Modern genomic tools reveal the structural and cellular diversity of cnidarian nervous systems
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Cnidarians shared a common ancestor with bilaterians more than 600 million years ago. This sister group relationship gives them an informative phylogenetic position for understanding the fascinating morphological and molecular cell type diversity of bilaterian nervous systems. Moreover, cnidarians display novel features such as endodermal neurogenesis and independently evolved centralizations, which provide a platform for understanding the evolution of nervous system innovations. In recent years, the application of modern genomic tools has significantly advanced our understanding of cnidarian nervous system structure and function. For example, transgenic reporter lines and gene knockdown experiments in several cnidarian species reveal a significant degree of conservation in the neurogenesis gene regulatory program, while single cell RNA sequencing projects are providing a much deeper understanding of cnidarian neural cell type diversity. At the level of neural function, the physiological properties of ion channels have been described and calcium imaging of the nervous system in whole animals has allowed for the identification of neural circuits underlying specific behaviours. Cnidarians have arrived in the modern era of molecular neurobiology and are primed to provide exciting new insights into the early evolution of nervous systems.

Introduction
Cnidarian nervous systems have attracted the interest of zoologists and neurobiologists since the middle of the 19th century, when first ‘neuro-muscular-epithelial cells’ and nematocytes [1,2], and a few decades later neurons [3–5] were identified. Central to this interest was the apparent simplicity of the cnidarian body plan, which was soon realized to reflect a long evolutionary distance to other animal groups. It is now well established that cnidarians are the sister group to the bilaterians [6–8] and thus occupy a key position in the animal tree of life for understanding early stages in nervous system evolution. Cnidarians are carnivores that are found in two strikingly different morphotypes: sessile polyps, generally attached to a substrate, and free-swimming medusae. In a typical cnidarian life cycle, a swimming planula larva is derived from sexual reproduction and continues to develop into a sessile polyp (Figure 1). Cnidarians comprise two principle groups, anthozoans and medusozoans; the polyps of these groups share a similar anatomy but differ in their developmental potential and their role in the life cycle. In anthozoans, polyps are the sexually mature stage that completes the life cycle by generating gametes that give rise to new planulae (Figure 1a). In medusozoans, polyps asexually generate medusae, and the medusae are the sexually mature stage that produces gametes to close the life cycle (Figure 1b). However, there is variation in the life cycle among medusozoan species, many of which have a truncated life cycle, having lost one or multiple stages [9].

Nervous system complexity and organization differ between the sessile polyp and free-swimming medusa, which is likely due to differences in life style. Polyps have a bi-layered, tube-shaped body with a single opening, surrounded by a ring of tentacles. The tentacles are used for catching prey and the single opening serves as both mouth and anus. The nervous system in the outer layer, named epidermis or ectoderm, is organized as a nerve net, though the density of neurons is typically higher at both the oral and aboral ends. In some species, a nerve ring at the oral end coordinates the feeding response, which involves tentacle motility and the ‘mouth’ opening. The inner tissue layer, named gastrodermis or endoderm, can be a plain epithelial sheet (as in many medusozoans), or it can bear folds (the mesenteries) that carry gonadal tissue and longitudinal musculature (as in many anthozoans). The gastrodermal nervous system is also a nerve net, but in some species prominent tracts of neurites run along the base of the mesenteries [10–13].
Medusae display a much higher degree of nervous system centralization as compared to polyps. A prominent nerve ring is often located at the margin of their bell and many medusae contain well-developed eyes that can be integrated into sophisticated light and gravity-sensing organs, the rhopalia [14,15]. The nerve ring and the sensory structures contribute to the control of swimming behavior, for example the avoidance of obstacles or escape from predators [16–18]. Studies of different medusae revealed interesting features in the regulation of their locomotion. In scyphomedusae, contraction of the bell musculature is coordinated with the help of bidirectional synapses in the motor nerve net [19,20]. In the hydrozoans Aglantha digitale, slow swimming and escape swimming are mediated by the same motor neurons, which can generate two types of action potentials. Weak depolarization triggers small and slow, calcium-driven spikes, whereas strong depolarization causes the large and fast, sodium-driven spikes that result in escape swimming [21,22]. The ongoing characterization of neuronal ion channels in several cnidarian species will help to relate such physiological properties of neurons to their molecular constitution [23–26].

