians (Dunn et al., 2008), are of particular import for investigations pertaining to the evolution of the nervous system. Cnidarian nervous systems are found to be composed of two diffuse nerve nets (one associated with the endoderm and the other with the ectoderm) with both bi and multipolar neurons able to conduct in either direction, allowing the transmission of a stimulus

from any one part of the nerve net to the other (Westfall, 1971; Anderson, 1985, 1988; Satterlie, 2002; Brusca, 2003). These neurons serve to coordinate the input from sensory cells and structures with the output of the epitheliomuscular cells (cnidarians do not have definitive muscle cells). Sensory structures such as eyes and statocysts are likely to have been independently evolved in the medusozoan lineages to which they are confined (scyphozoa and cubozoa) and were not present in the cnidarian-bilaterian ancestor. Single epithelial sensory cells and unique stinging cells called cnidae are found throughout the cnidaria. Cnidae also act as sensory cells by responding to stimuli (Pantin, 1942) and are integrated with the nervous system through synapses (Westfall, 2004), and like neurons, originate from multipotent “i” cells in *Hydra* (Bode, 1996).

Nervous system organization differs significantly between cnidarian and bilaterian taxa, but much is conserved at the individual neuronal unit. Interneuronal communication in cnidarians employs transmission through both chemical synapses and electrical synapses mediated by gap junctions (Satterlie and Spencer, 1987), which is further supported by the discovery of a pannexin-like gene in the *N. vectensis* genome (Putnam et al., 2007) and innexins in hydrozoans (Alexopoulos et al., 2004). Both excitatory and inhibitory postsynaptic potentials have been reported (Anderson and Spencer, 1989). Moreover, there is also evidence for epithelial conduction through junctions between epithelial cells in some cnidarians (Mackie, 1965, 1976; Mackie and Passano, 1968). Studies on swimming behavior in jellyfish such as *Aglantha digitale* have revealed the use of calcium and sodium action potentials as well as their regulation by potassium channels (Mackie, 1980, 2004; Anderson and Spencer, 1989; Meech and Mackie, 1993a,b; Mackie and Meech, 2000; Greenspan, 2007). Cnidarians utilize both classical fast (acetylcholine, glutamate, GABA, and glycine) and slow (catecholamines and serotonin) transmitters and neuropeptides (especially of the RFamide and RWamide families) for synaptic neurotransmission (Satterlie, 2002; Kass-Simon and Pierobon, 2007). Recently, it has been hypothesized, with evidence from newly available basal metazoan genomes, that the postsynaptic scaffold common to all metazoans was present prior to the divergence of the cnidarian-bilaterian ancestor (Sakarya et al., 2007). Although previous work illustrates that morphology and transmission mechanisms of neural cells appear quite similar in bilaterians and cnidarians, it is unclear how the overall neural architecture and patterning of cnidarian nervous systems relates to the main body axis or if

patterning occurs by way of a bilaterian-like mechanism. Much of cnidarian neurobiology has been conducted in adult medusozoans, including the model *Hydra*. Questions regarding the development of neural organization during embryogenesis remain to be answered.

Nematostella vectensis is an emerging cnidarian model for investigations of the nervous system during embryonic development (Darling et al., 2005) and is therefore particularly suited for studies regarding the evolution of the nervous system. Like all other anthozoans, *N. vectensis* has both a larval and polyp stage [Fig. 1(B,C)], and lacks the derived pelagic medusa (jellyfish) stage found in hydrozoans, scyphozoans, and cubozoans (Collins et al., 2006). Development in *N. vectensis* is well characterized to include an early chaotic cleavage stage, a hollow blastula which gastrulates by unipolar invagination to a swimming planula, which settles and grows tentacles surrounding its oral opening to the gut, finally forming a juvenile polyp (Hand and Uhlinger, 1992; Lee et al., 2007; Magie et al., 2007). *Nematostella vectensis* has many practical advantages such as ease of culture, access to developmental material (Fritzenwanker and Technau, 2002), and a sequenced and annotated genome (Putnam et al., 2007). These technical advances in addition to a growing interest in basal metazoan taxa have fueled a rapidly accumulating body of work centering on the molecular and cellular basis of development in *N. vectensis*.

Cnidarians have traditionally been viewed as simple diploblasts with a loosely organized nervous system and little axial organization. Recent work emerged to show that cnidarians possess a great deal of genomic and molecular complexity (Putnam et al., 2007) much of which is found in discrete cell populations and indicates a greater number of cell types than previously described. The *N. vectensis* genome contains nearly 18,000 protein coding genes and encompasses all of the major signaling pathways (*notch*, *wnt*, *TGFbeta*, *FGF*, and *Hedgehog*), as well as components of virtually all transcription family members used in bilaterian patterning (Putnam et al., 2007). This molecular and genomic complexity is used for cryptic patterning of the *N. vectensis* body plan during development by way of complex expression patterns for these transcription factors and signaling pathways (Kusserow et al., 2005; Ryan et al., 2007). Functional studies have begun to confirm conserved roles for these genes in germ layer segregation (Wikramanayake et al., 2003) and in formation of larval structures (Rentzsch et al., 2008), and although a subset of these infer a role for these pathways in neurogenesis, virtually nothing is known about the

development or complexity of the nervous system of *N. vectensis*.

Here, we determine the neural architecture of *N. vectensis* at various developmental stages, present evidence for potentially neurogenic regions and interpret these data with respect to the biphasic life history (swimming planula versus benthic polyp). Using antibodies directed against GABA, FMRFamide, and serotonin and *in situ* hybridization for *antho-Rfam* and *dopamine beta hydroxylase*, we have identified populations of neurons with distinct morphology and localization within gastrula, planula, and polyp stages. We have also identified regions of both the endoderm and ectoderm that express transcripts of putative markers of stem cells and early neurons (*ELAV* and *Musashi*) as well as those specific for subsets of developing neurons (*GCM*, *repo*, and *NCAM*). These results suggest that although *N. vectensis* does not possess a centralized nervous system, its neural morphology is patterned along the oral–aboral axis and correlates with previously identified molecular domains of gene expression, which are temporally associated with the planula and polyp stage.

METHODS

Identification and Amplification of Target Genes

Orthologous genes were identified by performing tblastx, tblastn, and blastp searches (NCBI) of the assembled *N. vectensis* genome and predicted gene models (JGI) using publicly available protein and DNA sequences from GenBank. Prior to the completion of the *N. vectensis* genome assembly, searches of sequenced scaffolds and ESTs (NCBI) were conducted using tblastn searches. Nested RACE PCR (Smart Race cDNA amplification kit, BD Biosciences Clontech) was used to amplify the 5' and 3' regions of target genes from mixed stage pools of *N. vectensis* cDNA using sequence specific primers. Sequencing of the PCR products cloned into the p-GEM T Easy vector (Promega) was conducted by Macrogen (South Korea). Open reading frames for submission to GenBank were determined by comparing RACE PCR products, EST data, and JGI gene predictions (Supp. Info. Fig. 1).

Alignment and Tree Construction

Protein sequences were aligned in MacVector using the default settings of the ClustalW alignment program and were trimmed by eye (Supp. Info. Fig. 2). Protein domains were identified using the SMART database (Ponting et al., 1999). Transmembrane, intracellular, and extracellular domains of the NvNCAM predicted proteins were predicted with Phobius prediction program (Kall et al., 2004). Orthol-

ogy of *Nematostella* proteins with bilaterian proteins was assessed with phylogenetic trees made with MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Additional information regarding the MrBayes analysis, relevant outgroups, and protein models employed in the analysis are available in supporting Information Figure 3.

In Situ Hybridization

In situ hybridization of probes to mRNA was performed, as previously described (Martindale et al., 2004). DIG labeled RNA probes of 750–2000 nucleotides were constructed from RACE products or expressed sequence tags from a previously constructed *N. vectensis* EST library using Ambion MEGAscript reverse transcriptase kit (AM1330 and AM1334).

Antibody Staining

Early embryos (0–48 hpf) were dejellied in 4% cysteine (pH 7.4), and polyp stages were relaxed in 7% MgCl₂ prior to fixation. Samples for antibody staining were fixed for 90 s in 4% paraformaldehyde and 0.2% glutaraldehyde in 1/3× filtered seawater (FSW). Samples were then fixed for 1 h at 4°C in 4% paraformaldehyde in FSW. Embryos were washed three times quickly and three times for 30 min in phosphate buffered saline (PBS) and 0.1% Tween-20 (PTw). Some samples were washed in PBS and 0.1% Triton X-100 (PBT). Samples were stored in either PBS or methanol.

