# Role of *Prdm* genes in ectodermal and non-ectodermal neurogenesis in *Nematostella vectensis*

Quentin I. B. Lemaître

Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
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UNIVERSITY OF BERGEN

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# SCIENTIFIC ENVIRONMENT

The research presented in this thesis for the *Philosophiae* Doctor Degree has been performed in the group of Prof. Fabian Rentzsch at the Sars International Centre for Marine Molecular Biology. The Sars Centre is a partner of the European Molecular Biology Laboratory (EMBL) and is affiliated to the University of Bergen (UiB, Bergen, Norway). The present thesis is part of the PhD training program provided by the University of Bergen via the Molecular and Computational Biology Research School. Funding from the Sars core budget and from the Research Council of Norway together with UiB (Toppforsk grant #251185/F20) supported the achievement of this work.




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*Nothing in biology makes sense*
*Except in the light of evolution*
*(Theodosius Dobzhansky, 1973)*

# TABLE OF CONTENTS

# ABSTRACT

The origin and evolution of the nervous system is a long-standing question in biology to understand the complexity observed today. Developmentally, the nervous system arises from the outer germ layer: the ectoderm. Nevertheless, several examples of mesodermal and endodermal contributions to neurogenesis have been described in bilaterians. In cnidarians, the nervous system is, completely or partly, derived from the internal germ layer, the mesendoderm. As the sister group to bilaterians, cnidarians hold an informative position to understand the cellular and molecular mechanisms underlying early aspects of neurogenesis, as well as common features shared with bilaterians.

In the present thesis, I have investigated mesendodermal neurogenesis in the anthozoan cnidarian *Nematostella vectensis*, the starlet sea anemone. Previous work has identified *NvPrdm14d* as a candidate gene potentially involved in mesendodermal neurogenesis. *Prdm* genes encode transcription factors playing a role in diverse developmental processes, notably in neurogenesis. Expression analyses together with cell proliferation assays revealed that *NvPrdm14d* defines a subpopulation of proliferating mesendodermal neural progenitor cells. Moreover, the generation and analysis of a reporter transgenic line for *NvPrdm14d,* crossed with different existing reporter lines, showed that these progenitors generate mesendodermal neurons in the body wall and the mesenteries, including potential motoneurons. Lastly, the analysis of the reporter line transcriptome provided a panel of genes potentially involved in mesendodermal neurogenesis. These findings uncovered that mesendodermal neurogenesis occurs from a molecularly heterogenous population of neural progenitors in *Nematostella*.

Since the role of *Prdm* genes have not been investigated in *Nematostella*, I have screened the temporal and spatial expression of all *NvPrdm* genes. This analysis indicated that *NvPrdm6d* and *NvPrdm13b* are potentially involved in neurogenesis, notably in cnidogenesis, the development of cnidocytes, a cnidarian-specific neural cell type.

Altogether, this thesis offers new perspectives on the evolution of non-ectodermal neurogenesis, and on the evolution of the role of *Prdm* genes, notably in neurogenesis.

# LIST OF PUBLICATIONS

**PAPER I:**

***NvPrdm14d* identifies a population of non-ectodermal neural progenitor cells in *Nematostella vectensis***

Quentin I. B. Lemaître, Ivan U. Kouzel, Henriette Busengdal, Patrick R. H. Steinmetz, and Fabian Rentzsch

*Manuscript in preparation*

**PAPER II:**

**The expression patterns of *NvPrdm6d* and *NvPrdm13b* suggest roles in cnidogenesis in the cnidarian *Nematostella vectensis***

Quentin I. B. Lemaître and Fabian Rentzsch

*Manuscript in preparation*

# LIST OF ABBREVIATIONS

**5mC:** 5-methylcytosine

**5hmC:** 5-hydroxymethylcytosine

**Ash:** gene family of *achaete-scute* homologs

**Ath:** gene family of *atonal* homologs

**bHLH:** basic helix-loop-helix domain

**BMP:** bone morphogenetic protein

**CaP:** caudal primary motor neuron

**CLE:** caudal-lateral epiblast

**CNH:** chordoneural hinge

**CNS:** central nervous system

**CtBP:** C-terminus binding protein

**DAPI:** 4',6-diamidino-2-phenylindole

**DFISH:** double fluorescent *in situ* hybridization

**Dnmt:** *de novo* methyltransferase

**dpf:** days post-fertilization

**EdU:** 5-Ethynyl-2'-deoxyuridine

**ENS:** enteric nervous system

**ESC:** embryonic stem cell

**FACS:** fluorescence-activated cell sorting

**FGF:** fibroblast growth factor

**GMC:** ganglion mother cell

**GRN:** gene regulatory network

**H3p:** phospho-histone 3

**HAT:** histone acetyltransferase

**HDAC:** histone deacetylase

**HMT:** histone lysine methyltransferase

**HnKn:** histone n at lysine n

**HnRn:** histone n at arginine n

**hpf:** hours post-fertilization

**HyEED:** Hydra Embryonic Ectoderm Development (Polycomb group gene)

**i-cell:** interstitial stem cell

**iPS:** induced pluripotent stem cell

**ISH:** *in situ* hybridization

**MAPK:** mitogen activated protein kinase

**MEK:** MAPK kinase

**Ngn:** Neurogenin

**NMp:** neuromesodermal progenitor

**NPC:** neural progenitor cell

**NS:** nervous system

**NSB:** node-streak border

**PGC:** primordial germ cell

**pMN:** primary motoneurons

**PIT1:** pituitary-specific positive transcription factor 1

**PNS:** peripheral nervous system

**PR:** PRDI-BF1 and RIZ1 homology domain

**PRC2:** Polycomb repressor complex 2

**Prdm:** family of PR domain-containing transcription factors

**RA:** retinoic acid

**scRNAseq:** single cell RNA sequencing

**SET:** Suppressor of variegation 3-9, Enhancer of zeste, Trithorax homology domain (carrying an HMT activity)

**Sip1:** Smad-interacting-protein 1

**sgRNA:** single guide RNA

**Shh:** sonic hedgehog

**suH:** suppressor of Hairless

**SynB:** Synaptotagmin B

**Syt1:** synaptotagmin1

**T/Bra:** T-box/Brachyury

**TET:** Ten-Eleven Translocation protein

# INTRODUCTION

The topic of the present PhD thesis takes place in the broader context of the diversity and evolution of neurogenesis, with a focus on non-ectodermal neurogenesis. In order to introduce the subject, I will provide a brief description of the evolutionary history of the nervous system, a review of the current knowledge about neurogenesis (both ectodermal and non-ectodermal) and finally, I will state the aims of my project.

## 1-Brief evolutionary history of the nervous system

The field of Evolutionary-Developmental biology (Evo-Devo) aims to study developmental processes across the animal (or plant) phylogenetic tree by comparing them in evolutionary distinct species. It allows the global understanding of the biological processes as well as their evolutionary history. It is, therefore, important to place the topic of the present thesis back into its evolutionary context. To this purpose, I will start by briefly introducing the phylogeny of metazoans and illustrating the evolutionary history of the nervous system(s).

Metazoans correspond to the phylogenic group representing the entirety of the animal kingdom. It is composed of bilaterians (animals with bilateral symmetry, *e.g.* vertebrates, arthropods, mollusks, etc.), cnidarians (jellyfish, sea anemones, corals), poriferans (sponges), ctenophores (comb jelly) and placozoans (**Figure 1**). While the phylogenetic relationship between cnidarians and bilaterians as sister groups is clear (Dunn et al., 2008; Hejnol et al., 2009; Ryan et al., 2013), the phylogenetic position of ctenophores and poriferans is still debated (Hejnol et al., 2009; Jékely et al., 2015; Moroz et al., 2014; Pisani and Liu, 2015; Ryan et al., 2013; Srivastava et al., 2008). This leaves the phylogeny of basal metazoan lineages uncertain and affects evolutionary models.

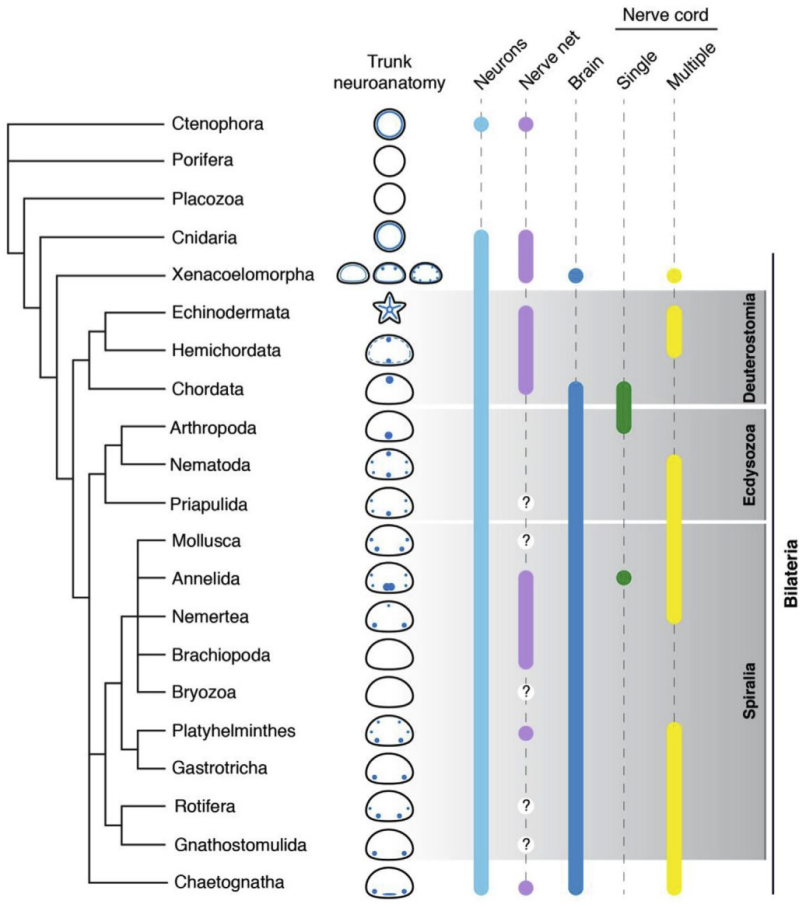



***Figure 1:* The diversity of neural anatomies across metazoans.** *Distribution of the nervous system and its anatomical characters in representative metazoan lineages. Note that the phylogenetic relationship between ctenophores, poriferans and poriferans is still debated. From* (Martín-Durán and Hejnol, 2019)

The nervous system is one of the defining features of metazoans and it is present in most animals, apart from poriferans and placozoans. The emergence of neurons played an important role during metazoan evolution and allowed fast behaviors in response to environmental stimuli. Today, there is a huge diversity of the nervous system across metazoans in term of cell number, functions, and anatomies (**Figure 1**). For example, the nematode *C. elegans* possesses exactly 302 neurons while the mammalian brain is composed of multiple billions of neurons. Moreover, neural cells likely form the

most diverse cell type (Burkhardt, 2015; Burkhardt and Sprecher, 2017; Martín-Durán and Hejnol, 2019).

Deciphering the origin of the nervous system(s) as a single or multiple event(s) is difficult due to the unresolved phylogenetic relationship between ctenophores, sponges and placozoans. If ctenophores are the sister group to all other metazoans, the nervous system could have arisen once, followed by a loss in sponges and placozoans. Alternatively, the nervous system could have evolved independently in ctenophores and eumetazoans (cnidarians and bilaterians) from the same basal precursor system. However, if ctenophores are the sister group to eumetazoans, the nervous system could have a unique origin (Burkhardt and Sprecher, 2017; Jékely et al., 2015).

### 1.1-The pre-nervous system machinery

Despite the nervous system being a structure exclusively found in metazoans, its basic molecular and cellular machinery pre-dates the emerge of metazoans. Indeed, the closest relatives to all metazoans, *i.e.* the protist choanoflagellates, possess a rich synaptic homologue repertoire in their genome (Burkhardt, 2015). This repertoire encodes several key proteins involved in post-synaptic scaffolding, such as Homer, Shank and PSD-95, as well as proteins involved in synaptic transmission with voltage-gated sodium and calcium channels (**Figure 2**). Moreover, choanoflagellates possess a primordial neurosecretory apparatus containing the neuronal SNARE complex. Some species, such as *Salpingoeca rosetta*, can form multicellular colonies in which voltage-gated channels and secretory SNARE are upregulated, but these colonies do not form synapses. Nonetheless, this indicates that the ability to communicate via electrical and/or chemical signaling using proto-synaptic proteins might have evolved before the appearance of metazoans (Burkhardt, 2015).

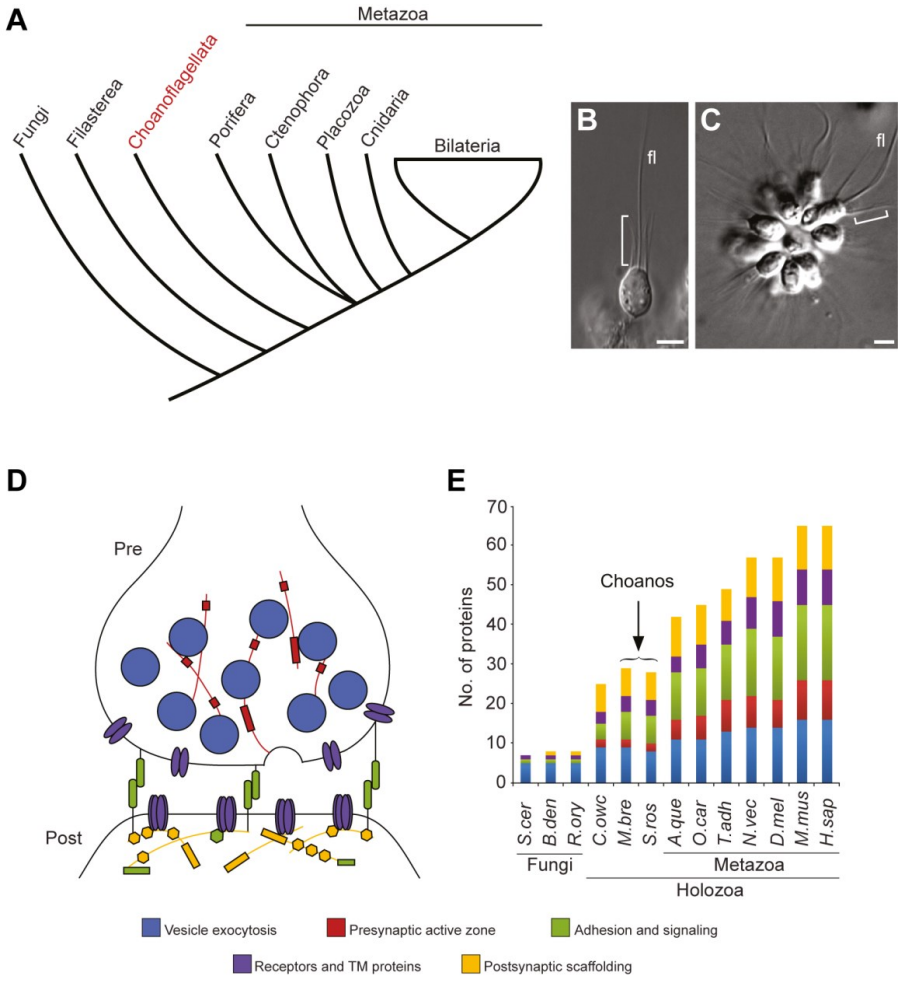



***Figure 2:*** ***A rich synaptic protein homologue repertoire pre-dates metazoans.*** *(**A**) Phylogeny showing that choanoflagellates are the closest living relatives to all metazoans. (**B**) Choanoflagellates, here represented by Salpingoeca rosetta, are unicellular aquatic protists with a single apical flagellum (fl) surrounded by an apical collar of actin-filled microvilli (bracket). Undulations of the flagellum allows the capture of bacteria used as food source. (**C**) Colonial stage of S. rosetta. Scale bar: 2 µm. (**D**) Schematic representation of a eumetazoan glutamatergic synapse. Protein types are defined in the key below the scheme. TM: transmembrane. (**E**) Abundance of synaptic protein families in diverse eukaryotes. Many proteins evolved before the divergence of C. owczarzaki, choanoflagellates and metazoans. Choanos: choanoflagellates; S. cer: Saccharomyces cerevisae; B. den: Batrachochytrium dendrobatidis; R. ory: Rhizopus oryzae; C. owc: Capsaspora owczarzaki; M. bre: Monosiga brevicollis; S. ros: Salpingoeca rosetta; A. que: Amphimedon queenslandica; O. car: Oscarella carmela; T. adh: Trichoplax adherens; N. vec: Nematostella vectensis; D. mel: Drosophila melanogaster; M. mus: Mus musculus; H. sap: Homo sapiens. Modified from* (Burkhardt, 2015)

Similarly, metazoans lacking a nervous system, *i.e.* poriferans and placozoans, also possess a rich synaptic homologue repertoire [**Figure 2E**; (Arendt, 2020; Burkhardt, 2015; Burkhardt and Sprecher, 2017; Leys, 2015)]. This includes important proteins required for the structure of synapses, for example Liprin-α, CASK, ephrin receptors and neurexin. Additionally, the poriferan genome encodes ionotropic glutamate and GABA receptors, as well as proteins composing the post-synaptic density. Interestingly, placozoans possess some gland cells carrying neuron-like features, as secretory SNARE proteins, complexin, synapsin and vesicular glutamate transporters (VGluT), and are stained with FMRFamide antibodies (Arendt, 2020; Leys, 2015).

Altogether, it means that the molecular and cellular machineries required to establish synaptic transmission already existed before the appearance of the nervous system. Therefore, neurons arose from the recruitment of these different pre-existing machineries into specialized units.

### 1.2-Origin of the first nervous system

Behaviors appeared before the nervous system as cell communication, phototaxis, chemotaxis and gravitaxis can be observed in bacteria, plants, fungi, and protists. The presence of a nervous system is therefore dispensable for the establishment of behaviors. Instead, evolutionary constraints to reduce the energetic cost and improve the efficiency of such behaviors might have led to the emergence of the first nervous system. Two main models have been proposed to explain the origin of the first nervous system: the input-output model and the skin brain hypothesis.

The input-output model postulates that the first neural circuit appeared in ciliated larvae to modulate and coordinate ciliary beating-mediated locomotion (Jékely, 2011). In this scenario, the first neural cell would have evolved from ciliated sensory cells to amplify the signal and reduce the energetic cost. This pre-neuron sensory cell would have modulated the beating of its own cilium or the beating of neighboring ciliary cells. Then, the first neuron would have evolved from this pre-neuron sensory cell to establish direct connections with ciliary cells. These direct connections would have allowed a

coordination of ciliary beating and improved the efficiency of behavioral responses. Finally, the co-evolution of neural and muscle cells would have led to a diversification of neural cell types and to a complexification of the neural circuit.

By contrast, the skin brain hypothesis postulates that the first neural circuit appeared to improve the coordination of contractile tissues such as muscles (Keijzer, 2015; Keijzer et al., 2013). As poriferans and placozoans exhibit contractile behaviors without a nervous system (Jékely, 2011; Leys, 2015), it is the necessity of coordinating tissue contraction that would have led to the emergence of the first nervous system. This original neural circuit would have patterned and coordinated the contraction of tissues to drive efficient locomotion. Since a proper coordination requires a sensitivity toward the contractions that are generated, neurons would have acquired a sensory function and they would have been subsequently recruited to probe the external environment.

Despite both models are appealing, it is still difficult to identify the selective pressure that drove the emergence of the first nervous system as well as its initial function. Nevertheless, the acquisition of a nervous system clearly provided a selective advantage to metazoans.

### 1.3-Architecture of the first nervous system

The first nervous system likely had a simple organization to connect sensory to effector cells (input-output model) or coordinate contractile tissues (skin brain hypothesis). Today, the simplest nervous system is shaped as a nerve net, *i.e.* a mesh-looking network of neural cells in which signals can be transmitted in any direction. Such nets are organized as a planar sheet connecting epithelial sensory cells to effector cells. These nerve nets are sufficient to regulate locomotion, rhythmic contractions, peristaltic movements, to sense the environment, and for the capture of prey. Therefore, the first nervous system was likely shaped as a nerve net (Hejnol and Rentzsch, 2015).

## 1.4-Centralization of the nervous system

During evolution, the complexification of behaviors led to an increase in predation and thus, to a necessity of improving the signal integration and the speed of response. Such improvements drove the centralization of the first nervous system from a nerve net to local condensations and later, to mainly centralized nervous systems with nerve cords and brain-like structures (Hejnol and Rentzsch, 2015; Jékely, 2011; Keijzer et al., 2013). Centralized nervous systems are mainly observed in bilaterians and those exhibit a diversity of this centralization (**Figure 1**). Similarly to the emergence of the nervous system, centralization may have occurred once or multiple times during evolution, however most models explaining centralization assume that it happened once and later progressively diversify [reviewed by (Martín-Durán and Hejnol, 2019)].

However recent phylogenetic analyses, developmental and molecular data have challenged all these scenarios. Indeed, the central nervous system (CNS) shows a large diversity among bilaterians (**Figure 1**) making difficult to identify the ancestral anatomy of the CNS, considering that such ancestral anatomy can still be observed in some extant species. Moreover, there is still a conflict between models integrating a stepwise centralization and complexification of the nervous system versus models based on an ancestral centralization and complexity followed by several simplification events in distinct lineages (Martín-Durán and Hejnol, 2019; Martín-Durán et al., 2018).

Interestingly, it seems that the centralization affected the portion of the nervous system derived from the ectoderm and relate a loss of non-ectodermal neurogenesis (Martín-Durán and Hejnol, 2019). However, despite non-ectodermal neurogenesis appears to play a major role exclusively in the generation of the cnidarian nervous system, it has also been observed in bilaterians (see the following chapter **2.2** page 41 for more details). It is possible that the generation of the CNS caught all our attention and that we have missed the contribution of non-ectodermal neurogenesis in the bilaterian species used as models. In line with this idea, new cases of non-ectodermal neurogenesis have been recently described (Brokhman et al., 2019; Fabian et al., 2020), hence the advance of experimental approaches combined with the desire to explore this aspect might lead to the characterization of a higher number of similar cases in the future.

# 2-Neurogenesis

## 2.1-An ectodermal origin of the nervous system

Neurogenesis is the developmental process leading to the formation of the nervous system (NS). The cells involved in this process can be traced back to the end of gastrulation when the three germ layers (in bilaterians) are formed: the ectoderm (outer layer), the mesoderm (intermediate layer) and the endoderm (inner layer).

In vertebrates, cells with a neurogenic potential are specified in the dorsal-most part of the ectoderm, called the neuroectoderm. This region thickens to form the neural plate along the antero-posterior axis. At the stage of neurulation, cells at the edge of the neural plate form folds and the neural plate is internalized by invagination (**Figure 3**). At the end of neurulation, the internalized neural plate forms the neural tube that closes through the fusion of the neural folds. Some cells from these folds, called the neural crest cells, undergo an epithelial to mesenchymal transition and migrate out to produce diverse tissues, such as the peripheral nervous system (PNS). Additionally, some ectodermal cells located directly ventrally to the anterior neural plate give rise to different placodes that later form the sensory structures. By contrast, the rest of the ectoderm becomes skin and epidermal tissues (Squire et al., 2008).

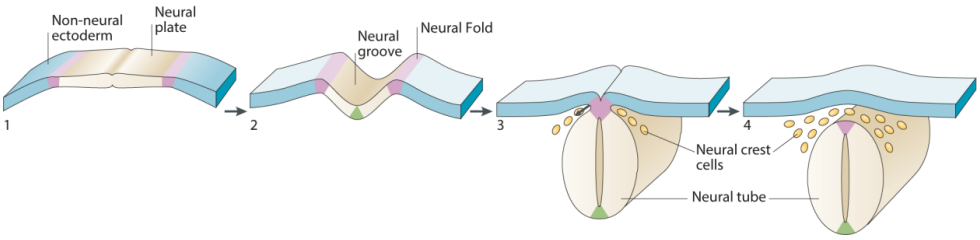



***Figure 3:* Formation of the neural tube by neurulation.** *(1) The CNS arises from a specialized epithelium, the neural plate. (2) The internalization of the neural plate by invagination is accompanied by the formation of neural folds. (3) At the end of neurulation, the neural folds fuse and segregate from the non-neural epithelium to form a neural tube (4). Neural crest cells derived from the neural folds migrate out to form the PNS, as well as melanocytes and cartilage in the head. Modified from* (Liu and Niswander, 2005)

In *Drosophila*, the neuroectoderm is specified on the ventral side of the ectoderm and the cells generating the NS (the neuroblasts) are internalized individually by delamination, while the remaining cells produce epidermis (Squire et al., 2008).

### *2.1.1-Neural induction*

The concept of neural induction, the process driving ectodermal cells to acquire a neural over an epidermal fate, comes from the experiments of Spemann and Mangold who showed that the ectopic graft of the dorsal blastopore lip induces a second body axis with a second CNS in amphibian embryos (Spemann, 1921; Spemann and Mangold, 1924). The dorsal blastopore lip acts as an organizer because in the aforementioned experiments, the second body axis is derived from the host tissues and not from the graft. Later, this organizer has also been identified in other vertebrates: the shield of teleost fish (Oppenheimer, 1936), and the node (distal tip of the primitive streak) in birds and mammals (Stern, 2005). Neural induction is mediated by the inhibition of Bone Morphogenetic Proteins (BMPs) as ectodermal cells cultured *in vitro* differentiate into neural cells rather than into epidermal cells when the BMP signaling is inhibited. However, when ectodermal cells are dissociated before being cultured, they acquire a neural identity independently from a BMP inhibition (Kuroda et al., 2005). This led to the "default model" for neural induction as ectodermal cells appear to differentiate into neurons in absence of BMP signal (**Figure 4**). This model was further supported with the identification of several BMP antagonists (*e.g.* Follistatin, Noggin and Chordin) secreted by the organizer. These antagonists are sufficient to induce ectodermal cells to acquire a neural fate *in vitro* (Lamb et al., 1993).

Nevertheless, neural induction is more complex than the "default model" suggests. More recently, additional pathways have been described as playing a role in neural induction. One of the most important of these additional pathways is the Fibroblast Growth Factor (FGF) pathway. Indeed, the FGF signaling also participates to the BMP inhibition by activating the expression of *Churchill* and *ERNI* (early response to neural induction). First, it makes the neuroectoderm competent to neural induction (Sheng et al., 2003;

Streit et al., 2000; Wilson et al., 2000) and then, Churchill induces the expression of Sip1 (Smad-interacting protein 1) which in turn, represses the expression of Smad1, a BMP effector (Stern, 2005). In parallel, the FGF signaling activates a MAPK cascade (Mitogen Activated Protein Kinase) leading to the phosphorylation of Smad1 (Pera et al., 2003). Together, these two pathways initiated by the FGF signaling result in an additional inhibition of BMPs (Stern, 2005; Stern, 2006). The FGF signaling is, therefore, playing an important role in neural induction by establishing the competency to neural induction (independently from a BMP inhibition) and reinforcing the BMP inhibition. Outside vertebrates, the FGF signaling rather than the BMP inhibition is required for the generation of the NS in ascidians (Bertrand et al., 2003; Hudson and Lemaire, 2001; Inazawa et al., 1998; Kim and Nishida, 2001).

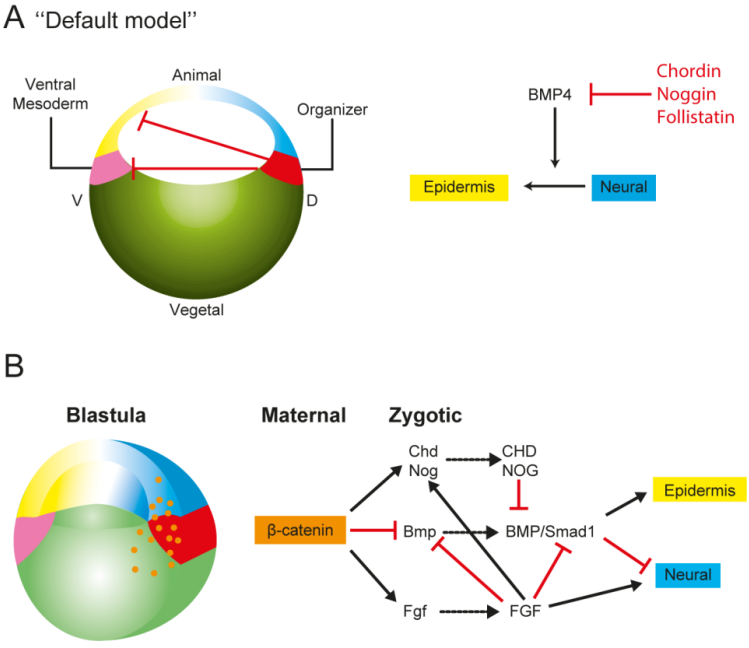



***Figure 4:*** ***Models for neural induction in vertebrates.*** *(**A**) The "default model". On the fate map, the red lines represent the BMP antagonist activity emanating from the organizer. (**B**) Model based on more recent studies to reconcile findings on the role of BMP, FGF and Wnt signaling pathways in neural induction. The neural ectoderm is shown in blue and the epidermal ectoderm in yellow. The endoderm is shown in green. Modified from* (Stern, 2005)

The inhibition of the Wnt signaling also plays a role in neural induction by predisposing the neuroectoderm to respond to FGF signaling and BMP inhibition (Heeg-Truesdell and LaBonne, 2006; Kuroda et al., 2004; Wilson et al., 2001).

The most recent model for neural induction suggests that it starts before gastrulation with inductive signals, such as FGF signaling and inhibition of Wnt, released by the organizer precursor cells to predispose the neuroectoderm to BMP inhibition. Then, FGF signaling and the BMP antagonists secreted by the organizer act in synergy to inhibit BMPs during gastrulation (**Figure 4**). After gastrulation, the competence to neural induction is lost (Stern, 2005). In order to allow neural induction, BMPs are actually inhibited three times: first, in the blastula to establish the dorso-ventral axis (BMPs are inhibited dorsally by Wnt signaling), then in mid-gastrula to determine the boundaries of the neural plate, and finally in the late gastrula to ensure a continuous expression of *Sox2* (marker of the neural plate) (Stern, 2005).

### *2.1.2-Cellular regulation of neurogenesis*

Cells composing the NS are produced by neural progenitors that are dividing cells with the ability to self-renew and to produce cells with a fate restricted to certain lineages (pluripotency). These cells can either divide asymmetrically to produce two distinct daughter cells, generally another neural progenitor to maintain the progenitor pool and, either a neural progenitor with a more restricted fate or a post-mitotic neural precursor which initiates the differentiation program. As neural progenitors have a limited mitotic potential, they can also divide symmetrically to produce two neural precursor cells. This last division can also be asymmetric if the two precursors are molecularly distinct and acquire different fates (**Figure 5**). By contrast, neural stem cells have an unlimited division potential and they generally divide asymmetrically to self-renew and increase the pool of specific cells with a fate restricted to certain lineages (Hartenstein and Stollewerk, 2015).

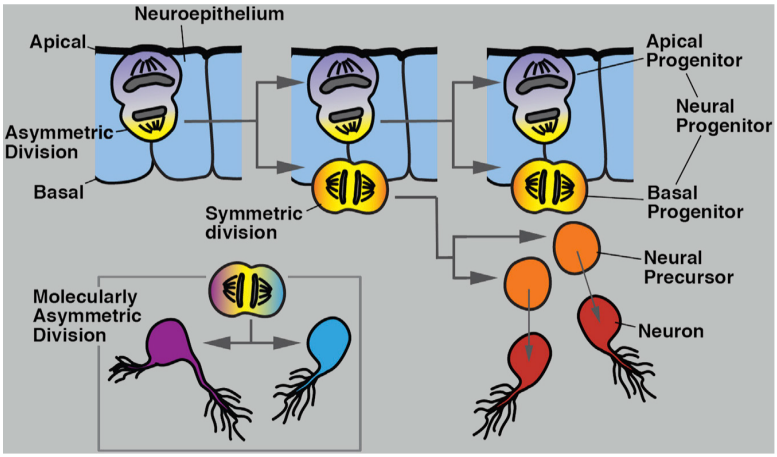



***Figure 5:* Different modes of cell division.** *Progenitor cells can divide either asymmetrically or symmetrically to self-renew and generate a precursor cell, or to generate two identical precursor cells, respectively. Progenitors can also divide asymmetrically to generate two molecularly distinct precursor cells. From* (Hartenstein and Stollewerk, 2015)

In vertebrates, the neural tube is a continuous epithelium in which neural progenitors divide first symmetrically along the plane of the epithelium to increase the pool of progenitors. Later, they divide asymmetrically to renew themselves and produce a neural precursor initiating neural differentiation to become a neuron (Götz and Huttner, 2005). In the cortex, cells are located at different positions along the apical-basal axis of the epithelium depending on their fate. Apical neural progenitors divide asymmetrically with one daughter cell remaining at the apical side and preserving the progenitor identity, and the other migrating to the basal side to differentiate (McConnell, 1995). In vertebrates, neurons and glial cells are generated by the same neural progenitors, although at different timepoints (Qian et al., 2000). Indeed, neurons are produced first by progenitors expressing both *Neurogenin2* and *Olig1/2.* Later in development, the expression of *Neurogenin2* moves dorsally and does not overlap with the expression domain of *Olig1/2* anymore. The progenitors that still express *Olig1/2* but not *Neurogenin2* generate glial cells (Zhou et al., 2001).

Unlike in vertebrates, there is no migration of neural progenitors (neuroblasts) after their internalization by delamination in *Drosophila*. Individual neuroblasts divide

asymmetrically to produce a bigger apical cell retaining the neuroblast identity, and a smaller basal cell becoming an intermediate progenitor called the Ganglion Mother Cell (GMC) (Hartenstein and Wodarz, 2013). The asymmetry of this division is driven by the unequal distribution of Numb (negative regulator of Notch) and Prospero (homeodomain transcription factor specifying the GMC fate) that are both inherited by the GMC (Betschinger and Knoblich, 2004). Then, the GMC divides only once to produce two neural precursors differentiating either into neurons or glial cells. The decision between the neuronal versus the glial fate is made by the asymmetric distribution of Gcm proteins (Glial cell missing) either during the division of the neuroblast or during the division of the GMC. The cell inheriting the Gcm proteins will generate glial cells while the other will produce neurons. Thus, there are three types of progenitors in Drosophila: "pure" neuroblasts exclusively producing neurons, glioblasts producing glial cells only and neuroglioblasts giving rise to both neurons and glial cells (Hartenstein and Wodarz, 2013; Jones, 2005).

In the nematode *Caenorhabditis elegans*, neuroblasts are internalized by epiboly and they undergo few rounds of molecularly asymmetric divisions before differentiating as neurons (Hartenstein and Stollewerk, 2015; Sulston et al., 1983).

### *2.1.3-Molecular regulation of neurogenesis*

During neural induction, the inhibition of BMPs allows the specification of the neuroectoderm (Mizuseki et al., 1998). Within the neuroectoderm, genes of the *Sox* family are among the earliest to be expressed. They encode transcription factors containing a HMG-box (High-Mobility Group) corresponding to a DNA-binding domain (Reiprich and Wegner, 2015). Genes of the SoxB class, particularly, play an important role in neurogenesis to specify cells acquiring a neural identity.

In vertebrates, the SoxB class is subdivided into SoxB1 (*Sox1*, *Sox2* and *Sox3*) and SoxB2 (*Sox14* and *Sox21*). *SoxB1* genes are expressed first to establish the neuroectodermal fate and maintain progenitor cells in a proliferative state (Bylund et al., 2003; Zhao et al., 2004). Despite its repressive action on neural differentiation, SoxB1

proteins predetermine cells for their subsequent differentiation by binding to the promoter of neural differentiation genes and maintaining them in a poised state (Bergsland et al., 2011; Wegner, 2011). SoxB2 proteins are expressed in proliferative SoxB1$^+$ progenitors and promote the progression of neurogenesis by competing with SoxB1 proteins for the same binding sites to initiate neural differentiation (Sandberg et al., 2005). When cells initiate their differentiation, *SoxB1* genes are rapidly downregulated (Graham et al., 2003), hence the balance between the antagonist actions of SoxB1 and SoxB2 proteins determines whether progenitors remain in a proliferative state or differentiate. The expression of *SoxB2* genes continues during the early steps of differentiation but it is downregulated as differentiation proceeds (Sandberg et al., 2005).

By contrast, the *Drosophila* orthologs of *SoxB1* and *SoxB2*, respectively *SoxNeuro* and *Dichaete*, act redundantly to specify neuroblasts. Moreover, they are still both expressed during neural differentiation, hence the specification of neuroblasts and the initiation of differentiation are not segregated in *Drosophila* (Guth and Wegner, 2008).