The cells that comprise cnidarian nervous systems are traditionally grouped into three broad classes: sensory/sensory-motor neurons, ganglion neurons, and mechanosensory cells called cnidocytes. Sensory neurons are defined by their upright position in the epithelium and the presence of an apical cilium. Ganglion neurons are considered a morphological equivalent of interneurons; their somata are located basally within the epithelium. Consistent with their classification as neurons, both sensory and ganglion cells extend neurites on their basal side that form a basi-epithelial meshwork (Figure 2). Distinction of neurites as axons and dendrites has not been shown yet. Cnidocytes ('stinging cells') are cnidarian-specific cells that can contain structures resembling presynaptic sites [27,28], a cnidocil and a highly sophisticated extrusive organelle, the cnidocyst, that discharges by fast Ca²⁺-dependent exocytosis for catching prey [29,30]. These features have led to the suggestion that cnidocytes are highly derived neural cells (reviewed in Ref. [11]). Morphological and molecular analyses reveal that each neuronal class contains several subpopulations, characterized for example by the expression of particular neuropeptides or by the presence of a specific type of cnidocyst [31–34]. In recent years, the genomes of several cnidarians have been sequenced [35–39], transgenic reporter lines have been established [40*,41*,42*], and gene-knockdown or genome-editing technologies have been implemented for Hydra [43,44], Hydractinia [45,46*], Clytia [47*,48] and Nematostella [49,50*,51]. These technologies open the door to a comprehensive understanding of the composition, the development, and the function of cnidarian nervous systems [9,52,53].

Transgenic reporter lines and single cell RNA sequencing as new tools for studying the diversity of cnidarian neurons

Histological and ultrastructural observations have provided many insights into the morphology of neurons in different cnidarians [54–57], but identifying defined subpopulations of neurons and capturing the dynamics of their development has been a major challenge. The generation of transgenic reporter lines in several cnidarian species [40*,41*,42*] was an important step in overcoming this
problem. Through the use of general neuronal promoters, the first transgenic lines were used to obtain a broad picture of cnidarian nervous system structure and development [58,59]. However, there is now a growing number of transgenic lines with fluorescent proteins expressed in specific neural subpopulations [60,61,62,63**,64**]. These revealed, for example, that neurons with stereotypic projection patterns and positions in the body column contribute to the Nematostella nervous system, thus suggesting that the seemingly diffuse and random cnidarian nerve nets have reproducible elements [60**].

Understanding the complexity and organization of cnidarian nerve nets requires uncovering neuronal diversity, which is now possible using single cell RNA sequencing (scRNA-seq). This technology combined with transgenic reporter lines has the potential to provide a detailed picture of cnidarian nervous systems. Thus far, scRNA-seq has been applied to both the Nematostella and Hydra adult polyp to characterize neuronal diversity [63**,64**]. Using transgenic lines with neuronal GFP expression, several thousand neurons were collected and sequenced from each animal, giving significant insights into cnidarian nervous systems and providing rich datasets for future exploration. Analysis of sequenced Nematostella neurons revealed 32 clusters with unique gene markers. In addition, the Nematostella neurons could be split broadly into two unique transcriptional states defined by the expression of unique sets of transcription factors. Transgenic lines were created to study these two neuronal states and this revealed differences in morphology and position [63**]. In Hydra, 12 neuronal subtypes were identified with distinct molecular signatures. Using both transgenic reporter lines to highlight neural subtypes and in situ hybridization, the location of

