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The cellular and molecular basis of cnidarian neurogenesis

Fabian Rentzsch,^{1*} Michael Layden² and Michaël Manuel³

Neurogenesis initiates during early development and it continues through later developmental stages and in adult animals to enable expansion, remodeling, and homeostasis of the nervous system. The generation of nerve cells has been analyzed in detail in few bilaterian model organisms, leaving open many questions about the evolution of this process. As the sister group to bilaterians, cnidarians occupy an informative phylogenetic position to address the early evolution of cellular and molecular aspects of neurogenesis and to understand common principles of neural development. Here we review studies in several cnidarian model systems that have revealed significant similarities and interesting differences compared to neurogenesis in bilaterian species, and between different cnidarian taxa. Cnidarian neurogenesis is currently best understood in the sea anemone *Nematostella vectensis*, where it includes epithelial neural progenitor cells that express transcription factors of the soxB and atonal families. Notch signaling regulates the number of these neural progenitor cells, achaete-scute and dmrt genes are required for their further development and Wnt and BMP signaling appear to be involved in the patterning of the nervous system. In contrast to many vertebrates and *Drosophila*, cnidarians have a high capacity to generate neurons throughout their lifetime and during regeneration. Utilizing this feature of cnidarian biology will likely allow gaining new insights into the similarities and differences of embryonic and regenerative neurogenesis. The use of different cnidarian model systems and their expanding experimental toolkits will thus continue to provide a better understanding of evolutionary and developmental aspects of nervous system formation. © 2016 The Authors. *WIREs Developmental Biology* published by Wiley Periodicals, Inc.

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INTRODUCTION

Cnidarians are an early offshoot in the evolution of animals, having separated from the lineage that led to the emergence of bilaterians more than

600 million years ago.¹ Their importance for understanding the evolution of nervous systems has long been recognized, based both on their phylogenetic position as an outgroup to bilaterians (Figure 1) and on the structure of their nervous system, which is predominantly organized as nerve nets. The cnidarians comprise two major clades, the anthozoans and the medusozoans (Figure 1),^{2,3} which separated before 550 million years ago and thus not long after the cnidarian lineage separated from all other animals.⁴ Two distinct body forms can be found in cnidarians: sessile polyps, which are tube-shaped animals with a single terminal body opening (called the mouth) surrounded by prey-catching tentacles;

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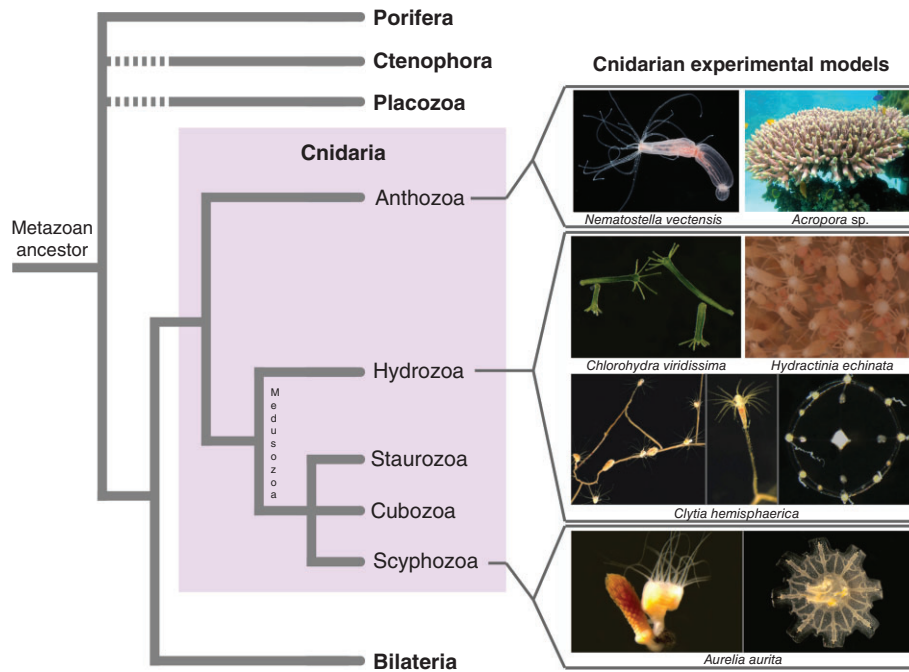


FIGURE 1 | Position of cnidarians in the animal tree of life and phylogenetic diversity within the phylum, with a selection of experimental models used for developmental biology studies. Photo credits: *Nematostella vectensis*: Chiara Sinigaglia; *Clytia hemisphaerica* (medusa): Michaël Manuel; *Acropora* sp.: www.aquaportal.com; *Chlorohydra viridissima* and *Aurelia aurita*: Thomas Condamine; *Hydractinia echinata*: Uri Frank; *C. hemisphaerica* (polyps): Muriel Jager.

and free-swimming medusae, which swim by rhythmic contractions of their bell. Polyps are present in both cnidarian clades, while medusae are only present in the medusozoans (Figure 1), suggesting that a polyp stage was present in the last common ancestor of cnidarians, whereas parsimony favors the evolution of the medusa stage after the separation of the two clades. The nervous system of polyps can in first approximation be described as a nerve net with more or less pronounced regionalisation, including in some taxa instances of local condensations of neurites (in the form of nerve cords or nerve rings), for example in the oral region, or along the internal musculature-bearing mesenteries.^{5–7} Medusae often possess sensory organs, in particular eyes and gravity sensors (lacking in polyps), associated with a considerable degree of centralized signal processing, best understood in hydrozoan medusae where it involves two peripheral and parallel nerve rings.^{8–11} These sense organs and CNS-like nervous system elements offer an excellent opportunity to study the independent evolution of advanced signal receiving and integrating structures and how they control the behavior and the unique mode of locomotion of jellyfish (see also Boxes 1 and 2).

In cnidarians, the nerve cell concept embraces three different but related classes of cells. Sensory

(or sensory-motor) cells generally have an elongated cell body, and always bear an apical cilium that emerges at the body surface. Ganglion cells have their cell body located in a deep, basi-epithelial position; they are often considered equivalent to interneurons, but they can also synapse on muscle cells and nematocytes.¹² Nematocytes are the cnidarian-specific stinging cells, characterized by a complex intracytoplasmic capsule (nematocyst) housing a coiled tubule, and an apical sensory ciliary cone. That nematocytes are modified nerve cells is supported by a vast array of data relating to their neurophysiological properties, ultrastructural features, and expression of neurogenic genes in nematocyte precursors.^{13,14} Sensory cells, ganglion cells, and some nematocytes bear neurites and establish synaptic contacts with other cells. Both morphological and molecular observations show that each of the three general neural cell types consists of several or many subtypes that can be characterized for example by the number of neurites or the expression of various neuropeptides.^{15,16} Glial cells have not been identified in cnidarians.