Nematostella vectensis embryos were blocked in 5% normal goat serum, NGS (Sigma, catalog no. G9023) in PBT, or PTw (blocking buffer) for 1 h prior to antibody staining. Samples were incubated overnight at 4°C in primary antibody: a commercially available rabbit anti-GABA antibody at 1:250–1:500, mouse anti-5HT at 1:200 (Dia Sorin catalog no. 20080), or rabbit anti-FMRFamide at 1:100 (Peninsula Laboratories, catalog no. 8755). Three quick washes (allowing the embryos to settle briefly) followed by three 30-min washes were done at room temperature with PBT or PTw. Embryos were reblocked for 30 min at room temperature and then incubated in secondary antibody in blocking buffer overnight at 4°C. Three quick washes followed by two long washes were performed prior to counterstaining in 2 U/mL (66 nM) 488 or 647 alexa-conjugated phalloidin (Molecular Probes, Eugene, Oregon, USA) and propidium iodide (1 µg/mL) in the presence of RNase (50 µg/mL). Following staining, embryos were dehydrated through an isopropanol:PBS series (50:50, 70:30, 90:10, and 100:0) then mounted in Murray clear (2:1 benzyl benzoate to benzyl alcohol).

DAPI Staining of Cnidocytes

Cnidocytes were stained according to Szczepanek et al. (2002). Embryos were fixed as for antibody staining with the modification that 10 mM EDTA was added to all fixa-

tives. *Nematostella vectensis* samples were stained with 140 mM DAPI for 30 min at room temperature and then washed three times for 30 min in PBS. Counterstaining with propidium iodide and phalloidin and mounting were carried out, as described above.

Imaging

In situ stained embryos were visualized under 20× differential interference contrast (DIC) microscopy on a Zeiss Z-1 imager microscope (Carl Zeiss, Jena, Germany) or Zeiss Axioscop2 and photographed with a Zeiss AxioCam HRC or Coolpix camera (Nikon, Tokyo, Japan). DAPI stained embryos were imaged on an Zeiss Z-1 imager microscope and photographed using an Orca-ER digital camera (Hamamatsu TOA Electronics, Shizuoka, Japan). Images were collected, processed, and false colored (DAPI cnidocytes) using Zeiss AxioVision or Volocity Acquisition software (Improvision, Coventry, UK). Neural antibody stained embryos were visualized using a Zeiss LSM510 confocal microscope using the LSM software. 3D projections were rendered using LSM or Volocity software and edited in Adobe Photoshop.

RESULTS

We have used crossreactive antibodies to neuropeptides and other neurotransmitters, staining of cnidocytes, and *in situ* hybridization of genes for neuropeptides and for neurotransmitter biosynthesis to reveal several subsets of neurons distributed in distinct regions of the planula and polyp. Neural organization of the animal changes with developmental time as the prominent neural region of the swimming planula, the apical tuft, is replaced upon settlement and polyp formation with the adult neural morphology consisting of the oral nerve ring, the pharyngeal nerve ring, continued elaboration of the innervation in newly forming mesenteries, and highly neuralized tentacle tips. Molecular markers of differentiating neurons indicate that neurogenesis occurs in both the ectodermal and endodermal tissue layers and is not limited to the ectoderm as is the case in many metazoans.

Anti-GABA Immunoreactivity

In the early planula, cells stained with anti-GABA antibody are first detected throughout the ectoderm along the oral–aboral axis and at high densities in the apical tuft at the aboral pole [Fig. 2(A–C)]. These early neurons are columnar in shape and span the entire width of the ectodermal epithelium, which like the endoderm, are one cell layer thick. As the planula develops, ciliary membranes of the apical tuft, cili-

ated sensory cells along the body column ectoderm [Fig. 2(C,D)], and a ring of large neurons at the base of the pharyngeal tissue, the pharyngeal nerve ring, become anti-GABA reactive [Fig. 2(H,I)]. At this stage, projections extend from the base of the cell bodies between the ectodermal and endodermal tissue layers and run parallel to the surface of the planula. As the tentacle buds form during polyp stages, new neurons form at the highest numbers in tentacle tips [Fig. 2(F,G)]. In the polyp stage, several distinct GABA-positive cell types are distinguishable that include, at a minimum, sensory neurons [Fig. 2(C,D)] pyramidal shaped neurons with long processes, which become bifurcated in some regions [Fig. 2(E)], and bipolar neurons in the tentacles [Fig. 2(G)]. Rather than a strictly columnar morphology, many neurons located at the base of the ectoderm adopt a flattened appearance, maintaining no contact with the external environment [Fig. 2(E,G)], whereas many presumed sensory cells remain positioned so that contact with the outer surface of the animal is maintained [Fig. 2(E,F)].

Neuropeptides of the RFamide Class

FMRFamide immunoreactivity, as with GABA immunoreactivity, is present in individual ectodermal cell bodies spread throughout the body wall by the early planula stage [Fig. 2(J)]. At later stages, strong staining is seen in both the body wall and in the pharyngeal region at the oral pole of the planula [Fig. 2(J,K)]. By the late planula stage, cell bodies are found throughout the ectoderm and endoderm and many cells, particularly those in the endoderm, extend long processes that run parallel to the surface of the animal in the mesoglea [Fig. 2(L,M)]. Polyp stage animals have an abundance of anti-FMRFamide staining neural processes along the oral–aboral axis at the interface between the ectoderm and endoderm [Fig. 2(L,M)] and many cell bodies are found in the oral region at the oral nerve ring and base of the tentacles [Fig. 2(O)]. In comparison to GABA-labeled polyps [Fig. 2(H)], there is a notably smaller number of cells in the tentacles and the pharyngeal nerve ring region of the FMRFamide-labeled polyps [Fig. 2(N)]. Many of the ectodermal neurons send projections laterally toward the mesenteries and project loosely in the oral–aboral orientation along the mesentery [Fig. 2(N–P)]. The aggregation of neural projections may allow the animal to quickly contract along the length of the body column by innervating the epitheliomuscular cells along the mesenteries (Ramsay, 1952).

