# Specification and differentiation of neural cells in *Nematostella vectensis*

Océane Tournière

Thesis for the degree of Philosophiae Doctor (PhD)
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## Scientific environment

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*À mes parents Sophie et Guy*

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# Contents

## List of abbreviations

| | |
|---|---|
| Acj6 | Abnormal Chemosensory Jump 6 |
| ALM | Anterior lateral microtubule neurons (*C.elegans*) |
| AP | Apical Progenitor |
| Ash | Achaete scute |
| ATAC-seq | Assay for Transposase-Accessible Chromatin with sequencing |
| Ath | Athonal |
| BDU | Interneurons (*C.elegans*) |
| BMP | Bone Morphogenetic Protein |
| BP | Basal Progenitor |
| BRN3 | Brain3 |
| CDK | Cyclin-dependent kinase |
| *C.elegans* | *Caenorhabditis elegans* |
| ChIP-seq | Chromatin Immunoprecipitation followed by DNA sequencing |
| CNS | Central Nervous System |
| CoRC | Core Regulatory Complex |
| DAPI | 4',6-diamidino-2-phenylindole |
| DEGs | Differentially expressed genes |
| EDTA | Ethylenediaminetetra acetic acid |
| EdU | 5-Ethynyl-2´-deoxyuridine |
| Egl-46 | Egg-laying deficiency-46 (*C.elegans*) |
| FACS | Fluorescence-activated cell sorting |
| FGF | Fibroblast Growth Factor |
| FLP | FMRFamide-like neuropeptide neurons (*C.elegans*) |

| | |
|---|---|
| GPCRs | G-Protein Coupled Receptors |
| HDAC | Histone deacetylase |
| HMG | High mobility group |
| HOB | Hook sensory neuron B (*C.elegans*) |
| HSN | Hermaphrodite specific motor neurons (*C.elegans*) |
| hpf | hour post fertilization |
| *Insm1* | *Insulinoma-associated 1* |
| MAPK | Mitogen-Activated Protein Kinase |
| MEK | MAPK/Erk Kinase |
| Myc | Myelocytomatosis oncogene cellular homolog |
| NCol3 | Minicollagen3 |
| NPC | Neural Progenitor Cells |
| NSM | Neurosecretory motor neurons (*C.elegans*) |
| Oct | Octamer-binding transcription factor |
| POU | Pit-1, Oct-1, Unc-86 |
| Pit-1 | Pituitary-specific positive transcription factor 1 |
| QL | Q neuroblast on the left side (*C.elegans*) |
| SNAG | Snail/Gfi-1 protein family |
| SOX | Sry-box transcription factors |
| UNC-86 | UNCoordinated-86 (*C.elegans*) |

## Abstract

During embryonic development, early neurogenesis can be divided into several components, such as the origin, proliferation and movement of neural stem cells and progenitor cells, which are regulated by conserved genes and signalling pathways. These fundamental aspects of neurogenesis have been extensively studied in only a few bilaterian model organisms, leaving many questions regarding the evolution of this process open.

The cnidarian and bilaterian lineages are sister groups that separated approximately 600 million years ago. Cnidarians have an informative position to study the early evolution of cellular and molecular aspects of neurogenesis and to understand common principles of neural development.

*Nematostella vectensis* is a sea anemone, member of the phylum Cnidaria. They possess epithelial neural progenitor cells that express *NvSoxB(2)* and *Atonal-like* transcription factors. The Notch signalling pathways regulates the number of progenitor cells and *achaete-scute* is involved in further development. While some aspects of neural progenitor cells have been identified, little is known regarding the specification and differentiation of neural subtypes. The present thesis focuses on those aspects of neurogenesis.

Through a candidate gene approach, two transcriptions factors were selected for further analysis. Expression analysis and generation of a transgenic reporter line for the single POU class 4 gene in *Nematostella vectensis,* revealed that this gene is expressed in diverse post-mitotic neural cell types. I analysed its function by first generating a mutant line with CRISPR/Cas9 and secondly by analysing and comparing transcriptomes derived from the mutants and from different neural cell populations. This study shows that *NvPOU4* is involved in the terminal differentiation program of different neural cells, a function conserved with many bilaterians. I further discuss the relevance of POU4 genes, and terminal selectors in general, for studying the evolution of cell types in metazoans.

The second candidate gene involved in neural differentiation is *Insulinoma associated 1 (Insm1).* Using expression analysis and a transgenic reporter line, I show that

*NvInsm1*-expressing cells give rise to sensory and ganglion neurons as well as to gland cells. In this study, I demonstrate that those cell types originate from a population of progenitor cells expressing *NvSoxB(2)*. I further discuss the implications of these results regarding the developmental and evolutionary origin of neural and gland cells in metazoans.

The new findings and molecular tools generated in this thesis provide the foundation for a better understanding of evolutionary and developmental aspects of nervous system formation.

# Chapter 1 Introduction

## 1. Evolutionary origin of the nervous system

In most metazoans, a population of specialized cells form an interconnected network, called the nervous system. This structure can detect multiple stimuli at once; the information is processed and then transmitted to specific effector organs, allowing the establishment of complex behaviours. Those precise and coordinated behavioural responses must have provided a powerful selective advantage, making the nervous system an evolutionary success within metazoans. The functional unit of any nervous system is the neuron; morphologically it is an elongated cell, which allows rapid signal transmission over large distances, beyond its immediate neighbouring cells (Bucher & Anderson, 2015; Marlow & Arendt, 2014; Miller, 2009). In the animal kingdom, the diversity of nervous system structures and functions is enormous, for example the human brain is estimated to have approximately 86 billion neurons whereas the nematode *Caenorhabditis elegans* only possesses a total of 302 neurons. Those variations have always fascinated scientists, and raise many questions: How did neurons first arise? What was their ancestral function? How did they organize themselves to give rise to the complex central nervous system of vertebrates?

The field of "Evolutionary Developmental Biology" ("evo-devo") integrates developmental biology and evolutionary biology to retrace the formation of new traits and explain how organismal diversity arose, for example in metazoans. Processes that take place early during embryonic development control the nervous system structure. A comparative analysis of those developmental processes within metazoans can help to unravel the origin of the nervous system.

Animals with a nervous system include all bilaterians, ctenophores and cnidarians (Marlow & Arendt, 2014; Moroz et al., 2014) **(Figure 1)**. Ctenophora and Cnidaria are the earliest branching animal phyla that possess a nervous system. They both possess a "nerve net", which describes an irregular arrangement of neurites connecting monopolar, bipolar or multipolar neurons. This flat neurite arrangement connect epithelial sensory cells to underlying contractile cells (Hejnol & Rentzsch, 2015). The emergence of the nerve net is connected with the emergence of the neuron. The

phylogenetic position of ctenophores makes the evolutionary origin of neurons an ongoing controversial debate. In the past, Ctenophora and Cnidaria were thought to be sister groups, and therefore the nervous system was thought to have evolved once, in the last common ancestor of ctenophores, cnidarians and bilaterians. This hypothesis has recently been largely disproven and currently two mutually exclusive hypothesis are debated, the so called Porifera sister and Ctenophora-sister hypothesis which place either Porifera or Ctenophora, respectively, as the sister to all other metazoans. This as well as other evidence has led to two possible scenarios for nervous system evolution. The first one suggests that neurons might have already been present in the last common ancestor of all metazoans and was lost in Porifera and Placozoa. The second hypothesis theorizes that neurons evolved independently in Ctenophora and in the cnidarian-bilaterian common ancestor (Marlow & Arendt, 2014; Miller, 2009; Moroz, 2015; Moroz et al., 2014; Ryan et al., 2013). While the evolutionary origin of ctenophore nervous system is still debated, a common origin of cnidarians and bilaterians nervous systems is on the other hand widely accepted among biologists (Hejnol et al., 2009; Pick et al., 2010). The cnidarian position, as sister group to bilaterians, makes it an extremely interesting group with which to compare neurogenic processes and try to understand the evolution of the nervous system.

The two major clades within Bilateria are the protostomes and the deuterostomes (Adoutte et al., 2000; Hirth & Reichert, 2007). Both possess a CNS, but the location of the nerve cord differs, with most protostomes having a ventral nerve cord whereas deuterostomes have a dorsal one. Since the eighteenth century, the evolutionary origin of the nerve cord has been highly debated. Due to divergent developmental mechanisms it was first believed that the nerve cord had independent evolutionary origins and that any similarities were due to common functions, not common ancestry i.e. convergent evolution. In 1830, based on morphological observation, Geoffroy Saint-Hilaire suggested that the dorsal-ventral axis was purely arbitrary, and that the nerve cord of protostome and deuterostome therefore had a common origin (Adoutte et al., 2000; Hirth & Reichert, 2007). Nowadays, developmental genetics has provided experimental evidence for this dorsoventral axis inversion hypothesis, which suggests

a common evolutionary origin of the CNS in bilaterians (Arendt & Nübler-Jung, 1994; De Robertis & Sasai, 1996; Nielsen et al., 2018). However, a recent study suggests that the bilaterian ancestor possessed a diffused nerve net and that the nerve cord would have evolved independently in protostomes and deuterostomes (Martín-Durán et al., 2018). This long-lasting debate remains a controversial question in evolutionary developmental biology.

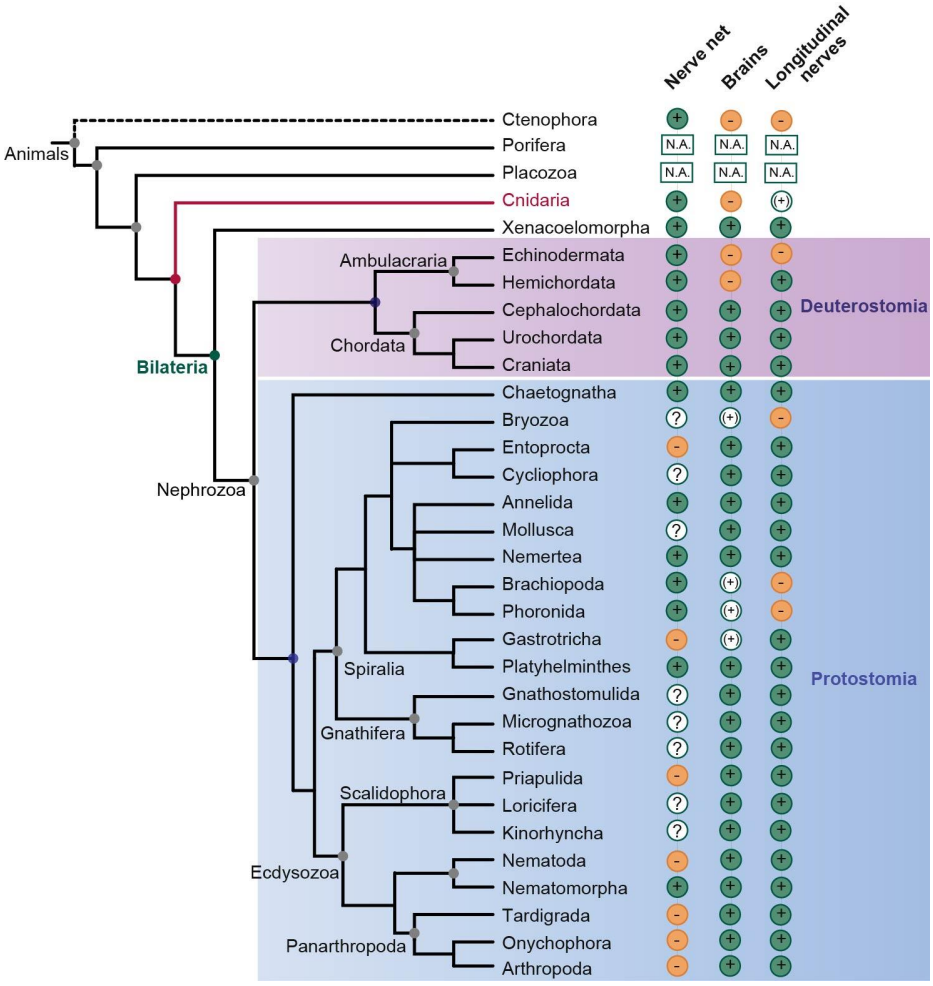


**Figure 1 : Phylogenetic relationship and nervous systems distribution of the major metazoan groups.** Phylogenic relationship among metazoans highlighting the presence/absence of nervous system (nerve net, brain and longitudinal nerves) (adapted from (Hejnol & Rentzsch, 2015))

In the next chapters, I will discuss the neurogenic mechanisms observed in bilaterians, including the origin, proliferation, specification and differentiation of neural cells. In the following section, I will then compare those processes to the closest outgroup of bilaterians, the cnidarians.

## 2. Nervous system development in bilaterians

### *2.1. Neurogenesis*

As mentioned previously, most bilaterians have a CNS composed of various neuronal cells. Neurogenesis is the process describing the formation of a neuron. It often starts during ontogeny with the generation of a neural stem cell, which is self-renewing and has the potential to generate all neuronal cell types **(Figure 2)**. Cells with a more limited proliferation capacity and progeny are often called neural progenitor cells (NPCs) (Hartenstein & Stollewerk, 2015; Taverna et al., 2014). Neural stem cells and progenitor cells can divide symmetrically or asymmetrically, depending on the identity of the daughter cells. Each division mode can also be associated with self-renewing or consumption of the progenitor cells, therefore making four types of cell division: symmetric proliferative, symmetric consumptive, asymmetric self-renewing and asymmetric consumptive **(Figure 2)**. Cells that do not anymore undergo division are called neural precursor cells. Following their formation, neural precursor cells can migrate and change their morphology along the way. Once they reach their final destination, they complete the differentiation program, form dendrites, axons, and synapses to establish connections and start expressing specific neurotransmitters and ions channels (Hartenstein & Stollewerk, 2015; Hippenmeyer, 2014; Taverna et al., 2014). During embryogenesis, stem cells and progenitor cells are therefore the first actors of neurogenesis and their division paces the formation of fully differentiated neurons, but how is this specific program molecularly orchestrated?

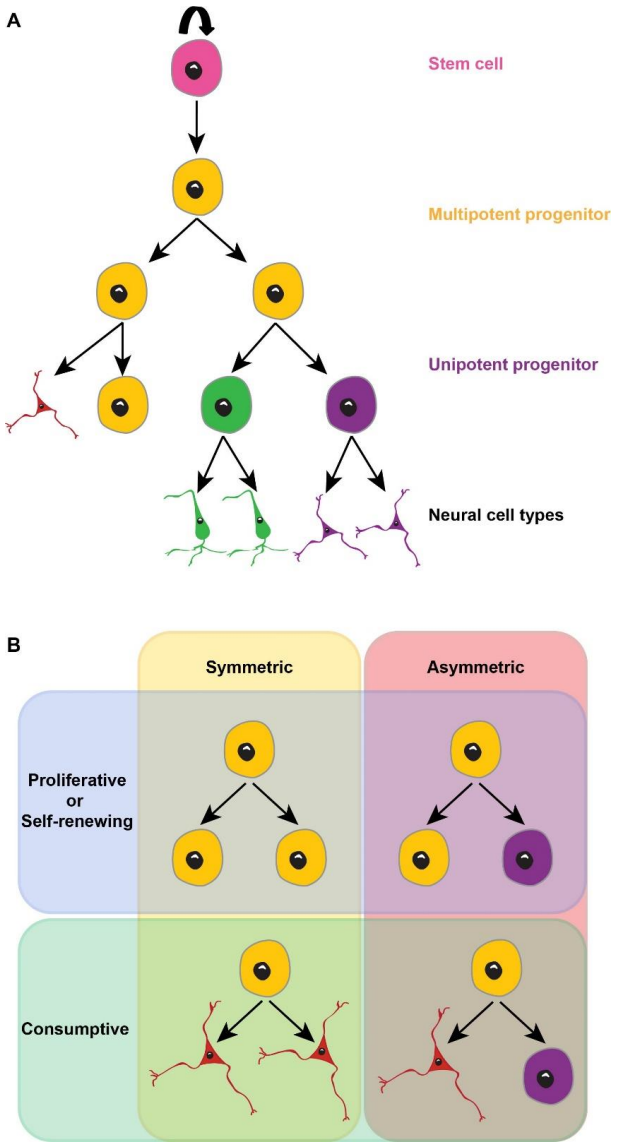


**Figure 2: The various modes of cell division during neurogenesis in bilaterian.** (A) Stem cells (pink) are self-renewing and can give rise to NPCs, that can be multipotent (if they give rise to various cell types, (yellow)) or unipotent (if they give rise to a specific cell type (purple/green)). (B) There are four types of cell division observed during neurogenesis: Symmetric proliferative; Symmetric consumptive; Asymmetric self-renewing and Asymmetric consumptive (Adapted from (Taverna et al., 2014).

## *2.2. Molecular control of neurogenesis*

### *2.2.1. Neural induction*

During early embryogenesis in vertebrates, the cells present in the ectoderm have the developmental potential to become neurons or epidermal cells. How do they choose their fate? In the early 1920s, Spemann and Mangold established the concept of organizer in amphibians embryos, based on the work of Ethel Brown who first described this principle in *Hydra* (Browne, 1909; Lenhoff, 1991; Spemann & Mangold, 2001). In salamander gastrulas, they transplanted the dorsal blastopore lips from one embryo to the ventral side of another embryos, which caused the formation of a complete second nervous system. Their study also suggested that in normal development, the dorsal blastopore lip of the embryo organizes and induces the formation of the nervous system, a region now called "Spemann's organizer" (Hemmati-Brivanlou & Melton, 1997). Similar organizing regions were observed in other vertebrates such as zebrafish (shield), chick (Henson's node) and mouse (node) (Beddington, 1994; Shih & Fraser, 1996; Smith & Schoenwolf, 1998). The conclusions from these experiments were that dorsal mesoderm induces neural tissue in the ectoderm via the emission of positive cues, and that in their absence the ectodermal cells would commit to become epidermal cells. Following this discovery, many studies tried to identify specific secreted signalling factors that could induce neurogenesis from this region, without great success. In 1989 various groups performed dissociation experiments on *Xenopus* embryos and showed that after re-aggregation, neural tissue could be detected. The authors could not really explain the observed cell autonomous neuralization and suggested the presence of putative inhibitor of neural induction in the intact ectoderm (Godsave & Slack, 1989; Grunz & Tacke, 1989; Sato & Sargent, 1989). Finally misexpression of a dominant-negative "activin" receptor in *Xenopus* embryos, blocked mesoderm formation but also generated ectopic neural tissue, which led to the so-called "default model" of neural induction (Hemmati-Brivanlou & Melton, 1994; Hemmati-Brivanlou & Meltont, 1997). In this model, the default state of the ectoderm is to develop as neural tissue, this process is blocked by the presence of BMP, which promotes epidermal fate (Hemmati-Brivanlou & Meltont, 1997;

Patthey & Gunhaga, 2014; Pera et al., 2014). Morpholino experiments targeting the four BMPs present in *Xenopus*, converted the entire ectoderm into neural tissue (De Robertis & Kuroda, 2004). In addition many molecules detected in the neural ectodermal region of the embryos, were BMP signalling antagonists (such as Chordin, Noggin, and Follistatin) (De Robertis, 2009; Harland, 2000; Weinstein & Hemmati-Brivanlou, 1997). Similar roles for BMP signalling were also observed in other bilaterians (Holley et al., 1995; Lowe et al., 2006).