Today, several cnidarian model species can be reared in the laboratory to provide access to embryos and the availability of new molecular tools allows addressing longstanding questions about the development and architecture of their nervous system

BOX 1

SENSE ORGANS IN CNIDARIANS

In the medusae of scyphozoans and cubozoans, the multifunctional neuro-sensory organ known as the rhopalia (which is a highly modified tentacle) contains at least seven groups of sensory cells arranged in complex bilateral pattern.¹⁵⁰ The rhopalia nervous system is primarily ectodermal and its development is a highly ordered process.¹⁵¹ Rhopalia are involved in gravity sensing, chemoreception, and in some cases, photoreception. Hydrozoan medusae do not have rhopalia; they either lack sensory organs or possess isolated statocysts (equilibration organs) and in some instances, simple eyes (e.g., in *Cladonema*).

Apical organs in anthozoan planulae are characterized by a tuft of long cilia, which are thought to have a chemo- and/or mechanosensory function that might be required for the transition from planula to polyp stage.^{36,140} The tuft region, however, does not contain neurons^{6,152} and apical tuft formation in *Nematostella* is not affected by knockdown of *NvSoxB* (2) or *NvAth-like*.^{79,89} Developmentally, there is thus no evidence that the cells of the apical tuft region are part of the nervous system.

(Table 1). Transgenic reporter lines can be used to label the nervous system in unprecedented detail and to trace the progeny of cells that express a given gene at a particular time point.^{6,25,30,35} Inhibition of gene function by RNA interference or morpholino antisense oligonucleotides has been established and can be complemented by overexpression of *in vitro* synthesized mRNAs.^{23,26,31,36–38,43} Heritable genome manipulations by TALENs and the CRISPR/Cas9 system have been reported³⁹ and the availability of genome and transcriptome resources allows analyzing the effects of gene manipulations and identifying new regulators of neural development beyond candidate genes.

In this review, we focus on the generation of neurons and the patterning of the nervous system in cnidarians during embryogenesis and in adult polyps and we briefly discuss aspects of the establishment of neural connectivity. Among cnidarian model organisms, neural development is currently best understood in the anthozoan *Nematostella vectensis*, and accordingly this sea anemone is central to our discussion on the cellular and molecular basis of neurogenesis, particularly during embryonic development.

BOX 2

NEUROGENESIS IN MEDUSAE

In hydrozoan medusae, nematogenesis appears restricted to the manubrium and the tentacle bulbs.¹⁵³ Tentacle bulbs are basal swellings of the tentacles, attached to the medusa bell periphery. Stem cell marker genes are expressed in two restricted symmetrical areas at the proximal extremity of the tentacle bulb ectoderm (presumably populations of interstitial stem cells) in *Clytia hemisphaerica*^{76,153} and in *Podocoryne carnea*.¹⁵⁴ There is some degree of spatial sorting of nematogenesis stages along the tentacle bulb axis, reflected by staggered expression of nematogenesis genes associated with different stages from stem cells to early differentiation and maturation (the latter restricted to the distal third of the bulb).^{24,77,153} Therefore, the tentacle bulb ectoderm behaves as a cellular conveyor belt,^{155,156} which can facilitate experimental approaches to understand the temporal progression of nematocyte development. Formation of sensory and ganglion cells has not been described in hydrozoan medusae.

THE GENETIC TOOLKIT FOR NEUROGENESIS IS CONSERVED IN CNIDARIANS

The genetic toolkit that controls the generation of nerve cells and the patterning of the nervous system in bilaterian animals is represented in cnidarian genomes by an almost full repertoire of orthologues. A few families of neurogenic genes are of more ancient origin than the metazoan lineage itself as deduced from their presence in unicellular holozoan genomes, e.g., several families of bZIP transcription factors (TFs) including CREB; putative members of the paired-like, POU and LIM classes of homeoboxes, and of the Sox/TCF family of TFs.^{44,45} However, the vast majority of genes with regulatory functions in neural development of bilaterians belong to gene families that were established either in a common metazoan ancestor after divergence of choanoflagellates but before divergence of sponges, or in an exclusive ancestor of cnidarians and bilaterians after divergence of sponges^{46,47} (leaving here apart ctenophores and placozoans, whose position in the animal tree

TABLE 1 | The Experimental Toolkit for Cnidarian Model Systems

Species	Genome/ Transcriptome	Gain-of- Function	Loss-of- Function	Trans- genics	Other	Refs
<i>Acropora millepora</i>	No/yes	No	No	No		17–19
<i>Aurelia aurita</i>	In progress/yes	No	dsRNA	No		20–22
<i>Clytia hemispherica</i>	In progress/yes	mRNA, plasmid	MO	No		23,24
<i>Hydra magnipapillata</i>	Yes/yes	Transgene	RNAi	Yes	FACS	25–29
<i>Hydractinia echinata</i>	In progress/yes	Transgene	MO, RNAi	Yes	FACS	30–34
<i>Nematostella vectensis</i>	Yes/yes	mRNA, plasmid	MO	Yes	BiFC, histone modifications, CRISPR/ Cas9, TALEN	35–42

Note that references for transcriptome resources are not comprehensive.

BiFC, bimolecular complementation fluorescence; CRISPR, clustered regularly interspaced short palindromic repeats; FACS, fluorescence activated cell sorting; MO, morpholino antisense oligonucleotide; TALEN, transcription activator-like endonucleases.

remains uncertain, Figure 1). Conservation of the neurogenic toolkit between cnidarians and bilaterians extends to all functional gene categories from TFs (see comprehensive lists compiled in Refs 14,47,48) and components of signaling pathways (Notch, Wnt, TGF- β , FGF, Hedgehog, and Jak/Stat) to post-transcriptional regulators acting at the mRNA level (e.g., Elav and Musashi).^{6,49}

As a general rule, multigenic families of neurogenic genes diversified before the last common ancestor of cnidarians and bilaterians, but many of them underwent further diversification within the bilaterian lineage(s). As a result, there are examples of well-known bilaterian neural development genes with no strict orthologue in cnidarians, such as Neurogenin and NeuroD (bHLH TFs),⁵⁰ SoxD (HMG domain-containing TFs),^{46,51,52} or Engrailed (Antp-class homeodomain-containing TFs).⁵³ These deductions rely on gene phylogenies that are often difficult to interpret, however, such that in most cases it is hard to say if absence of a given orthologue in cnidarians reflects a primitive state (with respect to a genetic novelty of bilaterians) or is the result of gene loss. This difficulty also causes frequent discrepancies with respect to orthology assessments among studies (for example within the Hox, Sox, and Wnt multigenic families). Finally, it must be recognized that our view of the cnidarian neurogenic toolkit is strongly biased toward searching for homologues of known bilaterian genes, with the consequence that the extent of cnidarian-specific genetic innovations associated with the nervous system remains entirely unevaluated, except for nematocyte-specific genes.^{54,55}