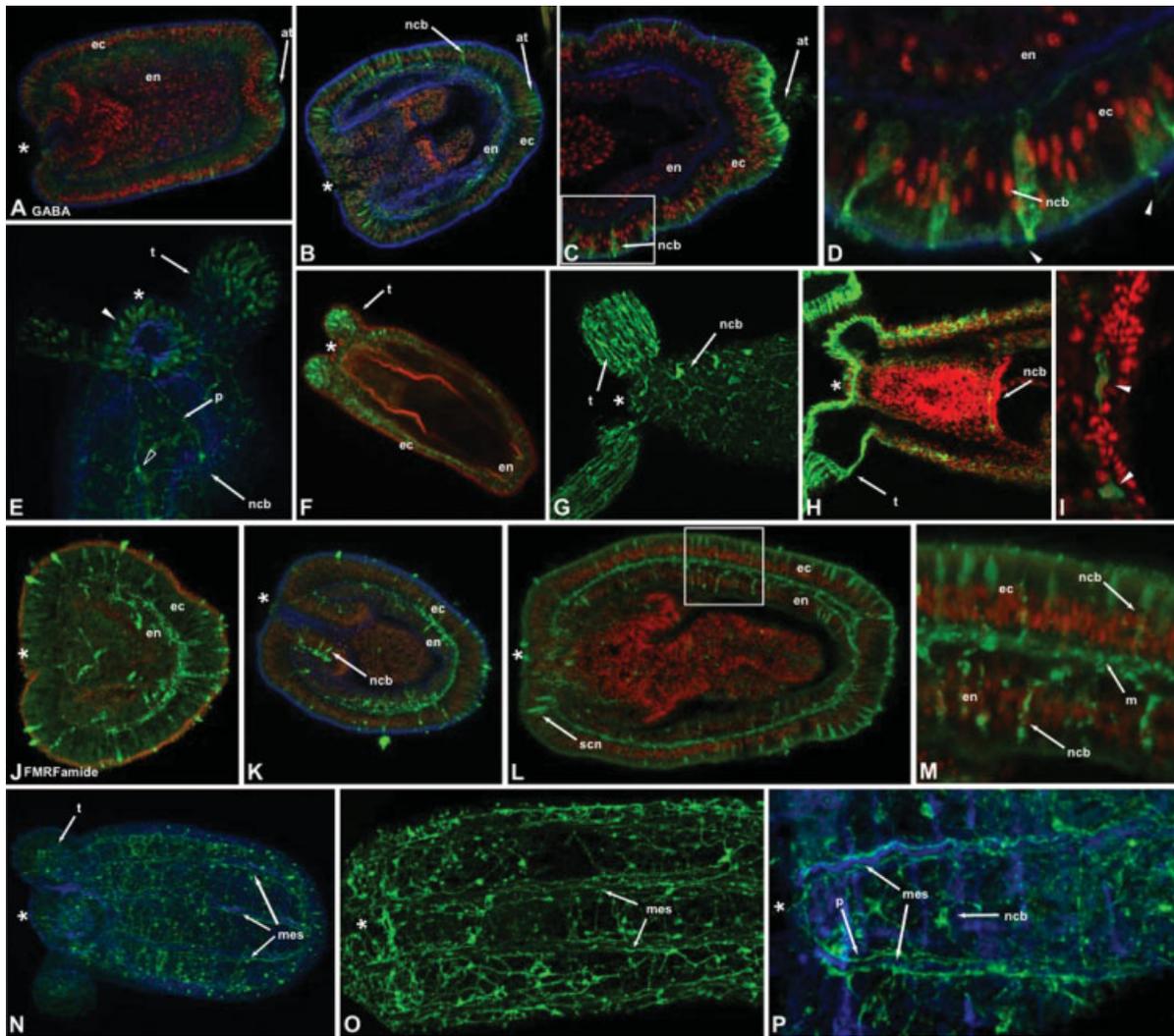


Figure 2 Anti-GABA and anti-FMRFamide immunoreactivity Nuclei are stained with propidium iodide (red), f-actin, a component of cell membranes, is stained with phalloidin (blue), and anti-GABA (A-H) and anti-FMRFamide (I-N) antibodies are detected with a green secondary antibody. Images are 3D renderings of confocal stacks or single slices. The oral pole is marked by an asterisk and mesenteries (mes), endoderm (en), ectoderm (ec), tentacles (t), neural cell bodies (ncb), apical tuft (at), mesoglea (m), and neural processes (p) are labeled. (A) Early planula with anti-GABA staining in the apical tuft at the aboral pole. (B, C) Later stage planula with staining in the body wall ectoderm (~10% of cells) and apical tuft (~25% of cells). (D) High magnification image of labeled ectodermal neurons and associated cilia (arrowheads) in (C). (E, F) Neurons extend long processes by the early polyp stage and the tentacles become heavily innervated. Sensory cells which maintain surface contact (arrowhead) and sub-ectodermal neurons (open arrowhead) are visible. (G) Neurons in the body wall extend processes in the oral–aboral direction and laterally, and those in the tentacles extend processes to the proximal and distal ends. Cell bodies are primarily basal within the ectoderm. (H) An optical cross section through the polyp pictured in (G), picturing a group of large neurons at the base of the pharyngeal ectoderm. (I) High magnification of neurons (arrowheads) in the pharyngeal nerve ring (pnr) in (H). (J) FMRF-amide staining in both endodermal and ectodermal tissue in an early planula. (K) Neural cell bodies are visible in both the ectoderm and endoderm (~5% of cells) and are concentrated in the oral and the apical tuft ectoderm (~10% of cells). Stained neural processes occur in the region between the two tissue layers, the mesoglea (m). (L, M) Cell bodies are primarily at the base of the ectoderm and extend processes parallel to the surface of the polyp. Processes are organized to run oral–aboral along the eight mesenteries. Spirocytes (scn) in the presumptive tentacles are also visible. (N, O) FMRFamide stained cell bodies in the body walls of polyps. (P) High magnification image of FMRFamide positive neural cell bodies in the body wall that send projections along the mesenteries. Levels of individual fluorescent channels were adjusted in Adobe Photoshop, but the relative relationship of pixels within those channels was not altered.

expression of *dopamine beta hydroxylase* is localized to only those cell bodies lying closest to the oral opening (apical within the ectoderm) and not all cells of the pharynx. These data suggest the presence of noradrenergic neurons in *N. vectensis*, though it remains to be determined if the expression of *dopamine beta hydroxylase* shows neurons that produce epinephrine or norepinephrine (since epinephrine is derived from norepinephrine by the enzyme phenylethanolamine-N-methyltransferase). Only one member of the NNMT/PNMT/TEMT methyltransferase family is found in the *N. vectensis* genome, so further experimentation is needed to know if this enzyme converts norepinephrine into epinephrine in anemones.

Cnidocyte Distribution and Anti-Serotonin Immunoreactivity

Cnidocytes are specialized cells containing collagenous organelles unique to cnidarians, the cnidocysts (Engel et al., 2001). Discharge occurs at “bullet speed” through the release of osmotic pressure created by the sequestering of poly- γ -glutamate within the capsule (Szczechpanek et al., 2002). At least, 28 different types of cnidocyst capsules have been described and are specialized for feeding, locomotion, and defense and are classified into three different structural groups; nematocysts, spirocysts, and ptychocysts (Kass-Simon and Scappaticci, 2002). Cnidocyte cells are considered to be components of the nervous system because they have been shown to share a common developmental precursor with the neural cells in hydrozoans (the interstitial stem cell population) (Bode, 1996; Bosch, 2007), synapse with ganglion and sensory cells, and stain with antibodies to various neuropeptides (Westfall et al., 2000; Anderson et al., 2004; Westfall, 2004). Cnidocytes

may act as both sensory (detect external stimulus through a sensory projection called the cnidocil)/ciliary cones) and effector cells (fire a barbed hollow thread in response to the stimulus) (Kass-Simon and Scappaticci, 2002). Anthozoan cnidarians have cnidocyte cells of several types including spirocytes, which house spirocyst capsules, which lack a shaft and spine, and six types of nematocytes with nematocyst capsules (Cutress, 1955; England, 1991; Kass-Simon and Scappaticci, 2002). *Nematostella vectensis* adults have cnidocyte cells that contain spirocysts, spirocytes, (in the tentacles) and two types of nematocytes; those with basitrichous isorhiza capsules (in the body walls) and those with microbasic *p-mastigophore* capsules (in the pharynx and mesenteries) (Williams, 1975; Frank and Bleakney, 1976).

Staining *N. vectensis* cnidocyst capsules with DAPI in the presence of a calcium chelator and visualizing specimens using a Cy2 filter (see Methods) allows us to detect cnidocyte cells and trace their appearance in developmental time. Nematocyte cells appear between 24 and 48 hpf (hours post fertilization) throughout the planula ectoderm [Fig. 4(A,B)]. During the tentacle bud and polyp stages [Fig. 4(D–F)], spirocyst cnidocytes form in the tentacles and additional nematocysts are generated in the body column and pharyngeal ectoderm. All cnidocytes appear restricted to the ectoderm as opposed to other components of the nervous system, which are found in both the ectoderm and the endoderm. Serotonin immunoreactivity labels nematocysts throughout the body column, but not spirocyst cnidocytes found in the tentacles [Fig. 4(L,M)]. 5HT-positive nematocysts first appear aborally and in small numbers throughout the body wall ectoderm [Fig. 4(J,K)]. Only a small number of nematocysts in the pharynx and mesenteries are labeled with 5HT antibody when compared with the pan-cnidocyst DAPI stain [Fig. 4(M,N)], indicat-

Figure 4 DAPI stained cnidocytes and 5HT like immunoreactivity DAPI stained cnidocyte capsules (A–F) and anti-5HT antibody (J–N) are shown in green, phalloidin staining of f-actin is blue (J, K, and N) and nuclei are stained red with propidium iodide (J–N) and DAPI staining of nuclei is blue (B–F). (E–H) (A) At 24 hpf, cnidocytes have not yet formed in the gastrula. (B, C) Cnidocytes (cn) occur throughout the ectoderm of the early planula. (D) By the late planula stage, basitrich cnidocytes (cn) are present throughout the body wall and in the newly forming tentacle buds. (E, F) Newly settled polyps have many nematocytes (cn) in the body wall and spirocytes (scn) in the tentacle. (G) Basitrich cnidocytes in the body wall of the pharyngeal region. (H) Mastigophore cnidocytes aligned in the ectodermal portion of the mesenteries (red line). (I) High densities of spirocytes (scn) in the tentacle tips. (J–N) Anti-5HT like immunoreactivity. (J) A small number of cnidocytes (cn) in the body wall stain with anti-5HT antibody. (K, L) The number of cnidocytes stained increases dramatically during the planula and late planula stage. (M) Only cnidocytes (cn) in the body wall stain with anti-5HT antibody, not spirocytes in the tentacle tips. (N) A high magnification image of nematocytes in the body wall. Levels of individual fluorescent channels were adjusted in Adobe Photoshop, but the relative relationship of pixels within those channels was not altered.