Genes of the bHLH (basic Helix-Loop-Helix) class also play an important role in neurogenesis. The bHLH domain is a structural motif composed of two α-helices linked by a loop. It mediates protein dimerization and allows the binding of DNA via the adjacent basic domain (Bertrand et al., 2002). These genes are involved in several steps of neurogenesis, such as the commitment of neural progenitors to a neural fate through the repression of alternative fates (known as the proneural function), the specification of neural progenitors to certain neural subtypes by integrating positional information, and the induction of neural differentiation by regulating differentiation genes. These genes belong to two main families: the achaete-scute (Ash) and atonal (Ath) homologs (Bertrand et al., 2002). The proneural bHLH genes are required for the selection of neural progenitors and their commitment toward specific neural lineages in both vertebrates and *Drosophila.* Their downregulation leads to a reduced number of neurons, while their upregulation increases the number of neurons. Despite these similarities, some differences exist between vertebrates and *Drosophila*.

In vertebrates, *SoxB1* genes counteract the activity of proneural bHLH genes to maintain progenitors in a proliferative and undifferentiated state. When overcoming this repression, proneural bHLH genes are transiently expressed in selected proliferative progenitors to refine the neural identity and they are downregulated before the exit of the cell cycle preceding differentiation (Bertrand et al., 2002; Bylund et al., 2003). For example, *Neurogenin2* (*Ngn2*, Ath family) specifies the neural over the glial precursor fate and activates the expression of the bHLH differentiation factor *NeuroD* (Bertrand et al., 2002; Hartenstein and Stollewerk, 2015). In addition, several bHLH genes, such as *Math1* (mouse Atonal homolog 1), *Ngn1* and *Mash1* (mouse Achaete-Scute homolog 1), are expressed in different domains along the dorso-ventral axis of the neural tube to confer distinct identities to interneurons. This function in the specification of the neural identities is mediated via either cross inhibition or various combination of bHLH genes (Bermingham et al., 2001; Gowan et al., 2001; Mizuguchi et al., 2001).

In *Drosophila*, proneural bHLH genes are expressed in quiescent cells, they act in the selection of neuroblasts delaminating from the neuroectoderm to produce neural cells, and they are downregulated before neuroblasts start dividing (Bertrand et al., 2002). In this process, the ortholog of *Ngn2* (*Tap/Biparous*) does not seem to act as a proneural gene. Instead, genes of the Achaete-Scute complex appear to fulfill this function (Bertrand et al., 2002; Gautier et al., 1997). These genes are expressed at a basal level in distinct groups of neuroectodermal cells, called proneural clusters, that become competent to the neural fate. Within these clusters, the future neuroblasts are chosen through a process named lateral inhibition (**Figure 6**). All cells of the proneural clusters express the neurogenic genes *Delta* (encoding the ligand) and *Notch* (encoding the receptor) under the regulation of proneural genes and exert a mutual lateral inhibition. Stochastically, some cells (the future neuroblasts) produce more Delta ligand than their neighbors, which initiates a negative feedback loop resulting in the inhibition of the neural fate in direct neighboring cells. In these lateral cells, Notch signaling activates the expression of other neurogenic genes, such as *Su(H)* (*Suppressor of Hairless*) and *E(spl)-C* (*Enhancer of Split C*), repressing proneural genes. Thus, these cells acquire an epidermal fate and stop expressing the Delta ligand. This leads to a reduction of the mutual lateral inhibition exerted on the future neuroblasts, resulting in an increase of

proneural gene expression and in the initiation of neural cell production through the activation of different bHLH genes (Bertrand et al., 2002; Campos-Ortega, 1985; Hartenstein and Stollewerk, 2015). This lateral inhibition also exists in vertebrates but controls the switch either between cells initiating neural differentiation and cells maintaining their neural progenitor state, or between cells acquiring different neural identities (Lewis, 1998).

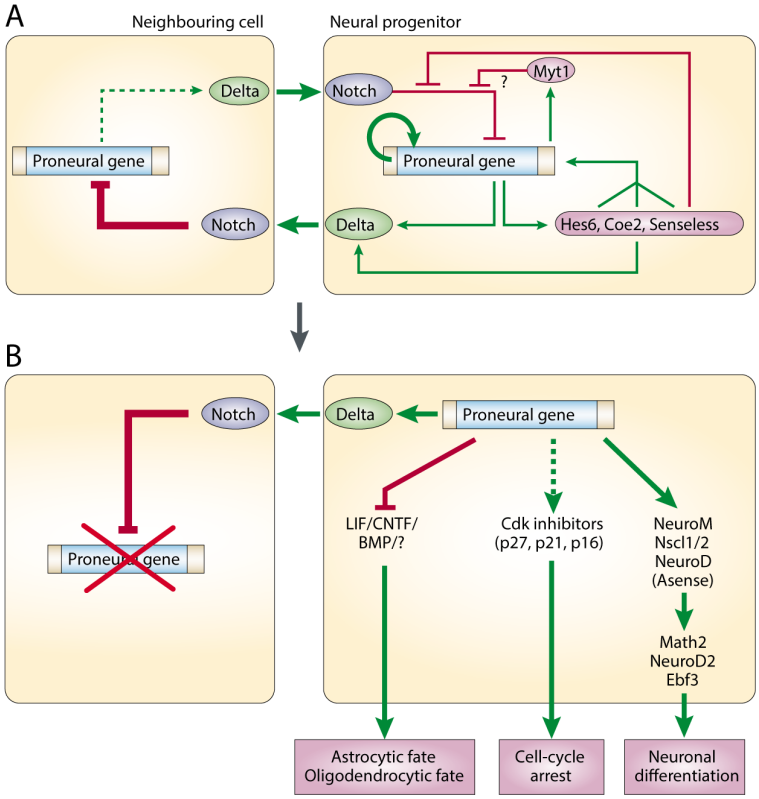



***Figure 6:* Model of lateral inhibition mediating the expression of proneural and neurogenic genes.** *(**A**) Proneural clusters in Drosophila initially express proneural genes and Delta at similar levels. A slightly elevated level of Delta in some cells (future neuroblasts) leads to the repression of proneural genes in the neighboring cells, and to a further increase in the same cells. (**B**) Proneural genes are highly expressed in neuroblasts, where it initiates the program for neuronal differentiation. Proneural genes are repressed in neighboring cells differentiating as epidermal cells. In vertebrates, proneural genes induce the expression of other bHLH genes involved in neuronal differentiation, cell cycle arrest and repression of the glial fate. From* (Bertrand et al., 2002)

### *2.1.1-Neural patterning*

Similar to the establishment of the different polarity axes in the bilaterian embryo, the CNS is also polarized along the antero-posterior and dorso-ventral axes. During the different morphogenetic events shaping the CNS, a regionalization into smaller domains occurs along these two axes.

*Patterning along the antero-posterior axis*

In *Drosophila*, the establishment of the antero-posterior axis of the CNS is linked to the general polarization of the embryo with the maternal Bicoid forming a gradient from the anterior to the posterior pole. Then, pair-rules genes together with the *antennapedia/bithorax* homeotic complex specify the identity of the different fragments along the antero-posterior axis (Squire et al., 2008). By contrast, the vertebrate CNS acquire its antero-posterior polarity through the action of different signals emanating from the subjacent mesoderm. Indeed, the transplant experiments of Spemann and Mangold revealed that at blastula stage, the organizer is subdivided into regions specifying the neural plate in an antero-posterior manner (Spemann, 1921; Spemann and Mangold, 1924). During gastrulation, the organizer is internalized and forms the chordal mesoderm located below the neural plate and later, the neural tube. The neural plate is "posteriorized" by different signals secreted by the notochord, such as retinoic acid (RA), Wnt and FGF (Del Corral and Storey, 2004; Niehrs, 2010). In the anterior side of the neural plate, Wnt signaling is inhibited by Dickkopf to maintain the anterior identity (Niehrs, 2004).

Following this broad antero-posterior patterning of the CNS, the different domains along this axis are defined by the expression of different Homeobox (Hox) genes in both vertebrates (**Figure 7**) and *Drosophila.* Hox genes are clustered in the genome and they have the particularity to have a spatial and temporal colinear expression. Indeed, genes located at the 3' of the cluster are expressed more anteriorly and earlier than genes located at the 5'. The precise expression domains of Hox genes along the antero-

posterior axis establishes a “Hox code” defining specific identities along the axis (Kessel and Gruss, 1991; Stern et al., 2006).

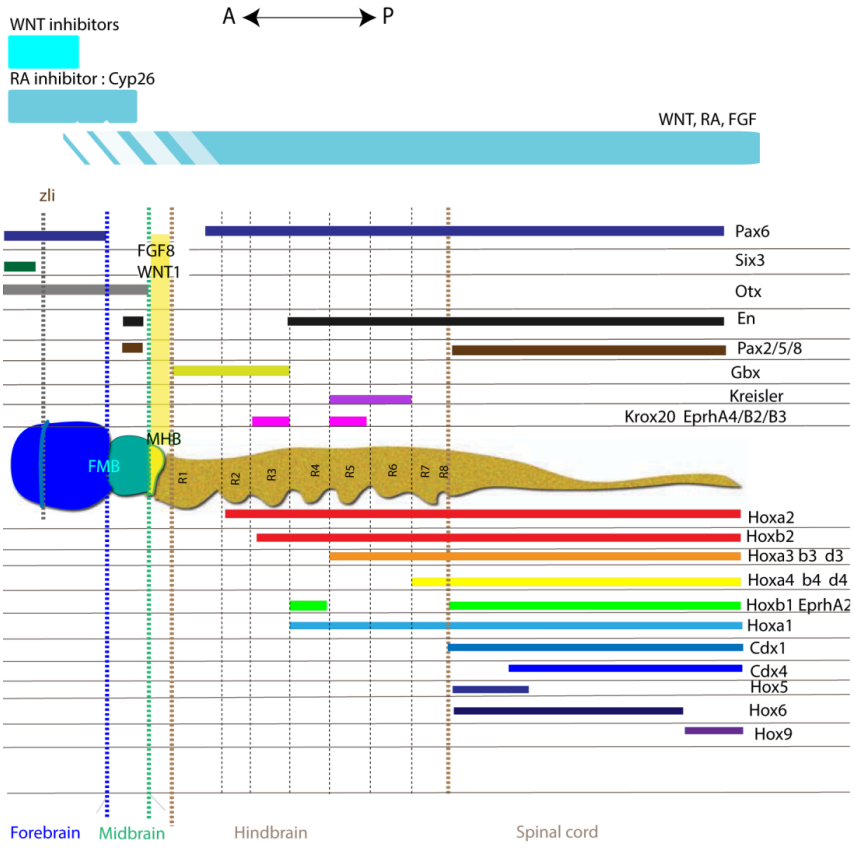



***Figure 7:* Patterning of the vertebrate CNS along the antero-posterior axis.** *The CNS is posteriorized under the action of retinoic acid (RA), the FGF and Wnt signaling pathways. Depending on the concentration of RA, different homeodomain (up) and homeobox (down) genes are expressed along the antero-posterior axis and define different domains of neural identities. FMB: fore-midbrain border, MHB: mid-hindbrain border, zli: zona limitans intrathalamica. From* (Lauri, 2013)

In the vertebrate CNS, other types of homeodomain genes are also involved in the antero-posterior patterning of the brain (**Figure 7**). Among these genes, *engrailed* is expressed in a gradient with higher levels medially at the mid-hindbrain border and lower levels at both anterior and posterior extremity of the brain (Wurst et al., 1994).

The forebrain is specified by *Emx* genes (*Empty spiracles* homologs), while *Otx* genes (*Orthodenticle* homologs) are expressed in both the forebrain and midbrain, and the anterior expression domain of *Gbx* genes (*Gastrulation brain homeobox*) defines the mid-hindbrain border. These genes interact with each other to establish the mid-hindbrain border (Rhinn and Brand, 2001). This mid-hindbrain border acts as an organizer inducing the expression of *Wnt1* in the adjacent anterior domain to participate in the specification of the midbrain. It also restricts the expression of *Fgf8* posteriorly to specify and polarize the hindbrain (Crossley et al., 1996; Squire et al., 2008). There is another organizer in the forebrain, called the zona limitans intrathalamica, which secretes Shh (Sonic hedgehog) to induce the expression of *Dlx* anteriorly in the prethalamus and *Gbx2* posteriorly in the thalamus (Kiecker and Lumsden, 2005).

Posteriorly, the gradient of RA defines which Hox genes are expressed along the hindbrain and the spinal cord. It determines the identity of the different rhombomeres and regions of the spinal cord. There is no RA in more anterior structures as the fore- and midbrain express the enzyme Cyp26 inhibiting RA (Glover et al., 2006).

*Patterning along the dorso-ventral axis*

In vertebrates, the dorso-ventral axis of the NS is established after internalization of the neural tube. This patterning occurs under the action of two organizers: the floor plate ventrally and the roof plate dorsally. Initially, the notochord acts as the ventral organizer and induces the floor plate in the ventral most part of the neural tube. Similarly, the epidermal ectoderm flanking the neural plate acts as the dorsal organizer before inducing the roof plate in the dorsal most part of the neural tube. The floor and the roof plates secrete two signals forming opposite and counteracting gradients: Shh and BMPs, respectively. The different concentrations of both signals specify the different domains along the dorso-ventral axis of the neural tube (Briscoe and Ericson, 2001; Helms and Johnson, 2003; Liu and Niswander, 2005). During neural differentiation, the ventral domains are defined by the combinational expression of different homeobox genes induced (*i.e. Nkx2.2*., *Nkx6.1*, *Nkx6.2* and *Olig2*) or repressed

(*i.e. Pax3/7*, *Pax6*, *Irx3*, *Dbx1* and *Dbx2*) by Shh. These different homeobox genes also repress each other in pairs. This combinatorial expression leads to the formation of the following ventral domains (ventral to dorsal): V3 interneurons, pMN motoneurons, V2, V1 and V0 interneurons. Dorsally, more interneuron domains are defined (**Figure 8**). As neural differentiation progresses, more genes (notably Hox and bHLH) are differentially expressed in each domain to refine the neural identities along the dorso-ventral axis (Briscoe and Ericson, 2001; Helms and Johnson, 2003; Zannino and Sagerström, 2015).

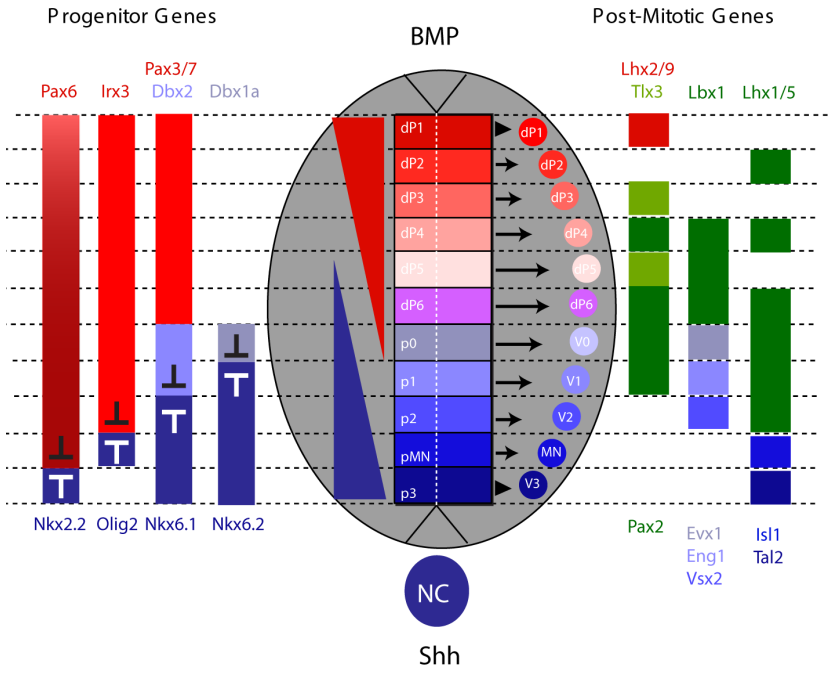



***Figure 8:* Patterning of the vertebrate CNS along the dorso-ventral axis.** *Dorso-ventral domains are established by opposing concentration gradients of Shh and BMPs, which regulate progenitor gene expression (left). The progenitor genes cross-repress each other to establish domain boundaries. Each domain will give rise to a specific cell type that expresses various post-mitotic differentiation genes. NC: notochord, dP: dorsal progenitor, p: progenitor: pMN: primary motoneurons, V: V interneurons. From* (Zannino and Sagerström, 2015)

In *Drosophila*, the dorso-ventral patterning is quite similar to the situation observed in vertebrates. Indeed, *vnd* (*ventral nervous system defective*, homolog to *Nkx2.2*), *ind* (*intermediate neuroblast defective*, homolog to *gsh*: *genomic screen homeobox*) and *msh* (*muscle segment homeobox*, homolog to the vertebrate *msx*) are expressed in the ventral, intermediate and dorsal columns of the neuroectoderm, respectively, and are required for the dorso-ventral patterning of neuroblast identities in the nerve cord (Cornell and Von Ohlen, 2000).

Both the antero-posterior and dorso-ventral axes patterning gene sets interact with each other to diversify the number of neural identities, as well as defining different dorso-ventral identities depending on the position along the antero-posterior axis (Simon et al., 1995).

### 2.2- Non-ectodermal neurogenesis

Despite the traditional view of neurogenesis taking place in the neuroectoderm originating within a sub-domain of the ectoderm, it is also known that neurogenesis is not restricted to a single germ layer. Indeed, several examples of non-ectodermal neurogenesis have been described over the past decades and it seems that both the mesoderm and endoderm have the potential to generate neural cells depending on the studied organism (**Figure 9**). Non-ectodermal neurogenesis seems to generally have a smaller contribution to the adult NS compared to the canonical ectodermal neurogenesis. However, it might be a more common feature than initially thought and be required for the acquisition of specific neural cell types.

In this thesis chapter, I will present a sample of non-ectodermal neurogenesis examples across metazoans in order to provide an overview of how this process occurs.

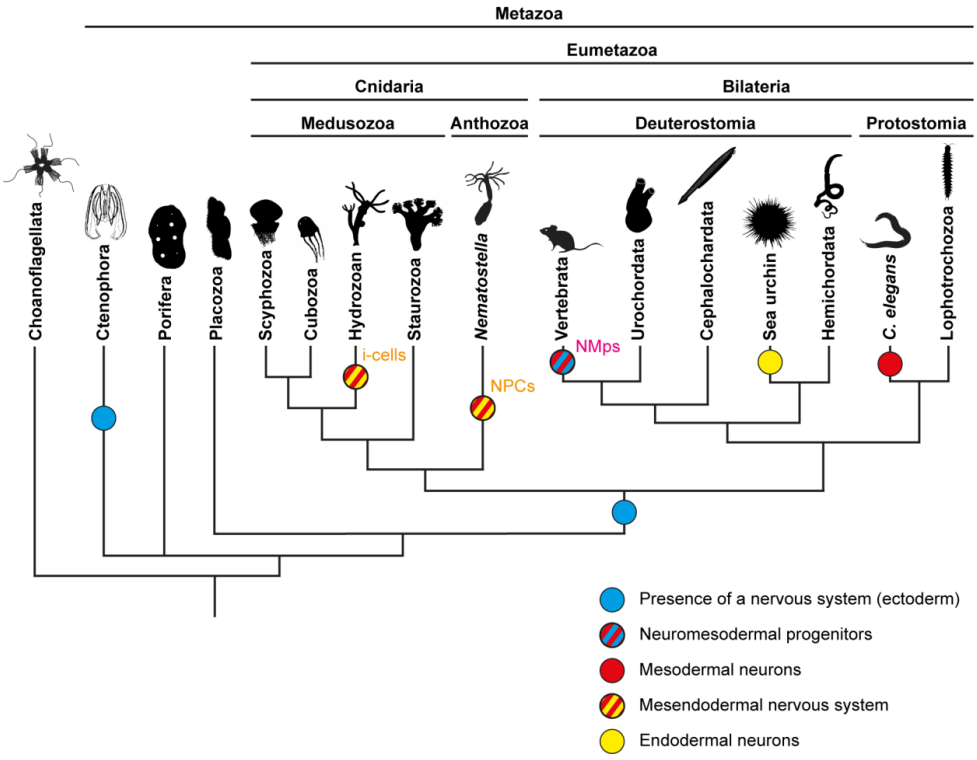



***Figure 9:*** ***Distribution of non-ectodermal neurogenesis across metazoans.*** *Simplified phylogeny of metazoans rooted with choanoflagellates as outgroup. Presence of a nervous system is indicated by a blue dot, independently from a single or multiple origin(s) of the nervous system. Non-ectodermal neurogenesis is indicated with dots of various colors illustrating the germ layer producing non-ectodermal neurons. Except for hydrozoan cnidarians, all cases of non-ectodermal neurogenesis are occurring in addition to canonical ectodermal neurogenesis. When applicable, cartoons show representative species in which non-ectodermal neurogenesis have been studied.*

### *2.2.1-Neuromesodermal progenitors in vertebrates*

In vertebrates, neuromesodermal progenitors (NMp) are bipotent cells involved in the embryonic axial elongation and they have the potential to give rise to both neural and paraxial mesoderm progenitors. The neurons derived from these progenitors will contribute to the posterior spinal cord while the mesodermal cells will contribute to the posterior somites (Henrique et al., 2015; Tzouanacou et al., 2009).

The embryonic origin of NMps remains unclear as it has not been investigated in detail. On the one hand, NMps could not be considered as part of either the ectoderm or the mesoderm as they appear to be part of a cell population which did not ingress through the primitive streak and did not commit to the ectoderm or the mesoderm yet. But since NMps are mainly found in tissues exhibiting a mesodermal identity, these progenitors could be alternatively considered as mesodermal cells capable of producing neural derivatives. This would imply that the portion of cells found in ectodermal tissues are NMps that have already been specified to a neural fate.

Nonetheless, neural progenitors derived from NMps follow a distinct neurogenic pathway from the one involved in the canonical ectodermal neurogenesis. The study of NMps is, therefore, of a particular interest in the context of non-ectodermal neurogenesis.

*Evidence for the existence of NMps*

As described in the previous section (see ***2.1*** page 28), neurulation is the developmental process leading to the internalization of the neural plate and formation of the neural tube in vertebrates. The neural tube is later patterned along the antero-posterior axis, although it mainly acquires anterior identities. Indeed, the most posterior identities are acquired during the body axis elongation forming the posterior structures at a later stage than the anterior structures.

The traditional view of the CNS patterning comes from the activation/transformation model (Nieuwkoop and Nigtevecht, 1954) claiming that the neuroectoderm is induced ("activated") under the inhibition of BMPs. In this model, the freshly internalized neural tube is first specified to anterior identities and it is only at a later stage that posterior identities are induced by a gradient of posteriorizing signals, such as RA, FGF and Wnt ("transformation", **Figure 10A**). Nieuwkoop's experiments have been performed in amphibian embryos and the activation/transformation model has been later applied to all vertebrates (Niehrs, 2010).

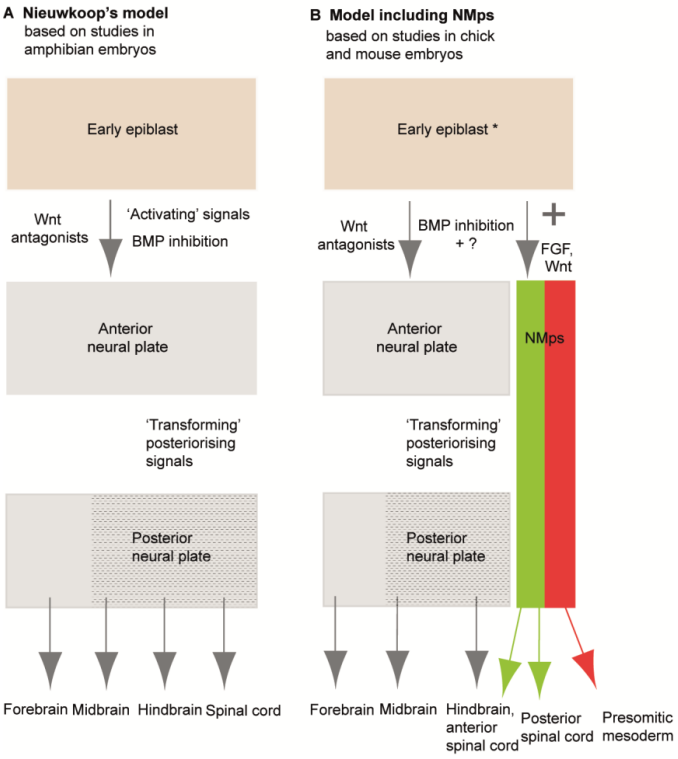



***Figure 10:*** ***Comparison of Nieuwkoop's and NMp-mediated models of neural induction.*** *(**A**) Classic 1954 Nieuwkoop's model. The activation - transformation model involves the induction of an initial anterior neural plate that is later polarized by posteriorizing signals to form the posterior neural plate. (**B**) Modern model involving neuromesodermal progenitors (NMps). Epiblasts acquire a neural fate in the anterior neural plate (which is then subdivided as proposed by Nieuwkoop) or through the induction of primitive streak-associated NMps contributing to the anterior and posterior spinal cord and to flanking pre-somitic mesoderm. From* (Henrique et al., 2015)

In amphibian embryos, the prospective regions of the CNS are proportional to their relative size in the adult. By contrast, the prospective region of the spinal cord in amniote embryos is much smaller than the anterior regions (**Figure 11**). After gastrulation, amniote embryos undergo a posterior growth, called body axial elongation, to generate all posterior structures from the remnant of the primitive streak and from the adjacent epiblasts (embryonic pluripotent cells) leading this posterior growth (Stern et al., 2006). Posterior somites develop progressively from the paraxial mesoderm produced by the caudal part of the primitive streak remnant, while its rostral tip, also known as the node (organizer corresponding to the dorsal lip of the amphibian blastopore), elongates the notochord (Brown and Storey, 2000; Cambray and Wilson, 2002; Psychoyos and Stern, 1996; Selleck and Stern, 1991) (**Figure 14**). Meanwhile, posterior neural structures (mid-hindbrain and spinal cord) originate from the surrounding epiblasts, called caudal-

lateral epiblasts (CLE) due to their location [**Figure 14**; (Wilson et al., 2009)]. At later stages, these two structures (the primitive streak and the CLE) will together form the tailbud that regresses in some species (Stern et al., 2006).

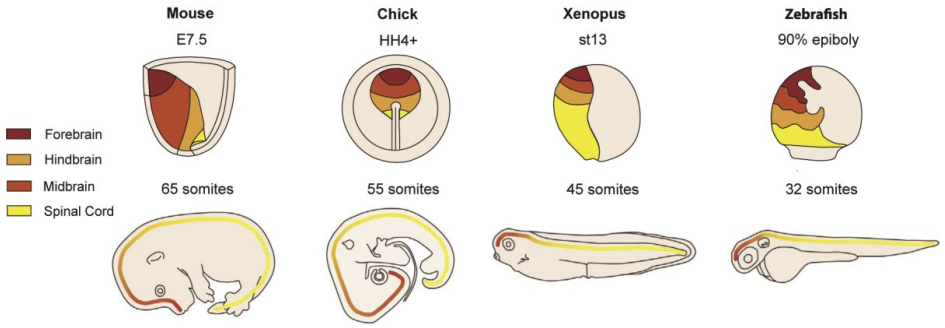



***Figure 11:*** ***Fate maps of the prospective CNS regions in four key vertebrate experimental models: mouse, chick, Xenopus and zebrafish****. (**Upper panel**) Fate maps for each species at roughly equivalent stages corresponding to the end of primary gastrulation. The entire region fated to generate the CNS is shown color coded according to prospective antero-posterior levels. (**Lower panel**) The NS regions are mapped onto embryos after the completion of somitogenesis. From* (Steventon and Martinez Arias, 2017)

For a long time, the presence of stem cells with the ability to produce both mesodermal and neural cells in the tailbud has been suspected. Indeed, transplantation experiments have shown that grafted nodes or primitive streaks (mesodermal tissues) can produce neural cells in addition to mesodermal derivatives. Moreover, this ability was conserved after three successive passages of the same ectopic tissues on different host embryos (Cambray and Wilson, 2002). Cells with this hypothetical bipotency were then mapped to the posterior part of the node and the anterior part of the primitive streak, structures that are in contact with each other. The contact zone between these two structures is called the node-streak border (NSB) (**Figure 14**) and it becomes the chordoneural hinge (CNH, contact zone between the neural tube and the notochord) at the tailbud stage. Therefore, the existence of such cells challenged the activation/transformation model and suggested that the posteriorization of the CNS was occurring through a different mechanism in non-amphibian vertebrates.

The existence of bipotent neuromesodermal progenitors exhibiting self-renewing features has been finally confirmed by lineage tracing in the mouse embryo (Tzouanacou et al., 2009). This also confirmed the localization of NMps in the NSB as well as their contribution to the posterior CNS. Additionally, NMps could be traced back to late gastrulation (stage E8.5).

The bipotency of NMps has been further confirmed by the co-expression of the neural progenitor marker *Sox2* and the early mesodermal marker *T/Brachyury* in the mouse NSB and CLE (Garriock et al., 2015; Tsakiridis et al., 2014). *Sox2* is generally expressed along the neural tube and in the CLE (Uchikawa et al., 2003), while *T/Bra* is expressed in the node, the primitive streak and the prospective mesoderm (Burtscher and Lickert, 2009; Kispert and Herrmann, 1994; Rivera-Pérez and Magnuson, 2005; Wilkinson et al., 1990) and they are both supposed to mutually repress each other to favor either neural or mesodermal fate (Takemoto et al., 2011; Thomson et al., 2011). Nonetheless, NMps co-express these two markers, although at lower levels than cell types expressing only one of them (Wymeersch et al., 2016). Since these two markers are also co-expressed in cells of the CLE that are not NMps, a specific marker for MNps is still missing.

*Induction and maintenance of NMps*

In the different lineage tracing studies, NMps have been observed during late gastrulation at the E8.5 stage in mouse; and they persist until the completion of the axial elongation (Cambray and Wilson, 2002; Tzouanacou et al., 2009). This suggests that NMps likely originate during gastrulation to expand their pool during organogenesis when the body axial elongation occurs. Additionally, the co-expression of *Sox2-T/bra* has also been observed at this stage, supporting the timing of NMp generation around stage E8.5 (Tsakiridis et al., 2014). In comparison to the understanding of the gene regulatory network leading to the modulation of the NMps cell state, little is known about the proper timing of NMps generation.

Indeed, *Sox2* and *T/Bra* are the molecular markers of two distinct progenitor pools, respectively neural and mesodermal. As they have been shown to repress each other (Takemoto et al., 2011; Thomson et al., 2011), the co-expression of these two transcription factors in NMps implies a complex regulation. Unsurprisingly, RA, the FGF and Wnt signaling pathways, known to promote posterior neural identities, are core regulators of NMps.

In the CLE, RA, the Wnt and FGF signaling pathways act in synergy to activate the expression of *Sox2* and participate to the induction of *Sox2* expression in NMps (Ribes et al., 2009; Takemoto et al., 2006). By contrast, BMP represses *Sox2* to restrict its expression to the neurogenic tissues, including the CLE [**Figure 12**; (Takemoto et al., 2006)].

In parallel, the expression of *T/Bra* is also promoted by the Wnt and FGF signaling in the NSB (Isaacs et al., 1994; Stern, 2005; Tsakiridis et al., 2014; Yamaguchi et al., 1999). Moreover, it has been shown that *Cdx2* was required to activate the Wnt signaling and induce the expression of *T/Bra* in NMps (Henrique et al., 2015; Savory et al., 2009; Shashikant et al., 1995; Subramanian et al., 1995). By contrast, RA represses the expression of *T/Bra* which is restricted to cells in the tailbud posteriorly to the somites, source of RA [**Figure 12**; (Savory et al., 2009)].

Furthermore, FGF signaling promotes the expression of *Nkx1.2* in the CLE and the pre-neural tube where it maintains the neural competency of NMps by reinforcing the FGF signal. By contrast, both *Nk1.2* and FGF are repressed by RA which favors the expression of the transcription factor *Pax6* in neural progenitors (Sasai et al., 2014). In addition, *Nkx1.2* is also activated by Wnt which is, in turn, reinforced by a positive feedback loop (**Figure 12**).

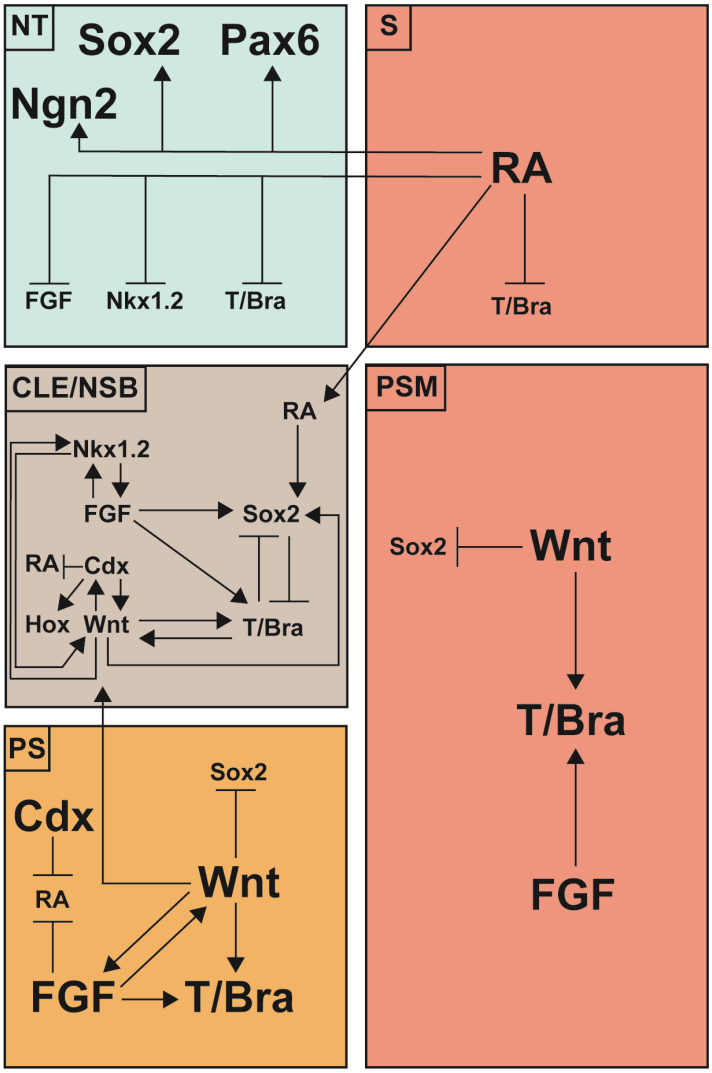



***Figure 12:*** ***Gene regulatory network for the induction of NMps in the CLE.*** *FGF and Wnt signals provided by the primitive streak (PS) and caudal-lateral epiblasts (CLE) induce the expression of T/Bra and Sox2, and T/Bra in turn promotes Wnt signaling. FGF signaling also promotes the expression of Nkx1.2 and this transcription factor in turn induces FGF transcription; it also indirectly promotes Wnt signaling. Wnt signaling induces the expression of Cdx genes, which act both to promote Wnt signaling and to regulate caudal Hox genes. RA signals produced by the somites (S) induce the expression of Sox2. The co-expression of T/Bra and Sox2 is a central feature of NMps and they are mutually repressive. This mutual repression might underpin the creation of a state in which cells are poised to adopt either neural or mesodermal cell fate. In the PS, the production of FGF and Wnt signals repress Sox2 and increase the expression of T/Bra. In the pre-somitic mesoderm (PSM), the FGF and Wnt signals also repress Sox2 to maintain T/Bra expression. In somites, produced RA signals repress T/Bra, in the somites and neural tube (NT). RA signals repress mesodermal genes in the NT: FGF and T/Bra as well as Nkx1.2, while it induces the expression of neurogenic genes: Sox2, Pax6 and Neurogenin2 (Ngn2). NSB: node-streak border. Modified from* (Henrique et al., 2015)

Once specified, NMps need to be maintained during axial elongation to allow a proper development of the posterior structures. FGF signaling is necessary for keeping NMps in a proliferating state (Mathis et al., 2001). RA is also necessary for the maintenance of the NMp state as the removal of RA *in vitro* prevents cells to co-express *Sox2* and *T/Bra* (Gouti et al., 2017), yet high levels of RA repress the expression of *T/Bra*, hence the maintenance of NMps is performed through low levels of RA. The expression of RA in NMps is maintained at low levels due to the repression exercised by Cdx proteins present in the primitive streak (Gouti et al., 2017).

As claimed by Nieuwkoop in the activation/transformation model, RA, FGF and Wnt signaling pathways are involved in the development of the posterior NS. However, these pathways initiate the development of the posterior neural structures rather than posteriorizing them (**Figure 10B**). It is now possible to derive NMps *in vitro* from mouse and human embryonic stem cells (ESC) by the application of FGF and Wnt inducing the co-expression of *Sox2* and *T/Bra* in NMp-like cells. The addition of low levels of RA enhances the induction of these cells and maintains their pluripotent state. These *in vitro* derived NMps produce neural and mesodermal derivatives with respectively a spinal cord and somitic identity corresponding to their native fate *in vivo* (Gouti et al., 2014; Gouti et al., 2017).