THE CELLULAR BASIS OF CNIDARIAN NEUROGENESIS

Interstitial Stem Cells Function in Hydrozoan Neurogenesis

Until recently, neurogenesis in cnidarians had been mainly studied in the context of the adult polyp of the freshwater *Hydra*, in which production of new nerve cells takes place continuously for tissue homeostasis and is also needed for budding or regeneration of lost body parts.^{56–58} In *Hydra*, the three types of nerve cells derive from a common pool of stem cells known as interstitial stem cells, located in the interspaces between ectodermal epithelial cells of the body column. The cell lineage deriving from interstitial stem cells, which also comprises glandular cells and germ cells, is in *Hydra* independent from the ectodermal and endodermal epithelio-muscular cell lineages,^{59–62} although in *Hydractinia*, i-cells can also generate epithelio-muscular cells.^{63,64} Within the *Hydra* interstitial cell lineage, there is a subset of i-cells that is restricted to the generation of nematocytes and other nerve cell types,⁶⁵ although it is not known whether individual i-cells in this population can give rise to different neural cell types. In *Hydra*, stem cells are exclusively found in the body column where they divide, and progenitor cells are then displaced (together with epithelial cells) toward one of the body extremities (either the oral or basal pole).^{66,67} They sequentially differentiate and trans-differentiate into particular subtypes of sensory or ganglion cells depending on their position along the body axis^{68–71} and are eventually eliminated at the extremities. Production of nematocytes follows a similar process but nematoblasts, arranged in clusters

resulting from several synchronous cell cycle divisions in the body column, differentiate (capsule formation) before actively migrating toward their final destination, i.e., mainly to the tentacles.⁶¹

There are experimental indications of significant variation concerning cellular aspects of neurogenesis in hydrozoans other than *Hydra*. Sensory cells (but not ganglion cells or nematocytes) can form in the absence of interstitial cells, and thus probably from epithelial cells, in the planula larvae of *Pennaria tiarella* and *Clytia* (formerly *Phialidium*) *gregaria*.^{72,73} Isolated striated muscular cells of the medusa of *Podocoryne carnea* were observed in Petri dishes to transdifferentiate into smooth muscle cells and then into nerve cells.⁷⁴

During embryonic development in hydrozoans, interstitial cells first appear in the endoderm shortly after gastrulation.^{75,76} In the early planula, the endodermal interstitial cells give rise to nematoblasts and neuroblasts, which then migrate to the ectoderm (Figure 2).⁷⁵ Therefore in hydrozoans, whereas adult interstitial stem cells are located in the ectoderm, the nervous system is of endodermal embryonic origin.

Dedicated Neural Progenitor Cells Contribute to Neurogenesis in *Nematostella*

Neurogenesis in *Nematostella* commences at blastula stage with the emergence of neural progenitor cells (NPC); differentiation of neural cells can first be observed in the ectoderm during gastrulation and subsequently also in the endoderm^{6,49,79} (Figure 2). Transplantation experiments have been used to generate chimeric embryos in which the endoderm carries the neuron-specific *NuElav1::mOrange* transgene and these embryos revealed that the endoderm itself can generate neurons.⁶ In *Nematostella*, no morphological equivalent of i-cells has been identified; instead, neural cells are derived from epithelial progenitors. A transgenic line expressing mOrange under the control of regulatory elements of the *NuSoxB(2)* gene revealed that the population of *NuSoxB(2)*-expressing cells gives rise to sensory cells, ganglion cells, and nematocytes but not to non-neural cell types.⁷⁹

In bilaterian neurogenesis, differences in cell cycle characteristics often reflect functionally asymmetric cell divisions that result in different fates of the daughter cells, generating for example one neuron and one intermediate progenitor cell or two different types of intermediate progenitor cells.^{80–82} A similar situation can be observed in *Nematostella*. Small clusters of *NuSoxB(2)::mOrange* positive cells (assumed to be clones derived from one NPC) can contain both even and odd numbers of cells,

suggesting that there is no strictly synchronous cell division in the progeny of an NPC. This is further supported by EdU pulse labeling experiments, which showed that even after a 2 h pulse, only one cell in pairs of *NuSoxB(2)::mOrange* positive cells has incorporated EdU and thus been in S-phase.⁷⁹ These observations suggest that NPCs in *Nematostella* can divide more than once and that their developmental program includes asymmetric divisions. The developmental potential of individual *NuSoxB(2)* expressing cells is currently not known, e.g., whether a single cell can give rise to all three principal classes of neural cells or whether different *NuSoxB(2)*⁺ NPCs are limited to the generation of either sensory cells, ganglion cells or nematocytes (Figure 3). It is also not clear whether *NuSoxB(2)*⁺ cells self-renew, i.e., whether they are stem cells. Alternatively, they might represent progenitor cells that after a series of divisions differentiate into neurons and that are continuously replenished from an as yet unidentified pool of self-renewing stem cells or directly from epidermal cells (Figure 3).

Neurogenesis in *Scyphozoa*

As in anthozoans, there is no evidence for the existence of an interstitial cell lineage in scyphozoans.⁸³ During embryonic development, differentiating nerve cells are first observed in the ectoderm of the planula and there is no indication that their progenitors would originate in the endoderm.⁸⁴

THE MOLECULAR REGULATION OF CNIDARIAN NEUROGENESIS

As discussed above, cnidarian genomes contain the bilaterian complement of 'neurogenic' genes. A table summarizing expression of known bilaterian neural genes across cnidarian species has been provided in a previous review paper.¹⁴ Here, we focus on functional evidence that suggests that neurogenesis in cnidarians and bilaterians is likely conserved beyond the superficial observation that they possess a similar complement of genes.

Sox Action Upstream of bHLH Proneural Gene Transcription Factors Represents a Conserved Neurogenic Cascade

Most functional data has come from disruption of candidate neurogenic genes (Table 2). bHLH proneural genes belonging to the *achaete-scute* and *atonal* families have been the focus of study in multiple cnidarians because of their highly conserved roles