ing that some nematocysts are unlabeled. Spirocysts of the tentacles are not labeled with the serotonin antibody [Fig. 4(M)], even though neuro-spirocyte

synapses of the cnidarian *Aiptasia pallida* have been shown to be serotonin immunoreactive (Westfall, 2004). In *N. vectensis*, the spirocysts of the late

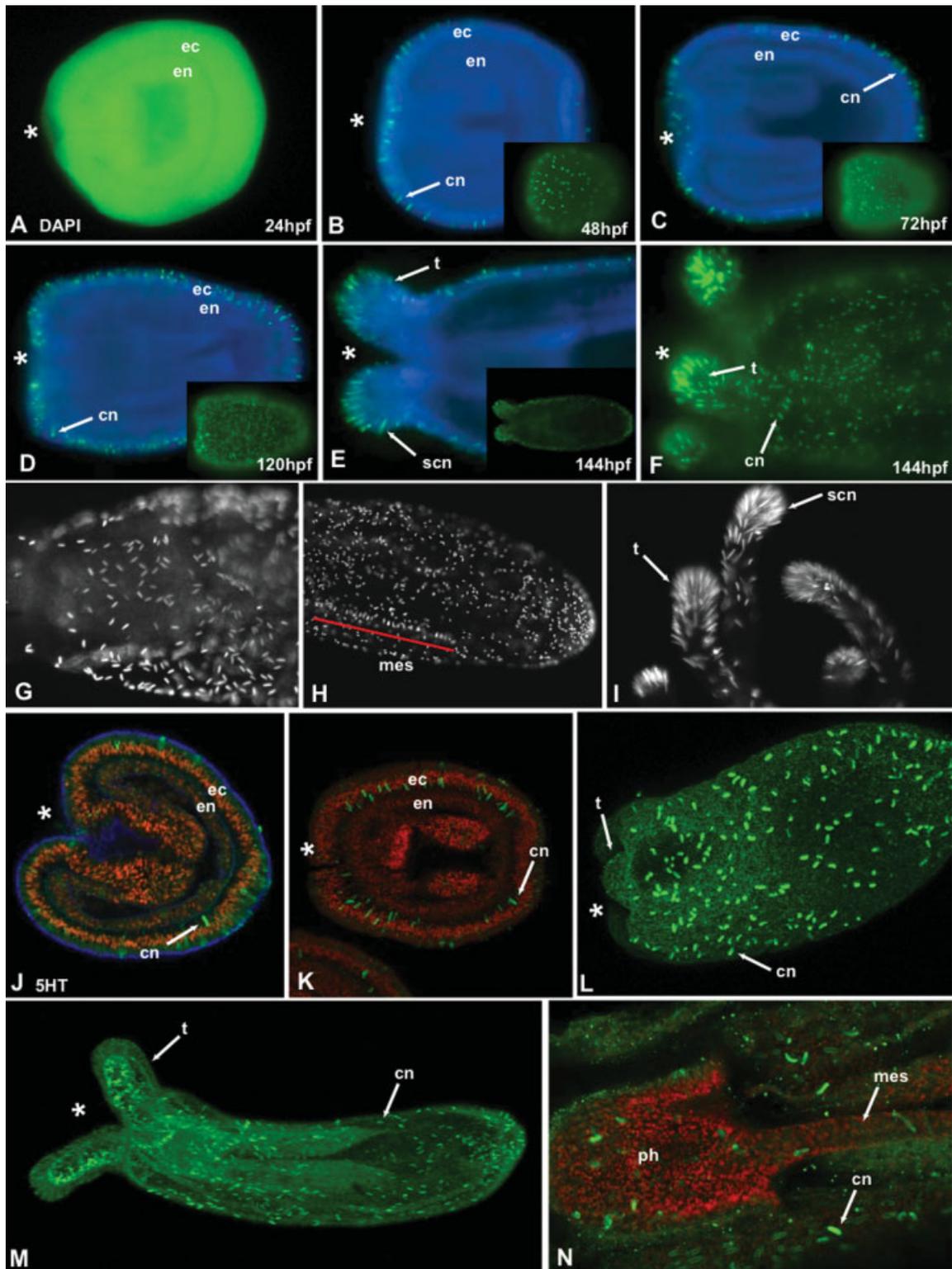


Figure 4

planula and polyp stage show FMRFamide reactivity [Fig. 2(L)], consistent with previous findings of FMRFamide and RFamide immunoreactivity of cnidocytes in the tentacles of many cnidarian species (Anderson et al., 2004).

ELAV and Musashi Mark Neurogenic Regions of the Planula and Polyp

The RNA binding proteins (RNAbps), *embryonic lethal abnormal vision (ELAV)*, and *musashi (msi)* are most notably expressed in the developing nervous system in animals as distantly related as *Drosophila* and vertebrates and have been implicated in neurogenesis and stem cell maintenance, respectively (Robinow and White, 1991; Good, 1995; Sakakibara and Okano, 1997; Akamatsu et al., 1999; Kawashima et al., 2000; Koushika et al., 2000; Okabe et al., 2001; Lowe et al., 2003; Okano et al., 2005; Denes et al., 2007). Due to the widespread conservation of expression and function of *ELAV* and *msi* in bilaterians, we investigated their potential role during embryonic development in *N. vectensis*.

Out of several related RNA-binding proteins found in the *N. vectensis* genome, one NvMsi homolog falls

within a monophyletic group of previously identified NvMsi proteins in Bayesian phylogenetic analyses (Supp. Info. Fig. 3), and when aligned with other NvMsi protein sequences clearly shares both RNA binding motifs (RRMs) with them (Supp. Info. Fig. 5). RNA binding proteins like MSI and ELAV regulate gene expression post-transcriptionally by interacting with the 3' UTR of mRNA and regulating the stability, splicing, and translation of the targeted gene (Sakakibara and Okano, 1997; Keene, 1999; Koushika et al., 2000; Okano et al., 2002; Okano et al., 2005; Siddall et al., 2006; Zhu et al., 2006). NvMsi mRNA can be detected by *in situ* hybridization in developing oral and tentacle bud ectoderm of the planula and later in pharyngeal ectoderm and scattered cells of the tentacle ectoderm of the juvenile polyp [Fig. 5(A–D)].

Two *ELAV*-like RNA-binding proteins identified in *N. vectensis* cluster with *ELAV/Hu* genes identified from other metazoans using phylogenetic analyses (Supp. Info. Fig. 3). Predicted proteins of the NvELAV1 and NvELAV2 genes both share the three RRM motifs common to all *ELAV/Hu* family genes (Good, 1995; Yim et al., 2006) and do not appear to be recent duplications as demonstrated by their relationship to bilaterian *ELAV* genes (Supp. Info. Fig. 4). *In situ*

Figure 5 Early onset of expression in the RNA-binding proteins *Musashi* and *ELAV*. (A, B) *Msi* is expressed in the oral ectoderm of the planula stage in epithelial cells which contact both the surface of the embryo and the endoderm. (C, D) *Msi* expression is restricted to the developing tentacles by the polyp stage and the morphology of cells which express *Msi* shifts from those, which span the ectoderm to cells, which are found only at the base of the ectoderm. Indicating a change in position of expressing cells or that a different cell population now expresses *Msi*. (E) *ELAV* expressing cells are found throughout the gastrula and early planula following gastrulation in cells which span the ectoderm. (F) By the late planula stage, *ELAV* expression is predominantly endodermal with some cells at the base of the ectoderm expressing transcripts. (G) Cells in the endoderm (en) and at the base of the ectoderm (ec) in the tentacle express *ELAV*. (H) Expression is almost entirely restricted to the endodermal components of the body column and tentacles. Levels and white balance of images were adjusted in Adobe Photoshop.