**Figure 2**

Labeling of neurons in transgenic cnidarians. (a) A GFP-labeled bipolar neuron after dissociation of a Hydra polyp. (b) A sensory cell in the body wall of a Nematostella late planula (green). The apical surface of the ectoderm is oriented to the top. The capsules of cnidocytes (magenta) are labeled with a different transgene [62**]. (c) A multipolar neuron close to the oral opening of a young Nematostella polyp, labeled by an NvElav1::mOrange transgene. (d) The gastrodermal nervous system of a Nematostella polyp includes prominent tracts of neurites along the mesenteries and a nerve net between these tracts. The transgene is NvElav1::mOrange. Scale bars in (b, d) 20 μm, in (C) 10 μm. Image credits: (a) Stefan Siebert; (b–d) Océane Tournièr.
each neuronal subtype was identified thus creating a spatial and molecular map of the Hydra nervous system, including the identification of distinct neuronal subtypes in the epidermal and gastrodermal nerve nets [64**].

The application of scRNA-seq to uncover neuronal diversity in cnidarians is very promising; however, more work is required to determine whether the full transcriptional diversity has been uncovered in Nematostella and Hydra. Importantly, the clustering parameters, number of cells sequenced, and sequencing depth are all important factors contributing to the number of clusters reported in a given study. In addition, the degree of similarity between cell types is an important consideration. Sequencing a small number of transcripts from relatively few cells is sufficient to discern two very different cells types due to large transcriptional differences. Delineating cell types by cluster analysis is more difficult when considering cell types with similar transcriptional profiles; we don’t yet fully understand the level of sampling required to distinguish two neuronal subtypes, which likely have a high level of transcriptional overlap. Finally, it is unclear what level of transcriptional differences between two cell types warrants classification as two different neuronal subtypes. Unlike in cnidarians, the full diversity of Caenorhabditis elegans neurons is known from a large body of previous work and therefore attempts to identify the transcriptional signatures of C. elegans neurons using scRNA-seq are informative in defining benchmarks. In C. elegans, nearly 7000 single neurons were sequenced with a median of approximately 700 transcripts per cell; this revealed 40 of the 118 known neuronal subtypes [65]. It is likely that increasing the number of sequenced cells and/or increasing the sequencing depth would ultimately uncover the transcriptional signatures of all 118 subtypes. The Nematostella and Hydra single cell datasets were sequenced at different depths – a median of approximately 550 transcripts per cell for Nematostella and a median of approximately 5650 transcripts per cell for Hydra. In addition, different clustering methodologies were used, so it is likely too early to make direct comparisons between the neuronal diversity of Nematostella and Hydra [63**,64**]. Ultimately, scRNA-seq experiments describing the neuronal diversity of any organism should be validated at the bench. Regardless, scRNA-seq is clearly a very valuable tool that will allow us to uncover the neuronal diversity in a large array of cnidian species and life stages. This information can be used to gain an understanding of the organizational principles underlying the cnidian nervous system and will provide molecular handles for the functional manipulations required to test nervous system development and function.

The developmental basis for the generation of neural cell types
Approaches to manipulate gene function using morpholinos, CRISPR/Cas9, RNAi, and shRNAs have been implemented in several cnidian species [43–45,47,48,49,50,51,66,67]. In combination with transgenic lines and data collected from scRNA-seq, the ability to test gene function allows for detailed analysis of cnidian nervous system development and function, thus providing a basis for evolutionary comparisons.

Surprisingly, the stem cells that give rise to neurons and cnidocytes might be quite different between medusozoa and anthozoans. In hydrozoans (a class of medusozoa), the multipotent interstitial stem cells give rise to all cells of the nervous system, as well as gland cells and germ cells [68–72]. By contrast, in anthozoans, interstitial stem cells have not been found and the nervous system may instead arise from epithelial-like stem cells [59,73], suggesting that interstitial stem cells might be a hydrozoan-specific or medusozoa-specific innovation [74].