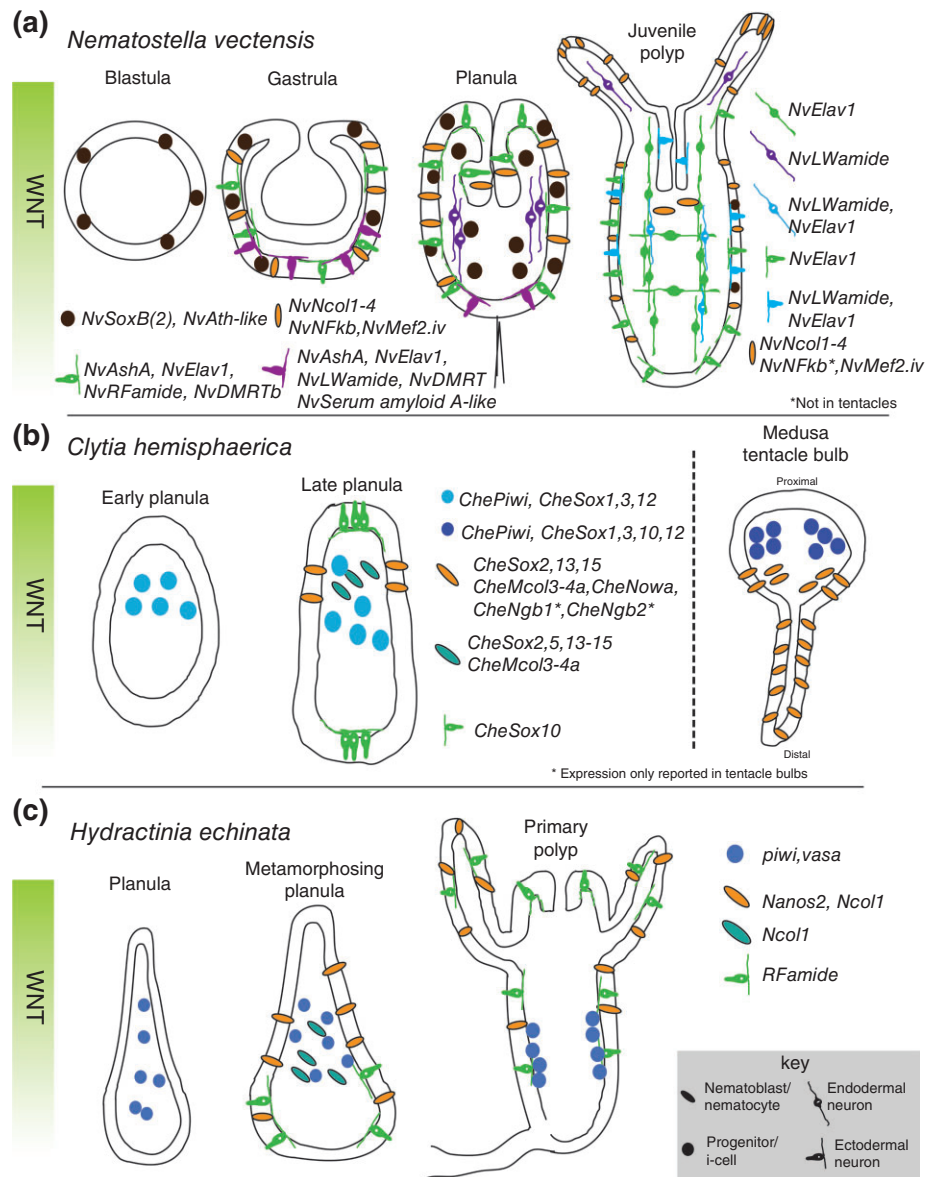


FIGURE 2 | Summary of neural gene expression during embryogenesis. Some of the known neural gene expression patterns for *Nematostella vectensis* (a), *Clytia hemisphaerica* (b), and *Hydractinia echinata* (c) are shown. Developmental time progresses from left to right with depicted stage indicated above each image. In all planulae and polyp stages, the images are oriented with oral pole facing up. The orientation of the *Clytia* tentacle (b, right side) is proximal up and distal down. Note that the expression patterns of *CheSox* genes are depicted in a simplified manner.⁷⁷ *CheNgb*, *neuroglobin*.⁷⁸

in bilaterian neurogenesis (reviewed in Ref 85). *Cnash*, a cnidarian *achaete-scute* homologue (*ash*) gene identified in *Hydra*, is expressed in developing nematocytes and sensory neurons.^{86,87} The endogenous neurogenic function of cnidarian *ash* genes was unclear until *NvAshA* was shown to be both necessary and sufficient for development of a subset of the *Nematostella* nervous system.⁸⁸ Although there is a conserved role for *ash* genes between multiple cnidarian species and bilaterians, some key expression

differences exist. Expression of cnidarian *ash* genes appears restricted to non-proliferative differentiating neurons,^{86,89} whereas in bilaterian species *ash* genes are expressed in both proliferative progenitor/stem cells and early differentiating neurons.^{85,90}

Nematostella has multiple *atonal-like* bHLH genes, but exact homology assignments are not clear.⁵⁰ Regardless, *NvAth-like* (also called *NvArp3*) promotes neural development in *Nematostella*.^{89,92} Another *atonal* family gene *NvArp6* is necessary for