Figure 6 Neural cell adhesion molecule (NCAM)-like gene expression in *N. vectensis*. (A, B) NvNCAM1 is expressed in the future aboral ectoderm of the late blastula and early gastrula but not invaginating endoderm. (C, D) NvNCAM1 is expressed in body wall ectoderm of the late planula and polyp stage and is not expressed in the apical tuft (at) of the planula (E). (F, G) NvNCAM2 is expressed in early cleavage stage embryos and in cells of both the endoderm and ectoderm of planula. (H–J) Late planula and polyp stages continue to express NvNCAM2 in scattered cells in the ectoderm and many cells in the endoderm. Expression in the pharynx, along mesenteries and tentacle tips is pronounced. (K) NvNCAM3 is not detected prior to gastrulation. (L) NvNCAM3 is expressed at the onset of gastrulation in a small number of cells at the future aboral pole. (M, N) In planula stages it is expressed at the apical tuft. (O) Following settlement, expression in the polyp is exclusively endodermal. (P–T) NCAM4 is expressed pan-endodermally in the early planula through the polyp. (U) Paralogy relationships between Ig domains (left) and FN3 domains (right) of the NvNCAM genes. Bootstrap values >0.5 label branches. (V) Domain structure of NvNCAM predicted proteins and genomic organization of linked genes NvNCAM1, NvNCAM3, and NvNCAM4. Levels and white balance of images were adjusted in Adobe Photoshop.

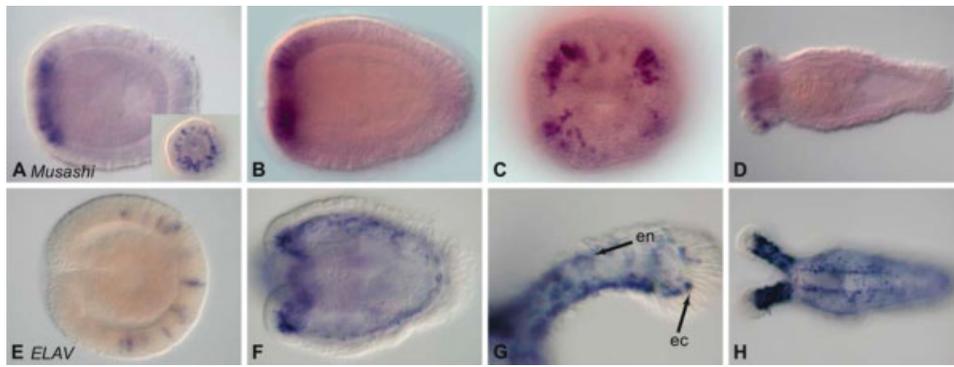


Figure 5

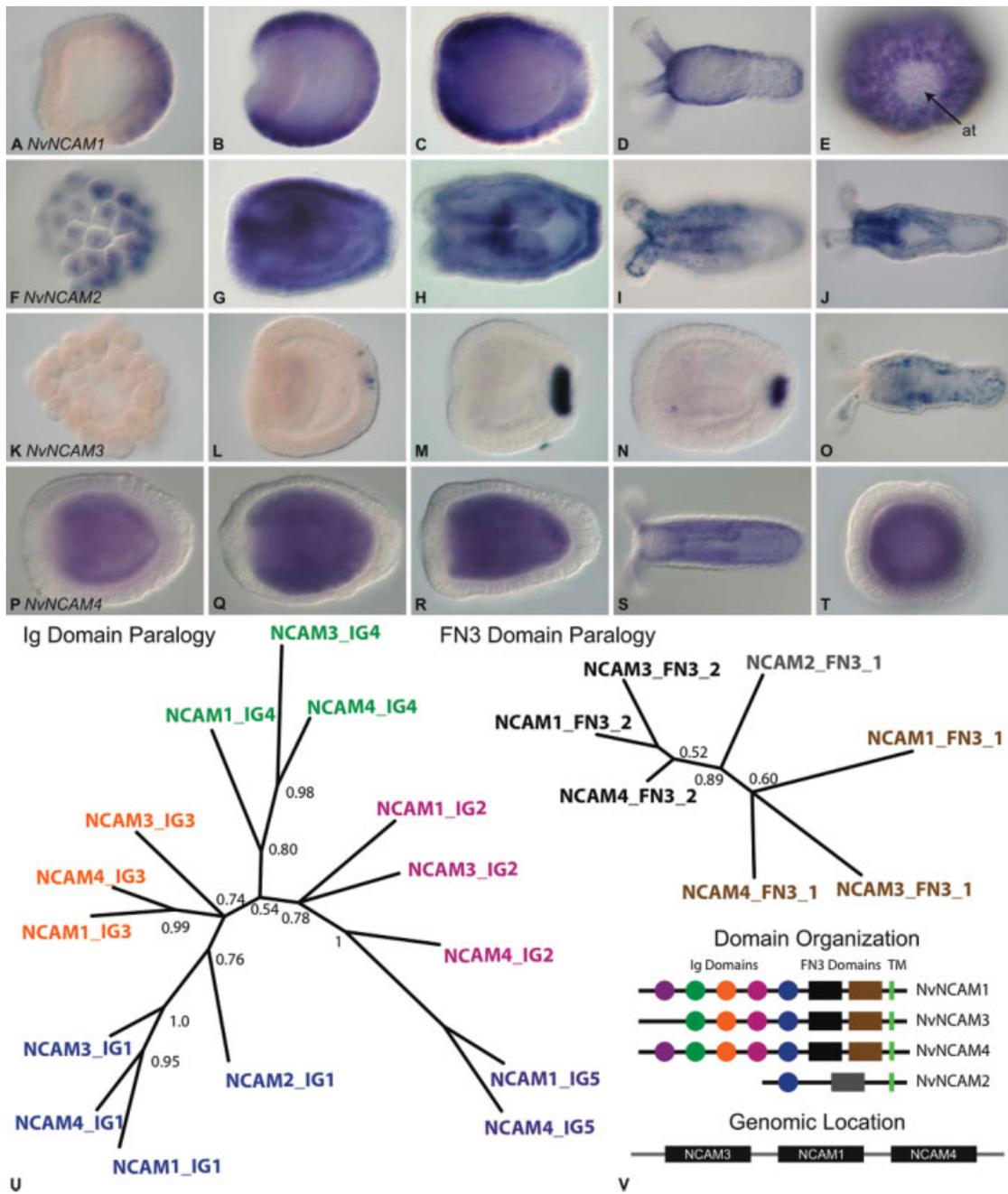


Figure 6

hybridization detects *NvELAV1* mRNA expression in scattered ectodermal cells at the aboral pole shortly after the onset of gastrulation [Fig. 5(E)]. This precedes the expression, but corresponds to the location of the earliest expressed classical markers of neural differentiation such as *NvNCAM1* and *NvNCAM3* (see below), *SoxB1* and *SoxB2* (Magie et al., 2005). Transcript expression expands to a large number of endodermal cells and to ectodermal cells in the body wall and tentacles [Fig. 5(F–H)] in the planula and polyp stages. Although we were able to clone *NvELAV2* from mixed stage pools of *N. vectensis* cDNA, we were not able to detect its expression by *in situ* hybridization.

Neural Cell Adhesion Molecule-Like Genes of the Ig Superfamily in *N. vectensis*

We have identified four immunoglobulin superfamily (Ig) genes in *N. vectensis*. Members of the Ig superfamily share the conserved Ig domain (often repeated) (Walsh and Doherty, 1997) and have been identified throughout bilaterian animals (Mayford et al., 1992; Vogel et al., 2003; Fusaoka et al., 2006), and are found to serve in various aspects of neural development (Rougon and Hobert, 2003; Maness and Schachner, 2007). Phylogenetic analyses place all four genes within the *NCAM* family to the exclusion of other Ig families (Supp. Info. Fig. 3). Domain architecture of three (*NvNCAM1*, *NvNCAM3*, and *NvNCAM4*) of the four *N. vectensis* immunoglobulin genes, consisting of 4–5 Ig domains followed by two FnIII domains is consistent with placement within the *NCAM/Fasciclin II* family [Fig. 6(V)]. These three genes are found linked within a 75 kb region of scaffold 47 in the recently sequenced *N. vectensis* genome (v1.0). The fourth gene (*NvNCAM2*) contains only one Ig domain and one FN3 domain and occupies a different genomic location in relation to the three *NCAM*-like genes.