Candidate gene approaches used to study the molecular control of neurogenesis suggest a significant degree of conservation in the broad specification of neurons between cnidarians and bilaterians (reviewed in Refs. [13,73]). For example, Notch and Wnt signalling, soxB, atonal/neurogenin and achaete-scute family genes play central roles in Nematostella neurogenesis [59*,75*,76–78] and soxB and nanos genes function in Hydractinia neurogenesis [79,80**]. However, in contrast to Nematostella and most bilaterians, Notch signaling appears not to be involved in the regulation of neurogenesis in both adult Hydra and embryonic Hydractinia, in which neurons derive from the non-epithelial interstitial cells [46*,81*,82*]. Currently, only a few functional studies have addressed the development of specific neural cell types. In adult Hydra, apical neurons require the Para-Hox gene gex/cnox-2 for their development [83] and in Nematostella, PaxA and Mef2 are involved in the formation of cnidocytes [84*,85]. Now, with new tools and resources, like those provided by scRNA-seq, it is possible to move beyond candidate gene approaches to obtain a less biased view of the regulatory networks that underlie cnidian neurogenesis.

In adult cnidian organisms, the nervous system is continuously replaced in homeostatic animals and is capable of regeneration, thus providing a platform for understanding the regulation of adult neurogenesis. In polyps, the whole nervous system readily regenerates after significant loss of the body column, while in medusae, regeneration is restricted to some organs such as the manubrium or the eyes [86–88]. Comparative analysis of progenitor behaviours between cnidarians and bilaterian model organisms showing similar regenerative potential (e.g. planarians), will allow us to determine possible common molecular mechanisms [89]. Such comparisons can be made utilizing amputation paradigms or ablation of specific structures like the eyes of jellyfish and planarians [88,90].
Conserved molecular basis for neurotransmission in cnidarians and bilaterians

Even though neuronic conduction, that is electrical conduction across non-neuronal cells, is observed across a wide repertoire of organisms, that is plants, protists, porifers, cnidarians and bilaterians [91], electrophysiological studies performed on the giant axon from the hydromedusae Aeglautha, have demonstrated that cnidarian and bilaterian synapses exhibit similar properties, with their activity relying on the formation of presynaptic and postsynaptic potentials [92,93]. Genomic sequencing from choanoflagellates, poriferans and placozoans has actually demonstrated that the molecular components of the post-synaptic density are already almost complete in phyla that do not differentiate nerve cells [94,95]. Genomic sequencing and transcriptomic sequencing from cnidarians have confirmed pharmacological studies showing that most chemical neurotransmitters used in bilaterians are also active in cnidarian neurons, acetylcholine, glutamate, GABA, glycine for fast transmission, catecholamines and serotonin for slow transmission [96,97,98]. Signaling through these neurotransmitters is required for coordinated behaviors such as the rather complex feeding response [99].

The role of peptides in neural signaling

Neuropeptides and epitheliopeptides are a prominent feature of the cnidarian nervous system. The expression of numerous G protein-coupled receptors and the discovery of peptide-gated ion channels suggests that these peptides play a key role in both slow and fast neurotransmission [31,100,101]. In mammals, peptidergic signaling is neuromodulatory, involved in slow neurotransmission and interacting with fast neurotransmission driven by small molecules such as GABA and glutamate. This neuromodulatory role of neuropeptides might represent a typical synapomorphy of nervous systems or alternatively, a convergent evolutionary trait in cnidarians and bilaterians.

Recent studies highlight the power of functional genomics to test the function of neuropeptides in cnidarians. For example, disruption of the expression of a GLWamide neuropeptide in Nematostella [102] resulted in a subtle delay in the progression from swimming to sessile life stages under laboratory conditions. Two other recent studies analyzed how light cues regulate the release of gametes, a feature common to many animals. A group of cells required for spawning in the gonad of the Cystia hemispherica medusa are both light sensitive and secrete neuropeptides. Opsin9 is expressed in these cells and mutating the Opsin9 gene blocks light induced oocyte maturation and spawning. The same cells express a neuropeptide that functions as the maturation-inducing hormone (MIH), and secretion of this neuropeptide requires the stimulation of Opsin9 by blue-cyan light [103,104]. These elegant experiments provide an interesting example of direct coupling of sensory and neurosecretory functions in one cell, a situation that may have been more common early in animal evolution [105]. Future studies should address the role of neuropeptides in nervous system function using similar approaches.