*Decision between neural versus mesodermal fate*

The bipotency of NMps depends on an equilibrium between RA, the FGF and Wnt signaling pathways. Any imbalance between these different signals will drive the NMps specification toward either a neural or mesodermal fate. For example, while high levels of FGF favor the co-expression of *Sox2* and *T/Bra* in NMps, a reduction of FGF levels leads NMps towards a neural differentiation. Somites produce RA that inhibits FGF signaling, thus drive the progenitors in the vicinity of somites to the acquisition of a neural fate (Olivera-Martinez et al., 2012). On the contrary, RA is inhibited by the expression of *Cdx2* in the primitive streak (Savory et al., 2009). This maintains the FGF signaling and promotes a mesodermal over neural differentiation (Olivera-Martinez et

al., 2012). Moreover, *Sox2* and *T/Bra* are respectively inhibited by BMP and RA, thus the fate decision between neural versus mesodermal is directed by external cues.

The neural differentiation of the NMp descendants is mainly driven by RA which is known to repress mesodermal markers and differentiation factors such as T/Bra (Savory et al., 2009), FGF (Diez et al., 2003) and particularly Wnt whose downregulation solely leads to the acquisition of neural identities (Cunningham et al., 2015a; Jurberg et al., 2014). Moreover, RA is required to initiate the expression of *Pax6* and *Neurog2* in neural progenitors (Diez et al., 2003; Sasai et al., 2014). This role of RA in neural differentiation of NMps derivatives is further confirmed by *in vitro* differentiation of NMps from mouse and human ESCs. The adjunction of RA drives these *in vitro* derived NMps towards a neural differentiation through an increase in *Sox2* expression as well as an increase of *Nkx1.2*, among other genes known to be involved in neurogenesis (Cunningham et al., 2016; Gouti et al., 2017). All these different genes are downregulated in mutants lacking RA (Cunningham et al., 2016). By contrast, the mesodermal fate depends on Wnt signaling (Garriock et al., 2015; Gouti et al., 2014; Gouti et al., 2017; Takada et al., 1994; Tsakiridis et al., 2014; Yoshikawa et al., 1997).

The fate mapping analysis of the CLE resulted in the determination of different fate regions within the NMp niche (Cambray and Wilson, 2002; Selleck and Stern, 1991; Wymeersch et al., 2016). This revealed that NMps acquiring a neural fate are localized in the rostral and rostral-lateral regions. Knowing that RA is produced by somites found rostrally to the NMp niche, the existence of a RA gradient is relevant. Therefore, NMps in the most rostral regions are exposed to high levels of RA and initiate neural differentiation. NMps located more caudally are exposed to low concentrations of RA and Wnt, which maintain their bipotency. Inversely, NMps in the most caudal regions are exposed to high levels of Wnt and initiate mesodermal differentiation (**Figure 13**). NMps are induced by RA, FGF and Wnt signaling and the maintenance of their proliferating uncommitted bipotent state depends on the proper balance between these pathways. The commitment and specification of these progenitors to either neural or mesodermal progenitor is also controlled by these signaling pathways, hence a tight regulation is necessary to produce the proper amount of NMps, neural and mesodermal

progenitors during axial elongation of the vertebrate embryo. Moreover, the NMp niche is a very dynamic structure with cells ingressing through the primitive streak and, later, with cells exiting the niche for contributing to tail growth. NMps are proliferating cells dividing symmetrically (Mathis et al., 2001), indicating that their progeny remains bipotent. Despite the growing pool of NMps, the niche keeps a constant size. NMps leaving the niche are then exposed to different environmental signals: rostrally, they receive a RA gradient and get specified into neural progenitors, whereas caudally, they receive a Wnt gradient and get specified into mesodermal progenitors (**Figure 13**). In the NMps niche, the RA and Wnt gradients meet at low levels and maintain the bipotency and self-renewing of NMps as NMps producing both neural and mesodermal progenitors are found in the medio-lateral region (Cambray and Wilson, 2002; Selleck and Stern, 1991; Wymeersch et al., 2016).

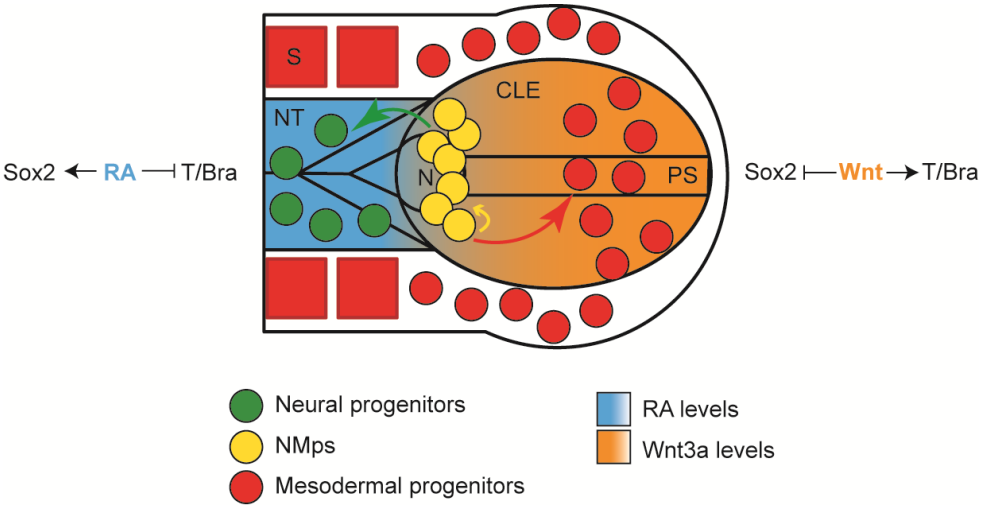



***Figure 13:* Model for the role of RA and Wnt in the fate decision of NMps in amniote vertebrates.** *The levels of Wnt expression in the epiblasts (represented by the orange color intensity) are lower next to the anterior part (left) of the primitive streak than in more posterior areas (right). Progenitors exposed to moderate levels of Wnt are involved in maintaining the NMp niche for further axial growth. Rostral to this area, progenitors exposed to high levels of RA (represented by the blue color intensity) switch on a neural program and contribute to the growing neural tube. Lower levels of RA in more caudal areas maintain the stemness of NMps. S: somite, NT: neural tube, N: node, CLE: caudal-lateral epiblasts, PS: primitive streak. Modified from* (Jurberg et al., 2014; Wymeersch et al., 2016)

*Contribution of NMps derivatives*

NMps are involved in the body axial elongation from stage E8.5 to produce the posterior neural structures and posterior somites. But to what extent are these bipotent progenitors contributing to these structures? The Wnt signaling directs these NMps to a mesodermal specification and an upregulation of this pathway in the NMp niche causes the truncation of the body axis with malformations and an absence of neural tube from the forelimb buds to the posterior-most extremity of the body (Jurberg et al., 2014). This demonstrates that NMps contribute largely to the trunk and tail development. Truncation of the body axis is also observed in mutants lacking RA and Wnt supporting this important contribution of NMps to the trunk and tail development (Cunningham et al., 2015b; Garriock et al., 2015).

Indeed, lineage tracing studies have shown that NMps participate to the formation of the hindbrain and the spinal cord (Brown and Storey, 2000) with contribution to the ventral part of the anterior spinal cord (Cambray and Wilson, 2007) and an increasing contribution to the posterior spinal cord, including across the dorso-ventral axis (Henrique et al., 2015; Tzouanacou et al., 2009).

In a more recent study, the derivatives of NMps have been tracked more precisely and it revealed a contribution of NMps from the posterior hindbrain to the NSB/CLE, spinal cord included. As previously described, NMps contribute to the floor plate of the trunk neural tube and their contribution increases posteriorly throughout the dorso-ventral extent of the neural tube from the forelimbs. Regarding the mesodermal tissues, NMps contribute to somites (except the first five) and their derivatives. In the tail, most of the neural tube and mesoderm are derived from the axial progenitors (Albors et al., 2018).

These lineage tracing studies have illustrated the important contribution of NMps for generating the hindbrain, the spinal cord, and the somite derivatives in the mouse embryo.

*Conclusion*

The existence of NMps producing both ectodermal (neural progenitors) and mesodermal (somitic progenitors) tissues during the embryonic axial elongation indicates that tissues of presumably mesodermal origin retain the potential to produce neural cells.

Evidence for such bipotent progenitors have been initially shown in the mouse embryo, but their presence were also suspected in the chick embryo (Selleck and Stern, 1991). Despite NMps were thought to be involved in the generation of the posterior neural structure exclusively in amniote vertebrates, these cells have been identified in zebrafish (Attardi et al., 2018; Martin and Kimelman, 2012). By contrast to the amniote vertebrate NMps, they form two distinct populations involved at different steps of neural development. Moreover, NMps are not self-renewing in zebrafish (Attardi et al., 2018). Surprisingly, NMps have also been described in some amphibians, such as *Xenopus* (Davis and Kirschner, 2000; Gentsch et al., 2013) and in the axolotl (Taniguchi et al., 2017), indicating that Nieuwkoop's activation/transformation model explains only part of the mechanisms driving the development of the posterior nervous system and that NMps are likely a feature shared by all vertebrates.

Interestingly, cells with a neuromesodermal potential involved during a posterior growth have been identified in some urochordates, the sister group to vertebrates. Indeed, during the secondary muscle lineage specification of the tail of such animals, a mesodermal blastomere give rise to both muscle and neural cells of the most caudal part of the tail (Lemaire et al., 2002; Razy-Krajka and Stolfi, 2019; Zalokar and Sardet, 1984). Furthermore, short germ-band insects are known to undergo a posterior elongation by addition of successive segments (Martin and Kimelman, 2009) and some annelids, *e.g. Platynereis dumerilii*, exihibit stem cells involved in the posterior elongation (Gazave et al., 2013). Although the existence of bipotent stem cells producing both ectodermal and mesodermal progenitors in protostomes remains to be elucidated, it appears that bipotent NMps-like cells might be an ancestral feature of bilaterians correlating with the presence of posterior elongation.

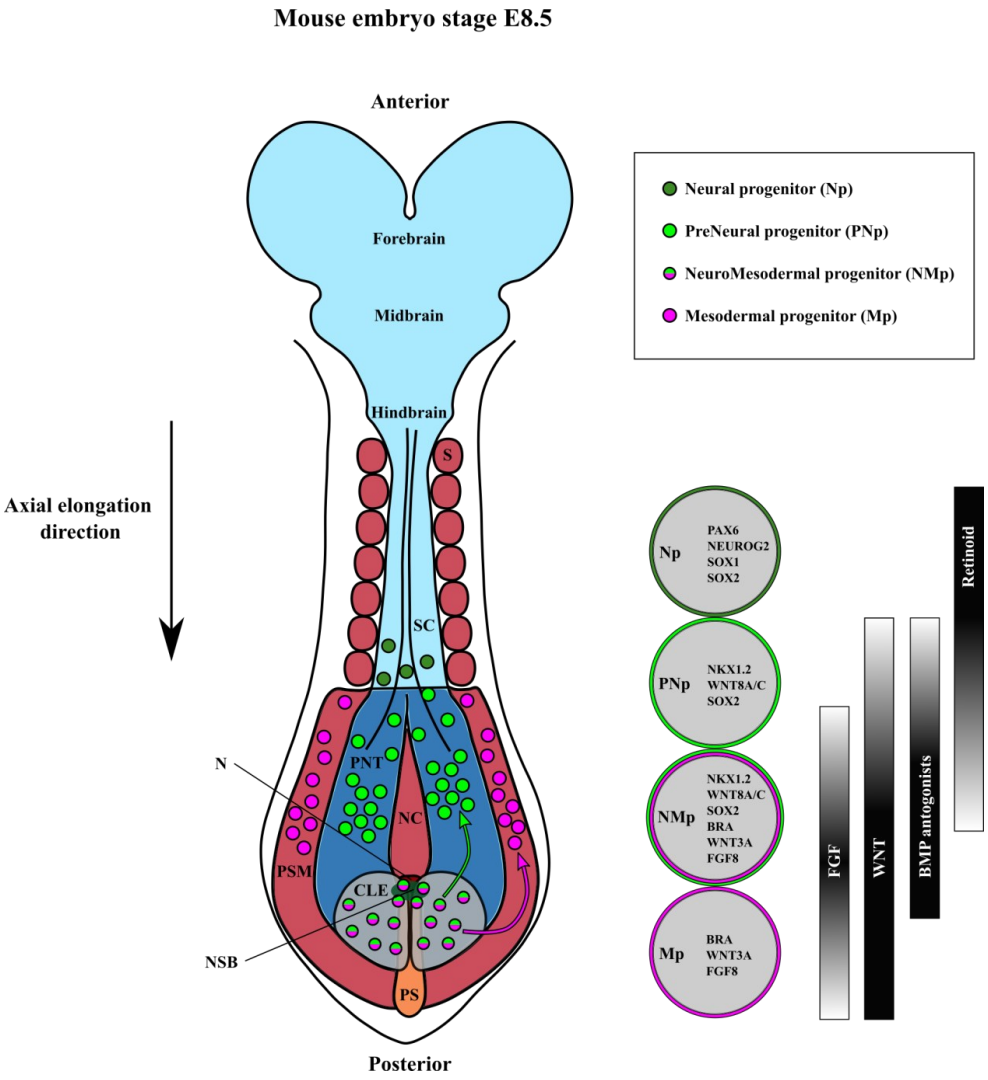



***Figure 14:* Summary of the NMps-mediated axial elongation in amniote vertebrates.** *Example of the mouse embryo at stage E8.5. NMps reside in the NSB and the CLE where they co-express Sox2 and T/Bra. Under the action of different signals, the progeny of these progenitors differentiates toward a neural or mesodermal fate, respectively contributing to the posterior spinal cord and somites. S: somite, SC: spinal cord, PNT: pre-neural tube, NC: notochord, PSM: pre-somitic mesoderm, N: node, CLE: caudal-lateral epiblasts, NSB: node-streak border, PS: primitive streak. Modified from* (Henrique et al., 2015; Verrier et al., 2018)

### *2.2.2- Mesodermal neurogenesis in Caenorhabditis elegans*

Other types of non-ectodermal neurogenesis are much less described than the vertebrate NMps, leaving a wide field open for future investigations. The second example I will present is the case of mesodermal neurogenesis taking place in the nematode *Caenorhabditis elegans*. Indeed, some pharyngeal neurons arise from a mesodermal lineage in this worm. Unlike NMps that keep the potential to give both neural and mesodermal progenitors after the segregation of the three germ layers, these specific neurons in *C. elegans* originate from cells that have already been committed to the mesodermal lineage. These neurons have been identified quite early as *C. elegans* is composed of exactly 959 cells, including 302 neurons, and that the complete cell lineage is known since 1983 (**Figure 15A**) (Sulston et al., 1983).

The pharynx of *C. elegans* is a neuromuscular tubular organ corresponding to the foregut of the animal. This structure pumps, filters and crushes bacteria that are the main food source of the worm (Pilon and Mörck, 2005). It is thought that the pharynx evolved from the same ancestral organ than the vertebrate heart due to physiological and molecular similarities (Pilon and Mörck, 2005). Indeed, both the heart and the pharynx are neuromuscular pumps contracting rhythmically (Avery and Shtonda, 2003), the muscle cells of the pharynx can contract autonomously even in the absence of neurons innervating them (Avery and Horvitzt, 1989) and the *C. elegans* homolog to the vertebrate homeobox gene *Nk2.5* (*i.e. ceh-22*) involved in heart specification is also involved in the pharynx development (Haun et al., 1998).

Interestingly, the pharynx in *C. elegans* has its own nervous system and it is almost completely isolated from the rest of the nervous system in the animal (Albertson and Thomson, 1976). Among the 62 cells composing the pharynx, 20 are part of the pharyngeal nervous system which is composed of one dorsal nerve cord, two ventral nerve cords and a nerve ring. Despite most of *C. elegans* neurons are derived from the ectoderm (AB lineage), the analysis of the cell lineage revealed that 6 pharyngeal neurons are derived from the mesoderm (MS lineage) (Sulston et al., 1983). These

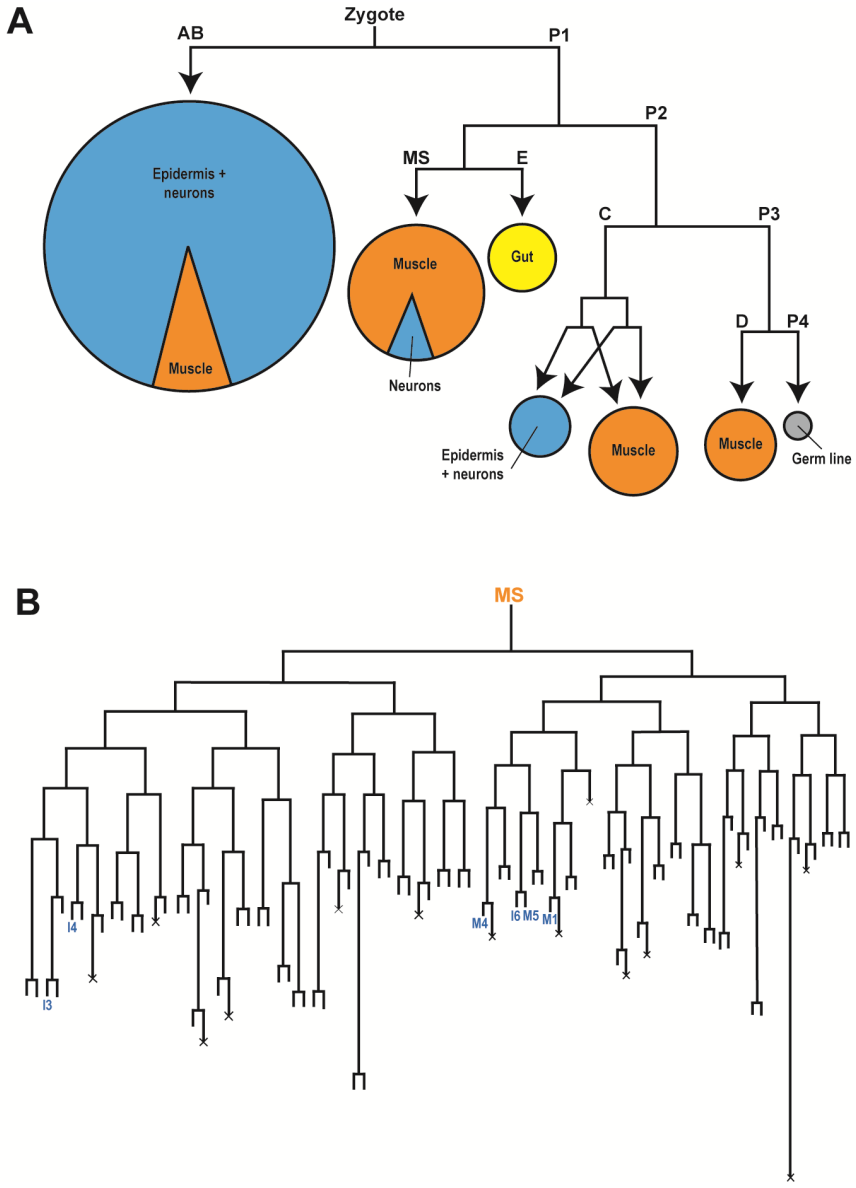



***Figure 15:*** ***The embryonic cell lineage of Caenorhabditis elegans.*** *(**A**) Generation of the founder cells and summary of cell types derived from them. Areas of circles and sectors are proportional to number of cells. The blue color typically represents ectodermal tissue, the orange color represents the mesodermal tissue, the yellow color represents the endodermal tissue, and the grey color represents the germ line. Six neurons develop from the mesodermal lineage MS. (**B**) Cell lineage of the mesodermal MS founder cell. The 6 mesoderm-derived pharyngeal neurons are indicated in blue at their own position. Note that they are all developing independently from each other, except I6 and M5 that are sister cells. Terminal branches labeled with an X indicates cells undergoing programmed cell death. Length of branches corresponds to the time at which cells are dividing, dying or differentiating. Modified from* (Sulston et al., 1983)

mesodermal neurons are the interneurons I3, I4 and I6; and the motor neurons M1, M4 and M5 (**Figure 15B**). All these neurons are unpaired single neurons, meaning that they are all unique cells (Albertson and Thomson, 1976). Some of these neurons are specified after the terminal cell division and are sister cells to muscle cells, indicating that the mesodermal lineage retains the capacity to produce neural cells (Sulston et al., 1983). However, the mechanisms underlying this ability are still poorly understood.

*Specification of the I4 neuron*

The pharyngeal I4 interneuron is a dorsal cell carrying two equivalent branches and it is the only mesoderm-derived pharyngeal neuron that has been extensively studied in terms of neuronal specification compared to most studies focusing on the differentiation and the function of the different pharyngeal neurons. As all other pharyngeal neurons, I4 exhibits basic neuronal molecular attributes such as expressing the GTPase RAB-3 and the guanine nucleotide exchange factor homolog RGEF-1, indicating that mesodermal and ectodermal neurons share a molecular signature (Luo and Horvitz, 2017; Stefanakis et al., 2015). However, the I4 neuron and its progenitor are labeled by a *hlh-1* reporter (Luo and Horvitz, 2017). HLH-1 is the *C. elegans* homolog of the mammalian muscle differentiation factor MyoD, hence it supports the mesodermal origin of the I4 neuron.

A genetic screen has been performed in order to identify mutants losing the I4 neuron identity (Luo and Horvitz, 2017). Once identified, such mutants exhibited a mutation in the *hlh-3* gene, a homolog to the mammalian proneural gene *Ascl1*/*Mash1* known to be able to reprogram mesodermal and endodermal cells into neurons *in vitro* (Amamoto and Arlotta, 2014; Marro et al., 2011). In these *hlh-3* mutants, the I4 cell is labeled by the *myo-2* and *ceh-22* muscle marker reporters, and expresses an acetylcholine esterase *ace-1* reporter that usually labels the I4 sister muscle cell pm5, indicating that I4 becomes a muscle cell in *hlh-3* mutants (Luo and Horvitz, 2017). The overexpression of the wild-type copy of *hlh-3* in the mutant allowed the rescue of the I4 defects indicating that *hlh-3* is necessary for the neural specification of the I4 cell (**Figure 16**). The loss of

*hlh-3* also affects few GABAergic neurons but does not affect neurogenesis in general, suggesting that this gene has a precise role in the specification of few neurons in *C. elegans*. Moreover, *hlh-3* acts cell autonomously to specify I4 as the ablation of the neighboring cells does not impair the I4 specification (Luo and Horvitz, 2017).

As the neurogenesis of I4 is not completely impaired in *hlh-3* mutants, Luo and Horvitz (2017) tested the potential role of *hlh-2*, a homolog of the *E2A*/*Tcf3*/*Daughterless* gene. HLH-2 is a molecular partner of HLH-3 (Grove et al., 2009; Krause et al., 1997) and they possibly act together to specify the I4 neuron. However, even though the loss of *hlh-2* affects the generation of I4, double mutants for both *hlh-3* and *hlh-2* show a more severe phenotype with the I4 neuron adopting a muscle cell fate in most mutant animals. This suggest that HLH-3 and HLH-2 acts in parallel and not together to specify I4 (**Figure 16**).

In addition, the *dpy-22* and *let-19* genes, respectively coding the mediator subunits Med12 and Med13 bridging DNA-binding proteins with the RNA polymerase II, are involved in the I4 specification as such mutants have been identified in the screen for worm strains lacking a proper I4 development (Luo and Horvitz, 2017). The expression of RNAi for these genes specifically in the I4 neuron, *i.e.* after its specification, did not have any effect, hence these genes are required for the specification of the I4 neuron but not for its maintenance. Double mutants of either one of these genes with the *hlh-2* mutation did not enhance the defective I4 phenotype, whereas it did when combined with the *hlh-3* mutation. Moreover, a yeast two-hybrid assay showed that HLH-2 and DPY-22 interact with each other (Luo and Horvitz, 2017). Taken together, these data show that HLH-2 acts with DPY-22 and LET-19 in parallel of HLH-3 to specify the I4 neuron (**Figure 16**).

The mediator complex is composed of four modules with the cyclin dependent kinase CDK8 and the cyclin C interacting with Med12 and Med13 (Malik and Roeder, 2010; Yin and Wang, 2014). Mutants for these genes in *C. elegans*, respectively *cdk-8* and *cic-1*, display a mild misspecification of I4 and this phenotype is not enhanced when these mutations are independently coupled with either *dpy-22* or *hlh-2* mutations, while

it is enhanced when coupled with *hlh-3* mutations (Luo and Horvitz, 2017). This indicates that the full mediator complex is interacting with HLH-2 in parallel of HLH-3 to induce the I4 neuron in *C. elegans* (**Figure 16**). This mediator complex acts through the inhibitory phosphorylation of the CDK-7/cyclin-H complex as the overexpression of a kinase dead CDK-7 mimics the *cdk-8*;*hlh-3* double mutant phenotype and the constitutively active form of CDK-7 does not (Luo and Horvitz, 2017). The CDK-7/cyclin-H complex could promote muscle specification by repressing neural specification (**Figure 16**).

The specification of the mesodermal-derived pharyngeal I4 neuron is therefore performed through the action of the proneural protein HLH-3 in parallel of the HLH-2/Mediator complex repressing the CDK-7/cyclin-H complex (**Figure 16**). However, HLH-2 and HLH-3 do not interact with each other in the specification of the I4 neuron, whereas they can form heterodimers (Grove et al., 2009; Krause et al., 1997). Moreover, the ectopic overexpression of both *hlh-2* and *hlh-3* induces a partial neural transformation of muscle cells as *myo2* is still expressed (Luo and Horvitz, 2017), indicating that additional factors are necessary to specify the I4 neuron from a mesodermal cell.

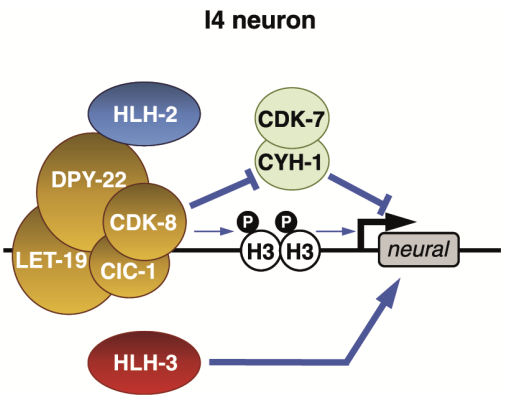



***Figure 16:* Model for the specification of the I4 neuron.** *The HLH-2 proneural protein and the CDK-8 Mediator complex kinase module act with the HLH-3 proneural protein to promote I4 neurogenesis. HLH-2 and CDK-8 likely act by inhibiting the CDK-7/CYH-1 complex and might also act secondarily by phosphorylating serine 10 of histone H3. CDK-7/CYH-1 might negatively regulate I4 neurogenesis by promoting a myogenic program, whereas H3S10 phosphorylation might facilitate neurogenic gene expression. From* (Luo and Horvitz, 2017)

*Specification of the other mesoderm-derived pharyngeal neurons*

Little is known about the specification of the other mesoderm-derived pharyngeal neurons in *C. elegans*. In their study focusing on the specification of the I4 neuron, Luo and Horvitz (2017) showed that the proneural gene *cnd-1* (NeuroD) is required for the neural specification of the I3 interneuron as *cnd-1* mutants exhibit I3 neurons adopting a gland cell fate. Similarly, they showed that the proneural gene *ngn-1* (Neurogenin) is necessary for the development of the M1 motor neuron as this neuron is missing in *ngn-1* mutants. The loss of *let-19* also affects the specification of the M1 neuron but the effects are much milder than for the I4 neuron. In addition, the unknown mutation *mnm-8* (M Neuron Morphology abnormal) induces the loss of the M1 neuron (Refai et al., 2013). Even though *mnm-8* has been mapped on the chromosome I of *C. elegans* in the vicinity of the Wnt pathway gene *pry-1*, it is not a mutation of *pry-1* and it is not known which gene is affected by this mutation (Refai et al., 2013). For the remaining I6, M4 and M5 mesoderm-derived pharyngeal neurons, the mechanisms driving their specification have not been investigated yet.

*Conclusion*

The existence of these mesoderm-derived pharyngeal neurons in *C. elegans* demonstrate that mesodermal tissues and/or cells can retain the ability to produce neurons. These mesodermal neurons share a common molecular identity with ectodermal neurons and the specification of both neuron types requires proneural genes (Luo and Horvitz, 2017; Stefanakis et al., 2015). However, mesodermal neurons require additional factors such as the I4 neuron specification depending on the Mediator complex associated with CDK-8. It also seems that the different mesodermal neurons of *C. elegans* need different proneural genes as *cnd-1* seems to be necessary for the I3 neuron, *hlh-3* for I4 and *ngn-1* for M1 (Luo and Horvitz, 2017). Additionally, the role of CDK-8 appears to be specific to I4 as the lack of this kinase does not affect the specification of the I3 and M1 neurons.

The work of Luo and Horvitz (2017) is pioneer in unveiling the developmental process leading to the development of mesodermal neurons in the nematode *C. elegans* and similar investigations await to be extended to the other mesodermal neurons. Their study allows a better understanding of the specification of the I4 neuron from a mesodermal progenitor, but information on how the neural potential is conserved all along the mesodermal lineage is still missing.

### *2.2.3- Endodermal neurogenesis in the sea urchin*

After the presentation of the two previous examples of non-ectodermal neurogenesis originating from a tissue related to the mesoderm, I will now introduce the case of endodermal neurogenesis occurring in the sea urchin S*trongylocentrotus purpuratus*. Interestingly, these endodermal neurons are found in the foregut which is part of the pharynx. Even though the sea urchin and *C. elegans* pharynxes are not homologous tissues, it is very intriguing that both species have non-ectodermal neurons in structures involved in the feeding behavior.

*Evidence for endodermal neurogenesis*

The sea urchin pharynx is formed at the end of gastrulation when the foregut endoderm fuses with the oral ectoderm. In the sea urchin, the foregut neurons are induced by the neural gene *Six3* involved in ectodermal neurogenesis, and it has been assumed that these neurons arise in the ectoderm before migrating to populate the pharynx (Wei et al., 2009).

In order to determine whether these neurons are indeed migrating from the ectoderm to the endoderm before the onset of gastrulation, Wei et al. (2011) have used the photoconvertible protein KikGR (Tsutsui et al., 2005) to specifically label the ectoderm or the endoderm. These experiments showed that no cell migration occurs from the

ectoderm to the endoderm indicating that the foregut neurons directly develop from the endoderm.

This endodermal neurogenesis is not specific to one sea urchin species as it also occurs in *Lytechinus variegatus* in which recombinant cell transplantation experiments revealed that only endodermal grafted cells gave rise to these foregut neurons (McClay et al., 2018).

*The sea urchin endodermal neurogenic pathway*

As mentioned above, foregut neuron precursors express the neural gene *Six3* at blastula stage, indicating that this gene is involved in the endodermal neurogenic pathway (**Figure 17**). However, *Six3* is not co-expressed with the pan-neuronal marker *SynaptotagminB* (*SynB*) (Wei et al., 2011). Indeed, their respective expression is separated by one day suggesting that *Six3* indirectly activates *SynB* through more steps of signaling pathway. The gene *Nkx3-2* is part of the *Six3* gene regulatory network (GRN) (Wei et al., 2009) and it is expressed in the foregut of gastrulating embryos indicating that it could be an intermediate factor linking *Six3* and *SynB* expression (Wei et al., 2011). The downregulation of *Nkx3-2* by morpholino injection induces the loss of $SynB^{+}$ foregut neurons and the *Nkx3-2* expression in the foregut neurons is lost in *Six3* morphants confirming that both genes are involved in the same cascade of the endodermal neurogenic pathway (Wei et al., 2011). Whereas *Nkx3-2* and *SynB* are co-expressed and their interaction could be direct, *Six3* and *Nkx3-2* are not expressed in the same cells meaning that more factors are present to mediate the activation of *Nkx3-2* by *Six3* (**Figure 17**).

At the time when *Nkx3-2* is expressed in the foregut, the expression of *SoxB1* is also detected in this region in addition to its broad expression across the ectodermal tissue (Kenny et al., 2003; Wei et al., 2011). *SoxB1* is related to the murine *Sox* genes involved in the maintenance of neural precursors (Bylund et al., 2003; Graham et al., 2003) suggesting that it could play a similar role in the sea urchin. *SoxB1* is not found in

differentiated neurons as it is not co-expressed with *SynB*. Moreover, the expression of *SoxB1* is not affected in *Six3* morphants, indicating that it acts earlier in the neurogenic pathway (**Figure 17)** (Wei et al., 2011). Additionally, the proneural Smad-interacting-protein 1 (Sip1) known to regulate *Sox2* in chick (Sheng et al., 2003; Uchikawa et al., 2003), activates *SoxB1* expression in the sea urchin endoderm as *Sip1* knockdowns exhibit a strong reduction of *SoxB1* mRNA levels in the endoderm (McClay et al., 2018). Altogether, these data suggest that *SoxB1* might allow the foregut endoderm to retain a neural competency (Wei et al., 2011).

Another gene of the *Sox* family, *SoxC*, is activated in all the neurogenic regions of the embryo at gastrula stage, including the foregut endoderm, implying its role in endodermal neurogenesis as well (Wei et al., 2016). Unlike *SoxB1*, *SoxC* acts downstream of *Six3* in the endodermal neurogenic pathway (**Figure 17**) as its expression is reduced in *Six3* morphants (Wei et al., 2016). However, *SoxC* expressing cells are cycling neural progenitors as they do not express *SynB* and are labeled by an anti-phospho-Histone 3 (H3p) antibody (Wei et al., 2016) specifically recognizing chromatin of dividing cells in prophase and anaphase (Hendzel et al., 1997). There is, therefore, another factor linking *SoxC* and *SynB* expression in the signaling cascade. The class III POU gene *Brn1/2/4* is co-expressed with both *SoxC* and *SynB* despite these two factors are expressed in different cells, suggesting that *Brn1/2/4* could connect *SoxC* to the terminal differentiation of neurons (**Figure 17**). This hypothesis is reinforced by the downregulation of *SoxC* reducing the *Brn1/2/4* expression levels and the stability of *SoxC* mRNA levels in *Brn1/2/4* morphants (Wei et al., 2016). Nevertheless, the overexpression of *Brn1/2/4* in *SoxC* morphants does not rescue the generation of neurons suggesting the existence of an additional pathway downstream of *SoxC* and in parallel of the *Brn1/2/4*-mediated neuronal differentiation (Wei et al., 2016). Similarly, the ectopic expression of *SoxC* is not sufficient to induce ectopic neurogenesis (Garner et al., 2016; Wei et al., 2016), hence other factors are acting in parallel of *SoxC* (possibly the *Nkx3-2*-mediated cascade).

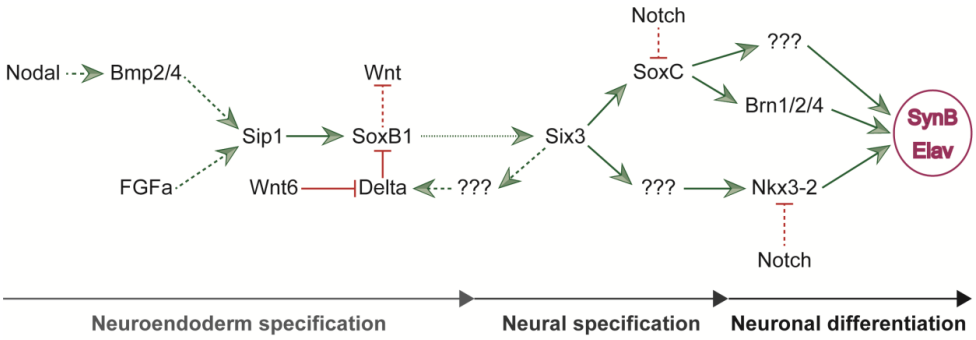



***Figure 17:* Endodermal neurogenic pathway of the sea urchin.** *The neurogenic potential of the foregut endoderm is induced by Nodal, Bmp, Wnt and FGF signals. This allows the expression of SoxB1 which, in turn, maintains a fate plasticity and a pluripotent state in the foregut endoderm. The foregut endoderm is therefore a neuroendoderm. Then, Six3 initiates the specification of neural precursors and SynB⁺;Elav⁺ neurons differentiate via the action in parallel of Brn1/2/4 and Nkx3-2. Unbroken arrows indicate confident connections. More intermediate factors could exist between two factors connected by an unbroken arrow. Dashed arrows indicate suspected connections that need to be properly located in the pathway. The dotted arrow for SoxB1 toward Six3 indicates a hypothetical connection as SoxB1 induces neural specification but its targets have not been investigated in detail. It is possible that SoxB1 activates Six3 through the inhibition of canonical Wnt signaling (β-catenin repression). It is important to keep in mind that this current model is incomplete. Color code: green for activation, red for repression, magenta for differentiated neurons. Compilation of data made from* (Garner et al., 2016; Kenny et al., 2003; McClay et al., 2018; Wei et al., 2009; Wei et al., 2011; Wei et al., 2016)

The expression domain of all these different factors inducing neurogenesis in the foregut endoderm is broader than the final number of cells becoming neurons and this is due to a regulation of neurogenesis by the Delta/Notch signaling pathway likely acting through lateral inhibition in order to select which cells will become neurons (McClay et al., 2018; Wei et al., 2011; Wei et al., 2016). Indeed, the inhibition of the γ-secretase by DAPT treatment blocks the Notch pathway and induces an increase of the number of neurons all over the embryo, foregut endoderm included. However, the possibility that other factors regulate neurogenesis negatively, in all neurogenic regions or in some of them, remains (Wei et al., 2011).