Studies have also implicated the FGF signalling pathway in anterior-posterior patterning of the nervous system. The FGF pathway activates the MAPK cascade, which phosphorylates Smad1 (an effector of the BMP signalling), therefore acting as an inhibitor of the BMP pathway (Kuroda et al., 2005; Linker & Stern, 2004). The border of the two signals allows the generation of the placodes and the neural crest (Patthey et al., 2009; Patthey & Gunhaga, 2014). Inhibition of the FGF pathways leads to the generation of epidermal cells (Stuhlmiller & García-Castro, 2012). Many studies show a function of the FGF pathway in neural induction through the inhibition of the BMP signalling. However, in Zebrafish embryos, the FGF signalling pathway is required for the expression of posterior neural markers during gastrulation, a function which is independent of the organizer and of the inhibition of BMP (Rentzsch et al., 2004).

Additionally, by establishing the dorsal-ventral axis, the Wnt/β-catenin signalling pathway patterns the nervous system. A study in *Xenopus* shows it inhibits *BMP-4*, making the dorsal ectoderm more sensitive to BMP antagonists and therefore inducing the neural fate (Baker et al., 1999). Mechanisms independent from BMP signalling were also shown; Wnt signalling positively regulates cell cycle progression and inhibits cell cycle exit of neural precursors in the spinal cord of chick (Megason & McMahon, 2002). Various studies show evidence, in deuterostomes, that Wnt signaling plays a role in the maintenance of neural progenitor identity, by stimulating their proliferation (Megason & McMahon, 2002; Ulloa & Briscoe, 2007; Zechner et al., 2003) but also their differentiation (Hirabayashi et al., 2004; Patapoutian & Reichardt, 2000; Valenta et al., 2011). In *Platynereis dumerilli* functional analysis show that the Wnt signalling

pathway is necessary for the transition between proliferating NPCs to differentiating neurons, suggesting a conserved function in protostomes (Demilly et al., 2013).

BMP, Wnt and FGF signalling pathways are playing major roles during neurogenesis, however due to their broad expressions and roles in various developmental processes, it can be difficult to understand their precise relationship during early neurogenesis (Rogers et al., 2009; Stern, 2006).

### *2.2.2. Neural cell specification and differentiation*

Following neural induction, a large number of transcriptions factor start to be expressed in the forming neural ectoderm and ultimately in the developing neural stem cells and progenitor cells. The SoxB family and the proneural basic helix loop helix proteins (bHLH) are among the most studied transcription factors and the earliest ones to be expressed during neurogenesis.

Sox proteins have an evolutionary conserved high-mobility group (HMG) box protein that mediates DNA binding and therefore they act as transcription factors. Based on phylogenetic analysis, SOX proteins are classified into groups, termed A to H, in mammals. The *SoxB* group is further divided into two groups, *SoxB1* and *SoxB2* (Bowles et al., 2000). Members within a group appear not only to have similar sequences but also to have similar biological functions (Bowles et al., 2000; Wegner, 2011). Following neural border specification established by BMP, Wnt and FGF signalling, *SoxB* genes are expressed in the ectoderm and induce neurogenic potential by maintaining the neuroectoderm in a proliferative state. They are expressed transiently in NPCs and are downregulated prior to terminal differentiation (Buescher et al., 2002; Bylund et al., 2003; Kerner et al., 2009).

In vertebrates, *SoxB1* genes are involved in the maintenance of embryonic pluripotent stem cells. One of its members *Sox2*, in combination of a cocktail of three other transcription factors (*Oct4*, *Klf4* and *c-myc*) was shown to be sufficient to induce pluripotent stem cell (Takahashi & Yamanaka, 2006). Further studies showed that all three SoxB1 members can substitute each other to generate induce pluripotent stem

cell whereas other member of the Sox family cannot (Nakagawa et al., 2008). While embryonic development proceeds, *SoxB1* genes allow the commitment of pluripotent stem cells to neural progenitor cells (Loh & Lim, 2011; Thomson et al., 2011) and play a role in NPCs maintenance (Graham et al., 2003; Holmberg et al., 2008). As the cells differentiate, the expression of SOXB1 protein is rapidly downregulated (Graham et al., 2003). The *SoxB2* genes have a trans-repression activity and co-express in NPCs with *SoxB1* genes in the developing CNS (Maria et al., 1997). They promote neural differentiation, antagonising the effect of *SoxB1* genes (Sandberg et al., 2005). The precise balance between *SoxB1* and *SoxB2* genes determines whether cells are maintained in a progenitor state or undergo differentiation.

In *Drosophila* two *SoxB* genes (*SoxN* and *Dichaete*) are expressed in the neuroectoderm and involved in the formation of neuroblasts. Their expression is however, maintained while neurogenesis progresses, suggesting there is no separation into neuroectoderm-promoting *SoxB1* expression and neural differentiation-promoting *SoxB2* expression (Buescher et al., 2002; Overton et al., 2002; Zhao & Skeath, 2002).

In *Platynereis dumerilli*, *Pdu-SoxB* is expressed in the developing neuroectoderm but stops being expressed during early neuronal differentiation (Simionato et al., 2008). This suggest a potential role in neuroectodermal specification, which would be a similar function to the vertebrate *SoxB1* gene.

In vertebrates, as NPCs exit the cell cycle and migrate, the *SoxC* genes start to be expressed in overlapping patterns. They were shown to act downstream of bHLH and activate pan-neuronal genes (such as *Tubb3* and *Map2*) (Bergsland et al., 2006). The existence of *SoxC* genes has been reported in many species (Bowles et al., 2000; Crémazy et al., 2001; Larroux et al., 2008; Magie et al., 2005). In the sea urchins *Strongylocentrotus purpuratus* and the sea star *Patiria miniata larvae, SoxC* is expressed in proliferating neural progenitors (Cheatle Jarvela et al., 2016; Garner et al., 2016). In sea urchins, its expression coincides with the transition from proliferating neural progenitors to undifferentiated post-mitotic neural precursors (Garner et al., 2016). In protostome models, the single *SoxC* gene in *Drosophila* does not seem to play a function in neurogenesis (Crémazy et al., 2001; Sparkes et al., 2001). On the

other hand, in *Platynereis dumerilli*, *Pdu-SoxC* is expressed in late stages of neurogenesis (Kerner et al., 2009), suggesting that *SoxC* genes have an ancestral function in neural differentiation in bilaterians.

Another large family of transcription factors involved in various steps of neurogenesis are the basic Helix-Loop-Helix (bHLH) genes. They are composed of two α-helices that mediate dimerization, and the adjacent basic region is required for DNA binding. There are six groups of bHLH genes; group A have their bHLH region binding to core DNA sequences called E boxes (Ledent & Vervoort, 2001; Simionato et al., 2007). Members of this group play various functions during neurogenesis, they commit neural precursors to a neural fate (proneural function), they specify particular neuronal identity and induce neuronal differentiation (Bertrand et al., 2002).

Their role as proneural genes has mainly been studied in vertebrate and *Drosophila*. The *achaete-scute* and *atonal* families play such a role in both (Bertrand et al., 2002; Ledent & Vervoort, 2001; Quan & Hassan, 2005), whereas the *neurogenin* family induce the formation of neural precursors in the nervous system in vertebrates only (Ma et al., 1998, 1999, 1996). In *Drosophila* its ortholog is rather expressed in differentiating neurons (Bush et al., 1996; Gautier et al., 1997). In sea urchin *Lytechinus variegatus, Achaete-scute* and *neurogenin* function in three neuronal subtypes, and their apparent function is similar to the function of their vertebrate orthologs (Slota & McClay, 2018). In *Platynereis dumerilli*, the expression of *Pdu-Ngn* (*neurogenin* ortholog) suggests a potential function as proneural gene (Simionato et al., 2008).

Functional work of bHLH genes in vertebrates and *Drosophila* first focused on their proneural function. However, they are often expressed in more restricted populations of progenitor cells, which correlate with the formation of sub-populations of neural cells. An interesting example to illustrate this idea, is the role *Math1* (member of the *atonal* family), *Mash1* (member of the *achaete-scute* family) and *ngn1* in contributing to the specification of dorso-ventral progenitor cells that give rise to distinct types of

interneurons in the mouse embryonic spinal cord (Bermingham et al., 2001; Bertrand et al., 2002; Gowan et al., 2001). Finally, many bHLH genes which have a function in vertebrates neurogenesis do not have *Drosophila* orthologs. For example *NeuroD* and *Olig* families play major roles in the specification and in the differentiation of neural cells in vertebrates (Bertrand et al., 2002; Mizuguchi et al., 2001; Novitch et al., 2001; Schwab et al., 2000). By interacting with different co-factors they activate different subprograms of neurogenesis (see Chapter1.2.3) (Hartenstein & Stollewerk, 2015; Powell & Jarman, 2008).

Many studies focused on understanding the genes and pathways that are controlled by proneural genes and allow the progression of specific neuronal lineages. An important event for proneural genes is the restriction of their activity to specific progenitor cells. This process is achieved through the activation of the Notch signalling pathway (Bertrand et al., 2002).

Work in *Drosophila* led to uncover the principle of "lateral inhibition" (Heitzler et al., 1996; Hinz et al., 1994; Kunisch et al., 1994). In this model, delaminating NPCs called neuroblasts form the future CNS. Neuroblasts are specified in clusters of ectodermal epithelial cells, called "proneural clusters". Originally bipotent, these cells have the developmental potential to become epidermal cells or neurons (Egger et al., 2008). Their fate is determined through the Notch signalling pathway. In neuroblasts, a higher concentration of proneural genes activates the Notch ligand Delta that in turn activates in the neighbouring cell downstream target genes (such as *Hes* genes*)* **(Figure 3)**. This will inhibit the expression of Delta and proneural genes in the neighbouring cell, leading these cells to adopt a non-neural fate (Bertrand et al., 2002; Quan & Hassan, 2005). Through this process, proneural genes expression is refined and restricted to single cells that then enter a differentiation pathway (Bertrand et al., 2002).

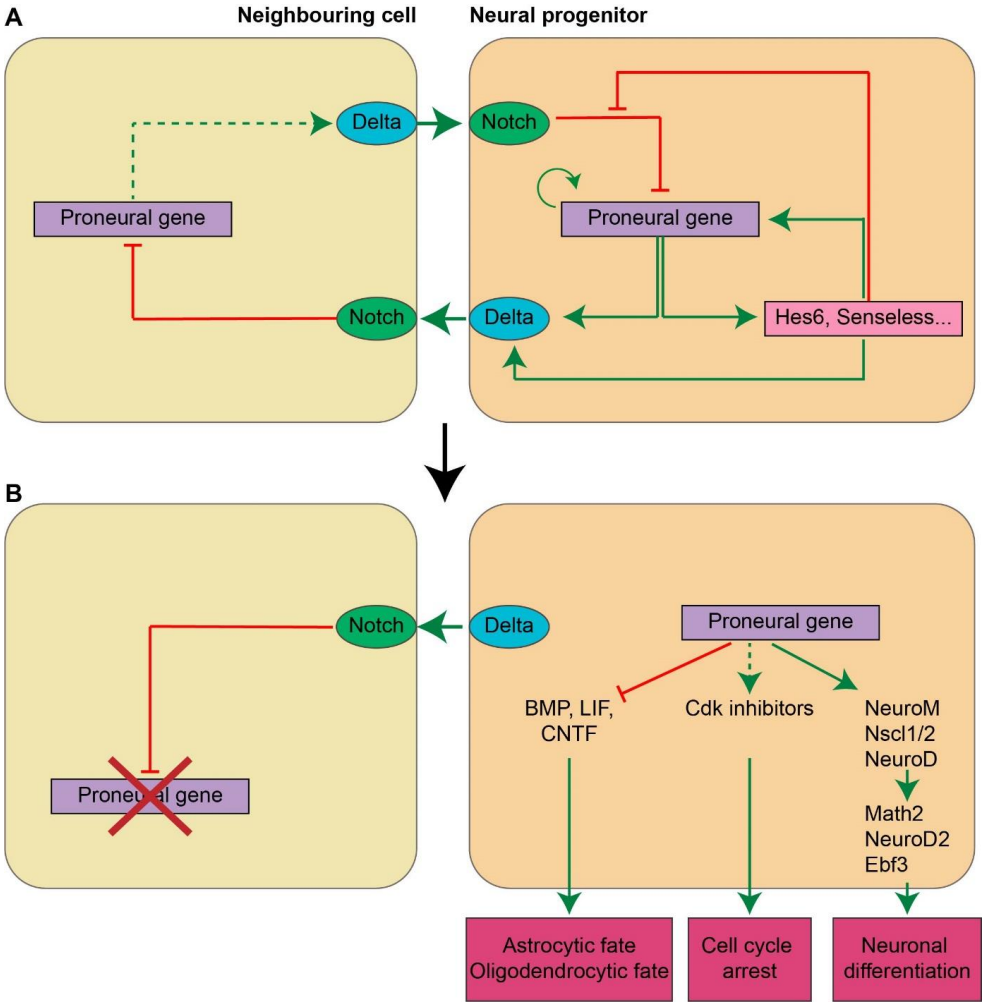


**Figure 3: Schematic representation of the role of proneural genes and the Notch signaling pathway during neuronal specification.** In *Drosophila*, through lateral inhibition, cells expressing proneural genes and the Delta ligand lead to the repression of proneural genes in the neighboring cell, promoting the expression of proneural gene in the same cell, the neural progenitor (Adapted from (Bertrand et al., 2002)).

In the neural tube of vertebrates, a gradient of *Notch1* is formed (Murciano et al., 2002). The higher expression on the apical side induces neurogenic specification, whereas the lower expression on the ventricular side allows the migration of differentiating neurons. On the apical side, like in *Drosophila*, the process of lateral inhibition regulates NPC formation (Formosa-Jordan et al., 2013; Latasa et al., 2009). The Notch signalling pathway is an important factor in early neurogenesis by regulating cell fate specification of many bilaterians (Hartenstein & Stollewerk, 2015). A recent study in the lophotrochozoan *Platynereis dumerilli* shows the Notch signalling pathway does not seem to play a major role in early neurogenesis in this model (Gazave et al., 2017). This study suggests the process of lateral inhibition might have been co-opted in insects and in vertebrates or it might have been present in the last common ancestor of bilaterians and secondarily lost in some groups.

### *2.3. Terminal selector concept*

In 1975 Garcia Bellido introduced the term "selector gene" which are genes that define the identity of specific domains of a developing organism and act transiently during specific phases of development (Garcia-Bellido, 1975, 1985). The final differentiation of a cell into a fully mature neural cell requires the activation of specific effector genes. They are responsible for the determination of specific cell type identity features such as neurotransmitter and neuropeptide identity, electrophysiological properties, or even the establishment of specific synaptic connections. Terminal selector genes control the transcriptional regulation of effector genes (Allan & Thor, 2015; Hobert, 2008, 2016b) and therefore initiate and maintain the terminally differentiated state of a neuron **(Figure 4)**. However only very few terminal selectors appear to be expressed specifically and only in one neuronal cell type, and often do not act as single, isolated proteins but rather in combination. Cooperation between terminal selectors can be distinct in various cellular contexts. To illustrate this, in *C.elegans*, UNC-86 and PAG-3 homeodomain protein binds together to the *cis* regulatory elements of effector genes in BDU neurons (Gordon & Hobert, 2015). In ALM neurons, MEC-3 prevents the binding of UNC-86 and PAG-3, driving the BDU identity into ALM (Duggan et al.,

1998; Gordon & Hobert, 2015; Xue et al., 1993). It is important to mention that the nature of those neurons is relatively different, ALM neurons are glutamatergic whereas BDU neurons express neuropeptides (Gordon & Hobert, 2015). This concept explains how one transcription factor can control various aspects of a differentiated neuron and distinct neuronal fates.

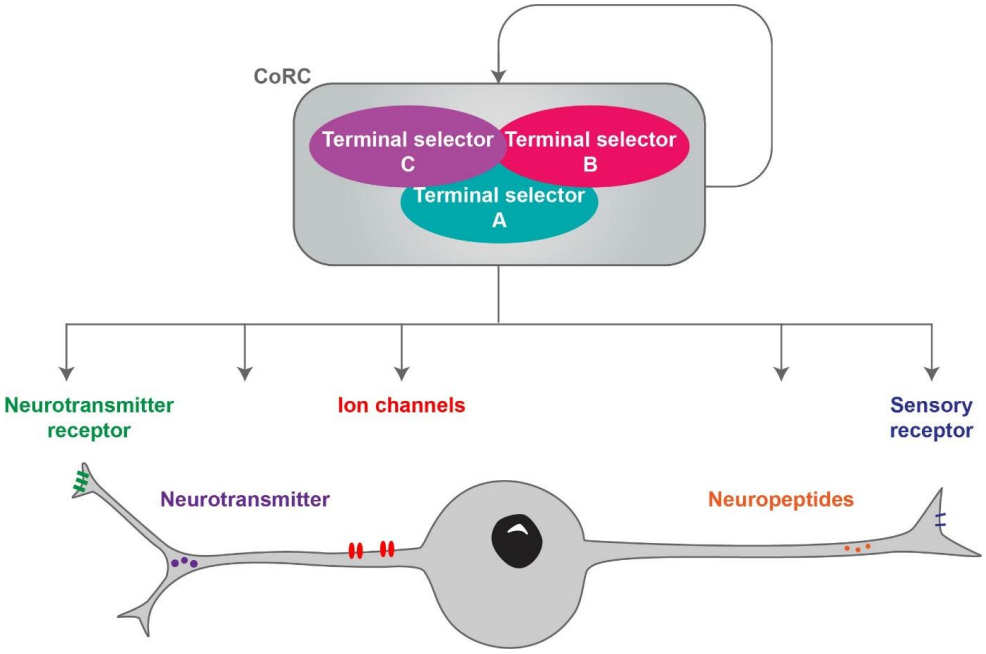


**Figure 4: Regulation of neural cell type identity.** Schematic illustration of terminal identity features controlled by transcription factors called terminal selector. Multiple terminal selectors form a Core Regulatory Complex (CoRC). The CoRC regulates the downstream effector genes and its own expression, regulating cell type-specific gene expression (modified from (Arendt et al., 2016; Hobert, 2016b)).