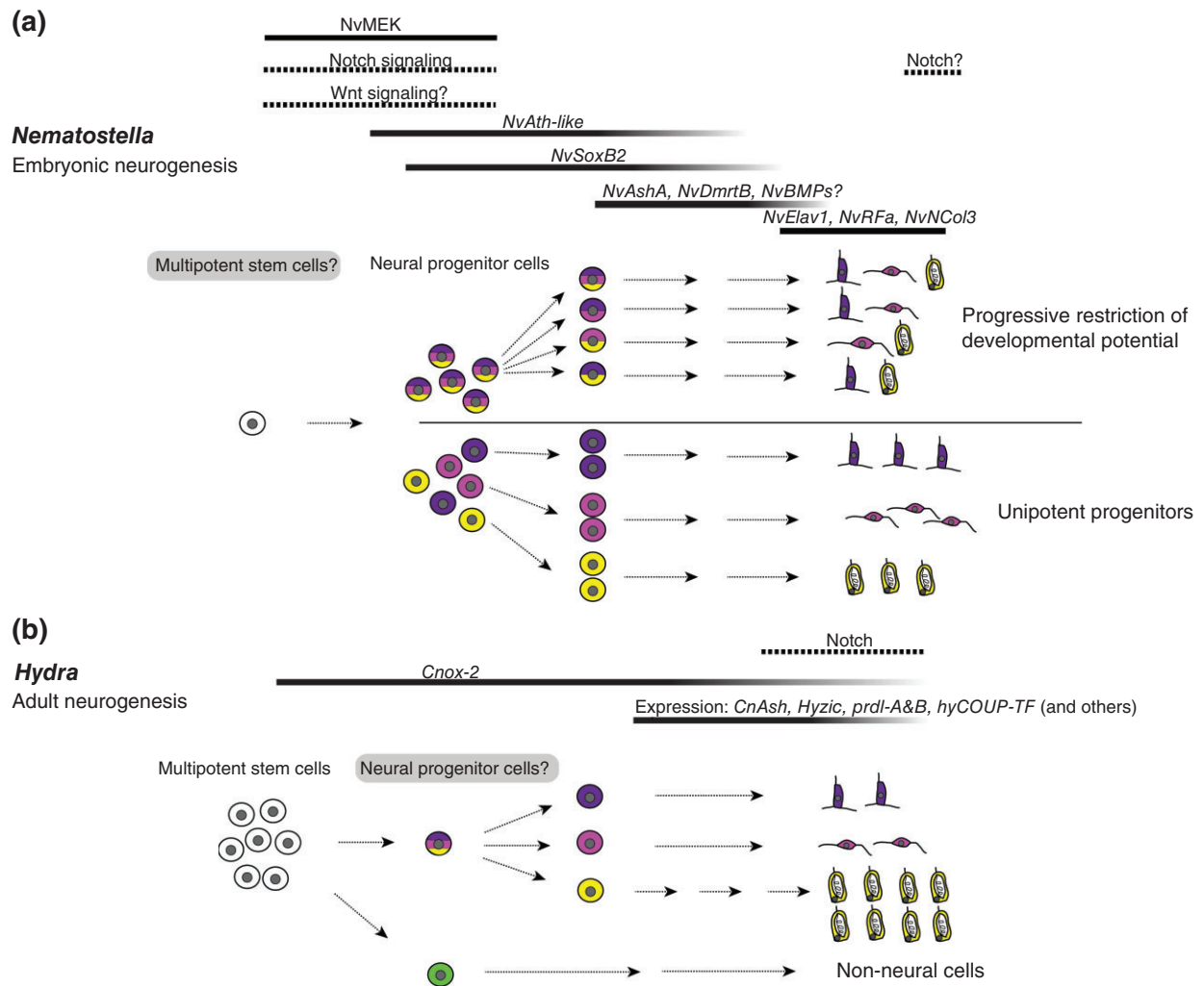


FIGURE 3 | Neurogenesis in *Nematostella* embryos and in adult *Hydra*. (a) In *Nematostella*, a pool of dedicated neural progenitor cells (NPCs) gives rise to the three major classes of neural cells (sensory cells, ganglion cells, and nematocytes) during embryogenesis. Individual NPCs may give rise to different classes (upper part) or to only one class of neural cells (lower part). Note that the existence of these two types of NPCs is not mutually exclusive. NPCs might be derived from multipotent stem cells, but experimental evidence for such stem cells is missing. Bars above the figure depict the stages at which the indicated genes act during the progression of neurogenesis, according to functional data described in the text (except for *NvNCoI3* and *NvRfA*). Notch signaling has a role in regulating the number of NPCs and likely in the differentiation of nematocytes. (b) In adult *Hydra*, multipotent interstitial stem cells (i-cells) give rise to the different classes of neural cells, but also to non-neural cells. As for *Nematostella* NPCs, the developmental potential of individual i-cells *in vivo* is not clear. The generation of neural cells may involve a dedicated NPC. Except for *cnox-2*, there are no functional data for the indicated genes.

development of GLWamide⁺ neurons during larval development of *Nematostella*. Unlike *NvAsh* genes, *NvAth-like* is clearly expressed in proliferating progenitor cells,⁸⁹ and loss of *NvAth-like* function results in a decrease in the expression of *NvAsh* and other neural markers such as *NvElav1*.^{89,92} It is still unclear if the exact function of *NvAth-like* is to promote neurogenesis by regulating the fate of already existing neural progenitors or whether it functions in their initial specification.

sox family TFs are one of the earliest expressed genes in the neural ectoderm of both *Drosophila* and

vertebrates^{98,99} and *sox* function is required for neurogenesis in both groups.^{98,100,101} Expression of *sox* genes has been characterized in both hydrozoans (*Clytia hemisphaerica*) and anthozoans (*N. vectensis*, *Acropora millepora*) during development^{51,79,102} and in an adult medusa.⁷⁷ Interestingly, *sox* genes are expressed in neural progenitor/stem cells in *Clytia* and *Nematostella* (Figures 2 and 3). Morpholino mediated knockdown of *NvSoxB(2)* reduces the number of neurons and the expression of *NvAshA* and *NvAth-like*.^{79,89} Additionally, knockdown of a different *soxB* gene, *NvSoxB2a*, reduces expression

TABLE 2 | Genes Required for Nervous System Development in Cnidarians

<i>Nematostella vectensis</i>				
Gene	Expression	Approach	Effect of Manipulation	Ref(s)
<i>NvAshA</i>	Scattered cells	lof—MO	Fewer SCs and GCs (ISH, qPCR)	88,91
		gof—mRNA	More SCs and GCs (ISH, qPCR)	88,91
<i>NvAth-like/NvArp3</i>	Scattered cells	lof—MO	Fewer SCs, GCs, and NCs (ISH, qPCR, <i>NvElav1::mOrange</i> , IHC)	89,92
<i>NvSoxB(2)</i>	Scattered cells	lof—MO	Fewer SCs, GCs, and NCs (ISH, qPCR, <i>NvElav1::mOrange</i> , IHC)	79
<i>NvNotch</i>	Scattered cells (gastrula)	lof—inhibitor (DAPT), MO	More NPCs, SCs, and GCs (ISH, qPCR, <i>NvElav1::mOrange</i>), more immature NCs, fewer mature NCs (ISH, IHC)	89,91,93
		gof— <i>NICD</i> mRNA	Fewer <i>NvAshA</i> ⁺ neural precursors, fewer SCs and GCs (ISH, qPCR)	91
<i>NvDelta</i>	Scattered cells (gastrula)	lof—MO	Increased <i>NvAshA</i> expression (qPCR)	91
		gof—mRNA	Fewer <i>NvAshA</i> ⁺ neural precursors (ISH, qPCR)	91
<i>NvSoxBa/NvSox1</i>	Broad in oral domain	lof—MO	Fewer <i>NvRFa</i> ⁺ and <i>NvGLWa</i> ⁺ neurons (IHC), no effect on <i>NvElav1::mOrange</i> ⁺ neurons	92
<i>NvAshB</i>	Broad in oral domain	lof—MO	Fewer <i>NvRFa</i> ⁺ and <i>NvGLWa</i> ⁺ neurons (IHC)	92
<i>NvArp6</i>	One sided in endoderm (planula)	lof—MO	Fewer <i>GLW</i> ⁺ neurons (IHC, qPCR)	92
<i>Nvβ-catenin</i>	nd	lof—inhibitor (iCRT14)	Fewer <i>NvRFa</i> ⁺ , <i>NvGLWa</i> ⁺ (IHC), and <i>NvElav1::mOrange</i> ⁺ neurons	92
		gof—GSK3 inhibitors	More <i>NvRFa</i> ⁺ and <i>NvGLWa</i> ⁺ neurons (IHC), no effect on <i>NvElav1::mOrange</i>	92,94
<i>NvElav1</i>	Scattered cells	lof—MO	Fewer <i>NvRFa</i> ⁺ , <i>NvGLWa</i> ⁺ (IHC), and <i>NvElav1::mOrange</i> ⁺ neurons	6
<i>NvDmrtB</i>	Scattered cells	lof—MO	Fewer endodermal <i>NvElav1::mOrange</i> ⁺ neurons	95
<i>NvBMPs</i>	One sided in oral domain	lof—MO	Fewer <i>NvRFa</i> ⁺ and <i>NvGLWa</i> ⁺ neurons (IHC)	92,96
		gof—protein	No effect at gastrula, fewer <i>NvRFa</i> ⁺ , and <i>NvGLWa</i> ⁺ neurons at planula (IHC)	92
<i>NvMEK</i>	nd	lof—inhibitor (U0126)	Fewer NPCs, SCs, and GCs (ISH)	97
<i>Hydra</i>				
<i>Cnox2</i>	Scattered cells (i-cells, neurons, nematoblasts)	lof—dsRNA	Fewer SCs and GCs (ISH, IHC)	65
<i>Hydractinia echinata</i>				
<i>nanos 2</i>	Scattered cells (nematoblasts, nematocytes)	lof—MO	Fewer NCs (IHC), more <i>RFa</i> ⁺ —neurons (ISH)	32
		gof—plasmid/transgene	More NCs (IHC), fewer <i>RFa</i> ⁺ —neurons (ISH)	32

GC, ganglion cell; gof, gain-of-function; ISH, *in situ* hybridization; IHC, immunohistochemistry; lof, loss-of-function; NC, nematocyte; MO, morpholino anti-sense oligonucleotide; qPCR, quantitative polymerase chain reaction; SC, sensory cell.

of *NvAshA*, *NvAshB*, and *NvAth-like*.⁹² The observations that two distinct *NvSoxB* genes act upstream of proneural gene TFs and that *NvSoxB(2)* is expressed in progenitor cells suggest that *soxB* function upstream of proneural genes at early steps in neurogenesis is a conserved feature of cnidarian and bilaterian neural programs.