Furthermore, the investigation of the GRN in the neurogenic regions of the sea urchin additionally revealed that Nodal signaling and *Bmp2/4* are required for endodermal neurogenesis, whereas they have repressing effects on ectodermal neurogenesis (McClay et al., 2018). It also appears that *Wnt6* is involved in both ectodermal and endodermal neurogenesis by repressing Delta in cells fated to become neurons (McClay et al., 2018) which is surprising as *Six3* has been shown to repress the canonical Wnt signaling (*Wnt1*, *Wnt8* and *Wnt16* in particular) (Wei et al., 2009). As both studies have tested the role of the Wnt pathway in two different species, this contradiction could be explained by inter-specific differences, albeit the role of the Wnt signaling could also depends on the molecule involved.

Finally, the FGF signaling seems to also play a role in initiating neurogenesis from both the ectoderm and the endoderm as FGFa is necessary for the expression of *SoxC* (Garner et al., 2016) and *Sip1* (McClay et al., 2018).

The neural induction of endodermal cells in the sea urchin embryo seems to depend on *Six3* which is necessary to generate neural precursors and appears to act as a master regulator of neural induction. These cells have been previously committed to an endodermal fate prior to the start of gastrulation (Peter and Davidson, 2010) and the expression of *SoxB1* in the foregut domain might allow the maintenance of the neural competency by repressing the canonical Wnt signaling [*SoxB1* represses β-catenin in the ectoderm (Kenny et al., 2003) and might play a similar role in the endoderm] and the endodermal fate. This retained fate plasticity leads to the expression of *Six3* and the generation of endodermal neurons through, at least, the *SoxC* and *Nkx3-2* signaling cascades (**Figure 17**).

*Conclusion*

The endoderm-derived neurons of the sea urchin illustrate that non-ectodermal neurogenesis is not restricted to mesodermal tissues and that it can also occur within the endoderm. In the ectoderm, neural progenitors express *SoxB1*, *SoxB2*, *Six3* and *SoxC* inducing the expression of *Brn1/2/4*, then neurons project neurites and start to express the neuronal markers *SynB* and *Elav* (Garner et al., 2016). However, *SoxB2* is not expressed in the endodermal lineage (Garner et al., 2016), indicating that *SoxB1* is in charge of maintaining the endodermal factors at low levels for specifying a neuroendoderm via the activation of the *Six3* signaling cascades (Wei et al., 2011).

Both ectodermal and endodermal neural specification depend on the *Six3→SoxC→Brn1/2/4* cascade but in parallel, endodermal neurons also require the *Six3→Nkx3-2* cascade. While *Nkx3-2* appears to be specifically involved in the endodermal neurogenic pathway, it is important to keep in mind that the ectopic overexpression of *SoxC* is not sufficient to induce the generation of ectopic neurons in the sea urchin embryo (Garner et al., 2016; Wei et al., 2016), suggesting that another cascade downstream of *Six3* and in parallel to *SoxC* is necessary for ectodermal neurons as well. This could be mediated via *Nkx3-2* as it is expressed in the oral animal pole ectoderm in addition to the foregut endoderm (Wei et al., 2011); or via other factors that still need to be identified. *Nkx3-2* might then not be specific to endodermal neurogenesis, nonetheless, this remains to be elucidated.

Furthermore, the Nodal, BMP, FGF, Wnt and Notch signaling are also involved in the endodermal neurogenic pathway (McClay et al., 2018; Wei et al., 2009; Wei et al., 2011; Wei et al., 2016), but their respective role remains unclear. Indeed, the *Six3→ Nkx3-2→SynB* and *Six3→SoxC→Brn1/2/4→SynB* cascades have been the most dissected in the context of endodermal neurogenesis even though they are still incomplete. These cascades are interacting with the Nodal, BMP, FGF, Wnt and Notch signaling (**Figure 17**), yet we are still lacking a proper description of their respective interaction within the endodermal neurogenic pathway.

### *2.2.4- Endodermal neurogenesis in cnidarians*

Until now, I have presented cases of non-ectodermal neurogenesis within bilaterians, but this phenomenon also occurs outside this group, notably in cnidarians. Being diploblastic, cnidarians are composed of two germ layers: the ectoderm and the endoderm [referred as mesendoderm in *Nematostella vectensis* because "mesodermal" genes are expressed in the endoderm (Fritzenwanker et al., 2004; Martindale et al., 2004)]. Therefore, non-ectodermal neurons develop from the (mes)endoderm and depending on the species, they originate from different types of cells such as interstitial cells (i-cells) in hydrozoans, and mesendodermal neural progenitor cells (NPCs) in the anthozoan *Nematostella vectensis*. Here, I will only present the case of i-cells as *Nematostella* mesendodermal neurogenesis will be presented later (see section **3.4.3** page 93).

*Generation of neurons from i-cells*

Hydrozoans have a specific type of stem cells lodged in the interstitial space between cells of the ectodermal epithelium, therefore called interstitial stem cells or shortly, i-cells. These i-cells have been mainly studied in *Hydra* and *Hydractinia* species lacking the medusa stage (Frank et al., 2009). In *Hydra*, these stem cells are multipotent migratory cells involved in generating and renewing secretory gland cells, neurons, cnidocytes and germ cells (when the animal undergoes sexual reproduction) throughout embryogenesis, adulthood and regeneration (**Figure 18A**) (Bode, 1996; Bosch and David, 1987). By contrast, *Hydractinia*'s i-cells are totipotent and can produce both germ and somatic cells, of all lineages including epithelia (**Figure 18B**) (Künzel et al., 2010; Müller et al., 2004). Nonetheless, i-cells have also been described in other hydrozoans such as the jellyfish *Clytia hemisphaerica* (Bodo and Bouillon, 1968; Denker et al., 2008; Leclère et al., 2012), *Podocoryne carnea* (Boelsterli, 1977), and the sea pen *Pennaria tiarella* (Martin and Archer, 1986).

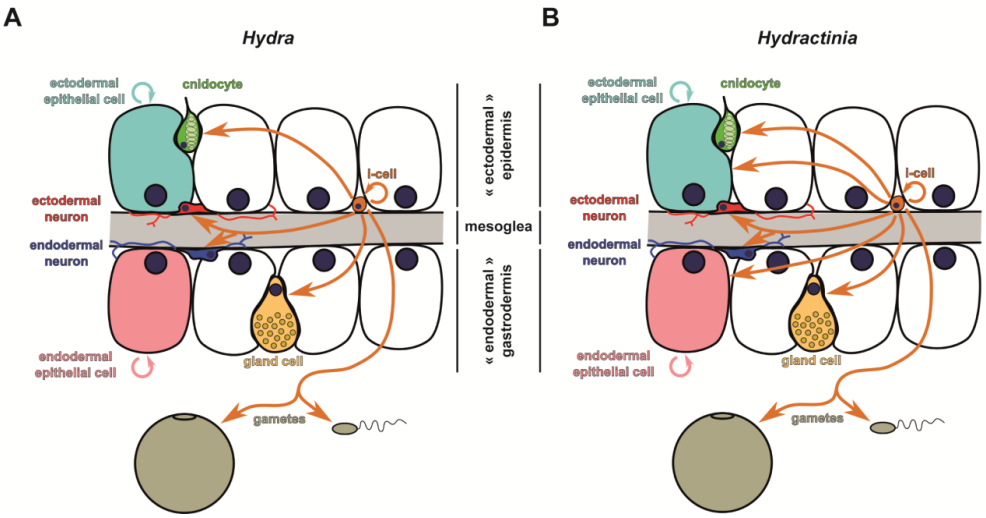



***Figure 18:* Schematic representation of cell lineages in hydrozoans.** ***(A)*** *In Hydra, interstitial stem cells (i-cells) produce neurons, cnidocytes, gland cells and gametes.* ***(B)*** *In Hydractinia, i-cells produce the same cell types, but they also generate epithelial cells from both the epidermis and the gastrodermis. Note that intermediate progenitors and differentiation stages are not represented here. The epidermis and the gastrodermis of the adult polyp are made of a mix of cells coming from both germ layers, explaining the quotes marks around the main origin of these epithelia. Adapted from* (Leclère et al., 2016a)

All neurons in hydrozoans are derived from i-cells and despite they are found in the ectodermal epithelium of polyps or medusae, they actually have an endodermal origin. Indeed, i-cells have been described during embryonic development within the endoderm of planula larvae of different hydrozoans such as *Hydractinia echinata* (Plickert et al., 1988; Weis et al., 1985), *Podocoryne carnea* (Gröger and Schmid, 2001) and *Pennaria tiarella* (Martin and Thomas, 1981a). These different studies revealed by electron microscopy, BrdU labeling of mitotic cells and colchicine-mediated elimination of i-cells that these i-cells appear in the endoderm before migrating to the ectoderm by crossing the mesoglea (extracellular matrix). This migration starts at late planula stage but mainly occurs during metamorphosis leading to the formation of the primary polyp. When all the endodermal i-cells are eliminated in the planula larvae, cnidocytes, ganglion neurons and gland cells are lacking in the resulting polyps, confirming the endodermal origin of these different cell types in hydrozoans. However, sensory neurons are still able to develop, suggesting that they can arise from ectodermal cells. While the

first developing cnidocytes are observed in the endoderm and migrate to the ectoderm simultaneously with i-cells, neurons do not develop before i-cells reach the ectoderm (Gröger and Schmid, 2001; Martin, 1987; Martin and Archer, 1986; Martin and Thomas, 1981b).

Additionally, the endodermal origin of i-cells is supported by molecular data. In *Hydra vulgaris*, the Polycomb group gene Embryonic Ectoderm Development (*HyEED*) has been shown to be expressed in i-cells and some of their undifferentiated derivatives (nematoblasts and spermatogonia) but not in the terminally differentiated derivatives (Genikhovich et al., 2006). The expression of *HyEED* is broad across the endoderm at the gastrula stage before being restricted to individual cells, shown to be i-cells, at the cuticle stage. In the hatched *Hydra* polyp, i-cells are found in the ectoderm, suggesting that they migrated from the endoderm to the ectoderm before hatching. Similarly, a study in *Clytia hemisphaerica* showed that maternally inherited *ChePiwi*, *CheNanos1*, *CheNanos2* and *ChePL10* mRNAs were localized in a small cluster of cells, which is internalized by ingression during gastrulation along with the presumptive endoderm (Leclère et al., 2012). In the planula, cells carrying these mRNAs are spread throughout the endoderm and were identified as i-cells. At later stages, *Clytia*'s i-cells are mainly found in the ectoderm (Bodo and Bouillon, 1968) suggesting again that a migration from the endoderm to the ectoderm occurs.

The best description of hydrozoan's neurogenesis is currently available for *Hydra* in which it was initially thought that i-cells were dividing asymmetrically to generate a daughter cell conserving the i-cell identity and a daughter cell acquiring a neural progenitor identity (or cnidocyte or gland cell progenitor) (Hager and David, 1997; Martin and Thomas, 1981a). However, more recent work suggested that neurons and cnidocytes share a common intermediate progenitor expressing the ParaHox gsx homolog gene *cnox-2* and the paired-like homeogene homolog *prdl-b* (Gauchat et al., 2004; Miljkovic-Licina et al., 2007). These bipotent neural progenitors are proliferating to increase the pool of neurons and cnidocytes produced during adult neurogenesis. Then, the expression of the COUP-TF nuclear receptor homolog gene *hyCOUP-TF*

initiates their exit of the cell cycle and their differentiation pathway (Gauchat et al., 2004). Furthermore, the analysis of *Hydra* single-cell transcriptomes unveiled the existence of a common progenitor for gland cells and neurons after the split with the cnidocyte lineage (Siebert et al., 2019). Therefore, an i-cell derived progenitor seemingly undergoes two rounds of cell fate decision: first, between the cnidocyte versus neuron/gland cell fates and second, between the neuronal versus gland cell fates (**Figure 19**).

By contrast, it has been shown that germ cells are derived from a distinct unipotent progenitor, indicating that the segregation between the somatic and germinal lineages is the first decision made by i-cells when exiting their multipotent stem cell state (**Figure 19**). Alternatively, i-cells could form a heterogeneous population with a portion of them specified to the somatic lineage and another specified to the germinal lineage (Littlefield, 1991; Nishimiya-Fujisawa and Sugiyama, 1993).

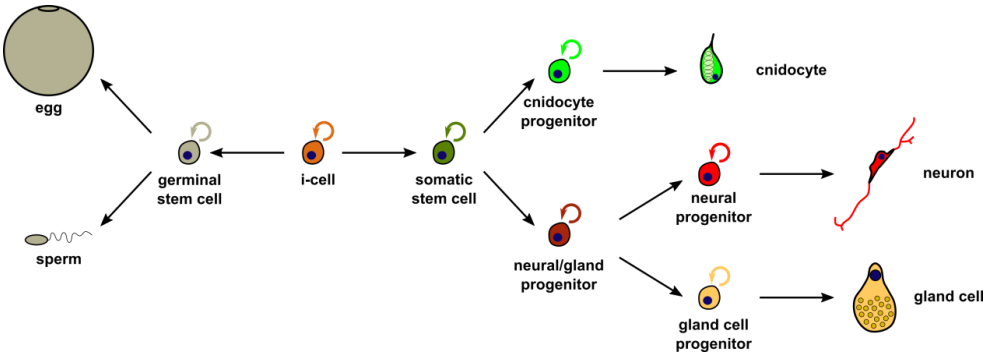



***Figure 19:* Lineage of i-cell derivatives in hydrozoans.** *The endoderm-derived interstitial stem cells generate both germinal and somatic stem cells. The somatic lineage is first split between the cnidocyte and the neural/gland cell fates, then the neural and gland cell fates are segregated.*

Despite this clear fate lineage of i-cell derivatives, the understanding of the mechanisms driving the fate decision remains unclear. Nonetheless, some scattered hints are already available.

In *Hydra*, other molecular cues have been shown to drive the commitment of i-cells into a neural fate. Indeed, some neuropeptides such as HeadActivator (Holstein and David, 1986; Holstein et al., 1986) and Hym355 (Takahashi et al., 1997) favor a neural fate, while Hym33H counteracts Hym355 to inhibit neural differentiation (Takahashi et al., 2000). Signaling pathways are also involved in i-cell differentiation. For example, the ectopic activation of Wnt signaling induces a decrease in the number of cnidocytes. The inhibition of Notch signaling has similar effects. This indicates that Wnt inhibits cnidocyte differentiation, whereas Notch drives cnidocyte differentiation (Khalturin et al., 2007). However, both signaling pathways do not affect the number of neurons suggesting that Wnt and Notch do not control the neural versus cnidocyte fate decision.

In *Hydractinia*, the Wnt signaling appears to play a different role than in *Hydra*. Indeed, an ectopic activation of Wnt signals by inhibition of GSK-3 induces an increase in the number of neurons and cnidocytes along with a decrease in the number of i-cells. As the reduction of the i-cell population is not due to apoptosis, it suggests that the Wnt signaling controls the pluripotent state of i-cells with high levels of Wnt driving their differentiation toward a neuronal and cnidocyte fate, while low levels of Wnt allow the maintenance of their stemcellness (Teo et al., 2006). The Wnt signaling acts by activating the expression of COUP-TF which initiates the differentiation of both neurons and cnidocytes (Duffy and Frank, 2011). Additional studies showed that the ectopic expression of *Nanos2* results in an increase of the number of cnidocytes and in a reduction of the number of neurons. Inversely, the downregulation of *Nanos2* by morpholino reduces the number of cnidocytes and increases the number of neurons (Kanska and Frank, 2013). Altogether, it indicates that in *Hydractinia*, the Wnt signaling favors the differentiation of i-cells into neurons and cnidocytes, while the downstream expression of *Nanos2* drive the differentiating cells toward the cnidocyte fate. However, the signal inducing the expression of *Nanos2* in some i-cells derivatives and not in others remains to be identified.

As all neurons in hydrozoans are derived from i-cells originating in the endoderm, it seems that hydrozoans do not exhibit canonical ectodermal neurogenesis. Nevertheless, the endodermal origin of hydrozoan's neurons can be questioned for two main reasons: first, despite the endodermal origin of i-cells, neurogenesis mostly takes place in the ectodermal tissue. This could be considered as ectodermal neurogenesis because neural progenitors do not appear before the migration of i-cells out of the endoderm. Second, the molecular markers of i-cells (*HyEED*, *ChePiwi*, *CheNanos1*, *CheNanos2* and *ChePL10*) are expressed in a subset of cells prior to gastrulation (Genikhovich et al., 2006; Leclère et al., 2012). These cells get internalized during gastrulation at the same time as the endoderm and the question whether i-cells are specified before gastrulation or within the endoderm after its internalization, remains.

The specification of the different i-cell derivatives follows several rounds of fate decision. First, i-cells are committed into a germinal or somatic stem cell fate. Germinal stem cells will produce sperm or eggs depending on the sex of the animals, while somatic stem cells produce progenitors that are specified either into cnidocyte, neuron or gland cell fates. As suggested for neuron and gland cells, some intermediate progenitors may exist. Finally, these different progenitors undergo some last rounds of commitment to segregate cell subtypes. However, the identity of the signals driving these successive rounds of fate decision is still unknown and let a wide field of investigation open.

*Conclusion*

It appears that non-ectodermal neurogenesis is a common feature of cnidarians, notably with hydrozoans developing neurons from an endoderm-derived cell type: the i-cells. The anthozoan *Nematostella vectensis* also generates non-ectodermal neurons from mesendodermal neural progenitors (see section **3.4.3** page 93 for more details). Like bilaterians, cnidarians show different mechanisms to produce non-ectodermal neurons. Despite our awareness of these processes, little work has been performed to investigate their regulation, hence information is still missing about the signals driving the fate decision in the common bipotent progenitors of neural and non-neural cells.

### *2.2.5- Concluding remarks about non-ectodermal neurogenesis*

In this chapter, I have described different cases of non-ectodermal neurogenesis spread across metazoans. It shows that this process might not be as atypical as initially thought and that the capacity to generate neurons from non-ectodermal tissues might be an evolutionary old feature of metazoans dating from the early steps of neurogenesis. Moreover, it seems that all three germ layers theoretically hold the potential to generate neurons.

The case of the vertebrate NMps is currently the best described and understood system and despite our knowledge about the other examples of non-ectodermal neurogenesis, we are still at the onset of understanding the mechanisms underlying this phenomenon.

The observation of non-ectodermal neurogenesis in cnidarians, *C. elegans* and the sea urchin has been facilitated by the accessibility, the simple body organization and the transparency of these organisms (Luo and Horvitz, 2017). Furthermore, neurogenesis takes place continuously during the cnidarian life cycle, thus it allows the study of this process in a wider time window.

These features do not apply to most vertebrates, which undergo much more complex developmental processes and whom embryos are protected by an eggshell or the maternal womb. The primitive streak and the tailbud are structures found at the surface of embryos and it might explain why we have been able to observe non-ectodermal neurogenesis in these structures and not in other tissues of the embryo. However, it does not mean that non-ectodermal neurogenesis is restricted to these tissues in vertebrates and we might discover more occurrences in the future.

The different cases I have presented in this chapter are difficult to compare because they come from different species spread across metazoans and each study focused on different aspects. However, some similarities and differences between them can be spotted.

In all these different examples, it appears that non-ectodermal neurons originate at a later stage than their ectodermal counterparts. Indeed, NMp-derived neurons, pharyngeal neurons from both *C. elegans* and the sea urchin can only be observed in a second wave of neurogenesis. This is also true for some cnidarians with the mesendodermal neurons of *Nematostella vectensis* developing at a later stage than the ectodermal neurons (Nakanishi et al., 2012). Moreover, these non-ectodermal neurons are produced by bipotent progenitors giving rise to neurons and to cells corresponding to the original germ layer identity (muscle or gland cells for instance). In several of these studies, it has been shown that signaling pathways such as Wnt and Notch were recurrently involved in the fate decision of bipotent progenitors and were driving the expression of proneural genes in the neural lineage.

By contrast, we can notice that in each studied species, the source tissue of non-ectodermal neurons is different, and they contribute to a diverse range of derivatives. Indeed, the mesoderm-derived neurons of *C. elegans* form the pharyngeal nervous system, while the vertebrate NMps contribute to the posterior CNS and the endoderm-derived i-cells of hydrozoan cnidarians generate most of the neurons.

Similarly, the comparison between the ectodermal and non-ectodermal neurogenesis reveals that both types of neurons follow the same molecular program. As both processes generate neurons, it makes sense. However, the main difference resides in the initiation of this developmental program. Indeed, the environment of each type of neurogenesis is different, hence the neurogenic potential is determined in different ways. For example, the sea urchin endoderm retains a neurogenic potential trough SoxB1, whereas ectodermal neurogenesis involves SoxB2. Moreover, some of the non-ectodermal neurons requires the activation of certain genes or pathways that are not involved in the development of ectodermal neurons, such as *cdk-8* or *hlh-3* in the I4 neurons of *C. elegans*. However, these genes are often restricted to a subpopulation of non-ectodermal neurons.

Altogether, this comparison raises the following question: are the differences between ectodermal and non-ectodermal neurogenesis due to different neurogenesis processes or to different functions fulfilled by the neurons generated by the respective processes?

In the sea urchin and *C. elegans*, the non-ectodermal neurons are associated with the pharynx. Both animals use their pharynx for feeding and we can wonder whether this similarity has any biological relevance or whether this is just a coincidence. We could make a parallel between the pharyngeal nervous system and the vertebrate enteric nervous system (ENS) from a functional point of view as they are both autonomous systems controlling the digestive system contractions and regulating the feeding behavior (Furness et al., 2014). Interestingly, despite the ENS develops from neural crest cells (Nagy and Goldstein, 2017), a recent study has shown by *in vitro* and *in vivo* lineage tracing that some neurons of the mouse ENS have an endodermal origin (Brokhman et al., 2019).

Unfortunately, to answer these questions, we need a better characterization of non-ectodermal neurogenesis in each of the systems presented in this chapter. It would allow a better understanding of the mechanism driving neuron development outside the ectoderm and it could provide potential insights for applied biomedicine.

# 3-Presentation of the research project

## 3.1-General scientific questions

The bilaterian CNS is a very complex organ with, notably, the human brain composed of a hundred billion neurons, with each of them able to make tens of thousands of connections. It controls a wide range of very complex behaviors throughout the animal kingdom. Despite our major advances in the understanding of its structure and function by using model organisms, such as *C. elegans*, *Drosophila* and mouse, we are still far from apprehending how such a complex system arose from a simple network of neural cells during evolution.

In order to investigate these aspects, we are studying the cellular and molecular mechanisms underlying the development of a relatively simple nervous system in an early branching metazoan, the cnidarian *Nematostella vectensis*. To this purpose, we are addressing questions such as: what are the mechanisms driving the extraordinary neurogenic potential of this seemingly simple animal? What is the developmental potential of individual neural progenitors? What is the diversity of neural cells?

Answering these questions will allow us to gather information about the early evolutionary steps leading to the formation of a simple system integrating external stimuli to induce a response from effector cells, about the structure of this first nervous system and about the evolutionary trajectory that led to the development of complex systems allowing complex behaviors as well as consciousness.

## 3.2-Cnidarians hold a key phylogenetic position in Evo-Devo

Cnidarians and ctenophores are the only non-bilaterian metazoans to possess a nervous system, howbeit ctenophores still occupy a controversial phylogenetic position among metazoans (**Figure 20**) (Jékely et al., 2015; Pisani and Liu, 2015). On the other hand, cnidarians are the sister group to bilaterians (**Figure 20**) (Dunn et al., 2008; Hejnol

et al., 2009; Ryan et al., 2013), and both groups diverged around 600 million years ago, before the Cambrian explosion (Dos Reis et al., 2015).

Despite their small and seemingly simple nervous system, cnidarians show a high plasticity in the structure of their nervous system, they perform neurogenesis continuously throughout their life cycle, they can regenerate the entire nervous system and they possess a cnidarian-specific neural cell type: the cnidocyte. Additionally, the cnidarian nervous system show some complexity with some remarkable neurophysiological specialization, such as bidirectional chemical synapses (Anderson, 1985; Horridge et al., 1962), signaling by a diversity of peptide-gated channels (Assmann et al., 2014; Golubovic et al., 2007), the rapid discharge of cnidocytes (Beckmann et al., 2015) and axons with two kinds of impulse (Mackie, 2004; Mackie et al., 2003; Takaku et al., 2014).

Furthermore, unlike ctenophores, cnidarians are very accessible organisms for experimental procedures as they have a simple body plan and they are transparent, which allow the direct visualization of cells. This explains why cnidarians have been used as laboratory models for more than a century. It led to the development of a good set of tools to study neurogenesis in cnidarians, such as the genomic and transcriptomic sequences, transgenesis, gene editing and functional assays with both loss and gain of function. Interestingly, it has been shown that cnidarians show a better conservation of the neurogenic toolkit with vertebrates than *C. elegans* or *Drosophila* that appear to be highly derived organisms. This neurogenic toolkit includes transcription factors (*e.g.* Sox, NeuroD), signaling pathways (Notch, Wnt, TGF-β, FGF, Hedgehog, and Jak/Stat) and post-transcriptional regulators acting at the mRNA level, such as Elav or Mashashi (Kortschak et al., 2003; Raible and Arendt, 2004; Rentzsch et al., 2016; Technau et al., 2005).

Cnidarians are, therefore, perfectly suitable model organisms to investigate ancient features of eumetazoan neurons by comparing them to bilaterians. It will help us to understand the processes permitting the coordination of several behaviors (feeding, swimming, reproduction) by a simple and not centralized nervous system. Moreover, cnidarians carry a neurogenic potential in both germ layers, and they carry extraordinary

capacities to regenerate. Thus, studying neurogenesis in cnidarians could provide new insights potentially interesting in the context of regenerative medicine.

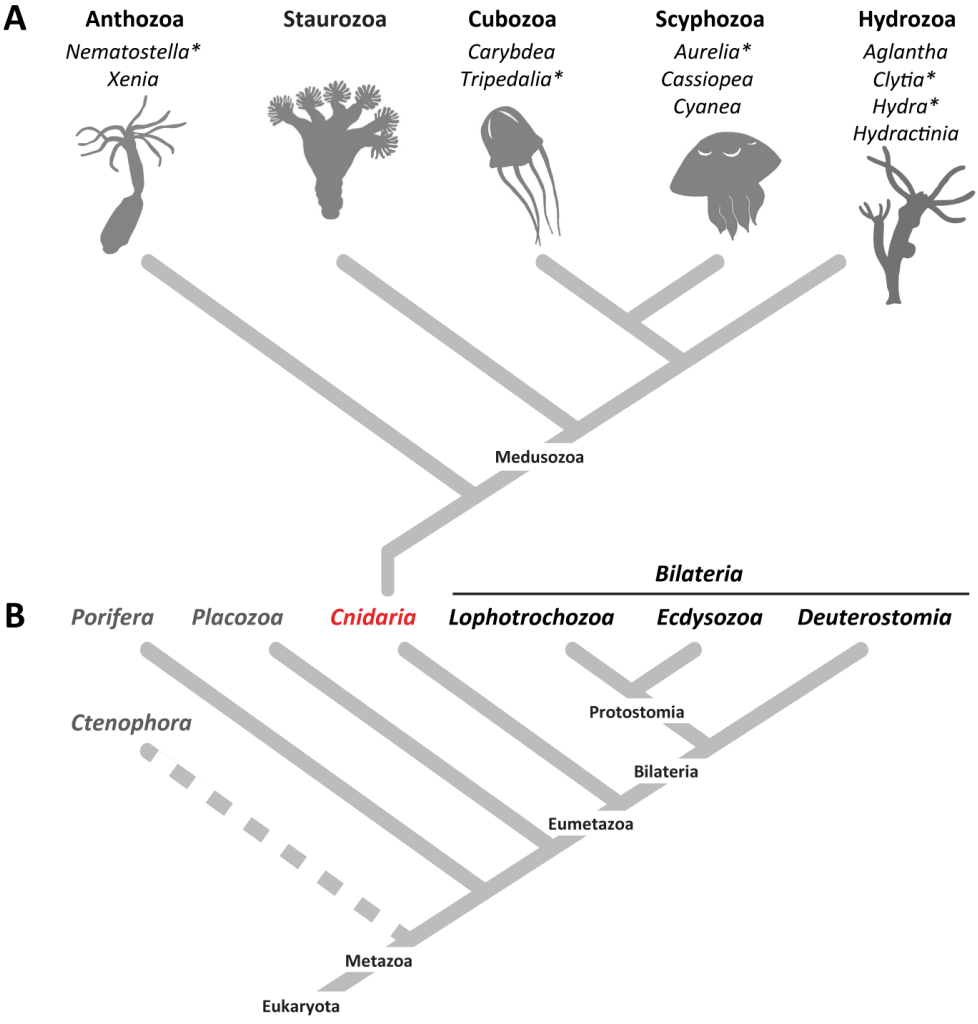



***Figure 20:*** ***Cnidarians hold a key phylogenetic position in Evo-Devo.*** *(**A**) Schematic phylogenetic tree showing the relationships of the five classes within the phylum Cnidaria. Species of cnidarians used for research on the nervous system are listed. Complete genome sequences are available for several cnidarian species (marked with asterisk). (**B**) Schematic phylogenetic tree showing main branches of metazoan evolution and the position of cnidarians among the non-bilaterian metazoans. As the phylogenetic position of ctenophores remains controversial, the branch leading to this group is represented by a dashed line. Modified from* (Bosch et al., 2017)

## 3.3- The sea anemone *Nematostella vectensis*: our model organism

*Nematostella vectensis*, also called the starlet sea anemone, belongs to the anthozoans, one of the two groups composing the cnidarians (**Figure 20**). It is a burrowing animal that lives in soft sediments and in plant debris within estuarine environments such as salt marshes and brackish water pools. It is distributed along the coast of England, on the North Atlantic coast from Nova Scotia to Georgia, in the Gulf of Mexico between Florida and Louisiana and on the North Pacific coast from California to Washington state (Hand and Uhlinger, 1992). In such environment, the temperature and the salinity undergo a wide range of variations, probably explaining why *Nematostella* shows great tolerance to environmental changes and can be easily cultured under laboratory conditions.

The morphology of the *Nematostella* polyp is rather simple with the body column shaped as a tube with a single oral opening surrounded by tentacles used to catch prey (**Figure 21A**). The number of tentacles varies with the age of the animal and is feeding-dependent (Ikmi et al., 2020). The oral opening corresponds to the site of gastrulation (Fritzenwanker et al., 2007; Lee et al., 2007). Internally, a pharynx is continuous with the oral opening and eight mesenteries run along the oral-aboral axis of the body column. Mesenteries are folds of the gastrodermis, the inner cell layer coating the gastric cavity (**Figure 21B**). From the body wall and in a proximal to distal direction, mesenteries are composed of: the parietal and retractor muscles controlling the contraction of the polyp along with the retraction of tentacles, the somatic gonad (oral half of the body column) or the trophic tract (nutrient storage, aboral half of the body column), and the septal filament containing gland cells involved in the digestion and uptake of nutrients (**Figure 21C**) (Extavour et al., 2008; Renfer et al., 2010; Steinmetz et al., 2017).

Perpendicularly to the oral-aboral axis, *Nematostella* possesses a directive axis corresponding to a sort of bilateral symmetry as attested by the disposition of the retractor muscles and the presence of a ciliated groove, the siphonoglyph, on one side of the pharynx (Berking, 2007; Layden et al., 2016a).

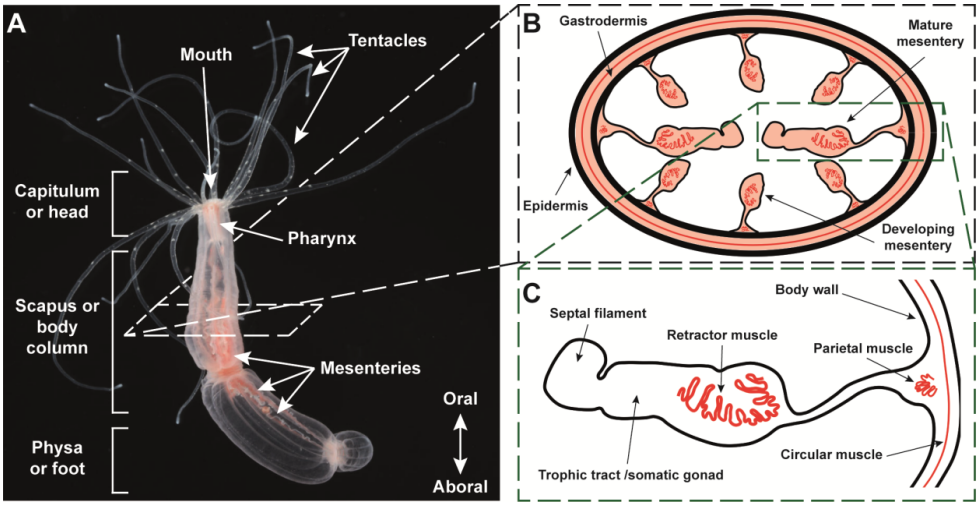



***Figure 21:*** ***The morphology of Nematostella vectensis.*** *(**A**) Adult Nematostella polyp. In the wild, polyps are around 1cm long while under laboratory conditions, they can reach a length of several centimeters. Picture from Fabian Rentzsch lab, ©Chiara Sinigaglia. Inspired from* (Stefanik et al., 2013) *(**B**) Schematic view of a cross section in the body column showing mature and developing mesenteries. (**C**) Schematic drawing of the morphology of a mature mesentery. Three types of muscle can be observed. Depending on the position along the oral-aboral axis, the mesentery contains either a trophic tract storing nutrients or a somatic gonad producing gametes. The septal filament is composed of exocrine cells involved in the digestion.*

As other cnidarians, *Nematostella* is a diploblastic animal formed of only two germ layers: the ectoderm and the endoderm. However, a recent study by (Steinmetz et al., 2017) challenged the homology between the cnidarian and bilaterian germ layers. Indeed, the cnidarian endoderm was considered as homologous to both the bilaterian endoderm and mesoderm (**Figure 22, left**) (Seipel and Schmid, 2005; Seipel and Schmid, 2006). Strikingly, the comparative analysis of bilaterian "endodermal" and "mesodermal" transcription factors and cell type markers expression with their profile in *Nematostella* suggested that *Nematostella*'s endoderm is homologous to the bilaterian mesoderm, while *Nematostella*'s ectodermal pharynx is homologous to the bilaterian endoderm (**Figure 22, right**) (Steinmetz et al., 2017). Due to this new model of germ layer homology and to prevent confusion, I will refer to *Nematostella*'s "endoderm" as mesendoderm and to *Nematostella*'s "endodermal neurogenesis" as mesendodermal neurogenesis in this thesis.

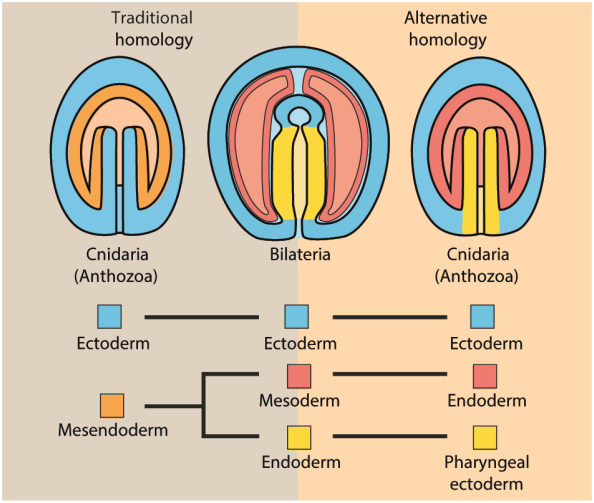



***Figure 22:*** ***Schematics of the traditional and alternative homology of germ layers between cnidarians and bilaterians.*** *An ectodermal origin of the gut-like tissue of Nematostella vectensis supports an alternative homology between cnidarian and bilaterian germ layers. Bilateria are represented by a schematized larva stage with through-gut. From (Steinmetz et al., 2017)*

The life cycle of *Nematostella* is completed in approximately 3 to 6 months under laboratory conditions at 21°C (**Figure 23**) (Fritzenwanker et al., 2007; Hand and Uhlinger, 1992; Lee et al., 2007). Being a dioecious species, both sexes are carried by different individuals that release their gametes in the environment, which leads to an external fertilization. In the laboratory, spawning is induced by light exposure and a temperature shift (Fritzenwanker and Technau, 2002; Stefanik et al., 2013). Females release eggs in a gelatinous package, while males release free-swimming sperm. The fertilized egg undergoes several rounds of division to form a blastula about 12 hours post-fertilization (hpf). Gastrulation starts around 20hpf by invagination of the animal pole to form a free-swimming planula larva composed of the two germ layers: the ectoderm and the mesendoderm, at approximately 48hpf. The planula elongates until 96hpf and finally settles down on a substrate where it performs a metamorphosis to become a primary polyp with four tentacles and two larger primary mesenteries after 7 days post-fertilization (dpf). From this stage, the polyp can feed and grows in a nutrient-dependent manner until it reaches sexual maturity.