In other contexts, the extent of cooperation between terminal selectors is not as clear, for example double mutants for unc-86 and ttx-3 fail to differentiate NSM neurons (serotonergic motor neurons), however in single mutants some identity features are

either not affected at all or as severely affected as in the double mutants (Zhang et al., 2014). This suggests that there might be a spectrum of various cooperation according to the cis regulatory elements of individual effector genes. This combinatorial action explains how one factor can act as terminal selector in one subpopulation of neurons and not have any effect on the same set of target genes in another neuronal cell type (Hobert, 2016b; Zhang et al., 2014). Most studies were performed in *C.elegans*, but this concept was shown to also apply in vertebrates and *Drosophila* (Cho et al., 2014; Serrano-Saiz et al., 2018; Wolfram et al., 2014). From these observations the concept of a Core regulatory complex (CoRC) was suggested and defines a protein complex made of terminal selectors that enables and maintains the specific gene expression programme of a cell (Arendt et al., 2016). The authors suggest that this concept is important in the study of cell type evolution.

## 3. Cnidaria

The formation of the CNS has fascinated scientists for centuries and has therefore been studied extensively, giving us a relatively good understanding of this process in bilaterians. In 2007, the first cnidarian genome was published and revealed that many genes involved in bilaterian neurogenesis are also present in cnidarians (Putnam et al., 2007). Traditionally described as “simple” or “primitive”, due to their lack of CNS, this discovery makes them interesting model organisms to study the origin of the nervous system formation.

### *3.1. Cnidarian model organisms*

In the beginning of the 19$^{th}$ century, the relationship between living organisms was the prime interest of many naturalists and philosophers. Most of the cnidarian species, by their lack of strong visible behaviour and their regeneration capacities were not considered as animals but as plants. It’s only in 1744 that Trembley noticed that *Hydra* actively captures its food and contracts its tentacles upon contact, suggesting the presence of a nervous and digestive system, which are specific traits of metazoans

(Galliot, 2012; Trembley, 1744). Later on, it became clear that Cnidaria is the sister group to the Bilateria. Cnidarians are divided into two main clades, the Anthozoa and the Medusozoa **(Figure 5)** (Bridge et al., 1995; Hejnol et al., 2009; Pick et al., 2010).

Anthozoans are sub-divided into two monophyletic groups, Hexacorallia (sea anemones and scleractinian corals) and Octocorallia (soft corals). Medusozoan are sub-divided into four groups, Scyphozoa (true jellyfish eg. *Aurelia*), Cubozoa (box jellies eg. *Morbakka*), Staurozoa (stalked jellyfish), and Hydrozoa (hydroids, hydromedusae, siphonophores eg. *Hydra*, *Hydractinia, Clytia*) (Zapata et al., 2015).

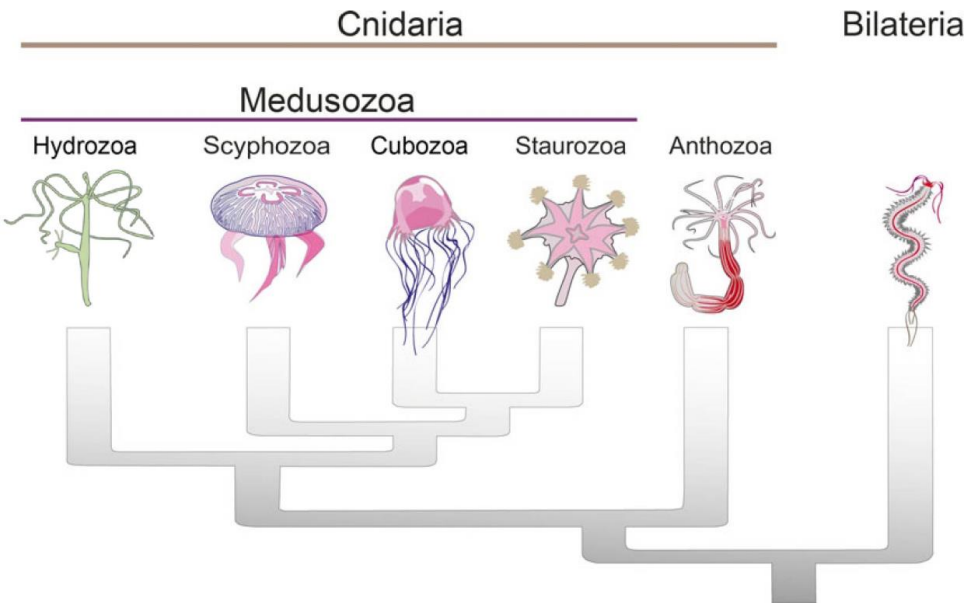


**Figure 5: Phylogenetic relationship among cnidarian species.** Schematic representation of the different cnidarian clades (adapted from (Wanninger, 2015) © Johanna Kraus)

Most cnidarians have a planula larval stage, which then settles and gives rise to a sessile polyp stage (which is the gamete producing form in anthozoan). Medusozoans, as

indicated by their name, also have an additional life stage, the free-swimming medusa stage, which is their gamete producing form (Technau & Steele, 2011). There are many described life cycle variations among medusozoan species with some lacking one or multiple stages (Cartwright & Nawrocki, 2010; Leclère et al., 2016).

Cnidarian body structure has traditionally been described as "simple". They indeed only have two cell layers (endoderm and ectoderm), one opening (which serves as both mouth and anus) and many of them lack a centralized nervous system. Many cnidarian genomes and transcriptomes are now available and show that this apparent morphological simplicity is not reflected at the genetic level. All the major signaling pathways and transcription factor families involved in bilaterian development, are also present in cnidarians (Baumgarten et al., 2015; Chapman et al., 2010; Gold et al., 2019; Leclère et al., 2019; Putnam et al., 2007). They also possess all the genes necessary for small RNA (microRNA, piRNA, etc) mediated gene regulation (Mauri et al., 2017; Modepalli et al., 2018; Moran et al., 2014, 2013; Praher et al., 2017) and a bilaterian-like gene regulatory landscape (Schwaiger, 2014; Sebé-Pedrós et al., 2018; Siebert et al., 2019). This makes cnidarians exciting model organisms to understand animal evolution and development.

Over the centuries, their unique traits have fascinated biologists, such as their asexual reproduction and ability to regenerate, their mechano-sensory cell type the cnidocytes (described in Chapter 1.4.1.2), but also their symbiosis capacity (observed for example in *Aiptasia* (Baumgarten et al., 2015). In this chapter, I will focus on cnidarian model organisms used for developmental and cell biology.

The earliest studied cnidarian was the medusozoan, *Hydra*, known to be the first model system for experimental developmental biology (Trembley, 1744) that led scientist to establish several basic biological concepts and terms. Its study has resulted in the discovery of important findings related to organizer activity, morphogen gradients, stem cells, ageing, cell death, signalling (Bode, 2012; Galliot, 2012). This has made *Hydra* one of the most studied non-bilaterian animals and with its genome published in 2010 (Chapman et al., 2010) and a recently published single cell RNA sequencing study (Siebert et al., 2019), *Hydra* remains a very promising and popular model

organism in cell biology. However, its rare reproduction makes it a difficult model to study embryogenesis and for the use of genome editing tools (such as CRISPR/Cas9). *Hydractinia,* on the other hand, fills this gap; it is a colony-forming member of the hydrozoan clade that can be manipulated easily during embryogenesis (Plickert et al., 2012). It was the first organism in which cells were described as "stem cells" (Slautterback & Fawcett, 1959), which makes it an interesting model organism in stem cell biology. In addition colonies of the same species in close contact can fuse or be rejected based on a genetic compatibility paradigm, which led to the discovery of the allorecognition complex, making it an excellent organism to study immunity (Nicotra et al., 2009). Another emerging hydrozoan model is *Clytia hemispherica*, which unlike *Hydra* and *Hydractinia* has a typical medusozoan life cycle including a medusa stage. Due to its easy culture and the total transparency of embryos, *Clytia* is a promising new model system (Houliston et al., 2010; Technau & Steele, 2011), with now a published genome (Leclère et al., 2019). The other medusozoan sub-groups are largely under-studied due to the difficulty to complete their life cycle in a laboratory. Previous studies of the scyphozoan *Aurelia* highlight a complex nervous and sensory system called the rhopalia (Galliot et al., 2009). This structure groups multiple sensory organs (an eye-cup; a mechanosensory touch plate and a geosensory statocyst). Its recently published genome, alongside that of the cubozoan *Morbakka* (Gold et al., 2019; Khalturin et al., 2019), provide an important resource for comparative studies within the medusozoan clade. They indeed show that genetic differences within the cnidarian phylum are equivalent to the variations in the protostomian and the deuterostomian clades taken together.

Within the Anthozoa, three model organisms are studied: the *Acropora* and *Aiptasia* species, are major models for coral ecology and the study of symbiosis (Baumgarten et al., 2015). *Nematostella vectensis* on the other hand, due to its ease of culture and the development of many genetic tools (Layden, Rentzsch, et al., 2016) has become an important model system for developmental biology in the past years.

### *3.2. Nematostella vectensis*

Discovered by Stefenson in 1935 (Frank & Bleakney, 1976) , *Nematostella vectensis* is a sea anemone that belongs to the anthozoan clade of cnidarians. In the wild, it is found on the Pacific coast of North America and also on the east and west cost of the North Atlantic (Darling et al., 2004). These past few years *Nematostella* has become an important new model organism. Its inducible spawning, controlled fertilisation and its relatively short generation time (3-6 months) make it a very suitable model organism for developmental studies (Darling et al., 2005). During the last 20 years, many techniques have been established on this model, such as morpholino mediated gene knockdown (Magie et al., 2007; Rentzsch et al., 2008), short hairpin RNA gene knock down (He et al., 2018), transgenesis (Renfer et al., 2010), over- and ectopic expression (Wikramanayake et al., 2003) and in vivo imaging (DuBuc et al., 2014). The recent optimization of an electroporation method, can potentially allow high throughput screening (Karabulut et al., 2019). Since 2007 the genome of *Nematostella* is available (Putnam et al., 2007) facilitating the use of genome editing tools such as the CRISPR/Cas9 system and TALENs (Ikmi et al., 2014; Putnam et al., 2007).

#### *3.2.1. Body structure description*

The adult polyp can measure about 10cm in laboratory condition due to the abundance of food, whereas in nature their size is generally 1,5cm long (Frank & Bleakney, 1976). The body structure of the adult polyp is composed of three regions: the oral opening which is surrounded by a variable number of tentacles that allow feeding behaviour; the body column and the aboral end which in nature allows the polyp to burrow into mud or sand (Williams, 1975) **(Figure 6A)**.

The body cavity is compartmentalised by eight longitudinal mesenteries. The distal part of each mesentery is called the septal filament and contains exocrine and insulinergic cells necessary for digestion and also some cnidocytes. The rest of the mesentery is essentially made of gonads, nutrient storage tissues and muscles (parietal, retractor and circular) (**Figure 6A**) (Steinmetz et al., 2017).

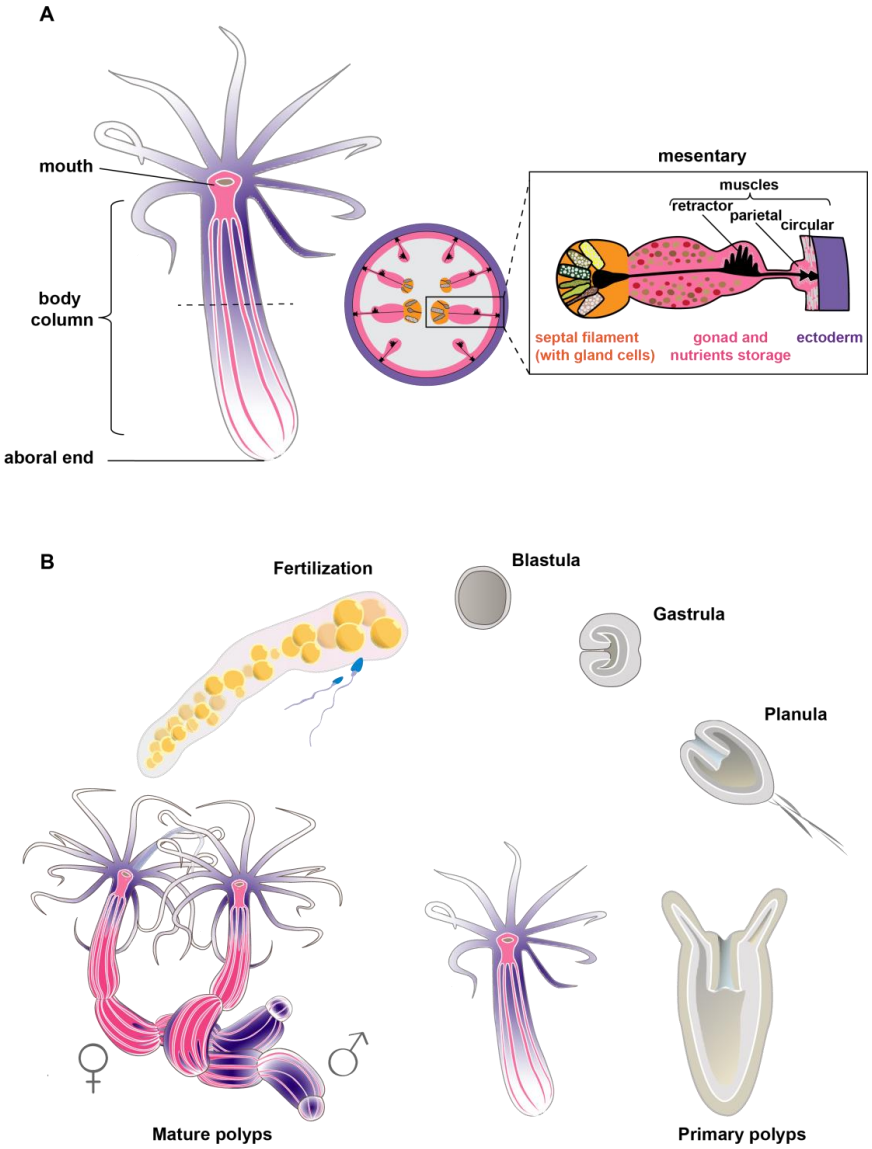


**Figure 6:** ***Nematostella vectensis*****.** (A) Schematic representation of a juvenile polyp. A cross-section of its body column highlights the presence of mesenteries, which are composed of the septal filament, the forming gonads and muscles (modified from (Steinmetz et al., 2017)). (B) Life cycle of *Nematostella vectensis* (modified from (Wanninger, 2015) © Johanna Kraus)

### *3.2.2. Development*

In *Nematostella*, the gonad tissue is located in the endodermal mesenteries. Upon maturation, eggs are squeezed into the gastric cavity and then released in the water through the oral opening (Fritzenwanker et al., 2007). Spawning can be induced by light and temperature shifts (Fritzenwanker & Technau, 2002). The size of one egg is between 170 to 240μm and upon release approximately 200 eggs are embedded into a jelly, forming egg packages (Hand & Uhlinger, 1992). Unfertilized eggs have a clear animal-vegetal polarity, established by the position of the female pronucleus in close proximity to the cell membrane, which defines the animal pole (Fritzenwanker et al., 2007; Lee et al., 2007).

The first two cleavages originate from the animal pole, at this stage, cytokinesis does not occur so the blastomeres are not fully separated. During cleavage stages, the cells are not polarized and the nuclei are located in the centre of the cells (Fritzenwanker et al., 2007; Ragkousi et al., 2017). At the 64 cell stage, the cells become polarized and form a blastula. Once the cells start to polarize, the nuclei are localized in close proximity to the apical surfaces, yolk granules move in the basal part of the cells and basophilic granules move apically. Those basophilic granules are suspected to play a role in the degradation of the jelly of the egg package at later stages (Fritzenwanker et al., 2007). Cycling invagination and evagination movements occur due to the synchronized cell divisions. The end of these cycles corresponds to the desychronization of the cell cycles (Fritzenwanker et al., 2007). At pre-gastrulation stage (17hpf at 21°C), the embryo begins to rotate due to the formation of cilia.

Gastrulation starts at approximately 20hpf, with the invagination of the epithelium. The animal-vegetal axis corresponds to the oral-aboral axis of the gastrula and planula, therefore the blastopore originates from the animal pole. A recent study shows that prior gastrulation the epithelium form cell-cell contacts on the apical and on the basal sides (via cadherin-3) (Pukhlyakova et al., 2019). As the pre-endodermal plate begins to invaginate, the cells adopt a partial epithelial to mesenchymal transition (EMT) phenotype, cadherin-3 disappears from the basal junctions of the invaginating cells, while it is retained on both sides in ectodermal cells. By losing their basal junctions,

the pre-endodermal cells become less rigid, modify their shape due to apical constriction, their nuclei migrate to a basal positions and the cells form filopodia. The blastopore lips will push the pre-endodermal plate deeper into the blastocoel, toward the blastodermal inner side of the epithelium (Fritzenwanker et al., 2004; Kraus & Technau, 2006; Magie et al., 2007; Pukhlyakova et al., 2019). After completion of the invagination process, cadherin-3 remains expressed exclusively in the ectoderm whereas it fully disappears from the cell junctions in the endoderm where it is replaced by the expression of cadherin-1 (Nathaniel Clarke et al., 2019; Pukhlyakova et al., 2019).