NCAMs may be expressed in a variety of tissues including muscle, glia, and neurons (Walsh and Doherty, 1997), but are primarily expressed in the nervous system and play conserved roles in synapse formation, neural migration, axon pathfinding, and vesicle cycling (Walsh and Doherty, 1997; Rougon and Hobert, 2003; Maness and Schachner, 2007). NCAMs, as cell adhesion molecules, regulate cell–cell and cell–ECM interactions, and through both hetero and homophilic interactions can activate second messenger cascades (Walsh and Doherty, 1997; Rougon and Hobert, 2003). Like the *NCAMs* of *Aplysia* and vertebrates, *NvNCAM1* has an mRNA expression

domain, which encompasses what we hypothesize to be both neurons, and nonneural cells of the ectoderm [Fig. 6(A–E)]. In contrast, *NvNCAM3* is expressed in the apical tuft, a neuralized chemosensory structure, and in what are likely to be components of the endodermal nerve net (based on immunostaining with antibodies to neurotransmitters) [Fig. 6(K–O)]. Interestingly, the expression of *NvNCAM3* overlaps with that of the FGF ligand *NvFGF1a* and the FGF receptor *NvFGFRa* (Matus et al., 2007b). Components of these two pathways are known to interact to regulate the growth of developing neurons (Maness, 2007). In contrast to *NvNCAM1* and *NvNCAM3*, *NvNCAM4* is expressed pan-endodermally in a non-neurally restricted manner [Fig. 6(P–T)]. *NvNCAM1* and *NvNCAM4* are sister paralogs (Supp. Info. Fig. 3), share an identical domain structure [Fig. 6(V)] and their predicted proteins are more distantly related to bilaterian NCAM proteins than the other *NvNCAMs*. *NvNCAM1* and *NvNCAM4* also show the most widespread expression. *NvNCAM2* is expressed by the early cleavage stage where it is localized in the nucleus of cells and is expressed throughout the early planula. Expression is later restricted to a large number of scattered cells in the endoderm as well as a few ectodermal cells. Although its genomic location and domain structure differ from *NvNCAMs1*, 3, and 4, the expression of *NvNCAM2*, *NvNCAM1*, and *NvNCAM3*, is consistent with a role in the nervous system [Fig. 6(F–J)]. As three of the four *NvNCAMs* are linked and possess similar domain architecture, we hypothesize that *NvNCAMs1*, 3, and 4 originated as the result of a duplication event from an ancestral *NCAM*-like gene. *NvNCAM1* and 3 maintain roles in patterning domains of the nervous system and *NvNCAM4* has subsequently diverged in its expression and potential role in development.

Bilaterian Glial and Neural Determinants in *N. vectensis* Development

Subsets of neural precursor cells in the developing nervous systems of both vertebrates (Wakamatsu, 2004) and invertebrates (Hosoya et al., 1995) have the potential to generate either neurons or glial cells. It is plausible that regulators of glial fate such as *GCM* may have initially evolved as more general regulators of neural precursor differentiation as evidenced from studies in chick and fly (Soustelle et al., 2007). To investigate the ancestral roles of bilaterian glial-specific genes in neural patterning, we looked for the expression of these genes in *Nematostella*.

Determination of the glial cell fate and subsequent differentiation in flies is regulated by glial cells miss-

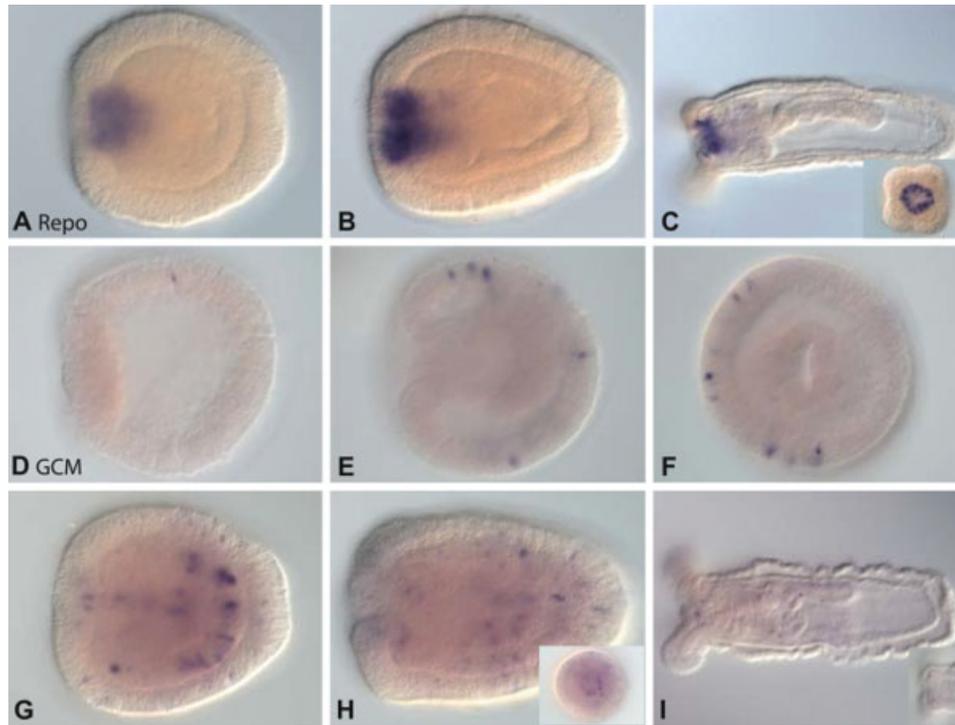


Figure 7 Bilateral glial genes are expressed in developing *N. vectensis* embryos. *Repo* expression during *N. vectensis* development. (A) *Repo* expression in the oral ectoderm of an early planula. (B) *Repo* expression remains in oral ectoderm in the late planula and in the early polyp (D) and late polyp (E) stages. (C) An oral view of *repo* expression in the ectoderm surrounding the mouth, the oral nerve ring. (G–L) Expression of the transcription factor GCM is restricted to a subset of cells in *N. vectensis* development. (G–I) Expression is in a small number of future ectoderm (early gastrula) and ectodermal cells (gastrula) during gastrulation. (J, K) Expression expands to include both ectodermal and endodermal cells of the planula, including an oral ring of expression. (L) Expression in the polyp includes ectodermal expression in the pharynx and mesenteries as well as endodermal cells which underly the oral nerve ring. Levels and white balance of images were adjusted in Adobe Photoshop.

ing (GCM) through the induction of glia-specific genes such as *pointed*, *tramtrack69*, and *repo* in flies (Hosoya et al., 1995; Yuasa et al., 2003; Lee and Jones, 2005), whereas in vertebrates it appears that *notch/delta* signaling in combination with glial inducing genes such as *neuregulin1* and *olig2* preferentially promote glial cell fates (Wakamatsu, 2004; Jessen and Mirsky, 2005). In both systems, it appears that a combination of positive regulation of glial fate and the negative regulation of neural fate is required for proper glial specification (Hosoya et al., 1995; Wakamatsu, 2004).

One glial specific gene in flies that has been shown to act in part by suppression of the neural fate is *repo* (Yuasa et al., 2003). *Repo* is a paired class homeodomain protein, which is found exclusively in a large subset of glial cells in flies (Xiong et al., 1994). We identified an ortholog of *repo* in *N. vectensis* indicating that the cnidarian-bilaterian ancestor possessed a

repo-like gene, and that this gene was later lost in the lineage that gave rise to vertebrates. *Repo* is expressed orally in the planula and polyp stages in the pharyngeal ectoderm at the site of the oral nerve ring [Fig. 7(A–C)].