*Nematostella* can also reproduce asexually by budding off pieces from its foot through transverse fission to produce clones (Hand and Uhlinger, 1995).

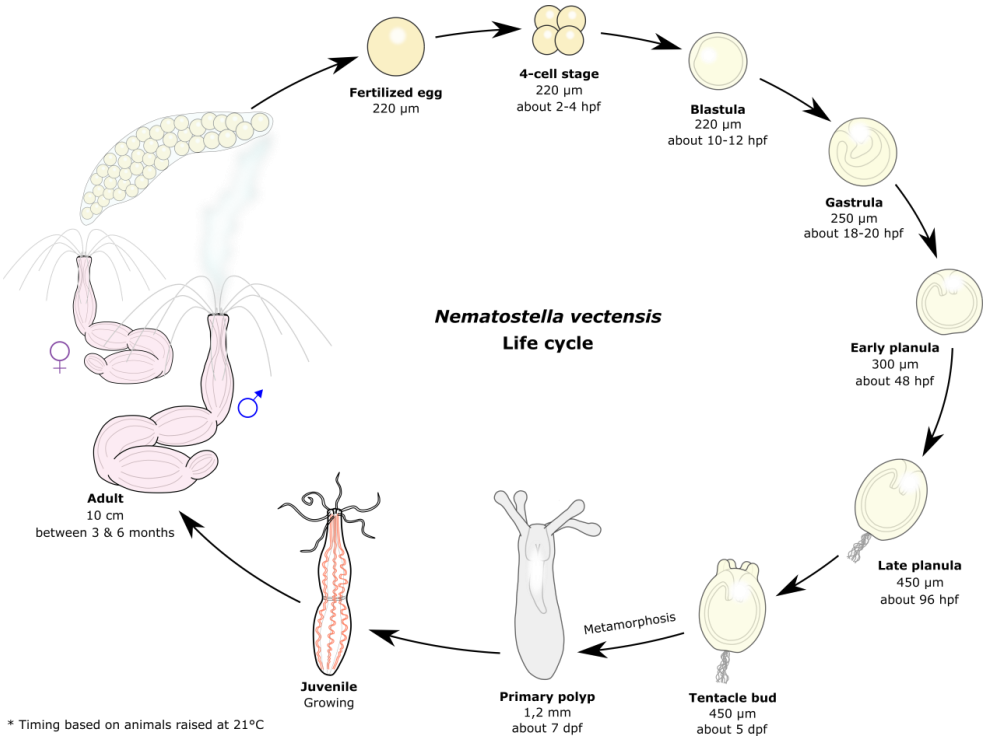



***Figure 23:* Sexual life cycle of the starlet sea anemone Nematostella vectensis.** *Gametes are released in the water and fertilization is external. The fertilized egg starts dividing after 2-4hpf. Gastrulation starts about 20hpf to form a planula larva around 48hpf. The planula larva settles and undergoes metamorphosis to become a primary polyp at 7dpf. It takes 3 to 6 months for the polyp to reach sexual maturity and complete the life cycle. The schematic is based on data reported by* (Fritzenwanker et al., 2007; Lee et al., 2007)

In the past 15 years, *Nematostella vectensis* has become an important model organism among cnidarians, especially since the release of its genome (Putnam et al., 2007). Surprisingly, it revealed that this seemingly simple animal has a complex genome with most of the gene families found in bilaterians. Moreover, it uncovered that gene syntenies and intron-exon boundaries are highly conserved between *Nematostella* and vertebrates indicating that these two groups are more similar to each other at the genomic level than any of them is to *C. elegans* or *Drosophila* (Putnam et al., 2007).

The access to the *Nematostella* genome together with a daily access to eggs opened great experimental opportunities with the development of many techniques that participated in establishing this cnidarian as a robust experimental model in the laboratory. Indeed, in addition to the utilization of mRNA *in situ* hybridization, antibody staining and mRNA injection for visualizing gene expression patterns and protein localization (Genikhovich and Technau, 2009; Wolenski et al., 2013), it allowed the refinement of gene models (Helm et al., 2013; Tulin et al., 2013) and the development of transgenics lines (Renfer et al., 2010). It is now possible to use these transgenic lines to sort cells by fluorescence-associated cell sorting (FACS) and perform either bulk or single cell RNA sequencing (scRNAseq) (Sebé-Pedrós et al., 2018). Moreover, the ability to knockdown gene function using morpholinos or shRNAs (He et al., 2018; Layden et al., 2013), and to perform gene editing via CRISPR/Cas9 (Ikmi et al., 2014), offers new possibilities to investigate the function of specific genes and retrace the ancestral function of these genes in the last common ancestor of cnidarian and bilaterians.

## 3.4- Neurogenesis in *Nematostella vectensis*

### *3.4.1-Description of the Nematostella nervous system*

*Nematostella vectensis* is the cnidarian model for which neurogenesis is best understood. Despite the apparent simplicity of its nervous system, the molecular regulation of neurogenesis appears more complex than initially suspected. Additionally, *Nematostella* carries a neurogenic potential in both germ layers. Therefore, this anthozoan is central for discussing the evolutionary origins of the nervous system and for comparisons with neurogenesis in bilaterians.

*Morphology of the nervous system*

The nervous system of *Nematostella* is shaped as a diffuse nerve net covering the entirety of the polyp. Despite the absence of centralization, some local condensations of neurites can be observed, such as in the longitudinal tracts following the musculature at

the base of mesenteries along the oral-aboral axis (Marlow et al., 2009; Nakanishi et al., 2012). In addition, an oral and a pharyngeal nerve ring have been described, respectively revealed through the immunostaining of the neurotransmitters Antho-RFamide and GABA labeling specific neuron populations (Marlow et al., 2009). However, the more recent use of transgenic lines, such as *NvElav1::mOrange*, do not report these oral and pharyngeal condensations (Nakanishi et al., 2012).

As in other cnidarians, *Nematostella* neurons are found in both the ectodermal and mesendodermal cell layers. Indeed, the longitudinal neurite tracts and a portion of the nerve net are in the mesendoderm (Nakanishi et al., 2012). The neuronal somas are scattered within the epithelia and mixed with other cell types.

*Diversity of neural cells*

In cnidarians, including *Nematostella*, neural cells are divided in three classes: sensory cells, ganglion cells and cnidocytes. No glial cells have been described in cnidarians so far.

Sensory neurons are defined by their elongated cell body standing from the basal to the apical pole of epithelia. In addition, they present an apical cilium at the body surface (Rentzsch et al., 2016).

Ganglion neurons are characterized by a cell body with a basal position within the epithelia. Most of them are thought to act as interneurons, while some others are likely innervating muscles and cnidocytes (Rentzsch et al., 2016).

Cnidocytes are mechanosensory stinging cells exclusively found in cnidarians. They are modified nerve cells containing a cytoplasmic capsule, the cnidocyst, enclosing a coiled tubule. They present an apical sensory cone that, upon activation, triggers the evagination of the tubule and the release of poisonous proteins. These cells are used as defense mechanism and as harpoons to catch preys (Galliot et al., 2009; Zenkert et al., 2011).

Despite the categorization of *Nematostella* neural cells in these three classes, many subclasses exist for each of them. Morphological, cellular and molecular evidences support this idea as neurons present a variable number of neurites and express different neurotransmitters (Marlow et al., 2009). In addition, some transcription factors seem to be specific for certain neural population. For example, *NvFoxQ2d* has been shown to be expressed in a unipotent neural progenitor generating a morphologically homogeneous population of FMRFamide-positive sensory neurons (Busengdal and Rentzsch, 2017). More recently, the single cell transcriptome of the whole adult *Nematostella* revealed the existence of 32 neuronal metacells (Sebé-Pedrós et al., 2018). These metacells can be divided in two groups based on shared expression profiles. Group 1 is characterized by the expression of the transcription factors *NvFoxL2* and *NvPOU4* as well as the expression of the ion channels *NvShaker1*, *NvShaker4* and *NvNav2.5*, while group 2 is defined by the expression of *NvGata*, *NvOtxC* and *NvIslet*. Additionally, this study confirmed that cnidocytes are composed of two populations, namely spirocytes and nematocytes (Frank and Bleakney, 1976; Zenkert et al., 2011).

Therefore, it is quite apparent that the three classes of neurons in *Nematostella* are composed of many subpopulations, however they are still poorly described. Furthermore, we are still missing information about the developmental programs leading to the generation of these different neural cell types. The characterization of these different neuronal populations will provide a better understanding of the neural cell repertoire of *Nematostella.* It will also allow the identification of which neurons are interneurons, motoneurons, as well as their own features (*e.g.* receptors, neurotransmitters, marker genes).

### *3.4.2- Neurogenesis*

In *Nematostella*, neurogenesis starts at the blastula stage when the first neural progenitor cells (NPCs) can be observed. Differentiated neurons appear first in the ectoderm during gastrulation and later, neurons start to appear in the mesendoderm at planula stage (**Figure 24**) (Marlow et al., 2009; Nakanishi et al., 2012; Richards and Rentzsch, 2014).

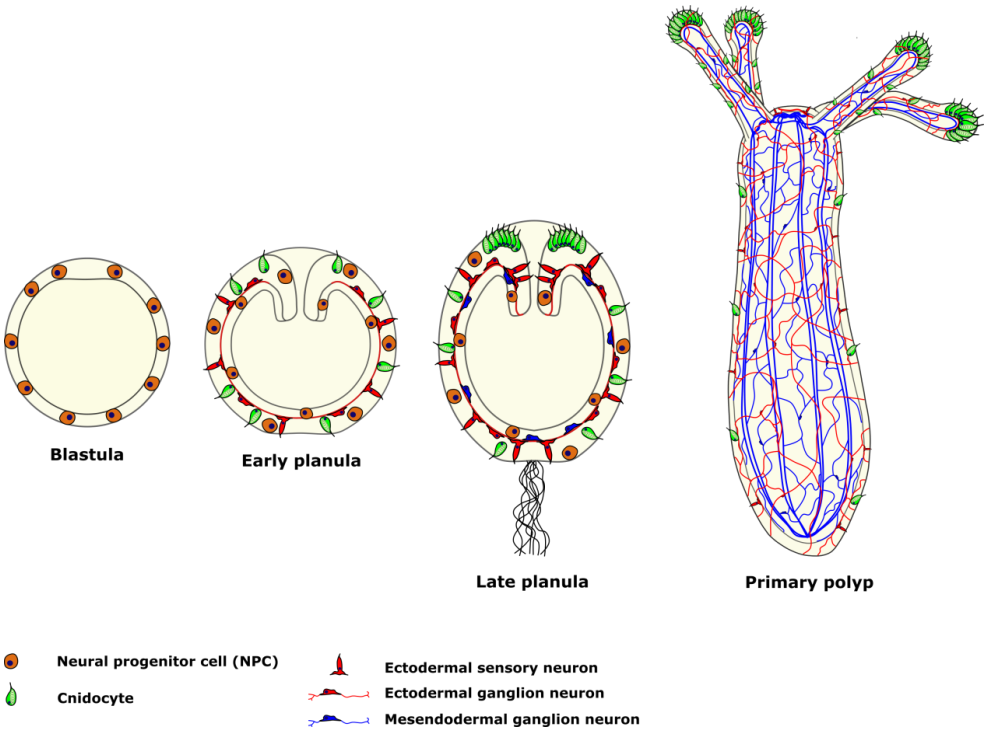



***Figure 24:*** ***Neurogenesis in the cnidarian Nematostella vectensis.*** *The first neural progenitor cells (NPCs) appear at blastula stage, while the first differentiating sensory neurons, ganglion neurons and cnidocytes first appear in the ectoderm at early planula stage. At the same time, NPCs appear in the mesendoderm. At late planula stage, mesendodermal neurons start to develop, with many of them condensing into longitudinal tracts which align to the musculature of the primary polyp. Schematics are not to scale; all stages have the oral pole up and show lateral cross sections, except for the polyp, which depicts a section across the mesoglea (extracellular matrix separating the ectoderm from the mesendoderm) showing both nerve nets. Adapted from* (Richards and Rentzsch, 2014)

*Cellular regulation of neurogenesis*

In *Nematostella*, no cell presenting a similar morphology to i-cells have been identified, suggesting that NPCs are derived from epithelial cells (Nakanishi et al., 2012; Richards and Rentzsch, 2014). These NPCs are characterized by the expression of *NvSoxB(2)*, a HMG-box *sox* family transcription factor related to the bilaterian *SoxB1* and *SoxB2* families expressed in developing neurons (Graham et al., 2003; Zhao and Skeath, 2002). The labelling of the NPC progeny by a transgene expressing the fluorescent protein mOrange under the control of the *NvSoxB(2)* promoter, reported that sensory neurons, ganglion neurons and cnidocytes, hence all neural classes, are derived from the *NvSoxB(2)* expressing NPCs (Richards and Rentzsch, 2014). However, the question whether the *NvSoxB(2)*$^+$ NPCs form a homogenous population or represent different subpopulations specifically producing one neural class, remains to be answered (**Figure 25**).

In the *NvSoxB(2)::mOrange* transgenic line, labelled cells are scattered across the embryo but small clusters of cells can be observed. These clusters are thought to represent the progeny of a single *NvSoxB(2)*$^+$ NPC. Interestingly, these clusters contain a different number of cells (odd and even number) suggesting that divisions are not synchronized. Moreover, the EdU labeling of these clusters showed that all cells in a single cluster do not proliferate, indicating that some cells are quiescent, post-mitotic or have a longer cell cycle. Therefore, NPCs divide asymmetrically. Nevertheless, it is still unclear whether *NvSoxB(2)*$^+$ NPCs are self-renewing (Richards and Rentzsch, 2014).

*Molecular regulation of neurogenesis*

The molecular mechanisms controlling neurogenesis in *Nematostella* rely on a similar set of genes playing similar functions than genes involved in bilaterian neurogenesis.

As described in the previous paragraph, proliferative NPCs express *NvSoxB(2)* (**Figure 25**). This gene is among the earliest genes expressed to initiate neurogenesis as its downregulation by morpholino results in the downregulation of proneural genes, such as *NvAth-like* and *NvAshA* (Richards and Rentzsch, 2015).

In *Nematostella*, the bHLH proneural genes *NvAth-like* (*atonal*-like) and *NvAshA* (*achaete-scute* homolog A) have been shown to act in early neurogenesis. *NvAth-like*, also known as *NvArp3*, is associated with the *neurogenin* and *neuroD* gene families (Simionato et al., 2007) and promotes neural development in *Nematostella* (**Figure 25**). Indeed, it is partially co-expressed with *NvSoxB(2)* in proliferative NPCs and the loss of *NvAth-like* expression induces a reduction of differentiated neurons (Richards and Rentzsch, 2015). However, whether *NvAth-like* initiates the specification of NPCs or whether it regulates the fate of existing NPCs remains to be elucidated. On the other hand, *NvAshA* is necessary and sufficient for the development of some aboral neurons as it affects the expression of neural genes in this domain of the embryo when up- or downregulated (Layden et al., 2012). Since *NvAshA* is expressed in non-proliferative *NvSoxB(2)*$^{+}$ NPCs, after the expression of *NvAth-like* ceased (Richards and Rentzsch, 2015) and along with some neural differentiation genes (Layden et al., 2012), it means that this *achaete-scute* homolog is involved in the early neural differentiation in *Nematostella* (**Figure 25**). Other genes of this family, *i.e. NvAshC* and *NvAshD*, are co-expressed with a subset of the neural differentiation genes regulated by *NvAshA* in non-proliferative cells, hence they are involved in the differentiation of some neural populations (Layden et al., 2012; Richards and Rentzsch, 2015). Therefore, the role of *NvAsh* genes is restricted to non-proliferative differentiating neurons (**Figure 25**). This role of *ash* genes in *Nematostella* differs from their role in bilaterians where they are predominantly expressed in proliferative neural progenitors and act in early neural differentiation (Bertrand et al., 2002).

Notch signaling is another regulator of neurogenesis conserved among bilaterians that also plays a role in *Nematostella* neurogenesis. The downregulation of *NvNotch* by morpholino or pharmacological DAPT treatment (inhibitor of the γ-secretase mediated activation of Notch) induces an increase in the expression of neural markers

(*e.g. NvRFamide*, *NvElav1*, *NvNcol3*) resulting in an increase of the total number of neurons (Richards and Rentzsch, 2015). By contrast, the over-expression of *NvNotch* suppresses neural differentiation (Layden and Martindale, 2014). Moreover, the perturbation of Notch signaling affects the expression of *NvSoxB(2)*, *NvAth-like* and *NvAshA* (Layden and Martindale, 2014; Richards and Rentzsch, 2015). This is consistent with *NvNotch* playing a role in the early regulation of the number of NPCs in *Nematostella* (**Figure 25**). However, this regulation of neurogenesis is likely mediated through the non-canonical Notch signaling pathway as the expression of *Nvhes* and *NvsuH* (*suppressor of Hairless*) genes is not affected by the perturbation of *NvNotch* and they do not affect the expression of neural genes (Layden and Martindale, 2014). Despite the downregulation of *NvNotch* by DAPT treatment induces an increase in the expression of the cnidocyte marker *NvNcol3*, it prevents the maturation of the capsule, suggesting that *NvNotch* could play an additional role at later steps of neurogenesis but it still needs to be demonstrated (Richards and Rentzsch, 2015).

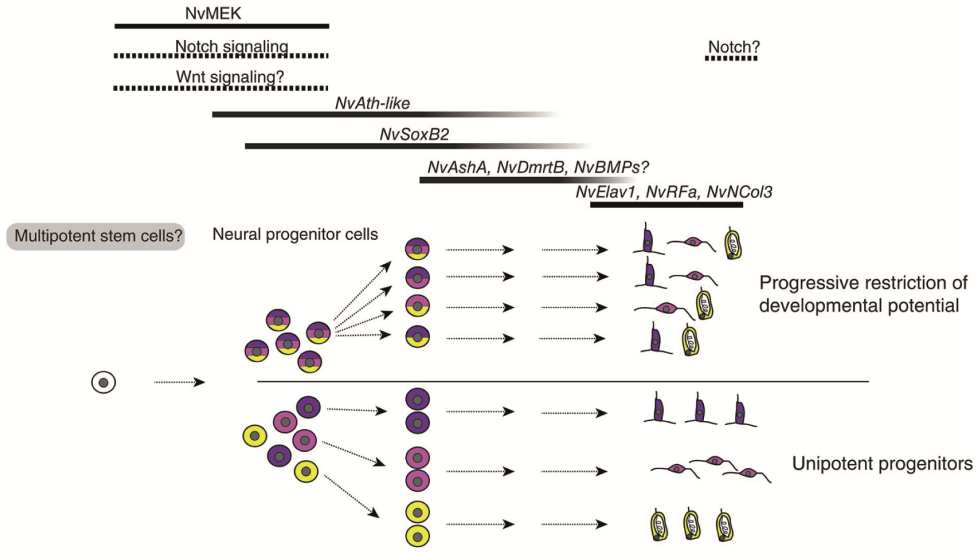



***Figure 25:* Cellular and molecular regulation of Nematostella neurogenesis.** *A pool of dedicated NPCs gives rise to the three major classes of neural cells (sensory cells, ganglion cells and cnidocytes) during embryogenesis. Individual NPCs may give rise to different classes (upper part) or to only one class of neural cells (lower part), but the existence of these two types of NPCs is not mutually exclusive. NPCs might be derived from multipotent stem cells, but experimental evidence for such stem cells is missing. Bars above the figure depict the stages at which the indicated genes act during the progression of neurogenesis. From* (Rentzsch et al., 2016)

*Acquisition of the neural potential*

As I have mentioned above, the overexpression of *NvAshA* increases the number of cells expressing neural genes in the aboral half of the embryo. Nevertheless, it does not commit all aboral cells into neurons (Layden et al., 2012). Similarly, the inhibition of *NvNotch* does not induce a ubiquitous expression of neural marker genes over the embryo, neither does the simultaneous inhibition of *NvNotch* and the overexpression of *NvAshA* (Layden and Martindale, 2014; Richards and Rentzsch, 2015). This indicates that some cells possess a neurogenic potential prior to the neurogenesis onset and suggests that this potential is either a feature of a distinct population of cells or induced by external cues.

In bilaterians, the inhibition of BMP2/4 by Noggin, Chordin and Follistatin is the signal inducing the specification of the neuroectoderm from the ectoderm (De Robertis and Kuroda, 2004; Mieko Mizutani and Bier, 2008; Ozair et al., 2013; Rogers et al., 2009). The inductive role of BMPs have been tested in *Nematostella* by treating embryos with the human BMP2 (Watanabe et al., 2014). While short early treatments until mid-blastula stage did not have any effect on the expression of neural marker genes, longer treatments until planula stage induced a reduction in the expression of these markers. Surprisingly, the injection of *NvBmp2/4* morpholinos had the same effect, indicating that BMPs have both a positive and negative role in *Nematostella* neurogenesis. However, it does not seem to have an inductive function (Saina et al., 2009; Watanabe et al., 2014).

FGF signaling induces neurogenesis in bilaterians by promoting the expression of *noggin* and *chordin,* both inhibiting BMPs (Guillemot and Zimmer, 2011; Stern, 2005), hence FGF could also induce neurogenesis in *Nematostella*. Consistent with BMPs not playing a role in *Nematostella* neural induction, the downregulation of *NvFgfRa* by morpholino injection showed no effect on neural genes expression such as *NvAshA* (Layden et al., 2016b). Thus, the FGF signaling does not induce neurogenesis in *Nematostella*.

However, the pharmacological inhibition of the MAPK MEK (Mitogen Activated Protein Kinase Kinase) disrupted neurogenesis by strongly reducing the expression level of the proneural genes *NvSoxB(2)*, *NvAth-like*, *NvAshA* as well as the number of cells expressing neural markers (Layden et al., 2016b). A MEK activity is therefore necessary for neurogenesis to occur in *Nematostella* (**Figure 25**). As the overexpression of *NvAshA* increases the number of cells undergoing neurogenesis, this gene has been upregulated by mRNA injection in embryos in which the MEK activity has been inhibited. The overexpression of *NvAshA* did not rescue neurogenesis in this context, indicating that cells are not competent to become neural (Layden et al., 2016b). Therefore, neurogenesis is promoted under a MEK-mediated induction in *Nematostella*, howbeit the signal activating the MEK signaling pathway remains unknown as FGF is not involved.

In addition, the Wnt/β-catenin signaling pathway seems to be involved in neural induction as well. An increase in the activity of this pathway induces an augmentation in expression of *NvSoxB(2)* and *NvAshA*, while an inhibition of the pathway have an opposite effect (Watanabe et al., 2014). Despite this effect on early neurogenesis, the Wnt/β-catenin pathway favors the acquisition of oral identities; hence it does not regulate neurogenesis in its entirety.

Altogether, these data indicate that neural induction occurs in *Nematostella*. Nevertheless, the inductive cue still needs be uncovered.

*Neural patterning*

The planula larva of *Nematostella* is patterned along the oral-aboral and directive axes with different genes expressed in specific domains. The Wnt signaling is a major regulator of the oral-aboral axis patterning as the disruption of some components of this pathway impairs the identity of the different domains. Indeed, the pharmacological ectopic activation of Wnt activity results in a shift of oral identities toward the aboral side, while the inhibition of the Wnt signaling have the opposite effect (Marlow et al., 2013). The Wnt signaling is also involved in the early specification of the aboral side as

knocking down *Nvβ-catenin* results in the total absence of oral-aboral patterning (Leclère et al., 2016b).

Consistent with the patterning of the embryo, some neural marker genes are expressed in separate areas. For example, *NvArp6* is only expressed in one side of the embryo, while RFamide and GLWamide expressing neurons are found in the oral region (Watanabe et al., 2014). Similarly, the different types of cnidocytes are distributed along the oral-aboral axis of polyps, with spirocytes concentrated in the head region (tentacles and mouth) and nematocytes concentrated on the body column (Zenkert et al., 2011). The nerve net of *Nematostella* is therefore patterned along the different body axes. This is confirmed by the fact that the overexpression of *NvAshA* increases the number of neurons but not the distribution of each neuronal type (Layden et al., 2012).

The neural patterning seems to be linked to the oral-aboral patterning as the Wnt signaling is required for the development of RFamide and GLWamide neurons in the oral territory (Watanabe et al., 2014) and the pharmacological disruption of Wnt activity shifts neural gene expression domains (Marlow et al., 2013). It appears that the neurogenic program is the same for the entirety of the nervous system in *Nematostella,* but the acquisition of the different neural identities depends on local body patterning signals, similarly to what has been described in bilaterians. However, the RFamide and GLWamide expressing neurons are not restricted to a specific domain, they are rather distributed in a gradient manner across several domains. The characterization of neurons subtypes is, therefore, necessary to completely unravel the nervous system patterning in *Nematostella* (Layden et al., 2016a; Rentzsch et al., 2016).

### *3.4.3- Mesendodermal neurogenesis*

Most cnidarians possess a nervous system in both the ectoderm and the endoderm, including *Nematostella* (Marlow et al., 2009; Nakanishi et al., 2012). I previously described that hydrozoans generate all their neurons from a endoderm-derived cell population, the i-cells (see **2.2.4** page 67). In the previous part, I mentioned that ectodermal neurons in *Nematostella* were generated by *NvSoxB(2)* expressing NPCs and this is also the case of mesendodermal neurons as revealed by the analysis of the *NvSoxB(2)::mOrange* transgenic reporter line (Richards and Rentzsch, 2014).

In order to determine whether mesendodermal neurons develop from ectodermal cells migrating to the mesendoderm or directly from mesendodermal cells, some transplantation experiments have been conducted (Nakanishi et al., 2012). Small pieces from the mesendodermal plate of *NvElav1::mOrange* transgenic embryos, at early gastrula stage, have been grafted into the mesendoderm of wild-type host embryos at equivalent stage. It resulted in the generation of chimeric embryos expressing the *NvElav1::mOrange* transgene in some mesendodermal neurons at planula stage (**Figure 26**). This experiment demonstrated that *Nematostella*'s mesendoderm possesses the potential to generate neurons, therefore NPCs develop not only from the ectoderm but also directly from the mesendoderm.

By contrast to the canonical ectodermal neurogenesis, the mesendodermal neurogenesis taking place in *Nematostella* has been poorly investigated. Very little is currently known, and this information usually comes from few observations made while investigating neurogenesis in general. It is therefore difficult to make a proper comparison between the ectodermal and mesendodermal neurogenesis processes.

However, several observations indicate that mesendodermal neurogenesis seems to begin with a similar genetic program than ectodermal neurogenesis. Indeed, NPCs localized in the mesendoderm also co-express *NvSoxB(2)* and *NvAth-like* even though there are more *NvSoxB(2)*$^{+}$ than *NvAth-like*$^{+}$ cells in the mesendoderm (Richards and Rentzsch, 2015). The proneural gene *NvAshA* involved in early neural differentiation is expressed in the planula mesendoderm as well (Layden et al., 2012). Later, the

GLWamide expressing neurons are mostly found on one side of the mesendoderm at planula stage. Although *NvBmp2/4* does not seem to have an inductive role, it might be involved in the development of GLWamide$^{+}$ neurons as they are absent in *NvBmp2/4* morphants (Watanabe et al., 2014).

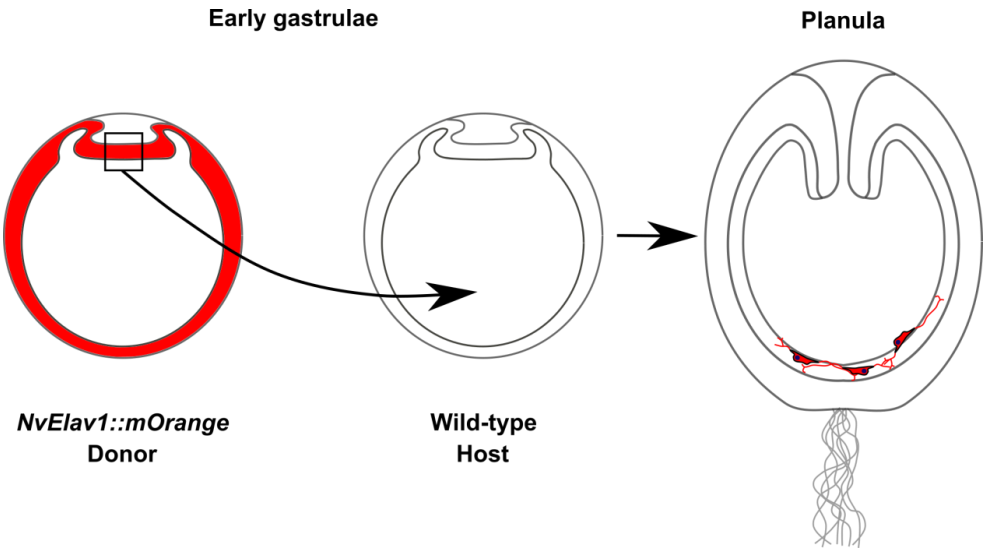



***Figure 26:*** ***The transplantation of mesendodermal pieces.*** *Small pieces of the invaginating mesendodermal plate from early NvElav1::mOrange transgenic gastrulae are grafted into equivalent wild-type gastrulae. When observed at planula stage, host larvae carry NvElav1::mOrange expressing mesendodermal neurons, demonstrating that neurons develop directly from the mesendodermal epithelium in Nematostella vectensis. Adapted from* (Nakanishi et al., 2012)

Despite the apparent similarities between ectodermal and mesendodermal neurogenesis in *Nematostella*, it seems that ectodermal and mesendodermal neurons express different neuropeptides and/or neurotransmitters as GLWamide is mainly found in mesendodermal neurons. Moreover, mesendodermal neurogenesis starts later (at planula stage) than ectodermal neurogenesis (at the end of gastrulation). It suggests that there could be different inductive cues or different sources of inductive cues for both processes. Therefore, the characterization of the mesendodermal neuronal subtypes as well as their developmental program is primordial to provide new comparative elements and better understand mesendodermal neurogenesis in *Nematostella.*

## 3.5- Aims of the PhD project

The general aim of my PhD project is to investigate mesendodermal neurogenesis in *Nematostella vectensis* in order to improve our knowledge about this non-canonical process.

To this purpose, we have used the data generated by a microarray experiment comparing the gene expression levels in two conditions: first, in *NvSoxB(2)* morpholino injected embryos in which neurogenesis is downregulated and second, in DAPT treated embryos in which neurogenesis is upregulated. This provided a large list of genes that are respectively down- and upregulated in these conditions and therefore, potentially involved in *Nematostella* neurogenesis (G.S. Richards, J. Blommaert and F. Rentzsch, unpublished). Within this list, transcription factors have been selected for preliminary screening by colorimetric *in situ* to identify genes presenting interesting expression patterns for further analysis. Among these genes, the transcription factor encoded by the PR domain (PRDM) zinc finger family gene *NvPrdm14d* exhibited an expression pattern suggesting a role in mesendodermal neurogenesis.

The present thesis aims to characterize in details the identity of the mesendodermal cells expressing *NvPrdm14d* by analyzing a stable transgenic line expressing a *NvPrdm14d::GFP* transgene (**Paper I**).

As genes of the PR domain family are transcription factors involved in diverse developmental processes in metazoans, the second aim of this thesis is to document the spatial expression of *NvPrdm* genes and to assess their potential role in *Nematostella vectensis* (**Paper II**).

# 4-Genes of the PR domain zinc finger protein family

## 4.1-Introduction of *Prdm* genes and phylogeny

Genes of the *Prdm* family encode transcription factors containing a subtype of SET domain at their N-terminal, the PR domain (**Figure 27**). This domain has been initially described in PRDI-BF1 (Positive Regulatory Domain Interferon-Binding Factor 1) and RIZ1 (Retinoblastoma protein-Interacting Zinc finger gene 1), now respectively known as Prdm1 (or Blimp1) and Prdm2 (Buyse et al., 1995; Hohenauer and Moore, 2012; Keller and Maniatis, 1991).

The SET domain is classically defining a large group of histone lysine methyltransferases (HMT) but an enzymatic activity of the PR domain has been only described for the vertebrate Prdm2, Prdm3, Prdm6, Prdm7/9, Prdm8, Prdm13 and Prdm16 in vertebrates (Hanotel et al., 2014; Hohenauer and Moore, 2012; Huang, 2002; Pinheiro et al., 2012; Wu et al., 2010). Mutations in the conserved H/RxxNHxC motif of SET proteins is responsible for a loss of catalytic activity but none of the Prdm proteins carries this motif nor the essential histidine in the NHxC (Rea et al., 2000). In Prdm2, the mutation of cysteine 206 reduces its HMT activity, however this residue is not conserved in other Prdm proteins exhibiting an intrinsic HMT activity (Hohenauer and Moore, 2012). Instead, the PR domain of other Prdm proteins is involved in protein-binding interactions to recruit co-factors. These catalytically dead Prdm proteins recruit other HMTs such as Prmt5, for example, but also some histone deacetylases (HDAC) and some histone acetyltransferases (HAT) (Hohenauer and Moore, 2012).

Additionally, all Prdm associate a variable number of zinc finger motifs at their C-terminal (**Figure 27**), except Prdm11 (Fumasoni et al., 2007; Kinameri et al., 2008; Sun et al., 2008). Zinc fingers are DNA- or protein-binding motifs involved in the binding to the regulatory regions of target genes or in the recruitment of co-factors. Nevertheless, a direct DNA-binding interaction has been only demonstrated for the vertebrate Prdm1, Prdm3, Prdm5, Prdm9, Prdm14 and Prdm16 (Bard-Chapeau et al., 2012; Baudat et al., 2010; Chia et al., 2010; Delwel et al., 1993; Duan et al., 2007; Hohenauer and Moore, 2012; Kuo and Calame, 2004; Ma et al., 2011; Seale et al., 2007).

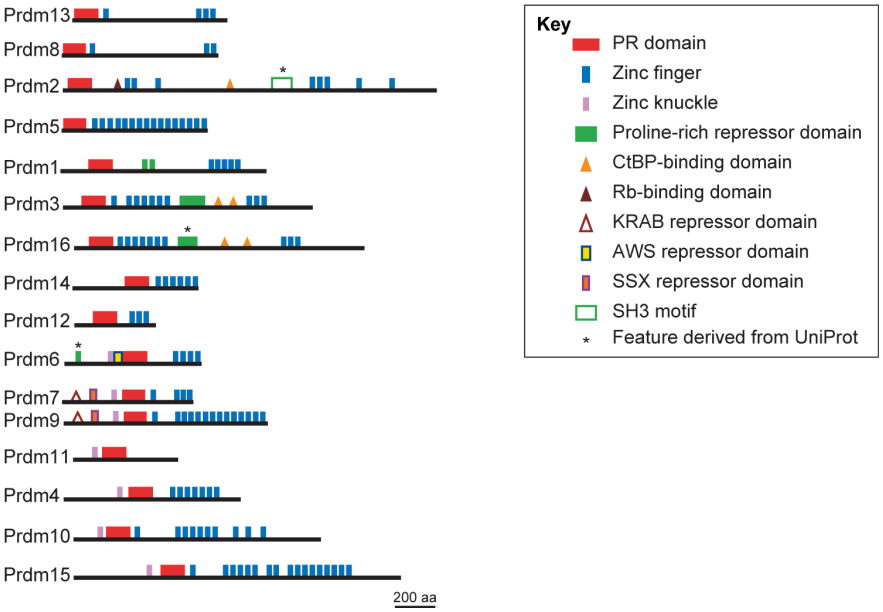



***Figure 27:* The Prdm family domain structure.** *The domain structure for each of the human Prdm family member is illustrated. Only the longest reported isoform is shown. Prdm11 alone does not contain zinc fingers, instead it has a smaller protein-protein interaction motif known as a zinc knuckle that is also present in several other family members. From* (Hohenauer and Moore, 2012)

### *4.1.1-Phylogeny of Prdm genes*

The phylogenetic analysis of 976 *Prdm* genes from 93 species whose genomes are sequenced, revealed the existence of 14 different subfamilies of *Prdm* genes in metazoans (**Figure 28**) (Vervoort et al., 2015). The different subfamilies have been named after the human genes and as several duplications are vertebrate or primate specific, some families are named after two human genes (*e.g.* Prdm7/9). The Prdm family is specific to metazoans as no ortholog has been found in non-metazoans species. However, some non-metazoan opisthokonts from different lineages (*Capsaspora owczarzaki*, *Sphaeroforma arctica* and *Spizellomyces punctatus*) possess proteins containing a SET domain related to the PR domain. It is thought that the PR domain of *Prdm* genes originated from a unique gene ancestral to all metazoans. By contrast, the zinc finger motifs have been recruited from different ancestral genes during metazoan evolution (Vervoort et al., 2015).