The newly formed two cell layered gastrula will also internalize ectoderm to form the pharynx, as a continuation of the initial endoderm internalization. The oral-most ectodermal cells invaginate while the endodermal cells flatten, forming an epithelial tube of ectodermal origin (pharyngeal ectoderm) (Magie et al., 2007; Steinmetz et al., 2017).

A free-swimming planula larva emerges out of the egg package at around 48hpf and generates an apical tuft on its aboral pole. After several days this planula will gradually elongate, loose its apical tuft, settle on the aboral pole and form four tentacles around its oral opening, this mark its transformation into primary polyp. The number of tentacles will gradually increase allowing the polyp to feed and grow (Fritz et al., 2013). Sexual maturity is reached after 4 months **(Figure 6B)**.

## 4. Cnidarian nervous system

Does the cnidarian nervous system arise via the same pathways and the same mechanisms characterized in bilaterians? In this chapter, I will first describe the nervous system and the different neural cells observed in cnidarians. I will then focus on the neurogenic mechanisms reported in hydrozoans, and finally on the embryonic neurogenesis processes characterized in the anthozoan *Nematostella vectensis.*

### *4.1. Organization of the cnidarian Nervous system*

#### *4.1.1. Nervous system description*

Cnidarian possess a simple nerve net based nervous system, generally lacking centralization (Galliot et al., 2009; Rentzsch et al., 2017). Some medusozoan species present a more complex nervous system with sensory structures, organized in rhopalia and statocysts. The rhopalium is an integrative center for sensory inputs and motor outputs and removal of this structure paralyzes the swimming of the animal (Kelava et al., 2015; Satterlie, 2011). The nerve net of many cnidarians also shows regional differences in the number of neurons, for example in hydra there is a higher density of differentiated neurons in the oral and aboral ends of the polyp (Galliot et al., 2009).

Neural cell types can be described based on their morphology, function or molecular identity. Those three features, and combinations of them, have been used to describe neural cells in cnidarians. Three main morphological classes have been described, sensory and ganglion neurons and cnidocytes. By being only present in cnidarians, cnidocytes are more derived and are referred to as “neural cells”, whereas “neuronal cells” only apply to the more “typical” neurons: sensory and ganglion neurons. Neural cells possess neurites and establish connection with each other and other cell types (such as muscle cells) (Rentzsch et al., 2017).

Sensory cells have an elongated cell body, with a single apical cilium and processes on the basal side. Ganglion cells are located in a basal position in the epithelium in close proximity to the mesoglea (extra cellular matrix localised between ectoderm and endoderm). They are thought to play an analogous role to interneurons. Markers used for mature/differentiated sub-population of neurons are, for example, the neuropeptides *NvRFamide* and *NvGLWamide* (Marlow et al., 2009; Nakanishi & Martindale, 2018; Nakanishi et al., 2012; Watanabe et al., 2014) and the neurotransmitter GABA (Kelava et al., 2015).

### *4.1.2. The cnidarian specific cell type, the cnidocyte*

Cnidocytes are the famous stinging cells of cnidarians, mainly used in prey capture and defence. Cnidocytes develop continuously throughout the lifetime of the animals. In most cnidarians, they are localized in the epidermis and in high density on the tips of the tentacles. Three major types have been identified based on morphological traits: Spirocytes, Ptychocytes and Nematocytes, but many more subtypes exists (defined by more morphological descriptions or by the production of different toxins for example). Spirocytes are only found in anthozoans, their capsule wall is thin and the encapsulated tubule is strongly coiled, additionally their tubule lack spines (Östman, 2000). Ptychocytes are found exclusively in Ceriantharia, a subclass of anthozoans, they are much larger in size than any other cnidocytes and once discharged, their tubule will be everted and inserted into their body tubes (Mariscal et al., 1977; Östman, 2000). Nematocytes are found in all cnidarians and are much more diverse, based on morphological analysis more than thirty categories have been identified (Babonis & Martindale, 2017; Özbek et al., 2009).

First described in *Hydra* in 1744 by Abraham Trembley, it's only in the beginning of the 20$^{th}$ century that scientists hypothesized that they are unusual types of neurons with both sensory and effector functions (Beckmann & Özbek, 2012; Lenhoff & Lenhoff, 1988; Pantin, 1942). They are indeed mechano-/chemoreceptor cells that contain an apical sensory ciliary cone, called cnidocil, and an extrusive organelle, the cnidocyst. The mature cnidocyst consist of a capsule with an invaginated internal tubule, which can be covered with spines. Under mechanical and chemical stimuli, the cnidocyst will create an explosive exocytosis that will harpoon and release toxins into the prey or into a potential predator (Hausmann & Holstein, 1985; Holstein & Tardent, 1984). This specialized exocytosis is one of the fastest events in nature (Nüchter et al., 2006; Weber, 1990) (**Figure 7**). Additionally, the mechanosensory responses of the cnidocil have been compared to the ones of hair cells found in the lateral line of vertebrates (Brinkmann et al., 1996; Hausmann & Holstein, 1985).

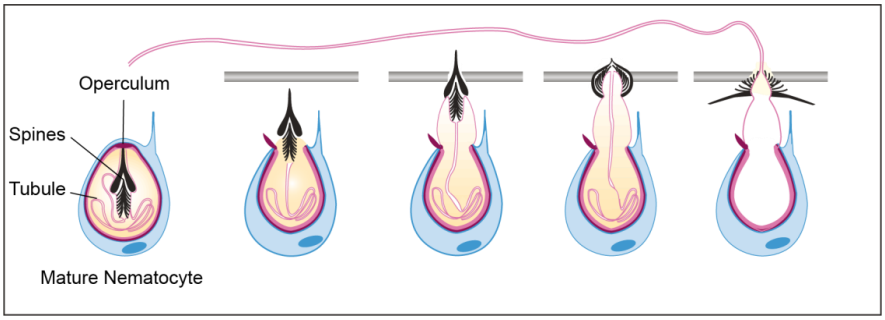


**Figure 7: Structure and discharge of a Cnidocyte.** Schematic representation of a nematocyte (blue; cell and vesicle membranes in dark blue) harboring one cyst (pink; minicollagen wall, tubule, and operculum) punching a hole into prey with its stylets (black) (Nüchter et al., 2006).

The first identified structural elements of the cnidocysts were minicollagens due to their short collagen helices (Kurz et al., 1991; Lenhoff et al., 1957). Minicollagens start being expressed from the beginning of cnidocyst morphogenesis until capsule maturation, allowing us to follow the capsule development (Engel et al., 2001). Minicollagen molecules have a common structure: the central collagen ends into polyproline stretches and is terminated at both ends by a short cysteine rich domain (called CRD) with a conserved cysteine pattern of six cysteines in a total number of 18 amino acids (Beckmann & Özbek, 2012). In *Hydra* 17 members of this protein family have been isolated whereas in *Nematostella* only five have been identified (David et al., 2008). After cnidocyte maturation, the minicollagens are highly compacted via a disulphide reshuffling process (Beckmann & Özbek, 2012; Özbek, Engel, et al., 2002; Özbek, Pertz, et al., 2002). In *Nematostella,* minicollagens are stabilized by further covalent cross-links (Zenkert et al., 2011). The tubule inside the cnidocyte is mainly composed of the galactose-binding lectin nematogalectin and chondroitin (Hwang et al., 2010).

### *4.2. Neurogenesis in hydrozoans*

Cnidocytes, sensory and ganglion neurons form an interconnected nerve net, but how are they generated in different cnidarian species? What are the genes controlling their specification and their differentiation during neurogenesis?

#### *4.2.1. Interstitial stem cells*

As mentioned earlier, one of the most intensively studied cnidarians is *Hydra*. They possess a population of interstitial stem cells called i-cells, which, morphologically, are small cells with large nuclei located in the interstitial spaces between ectodermal epithelial cells. They are described as highly proliferative, can migrate and express conserved stem/germ cell marker genes (such as *Nanos* and *Piwi*) (Mochizuki et al., 2000). *Hydra* i-cells were shown to be self-renewing by repopulating interstitial cell free tissue (David & Murphy, 1977) and to be multipotent by giving rise to somatic cells such as neurons, cnidocytes and gland cells but also to germ cells (Bode et al., 1987; Bosch & David, 1987; Davis & Bursztajn, 1974). It is however important to mention that they do not give rise to epithelial cells. Ectodermal and endodermal epithelial cells have their own stem cell populations (Bosch et al., 2010) which are unipotent and can that divide and create a continual displacement of cells towards the extremities. Transgenic reporter lines showed that those three stem cell lineages are completely independent from each other (Wittlieb et al., 2006).

Neurogenesis in *Hydra* therefore happens through the interstitial stem cell lineage. Commitment of neural cells occurs in the body column and neural progenitor cells migrate to the sites of terminal differentiation (often towards the oral and aboral ends) (Hager & David, 1997). In hydrozoans, during embryonic development, i-cells are formed in the developing endoderm at gastrulation stage, then divide and give rise to nematoblasts and neuroblasts (progenitor cells) that migrate to the ectoderm. In adult hydrozoans, i-cells are located in the ectoderm but they come from an endodermal embryonic origin (Leclère et al., 2012; Rentzsch et al., 2017). Single cell transcriptomic analysis in *Hydra* suggests the existence of a neuron/gland progenitor

cell population in adult, that is born from i-cells, in the ectodermal layer, and is able to go through the extracellular matrix to give rise to both gland cells and neurons in the endodermal layer (Siebert et al., 2019). This hypothesis differs from what has been reported before by Miljkovic-Licina and colleagues, in which gene expression analysis suggests the existence of a neuron/cnidocyte progenitor cell population (Miljkovic-Licina et al., 2007). Only one marker is exclusively clustered in i-cells (*Hy-icell1*), suggesting i-cells might be defined by an absence of cell type specific markers (Siebert et al., 2019).

### *4.2.2. Cnidocyte formation in hydrozoans*

Cnidocyte development and mechanism of action have been greatly studied by scientists to understand how new cell types arise during evolution (Babonis & Martindale, 2014; Beckmann & Özbek, 2012; David et al., 2008).

Their morphogenesis has mainly been studied in *Hydra*. Their formation takes place in the body column where i-cells undergo 3-5 divisions producing cluster of 8-32 cells connected to each other by cytoplasmic bridges (Slautterback & Fawcett, 1959). The cnidocyst formation takes place in the cytoplasm of the cell. The cnidocyte vesicles grow by addition of protein filled vesicles from the Golgi apparatus (Holstein, 1981) (**Figure 8**). Then at the apical site of the cell the tubule formation starts via membrane tabulation of the vesicles (Adamczyk et al., 2010). The newly formed tubule will then invaginate (by a mechanism which is still poorly understood) and will be tightly coiled within the capsule. At the same time the capsule is filled with poly-γ-glutamate, giving rise to a high intercapsular pressure (150 bar). The capsule will then become more and more compact (Engel et al., 2001) and the cytoplasmic bridges linking cnidocytes to each other will break down and allow the independent migration of each cnidocyte towards the tentacles (Campbell & Marcum, 1980). In other species such as *Hydractinia*, nematocyte formation occurs in a band near the base of the polypand migrate towards the head of the polyps (Teo et al., 2006). In this species, the gene *nanos 2* was shown to promote nematocyte formation at the expense of neuronal

formation (Kanska & Frank, 2013). In *Clytia*, nematogenesis was studied on the tentacle bulb ectoderm. In this model, the different phases of nematocyte differentiation are spatially separated and progress from the base to the tip of the bulb (Denker et al., 2008).

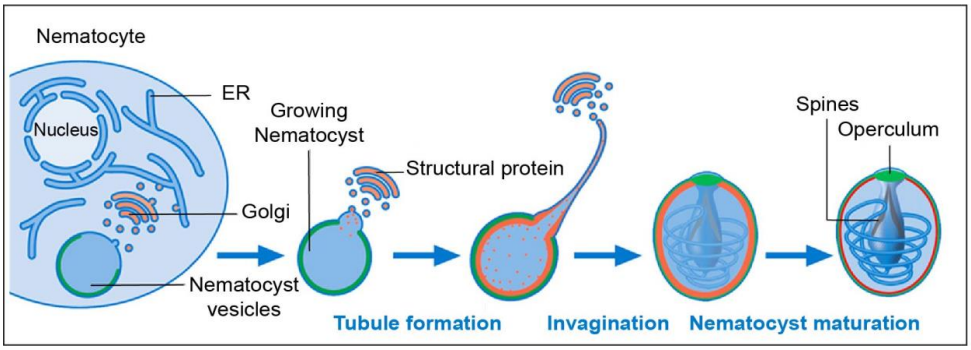


**Figure 8: Schematic representation of nematocyst morphogenesis.** Nematocyst formation takes place in the cytoplasm, the vesicle grows by addition of protein filled vesicles from the Golgi apparatus. After completion, the tubule is invaginated into the capsule matrix and the capsule is closed by a lid structure (operculum). Spines are added all over the tubule after invagination. Nematocyst maturation involves capsule wall compaction by polymerization of structural proteins (minicollagens) (Beckmann & Özbek, 2012).

### *4.2.3. Molecular control of neurogenesis*

In bilaterians, *SoxB* genes, proneural bHLH genes and the Notch signalling pathway play major roles during early embryonic neuronal regulation.

The phylogenetic relationship between the *Sox* genes has been extensively studied but remains not fully resolved (Bowles et al., 2000; Jager et al., 2011). In non-bilaterians, clear orthologues of *SoxB1* and *SoxB2* proteins have not been identified, but several *SoxB*-like genes were found in various species (Jager et al., 2006; Schnitzler et al., 2014; Shinzato et al., 2008). In *Hydractinia,* 12 *sox*-like sequences were identified with three potential *SoxB*-like genes (Flici et al., 2017). In *Hydractinia*, *SoxB2* is present in

progenitor cells, and *SoxB3* in differentiated neurons and nematocytes. Down regulation of these genes reduces the number of neurons and nematocytes formed, suggesting an important role in the regulation of neurogenesis (Flici et al., 2017). Expression analysis of *SoxB*-like genes was also reported in *Clytia,* where they are detected in i-cells and nerve cells, however, no functional work has been performed on this model so far (Jager et al., 2011).

Basic HLH genes were described as major regulators of neuronal specification in bilaterians. Phylogenetic analysis of the bHLH shows that most of the bilaterians families have orthologs present in cnidarians (Simionato et al., 2007). There are two classes of *Achaete-scute* genes (class A and B), in *Hydra*, the function of the class A ortholog, *CnASH*, was investigated and shows expression in sensory neurons and differentiating cnidocytes (Grens et al., 1995; Hayakawa et al., 2004; Lindgens et al., 2004). The ectopic expression of *CnASH*, in *Drosophila* larvae, leads to the ectopic formation of sensory organs, and can partially rescue the phenotype of *achaete* and *scute* double mutants (Grens et al., 1995). In *Podocoryn carnea*, a jellyfish, two *Achaete/Scute* genes were identified, the class A-like *Ash1* is expressed in differentiating cnidocytes (Müller et al., 2003). The class B-like *Ash2* is expressed in secretory cells (Seipel et al., 2004). These observations suggest that *Ash* class A seems to be involved in the differentiation of specific neural cells in hydrozoans.

The second family of bHLH genes highly studied in bilaterians, is the *Atonal* family. In hydrozoans it has only been studied in the jellyfish *Podocoryn carnea,* where the *Athonal-like* gene (*Atl1*) is expressed in endodermal neuronal precursors, suggesting a role as proneural gene in cnidarians (Seipel et al., 2004).

As mentioned in the previous chapter, the Notch signalling pathway is also an important neurogenesis regulator in bilaterians. In *Hydra*, under DAPT treatment, nematoblasts cannot differentiate properly and therefore undergo programmed cell death, showing that the Notch signalling pathway is important for cnidocyte

differentiation (Käsbauer et al., 2007; Khalturin et al., 2007). However, the treatment does not affect neuron differentiation, which differs from what has been observed in bilaterians. By being only performed at polyp stage, these studies do not address Notch signalling function during early neurogenesis in *Hydra*. A recent study in *Hydractinia* confirms this result not only in adult polyps but also during embryogenesis, suggesting that the function of Notch signalling as a negative regulator of neurogenesis must have been lost in the hydrozoan lineage (Gahan et al., 2017).

### *4.3. Neurogenesis in the anthozoan Nematostella vectensis*

#### *4.3.1. Neural development*

*Nematostella vectensis* is a more recent model for studies of neurogenesis in anthozoan cnidarians. No self-renewing multipotent stem cell population has been discovered so far, however a neural progenitor cell population has been described. NPCs are first observed all around the single cell layer of the embryos at blastula stage **(Figure 9)**. This population of NPCs has the developmental potential to give rise to the diverse neural cell types (Rentzsch et al., 2017; Richards & Rentzsch, 2014). By gastrula stage, differentiation of neural cells is already visible in the ectoderm (Marlow et al., 2009). At this stage, the endoderm also starts generating endodermal NPCs that later on give rise to the endodermal nervous system (Richards & Rentzsch, 2014). This trait set *Nematostella* aside from most metazoan as the endoderm was shown to be sufficient by itself to produce neurons (Nakanishi et al., 2012). At polyp stage, longitudinal tracts of neurites run along the mesenteries and oral and pharyngeal nerve ring have been reported (Watanabe et al., 2014). At this stage, cnidocytes are found all over the ectodermal epidermis, even in the pharynx and the septal filaments (Babonis & Martindale, 2014; Zenkert et al., 2011).

Cnidocyte formation, extensively studied in hydrozoans, has received relatively little studies in anthozoans. They appear to develop asynchronously, individually and locally in the tissue, without undergoing migration (Babonis & Martindale, 2014; Babonis et al., 2016). Throughout development, the epithelium is described as heterogeneous

containing various cnidocytes at different stages of cnidogenesis (Babonis & Martindale, 2017; Zenkert et al., 2011). Many of them come from this population of NPCs (Richards & Rentzsch, 2014) and are specified by the transcription factor *NvPaxA* (Babonis & Martindale, 2017).