Although *GCM*, the “genetic switch” that determines the glial cell fate, for the majority of glia in flies has not been shown to have a role in gliogenesis in vertebrates, it does play a role in the regulation of the neural fate (Soustelle et al., 2007). *GCM* was also identified in *N. vectensis* and, like *repo*, is expressed in a few cells of the oral ectoderm of the planula and in the endodermal portion of the oral nerve ring of the polyp [Fig. 7(K,L)]. *GCM* also has expression domains that do not overlap with *repo* in the gastrula and planula stages in scattered cells of the endoderm and ectoderm. This expression is coincidental with the appearance of other early neural markers such as *ELAV* and *Pax* genes and is likely to represent

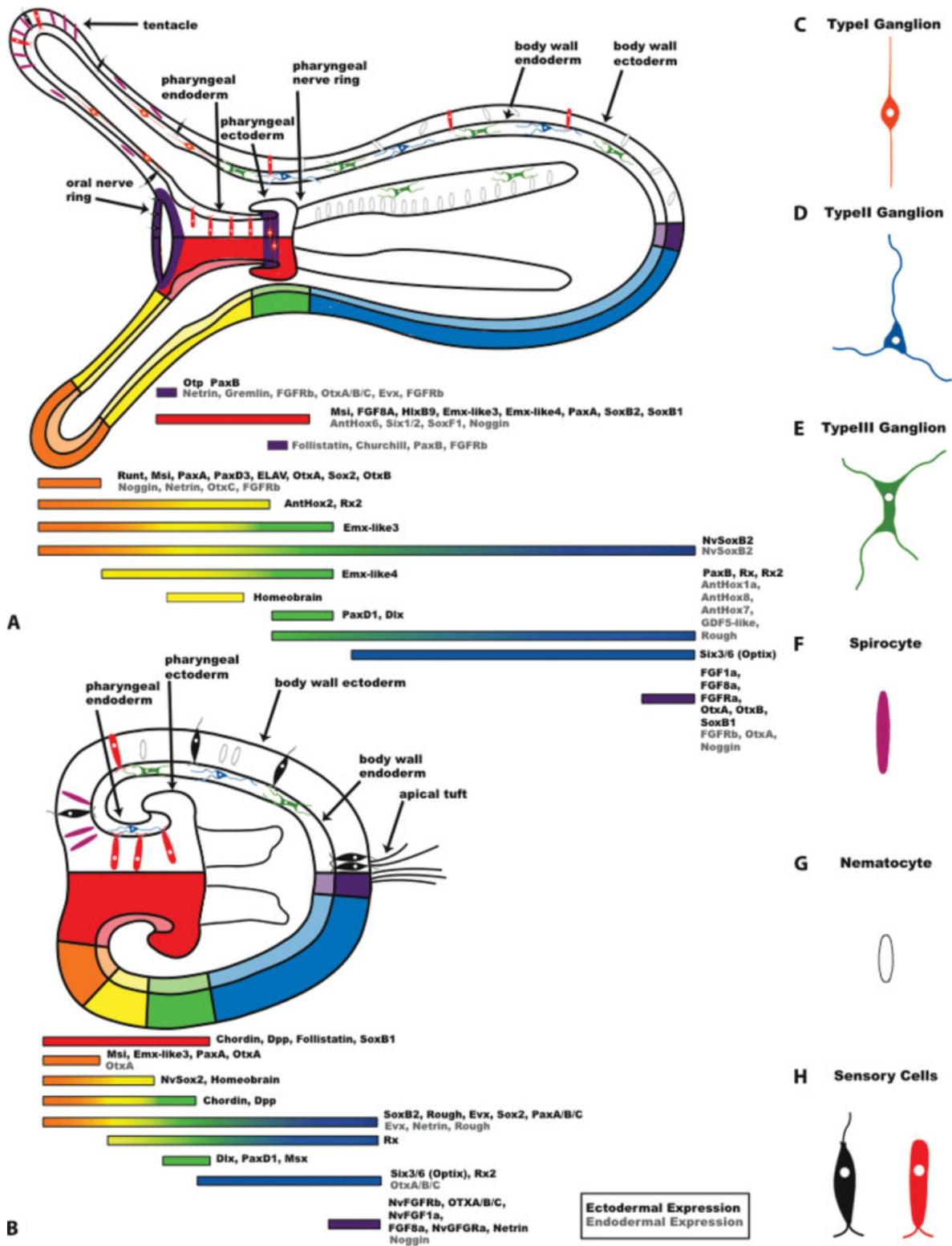


Figure 8

expression in predifferentiated neurons. Examining the role of *GCM* and *repo* in the early branching lineage giving rise to cnidarians provides insight into the ancestral roles of these genes. As glia have not been identified in basal metazoans, the ancestral role of *GCM* and *repo* are likely to be as regulators of differentiation in subsets of neurons, consistent with their expression in *N. vectensis*.

DISCUSSION

Here, we report a complex neural morphology for the anthozoan anemone, *N. vectensis*. Individual neural populations are organized along the oral–aboral axis and in discrete structures that are readily correlated to the behavior and life history of the animal. Cnidarians and ctenophores are likely the first metazoans to have evolved a recognizable nervous system. Anthozoan cnidarians (corals, sea pens, and anemones) represent the earliest branching class of cnidarians (Collins et al., 2006). Consistent with previous studies of anthozoans, we find the neural anatomy of the anemone *N. vectensis* to be considerably more complex than that of the classical model for cnidarians, *Hydra*. This morphological organization is accompanied by the similarly complex expression of molecular markers for neural specification and patterning. In addition to describing the neural anatomy, we report the origins and development of neural structures over the course of developmental time, information that has been often overlooked in investigations of Cnidaria. The significant morphological and molecular complexity of the neural system of *N. vectensis*, the absence of the seemingly derived “i” cell populations identified as progenitors in *Hydra*, and the evolutionary distance (~550 my) between hydrozoans and anthozoans as demonstrated by fossil (Chen et al., 2002) and molecular (Putnam et al., 2007) evidence highlights the necessity of this new developmental model and promises to lend considerable insight into evolution of

neural cell types and stem populations in both the cnidarians and early metazoans.

This study indicates that *N. vectensis* possesses a diverse assembly of neural elements, consisting of multiple classes of neural cells and cnidocyte classes. These can be identified based upon their morphology and neuropeptide or neurotransmitter expression and are localized to restricted domains in the endoderm and ectoderm and arise at specific developmental stages. Previous work, including the discovery of both dense and clear core vesicles (Kass-Simon and Pierobon, 2007), immunostaining, biochemical analyses, and behavioral response studies (Westfall et al., 2000; Grimmelikhuijzen et al., 2002; Kass-Simon and Pierobon, 2007) has suggested that cnidarians possess both chemical neurotransmitters (Grimmelikhuijzen and Graff, 1986; Westfall et al., 2000; Gillis and Aentil, 2001), as well as an abundance of neuropeptides (Grimmelikhuijzen et al., 2002; Kass-Simon and Pierobon, 2007). Here, we show evidence for the presence of monamines (5HT and dopamine synthesis pathway components), GABA, and neuropeptides in the two main classes of previously identified cnidarian neurons; sensory and ganglion cells, and in cnidocytes (Grimmelikhuijzen et al., 2002; Koizumi, 2002). As in other cnidarians, these transmitters can be associated with different subsets of the cnidocyte assemblages (anti-5HT reactive nematocytes versus anti-FMRamide reactive spirocytes) as well as GABA and FRMF-amide positive sensory and ganglion cells (Koizumi, 2002; Koizumi et al., 2004). Further spatial restriction of the neurotransmitter synthesis component dopamine beta hydroxylase pharyngeal ectoderm) and the specific RFamide family member Antho-RFamide (oral region and physa/foot region as in *Hydra*) suggests that expression profiles differentiate many neurons that may appear indistinguishable morphologically. These distinct neural elements are differentially positioned along both the oral–aboral axis and between the endoderm and ectoderm. Analyses of the genome of *N. vectensis* indi-

Figure 8 Developmental transcription factors, axial patterning genes and neural architecture. Expression domains of neural markers and developmental genes in *N. vectensis* planula and polyp stages. (A) A polyp stage with neural morphology shown in the upper portion of the diagram. The oral and pharyngeal nerve rings are highlighted in purple. Associated patterns of gene expression are shown in the lower region of the diagram. (B) A planula stage with neural morphology shown in the upper portion of the diagram and associated gene expression patterns in the lower region of the diagram. (C–H) Individual neural and cnidocyte morphologies shown schematically. Three ganglion, two cnidocyte, and two sensory type cells are represented. Developmental gene expression is drawn according to data presented in this paper and that previously published (Magie et al., 2005; Matus et al., 2006a,b; Matus et al., 2007a,b; Mazza et al., 2007; Ryan et al., 2007; Rentzsch et al., 2008), and currently unpublished data *Emx*-like genes (Matus et al., in prep) and *Homeobrain* and *Rx2* (Martindale et al., in prep).

cate the presence of cholinergic, adrenergic, and catecholamine pathways as well as several characteristic cnidarian propeptide families, and suggest an even larger number of specialized cell types (Supp. Info. Fig. 4).