Notch in Cnidarian Neurogenesis

A highly conserved bilaterian neural regulatory pathway is the Notch signaling pathway. Notch activity in cnidarians has been investigated using both pharmacological and gene specific functional analyses. During *Nematostella* development, treatment with DAPT, which inhibits Notch activity by disrupting

γ -secretase mediated cleavage of Notch, results in an increased neural marker expression at embryonic and larval stages.^{89,91,93} Specific knockdown of *NvNotch* also resulted in increased neurogenesis, and hyperactivation of *NvNotch* suppresses neural differentiation.⁹¹ These data are consistent with Notch having conserved neurogenic function between *Nematostella* and bilaterian animals. More specifically, *NvNotch* regulates the number of *NvAth-like*⁺ and *NvSoxB* (2)⁺ NPCs,^{89,91} which is also in line with the observed role for Notch regulation of neural progenitor fates in multiple bilaterian species.¹⁰³ Interestingly, Notch effects on neurogenesis in *Nematostella* are likely mediated by a *hes*- and 'suppressor of hairless'-independent pathway,⁹¹ which indicates an ancient role for the still poorly understood non-canonical Notch signaling mechanisms in neurogenesis. It is not yet known if Notch can act at multiple steps of neurogenesis in *Nematostella*. For example, in *Drosophila* Notch activity is first required to select neuroblast progenitor cells in the ventral neuroectoderm and then to regulate neuroblast and ganglion mother cell fates after each neuroblast division.^{104–107} Currently, there is no experimental evidence in *Nematostella* in support of or refuting the possibility of Notch acting at multiple levels of the neurogenic cascade. On its own the work in *Nematostella* suggests that Notch is a conserved neurogenic signaling pathway.

In contrast to the situation in *Nematostella*, *Hydra* polyps treated with DAPT generate normal numbers of neurons and nematocytes, but nematocytes fail to fully differentiate.^{108,109} Nematocyte maturation defects are also observed in *Nematostella* after DAPT treatment.⁸⁹ The lack of a clear neural phenotype in DAPT treated *Hydra* blurs the comparison between Notch activity in cnidarians and bilaterians. However, it is notable that the *Hydra* studies were done in adult polyps. Without a better comparison of Notch activity at embryonic stages in other cnidarians, it is difficult to establish that neurogenic phenotypes for Notch in cnidarians and bilaterians represent a deep conservation for this pathway in early neural development.

Does Neurogenesis in *Nematostella* Require an Inductive Cue?

Neurogenesis in many bilaterians requires molecular cues that confer the competence of a tissue to generate neurons; this process is termed neural induction.^{110–113} Whether comparable inductive signals exist in cnidarians is not irrevocably clear, but there is evidence that suggests cells in *Nematostella*

exhibit differential abilities to become neuronal. For example, ubiquitous misexpression of *NvAshA* is able to upregulate neural marker expression in *Nematostella*.⁸⁸ However, close examination reveals a number of cells that are unresponsive to the ectopic *NvAshA*. This is reminiscent of unilateral misexpression of *ash* genes in *Xenopus* expanding the nervous system, but not neuralizing an entire half embryo.¹¹⁴ Both the *Xenopus* and *Nematostella* proneural misexpression phenotypes indicate that cells require a signal to sensitize them toward competence to respond to proneural gene activity. Additional support that not all cells will generate neurons comes from studies investigating Notch signaling. Notch suppresses neurogenesis in *Nematostella*, but inhibition of Notch by either morpholino or DAPT, does not result in ubiquitous neural marker expression and neither does the simultaneous inhibition of Notch and overexpression of *NvAshA*.^{89,91} These data argue that Notch regulates the total number of neurons, but that it does not act on embryonic tissue that has a uniform neurogenic potential. Rather, Notch signaling appears to act on a distributed population of cells that has already acquired neurogenic potential. The question then remains whether the differential neurogenic potential is the result of some inductive cue or if it reflects some inherent properties of a subpopulation of embryonic cells.

There are a number of studies of candidate molecules that can provide some insight regarding the potential for neural induction in *Nematostella*. Inhibition of BMP2/4 signaling is perhaps the best-known neural induction mechanism in bilaterians.^{111,112,115,116} In *Nematostella* embryos, until gastrulation, BMP signaling activity is hardly detectable (as measured by phosphorylation of the signal transducer NvSmad 1/5/8) and the onset of neurogenesis at blastula stage thus occurs at very low levels of BMP signaling.^{92,117,118} Treatment with human BMP2 protein until mid-blastula stage does not affect the number of NvFMRF⁺ and NvGLW⁺ neurons at planula stage; however, it is not clear whether the onset of neurogenesis is delayed until the BMP protein is washed out, which would be expected if inhibition of BMP signaling is required for neurogenesis. Prolonged treatment with human BMP2 (until planula stage) reduces expression of neural markers at larval stages of *Nematostella* development,⁹² but injection of the *NvBMP2/4* morpholino has the same effect.^{92,96} Thus, it remains unclear whether the absence of BMP activity is a prerequisite for the initiation of neural development.

Reports from chicken and zebrafish indicate that FGF signaling promotes neurogenesis by both inducing the expression of the BMP2/4 inhibitors *chordin* and *noggin* as well as directly acting to promote expression of neural genes.^{113,119} Broad inhibition of FGF signaling with SU5402 does not impact expression of *NvashA* and thus does not appear to have a neurogenic role.⁹⁷ However, FGF independent activity of the Mitogen Activated Protein Kinase kinase MEK is necessary for the expression of *NvSoxB(2)*, *NvAth-like*, and *NvAshA*.⁹⁷ Additionally, ectopic *NvAshA* cannot promote neural fates when MEK activity is blocked with the pharmacological inhibitor U0126,⁹⁷ which taken together suggests that some instruction is necessary to impart neurogenic potential in embryonic cells.

Wnt signaling has also been linked to neural development in *Nematostella*. Inhibition of Wnt/ β -catenin signaling impairs neural development and reduces the expression of early markers of neurogenesis already at blastula stage.⁹² While the exact step at which Wnt/ β -catenin signaling regulates neurogenesis remains to be determined, it is interesting to note that the activity of this pathway is highest at the oral pole of the blastula and gastrula, whereas expression of neural markers becomes excluded from the high Wnt activity oral domain just prior to gastrulation.^{79,89} Loss of neurogenesis following disruption of Wnt, BMP, and MEK signaling suggests that external cues are able to promote neural fates, and the requirement of MEK activity for *NvAshA* to promote neural fates suggests that 'neural' is not an inherent/default state in some or all of *Nematostella* cells. Taken together current observations suggest that a process similar to neural induction is required to make cells in *Nematostella* competent to become neural, but the exact identity of the inductive cue remains elusive.