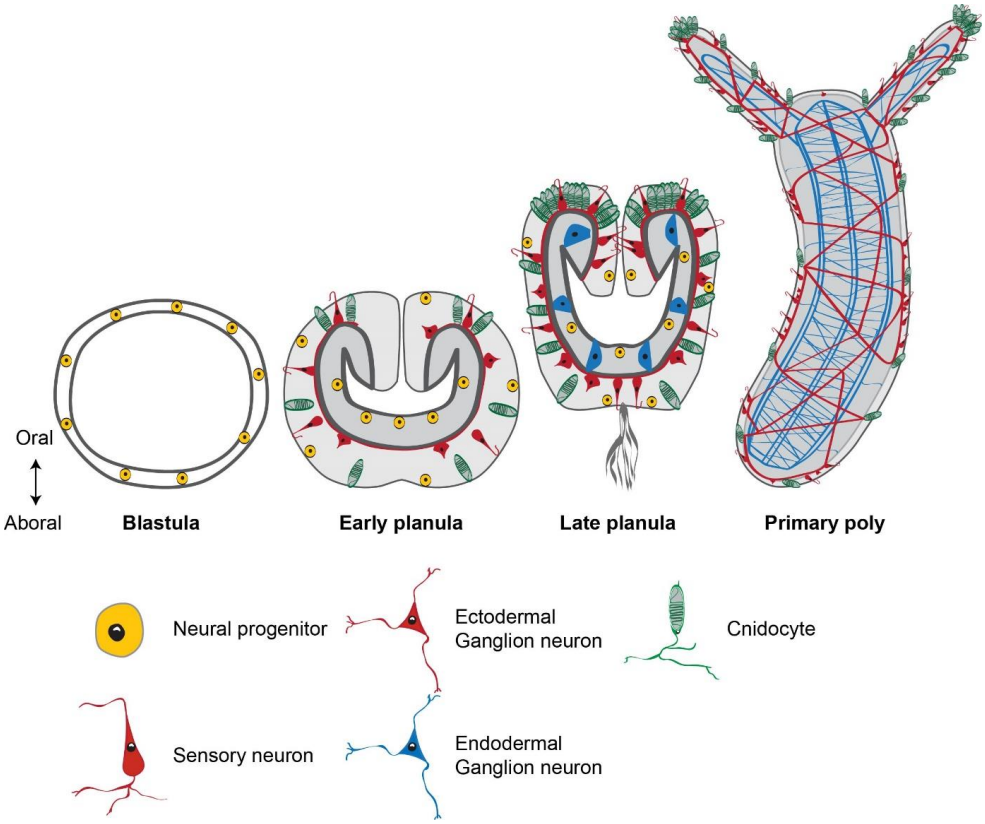


**Figure 9: Schematic representation of the nervous system development in *Nematostella vectensis.*** During embryogenesis, *Nematostella* possess NPCs in the ectoderm and endoderm that will give rise to sensory and ganglion neurons and to cnidocytes. Those neural cells have neurites connecting them to each other and forming a nerve net structure at primary polyp stage (modified from (Richards & Rentzsch, 2014).

### *4.3.2. Molecular control of early neurogenesis*

In *Nematostella*, *NvSoxB(2)* is one of the five genes closely related to the bilaterian *SoxB* transcription factor family (Magie et al., 2005). Many cells expressing *NvSoxB(2)* are proliferating and some of them divide asymmetrically suggesting a role as progenitor cells. To study the progeny of the *NvSoxB(2)*-expressing cells, a transgenic reporter line was generated, in which the *NvSoxB(2)* promoter drives the expression of mOrange (Richards & Rentzsch, 2014). It appears that this population of *NvSoxB(2)*-expressing cells has the developmental potential to give rise to sensory, ganglion neurons and cnidocytes (Richards & Rentzsch, 2014). Morpholino experiments showed that in its absence, those three neural cell types fail to develop properly. Many *NvSoxB(2)* expressing cells are not in mitosis, suggesting that it might be expressed differently in various populations of cells (before or during mitosis in some NPCs and postmitotically in other neurons). Overall, this data suggests that *SoxB* genes are ancient genetic components of neurogenesis and regulate the development of a NPC population in Cnidaria and Bilateria.

The expression and function of bHLH genes in *Nematostella* has also been investigated. There are four *Ash* homologs present in *Nematostella* and one of them, *NvAshA*, is expressed in multiple neural subtypes and its loss of function leads to the loss of neural markers (Layden et al., 2012). By not being expressed in proliferating cells (Richards & Rentzsch, 2015), *NvAshA* seems to function in early differentiation, which differs from the role of *Ash* genes in bilaterians. Another bHLH studied in *Nematostella* is *NvAth-like* and was shown to co-express with *NvSoxB(2)* in dividing progenitor cells (Richards & Rentzsch, 2015). Knockdown of *NvAth-like* decreases the expression of *NvAshA* and *NvElav1* (Richards & Rentzsch, 2015; Watanabe et al., 2014). Unlike *NvAshA*, *NvAth-like* is expressed in proliferating progenitor cells, suggesting it functions as a proneural gene. However the precise function of *NvAth-like* remains unclear. It might be involved in the early specification of NPCs or it could promote neurogenesis by regulating the fate of already present NPCs. Additionally, as many *NvAth-like* expressing cells are not in mitosis, it is possible that this gene is

expressed differently in various populations of cells (before or during mitosis in some NPCs and postmitotically in other neurons). Knockdown of *NvSoxB(2)* reduces the number of neurons and the expression of *NvAshA* and *NvAth-like* suggesting that *NvSoxB(2)* acts upstream of the bHLH transcription factors which is a common features of *Nematostella* with many bilaterians (Layden et al., 2012; Richards & Rentzsch, 2015).

In bilaterians, the Notch signalling pathway is another important regulator of neurogenesis. In a similar way, its inhibition in *Nematostella* (performed via DAPT treatment, a chemical inhibitor of γ-secretase) increases the expression of neurogenic genes and in some studies down regulates the expression of *Hes* genes (Layden & Martindale, 2014; Marlow et al., 2012; Richards & Rentzsch, 2015). Richards and Rentzsch also showed that the requirement for *NvSoxB(2)* is independent of the Notch signalling pathway as embryos in which Notch is inhibited do not develop nervous system without *NvSoxB(2)* (Richards & Rentzsch, 2015)). These data suggest a conserved role of the Notch signalling pathway during neurogenesis in anthozoans and in some bilaterians.

To conclude, it seems that *NvSoxB(2)* and the Notch signalling respectively act as positive and negative regulators of neurogenesis in *Nematostella* and potentially also in a broader scale in the last common ancestor of Cnidaria and Bilateria (Richards & Rentzsch, 2015).

### *4.3.3. Potential inductive cues in Nematostella*

As discussed previously, neurogenesis in many bilaterians is dependent on inductive cues that will provide to a given tissue the competence to generate neurons. Whether a similar process occurs in cnidarians is not entirely clear. In *Nematostella*, various studies showed that cells present different abilities to become neuronal. For example, I previously described that Notch is an inhibitor of neurogenesis in *Nematostella*, but the inhibition of the Notch signalling pathways, does not induce a ubiquitous

expression of neural markers (Layden & Martindale, 2014; Richards & Rentzsch, 2015). Similarly, misexpression of *NvAshA* up-regulates neural marker in a general pattern, however, some cells remain insensitive to this signal (Layden et al., 2012). This was also observed in *Xenopus*, in which misexpression of *ash* genes appeared to not induce neuralization of the entire embryo (Turner & Weintraub, 1994). These studies suggest that not all the cells have the ability to become neuronal; they seem to require some signals in order to be able to respond to neurogenesis regulators.

In *Nematostella*, a study showed that the inhibition of the FGF pathway (with pharmacological inhibitors) does not inhibit the expression of *NvAshA*, suggesting that the FGF pathway does not induces neurogenesis (Layden, Johnston, et al., 2016). However this same study demonstrates that MEK signalling is necessary for the correct expression of NPCs markers such as *NvSoxB(2)* and *NvAth-like*, suggesting a potential role in neural competency.

As mentioned previously, in bilaterians the inhibition of the BMP signalling leads to neural induction (Pera et al., 2014). In *Nematostella* the onset of neurogenesis, from blastula to gastrula stage, happens with a relatively low level of BMP signalling (Genikhovich et al., 2015; Leclère & Rentzsch, 2014; Watanabe et al., 2014). Embryos treated with BMP2 protein, until planula stage, have a reduced expression of neural markers at larval stage (Watanabe et al., 2014), which suggests that inhibition of BMP is necessary for neurogenesis to occur. However, injection of *NvBMP2/4* morpholino also reduces the expression of neural markers (Saina et al., 2009). It is therefore still unclear if the inhibition of BMP is necessary for initiation of neurogenesis in *Nematostella*.

The Wnt signalling pathway has also been studied in this model. Its inhibition reduces the expression of neural markers at blastula stage (Watanabe et al., 2014), suggesting a function in inducing neurogenesis. It is however important to keep in mind that these neural defects might be due to more general patterning defects rather than directly affecting neurogenesis.

Disruption of the BMP, Wnt and MEK signalling pathways reduces the expression of neuronal markers and these observations suggest the existence of a potential neural induction process in *Nematostella* (Rentzsch et al., 2017), however it is still unclear how exactly this would work and what inductive cues are necessary.

### *4.3.4. Differentiation and generation of neuronal diversity*

The neural diversity in *Nematostella* has been addressed by studying the morphology of cells and by the use of various molecular markers (e.g. RFamide, LWamide), which suggests the existence of various subtypes of neurons and cnidocytes. However, there is hardly any information regarding the developmental program that control the differentiation of neural subtypes

The increased generation of transgenic reporter lines intends to fill this gap and characterize in more details the neuronal diversity. For example the *NvElav1*::mOrange reporter line was shown to label sub-populations of sensory and ganglion neurons (Nakanishi et al., 2012). Used as a marker of differentiating neurons, it is widely accepted that it is however not pan-neural. Additionally, a population of homogeneous sensory cells has been characterized that are derived from unipotent progenitor cells expressing *NvFoxQ2d* (Busengdal & Rentzsch, 2017). *NvElav1* and *NvFoxQ2d*-transgenes characterize two non-overlapping neuronal populations. Other population of differentiated neurons have been characterize by the study of neuropeptides, for example the *NvLWamide*::mCherry line labels a large population of neuronal cells in the ectoderm (Havrilak et al., 2017; Layden, Johnston, et al., 2016), however the comparison of this line with the *NvFoxQ2D* and the *NvElav1* trangenic reporter lines has not been characterized so far.

Recently, single cell RNA sequencing has revealed the existence of many more neuronal cell types or cell states. In *Nematostella*, 32 metacells are described, in *Hydra* 15 neuronal clusters were identified, each of them with specific marker genes (Sebé-Pedrós et al., 2018; Siebert et al., 2019). By providing lists of potential marker genes, those studies are considerable resources for the cnidarian community. The generation

of new transgenic lines and functional work on these neuronal sub-populations has to be done in order to understand and describe in more detail the composition of cnidarian nervous systems.

Traditionally, cnidocytes have been classified according to morphological features (Zenkert et al., 2011), however each of these morphological class could contain several subtypes that only differ molecularly. Single cell data might allow us to study those putative subtypes. Different markers have been identified to trace cnidogenesis in *Nematostella* (Babonis & Martindale, 2017). RNA probe of *NvNCol3* can be used to label cells undergoing minicollagen transcription and therefore cells that are specified but are still in early stage of differentiation. The NvNCOL3 antibody (Zenkert et al., 2011) is used to label cells that are progressing in their differentiation, but have not completed the polymerisation of their developing capsules. The NvNCOL3 antibody no longer recognizes mature cnidocysts, once it is polymerized. High concentration of DAPI (143µM) with EDTA can then be used to label poly-γ-glutamate present in the matrix of mature, fully differentiated, cnidocytes (Babonis & Martindale, 2017; Marlow et al., 2009; Szczepanek et al., 2002). Various cnidocytes sub-populations have been suggested to exist and express different toxins (Moran et al., 2013), but their detailed characterization has not been done so far.

### *4.3.5. Open questions*

In the last 20 years the understanding of cnidarian neurogenesis has greatly improved due to the generation of various molecular techniques. Many questions remain, however, unanswered. In *Nematostella*, the origin of NPCs is not known, how are *NvSoxB(2)*-expressing cells specified in the embryo and in later stages? Additionally, the developmental potential of individual *NvSoxB(2)*-expressing NPCs is currently not clear. Finally, the developmental program leading to neural differentiation is poorly understood. How do NPCs give rise to the various neural cell types observed? Is it

through the same processes and concepts as the ones described in bilaterians or do they have mechanisms of their own?

## 5. Identification of genes involved in neural differentiation in *Nematostella*

*NvSoxB(2)* and the Notch signalling pathway have opposite roles in regulating populations of NPCs. In order to characterize genes that are specifically involved in neuronal differentiation in *Nematostella*, a microarray experiment has been performed comparing the gene expression in animals with expanded neurogenesis (induced by DAPT treatment) and individuals with inhibited neurogenesis (achieved by morpholino knockdown of *NvSoxB(2)*). A substantial catalogue of genes exhibited downregulated expression as a result of the *NvSoxB(2)* inhibition, as expected they include *NvElav1*, *NvRFamide* and *NvNcol3*, but also many others (Richards, Blommaert and Rentzsch, unpublished). From the downregulated genes, the two transcription factors, *NvPOU4* **(Paper I)** and *NvInsm1 (Insulinoma-associated 1)* **(Paper II),** were selected for more detailed analysis. In the next chapters, I will introduce both genes and retrace the various expression patterns and functions observed in their bilaterian orthologs.

### *5.1. POU4 class (Brain3) of transcription factors*

*Brain3* is a transcription factor that belongs to the *POU4* class. *POU* genes are broadly expressed within metazoan nervous systems. The structure of their protein consists of a POU domain and a homeodomain localized in the C terminal part of the protein. A short linker region separates those two domains from each other and allows the proper binding of the protein to the target DNA. The name POU comes from the initials of the first three proteins described with such a domain: Pit-1; Oct-1 and Unc-86 (Bodner et al., 1988; Clerc et al., 1988; Finney et al., 1988; Herr et al., 1988; Ingraham et al., 1990; Sturm et al., 1988). There are fifteen *POU* genes in mammals that have been classified into six classes (Gold et al., 2014). Cnidarians possess only four of them: classes 1, 3, 4 and 6 (Gold et al., 2014).

Vertebrates have three *POU4* genes called *Brain-3*a (or *Brn-3.0* or *Pou4f1*), *Brain-3b* (or *Brn-3.2* or *Pou4f2*) and *Brain-3c* (or *Brn-3.1* or *Pou4f3*). Those three genes share a highly conserved DNA binding POU domain with 95% amino acid sequence identity and 70% sequence identity in regions outside the POU domain (Xiang et al., 1995). In vitro DNA-binding assays showed that all BRN3 proteins bind to the same specific consensus DNA sequence (Gruber et al., 1997) and in vivo study suggests they are sufficient to rescue each other in case of knockdown (Pan, 2005). Expression analysis revealed that those three genes are expressed in distinct but overlapping patterns in the peripheral nervous system (overlapping subsets of visual, auditory and somatosensory neurons) (Badea et al., 2012) and also in the CNS (neurons in the midbrain, hindbrain and spinal cord) (Fedtsova & Turner, 1995). Functional analysis with mutant mice demonstrate that *Brn3b* is essential for axon growth, pathfinding and survival of retinal ganglion cells (Erkman et al., 1996; Gan et al., 1999). *Brn3c* is responsible for the correct differentiation of vestibular and auditory hair cells (Erkman et al., 1996; Wang et al., 2002; Xiang et al., 1997). *Brn3a* plays multiple roles in sensory cells and brainstem neurons (Huang et al., 2001; Ma et al., 2003; McEvilly et al., 1996; Raisa Eng et al., 2001; Xiang et al., 1996). *Brn3* genes seem to act in late stages of differentiation of sensory cells by inducing axonal growth and pathfinding, and by promoting the correct and final morphological features of various cell types (Badea et al., 2009, 2012; Erkman et al., 2000; Huang et al., 2001; Raisa Eng et al., 2001; Ryan & Rosenfeld, 1997).

In *Drosophila,* the only member of the POU4 family is *Acj6*, for *Abnormal Chemosensory Jump 6*. This gene was isolated in a behavioural screen for mutants lacking odour responses (Ayer & Carlson, 1991). Functional analysis suggests that *Acj6* is necessary for the correct establishment of synaptic connections in the CNS and in olfactory neurons (Certel et al., 2000; Clyne et al., 1999).

In the nematode *Caenorhabditis elegans*, the single *POU4* gene called *unc-86* is one of the first *POU* genes discovered (Finney et al., 1988). Functional studies showed that it is required for correct neuroblast progression and terminal differentiation of neurons (Chalfie, 1981; Duggan et al., 1998). It is indeed one of the first genes used to illustrate

the terminal selector concept by acting in the terminal differentiation of multiple neuronal subtypes, such as serotonergic and glutamatergic touch neurons (Duggan et al., 1998; Hobert, 2016b, 2016a; Serrano-Saiz et al., 2013) but also in three distinct cholinergic neurons (Zhang et al., 2014). This is achieved through cooperation with multiple co-factors (as mentioned in Chapter 1.2.3). This terminal selector function of *POU4* genes is often also required for the maintenance of the identity of these neurons, both in *C. elegans* and in mice (Serrano-Saiz et al., 2018).

Additional studies in diverse bilaterians, show expression of *POU4* genes in neurons of developing cephalic and sensory structures. These observations include the Acoel *Neochildia fusca* (Ramachandra et al., 2002), the Cephalochordate *Brachiostoma florida* (Candiani et al., 2006), the Ascidian *Ciona intestinalis* (Candiani et al., 2005), the Gastropod *Haliotis asinine* (O'Brien & Degnan, 2002), the Polychaete *Platynereis dumerilii* (Backfisch et al., 2013) and in the Cephalopod *Idiosepius notoides* (Wollesen et al., 2014). However, no functional work on these species has been done so far. Outside bilaterians, little is known about the role of *POU4* genes. In cnidarians, the Scyphozoa *Aurelia* and the Hydrozoa *Craspedacusta sowerbyi,* express it in sensory structures (such as the rhopalia) (Hroudova et al., 2012; Nakanishi et al., 2010).