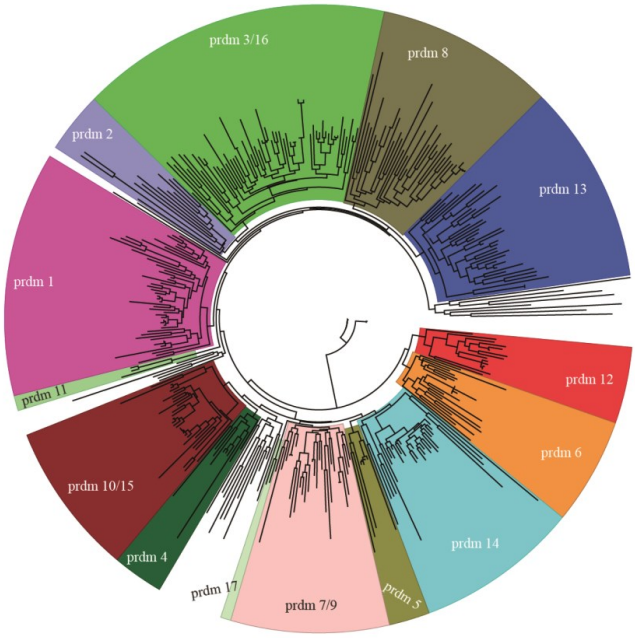



***Figure 28:* Phylogeny of the Prdm gene family.** *The Prdm gene family is specific to metazoans and is composed of 14 subfamilies. Each subfamily is represented in a different color, uncolored genes are labeled as "orphans". The name of subfamilies comes from the human gene(s), hence human genes originating from a vertebrate or primate specific duplication are in the same subfamily and both genes gave their name to the subfamily (as Prdm7/9, for example). From* (Vervoort et al., 2015)

The Prdm family underwent four expansion phases: first, in the last common ancestor to all metazoans with the production of 2 or 3 genes. Then, in the last common ancestor of cnidarians and bilaterians with 6 genes, followed by a third expansion in the last common ancestor to all bilaterians with 11 genes and finally, in early vertebrate evolution for the remaining genes (Vervoort et al., 2015). Nonetheless, there is a wide variation in the number of *Prdm* genes within species ranging from 2 to 19 in a single species. These variations are due to losses in certain lineages, such as in ecdysozoans with only 2 genes in *C. elegans* and *Drosophila* (**Figure 29**).

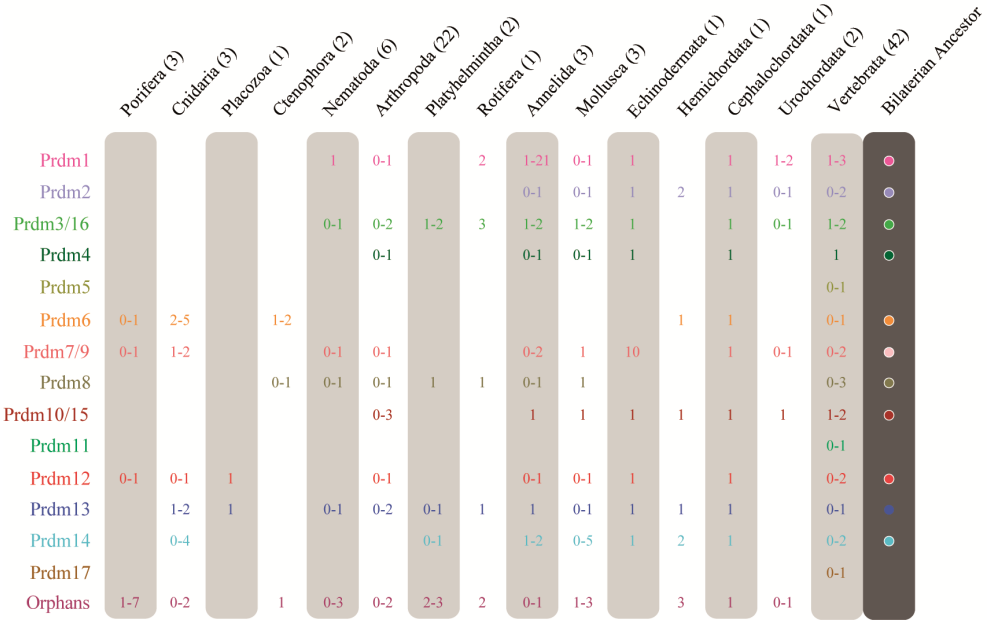



***Figure 29:*** ***Prdm subfamilies in the main metazoan groups.*** *The number (or range of numbers) of members of each Prdm subfamily and orphan genes is indicated (none if no member detected). The number of studied species in each phylogenetic group is indicated next to the group name. A final column summarizes the putative ancestral set of Prdm subfamilies in the bilaterian ancestor. From* (Vervoort et al., 2015)

### *4.1.2-Overview of Prdm gene functions*

*Prdm* genes are transcription factors that play important roles in diverse developmental processes, such as germ cell development, neurogenesis, vascular development, brown fat differentiation and hematopoiesis (Hohenauer and Moore, 2012). Moreover, a single *Prdm* gene can act in different processes, at different levels of the acquisition of the cell identity and as an activator or a repressor depending on the cellular context. For example, Prdm16 allows the acquisition of the brown adipose tissue identity by repressing other cell fates (Kajimura et al., 2008; Kajimura et al., 2009; Seale, 2010; Seale et al., 2007). In addition, it also maintains hematopoietic stem cells in a quiescent or proliferative state (Aguilo et al., 2011; Chuikov et al., 2010). Similarly, Prdm1 is involved in the regulation of the cell state and cell fates in different contexts. Indeed, it maintains neonatal mouse enterocytes in a juvenile state (Harper et al., 2011;

Muncan et al., 2011), participates in the generation of primordial germ cells (Kurimoto et al., 2008a; Ohinata et al., 2005; Vincent et al., 2005) and regulates the terminal differentiation of B cells into antibody secreting plasma cells (Turner et al., 1994).

Regarding neurogenesis, several *Prdm* genes have been described to be expressed in different populations of cells in the developing nervous system of several organisms (Kinameri et al., 2008). In *Drosophila* and *C. elegans*, *Prdm3* (respectively known as *Hamlet* and *EGL-3*) is necessary for the differentiation of sensory neurons (Garriga et al., 1993; Moore et al., 2002). In mouse, *Prdm3* is expressed in the peripheral nervous system (Perkins et al., 1991). In zebrafish, Prdm1 is required for the specification of neural crest and sensory neurons (Hernandez-Lagunas et al., 2005). In mouse, Prdm16 regulates the survival of neural progenitors through the modulation of the oxidative stress (Chuikov et al., 2010).

However, the more striking role of *Prdm* genes during neurogenesis is linked to their function in the dorso-ventral patterning of the vertebrate neural tube (Zannino and Sagerström, 2015). Indeed, several *Prdm* genes are involved in the specification of neural fates and are expressed in different domains along the dorso-ventral axis of the neural tube. In the ventral neural tube, *Prdm8* is expressed in the p0, p1, p2 and pMN domains (Kinameri et al., 2008; Komai et al., 2009); *Prdm12* in the p1 domain (Kinameri et al., 2008; Zannino et al., 2014); and *Prdm14* in the pMN domain (Liu et al., 2012). In the opposite dorsal pole of the neural tube, *Prdm13* is expressed from the domain dP2 to dP6 (**Figure 30**) (Chang et al., 2013; Hanotel et al., 2014). These different *Prdm* genes are likely under the regulation of Shh and BMP signaling as the zebrafish *Prdm12b* is downregulated when Shh is inhibited by cyclopamine treatments (Zannino et al., 2014). It is likely that *Prdm12* is also regulated by BMP as the establishment of the p1 domain depends on BMP signaling (Timmer et al., 2002) and *Prdm12* is positively regulated by BMP to specify the pre-placodal ectoderm and neural crests in *Xenopus* (Matsukawa et al., 2015). However, it remains to be demonstrated for the other *Prdm* genes mentioned here.

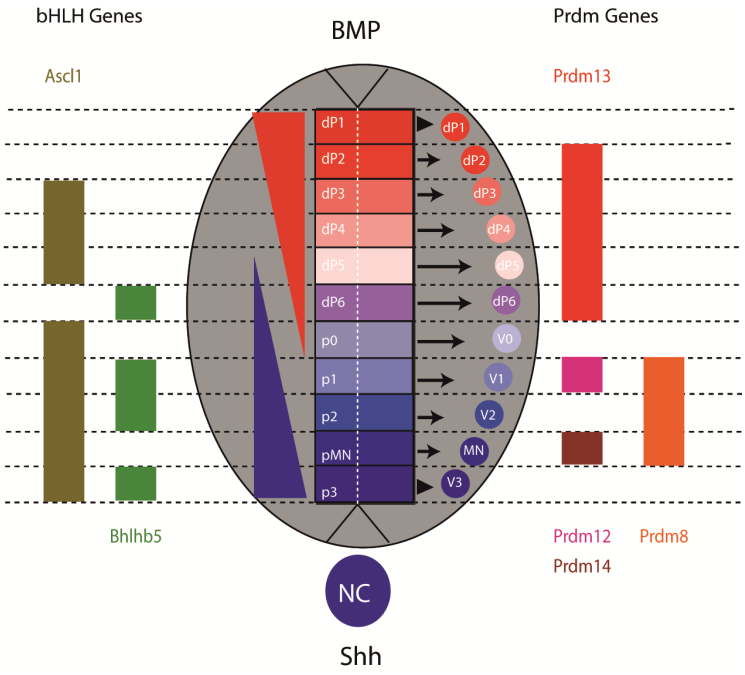



***Figure 30:* Prdm genes pattern the dorso-ventral axis of the vertebrate neural tube.** *Summary of Prdm genes and interacting bHLH expression domains along the dorso-ventral axis of the neural tube. Modified from* (Zannino and Sagerström, 2015)

These different *Prdm* genes play a role at different steps of the vertebrate nervous system development and interacts with proneural and bHLH genes. In zebrafish and mouse, *Prdm12* delimits the boundary between the p1 and p2 progenitor domains, and is involved in the specification of V1 interneurons (Kinameri et al., 2008; Zannino et al., 2014). In *Xenopus* and mouse, Prdm13 favors the inhibitory GABAergic identity over the excitatory glutamatergic identity in the dP4 domain by interacting with Ascl1 (Chang et al., 2013; Hanotel et al., 2014). In zebrafish, Prdm14 activates the expression of *Islet2* to promote axon outgrowth in caudal primary motor neurons (Liu et al., 2012). Finally, Prdm8 controls the proper connectivity of neural circuits in the mouse telencephalon through its interaction with Bhlhb5 (*a.k.a.* Bhlh22) to direct axon outgrowth (Ross et al., 2012). Therefore, *Prdm* genes are primordial for a proper development of the vertebrate nervous system.

## 4.2-The *Prdm* gene repertoire of *Nematostella vectensis*

The phylogenetic analysis of *Prdm* genes in metazoans revealed that the genome of *Nematostella vectensis* contains, at least, 13 *Prdm* genes (Vervoort et al., 2015). There are five *Prdm6* paralogs (namely *NvPrdm6a*, *NvPrdm6b*, *NvPrdm6c*, *NvPrdm6d* and *NvPrdm6e*), one *NvPrdm7/9* gene, one *NvPrdm12* gene, two *Prdm13* paralogs (*NvPrdm13a* and *NvPrdm13b*), and four *Prdm14* paralogs (*NvPrdm14a*, *NvPrdm14b*, *NvPrdm14c* and *NvPrdm14d*). As none of these genes have been studied in *Nematostella* or in any cnidarian species, I will introduce the *Prdm* genes that I have studied, through their role in bilaterians.

### *4.2.1-Prdm6*

The *Prdm6* gene underwent specific duplications in the cnidarian and ctenophore lineages, while bilaterian species, except protostomes who have lost the gene (**Figure 29**), only possess one *Prdm6* (Vervoort et al., 2015).

In mouse, this gene is essential for embryonic vascular patterning as it regulates genes involved in angiogenesis (Fog et al., 2012; Gewies et al., 2013; Wu et al., 2008). It acts by promoting the proliferation of smooth muscle progenitors and favors smooth muscle cell over endothelial cell differentiation (Davis et al., 2006; Wu et al., 2008). At the molecular level, Prdm6 carries an intrinsic HMT activity independently of the PR domain, and specifically methylates histone 4 at lysine 20 (H4K20) (Wu et al., 2008). Despite this intrinsic HMT activity, it also recruits other HMTs such as G9a, some class I HDACs and some HAT such as p300 (Davis et al., 2006). In addition to the PR domain and the zinc fingers, Prdm6 carries a AWS domain (associated with SET) required for the repressive function and the binding to HDAC3 (Davis et al., 2006).

Additionally, the expression of *Prdm6* has been detected in a small population of post-mitotic neurons in the ventral spinal cord and in putative sclerotomes in mouse (Kinameri et al., 2008). Nonetheless, its function in neurogenesis remains to be investigated.

### *4.2.2-Prdm13*

The *Prdm13* gene is present in both cnidarians and bilaterians, but it has been lost in several protostome lineages **(Figure 29)**. By contrast, it underwent some duplication events in specific species, such as in *Nematostella* (Vervoort et al., 2015). This gene acts as a transcriptional repressor and carry a HMT activity for the methylation of H3K9, however it remains unclear whether this activity is intrinsic or mediated by the recruitment of other HMTs (Hanotel et al., 2014; Mona et al., 2017). Interestingly, the zinc fingers appear to be more important to the proper function of Prdm13 than the PR domain (Chang et al., 2013).

The function of *Prdm13* has been mainly investigated in vertebrates where it plays a role in neurogenesis. As described in a previous paragraph, Prdm13 is expressed in the dorsal spinal cord from the dP2 to dP6 domains (**Figure 30**) where it interacts with Ascl1 to block its activity and to favor the development of inhibitory GABAergic over excitatory glutamatergic interneurons (Hanotel et al., 2014; Kinameri et al., 2008; Sun et al., 2008). Concomitantly, Prdm13 actively contributes to the dorso-ventral patterning of the neural tube as it represses ventral genes in the dorsal neural tube (Mona et al., 2017). Consistent with its role in fate decision, *Prdm13* is expressed in progenitors and postmitotic differentiating cells in which it negatively regulates proliferation (Hanotel et al., 2014).

In addition, *Prdm13* is also expressed in the retina where it promotes the development of inhibitory GABAergic and glycinergic amacrine interneurons involved in the processing of visual information (Bessodes et al., 2017; Watanabe et al., 2015). Mutant mice for *Prdm13* show an elevated sensitivity to visual stimuli caused by the loss of the inhibitory amacrine neurons (Watanabe et al., 2015).

The expression of *Prdm13* has also been detected in the dorsomedial nucleus of the hypothalamus, a structure regulating aging and longevity (among other processes) in mammals. In mice, the expression level of *Prdm13* naturally decreases with aging, while it increases under diet restriction enhancing longevity. In long-lived BRASTO (brain-specific *Sirt1*-overexpressing transgenics) mice, the *Prdm13* expression shows a diurnal

oscillation with higher expression levels in dark periods than in light periods. In concordance with these observations, *Prdm13* mutant mice harbor less sleep quality and higher adiposity (Satoh et al., 2015).

### *4.2.3-Prdm14*

The *Prdm14* gene is present in both cnidarians and bilaterians, however it has been lost in ecdysozoans, in rotifers and in urochordates (**Figure 29**). This gene underwent some duplication events in several lineages but surprisingly, all model organisms used to study the role of *Prdm14* only possess one paralog (Vervoort et al., 2015). In vertebrates, the PR domain and the zinc fingers, except the sixth one (=the last one), are well conserved, while the N-terminus of the protein is divergent between species (Nakaki and Saitou, 2014). No intrinsic HMT activity has been detected for the PR domain but a direct binding of the zinc fingers to the consensus sequence 5'-GGTCTCTAA-3', located within 10kb from the transcription start site (TSS) of target gene promoters, has been described in zebrafish, mouse and human (Chia et al., 2010; Liu et al., 2012; Ma et al., 2011; Tsuneyoshi et al., 2008; Yamaji et al., 2013).

*Prdm14 as a pluripotency factor*

One of the best described functions of *Prdm14* is its role in the maintenance of pluripotency in mammals. Indeed, *Prdm14* is expressed in mouse and human ESCs but not in differentiated cells. It is required for the maintenance of pluripotency as cells differentiate and lose their ability to self-renew when lacking Prdm14 (Assou et al., 2007; Chia et al., 2010; Ma et al., 2011; Nady et al., 2015; Nakaki and Saitou, 2014; Tsuneyoshi et al., 2008). In mouse, the BMP4 and Wnt3 signaling induce the expression of *Prdm14* (Aramaki et al., 2013; Ohinata et al., 2005; Ohinata et al., 2009; Yamaji et al., 2008). It has also been shown that Pou5f1 (*a.k.a* Oct4), Nanog and Sox2 (pluripotency factors) regulate the expression of *Prdm14* (Boyer et al., 2005; Chia et al., 2010). The role of Prdm14 in the maintenance of ESCs pluripotency is mediated through

both activation and repression of gene expression (**Figure 31**). Indeed, Prdm14 does not act alone but in protein complexes. The activating or repressing activity of the Prdm14-containing complexes depends on the other interacting proteins (Magnúsdóttir et al., 2013; Nakaki et al., 2013; Seki, 2018).

On one side, Prdm14 promotes the expression of genes involved in pluripotency, such as *Pou5f1*, *Sox2*, *Nanog* and *Klf5*, by directly binding to their promoter (Chia et al., 2010; Ma et al., 2011; Tsuneyoshi et al., 2008; Yamaji et al., 2013). Interestingly, Prdm14 seems to act in synergy with these exact same factors to induce the expression of additional genes required for the maintenance of pluripotency (Boyer et al., 2005; Chia et al., 2010). To activate the expression of such pluripotency genes, Prdm14 interacts with different molecular partners. One of them is the Ten-Eleven Translocation protein (TET), which oxidizes 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC) leading to a demethylation and expression of the target genes (Okashita et al., 2014; Wu and Zhang, 2014). Prdm14 also interacts with Prmt4 (Protein arginine methyltransferase 4) to induce the di-methylation of histone 3 at arginine 26 (H3R26me2) and promote gene expression (Burton et al., 2013). The estrogen-related receptor β (ESRRβ) is another known partner of Prdm14 promoting gene expression (Yamaji et al., 2013).

On the other side, Prdm14 is repressing a second set of genes to prevent cells from differentiating (Chia et al., 2010; Ma et al., 2011; Tsuneyoshi et al., 2008). The repressor complex contains CBFA2T, which is indispensable for downregulating differentiation genes (Nady et al., 2015; Tu et al., 2016). The pre-PR and PR domains of Prdm14 are essential for the interaction with CBFA2T and thus, for the gene repression activity (Nady et al., 2015; Yamamoto et al., 2020). However, CBFA2T by itself does not affect regulatory regions of target genes, instead it acts as a platform for recruiting additional partners, such as CtBP1/2 (C-terminus Binding Protein 1 and 2). In turn, CtBP1/2 recruit the Polycomb Repressor Complex 2 (PRC2) triggering the tri-methylation of H3K27 at target genes, notably genes of FGF signaling (*i.e.* FGFR encoding genes), and repress their expression (Margueron and Reinberg, 2011; Nakaki et al., 2013; Yamaji et al., 2013; Yamamoto et al., 2020). PRC2 is also required for the negative self-regulation

exerted by Prdm14 (Yamamoto et al., 2020). In addition, the Prdm14-containing repressor complex has been shown to interacts with other complexes, such as NODE, BRG1, esBAF and CRL4 (Tu et al., 2016; Yamamoto et al., 2020).

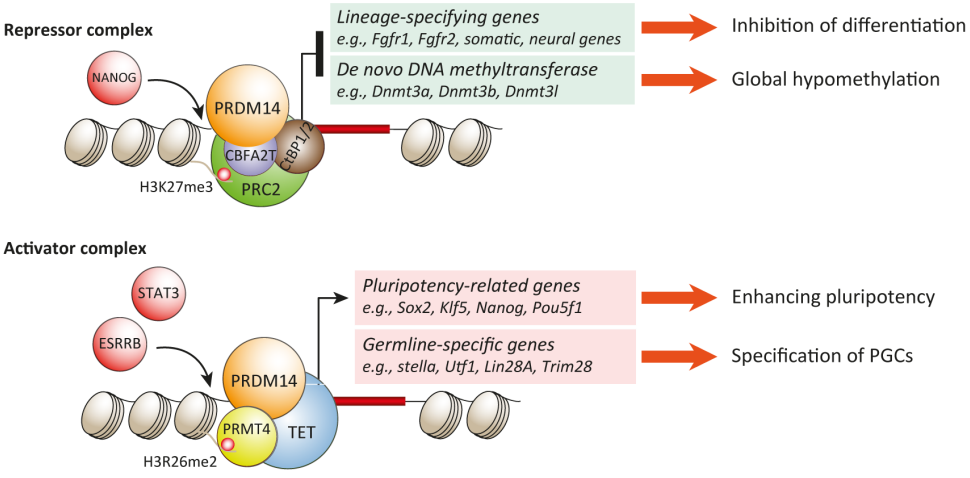



***Figure 31:* Mechanism of action of Prdm14 in vertebrate pluripotent cells and primordial germ cells (PGCs).** *Prdm14 recruits PRC2 at the promoter of target genes, including lineage-specific genes and de novo DNA methyltransferase genes. It results in the inhibition of differentiation as well as a global chromatin hypomethylation. Genes repressed by Prdm14 are often co-occupied by Nanog, which may also contribute to their repression. In parallel, Prdm14 recruits TET and Prmt4 for contributing to the activation of genes involved in pluripotency and PGCs specification. Genes activated by Prdm14 are frequently co-occupied by ESRRβ and Stat3, which may also contribute to their activation. PRC2: Polycomb Repressive Complex 2, H3K27me3: Histone 3 lysine 27 tri-methylation, H3R26me2: Histone 3 arginine 26 di-methylation, ESRRβ: estrogen-related receptor beta. Modified from* (Nakaki and Saitou, 2014)

Therefore, the Prdm14-containing complexes have either an enhancing or repressive effect on gene expression depending on which major binding partner is involved. Interactions with TET define the enhancing complex, while CBFA2T defines the repressive complex (Nakaki and Saitou, 2014; Yamamoto et al., 2020). Interestingly, it has been shown that the interaction between Prdm14 and CBFA2T is conserved among vertebrates to mediate the function of Prdm14 (Kawaguchi et al., 2019).

In line with this role of Prdm14 in the maintenance of pluripotency, it has been shown that its expression in differentiated cells is enough to induce pluripotency (Nakaki et al., 2013). It is, indeed, used in complement of the reprogramming cocktail (Sox2, Klf4, C-Myc and Oct4/Pou5f1) as it enhances its efficiency to induce pluripotent stem cells (iPS) *in vitro* (Gillich et al., 2012). Prdm14 can actually replace Klf4 in such cocktail (Chia et al., 2010). In this context, the recruitment of PRC2 by Prdm14 is necessary to induce pluripotency (Yamamoto et al., 2020).

*Prdm14 is required for the specification of primordial germ cells*

In amniote vertebrates, *Prdm14* has been shown to play an essential role in the reacquisition of pluripotency, which is indispensable for the specification of primordial germ cells (PGCs). Indeed, it is expressed in mouse, human and chicken gonads as well as induced PGCs (Okuzaki et al., 2019; Sybirna et al., 2020; Yabuta et al., 2006; Yamaji et al., 2008). Prdm14 is not required for the competence of cells to become PGCs but it acts early in their development through the reactivation of pluripotency genes (*e.g. Sox2* and *Nanog*) and the activation of germline-specific genes, while repressing somatic genes (particularly of neural lineages) (Kurimoto et al., 2008b; Kurimoto et al., 2008a; Okuzaki et al., 2019; Sybirna et al., 2020; Yamaji et al., 2008). However, it seems that genes targeted by Prdm14 are different in mouse and human (Sybirna et al., 2020). Another difference between mouse and human PGCs is that Prdm14 is enough to induce PGCs from mouse ESCs *in vitro*, whereas it is not for inducing PGCs from human ESCs (Nakaki et al., 2013; Sybirna et al., 2020).

In PGCs, the BMP4-Smad1 signaling induces the expression of Prdm1 that directly activates the expression of Prdm14 (Ohinata et al., 2005; Okuzaki et al., 2019; Sybirna et al., 2020; Yamaji et al., 2008). In turn, Prdm14 regulates the expression of Prdm1 in a feedback loop, but they also act together with TFAP2C (encoding AP2γ) to regulate gene expression and repress the Wnt signaling (Magnúsdóttir et al., 2013; Nakaki et al., 2013; Sybirna et al., 2020; Yamaji et al., 2008). In chicken, the expression of *Prdm14* in PGCs is additionally depending on FGF2 signaling (Okuzaki et al., 2019).

In mouse, mutants for *Prdm14* cannot form PGCs and the resulting individuals are sterile (Ohinata et al., 2009; Yamaji et al., 2008). In human cells, the knock-down of *Prdm14* reduces the number of PGCs differentiating *in vitro* (Sybirna et al., 2020). Similar results are obtained in chicken; however, embryos die early, suggesting an essential role in the early development of chicken (Hagihara et al., 2020; Okuzaki et al., 2019).

In anamniote vertebrates, the expression of *Prdm14* has been detected in the gonad of a teleost fish, the Japanese flounder (*Paralichthys olivaceus*). The promoter of *Prdm14* contains binding motifs for Pou5f1, Sox2, Nanog, Smad1/5 and Prdm1, suggesting their role in regulating *Prdm14* expression. Therefore, *Prdm14* is likely involved in the specification of PGCs in teleost fish as well (Fan et al., 2015).

*Prdm14 as an epigenetic reprogramming factor*

Despite Prdm14 lacks an intrinsic HMT activity, it recruits several molecular partners carrying a histone modifying capacity. As explained above, two major partners of Prdm14 are TET and PRC2. Together, they regulate the epigenetic state of target genes to control their expression. Nevertheless, Prdm14 is also involved in epigenetic reprogramming at a much broader scale as it demethylates the genome and reactivate the second X chromosome (**Figure 32**) (Burton et al., 2013; Kamikawa and Donohoe, 2015; Magnúsdóttir et al., 2013; Nakaki et al., 2013; Payer et al., 2013; Yamaji et al., 2008).

In mouse, Prdm14 is involved in the global DNA demethylation of preimplantation embryos, PGCs and ESCs as mutants do not show any reduction of the H3K9me2 levels (Burton et al., 2013; Ficz et al., 2013; Habibi et al., 2013; Hackett et al., 2013; Magnúsdóttir et al., 2013; Nakaki et al., 2013; Yamaji et al., 2008). The PRC2-containing Prdm14 repressor complex induces a passive hypomethylation of the genome by repressing the expression of *Ehmt1* (euchromatic histone methyltransferase 1) and *Dnmt3a/b/l* (*de novo* methyltransferase a, b, and l) (Burton et al., 2013; Leitch et al., 2013; Yamaji et al., 2008; Yamaji et al., 2013). In parallel, Prdm14 interacts with the

HMT G9a to degrade Dnmt3a/b/l proteins via lysine methylation-dependent polyubiquitination (Sim et al., 2017). By contrast, the recruitment of TET and PRC2 by Prdm14 induces an increase in, respectively, the 5hmC and H3K27me3 levels across the genome (Okashita et al., 2014; Okashita et al., 2015; Yamaji et al., 2008).

Concomitantly, Prdm14 participates in the reactivation of the second X chromosome in female cells. Indeed, the Prdm14-PRC2 repressor complex directly binds to the promoter of the E3 ubiquitin ligase *Rnf12* and represses its expression (Payer et al., 2013). Rnf12 is promoting the expression of *Xist*, a long non-coding RNA involved in the X chromosome inactivation (Jonkers et al., 2009), hence Prdm14 is indirectly repressing *Xist*. Nonetheless, Prdm14 also directly binds to *Xist* intron 1 in a *Tsix* (*Xist* antisense RNA) -dependent manner to enhance *Xist* repression (Payer et al., 2013).

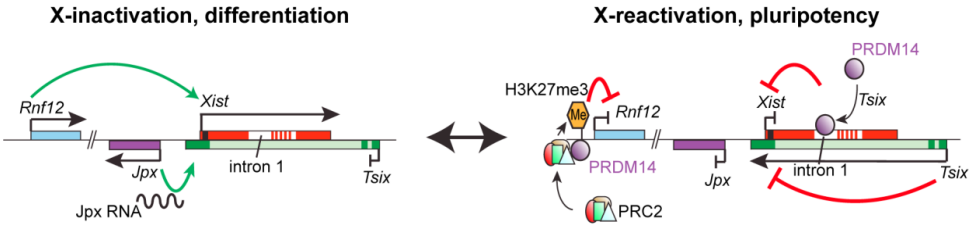



***Figure 32:* Model for the role of Prdm14 during X chromosome reactivation (XCR).** *In differentiated cells and before XCR, Prdm14 is absent, while Rnf12 and Jpx RNA activate Xist, leading to X chromosome inactivation. During XCR in pluripotent stem cells, Prdm14 is expressed and binds to the Rnf12 promoter. In turn, PRC2 is recruited and methylates H3K27, which leads to Rnf12 repression. Furthermore, Tsix is expressed and facilitates Prdm14 binding to Xist intron 1. The lack of Xist activators (Rnf12 and Jpx) and repressive effects of Tsix and Prdm14 on Xist lead to Xist repression, an important step for XCR. From* (Payer et al., 2013)

*Prdm14 is required for axon outgrowth*

As described in an earlier paragraph, *Prdm14* also plays a role in neurogenesis. Indeed, it is expressed in the pMN domain of the zebrafish spinal cord (**Figure 30**) where it is thought to be involved in the dorso-ventral patterning of the neural tube (Liu et al., 2012; Zannino and Sagerström, 2015).

In zebrafish, *Prdm14* is actually expressed in diverse neural structures, such as primary motoneurons (pMN), statoacoustic ganglion neurons, trigeminal neurons, olfactory sensory neurons, mid- and forebrain neurons, and reticulospinal neurons (Liu et al., 2012), suggesting a role in neurogenesis. *Prdm14* does not seem to be involved in early neurogenesis but rather in the control of axon outgrowth in caudal pMN (CaP) as mutants show shortened axons with abnormal branching and an impairment of embryonic movements (**Figure 33**) (Liu et al., 2012). In this context, Prdm14 does not exhibit any HMT activity, but it directly binds to the promoter of *Islet2* for promoting its expression and induce a proper axon outgrowth in CaP.

Similarly, *Prdm14* is expressed in the neural tube of amphioxus where it resembles the expression pattern of *Mnx*/*Hb9*, a motoneuron marker gene (Kawaguchi et al., 2019). Some of these *Prdm14* expressing cells co-express *Islet2*, suggesting that *Prdm14* is also involved in the generation of motoneurons in amphioxus. Additionally, it seems that the Prdm14-CBFA2T and Prdm14-TET complexes have been co-opted from motoneurons to pluripotent cells during vertebrate evolution. This shift in the *Prdm14* function is potentially due to the loss of pMN (secondary motoneurons still present) in amniote vertebrates (Kawaguchi et al., 2019). Furthermore, the expression of *Prdm14* has been detected in the nervous system of *Xenopus* embryos, reinforcing this hypothesis (Eguchi et al., 2015).

Moreover, *Prdm14* is expressed in the neural plate of the chicken embryo (Okuzaki et al., 2019), suggesting that Prdm14 might play different functions in amniote vertebrate development. Consistent with this idea, it has been shown that Prdm14 represses genes involved in neurogenesis in human ESCs (Chia et al., 2010; Sybirna et al., 2020), however it remains to determine whether *Prdm14* maintains the pluripotency of human

neural progenitors or simply represses differentiating genes in general, including neural ones.

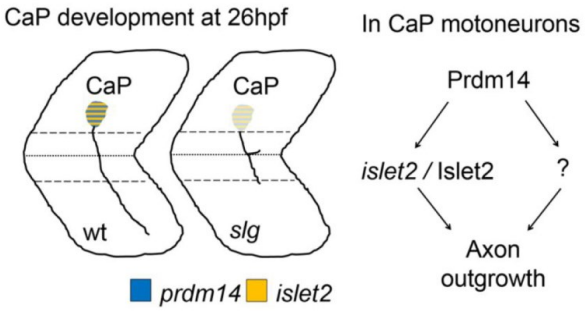



***Figure 33:* Model for the regulation of CaP axon outgrowth by Prdm14.** *Prdm14 regulates CaP axon outgrowth through Islet2 activation and yet unidentified factors (question mark). From* (Liu et al., 2012)

# RESULTS

## 1-*NvPrdm14d* identifies a population of non-ectodermal neural progenitor cells in *Nematostella vectensis* (Paper I)

### 1.1-Summary of results

The main aim of this thesis is to investigate the potential role of *NvPrdm14d* in *Nematostella* mesendodermal neurogenesis.

To this purpose, we first analyzed the spatial expression of *NvPrdm14d* by *in situ* hybridization (ISH). The expression of *NvPrdm14d* starts at early gastrula stage in few single ectodermal cells before being expressed in individual cells in the pharynx from mid-gastrula (**Figure 2A-E, Paper I**). From early planula stage, *NvPrdm14d* is additionally expressed in scattered mesendodermal cells and from mid-planula, it is mainly expressed in scattered mesendodermal and in some pharyngeal cells (**Figure 2D-H, Paper I**). In parallel, a small number of ectodermal cells express *NvPrdm14d* during gastrula and planula stages (**Figure 2B-H, Paper I**). This expression pattern in scattered cells coincides with the onset of the first neural progenitor cells (NPCs) in *Nematostella* mesendoderm, suggesting a potential role for *NvPrdm14d* in mesendodermal neurogenesis.

Next, we performed double fluorescent *in situ* hybridization of *NvPrdm14d* and *NvSoxB(2),* a marker for NPCs. It revealed that most of the *NvPrdm14d*$^{+}$ cells co-express *NvSoxB(2)*, while they only represent a small portion of *NvSoxB(2)*$^{+}$ cells (**Figure 3A-C, Paper I**). Moreover, some *NvPrdm14d*$^{+}$ cells incorporate EdU, indicating that some *NvPrdm14d*$^{+}$ cells are proliferating (**Figure 3D-G, Paper I**). Altogether, this shows that *NvPrdm14d* is expressed in a subset of mesendodermal NPCs.

To identify the type of neurons generated by the *NvPrdm14d*$^{+}$ NPCs, we generated a stable transgenic reporter line expressing a membrane-tethered GFP under the control of the *NvPrdm14d* promoter*,* referred to as *NvPrdm14d::GFP*. Transgenic animals

exhibit GFP expression in mesendodermal cells from early planula stage (**Figure 4, Paper I**). While some of these cells are round, divide and likely represent the *NvPrdm14d*$^{+}$ NPCs (**Figure 5A, Paper I**), other cells project neurites and correspond to the progeny of *NvPrdm14d*$^{+}$ NPCs (**Figure 4 & 5, Paper I**). These GFP$^{+}$ neurons label a portion of the mesendodermal nervous system (**Figure 4C-F, Paper I**) and most of these cells are, by their morphology, ganglion neurons showing a variable number of neurites (**Figure 5C-D, Paper I**). Together, it shows that the *NvPrdm14d* reporter labels a subset of the mesendodermal NPCs and their progeny. Thus, this transgenic line can be used to further characterize *NvPrdm14d*$^{+}$ cells.

We therefore generated double transgenic animals by crossing the *NvPrdm14d::GFP* line with other available reporter lines. We first crossed this line with the *NvElav1::mOrange* line labelling differentiated neurons in both the ectoderm and the mesendoderm (Nakanishi et al., 2012). Surprisingly, we did not detect any co-expression of the two reporters (**Figure 6A-F, Paper I**). However, *NvPrdm14d*::GFP$^{+}$ neurons seem entangled with *NvElav1*::mOrange$^{+}$ neurons (**Figure 6E-F, Paper I**). These double transgenic animals indicate that the *NvPrdm14d*::GFP and *NvElav1*::mOrange reporters label two distinct populations of neurons.

Next, we crossed the *NvPrdm14d::GFP* line with the *NvPOU4::mCherry* line labelling ectodermal and mesendodermal neurons, as well as cnidocytes (Tournière et al., 2020). In such double transgenic animals, the dividing *NvPrdm14d*::GFP$^{+}$ NPCs do not co-express *NvPOU4*::mCherry (**Figure 6H, Paper I**), while all differentiated *NvPrdm14d*::GFP$^{+}$ neurons co-express *NvPOU4*::mCherry (**Figure 6I & K-L, Paper I**). This fits with the role of *NvPOU4* in terminal differentiation of neurons (Tournière et al., 2020). However, the majority of mesendodermal neurons are *NvPrdm14d*::GFP$^{-}$. Thus, the *NvPrdm14d*$^{+}$ NPCs only generate a small subset of mesendodermal neurons.