THE RELATION OF ECTODERMAL AND NEURAL PATTERNING

Gene expression studies have identified distinct ectodermal territories with sharp boundaries along the oral–aboral axis of cnidarian planulae, most prominently in *Nematostella*. In *Nematostella*, *Clytia*, *Hydractinia*, and *Hydra* Wnt signaling has been shown to be an important regulator of oral–aboral patterning, with high levels of Wnt/ β -catenin signaling promoting the development of oral identity, and different Wnt genes having nested expression domains starting from the oral pole.^{31,94,120–125} In bilaterians, the nervous system is patterned along the

antero-posterior axis in register with the ectodermal expression domains of regulatory genes such as *otx* or the *hox* genes. Morphological regionalisation along the oral–aboral axis is not much pronounced in the cnidarian planula larva (except for the mouth at the oral pole and the apical organ at the aboral pole), but when looking at the precise distribution of cell types, it appears that there is oral–aboral regionalization of the planula nervous system. In hydrozoan planula larvae, RFamide and GLWamide immunoreactive sensory cells are concentrated in the aboral region, and nematocytes at the oral pole, at least in some species (e.g., *Clava multicornis*¹²⁶; *C. hemisphaerica*¹²⁷ and unpublished observations; *Hydractinia echinata*^{16,128–130}). There is also regionalized distribution of molecularly defined neural cell types in anthozoan and scyphozoan planulae,^{6,49,131} showing that patterning of the nervous system along the oral–aboral axis is common in cnidarians.

A correspondence of these domains of neural marker expression to those of ectodermal patterning genes remains to be established. Consistent with its function in the patterning of the oral–aboral axis, Wnt/ β -catenin signaling is required for the formation of RFamide and GLWamide immunoreactive neurons in the oral territory of *Nematostella*.⁹² Similarly, *NvSix3/6*, which regulates aboral development, is required for the expression of *NvDmrtB* in the aboral domain.¹³² However, the expression of RFamide, GLWamide and *NvDmrtB* is not strictly limited to oral and aboral domains, respectively; rather their expression can be detected in different densities along the oral–aboral axis. Thus, while regionalization of the nervous system along the oral–aboral axis is present in cnidarian planulae, the molecular mechanisms that control this regionalization remain to be explored. As a first step, a better molecular definition of distinct neural cell types (by the expression of e.g., receptors, neuropeptides, neurotransmitters, or related biosynthetic enzymes and transporters) will be required.

SUBPOPULATIONS OF NEURONS

Subpopulations of neurons can be characterized by their function (e.g., as chemo- or mechanosensory cells), their morphology (e.g., the pattern of neurite projections) and by molecular features (e.g., by neurotransmitter or gene expression) or by combinations of these characters. Each of these three categories of features has been used to describe neural cell types in cnidarians, but there is hardly any information on the developmental programs that control the

generation of these cell types. Nematocytes have mechanosensory properties and they contain a sophisticated extrusive organelle, the nematocyst.^{133,134} Despite the existence of several different types of nematocysts, the nematocytes might thus be considered a rather well-defined class of neural cells. In *Hydra* polyps, nests of nematoblasts derived from up to five rounds of synchronous divisions allow the relation of gene expression patterns to nematocyte development.¹³⁵ The zinc finger gene *HyZic* is expressed in proliferating nematoblast nests but not in sensory or ganglion cells, identifying it as a nematocyte-specific regulator that might be useful to elucidate the molecular basis of nematocyte development.¹³⁶ Recently, the *Hydractinia nanos 2* gene has been shown to promote the formation of nematocytes at the expense of neurons, thus acting as a switch between two classes of neural cells.³² In *Nematostella*, *in situ* hybridization combined with counterstaining using neural markers (e.g., neuropeptides) or the use of transgenic reporter lines can tie expression patterns to specific neural cell types. For the nematocyte lineage, *NvNF-κB* has been described as a specific regulator,¹³⁷ whereas *NvElav1* is expressed in and required for the development of subsets of sensory and ganglion cells, but not nematocytes.⁶

For a better understanding of the development of classes of neural cells, it will be necessary to find markers for subpopulations of mature neurons. Subpopulations of sensory cells can potentially be identified by genes related to sensory functions in bilaterians, even though it is often not straightforward to assign particular sensory modalities to these genes. Genes related to vertebrate chemoreceptors and to insect gustatory receptors have been identified in the *Nematostella* genome,^{138,139} but their expression patterns are either not known, or (in the case of the putative gustatory receptor *NvGr11*) do not suggest a role in chemoreception. For other candidate sensory cell receptors (e.g., TRP channels, TMCs, and Piezo) expression analyses are so far also limited. A *TRPV-like* gene is expressed in the apical organ of *Nematostella*,¹⁴⁰ which contains a tuft of long cilia that are thought to have mechano- and/or chemosensory functions; and an antibody against *NvTRPA1* labels potentially mechanosensory hair cells in the tentacles.¹⁴¹ The expression of some candidate regulators of neural development in *Nematostella* (e.g., *NvRough* and *NvEvx*¹⁴²) is confined to a small number of distributed cells, potentially identifying neural subpopulations defined by a shared developmental program. Functional analyses, however, have so far focused on early and broadly acting

regulators of neural development in *Nematostella* (and other cnidarians), and the knowledge derived from these studies can now be used to test whether a particular candidate gene is indeed expressed in the neural lineage. The generation of new transgenic lines will be essential to describe the composition of the *Nematostella* nervous system and to refine the cellular and molecular program that generates neural cell type diversity. This more detailed knowledge will in turn provide the basis for comparisons of cell type specific regulatory programs between cnidarians and bilaterians that can inform the reconstruction of the evolution of neural cell types.

IMPLICATIONS CONCERNING THE EVOLUTION OF NEURAL DEVELOPMENT

It is now clear that *Nematostella* shares several cellular and molecular features of neurogenesis with bilaterian model organisms. Epithelial NPCs divide repeatedly to give rise to different neural cell types; their number is regulated by Notch signaling; *soxB*, *atona*, and *achaete-scute* genes are broadly required for neural development and Wnt signaling is involved in the patterning of the nervous system. These features are strong candidates for being shared ancestral traits of cnidarians and bilaterians. A more detailed understanding of the transcriptional regulation of conserved neurogenic genes in cnidarians will likely help to understand how their expression became restricted to defined parts of the ectoderm in many groups of bilaterians and how this relates to the evolution of centralized nervous systems.