In **paper I**, I investigate the expression and function of the single *POU4* gene in the sea anemone *Nematostella vectensis*.

### *5.2. The zinc finger Insulinoma associated-1*

*Insulinoma-associated 1* (*Insm1*) is a transcription factor that was first identified in a substraction library from human insulinoma tissues (Goto et al., 1992). It encodes a DNA binding protein with a conserved SNAG domain and a high percentage of alanine and proline residues (40%) at its N-terminus, followed by five zinc finger domains symmetrically arranged at the C-terminus (Goto et al., 1992). Comparison of protein sequences between human and other species revealed that orthologs contain five zinc fingers except *C.elegans* and *Drosophila* that possess only three (Lan & Breslin, 2009). The second zinc finger is the most conserved (Lan & Breslin, 2009) and, in

combination with the third zinc finger, was shown to be sufficient for transcriptional activity (Breslin et al., 2002). During mammalian embryogenesis, it is expressed in the developing pancreas, duodenum, stomach, thymus, thyroid, adrenal gland and in the forming nervous system, more specifically in the forebrain, midbrain, hind brain, cerebellum, spinal cord, retina and olfactory bulb (Breslin et al., 2003, 2002; Duggan et al., 2007; Lan & Breslin, 2009; Mellitzer et al., 2006; Zhu et al., 2002). It decreases at later stages of development and is almost completely absent in adult tissues (Zhu et al., 2002). Tumour tissues, on the other hand, re-express it for example in medulloblastoma, retinoblastoma, pituitary tumor, pheochromocytoma, medullary thyroid carcinoma and small cell lung carcinoma (Breslin et al., 2002). Additionally, recent studies suggest a role for *Insm1* in neocortex developmental expansion in mammals. Indeed, by inducing the loss of adherens junctions in apical progenitors (APs), *Insm1* induces the delamination of those cells, promoting the formation of basal progenitor cells (BPs) (Farkas et al., 2008; Tavano et al., 2018; Taverna et al., 2014). Increased numbers of BPs has been proposed to participate in neocortical expansion in mammals, as their division is not limited to the ventricular zone. In Zebrafish and Medaka (*Oryzias latipes*), there are two *Insm1*-like genes (*Insm1a* and *Inam1b*). Their expression is limited to neural and pancreatic cells and their progenitors (Lukowski et al., 2006). Functionally, by acting on the cell cycle they ensure the transition from cycling progenitors to differentiating cells (Candal et al., 2007; Forbes-Osborne, Wilson, & Morris, 2013). In vertebrates, INSM1 was shown to be a transcriptional repressor by directly regulating NeuroD/β2, insulin and INSM1 itself, and by interacting with cyclin D1 (Zhang et al., 2009). The complex of cyclinD1/INSM1 binds to NeuroD1 and insulin promoters along with the co-repressors HDAC-1 and HDAC-3 (Liu et al., 2006; Monaghan et al., 2017). By interacting with cyclinD1, INSM1 directly suppresses the cyclinD1/CDK4 complex, inducing cell cycle arrest.

To our knowledge in other deuterostomes its expression has only been studied in the sea urchins *Lytechinus variegatus*, in which it is expressed in the neuronal domains, suggesting a role in neurogenesis (Mcclay et al., 2018).

The *Drosohpila Insm1* homolog*, Nerfin-1,* is also expressed in the developing nervous system and was suggested to function in axon guidance (Kuzin et al., 2005; Stivers et al., 2000), but also in the differentiation and maintenance of neurons (Froldi et al., 2015; Vissers et al., 2018; Xu et al., 2017).

In *C.elegans*, a screen for animals with egg-laying deficiency pointed out *egl-46*, the *Insm1* homolog, as important. *Egl-46* mutants have various neuronal defects, revealing a role in the specification of mechanosensory neurons (HSN, FLP and HOB neurons). Additionally, in *Egl-46* mutants, the QL neuroblast lineage presents extra divisions suggesting a potential role in cell cycle exit of progenitor cells (Desai et al., 1988; Desai & Horvitz, 1989; Yu et al., 2003). *Insm1* genes seem to share a conserved function in sensory and endocrine cell differentiation in bilaterians. Its strong expression in developing and tumoural neuronal and endocrine developing tissues, and its function in cell cycle exit, has made it an interesting candidate gene in the development of therapeutic treatments. To our knowledge, this gene has never been studied outside of bilaterians and in **paper II** I investigate its expression and function within the gene regulatory network involved in neurogenesis in *Nematostella vectensis*.

## Chapter 2 Aim of the study

Studies focusing on neurogenesis in the starlet sea anemone *Nematostella vectensis* are increasing in recent years. These data reveal unifying features with the neural progenitor cells observed in bilaterians, as well as non-conserved characters. What remains unclear are the mechanisms involved in the differentiation into various neural cell types. Identifying these mechanisms has implications for understanding the origin and diversification of the nervous system in Metazoa. With the experiments presented in this thesis, my colleagues and I have aimed to shed light on these open questions.

The presence of a *NvSoxB(2)*-expressing progenitor cell population, that give rise to the various neural cells is well documented in *Nematostella*. Among the genes identified by transcriptomic analysis of animals injected with *NvSoxB(2)* morpholino, were the two transcription factors *NvPOU4* and *NvInsm1*. The present thesis focuses on those transcription factors and their potential role in neural differentiation in a non-bilaterian model organism.

More specifically, we aim to:

- Characterize the expression and the functional role of the single *NvPOU4* gene. Orthologs of this gene are involved in the terminal differentiation of various neural cells in bilaterian. By studying this gene in a cnidarian model we want to test if the terminal selector concept is applicable outside bilaterian.

- Examine in detail the expression of *NvInsm1*. Vertebrate orthologs of this gene are involved in the development of neurons and endocrine cells. Through its analysis we aim to obtain new insights into the developmental potential of *NvSoxB(2)*-expressing progenitor cell population.

This thesis aims to unravel insights into the mechanisms regulating the specification and differentiation of neural cells in *Nematostella vectesis* and to improve the reconstruction of ancestral and derived aspects of cnidarian neurogenesis.

# Chapter 3 List of papers

Paper I:

Océane Tournière, David Dolan, Gemma Sian Richards, Kartik Sunagar, Yaara Y Columbus-Shenkar, Yehu Moran, Fabian Rentzsch

***NvPOU4/Brain3* functions as a terminal selector gene in the nervous system of the cnidarian *Nematostella vectensis***

*Cell Reports, Accepted*

Paper II:

Océane Tournière and Fabian Rentzsch

***Insulinoma associated-1* expressing cells give rise to neuronal and gland cells in *Nematostella vectensis***

*In preparation*

# Chapter 4 Summary of the results

## 1. *NvPOU4* functions as a terminal selector gene in the nervous system of the cnidarian *Nematostella vectensis* (Paper I)

In many bilaterians, POU4 genes are expressed in the developing nervous system and play a role in the terminal differentiation of various neuronal cell types (Certel et al., 2000; Serrano-Saiz et al., 2018, 2013). We decided to investigate the function of the single *POU4* gene in *Nematostella vectensis*. It is first expressed in single cells at blastula stage, then this pattern of expression expands to the endoderm at planula stage **(Figure 1A-C, paper I)**. From late planula to polyp stage its expression also highlights the forming pharynx and tentacles **(Figure 1D-F, paper I)**. This scattered single cell expression is reminiscent of the localization of neural cells in *Nematostella*. In order to test this hypothesis, we injected *NvSoxB(2)* morpholinos to inhibit the development of neural cells. *NvSoxB(2)* morpholino injected embryos had a weak *NvPOU4* expression compared to the control animals **(Figure 1G-K, paper I)**. This experiment shows that *NvPOU4* acts downstream of the neurogenesis regulator *NvSoxB(2)*.

Double fluorescent in situ hybridization demonstrates that *NvPOU4* is not expressed in the *NvSoxB(2)*-expressing neural progenitor cells. It is, however, co-expressed with *NvNCol3* and *NvRFamide* which label differentiating cnidocytes and sensory and ganglion cells, respectively **(Figure 2A-F, paper I)**. *NvPOU4*-expressing cells do not incorporate EdU, and therefore do not proliferate.

To study the progeny of the *NvPOU4*-expressing cells, we generated a transgenic reporter line, where the *NvPOU4* promoter region drives the expression of a membrane-bound GFP protein **(Figure 3A-C, paper I)**. Next, we generated double transgenic animals by crossing the *NvPOU4*::memGFP line with other previously characterized neuronal reporter lines. In *NvSoxB(2)*::mOrange; *NvPOU4*::memGFP animals, nearly all the *NvPOU4*::memGFP expressing cells are also expressing the *NvSoxB(2)* reporter (Richards & Rentzsch, 2014), suggesting that the two genes are expressed sequentially in the same cells as their mRNA expression does not overlap **(Figure 3D-E, paper I)**. The *NvNCol3*::mOrange2 transgene labels the developing

cnidocyte capsule (Sunagar et al., 2018). In *NvNCol3*::mOrange2; *NvPOU4*::memGFP animals, the *NvPOU4* transgene highlights the membrane surrounding each capsule, from planula to polyp stage, suggesting that *NvPOU4* is expressed in developing cnidocytes **(Figure 4A-F, paper I)**. The *NvElav1*::mOrange transgenic reporter line labels a subset of sensory and ganglion neurons (Nakanishi et al., 2012). *NvElav1*::mOrange; *NvPOU4*::memGFP, double transgenic animals reveal that the two transgenes co-express in single cells in the ectoderm at planula stage, and in the endodermal nerve net at primary polyp stage **(Figure 4G-L, paper I)**. *NvPOU4* is therefore expressed in developing neurons and cnidocytes.

Next, we decided to investigate the function of *NvPOU4* by generating a mutant line via CRISPR/Cas9 which led to the creation of a 31bp deletion at the beginning of the POU domain **(Figure 5A, paper I)**. Morphological analysis of the homozygous mutants revealed they lack cnidocyte capsules **(Figure 5B, C, paper I**). Further analysis showed they do not possess any mature capsules but still express NCOL3 protein, which labels differentiating cnidocytes **(Figure 5D-K, paper I**). These observations suggest that cnidocytes are, to a certain degree, specified but fail to differentiate properly. To examine the role of *NvPOU4* in neuron formation, we crossed the *NvPOU4* mutant with the *NvElav1*::mOrange transgenic line and counted the number of mOrange$^{+}$ cells **(Figure 6A-D; Figure S6, paper I)**. However, we could not observe any striking neuronal phenotype in the mutants, suggesting that *NvPOU4* does not have a major role in the specification of the *NvElav1*$^{+}$ neurons.

To characterize the *NvPOU*$^{-/-}$ mutants in more details, we used RNA sequencing to analyze transcriptional changes in the homozygous mutants compared to their sibling controls **(Figure 6E, paper I)**. An analysis of Gene Ontology (GO) terms identified 21 terms overrepresented among the genes down-regulated in the mutants, with “ion channels” or “neurotransmitters” highly represented in the GO domain “molecular function” **(Figure 6F, paper I)**. We then decided to compare the differentially expressed genes with a previously generated transcriptome of cnidocytes from the *NvNcol3*::mOrange2 line (Sunagar et al., 2018). Sunagar and colleagues identified two populations of cells, the mOrange2 positive differentiating cnidocytes (called

*NvNCol3*$^{+}$) and the mOrange2 super positive mature cnidocytes (called *NvNCol3*$^{++}$). We generated, for each population, a unique list of genes, and compared it to the *NvPOU4*$^{-/-}$ differentially expressed genes (DEGs) **(Figure 6G-I, paper I)**. Of the 132 DEGs present only in the differentiating cnidocytes but not the mature cnidocytes, 85.6% of them were up regulated and 14.4% were down regulated in the *NvPOU4* mutants **(Figure 6G, paper I)**. In contrast, of the 62 DEGs present only in the mature cnidocytes, 88.7% of them were down regulated and 11.3% were up regulated in the *NvPOU4* mutants **(Figure 6I, paper I)**. These observations suggest that loss of *NvPOU4* reduces the expression of genes involved in the terminal differentiation of cnidocytes and increases the expression of genes involved in earlier steps of their development.

Similarly, we compared the transcriptome of *NvElav1*::mOrange cells with the DEGs of the *NvPOU4* mutants **(Figure 6J, paper I)**. Among the 182 genes expressed in *NvElav1::*mOrange$^{+}$ cells and differentially expressed in the *NvPOU4* mutants, 73.7% were down regulated and 26.3% were up regulated in the homozygous mutants. Many of those down regulated genes, highly expressed in *NvElav1* neurons, are neurotransmitter receptors. We selected two neurotransmitter receptors (a putative ionotropic glutamate receptor and a putative $GABA_A$ receptor) and studied their expression by double fluorescent in situ hybridization with *NvPOU4* (**Figure 7, paper I)**. Each of them is expressed in a different subset of the *NvPOU4*-expressing cells suggesting that *NvPOU4* plays a role in the terminal differentiation of different neuronal cell types.

This study suggests that *NvPOU4* is expressed in cnidocytes, ganglion and sensory neurons, and plays a role in the terminal differentiation of those cell types.

## 2. *Insulinoma associated-1* expressing cells give rise to neuronal and gland cells in *Nematostella vectensis* (Paper II)

In many bilaterians, the transcription factor *Insulinoma-associated 1* (*Insm1*) is expressed in various neuronal and gland cell populations and leads progenitor cells to exit the cell cycle and to enter differentiation (Duggan et al., 2007; Farkas et al., 2008; Forbes-Osborne et al., 2013). By being differentially expressed after injection of *NvSoxB(2*) morpholino, *NvInsm1* appeared as a good candidate gene to address the generation of neural cell type diversity during neuronal differentiation in *Nematostella vectensis.*

First, we decided to study the expression *NvInsm1* via in situ hybridization **(Figure 1, paper II)**. At blastula stage, it is expressed in scattered single cells. During development, this pattern continues and is more prominent on the aboral side of the embryos, but by planula stage it is also present in the forming pharynx and endoderm **(Figure 1H-N, paper II)**. *NvInsm1* expression is similar to *NvSoxB(2)*, and indeed double fluorescent in situ hybridization shows that the two genes co-express in many cells from blastula to planula stage **(Figure 1O-V, paper II).** EdU incorporation experiments showed that most *NvInsm1*$^{+}$ cells are post-mitotic, suggesting that *NvInsm1* is potentially involved in neural differentiation in *Nematostella* **(Figure S2, paper II).**

To study the progeny of the *NvInsm1*-expressing cells, we generated a transgenic reporter line, where the *NvInsm1* promoter region drives the expression of a membrane-bound GFP protein **(Figure 2, paper II).** *NvInsm1* expressing cells give rise to a vast diversity of cell types; many possess neurites whereas others are relatively large cells containing many vesicle-like structures. In order to ensure that all those cells came from a population of progenitor cells expressing *NvSoxB(2)*, we generated double transgenic animals by crossing *NvInsm1*::memGFP with *NvSoxB(2)*::mOrange animals **(Figure 3, paper II)**. This experiment revealed that the memGFP$^{+}$ cells are a subset of the *NvSoxB(2)::*mOrange$^{+}$ cells.

However, the main question remained, what is the nature of those cells? Some of them do not possess neurites, but are they all neural cells? In order to unravel this cell type diversity, we first decided to test if some of them were neurons. Double fluorescent in situ hybridizations show that *NvRFamide* (a marker of sensory and ganglion cells) and *NvInsm1* partially co-express from blastula to planula stage **(Figure 4A-H, paper II)**. Next, we generated double transgenic animals by crossing the *NvInsm1*::memGFP with other previously characterized neuronal reporter lines. The *NvElav1*::mOrange line labels sensory and ganglion cells and highlights the endodermal nerve net (Nakanishi et al., 2012). The *NvFoxQ2d*::mOrange line labels a small population of ectodermal sensory cells which do not overlap with the *NvElav1:*mOrange$^{+}$ population (Busengdal & Rentzsch, 2017) **(Figure 4I-X, paper II).** These double crossings revealed that most of the *NvElav1::*mOrange$^{+}$ and *NvFoxQ2d::*mOrange$^{+}$ cells are part of the *NvInsm1:*:memGFP$^{+}$ population. However, many cells labeled by the *NvInsm1* transgene were expressing neither the *NvElav1* transgene nor the *NvFoxQ2d* one suggesting a larger diversity of cell types.

We then wondered if some of those cells types were cnidocytes and decided to use *NvNCol3* as a marker for differentiating cnidocytes (Sunagar et al., 2018) **(Figure 5 paper II)**. Double fluorescent in situ hybridization and double transgenic lines, showed that *NvInsm1*-expressing cells do not give rise to cnidocytes.

At this point, our results suggested that *NvInsm1*$^{+}$ cells are sensory and ganglion neurons but not cnidocytes. However, many other cell types were labeled by our transgene and remain uncharacterised. As mentioned, some of them are relatively large cells containing many vesicle-like structures and do not seem to harbor any neurites. Their general shape and their presence in the ectodermal body column, the forming pharynx and septal filaments suggested that those cells could be gland/secretory cells (Babonis et al., 2019; Steinmetz et al., 2017). A published single cell sequencing atlas of *Nematostella* confirmed our morphological observations by showing expression of *NvInsm1* in neuronal and in gland/secretory metacells (Sebé-Pedrós et al., 2018).

In a comparative approach, we decided to search for the single *Insm1* gene in a published single cell data set in *Hydra* (Siebert et al., 2019) **(Figure 6 paper II)**. We

found that in *Hydra*, *HvInsm1* is also expressed in ectodermal and endodermal neurons, as well as in gland cells and their progenitors.

Our study shows that *NvInsm1*-expressing cells represent various populations of sensory and ganglion neurons in the ectoderm and in the endoderm, but also putative gland cells in *Nematostella vectensis,* and potentially in the last common ancestor of cnidarians.