Although no distinct CNS or ganglia are present in anemones, *N. vectensis* has specialized neural structures with distinct populations of neurons located at the pharyngeal nerve ring, the oral nerve ring, innervating the mesenteries, and at the tentacle tips (see Fig. 8). Furthermore, stage specific structures, such as the apical tuft, possess additional neural subpopulations. Each neural subset in *N. vectensis* is likely to carry out its own function and may be related to behaviors such as feeding, coordination of swimming behavior (in larvae), defense or contraction for locomotion (burrowing and creeping) (Hand and Uhlinger, 1992; Williams, 2003). Cnidarian nervous systems form both local and multineuron circuits (Westfall, 2004) that may regulate behaviors such as feeding. Morphologically similar cells, such as ganglion cells, often express different neurotransmitters or a combination of transmitters (Grimmelikhuijzen et al., 2002; Koizumi et al., 2004; Westfall, 2004). These findings indicate that cnidarians do not possess a simple homogeneous nerve net but rather have complex subfunctionalized cell types in both ectodermal and endodermal tissue layers. This organization is dependent upon both cell type specific and position specific developmental cues for both an ectodermal and endodermal nerve net. Such sub-functionalization of the nerve net requires similarly complex molecular patterning.

Cnidarians have a central body axis (the oral–aboral axis) and a nerve net, but no nerve cord or distinct brain, yet many of the conserved bilaterian A-P neural patterning genes are expressed in distinct patterns along the cnidarian oral–aboral axis (Kusserow et al., 2005). These expression domains in many cases correspond to what we have identified as distinct neural structures (oral nerve ring, pharyngeal nerve ring, apical tuft, and tentacle tips).

Paired homeodomain genes, four classes of cnidarian pax genes *PaxA-D* as well as an *Rx* gene have previously been identified in *N. vectensis* (Matus et al., 2007a) and the coral *Acropora millepora* (Miller et al., 2000). Expression patterns of four classes of *Pax* genes in *N. vectensis* (which correspond to the three bilaterian pax classes *Pax2/5/8*, *Pox neuro*, and *Pax3/7*), demarcate distinct regions encompassing a large portion of the oral–aboral body axis. *PaxB* (The bilaterian *Pax2/5/8* ortholog) is expressed in an endodermal ring at the base of the pharynx (Matus et al., 2007a) corresponding to the location of the pharyngeal nerve ring. *Pax* genes, i.e., *Pax 2/5/8*, in bilaterian ani-

mals have been determined to be involved in the maintenance of boundaries in the developing nervous system, such as the midbrain-hindbrain boundary in vertebrates. The paired homeodomains *repo* and *NvPaxB* demarcate discrete structures, the oral and pharyngeal nerve rings, respectively [Fig. 8(A)].

TGF-beta and FGF signaling pathways are responsible for the promotion of neural fate through regulation of neural inhibitors. Expression of the *TGF-beta* antagonists *folliculin* (endodermal pharyngeal ring), *gremlin* (endodermal oral ring), and *noggin* (tentacle tips and pharynx) overlap with neuralized regions of *N. vectensis* in both the planula and polyp stage. This in addition to a conserved function of *NvGremlin* and *NvChordin* to block the ventralizing activity of *NvDpp*, respectively (Rentzsch et al., 2006). Expression of the TGF-beta antagonists and the dorsalizing activity of *NvChordin* and *NvGremlin* indicate that this pathway has a conserved role in axial (and possibly neural) patterning along a central body axis. FGF ligands and receptors are also localized in the main planula sensory structure, the apical tuft (Matus et al., 2007b; Rentzsch et al., 2008), and appear to regulate the boundaries of this structure (Rentzsch et al., 2008). NCAMs have been shown to interact with FGF receptors and regulate growth of neural processes (Maness and Schachner, 2007), and we hypothesize based on the role of FGFs for apical tuft formation (Rentzsch et al., 2008), and the presence of NCAM3 transcripts in this region that it is likely that a similar interaction occurs in *N. vectensis*. Thus, it appears likely that many of the bilaterian patterning pathways not only existed in the cnidarian-bilaterian ancestor, but were deployed to generate complexity of neural elements.

The organization of elements of the *N. vectensis* nervous system change over developmental time. Biphasic gene expression patterns are particularly common in *N. vectensis*. This shift in expression can be correlated with the change in the direction of locomotion of developmental stages: planula and newly settled polyps swim with the aboral end forward, whereas adult polyps creep with the aboral end trailing (Hand and Uhlinger, 1992). Cnidarian *HOX* gene expression (Finnerty et al., 2004) and the biphasic expression patterns of genes, such as the *OTX* genes (Mazza et al., 2007), are best considered with respect to cnidarian-specific traits including the endodermal component of the nerve net and the individual larval (apical tuft) and adult (tentacle tips, pharyngeal, and oral nerve rings) neural structures. It, therefore, appears that many of the same genes required for cell type specification are redeployed in distinct regions at different life history stages.

The developmental origin of neural stem cells and neural progenitor cells is still an open question in cnidarians. A significant amount of work surrounding the stem cell population of hydrozoans (i cells) has been conducted, their embryonic origin, and the molecular signals directing their specification and differentiation have yet to be worked out. As a first attempt at identifying potential stem or progenitor populations, we have utilized widely conserved molecular markers of developing bilaterian neurons. *ELAV* and *Msi* act as markers for early neurons or progenitors even in cases when developmental processes and pathways leading to neural specification among these organisms differ, making them particularly promising candidates for the identification of developing neural populations in many distantly related taxa (Robinow and White, 1991; Good, 1995; Kawashima et al., 2000; Perrone-Bizzozero and Bolognani, 2002; Lowe et al., 2003). *GCM*, while serving a dominant role in the decision of a cell to become a neuron or a glia in bilaterian taxa, also plays a role in determining whether a cell will differentiate into a neuron or remain in an undifferentiated state (Soustelle et al., 2007). It is likely that regulators of glial fate such as *GCM* may have initially evolved as more general regulators of cell fate as can be extrapolated from its many roles in vertebrate and fly development (Anson-Cartwright, 2000; Soustelle, 2007). In *N. vectensis*, *ELAVI* and *GCM* undergo a marked shift in expression from their early ectodermal expression domains in the gastrula to a predominantly endodermal pattern in the late planula and polyp. This shift may indicate that ectodermal neurons are specified prior to endodermal populations or that ectodermal populations present in planula larvae are replaced by an adult nervous system as has been recently described in scyphozoan jellyfish (Nakanishi, 2008 no. 2192). In contrast, *Msi* expression is limited to the ectoderm of tentacle precursors and the tips of tentacles in polyps. *Msi* regulates the division of neural precursor cells through repression of the neural inducer *tramtrack* (*ttk*) in *Drosophila* (Siddall et al., 2006), and the maintenance of neural stem cells by repressing the Notch signaling antagonist *numb* (Imai et al., 2001). *NvMsi* expression, particularly in the planula stage is coincident with expression with *NvNotch* (Marlow and Martindale, in preparation), and as *ttk*, the target of *Msi* in *Drosophila*, has not been identified in the *N. vectensis* genome, we hypothesize that *Msi* may act to maintain a specialized subset of neural precursors by way of a vertebrate-like molecular mechanism. Functional analyses are needed to test this model.

Nematostella vectensis has not been shown to possess a large number of migratory neurons in adult animals or an interstitially restricted stem population, but rather displays expression of neural progenitor markers throughout the body as well as in both ecto and endodermal tissue. We propose that neurogenesis occurs through a significantly different route than that described for the model *Hydra*, wherein stem populations are spatially restricted, migration of precursors occurs, and position dependent cues subsequently determine identity. Examination of neural precursor markers reveals a striking trend in the shift from ectodermal expression early in development to endodermal expression later in development (with the exception of a small *Msi*-regulated population). This pattern is indicative of either a scenario in which neural stem cells/progenitors are specified in the ectoderm and later migrate locally to an endodermal position or of a system in which two independent stem cell lineages (endodermal and ectodermal) are specified. To test these models, additional experiments to test the function of these stem cell markers as well as cell lineage experiments to test for migratory cells in early embryos are needed.

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