To determine whether *NvPrdm14d* is involved in the development of motoneurons in *Nematostella,* similarly to the situation observed in zebrafish, we performed vibratome sectioning to visualize expression of the *NvPrdm14d*::GFP reporter in the mesenteries (**Figure 7, paper I**). This allowed the detection of *NvPrdm14d*::GFP$^{+}$ neurons in the

close vicinity of retractor muscles (**Figure 7B & D-F, Paper I**). When we crossed the *NvPrdm14d::GFP* line with the *NvMyHC1::homer-mCherry* line presumably labelling post-synaptic sites in the retractor muscles (Cole et al., 2020), we observed that the *NvPrdm14d*::GFP$^+$ neurons are very close to *NvMyHC1*::homer-mCherry$^+$ putative post-synaptic sites via their soma and neurites (**Figure 7G-J, Paper I**). Together, this data suggests that the *NvPrdm14d*::GFP reporter highlights a population of previously undescribed potential motoneurons in the vicinity of retractor muscles.

Additionally, we crossed the *NvPrdm14d::GFP* line with the *NvFoxA::mCherry* line labelling the ectodermal portion of the pharynx (P.R.H. Steinmetz, unpublished) to verify the mesendodermal origin of the *NvPrdm14d*$^+$ cells. We observed that *NvPrdm14d*::GFP$^+$ and *NvFoxA*::mCherry$^+$ cells are found in two distinct domains of the pharynx at planula stages (**Figure 8A-F, Paper I**) and in two different regions of the mesenteries in primary polyps (**Figure 8G-J, Paper I**). These observations confirm that mesendodermal *NvPrdm14d*$^+$ cells originate from the mesendoderm, including the mesendodermal region of the pharynx, and not from ectodermal pharyngeal cells.

To gain further insights into the characteristics of neurons derived from *NvPrdm14d*$^+$ NPCs, we determined the transcriptome of *NvPrdm14d*::GFP$^+$ cells by RNA sequencing. We found a total of 5,535 differentially expressed genes in the *NvPrdm14d*::GFP$^+$ cells, including 2,153 upregulated and 3,382 downregulated genes (**Figure 9A-D, Paper I**).

We decided to compare the *NvPrdm14d*::GFP$^+$ transcriptome with the previously generated transcriptome for *NvElav1*::mOrange$^+$ cells (Tournière et al., 2020). We found that about 50% of the genes upregulated in *NvPrdm14d*::GFP$^+$ cells are significantly upregulated in *NvElav1*::mOrange$^+$ cells (**Figure 9E-F, Paper I**). We also found *NvElav1* as a significantly upregulated gene in our transcriptome (**Table S1, Paper I**). This was a surprising result as the reporters for *NvPrdm14d* and *NvElav1* are not co-expressed (**Figure 6A-F, Paper I**). Altogether, these data suggest that the *NvElav1*::mOrange reporter does not label the entirety of *NvElav1*$^+$ cells, notably *NvPrdm14d*$^+$ NPC-derived neurons.

Similarly, we compared our transcriptome with the one of *NvPOU4* mutants [referred to as *NvPOU4*$^{-/-}$ (Tournière et al., 2020)], as we have found a co-expression of the *NvPrdm14d* and *NvPOU4* reporters (**Figure 6G-I, Paper I**). We found a very small, but significant, overlap between genes upregulated in *NvPrdm14d*::GFP$^{+}$ cells and those downregulated in *NvPOU4*$^{-/-}$ (**Figure 9E & G, Paper I**). Thus, *NvPOU4* might regulate aspects of the terminal differentiation of *NvPrdm14d*$^{+}$ neurons.

Next, we performed a GO term analysis to search for a molecular signature of motoneurons in the *NvPrdm14d*::GFP$^{+}$ cells. Despite the upregulation of *NvIslet* (a direct target of Prdm14 in zebrafish motoneurons) in the *NvPrdm14d*::GFP$^{+}$ cells, we did not find additional support for a potential motoneuron identity of *NvPrdm14d*::GFP$^{+}$ neurons (**Table S1 & Figure 9I, Paper I**).

Finally, we focused on transcription factors upregulated in *NvPrdm14d*::GFP$^{+}$ cells. In the literature, we found that some of these genes exhibit an expression pattern restricted to the mesendoderm, whereas some others are expressed in both the ectoderm and the mesendoderm. This finding suggests a role for these genes in mesendodermal neurogenesis. Moreover, we performed ISH for three transcription factors whose expression pattern have not been documented yet: *NvAtonal/neuroD*, *NvAshD* and *NvPIT1* (**Figure 9J, Paper I**). These genes are mainly expressed in scattered mesendodermal cells at planula stage, although they display expression in some ectodermal cells at gastrula stage, which resembles the *NvPrdm14d* expression pattern. Therefore, we identified a panel of genes potentially involved in the development of mesendodermal neurons, notably *NvAtonal/neuroD* whose expression might be restricted to the *NvPrdm14d* lineage (**Figure S5, Paper I**).

In conclusion, this study shows that *NvPrdm14d* identifies a previously undescribed population of mesendodermal neural progenitors whose progeny includes potential motoneurons.

## 1.2-Additional results: generation of new reporter lines labelling *NvPrdm14d*$^{+}$ neurons, and generation of *NvPrdm14d* mutants

### *1.2.1- Generation of reporter lines labelling pre-synaptic junctions of NvPrdm14d*$^{+}$ *neurons*

In paper I, we have shown that *NvPrdm14d*::GFP$^{+}$ neurons are in direct contact with the *NvMyHC1*::homer-mCherry$^{+}$ post-synaptic neuromuscular sites of the retractor muscles, suggesting that these neurons are potential motoneurons establishing synaptic connections with muscles. However, this is not sufficient to claim with certainty that such connections exist, especially that we failed to identify a molecular signature of motoneurons in our *NvPrdm14d*::GFP$^{+}$ transcriptome analysis. Therefore, we decided to investigate further the existence of such synaptic connections between the *NvPrdm14d*::GFP$^{+}$ neurons and retractor muscles.

To this purpose, I generated new genetic cassettes consisting of the pre-synaptic protein synaptotagmin1 (syt1), fused to either a mWasabi or mCherry fluorescent reporter, expressed under the control of the same 5 kb sequence from the regulatory region of *NvPrdm14d* that I used for the *NvPrdm14d::GFP* cassette (**Figure 34**). I started injecting these cassettes into fertilized eggs in order to generate new transgenic lines. Once generated, these lines will allow me to determine whether *NvPrdm14d*$^{+}$ neurons establish synaptic connections with retractor muscles.

On the one hand, I started to inject the *NvPrdm14d::syt1-mCherry* plasmid into *NvPrdm14d::GFP* fertilized eggs. I did not find any double transgenic embryo yet, but once generated, such animals will allow me to confirm that both syt1-mCherry and GFP are expressed in the same neurons, as well as to determine whether *NvPrdm14d*::GFP$^{+}$ neurons possess pre-synaptic sites in contact with retractor muscles. If this is the case, I expect to observe syt1-mCherry$^{+}$ dots on the GFP$^{+}$ neurons and in contact with the retractor muscles. This would look similar to what I observed when analyzing the *NvPrdm14d::GFP*; *NvMyHC1::homer-mCherry* double transgenic animals, except that it would label pre-synaptic instead of post-synaptic sites (**Figure 34**).

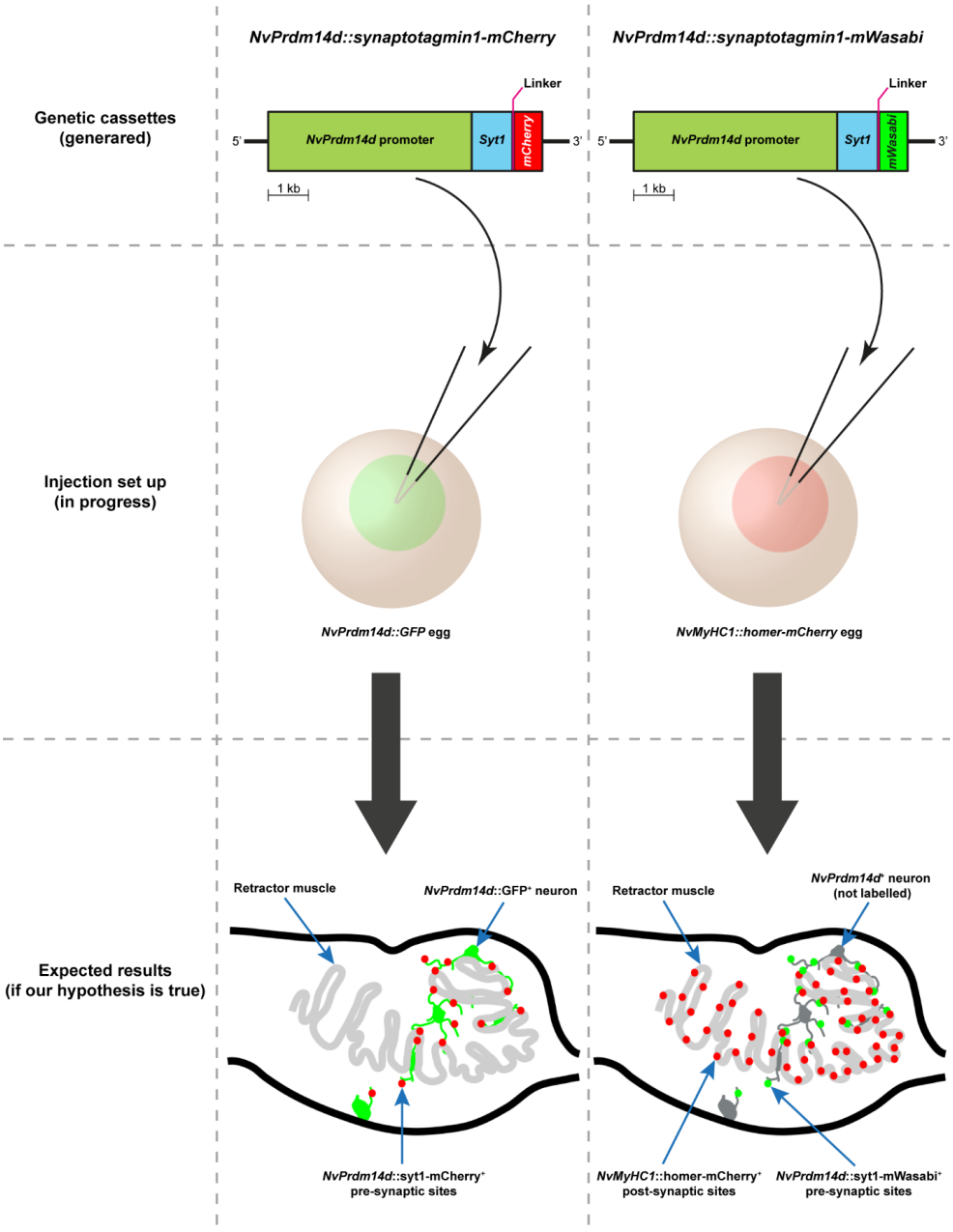



***Figure 34:*** ***Experimental design to generate NvPrdm14d::synaptotagmin1-(fluorescent protein) transgenic lines.*** *The methodology to generate new transgenic reporter lines is depicted here. These lines will allow the determination of whether NvPrdm14d+ neurons establish direct synaptic sites with the retractor muscles. The genetic cassettes have been generated and their injection is under progress. A schematic representation of expected results is shown and corresponds to what we could see if NvPrdm14d+ neurons are indeed connected to the retractor muscles. Syt1: synaptotagmin1*

In parallel, I also started to inject the *NvPrdm14d::syt1-mWasabi* plasmid into *NvMyHC1::homer-mCherry* fertilized eggs. I did not yet find any double transgenic embryo for this combination either, but such animals will allow me to see whether the pre-synaptic sites of *NvPrdm14d*$^{+}$ neurons are in close contact with post-synaptic sites of the retractor muscles. If this is the case, I expect to see mWasabi$^{+}$ and mCherry$^{+}$ pre- and post-synaptic sites overlapping or in close proximity along the retractor muscles (**Figure 34**).

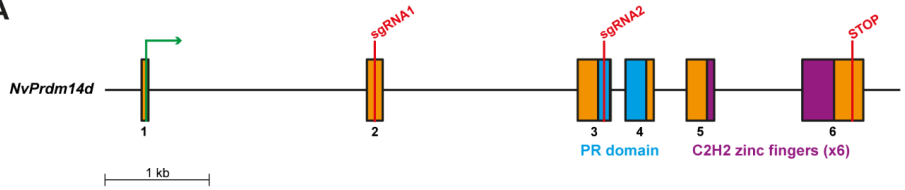
Altogether, the generation and analysis of such double transgenic animals could show that *NvPrdm14d*$^{+}$ neurons establish neuromuscular connections with retractor muscles, in the case where *NvPrdm14d*$^{+}$ neurons are indeed motoneurons.

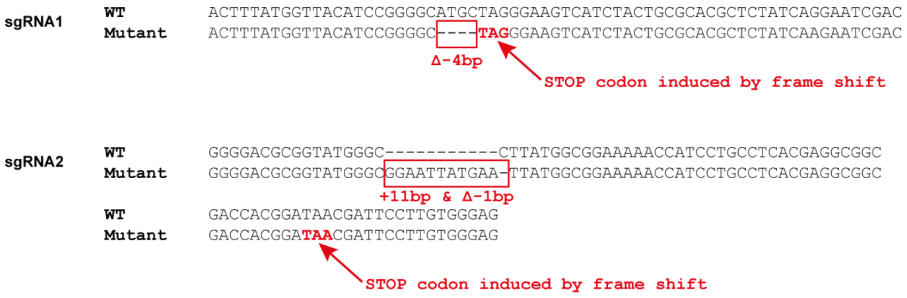
### *1.2.2- Generation of mutant lines for NvPrdm14d*

To further investigate the role of *NvPrdm14d* as well as the function of *NvPrdm14d* expressing neurons, we additionally decided to generate CRISPR/Cas9-mediated mutant lines.

To this goal, I synthetized two single guide RNAs (sgRNAs): the first one targeting the second exon to induce an early STOP codon, and the second one targeting the PR domain in exon 3 (**Figure 35A**). Upon the independent injection of these sgRNAs associated with the Cas9 protein in fertilized eggs, I could induce mutations in *NvPrdm14d* as attested by melting curve analysis. As F0 injected animals are likely mosaic for the mutation, I raised these animals to sexual maturity and crossed them with wild-type animals. Then, I sequenced the F1 heterozygous mutants to identify the type of mutation induced by each sgRNA. Among the different mutations induced by the first sgRNA (sgRNA1), I selected a deletion of 4 bp leading to a frame shift and a premature STOP codon immediately after the deletion (**Figure 35A**). Similarly, after injection of the second sgRNA (sgRNA2), I selected a mutation consisting in an insertion of 11 bp followed by a deletion of 1 bp, also leading to a frame shift. However, the premature STOP codon occurs 45 bp downstream of the mutation (**Figure 35A**).

A




B

**Juvenile polyps**

| Genotype | nb of embryos | Proportions |
|---|---|---|
| *NvPrdm14d*$^{WT}$ | 19 | 32.2% |
| *NvPrdm14d*$^{WT/-}$ | 40 | 67.8% |
| *NvPrdm14d*$^{-/-}$ | 0 | 0% |

C

**Primary polyps**

| Genotype | nb of embryos | Proportions |
|---|---|---|
| *NvPrdm14d*$^{WT}$ | 40 | 28.4% |
| *NvPrdm14d*$^{WT/-}$ | 98 | 69.5% |
| *NvPrdm14d*$^{-/-}$ | 3 | 2.1% |

D

**Early planula (48 hpf)**

| Genotype | nb of embryos | Proportions |
|---|---|---|
| *NvPrdm14d*$^{WT}$ | 7 | 23.3% |
| *NvPrdm14d*$^{WT/-}$ | 18 | 60% |
| *NvPrdm14d*$^{-/-}$ | 5 | 16.7% |

E

**Screened for 10 days**

| Genotype | nb of embryos | | Proportions | |
|---|---|---|---|---|
| *NvPrdm14d*$^{WT}$ | 2 | | 13.3% | + dead 11.1% |
| *NvPrdm14d*$^{WT/-}$ | 12 | | 80% | 66.7% |
| *NvPrdm14d*$^{-/-}$ | 1 | + dead 4 | 6.7% | 22.2% |

F

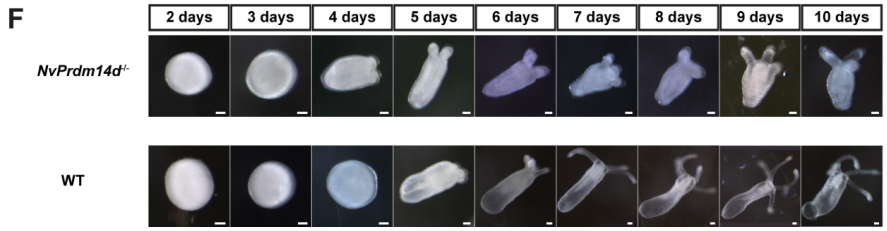


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**Figure 35: Generation and screening of NvPrdm14d mutants.** (**A**) Schematic representation of the NvPrdm14d gene. It shows the different protein domains and the binding sites of sgRNAs where the Cas9 cleaves the gene to induce mutations. The sgRNA1 induces a 4 bp deletion and an immediate downstream premature STOP codon. The sgRNA2 induces a 11 bp insertion and a 1 bp deletion as well as a premature STOP codon 45 bp downstream the mutation site. (**B**) Quantification of each genotype among F2 juvenile polyps derived from the intercross of animals injected with the sgRNA1. No homozygous mutant was found, suggesting that the lack of NvPrdm14d is lethal. (**C**) Quantification of each genotype among F2 primary polyps derived from the intercross of animals injected with the sgRNA1. Very few homozygous mutants are identified. (**D**) Quantification of each genotype among F2 early planula derived from the intercross of animals injected with the sgRNA1. A higher proportion of homozygous mutants are detected at this stage, but it is still lower than the Mendelian distribution. (**E**) Quantification of each genotype among F2 primary polyps derived from the intercross of animals injected with the sgRNA1. These animals were screened every day for 10 days. Counting dead animals as homozygous mutants almost allows to reach the Mendelian distribution, however the proportion of homozygous wild-type is incongruent. (**F**) Pictures of the unique homozygous NvPrdm14d mutants that survived over 10 days compared to a homozygous wild-type animal (same animals as in **E**).
*Scale bar: 50 µm*

←

As the animals derived from eggs injected with the sgRNA1 were available earlier and carry an early premature STOP codon, I started by screening this line for a phenotype. I intercrossed F1 heterozygous siblings (referred as *NvPrdm14d*$^{sg1/+}$) in order to get F2 homozygous mutants (referred as *NvPrdm14d*$^{sg1/sg1}$). First, I genotyped F2 animals at juvenile polyp stage (n=59, 1 month old) by sequencing to check the distribution of the different genotypes, However, I could not detect any homozygous mutant (**Figure 35B**). The proportion of heterozygous mutants and homozygous wild-type animals correspond to the Mendelian distribution when homozygous mutants do not survive. This suggests that the lack of *NvPrdm14d* is lethal. Therefore, I screened F2 animals at earlier stages to determine the time point when the homozygous mutants die. In primary polyps, only 2.1% of the animals were homozygous mutants (**Figure 35C**), while at early planula stage 16.7% were homozygous mutants (**Figure 35D**). Altogether, it indicates that homozygous *NvPrdm14d*$^{sg1/sg1}$ mutants die early in development.

In parallel, I followed 18 embryos and screened them every day for 10 days to determine the number of embryos dying during development. After 10 days, I sequenced the animals that survived and found 1 homozygous mutant (= 6.7%). Compared to other

survivors, this homozygous mutant showed morphological defects with shorter tentacles and body column as if completion of metamorphosis cannot be reached (**Figure 35E-F**). Within the 18 initial embryos, 3 of them died and if these animals are considered as homozygous mutants, we reach 22.2%, which is close to the Mendelian distribution (**Figure 35E**). However, I only identified 11.1% of homozygous wild-type animals meaning that these results are not significant. Moreover, two heterozygotes did not develop properly.

These results indicate that the mutation of *NvPrdm14d* induced by the sgRNA1 is lethal, hence that *NvPrdm14d* is required for a proper embryonic development, especially the completion of metamorphosis. However, these results are surprising as *NvPrdm14d*$^{+}$ neurons represent only a small population of mesendodermal neurons, including potential motoneurons (**Paper I**). Furthermore, there are several incongruencies within the screening results. This suggests that the observed lethality might be due to an off-target effect of the sgRNA1. Despite sgRNAs were blasted against the *Nematostella* genome and chosen among those having the smallest number of off-target sequences, the risk of obtaining off-targets still exists. Therefore, I will continue the screening with animals derived from the injection of the sgRNA2 to assess whether a similar phenotype is observed. Additionally, I could also cross F1 heterozygous mutants derived from the injection of both sgRNAs to remove of any off-target effects as those would be different for both sgRNAs, hence heterozygous in transheterozygous *NvPrdm14d*$^{sg1/sg2}$ mutants.

# 2-The expression patterns of *NvPrdm6d* and *NvPrdm13b* suggest roles in cnidogenesis in the cnidarian *Nematostella vectensis* (Paper II)

## 2.1-Summary of results

The second aim of this thesis is to document the spatial expression of *NvPrdm* genes and to assess their potential role in *Nematostella* development.

To this goal, we first searched for the temporal expression of the *NvPrdm* gene repertoire (apart from *NvPrdm14* paralogs that we characterized in **Paper I**) in the publicly available NvERTx database providing a developmental time course of gene expression in *Nematostella* (Warner et al., 2018). We saw that *NvPrdm6* paralogs are expressed during embryonic development with different levels and dynamics, except *NvPrdm6c* which is only expressed from the primary polyp stage (**Figure 1A, Paper II**). Similarly, *NvPrdm7/9*, *NvPrdm12* and *NvPrdm13* paralogs show a dynamic expression during embryonic development (**Figure 1B-D, Paper II**). This suggests that most *NvPrdm* genes potentially play a role in *Nematostella* embryonic development.

In order to characterize the spatial expression of these genes during developmental stages, we performed colorimetric *in situ* hybridization for each of these *NvPrdm* genes. However, we obtained clear and replicable expression patterns only for *NvPrdm6d* and *NvPrdm13b*.

From early gastrula stage, *NvPrdm6d* is expressed in scattered ectodermal cells (a "salt and pepper" pattern). It then starts to be additionally expressed in individual pharyngeal cells from mid-gastrula stage, and in a domain encircling the oral opening from early planula stage (**Figure 2A-F, Paper II**). In late planula and tentacle bud stage, *NvPrdm6d* is restricted to four domains around the oral opening corresponding to the four primary tentacle buds (**Figure 2G-H, Paper II**). The spatial expression in a "salt and pepper" pattern indicates that *NvPrdm6d* is potentially involved in neurogenesis, notably in the development of cnidocytes as suggested by the strong expression on the oral pole.

Similarly, we observed a “salt and pepper” expression pattern for *NvPrdm13b* from gastrula stage, an expression in individual pharyngeal cells from mid-gastrula stage and an expression encircling the oral opening from planula stage (**Figure 3, Paper II**). By contrast to *NvPrdm6d*, *NvPrdm13b* is expressed in scattered mesendodermal cells from early planula stage (**Figure 3D, Paper II**) and the ectodermal “salt and pepper” pattern persists until primary polyp stage (**Figure 3I, Paper II**). In the primary polyp, *NvPrdm13b* is mainly expressed in the tentacle tips (**Figure 3I, Paper II**). Altogether, these observations also suggest a role for *NvPrdm13b* in neurogenesis, notably in cnidogenesis.

In conclusion, this study provides new candidate genes for a role in the development of cnidocytes, the cnidarian-specific neural cell type, in the sea anemone *Nematostella vectensis*.

# DISCUSSION

The aim of the present PhD thesis was to investigate the molecular mechanisms underlying mesendodermal neurogenesis in *Nematostella vectensis*. To this purpose, I focused on characterizing the identity of mesendodermal cells expressing the transcription factor NvPrdm14d since it has been previously identified in a microarray experiment as potentially involved in mesendodermal neurogenesis (G.S. Richards, J. Blommaert and F. Rentzsch, unpublished). This study resulted in the identification of a previously undescribed subpopulation of mesendodermal neural progenitor cells whose progeny includes potential motoneurons (**Paper I**).

Since the role of genes belonging to the *Prdm* family has not been investigated in *Nematostella* before the work presented in this thesis, I additionally screened the spatial expression of the *NvPrdm* gene repertoire. This second study led to the identification of *NvPrdm6d* and *NvPrdm13b* as genes potentially involved in cnidogenesis (**Paper II**).

## 1-Mesendodermal neurogenesis in *Nematostella*

Despite the existence of mesendodermal neurogenesis in *Nematostella* has been demonstrated by transplantation experiments almost a decade ago (Nakanishi et al., 2012), no proper characterization of the molecular mechanisms underlying this process have been conducted. Thus, this particularly intriguing developmental process is currently poorly understood. However, previous studies have shown that ectodermal and mesendodermal neurogenesis in *Nematostella* are both regulated by similar transcription factors [*NvAth-like* and *NvSoxB(2)*] and signaling molecules (Notch) acting at the level of neural progenitor cells (Layden and Martindale, 2014; Richards and Rentzsch, 2014; Richards and Rentzsch, 2015). Similarly, the proneural gene *NvAshA* and the neural differentiation gene *NvDmrtB* regulate neural differentiation in both the ectoderm and the mesendoderm (Layden et al., 2012; Parlier et al., 2013). Although similarities have been detected between these two types of neurogenesis, some differences have also been

observed, such as the neuropeptide GLWamide mainly found in the mesendoderm and cnidocytes exclusively found in the ectoderm (Babonis and Martindale, 2017; Nakanishi and Martindale, 2018; Watanabe et al., 2014). Nevertheless, the absence of investigations specifically focusing on mesendodermal neurogenesis led to a lack of information about the degree of differences between ectodermal and mesendodermal neurogenesis.

### 1.1-*NvPrdm14d* identifies a population of mesendodermal NPCs

We have shown that the expression of *NvPrdm14d* is initially detectable in the pharynx, before expression in scattered cells throughout the mesendoderm becomes visible (**Figure 2, Paper I**). One explanation for these dynamics would be a migration of neural progenitors or precursors from the pharynx into the body wall mesendoderm. Since the pharynx consists of an ectodermal and mesendodermal part, the *NvPrdm14d*$^{+}$ neurons in the body wall mesendoderm could potentially be of ectodermal origin. However, we have made two observations arguing against this scenario. First, the expression of *NvPrdm14d* is predominantly found in the mesendodermal part of the pharynx (**Figure 3D, Paper I**) and second, the *NvPrdm14d*::GFP$^{+}$ cells in the body wall mesendoderm do not express *NvFoxA*::mCherry (**Figure 8, Paper I**), which labels the ectodermal pharynx and cells derived from it. This suggests that the *NvPrdm14d*$^{+}$ neurons are indeed of mesendodermal origin.

Additionally, we have shown that *NvPrdm14d* is expressed in *NvSoxB(2)*$^{+}$ proliferating neural progenitor cells (**Figure 3, Paper I**).

We have, therefore, demonstrated that the *NvPrdm14d*$^{+}$ neural progenitor cells have a mesendodermal origin and that at least some mesendodermal NPCs are molecularly distinct from ectodermal NPCs. Thus, the work presented in **Paper I** reinforces the idea of distinct molecular mechanisms driving ectodermal and mesendodermal neurogenesis in *Nematostella vectensis*.

### 1.2-Mesendodermal NPCs are heterogenous

The expression analysis reported in **Paper I** revealed that only a small portion of *NvSoxB(2)*$^{+}$ mesendodermal NPCs co-express *NvPrdm14d* (**Figure 3A, Paper I**). It suggests that mesendodermal NPCs form an heterogenous population of cells and that aspects specific to mesendodermal neurogenesis are not restricted to the post-mitotic differentiation of individual neural cell types. This is further supported by the analysis of the *NvPrdm14d::GFP* reporter line labelling a small fraction of the mesendodermal nervous system, compared to the *NvElav1::mOrange* and *NvPOU4::mCherry* lines seemingly labelling most mesendodermal neurons (**Figure 6, Paper I**). Taken together, our data suggest that the mesendodermal nervous system is generated by several populations of molecularly distinct neural progenitors.

This heterogeneity of mesendodermal NPCs opens new directions for pursuing the investigation of mesendodermal neurogenesis in *Nematostella*. Indeed, it would be of particular interest to identify the molecular markers characterizing those molecularly distinct populations of mesendodermal NPCs. Furthermore, the presence of heterogenous NPCs is not incompatible with the existence of a potential molecular marker shared by all mesendodermal neural progenitors, but not with ectodermal NPCs. The existence of such marker would facilitate the identification of a gene regulatory network allowing mesendodermal cells to acquire a neural potential. Subsequently, it would permit the comparison between mechanisms leading to the acquisition of this neural potential in both ectodermal and mesendodermal cells. Such comparison might unveil whether these processes are shared between NPCs originating from different germ layers.

So far, it is not clear whether the early *NvSoxB(2)*$^{+}$ NPCs form a homogenous population of multipotent progenitors, whose developmental potential is progressively restricted, or whether they form a heterogenous population of unipotent progenitors (Rentzsch et al., 2016). Through the analysis of the *NvPrdm14d::GFP* reporter line, we have shown that the *NvPrdm14d*$^{+}$ NPCs were giving rise to different types of neurons (**Figure 5, Paper I**), suggesting that mesendodermal NPCs might form a heterogenous population of multipotent progenitors and that they are molecularly distinct from the ectodermal

population. Regarding ectodermal NPCs, a population of unipotent *NvFoxQ2d*-expressing progenitors has been previously identified (Busengdal and Rentzsch, 2017). These *NvFoxQ2d*$^{+}$ NPCs are derived from *NvSoxB(2)*$^{+}$ NPCs and both genes show a temporal overlap. Since these cells appear to divide only once, they are considered as resulting from the restriction of the developmental potential occurring during the progression towards the production of neurons. However, it remains unclear whether the early ectodermal *NvSoxB(2)*$^{+}$ NPCs form a homogenous or heterogenous population. It would be particularly interesting to further investigate this aspect to determine whether ectodermal NPCs could form a heterogenous population, similarly to mesendodermal NPCs. Nonetheless, if such heterogeneity cannot be identified, it could mean that one difference between ectodermal and mesendodermal neurogenesis, in *Nematostella,* is the nature of the neural progenitors involved in these processes.

## 2-Perspectives on the evolution of non-ectodermal neurogenesis

### 2.1-Comparing ectodermal and non-ectodermal neurogenesis

In the present thesis, I have described different cases of non-ectodermal neurogenesis spread across metazoans (see **Introduction** chapter **2.2** page 41 and section **3.4.3** page 93, and **Results** part **1** page 113). However, it is not well understood to what extend non-ectodermal neurogenesis differs from ectodermal neurogenesis within a given species and whether there are specific similarities in the regulation of non-ectodermal neurogenesis in different taxa. Such comparisons are difficult because these cases come from distant species, each study focused on different aspects and each case of non-ectodermal neurogenesis is currently poorly characterized. Nevertheless, some similarities and differences can already be identified.

In the different cases mentioned here, the initiation of non-ectodermal neurogenesis occurs at a later stage than ectodermal neurogenesis. Indeed, the vertebrate NMps produce neural progenitors during secondary neurulation, and the pharyngeal

neurons of both *C. elegans* and the sea urchin originate later than the first ectodermal neurons (Henrique et al., 2015; Sulston et al., 1983; Wei et al., 2011). Similarly, the first mesendodermal neurons in *Nematostella* are observed at planula stage compared to ectodermal neurons starting to differentiate at gastrula stage (Nakanishi et al., 2012). In line with these observations, the data reported in **Paper I** showed that no *NvPrdm14d*::GFP$^{+}$ neuron could be detected in gastrulae (**Figure 4, Paper I**).

Moreover, these different non-ectodermal neurons often originate from a population of bipotent progenitors. Indeed, the vertebrate NMps produce both neural and mesodermal progenitors, the mesodermal pharyngeal neurons of *C. elegans* are produced by progenitors also producing muscle cells, and neurons derived from i-cells in *Hydra* are presumably generated by an intermediate progenitor producing gland cells as well (Henrique et al., 2015; Luo and Horvitz, 2017; Siebert et al., 2019). Moreover, it has been shown that the fate decision performed in several of those bipotent progenitors requires signaling pathways such as Wnt or Notch. In *Nematostella*, a bipotency of mesendodermal NPCs has not been described yet. However, the Wnt signaling is present and is involved in the oral-aboral patterning of the planula larva (Marlow et al., 2013; Watanabe et al., 2014), hence it could be involved in the fate decision of the bipotent mesendodermal NPCs. Nevertheless, the existence of such bipotent progenitors and of a heterogeneity of NPCs along the oral-aboral axis have not been demonstrated and thus remains speculative.

By contrast, the source of tissue from which non-ectodermal neurons originate is different and those neurons contribute to a diverse range of derivatives. Indeed, the vertebrate NMps contribute to the posterior CNS, the hydrozoan i-cells produce most neurons and *Nematostella* mesendodermal NPCs potentially generate a population of motoneurons (**Figure 7, Paper I**).

Similarly, both ectodermal and non-ectodermal neurogenesis appear to follow the same molecular program. For example, *SoxB* genes have been shown to be involved in ectodermal and non-ectodermal neurogenesis in the sea urchin, *Nematostella* and mice,

suggesting a general role in neurogenesis (Bylund et al., 2003; Garner et al., 2016; Garriock et al., 2015; Graham et al., 2003; McClay et al., 2018; Richards and Rentzsch, 2014; Richards and Rentzsch, 2015; Tsakiridis et al., 2014; Wei et al., 2011). However, the identification of *NvPrdm14d* as a gene involved almost exclusively in mesendodermal neurogenesis in *Nematostella* provides an example for germ-layer specific differences in neurogenesis (**Paper I**). While this observation is consistent with other cases of non-ectodermal neurogenesis, such as in the mesodermal pharyngeal neurons of *C. elegans* and the endodermal foregut neurons of the sea urchin (Luo and Horvitz, 2017; Wei et al., 2011), I am not aware of conserved regulators that would be specific for non-ectodermal neurogenesis. The role of *Prdm14* in zebrafish motoneuron development (Liu et al., 2012) shows that its function is not limited to non-ectodermal neurogenesis. However, the role of *Prdm14* in neurogenesis has been poorly described in general and it will be interesting to determine whether this gene is involved in non-ectodermal neurogenesis in other organisms.

Generally speaking, differences in the regulation of ectodermal and non-ectodermal neurogenesis could be due to the embryonic origin of the neurons or to the neural cell types that are produced. Broader investigations of non-ectodermal neurogenesis in cnidarians and bilaterians are, therefore, required to better understand the evolution of this process. The work presented in **Paper I** provided a panel of candidate genes with a role potentially restricted to mesendodermal neurogenesis in *Nematostella* and it may serve as a resource for such studies.

## 2.2-Relation between non-ectodermal nervous systems and enteric nervous systems

The comparison of the different cases of non-ectodermal neurogenesis led to the identification of an intriguing similarity between the potential role of the neurons generated through this process. Indeed, the mesendodermal neurons of *C. elegans* are part of the pharyngeal nervous system, the endodermal neurons of the sea urchin are located in the foregut and the mesendodermal neurons of *Nematostella* are found in the gastrodermis. Therefore, it appears that most cases of non-ectodermal neurogenesis produce neurons acting in close relationship with the digestive/gastric system of the respective species. This particularity reminds the enteric nervous system (ENS) of vertebrates.

The vertebrate ENS is a network composed of more than 100 million neurons subdivided into at least 18 subtypes (Brookes, 2001; Schemann, 2005), and it innervates the gastrointestinal tract to regulate the gastrointestinal functions, such as contraction, fluid exchange, blood flow and gut hormone release (Sasselli et al., 2012). It also communicates with the intestinal epithelial cells and the immune system to regulate the physiological responses of the gut (Rao and Gershon, 2018). The ENS represents the largest and the most complex portion of the peripheral nervous system. Moreover, it is autonomous as it does not require any input from the CNS to regulate enteric behaviors. Nevertheless, both the ENS and the CNS are interconnected and communicate with each other (Furness et al., 2014; Gershon, 1999; Rao and Gershon, 2018). From a developmental point of view, the ENS has an ectodermal origin as it is derived from vagal (at the level of the postotic hindbrain adjacent to somites 1-7) and sacral (adjacent to somites 28-33) neural crest cells (Nagy and Goldstein, 2017).

The enteric neurons and glial cells are distributed in two intramuscular plexuses: the myenteric plexus located between the outer longitudinal and inner circular muscle layers, and the submucosal plexus adjacent to the mucosal layer (**Figure 36A & B**). Enteric neurons are divided into four main classes that are themselves divided in different subtypes depending on whether they are excitatory or inhibitory, and based on

the neurotransmitters they use. Enteric motoneurons innervate the circular smooth muscles and the longitudinal muscles to control the contraction pattern of the gut, including peristaltic contractions, and induce a directive flow. Enteric interneurons act in synergy with the motoneurons to regulate and coordinate the contraction patterns. Intestinofugal neurons connect the different enteric ganglia along the gastrointestinal tract to the CNS and other organs involved in feeding and in the nutrient homeostasis. The intrinsic primary afferent neurons (IPAN) are sensory cells innervating the mucosal villi of the gut to detect mechanical and chemical stimuli originating from the gut lumen. These IPANs transmit the signals to effector cells via enteric interneurons (Hao et al., 2016; Nagy and Goldstein, 2017; Rao and Gershon, 2018; Sasselli et al., 2012).