### 3. Additional results: functional analysis of *NvInsm1* via CRISPR/Cas9

In addition to the results presented in **paper II**, we decided to investigate the function of *NvInsm1* by generating a mutant line via CRISPR/Cas9. 25 sgRNAs were synthesized and injected independently with Cas9 into fertilized embryos, however only one of them created a mutant profile (observed by melt curve analysis in F0). Injected embryos were raised and crossed to wildtype to produce heterozygous mutants. Genotyping of the F1 was performed via sequencing and revealed that the working sgRNA generated a 7bp deletion before the first zinc finger **(Figure 10)**. *NvInsm1*$^{+/-}$ animals were raised and crossed with previously described transgenic reporter lines, such as *NvSoxB(2)*::mOrange (Richards & Rentzsch, 2014), *NvFoxQ2d*::mOrange (Busengdal & Rentzsch, 2017) and *NvInsm1*::memGFP. Heterozygous mutants do not harbor any gross morpholgical phenotype. They have now reached sexual maturity and will soon be crossed with each other to generate *NvInsm1*$^{-/-}$ animals. The tools and techniques developed over the course of this study will facilitate understanding the function of this gene in *Nematostella vectensis.*

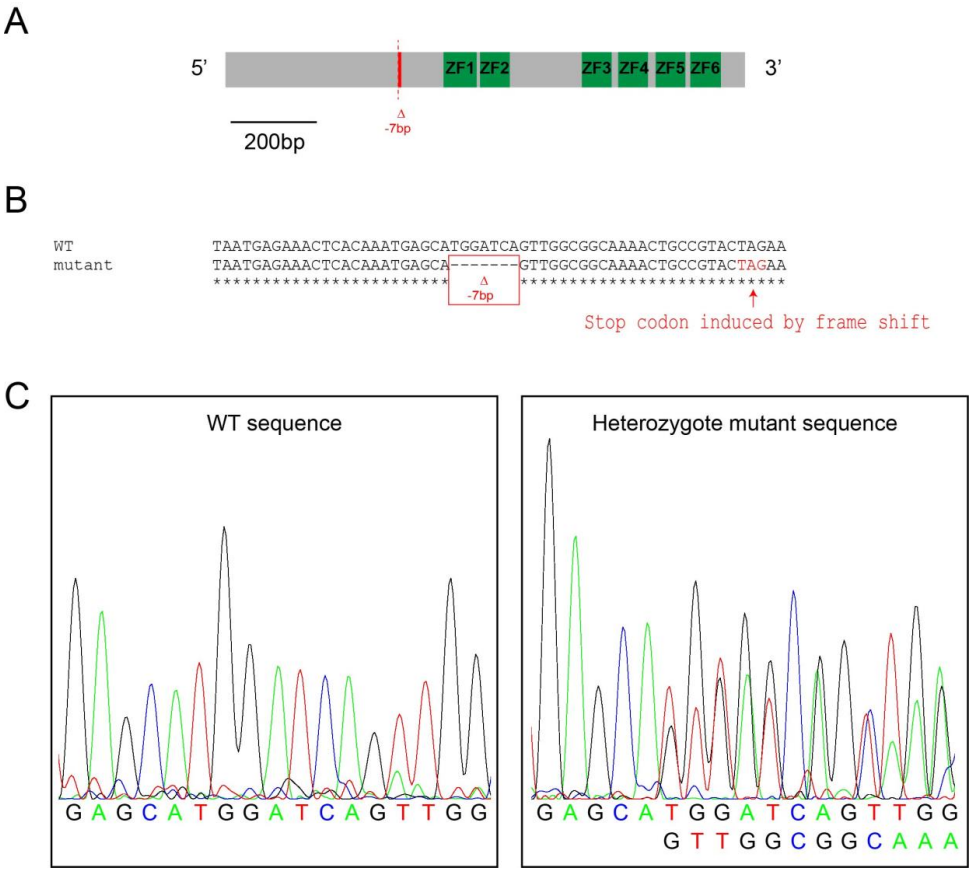


**Figure 10: Generation of *NvInsm1* mutant line via CRISPR/Cas9.** (A) Schematic of the targeting strategy. *NvInsm1* is an intronless gene, the coding sequences are in grey boxes, the zinc finger domains (ZF) are shown as a green box. The sgRNA targets before the first zinc finger domain (red dotted line) and generated a deletion of 7bp (red box) causing a frame shift and the appearance of a premature STOP codon. (B) Sequence alignment between the wildtype and the mutant sequences. The STOP codon is highlighted in red. (C) DNA chromatograms derived from individual animals with wildtype and heterozygous *NvInsm1* genotypes.

# Chapter 5 Discussion

The goal of the present thesis was to compare if mechanisms, processes and genes involved in neural differentiation in bilaterians are conserved in a non-bilaterian model organism, *Nematostella vectensis*. Two transcription factors, acting downstream of the neurogenesis regulator *NvSoxB(2),* and involved in neural differentiation in bilaterians, were selected and studied in detail in *Nematostella*.

## 1. Terminal selectors, a concept applicable outside bilaterians

### *1.1. NvPOU4 is involved in the terminal differentiation of neural cells in Nematostella*

In **paper I**, we studied the expression and function of the single *POU4* gene in *Nematostella vectensis*. Our analysis reveals that *NvPOU4* is expressed in a large population of post-mitotic neural cells, including sensory and ganglion cells and cnidocytes, the highly derived cnidarian-specific cell type. Functional studies demonstrate the pivotal role of *NvPOU4* in the terminal differentiation of those cells. *NvPOU4* mutants still produce NvNCol3 protein, which shows that the cnidocyte lineage is specified. Those animals, however, fail to correctly assemble the cnidocyst, which is characteristic of mature, fully differentiated cnidocytes. Our RNA sequencing experiment allowed the generation of a list of differentially expressed genes in *NvPOU4* mutants compared to their sibling controls. The transcriptomes of *NvNCol3*-expressing cells (Sunagar et al., 2018) at early stage of differentiation and after maturation, allowed us to study *NvPOU4* function in more detail. Genes expressed in mature cnidocytes appeared to be mostly down regulated in *NvPOU4* mutants. This finding matched our observations regarding the lack of mature cnidocysts in *NvPOU4* mutants. To our surprise, genes expressed at early steps of cnidocyte differentiation, including *NvPaxA* (Babonis & Martindale, 2017) and *NvNcol3*, were up regulated in *NvPOU4* mutants. Two hypotheses could explain this result; the first one suggests that genes involved in cnidocyte specification and early differentiation are up regulated to compensate for the lack of mature cnidocytes produced. The second one is that

*NvPOU4* is involved in cnidocyte maturation by reducing the expression of genes involved at early steps of cnidogenesis. ChIP-seq (chromatin immunoprecipitation followed by DNA sequencing) could be performed to analyse the binding of NvPOU4 genome wide and determine direct versus indirect targets. If NvPOU4 binds to the regulatory regions of both up and down regulated genes, this would suggests that it can act as both a transcriptional activator or repressor (most likely depending on different co-factors), and would therefore show that NvPOU4 represses the expression of genes involved in early cnidogenesis. However, if NvPOU4 does not bind to the regulatory regions of the up-regulated genes, it could suggest that it represses these genes indirectly. NvPOU4 could indeed activate the transcription of a repressor that represses those genes. Conceptually, it would be important to understand if there are more cells expressing those early cnidocyte markers (for example more cells expressing *NvPaxA*), however, even if it was the case it could still be due to more cells transcriptionally activating it, or that cells do not repress it. It would therefore be difficult to distinguish between the two possibilities.

In *C.elegans* and mouse a recent study showed that *POU4* genes are required not only for neural development but also for the maintenance of those cells at adult stages (Serrano-Saiz et al., 2018). A comparable function in *Nematostella* is possible, however, our present data do not allow us to test this hypothesis. Single cell RNA sequencing suggests that *NvPOU4* is expressed in neural cells at adult stage (Sebé-Pedrós et al., 2018), and our *NvPOU4*::memGFP transgenic reporter line remains expressed in sexually mature adult polyps. This argues for expression of *NvPOU4* in fully differentiated neurons, but we cannot exclude that the expression in adult polyps is due to the renewal of cells, which constantly occurs in cnidarians. In order to study the maintenance of those cells it would be necessary to develop methods for conditional and/or cell type specific loss of function in *Nematostella*.

Regarding the *NvElav1*$^{+}$ neurons, the transcriptome does not allow us to separate early differentiating neurons from mature ones but many genes up-regulated in *NvElav1::*mOrange$^{+}$ cells were down regulated in our RNA sequencing. In *NvPOU4* mutants, we could still observed the presence of mOrange$^{+}$ cells, which suggests that

*NvPOU4* is mainly involved in the terminal differentiation of these cells. Those cells are specified but lack specific identity features such as neurotransmitter receptors. It would be interesting to search for consequences not only at the cellular level but also at the entire organismal level, by searching for any behavioral changes. This would be challenging, as it is not clear what the function of the *NvElav1*$^{+}$ neurons is in wild-type animals. It would first be necessary to generate animals which clearly lack the *NvElav1*$^{+}$ neurons (neurons not specified) or lack the activity of *NvElav1*$^{+}$ neurons in order to compare them with wild-type animals and understand their biological function. Comparing then the *NvPOU4*$^{-/-}$ would become relevant to understand how the animals cope with the absence of this terminal selector. In the absence of *NvPOU4*, are the *NvElav1*$^{+}$ neurons still producing some kind of response or are they completely ineffective to the animal? It seems to me important to understand how neurons are formed and to integrate it within the context of their future function in the organism, however, more tools and behavioral assays need to be established to characterize those responses properly. In *Hydra*, simple protocols to characterize contraction rates in response to various stimuli have been tested (Rushforth et al., 1964) and more recently, machine learning techniques have been used to identify both known and unannotated behaviors (Han et al., 2018). As mentioned in the introduction, *Nematostella* has three types of muscles along its body column, circular, parietal and retractor muscles and also some retractor muscles in its tentacles. Upon touching, the animals respond by contracting those muscles and sometimes by hiding its upper, oral body part inside its body cavity suggesting that those muscles might be innervated by neurons. A recent study shows that acetylcholine regulates tentacle contraction (Faltine-Gonzalez & Layden, 2019). Establishing more protocols to characterize contraction rates in response to various stimuli but also calcium imaging, via the generation of neuronal and muscular GCaMPs transgenic reporter lines (Dupre & Yuste, 2017; Szymanski & Yuste, 2019), might help to unravel the diversity of behaviors observed in *Nematostella* and could be used to elucidate the link between neurogenesis, neuronal activity, and behavior.

*1.2. The genetic control of terminal differentiation*

It is fascinating to see how a single transcription factor can be activated in various cell types and regulate the transcription of different effector genes, according to the cell type it is expressed in. It would be particularly interesting to study the initiation and regulation of *NvPOU4* expression in those various cell types by studying its regulation in more detail. Our morpholino experiments and our analysis of the double transgenics show clearly that *NvPOU4* is acting downstream of *NvSoxB(2)*. It remains unclear however if *NvPOU4* is a direct or an indirect target. The observation that those two genes are not co-expressed in the same cells and that not all the *NvSoxB(2)*-expressing cells activate *NvPOU4* suggests that there is likely an additional step in between or that, at least, NvSoxB(2) is not sufficient on its own to activate *NvPOU4* transcription (**Figure 11**).

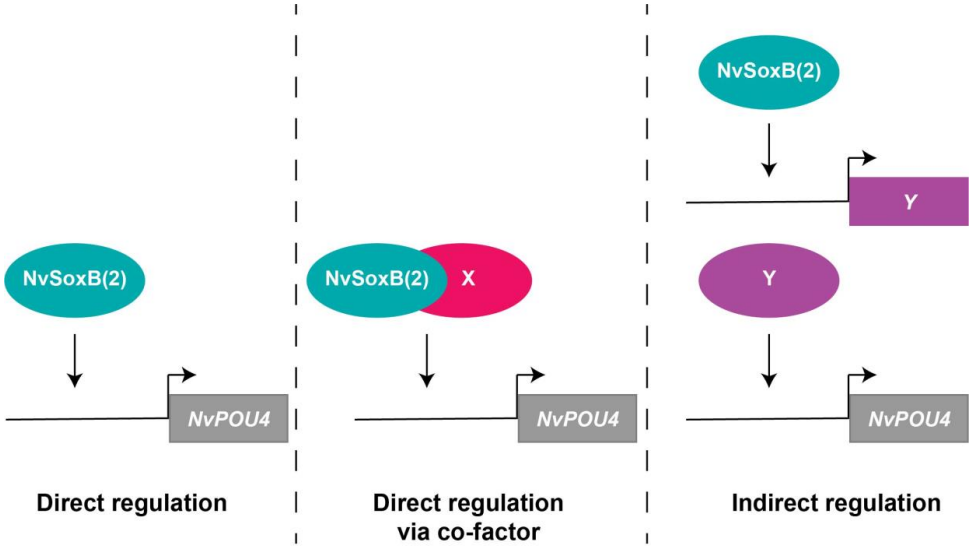


**Figure 11: NvSoxB(2) regulates *NvPOU4* activation via direct or indirect binding.** NvSoxB(2) positively regulates *NvPOU4* transcription via direct binding, on its own, or by forming a heterodimer with another transcription factor (X), or indirectly by activating an intermediary transcription factor (Y). These hypotheses are not mutually exclusive.

Those hypothesis illustrated in figure 10 are not mutually exclusive, for example NvSoxB(2) could activate the transcription of another transcription factor which can then co-operate with it to activate *NvPOU4* transcription. However, only the analysis of the binding of NvSoxB(2) genome wide can solve this question. More broadly speaking it would be interesting to dissect the regulation of *NvPOU4* in these different cells. By using available ATAC seq (Assay for Transposase-Accessible Chromatin with highthroughput sequencing) data sets, and given that we have defined a region sufficient for *NvPOU4* expression, it should be possible to identify cis regulatory elements responsible for its expression. These elements could then be analysed for transcription factor binding sites to generate a list of potential upstream factors which could be tested experimentally. This would allow us to identify the GRN acting upstream of *NvPOU4* in both neurons and cnidocytes.

According to the cell type it is expressed in, a terminal selector can activate different targets, effectors genes and therefore determine the cellular identity of a neuron. This process is often regulated via the combinatorial action of other terminal selectors (Hobert, 2016b). Direct binding of terminal selectors was studied in bilaterians (Cho et al., 2014; Duggan et al., 1998; Xue et al., 1992). In *Nematostella* single cell-sequencing (Sebé-Pedrós et al., 2018) shows that *NvPOU4* is expressed in 14 neural metacells, and each metacell expresses a unique combination of transcription factors. It is likely that some of them act, together with *NvPOU4*, to regulate the terminal identities of these cell types. Combining ATAC-seq data with target binding site prediction of NvPOU4 and with our RNA sequencing results could allow us to build the Gene Regulatory Networks (GRNs) of neuron and cnidocyte terminal differentiation.

In our analysis *NvPOU4* is involved in the terminal differentiation of neurons and cnidocytes. Why is *NvPOU4* expressed in those two cell types? The first hypothesis is cnidocytes and neurons might have a common evolutionary origin. This gene might have been expressed in the ancestral cell that gave rise to both cell types. The second hypothesis is that *NvPOU4* is regulating a common feature of cnidocytes and neurons that evolved independently in these two classes of cells. The independent evolution of the *NvPOU4* function does not mean that the two cell classes have no common origin.

Within the down-regulated genes in *NvPOU4*$^{-/-}$, there are 20 genes that are commonly expressed in *NvElav1*$^{+}$ neurons and in mature cnidocytes (**Table 1**). Among, those genes, half of them are neurotransmitter receptors or proteins important for calcium signaling, the other half remains un-annotated.

| **Genes** | **Annotation** |
|---|---|
| NVE22390 | NA |
| NVE13383 | NA |
| NVE18817 | calmodulin |
| NVE6937 | sodium channel protein 60e-like |
| NVE21922 | gamma-aminobutyric acid receptor subunit beta-4-like |
| NVE9613 | diamine acetyltransferase 2 |
| NVE8353 | NA |
| NVE9474 | NA |
| NVE6049 | cub and zona pellucida-like domains 1 |
| NVE18050 | NA |
| NVE13502 | low density lipoprotein receptor adapter protein 1 |
| NVE16899 | NA |
| NVE11189 | NA |
| NVE22611 | NA |
| NVE4432 | oxoglutarate (alpha-ketoglutarate) dehydrogenase |
| NVE18474 | cadherin egf lag seven-pass g-type receptor 1 |
| NVE3922 | lysophosphatidylcholine acyltransferase 2 |
| NVE19357 | ligand-gated chloride channel homolog 3 |
| NVE14446 | NA |
| NVE3646 | gamma-aminobutyric acid receptor subunit beta-3 |

**Table 1: Genes expressed in *NvElav1*$^{+}$ neurons and in mature cnidocytes that are down-regulated in the *NvPOU4* mutants.**

This present list of genes does not allow us to favor a specific common function in those two cell types, however, it is important to keep in mind that they might reflect some commonalities between cnidocytes and *NvElav1*$^{+}$ neurons, common aspect regulated by *NvPOU4*. Studying the expression and function of those genes in more details and in those two cell types could bring some more insights in this regard. Previous studies in bilaterians have shown the involvement of *POU4* genes in the formation of mechano-sensory cells, including hair cells, sensory neurons and merkel cells (Arendt et al., 2016; Fritzsch et al., 2007; Masuda et al., 2011; Schlosser, 2015; Xiang et al., 1997). **Paper I** shows its involvement in the formation of cnidocytes, which are cnidarian specific mechano-sensory cells. This raises two hypotheses: first, *POU4* genes might have been expressed in an ancestral mechano-sensory cell. The second hypothesis suggests that *POU4* genes might have been co-opted and integrated within new CoRC in different cell types. Arendt and colleagues define a Core Regulatory Complex as a protein complex made of terminal selectors that enables and maintains the specific gene expression programme of a cell (Arendt et al., 2016). A terminal selector by itself is often not linked to the development of a specific cell type. It is therefore only by studying POU4 gene in a specific context, within a specific GRN or a specific Core Regulatory Complex that cells expressing it could be compared (Arendt et al., 2016). Comparing CoRCs between various mechano-sensory cells would therefore be relevant to study the evolution of these cell types within metazoans.