In Hydra, epitheliopeptides can act either positively or negatively on neurogenesis and/or neurotransmission [101]. This epitheliopeptide signaling points to a tightly regulated cross-talk between the myoepithelial cells and the nervous system. The physiological function and the regulation of this crosstalk are currently not well understood. The elimination of the interstitial stem cells, and consequently neurons, leads to the up regulation of taxon-specific epitheliopeptides in the epithelial cells in Hydra [97]. This may suggest a proto-neuronal function for the epithelial cells, which are widely recognized as the cells from which synaptic conduction likely emerged [106]. A differentiated nervous system could repress the proto-neuronal function of the epithelial cells, whereas in the absence of neurogenesis, this potential could be expressed. While this hypothesis remains to be tested, one possible interpretation is that peptides played a key role in the emergence of neurons from epithelial cells, that is cells that integrated three major functions: secretion, integration, and conductivity (Figure 3).

The wiring of cnidarian nervous systems

Electrophysiological recordings have been instrumental for understanding conduction and function in cnidarian nervous systems [22,107,108]. Genetically encoded reporters of neural activity and tools for optogenetic manipulations now also allow system-wide analyses. This has recently been accomplished in Hydra by transgenic expression of the fluorescent calcium sensor protein GCaMP6s in the entire nervous system [109]. This allowed for imaging of nervous system activity in a whole animal and the identification of neuronal populations whose activities correlate with two main types of previously described electrical activity – contraction bursts (CBs) and rhythmic potentials (RPs) [107,110,111]. As previously postulated, CBs are associated with longitudinal contraction. Unexpectedly, two non-overlapping RP networks were identified, one in the epidermis and one in the gastrodermis. While the gastrodermal RP is related to radial contraction as previously thought, the epidermal RP is related to longitudinal elongation as a response to light stimulation. Apart from their functional and spatial separation, the two RP neuron populations also displayed differences in cellular morphology [109]. These findings coupled with the new molecular and spatial map of the Hydra nervous system [64], the recent classification of the Hydra behavioral repertoire [112], and the development of new technologies to measure Hydra nervous system activity [113], means we are now
poised to gain a comprehensive understanding of the *Hydra* nervous system form molecules to behavior.

**Conclusions and outlook**

Cnidarian neurobiology is enjoying a renaissance with several species being amenable to genetic manipulations, thus allowing for the visualization and interrogation of nervous system development and function. Calcium imaging will likely soon be used together with optogenetic tools for the activation and inhibition of individual neurons, which will lead to new insights into the logic of neural circuits in nerve net-based nervous systems. Studies of neural development currently focus on the specification of different neural cell types; in the near future, we expect these studies to expand to cellular aspects of the formation of neural connectivity via neurites and synapses. Basic questions about the nature of cnidarian neurons, including the identification of distinct dendrites and axons and the molecular composition of chemical synapses remain unanswered. The development of cell culture protocols has so far eluded cnidarian researchers, but would be useful for addressing these questions. Another emerging topic is the crosstalk between neurons and the microbiome that recently has been evidenced in *Hydra*, pointing to previously overlooked functions of the nervous system [114], possibly maintained in bilaterians [115,116]. At the organismal level, adding genetically tractable model systems from other classes of cnidarians would allow, for example, studying the development of convergently evolved eyes and centralizations of the nervous system (in scyphozoans and cubozoans), or the neural basis of the exquisite behavioral repertoire of box jellies (cubozoans). Extrapolating from the recent progress summarized here, it is likely that many new insights into the fascinating neurobiology of this diverse group of animals are on the horizon.

**Conflict of interest statement**

Nothing declared.

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Cnidarian nervous systems Rentzsch, Juliano and Galliot

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