Comparison of neurogenesis in hydrozoans and *Nematostella* reveals similarities at a general level, like the broad neurogenic potential during development and regeneration, but also clear differences. The generation of hydrozoan neurons by interstitial stem cells and the lack of an effect of Notch inhibition on the number of neurons^{108,109} indicate substantial differences in the cellular source and the molecular regulation of neurogenesis (Figure 3). Such differences may provide an opportunity to study the evolutionary plasticity of neural development, but such attempts will require a much improved understanding of neurogenesis in hydrozoans, *Nematostella* and other cnidarians. Current comparisons are to a large extent based on observations in adult *Hydra* polyps, whereas data for *Nematostella* is exclusively derived from embryonic neurogenesis. Other important questions that need to be addressed are the origin of the *NvSoxB(2)*⁺ NPCs in

Nematostella and the developmental potential of individual i-cells during unperturbed hydrozoan development. Hypothetically, the *NuSoxB(2)*⁺ NPCs could be derived from multipotent epithelial stem cells (functionally resembling i-cells) and the i-cells might contain subpopulations that are dedicated to the generation of neural cells (resembling NPCs, Figure 3). New data on these questions will improve the reconstruction of ancestral and derived aspects of cnidarian neurogenesis and in consequence that of shared features of cnidarian and bilaterian neurogenesis.

DEVELOPMENT OF CONNECTIVITY IN CNIDARIAN NERVOUS SYSTEMS

Establishing functional neural circuits requires the outgrowth of neurites and the formation of synaptic connections to other neurons and/or to effector cells, e.g., contractile or secretory cells. In bilaterians, neurites are usually distinguished into dendrites, which receive signals at their postsynaptic sites, and axons, which transmit signals to other cells via presynaptic sites. The mechanisms that control neurite outgrowth differ for dendrites and axons and are better understood for the latter.^{143,144} In cnidarians, it is not known whether a clear separation of neurites into axons and dendrites exists. Neurons, in particular ganglion cells, can have multiple neurites, but they usually do not display obvious morphological features (e.g., large synaptic terminals or dendritic spines) that would identify them as dendrites or axons. Cnidarian chemical synapses can be unidirectional or bidirectional (i.e., with synaptic vesicles on both sides of the synaptic cleft), with varying relative abundance,^{145–147} but neither the distribution of pre- and postsynaptic sites nor the polarity of microtubules (which differs between bilaterian dendrites and axons¹⁴⁸) have been mapped systematically.

Cnidarians also differ from bilaterians with respect to the development of neural connectivity. Neurons (including ganglion cells) are generated throughout most of the body column in *Nematostella* and the distribution of i-cells in *Clytia* and *Hydractinia* planulae suggests that this is also the case in hydrozoans. This widely distributed origin of neurons contrasts with the spatially more restricted generation of neurons (in particular interneurons) in the main bilaterian model organisms. In these animals, neurites are guided by a combination of permissive and instructive cues that are provided by the extracellular matrix and by intermediate ‘signpost cells’ or the eventual targets for innervation.^{143,144} The

formation of a nerve net starting from distributed neurons is, however, also conceivable without target-derived guidance cues. Neurites might grow out randomly and the formation of stable synaptic contacts could be determined by the ‘availability’ of target cells, i.e., target cells would accept only a limited number of synaptic contacts. In such a scenario, neurites would compete for the available target sites and the consolidation of synapses would potentially depend on activity that reflects integration into neural circuits. Two observations, however, argue that the outgrowth of neurites in *Nematostella* is not an entirely random process. At the early planula stage, a dense net of basi-ectodermal neurites is present but they are almost entirely excluded from a small region at the aboral pole.⁷⁹ In contrast, at a slightly later timepoint, the neurites of the NvElav1 and GLWamide expressing sensory neurons all project in an aboral direction.⁶ While this is a transient phenomenon (later born NvElav1 and GLWamide neurons project rather in transverse orientation), it suggests that the aboral pole may have a role in regulating the orientation of neurite outgrowth, first negatively and subsequently positively for a subpopulation of neurons.

In anthozoans, bundles of neurites run along the base of the mesenteries, which are endodermal infoldings that structure the gastric cavity and bear the retractor muscles and the gonads.^{6,49} While these neurite bundles are the most prominent morphological feature of the nervous system, the neurites within these bundles can project in oral or aboral direction, suggesting that there is no uniform mechanism that regulates their formation.⁶ Molecularly, genes encoding for several of the major receptor-ligand pairs involved in bilaterian neurite guidance (Semaphorin and Plexin, Ephrin and Eph, Wnt and Ryk, Netrin and Neogenin/DCC, Unc5, RGM) are present in the *Nematostella* genome.⁴⁸ Interestingly, Netrin and RGM are expressed in different subdomains in the aboral territory, consistent with a possible role in the attraction and/or repulsion of neurites in this area.^{117,149} Functional characterization of these conserved candidate genes will likely provide interesting first insights into the mechanisms that direct the establishment of the nervous system architecture in cnidarian polyps.

CONCLUSIONS AND FUTURE PERSPECTIVES

Continued study of cnidarian neurogenesis will impact our understanding of nervous system function,

evolution of nervous systems, and potentially provide critical clues about mechanisms regarding neural regeneration. Now that tools have been developed (namely transgenesis) in multiple cnidarian species it will be possible to begin to unravel the connectivity of cnidarian nerve nets. Use of neural specific promoters to express calcium sensitive fluorescent proteins, light-controlled ion channels, and anterograde and retrograde labeling reagents, such as the C-terminal fragment of the tetanus toxin (TTC) and wheat germ agglutinin (WGA),¹⁵⁷ will allow us to assemble wiring maps which in turn can help to understand how neural patterning in neurogenesis might contribute to the formation of specific neural circuits.

Functional studies demonstrate that some cnidarian nervous systems are using the same generic neurogenic programs that bilaterian animals deploy. This links cnidarian nerve nets and bilaterian nervous systems to a common origin. Expression profiling of animals in which neurogenesis has been enhanced or decreased experimentally or of fluorescently labeled neural cells now allows looking at cnidarian neural development at broader scale. Such studies will likely also identify roles of non-conserved, taxonomically restricted regulators of neural development. More detailed knowledge of the neurogenic program in cnidarians will likely provide insight as to what evolutionary modifications of neural gene regulatory networks gave rise to bilaterian nervous systems and in particular to the brain(s) and central nervous system(s).

While neuronal patterning in both cnidarians and bilaterians is tied to axial patterning, the identity of neurons within distinct domains cannot be easily homologized and there is no adequate description of cnidarian neuronal cell types that would allow specific comparisons between cnidarian nerve nets and bilaterian nervous systems. Thus, there is currently insufficient data to make definitive statements about potential homology of neural cell types or particular regions of the nervous systems of cnidarians and bilaterians.

An exciting aspect of cnidarian neurogenesis is the potential to utilize this highly regenerative group of animals to better understand how nervous systems regenerate. During regeneration, new neurons must reintegrate with existing neurons to reform a functional system. This process is still poorly understood in animals. One of the challenges of neural regeneration is neurite pathfinding and re-establishing connectivity in an adult environment. Data about how this occurs naturally in animals are relatively limited, but one study in zebrafish suggests that axon pathfinding during regeneration requires molecular programs distinct from those used during development.¹⁵⁸ This observation implies that even if research on regenerative neurogenesis can be guided by development, independent studies specifically focused on understanding regeneration must be carried out. Multiple cnidarian species are now accessible to experimental manipulation during development and regeneration and they are poised for studies identifying and comparing developmental and regenerative neurogenic mechanisms.

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FURTHER READING

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