## 2. The origin of neurons and gland cells

### *2.1. NvInsm1 is expressed in neurons and gland cells in Nematostella*

In **paper II**, we studied the expression of *Insulinoma-associated 1* in *Nematostella vectenesis*. *NvInsm1* acts downstream of *NvSoxB(2),* and is present in many post-mitotic *NvSoxB(2)*-expressing cells. Double fluorescent in situ hybridization and analysis of its transgenic reporter line identify sensory and ganglion neurons. *NvInsm1*-expressing cells are a heterogeneous cell population; it indeed comprises the *NvFoxQ2d* and the *NvElav1* neurons, which are two non-overlapping sensory/ganglion

cell populations (Busengdal & Rentzsch, 2017; Nakanishi et al., 2012). It is, however, not present in all neural cells and indeed we could show *NvInsm1*$^{+}$ cells do not give rise to cnidocytes.

Its expression is not restricted to sensory and ganglion neurons; gland cells present in the ectodermal body column, the pharynx and in the septal filaments also express it. We were surprised to notice gland cells in this line, and even more to notice that those gland cells are also labelled by the *NvSoxB(2)* transgene, meaning that they come from a population of *NvSoxB(2)*$^{+}$ cells. The *NvSoxB(2)*::mOrange transgene was previously shown to label cnidocytes, sensory and ganglions neurons (Richards & Rentzsch, 2014). Mainly studied at early stages, it has a very broad expression with various intensities. These variations of transgene intensities in different cell types might be due to different *NvSoxB(2)* expression levels in different cells, or to successive cell divisions making the transgene weaker, or due to the degradation of the mOrange protein. The GFP protein is brighter than the mOrange one and, by being present in fewer cells, it makes it easier to identify the cell type diversity. It allowed us to notice large cells containing many vesicle-like structures, visible via the membrane-tagged GFP, which also labels membranes inside the cell.

Double transgenic animals expressing both the *NvInsm1* and *NvSoxB(2)* transgenes, revealed that those cells also come from a *NvSoxB(2)*$^{+}$ cell population. This observation suggests that the *NvSoxB(2)*-expressing cells contain a population of progenitor cells that will give rise to cnidocytes, sensory and ganglion neurons and also gland cells. From these observations, two hypotheses can be raised regarding the specification of those cell types in *Nematostella*. The first one suggests that there is a population of *NvSoxB(2)*$^{+}$ progenitor cells that are composed of lineage restricted progenitor cells. The first one with the developmental potential to only give rise to sensory neurons, the second one only to ganglion neurons, the third one only to cnidocytes and the last one only to gland cells. In this scenario, *NvInsm1* would be expressed independently in sensory, ganglion neurons and gland cells, as we showed it does not give rise to cnidocytes. The second hypothesis suggests that there is a population of *NvSoxB(2)*$^{+}$ progenitor cells that eventually give rise to one lineage

restricted cnidocyte progenitor cell population and one neuron/gland progenitor cell population. The latter would activate *NvInsm1* after its terminal division at the beginning of the differentiation, and exclusively give rise to neurons and gland cells (**Figure 12**).

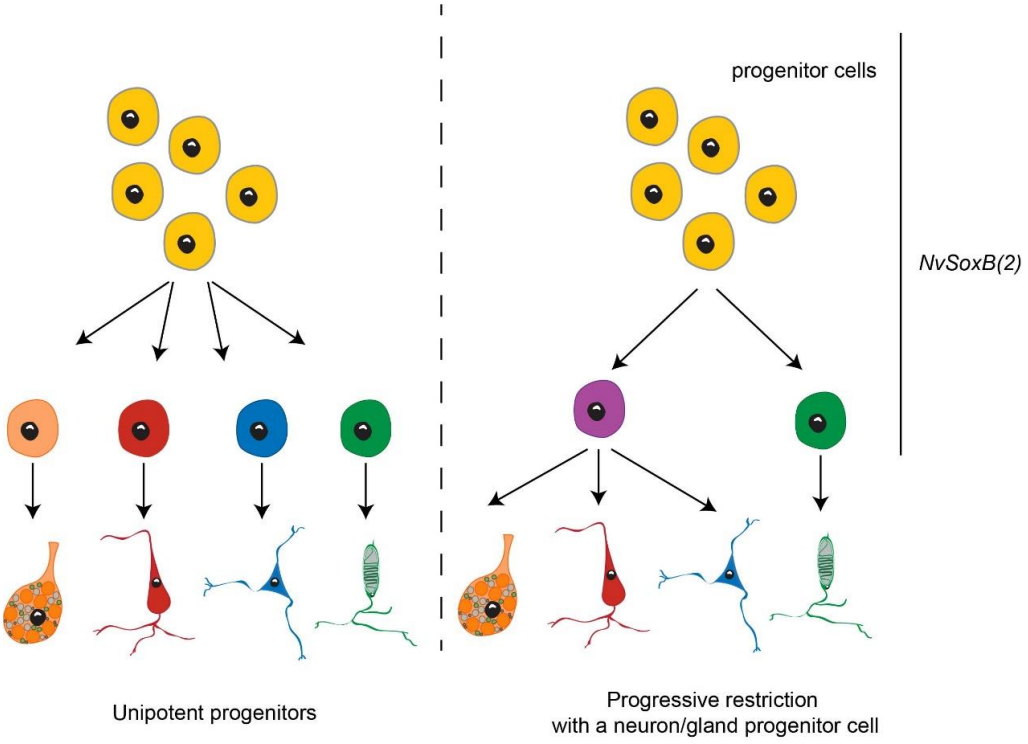


**Figure 12: Specification and differentiation of neural and gland cells during embryogenesis in *Nematostella vectensis.*** A pool of progenitor cells (which is potentially heterogeneous) gives rise to the three major neural cell types (sensory, ganglions and cnidocytes) and to gland cells during embryogenesis. Individual progenitor may give rise to only one class of cells (left part) or to different classes (right part). The bar on the right shows the stages at which *NvSoxB(2)* is expressed. (modified from (Rentzsch et al., 2017)

Our present results do not allow us to favour one of the two hypotheses and currently the lineage tracing tools necessary to address such questions have not been applied in *Nematostella*. It would be interesting to study in more detail this gene and especially those few cycling cells, expressing it. Further EdU incorporation experiments will be necessary; with for example longer pulses to catch every cycling cell. Besides, are those *NvInsm1*$^{+}$ cells in the pharynx and in the mesenteries cycling or are they post-mitotic? In order to address this question, it might be necessary to generate cross-sections of juvenile mesenteries and pharynx to have better resolution. However, if those *NvInsm1*$^{+}$ cells are not cycling in the juvenile, it does not necessarily mean that they were not cycling at earlier developmental stages. Additionally, one could also analyse *NvInsm1* expression with other known markers such as *NvAth-like*, or *NvAshA* (Layden et al., 2012; Richards & Rentzsch, 2015) which provide a better resolution of the timing of *NvInsm1* expression.

The development of single cell sequencing techniques captures the transcriptome of cells in the process of differentiation. Ordering those cells into differentiation trajectories could help in understanding cell type specification in *Nematostella* and could give us a list of candidate genes potentially expressed in putative intermediate progenitors. This analysis has indeed been done recently in *Hydra* (Siebert et al., 2019) and suggests the existence of a previously undescribed common neuron/gland progenitor cell population. This hypothesis contrasts with a previous one that suggested the existence of a common cnidocyte/neuron progenitor population (Miljkovic-Licina et al., 2007).

In a comparative approach, we decided to search for the single *Insm1* gene in *Hydra,* within the single cell dataset, and found that *HvInsm1* is also expressed in ectodermal and endodermal neurons, as well as in gland cells and their progenitors. This observation suggests that the expression of *Insm1* in *Nematostella* and in *Hydra* might be similar, and it would now be highly relevant to study its function in both clades.

*2.2. Neurons and gland cells, an evolutionary perspective*

Many bilaterians possess entero-endocrine cells, which are specialized gland cells of the intestinal epithelium. Located basally they possess a neck reaching towards the lumen and often have a basal process that interacts with the enteric nervous system (Hartenstein et al., 2017). They derive from the endoderm whereas neurons from the enteric nervous system derive from the neuroectoderm (the neural crest) (Hartenstein et al., 2017). In *Hydra*, neuron/gland progenitor cells come from interstitial stem cells present in the ectoderm and migrate through the extracellular matrix to provide gland cells and neurons in the endodermal layer (Siebert et al., 2019). In *Nematostella*, gland cells have been observed in the ectodermal body column, the pharynx and the septal filaments, all of which have an embryonic ectodermal origin (Babonis et al., 2019; Sachkova et al., 2019; Steinmetz et al., 2017). In their paper, Steinmetz and colleagues investigated the expression of bilaterian foregut, midgut and hindgut marker genes in *Nematostella*, and found them expressed in the pharynx and the septal filaments. They suggest homology between these regions and the bilaterian gut (Arendt, 2019; Steinmetz et al., 2017). By being expressed in gland cells in bilaterians and cnidarians, *NvInsm1* expression brings additional evidence that in *Nematostella*, the ectodermal pharynx and septal filaments are potentially homologous to the bilaterian endoderm. However, one could also argue that only the *Insm1*-expressing gland cells are homologous but not the entire tissue.

At the end of this study, one remaining question is the function of *NvInsm1* in *Nematostella*. Why is *NvInsm1* expressed in neurons and in gland cells? The first hypothesis is that those two cell types share common features that are regulated by *NvInsm1*. Another possibility is that *NvInsm1* was expressed in an ancestral neuro-secretory cell which then diversified to give rise to both neurons and endocrine cells, wither in the cnidarian-bilaterian ancestor or independently in both lineages. In the following paragraph we will discuss the commonalities between sensory and gland cells observed in metazoans before addressing these two hypothesis.

In many bilaterians, sensory cells and endocrine cells present in the intestinal epithelium or pancreas, share many functional and structural properties. Endocrine

cells possess two types of vesicles, large dense core vesicles that can contain neuropeptides, and synaptic like microvesicles that can contain neurotransmitters like sensory cells (Hartenstein et al., 2017; Rindi et al., 2004). They possess similar proteins and mechanisms for vesicle trafficking and release of peptides such as synaptophysin or vesicular monoamine transporter (Hartenstein et al., 2017). Receptors on their apical membrane, such as GPCRs, induce intracellular calcium waves leading to the release of peptides (Jang et al., 2007; Wu et al., 2002). Various neuropeptides can be identified in the gut of bilaterians, 15 different ones in mammals (Habib et al., 2012) and 10 in Drosophila (Veenstra & Ida, 2014). In sensory and endocrine cells those neuropeptides are secreted from their basal membrane whereas in exocrine cells the secretion process happens on the apical side.

Sensory and secretory cells not only share structural and functional properties, they also show common developmental features (Hartenstein et al., 2010, 2017). In vertebrates, secretory cells originate from slowly cycling and self-renewing intestinal stem cells located in the crypt, which will give rise to progenitor cells. Those progenitor cells divide and differentiate into various types of gland cells that move upwards into the folded villi, which is where the final step of differentiation occurs (Hartenstein et al., 2017). Sox and bHLH transcription factors regulate their specification and early differentiation processes, for example, Ngn3 specifies entero-endocrine cell fate and NeuroD1, Pax4, Pax6, Nkx2.2 are expressed in enteroendocrine sublineages (Ernsberger, 2015; Li et al., 2011). To our knowledge *Insm1* is only required for the differentiation of neuronal and endocrine cells (Bastidas-Ponce et al., 2017; Gierl et al., 2006; Osipovich et al., 2014), not for exocrine cells.

In *Nematostella vectensis*, various gland cell have been described (Babonis et al., 2019; Sachkova et al., 2019; Steinmetz et al., 2017). Located in the pharynx and the septal filaments they secrete enzymes apically into the gastric cavity and are thought to be responsible for digestion. It is however not entirely clear if some of those gland cells secrete basally (suggestive of an endocrine function).

Described above, by being expressed in neurons and gland cells, it is possible that *NvInsm1* regulates some common aspects of this secretory function. If it was the case,

it would have to be an aspect of secretion that are not shared with cnidocytes. Indeed cnidocytes have a large post-Golgi vesicle that is discharged, i.e. secreted by exocytosis, from their apical membrane. At the present time we do not have any evidence regarding a specific shared function between those two cell types which might be under the control of *NvInsm1*. The detailed study of the *NvInsm1* mutants will allow us to approach this question. If they fail to generate neurons and gland cells this would suggest that the function of this gene is probably not to control certain aspects of secretion but probably earlier aspects of the specification which may, of course, also be shared. If the cells are specified, it would be possible to cross *NvElav1::*mOrange, *NvInsm1::*memGFP double transgenics with *NvInsm1* mutants. By using FACS (Fluorescence-activated cell sorting), the double positive cells (labeling the neurons) could be sorted out from the *NvInsm1* single positive cells (labeling the gland cells), and then RNA sequencing could be used to characterize and study the impact of the mutation in the two cell types independently. In parallel, ChIP-seq could be used to study the NvInsm1 target genes in the different cell types. Together these approaches would enable us to see if in *Nematostella*, *NvInsm1* has the same target genes in those two cell types which could point towards a common function, evolutionary origin or both.

The overlap in transcription factors and secretory apparatus between neuronal and gland cells has raised the question regarding the evolutionary origin of those two cell types (Hartenstein et al., 2017). This led to the hypothesis of "protoneurons", which suggests the existence of a sensory-secretory epithelial cell in the last common ancestor of metazoans that later on diversified and gave rise to both neurons and gland cells in bilaterians (Hartenstein et al., 2017).

In the non bilaterians, poriferans and placozoans which lack a nervous system, possess gland cells containing secretory vesicles. Gland cells in placozoans, for example, express synaptic proteins such as SNAP-24, synapsin and syntaxin but also neuropeptides such as FMRFamide (Smith et al., 2014). Similarly, in sponges gland cells were shown to respond to calcium stimuli (Nakanishi et al., 2015). This suggests

that before the formation of the nervous system, some cells might already have had some specific secretory features.

The existence of a common progenitor cell that migrates and gives rise to both neurons and gland cells in the endoderm, has been hypothesized in *Hydra* (Siebert et al., 2019). If this hypothesis is correct, it would suggests that the development of those two cell types is compatible with the idea of a shared origin. However, the fact that those two cell types are linked developmentally does not necessarily mean that they are linked evolutionarily and a better understanding of the developmental potential and genetic regulation of these cells is required. The recently published single cell RNA sequencing of placozoans, sponges and ctenophores (Sebé-Pedrós, Chomsky, et al., 2018) could be used and combined with morphological analysis to bring some insights regarding the putative existence of a sensory-secretory cells in the last common ancestor of metazoans.

### 3. *NvInsm1* and *NvPOU4,* major actors of neurogenesis in *Nematostella*

It is intriguing how, together, the two transcription factors analysed in this study label most of the neural and gland cells observed in *Nematostella*. *NvPOU4* is expressed in a subpopulation of neuron and in cnidocytes while *NvInsm1* is expressed in a subpopulation of neurons and in gland cells. They are both acting downstream of *NvSoxB(2)* and both start being expressed around the same time at early blastula stage, *NvPOU4* mainly at the oral side whereas *NvInsm1* mostly at the aboral side. It is interesting to mention that in *C.elegans* both genes are major components of the terminal differentiation program of touch cell and FLP neurons. MEC-3 and UNC-86 (POU4) heterodimer activates the expression of touch-cell specific genes in the six touch neurons but also in FLP neurons. In FLP neurons, this action is prevented by EGL-44 and EGL-46 (INSM1) heterodimer, which represses these touch-cell specific genes (Wu et al., 2001). By acting on the same target genes, UNC-86 and EGL-46 regulate neuronal fate in FLP neurons. In *Nematostella*, we showed that both genes are expressed in *NvElav1*$^{+}$ neurons, it would be interesting to analyse their function in

those cells and see if like in *C.elegans* they might have an opposite role in the terminal differentiation of those cells. It is indeed possible that *NvInsm1*, as *NvPOU4*, is also a terminal selector in *Nematostella*, that drives the terminal differentiation of neuronal and gland cells depending on its action with different co-factors. Analysis of the *NvInsm1* mutant line will bring crucial information regarding this last point and will also reveal putative target genes that might be commonly targeted by *NvPOU4*. If it was true, crossing the *NvPOU4* transgenic reporter line with the *NvInsm1* mutant line might show some changes in neuronal fate in the absence of *NvInsm1*.

## 4. Conclusions and Future perspectives

In this study, we intended to obtain new insights into the GRNs involved in neural cell specification and differentiation during embryogenesis in *Nematostella vectensis*. We identified two transcription factors, *NvPOU4* and *NvInsm1*, acting downstream of *NvSoxB(2)*. Together, these two transcription factors label most of the neural cells described in *Nematostella*, as well as gland cells.

Although the expression of *NvInms1* has been studied in detail and brought new insights regarding the developmental origin of neurons and gland cells in *Nematostella*, many questions remain. They include the detailed temporal and spatial expression pattern of *NvInsm1* with other neuronal and secretory markers and further characterization of the proliferation state of these cells. Its functional role in neurons and gland cell differentiation also remains to be addressed. The tools and techniques developed over the course of this study will facilitate addressing these questions using *Nematostella vectensis* as a model organism.

Finally, the finding that *NvPOU4* plays a conserved function in the terminal differentiation of neural cells in *Nematostella*, suggests that terminal selectors were already present and active in the last common ancestor of cnidarians and bilaterians. POU homeobox genes, and terminal selectors in general, do not just control some sub-aspects of neuronal differentiation, but rather regulate the expression of the entire differentiation program of a neural cell. Although this thesis devoted a lot of work to

the expression and function of the *NvPOU4* gene in *Nematostella*, a number of questions are still outstanding. They include the analysis of the genetic control and regulation of *NvPOU4,* but also its possible interactions with other transcription factors leading to the differentiation of various neural cells. More than 600 million years after the divergence of cnidarians and bilaterians, its conserved function as a terminal selector, in two completely different populations of cells, suggests that the biological processes responsible for the specification and differentiation of neural cells were present before the appearance of a centralized nervous system. This process, among others, set the foundation to form the complex and sophisticated nervous system observed in many bilaterians today.

# Chapter 6 References


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