Interestingly, cross-sections of the vertebrate gut and of *Nematostella* body column show resembling structures (**Figure 36**). Both the vertebrate gut and *Nematostella* gastrodermis exhibit folds (villi and mesenteries, respectively) and are associated with circular (in both species) and longitudinal muscles (parietal and retractor muscles are longitudinal in *Nematostella*). Similar to the vertebrate gut, *Nematostella* body column undergoes waves of peristaltic contractions and expels food residues after digestion. Moreover, the existence of potential motoneurons revealed by the *NvPrdm14d::*GFP expression in the vicinity of retractor muscles (**Figure 7, Paper I**) suggests that part of *Nematostella* mesendodermal nervous system contains motoneurons. Although we have shown potential synaptic connections between *NvPrdm14d*::GFP$^{+}$ neurons and retractor muscles only, *NvPrdm14d*::GFP$^{+}$ neurons in the body wall are located in proximity of the circular and parietal muscles (**Figure 7, Paper I**). All the *NvPrdm14d*::GFP$^{+}$ neurons could therefore be motoneurons regulating the contraction of the different muscles. In line with these observations, we could speculate that the mesendodermal nervous system in *Nematostella* may play a similar role as the vertebrate enteric nervous system.

Furthermore, the ENS is shaped as a nerve net wrapped around the gut and is thought to have potentially been the first autonomous integrative neural circuit to evolve in bilaterians before and independently from the CNS (Furness and Stebbing, 2018). Knowing that the first nervous system was likely shaped as a diffuse nerve net (see **Introduction** chapter **1.3** page 26), it is probable that the appearance of the first

digestive tract has been accompanied by a recruitment of a portion of the nervous system to regulate its activity. This would imply that a portion of the nervous system was already allocated to the control of digestive functions in the last common ancestor of eumetazoans. This ancestral nervous system would have later evolved to give the enteric nervous system and the mesendodermal nervous system in vertebrates and *Nematostella*, respectively.

Strikingly, a recent study revealed that a subpopulation of the mouse enteric neurons have an endodermal origin (Brokhman et al., 2019). This new data reinforces the apparent homology between *Nematostella* mesendodermal nervous system and the vertebrate ENS as both systems are playing similar functions and have, at least in part, a non-ectodermal origin. It is possible that the ancestral ENS had an endodermal origin but that the centralization of the ectodermal nervous system together with the appearance of neural crest cells led to a switch of tissue producing the ENS.

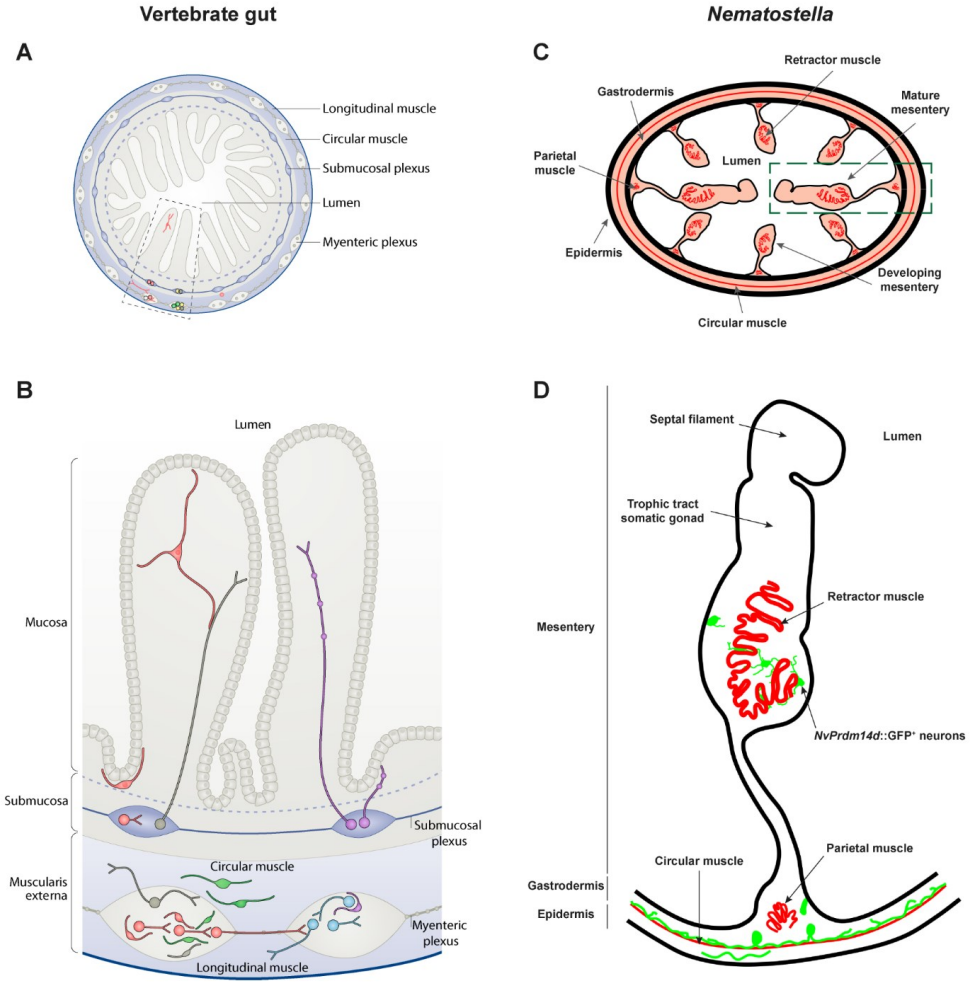


***Figure 36:*** ***Comparison between the vertebrate enteric nervous system and Nematostella mesendodermal nervous system.*** *(**A**) Schematics representing a cross-section of the vertebrate gut. It illustrates the villi in the lumen, the circular and longitudinal muscles, and the different plexuses composing the ENS. (**B**) The boxed area in (**A**) is expanded to illustrate better the organization of the ENS. Enteric neurons encircle the gut and connect to both circular and longitudinal muscles. They also innervate the villi. (**C**) Schematics representing a cross-section of Nematostella body column. It illustrates the mesenteries in the lumen, the circular muscle and the two types of longitudinal muscles (parietal and retractor). (**D**) The boxed area in (**C**) is expanded to illustrate better the organization of the mesendodermal nervous system. Mesendodermal NvPrdm14d::GFP⁺ neurons (green) project neurites towards the retractor muscles. They are also found in close proximity of the parietal and circular muscles.*

*When comparing the vertebrate ENS organization and Nematostella mesendodermal organization, some similarities are apparent.*

*(**A & B**) are from* (Rao and Gershon, 2018)

## 2.3-Non-ectodermal neurogenesis, an innovation or a common feature?

Despite there is only a small number of non-ectodermal neurogenesis cases that have been described so far, they are distributed across the metazoan phylogenetic tree, suggesting that this process might be more common than initially thought (**Figure 9**). Furthermore, the different observations of non-ectodermal neurogenesis have been mainly made in easily accessible, simple, and transparent organisms (apart from vertebrate's NMps), suggesting that more cases may be described in the future with the advance of experimental approaches. In line with this hypothesis, recent advances in cell lineage tracing methods allowed the identification of the aforementioned subpopulation of enteric neurons originating from the endoderm in mouse (Brokhman et al., 2019).

The fact that non-ectodermal neurogenesis exists in addition to ectodermal neurogenesis in diverse species among eumetazoans, suggests that both processes might have been present in the last common ancestor of eumetazoans. Subsequently, it could have been used as the main source of neural cells as observed in some cnidarians (*e.g.* hydrozoans) and mainly lost in bilaterian lineages, apart from few exceptions (*e.g. C. elegans*, sea urchins and vertebrates). Alternatively, non-ectodermal neurogenesis could still exist in most eumetazoans and it might have been missed as the CNS is mainly of ectodermal origin. In the latter case, the smaller contribution of non-ectodermal neurons might have led them to be more difficult to detect in the main laboratory model organisms possessing a complex and centralized nervous system, such as vertebrates and *Drosophila*.

Lastly, investigating non-ectodermal neurogenesis in diverse species will provide a better understanding of the process initiating neurogenesis from various cellular origins.

## 3-*NvPrdm14d* reveals previously undescribed putative motoneurons

Cnidarian neural cells are classically divided in three classes: sensory cells, ganglion cells and cnidocytes. In jellyfish, sensory neurons have been shown to establish synapses with effector cells, *i.e.* myoepithelial cells and cnidocytes. Similarly, sensory neurons have been observed in association with smooth muscle fibers in sea anemones. Together, it suggests that some sensory neurons behave as sensory-motoneurons (Galliot et al., 2009). However, a proper description of neurons controlling motor functions in cnidarians is currently lacking.

In the sea anemone *Nematostella vectensis*, some ganglion neurons are suspected to innervate muscles (Rentzsch et al., 2016). In **Paper I**, we have reported *NvPrdm14d*::GFP$^+$ neurons in the close vicinity of retractor muscles (**Figure 7, Paper I**). Moreover, these neurons are in contact with putative post-synaptic sites of the retractor muscles (**Figure 7, Paper I**) and they express *NvIslet* (**Table S1, Paper I**), a direct target gene of Prdm14 in zebrafish motoneurons (Liu et al., 2012). Together, these data suggest that *NvPrdm14d*$^+$ NPCs generate a population of motoneurons in *Nematostella*. We have shown that most *NvPrdm14d*::GFP$^+$ neurons are ganglion cells, including neurons in close proximity of the retractor muscles (**Figure 5 & 7, Paper I**), indicating that these potential motoneurons are not sensory-motor cells. Thus, it suggests that some neurons fulfilling motor functions in *Nematostella* could be "pure" motoneurons, while some others would be sensory-motoneurons.

Furthermore, we did not observe any connection between the *NvPrdm14d*::GFP$^+$ cells in the vicinity of retractor muscles with the *NvPrdm14d*::GFP$^+$ neurons of the body wall (**Figure 7, Paper I**), suggesting that *NvPrdm14d*$^+$ neurons from the body wall and the mesenteries are not directly connected to each other. It is possible that such connections between *NvPrdm14d*::GFP$^+$ neurons are made at a different level along the oral-aboral axis of the animal and that we have missed them in the sections that we analyzed. Despite the lack of direct evidence, retractor muscles are thought to respond to external stimuli (*e.g.* to touch). In order to respond to such stimuli and induce muscle contraction,

the *NvPrdm14d*::GFP$^{+}$ neurons in the vicinity of retractor muscles need to receive signals from sensory stimulation. Since these neurons do not project neurites towards neurons of the body wall or towards the surface of the mesenteries, we suspect that some sensory neurons from either the body wall, the mesenteries or both, establish direct or indirect synapses with *NvPrdm14d*::GFP$^{+}$ neurons. However, such neurons remain to be identified as the *NvElav1*::mOrange reporter did not allow their visualization (**Figure S4D, Paper I**).

The work presented in **Paper I** might, therefore, represent the first step toward the identification of a population of neurons exclusively fulfilling motor functions in *Nematostella*. Similarly, a very recent study has identified two populations of neurons inducing body contraction in *Hydra* (Noro et al., 2020), hence further investigations of these neurons would allow the determination of whether those cells are sensory-motor or exclusively motor cells in another cnidarian species.

# 4-The role of *NvPrdm* genes in *Nematostella* neurogenesis

## 4.1-*NvPrdm14* paralogs

### *4.1.1-The role of NvPrdm14 paralogs in different neural populations*

In **Paper I**, we have screened the spatial expression of the four *NvPrdm14* paralogs, but we only obtained an informative expression pattern for *NvPrdm14d* (**Figure 2 & S2, Paper I**). The expression of *NvPrdm14d* in scattered individual cells in the mesendoderm and in the pharynx, suggested a role in neurogenesis. Initially, *NvPrdm14d* was identified in the microarray experiment as potentially involved in mesendodermal neurogenesis (G.S. Richards, J. Blommaert and F. Rentzsch, unpublished). Later, it has been identified as one of the genes defining two neuronal metacells (35 & 36) in the whole-organism single cell transcriptome atlas (Sebé-Pedrós et al., 2018). Moreover, *NvPrdm14d* is detected in the transcriptome of two neural populations: *NvSoxB(2)*::mOrange$^{+}$ (neural progenitor cells and their progeny; J.M. Gahan, I.U. Kouzel and F. Rentzsch, unpublished) and *NvNCol3*::mOrange2$^{+}$

[cnidocytes, (Gahan et al., 2020)]. By investigating in more details the role of this gene, mainly via the generation and the use of the *NvPrdm14d::GFP* reporter line, we were able to confirm the role of *NvPrdm14d* in neurogenesis as it is involved in the generation of a subpopulation of mesendodermal neurons, including potential motoneurons (**Paper I**). However, the analysis of the *NvPrdm14d::GFP* reporter line did not bring additional support in favor of a role in cnidocyte as suggested by the detection of *NvPrdm14d* in the *NvNCol3*::mOrange2$^{+}$ transcriptome (**Table 1**). It is, therefore, possible that the presence of *NvPrdm14d* in the cnidocyte transcriptome is artifactual, but it could also mean that the *NvPrdm14d::GFP* transgene does not label the entirety of the *NvPrdm14d*$^{+}$ NPCs and their progeny, notably cnidocytes.

Similar to what we did for *NvPrdm14d* in **Paper I**, we took advantage of available resources in search of potential hints about the role of the other *NvPrdm14* paralogs.

First, we have explored these resources to collect information about *NvPrdm14a*. In the NvERTx gene expression database (Warner et al., 2018), we saw that this gene is not expressed during *Nematostella* embryonic development. According to this temporal expression, we were not able to detect a spatial expression for this gene by colorimetric *in situ* hybridization (ISH; **Figure S2, Paper I**). This did not allow us to emit any hypothesis about the potential role of *NvPrdm14a*. However, this gene is detected in the transcriptome of *NvPrdm14d*::GFP$^{+}$ (**Paper I**), *NvSoxB(2)*::mOrange$^{+}$ (J.M. Gahan, I.U. Kouzel and F. Rentzsch, unpublished), *NvElav1*::mOrange$^{+}$ (Tournière et al., 2020) and *NvNCol3*::mOrange2$^{+}$ (Gahan et al., 2020) cells (**Table 1**). Additionally, we saw that *NvPrdm14a* is downregulated in mutants for *NvPOU4*, a terminal selector gene in the nervous system of *Nematostella* (Tournière et al., 2020). Altogether, the combination of data provided by these different resources suggests that *NvPrdm14a* plays a role in neurons and cnidocytes, potentially in the late stages of their differentiation.

Next, we have sought for *NvPrdm14b* within these different databases. The NvERTx database shows that *NvPrdm14b* is highly expressed during developmental stages, however we detected a weak expression in the pharynx at early and mid-gastrula stage, and a broad expression in the mesendoderm from late gastrula to tentacle bud stage (**Figure S2, Paper I**). This spatial expression differs from the temporal expression and

is not informative about the potential role of *NvPrdm14b*. Nevertheless, we saw that *NvPrdm14b* is detected in the *NvElav1*::mOrange$^{+}$ transcriptome, suggesting a role for this gene in neurons (**Table 1**).

Finally, we have looked for *NvPrdm14c* and despite it is more highly expressed at early embryonic stages than at planula stage, we did not detect any signal by ISH in gastrula and early planula stage, but a weak broad expression in the mesendoderm from mid-planula to tentacle bud stage (**Figure S2, Paper I**). Albeit it does not provide any insight about the potential role of *NvPrdm14c*, this gene is detected in the *NvElav1*::mOrange$^{+}$ transcriptome, as well as in the neuronal metacell 34 from the whole-organism single cell transcriptome atlas (**Table 1**). This suggests that *NvPrdm14c* may also play a role in the nervous system.

Altogether, the data collected from different resources for neural cells in *Nematostella* indicate that all the *NvPrdm14* paralogs apparently play a role in neural cells. Since *Prdm14* plays a role in neural development in zebrafish (Liu et al., 2012) and that the Prdm14 complex is thought to have been recruited from neurons to pluripotent cells during vertebrate evolution (Kawaguchi et al., 2019), these data suggest that *Prdm14* might have played a role in neurogenesis in the last common ancestor of both cnidarians and bilaterians. Moreover, the expression of *Prdm14* has been detected in the developing nervous system of *Xenopus* and chicken (Eguchi et al., 2015; Okuzaki et al., 2019). It would therefore be informative to investigate the role of this gene during neural development in these species. Furthermore, investigating the role of *Prdm14* in protostomes would provide a more complete evolutionary perspective on its potential ancestral role.

### *4.1.2-Perspectives on the evolution of the role of Prdm14*

Despite the *Prdm14* gene is present in a variable number of copies across eumetazoans, the function of this gene has been only studied in species carrying a single *Prdm14* gene. While *Nematostella* possesses four *NvPrdm14* paralogs, the anthozoan *Acropora* only possesses one *Prdm14* gene and the hydrozoan *Hydra* does not possess any. It suggests that this gene underwent several duplication events specifically in *Nematostella*'s lineage (Vervoort et al., 2015).

In mammals, the single *Prdm14* is involved in the induction and the maintenance of pluripotency (Chia et al., 2010; Yamaji et al., 2008). Since *NvPrdm14d* is expressed in neural progenitor cells (**Figure 3, Paper I**), it could play a role in the maintenance of the progenitor state of these cells. However, only a subpopulation of mesendodermal NPCs express *NvPrdm14d*, hence it is more relevant to think that *NvPrdm14d* is involved in the specification of a neural lineage rather than in the induction and maintenance of the progenitor state. Nevertheless, *NvPrdm14d* could maintain those specific NPCs in a proliferative state while binding differentiation genes to keep them in a poised state, similar to the situation observed with *SoxB1* genes in the vertebrate neural plate (Bergsland et al., 2011; Wegner, 2011). Investigating the target genes of *NvPrdm14d* in *Nematostella* would surely help decipher these hypotheses.

More broadly, this single *Prdm14* gene is also involved in the specification of primordial germ cells in vertebrates (Yabuta et al., 2006; Yamaji et al., 2008). Despite the detection of *NvPrdm14b* in the *NvElav1*::mOrange$^{+}$ transcriptome (**Table 1**) suggesting a role in neurogenesis, this gene seems to be broadly expressed in the mesendoderm (**Figure S2, Paper I**). This spatial expression pattern resembles those of *NvNanos2*, *NvPiwi1*, *NvPiwi2*, and *NvVasa2* (Extavour et al., 2008; Praher et al., 2017), genes that are assumed to play a role in stem cells and/or in PGCs. Thus, the *NvPrdm14b* paralog might appear as a candidate potentially involved in such cells in *Nematostella*.

Another role described for *Prdm14* in mouse is the induction of a genome-wide epigenetic reprogramming (Yamaji et al., 2008; Yamaji et al., 2013). The knowledge

about the precise molecular role of *NvPrdm14* paralogs is still missing, hence we cannot emit any hypothesis on this aspect.

Lastly, *Prdm14* has been shown to play a role in the axon outgrowth of caudal primary motoneurons in zebrafish (Liu et al., 2012). The localization of *NvPrdm14d*::GFP$^{+}$ neurons in the close vicinity of retractor muscles, their contact with putative post-synaptic sites of the retractor muscles and the expression of *NvIslet* (a direct target of Prdm14 in zebrafish motoneurons), indicate that *NvPrdm14d* is also involved in the generation of motoneurons in *Nematostella* (**Figure 7 & Table S1, Paper I**). Furthermore, the expression of *Prdm14* has been detected in the developing nervous system of both *Xenopus* and chicken (Eguchi et al., 2015; Okuzaki et al., 2019), indicating that a role for *Prdm14* in neurogenesis is conserved between vertebrates and *Nematostella*.

Since all *NvPrdm14* paralogs seem to play a role in the nervous system (**Table 1**) and the single vertebrate ortholog is also expressed in the nervous system (Eguchi et al., 2015; Kawaguchi et al., 2019; Liu et al., 2012; Okuzaki et al., 2019), it suggests that the ancestral *Prdm14* gene played a role in the nervous system already in the last common ancestor of eumetazoans. However, a function for this ancestral gene in pluripotency, PGCs specification and epigenetic reprogramming is not certain. It has been postulated that *Prdm14* has been recruited from motoneurons to pluripotent cells during vertebrate evolution (Kawaguchi et al., 2019). In line with this idea, none of the *Nematostella NvPrdm14* paralogs appear to be involved in such processes, hence these functions might be vertebrate-specific innovations. In *Nematostella*, the duplication of the ancestral *NvPrdm14* gene into four paralogs might have allowed a diversification of its role in specific neural populations, such as *NvPrdm14a* that have apparently been recruited to the cnidocyte lineage (**Table 1**). A better understanding of the evolutionary history of the *Prdm14* function would be obtained by investigating the role of this gene in a larger sample of species, notably in those carrying several *Prdm14* paralogs in their genome, *e.g.* the annelid *Capitella teleta*, the mollusk *Crassostrea gigas* and the hemichordate *Saccogliossus kowalevskii*.

## 4.2-*NvPrdm6d* and *NvPrdm13b*

### *4.2.1-General role in neurogenesis*

In **Paper II**, we have characterized the spatial expression of *NvPrdm6d* and *NvPrdm13b* by ISH. Both genes are expressed in a "salt and pepper" pattern typically observed for neural genes. Moreover, they are expressed in a domain encircling the oral opening at gastrula stage and in the primary tentacle buds (**Figure 2 & 3, Paper II**). Therefore, this suggests that these genes are potentially involved in *Nematostella* neurogenesis, notably in cnidogenesis, the generation of cnidocytes, a cnidarian-specific neural type.

These conclusions are confirmed by the different available databases that we previously used for the *NvPrdm14* paralogs. Indeed, both *NvPrdm6d* and *NvPrdm13b* have been identified in the microarray experiment (G.S. Richards, J. Blommaert and F. Rentzsch, unpublished), initially suggesting their potential role in neurogenesis (**Table 1**). Moreover, both genes are detected in the *NvNCol3*::mOrange2$^{+}$ transcriptome (Gahan et al., 2020), which further support their role in the development of cnidocytes. The potential role for *NvPrdm13b* in cnidogenesis is additionally reinforced by the whole-organism single cell transcriptome atlas (Sebé-Pedrós et al., 2018), as it is one of the genes defining the cnidocyte metacell 8 (**Table 1**). By contrast to *NvPrdm13b*, *NvPrdm6d* is upregulated in the *NvPOU4* mutants (Tournière et al., 2020). It indicates that *NvPOU4* may negatively regulate the expression of *NvPrdm6d* and suggests that *NvPrdm6d* might play a role in the early stages of cnidocyte development. This matches with the observations we have made in **Paper II** based on the expression pattern of *NvPrdm6d*.

Furthermore, we have observed that *NvPrdm13b* is additionally expressed in some scattered mesendodermal cells (**Figure 3, Paper II**), while such expression could not be detected for *NvPrdm6d*. This difference suggests that the expression of *NvPrdm13b* may not be restricted to cnidocytes. In line with this observation, we found *NvPrdm13b* in the *NvSoxB(2)*::mOrange$^{+}$ (J.M. Gahan, I.U. Kouzel and F. Rentzsch, unpublished), *NvElav1*::mOrange$^{+}$ (Tournière et al., 2020), *NvFoxQ2d*::mOrange$^{+}$ (population of

sensory neurons, J.M. Gahan, I.U. Kouzel and F. Rentzsch, unpublished) and *NvPrdm14d*::GFP$^{+}$ (**Paper I**) transcriptomes (**Table 1**). Moreover, *NvPrdm13b* is one of the genes defining the neuronal metacell 4 in the whole-organism single cell transcriptome atlas. Taken together, the detection of *NvPrdm13b* in these different transcriptomes suggests that this gene may play a role in a diverse group of neurons, including sensory neurons, ectodermal and mesendodermal neurons in addition to cnidocytes.

Despite a proper investigation of both *NvPrdm6d* and *NvPrdm13b* is required to confirm these potential roles, we already collected a substantial set of evidence in favor of a function for these genes in *Nematostella* neurogenesis, including cnidogenesis.

### *4.2.2-Hypothesis on their role in cnidogenesis*

Our observations in **Paper II** and the detection of both *NvPrdm6d* and *NvPrdm13b* in the cnidocyte transcriptome, provide strong evidence for a potential role of these genes in cnidogenesis. However, we have noticed some differences. For example, *NvPrdm13b* seems to be expressed in a higher number of ectodermal cells than *NvPrdm6d,* with this expression of *NvPrdm13b* persisting until primary polyp stage, whereas this expression of *NvPrdm6d* ceases in mid-planulae (**Figure 2 & 3, Paper II**). This difference combined to the fact that *NvPrdm6d* is upregulated in *NvPOU4* mutants and that *NvPrdm13b* is one of the genes defining the cnidocyte metacell 8, suggest that these two genes are likely involved in different aspects of cnidogenesis.

Indeed, both genes might play a role at different timepoints during the development of cnidocytes. Since *NvPrdm6d* is potentially negatively regulated by *NvPOU4* (see previous section **4.2.1** page 142), it could play a role in early steps of cnidocyte formation and it might occur before the action of *NvPrdm13b* is required. Similarly, the role of *NvPrdm6d* could be restricted to early steps, while *NvPrdm13b* could be involved in more steps, hence still be expressed after the expression of *NvPrdm6d* stops.

Moreover, the detection of *NvPrdm13b* in the cnidocyte metacell 8 suggests that this gene might still be expressed in some differentiated cnidocytes, hence reinforce the idea that *NvPrdm13b* may play a role at later stages of cnidocyte formation than *NvPrdm6d*.

Alternatively, both genes could be involved in the development of two subpopulations of cnidocytes, which may overlap or not. Indeed, *Nematostella* possesses three types of cnidocytes characterized by distinct cnidocysts: the basitrichous haplonema (also called isorhiza), the microbasic mastigophores and the spirocytes. These different types of cnidocytes can be easily distinguished by their distinct morphologies. Moreover, these three populations are detected in different proportions that vary during the embryonic development but also along the oral-aboral axis of the adult polyp (Zenkert et al., 2011). Based on these data combined with the expression patterns of these two genes (**Figure 2 & 3, Paper II**), we could speculate that *NvPrdm6d* might be involved in the development of spirocytes mainly found in the head region (including tentacles), while *NvPrdm13b* might be involved in the development of mastigophores present in higher numbers than spirocytes at embryonic stages, and in both the head region and body column of the polyp. By contrast, the number of isorhiza is important on the polyp body column. From our observations, the spatial expression of both *NvPrdm6d* and *NvPrdm13b* does not fit with a role in the development of this class of cnidocytes. Indeed, *NvNCol3* is expressed in most cnidocytes and this gene exhibits a much broader expression pattern than either *NvPrdm6d* or *NvPrdm13b*, hence these genes are not pan-cnidocyte markers in *Nematostella* (Babonis and Martindale, 2017; Richards and Rentzsch, 2014; Richards and Rentzsch, 2015; Zenkert et al., 2011).

### 4.3-Expression of other *NvPrdm* genes in the nervous system

In parallel of characterizing the spatial expression of both *NvPrdm6d* and *NvPrdm13b*, we additionally screened the spatial expression of the remaining *NvPrdm* genes by ISH. However, we have not obtained clear and replicable expression patterns for any of these genes. Nonetheless, we have shown in **Paper II** that all the other *NvPrdm* genes are expressed during *Nematostella* embryonic development, apart from *NvPrdm6c* whose expression starts in the primary polyp (**Figure 1, Paper II**).

Similar to what we have done for the genes studied in **Paper I** and **Paper II**, I have sought for the remaining *NvPrdm* genes in the available databases.

First, I have sought for these genes in the data from the microarray (G.S. Richards, J. Blommaert and F. Rentzsch, unpublished) and have found that both *NvPrdm12* and *NvPrdm13a* are detected, hence potentially involved in neurogenesis (**Table 1**). Additionally, I have seen that both genes are among those defining some neuronal metacells from the whole-organism single cell transcriptome atlas (Sebé-Pedrós et al., 2018), with *NvPrdm12* in metacells 1 and 37, and *NvPrdm13a* in the metacell 13. These genes are also detected in the transcriptome of several neural populations (**Table 1**). *NvPrdm12* is detected in all of these transcriptomes (*i.e* *NvSoxB(2)*$^{+}$, *NvElav1*$^{+}$, *NvFoxQ2d*$^{+}$, *NvNCol3*$^{+}$ and *NvPrdm14d*$^{+}$ cell populations), suggesting a broad function for this gene in the nervous system. By contrast, *NvPrdm13a* is only detected in the *NvSoxB(2)*::mOrange$^{+}$ transcriptome (J.M. Gahan, I.U. Kouzel and F. Rentzsch, unpublished), also suggesting a role for this gene in neurogenesis.

Regarding the *NvPrdm6* paralogs (excluding *NvPrdm6d* discussed in the section **4.2** page 142), I have seen that none of them are detected in the microarray experiment or in the whole-organism single cell transcriptome atlas. Nevertheless, they are detected in the transcriptome of different neural populations (**Table 1**). Both *NvPrdm6a* and *NvPrdm6e* are found in the *NvPrdm14*::GFP$^{+}$ transcriptome (**Paper I**), suggesting a role in at least a subpopulation of mesendodermal neurons. As *NvPrdm6e* is also present in the *NvElav1*::mOrange$^{+}$ transcriptome (Tournière et al., 2020), it could be involved in a larger number of neurons. Similarly, *NvPrdm6a* is found in the *NvElav1*::mOrange$^{+}$

transcriptome but also in the *NvNCol3*::mOrange2$^{+}$ transcriptome (Gahan et al., 2020), suggesting a role in cnidocytes. By contrast, both *NvPrdm6b* and *NvPrdm6c* have been identified in the *NvNCol3*::mOrange2$^{+}$ transcriptome but not in any other differentiated neural cell population (**Table 1**). It suggests that these two genes might have a role restricted to cnidocytes. Additionally, I have seen that none of the *NvPrdm6* paralogs are detected in the *NvFoxQ2d*::mOrange$^{+}$ transcriptome (J.M. Gahan, I.U. Kouzel and F. Rentzsch, unpublished), indicating that, so far, there is no evidence in favor of a role for these genes in sensory neurons.

Finally, I have noticed that *NvPrdm7/9* is absent from all the aforementioned databases (**Table 1**). Therefore, there is currently no evidence of a role for this gene in neurogenesis.

Taken together, the search for the *NvPrdm* gene repertoire of *Nematostella* in the different available databases indicates that all *NvPrdm* genes, apart from *NvPrdm7/9*, play a role in the nervous system. Since these genes are expressed during embryonic stages, except *NvPrdm6c* whose expression starts in the primary polyp, it further suggests a role in the nervous system development. It makes the *Prdm* gene family a good candidate to further investigate neurogenesis in *Nematostella* and better understand this process in this cnidarian model.

***Table 1:* Detection of each of the NvPrdm genes in available transcriptomic data.** *The green color indicates where each gene is detected. For the transcriptome of NvPOU4 mutants, it is indicated whether the gene is up- or downregulated. The ID of clusters from the single-cell atlas is indicated when genes are detected.*

| *NvPrdm* genes | Transcriptomes | | | | | | Single-cell clusters | | | | Micro-array |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *NvSoxB(2)*$^{+}$ | *NvElav1*$^{+}$ | *NvFoxQ2d*$^{+}$ | *NvNCol3*$^{+}$ | *NvPrdm14d*$^{+}$ | *NvPOU4*$^{-/-}$ | Neuronal | Cnidocyte | Muscle Gastrodermis | Gland Secretory | |
| ***NvPrdm6a*** (NVE1591, v1g112691) | | | | | | | | | | | |
| ***NvPrdm6b*** (NVE9894, v1g200651) | | | | | | | | | | | |
| ***NvPrdm6c*** (NVE21126, v1g54602) | | | | | | | | | | | |
| ***NvPrdm6d*** (NVE25286, v1g84066) | | | | | | UP | | | | | |
| ***NvPrdm6e*** (NVE1588, v1g41788) | | | | | | | | | | | |
| ***NvPrdm7/9*** (NVE2255, v1g113856) | | | | | | | | | | | |
| ***NvPrdm12*** (NVE5655, v1g119550) | | | | | | | C1 & C37 | | C29 | | |
| ***NvPrdm13a*** (NVE1665, v1g40616) | | | | | | | C13 | | | | |
| ***NvPrdm13b*** (NVE7535, v1g66221) | | | | | | | C4 | C8 | | | |
| ***NvPrdm14a*** (NVE22869, v1g104327) | | | | | | DOWN | | | | | |
| ***NvPrdm14b*** (NVE19092, v1g197426) | | | | | | | | | | | |
| ***NvPrdm14c*** (NVE9426, v1g61034) | | | | | | | C34 | | | | |
| ***NvPrdm14d*** (NVE17327, v1g96522) | | | | | | | C35 & C36 | | | | |

# 5-Future directions

## 5.1-Further investigations of *NvPrdm14d*

The identification of a subpopulation of mesendodermal neural progenitor cells expressing *NvPrdm14d* described in **Paper I** represents the first investigation of the molecular mechanisms specifically underlying mesendodermal neurogenesis in *Nematostella*. However, these data do not provide information about the function of *NvPrdm14d* in these cells, hence further investigations are required to better understand the role of this gene in mesendodermal neural progenitor cells.

As mentioned in the result section (see **Results** section **1.2.1** page 117), two new reporter transgenic lines labelling the pre-synaptic sites of *NvPrdm14d*$^{+}$ neurons are currently in the generation process. Once generated, these two additional reporter lines, differing by their fluorescent reporter, can be combined with the already existing reporter lines used in **Paper I**, *i.e. NvPrdm14d::GFP* and *NvMyHC1::homer-mCherry*. This will demonstrate whether *NvPrdm14d*$^{+}$ neurons indeed establish direct synaptic connections with retractor muscles.

Similarly, heterozygous *NvPrdm14d* mutant animals (F1) derived from the independent injection of two distinct sgRNAs are available (see **Results** section **1.2.2** page 119). However, the screening of animals derived from the injection of the first sgRNA did not provide significant outcomes. Indeed, the loss of *NvPrdm14d* function seems to be lethal from early embryonic stages in F2 homozygous mutants, while the small number and the localization of *NvPrdm14d*::GFP$^{+}$ neurons does not suggest such strong effect (**Paper I**). As the observed lethality of homozygous mutants could be triggered by off-target effects of the sgRNA1-Cas9 complex, it is required to screen the phenotype of homozygous mutants derived from the injection of the second sgRNA. If this second set of animals exhibits a different genotype distribution than the first one and an absence of lethality, those animals will be used for searching a phenotype specifically induced by the *NvPrdm14d* mutation. This screen could be initially done by following embryos

every day and genotyping them after 10 days in order to determine whether homozygous mutants can be linked to any morphological defect. Additionally, the mutant line could be crossed to the *NvPrdm14d::GFP* reporter line to check whether the presence, the localization or the morphology of *NvPrdm14d*::GFP$^{+}$ neurons are affected by the mutation of the gene. Ideally, it would also be interesting to test whether homozygous mutants display a retractor muscle contraction defect, as we suspect some *NvPrdm14d*::GFP$^{+}$ cells to be motoneurons (**Paper I**).

In the eventuality that homozygous mutants derived from the injection of the second sgRNA exhibit an early lethality, as observed for those derived from the injection of the first sgRNA, we would have to investigate the potential motoneuron identity of the *NvPrdm14d*::GFP$^{+}$ neurons via a different set of tools.

First, we could use the nitroreductase cell ablation system to specifically kill neurons derived from *NvPrdm14d*$^{+}$ NPCs. The nitroreductase is a bacterial enzyme converting the non-toxic prodrug nifurpirinol into a cytotoxic metabolite. Expressed under the control of a tissue or cell type-specific promoter, the nitroreductase is used as a cell ablation system to kill a specific cell population upon exposure to nifurpirinol (Bergemann et al., 2018; Curado et al., 2008). Thus, we could use this system to generate a new transgenic line expressing the nitroreductase, fused to a fluorescent protein, under the *NvPrdm14d* promoter. With such line, we would be able to specifically remove all the neurons derived from *NvPrdm14d*$^{+}$ NPCs and test whether transgenic animals show contraction defects upon metrodinazole exposure. The advantage of this method is that nifurpirinol is non-toxic and that it can be directly added to the *Nematostella* medium. As animals are relatively small, the prodrug will rapidly and efficiently reach any cell type.

Alternatively, we could use optogenetic tools to either activate or silence neurons derived from *NvPrdm14d*$^{+}$ NPCs. Optogenetics is the use of light-activated proteins to study cell function. The algal channelrhodopsin-2 is the most used protein for activating neurons. Cell expressing this cation channel will emit an action potential upon exposure to blue light (Britt et al., 2012; Cardin et al., 2010). We could, therefore, generate a transgenic line expressing the channelrhodopsin-2 under the *NvPrdm14d* promoter and

subsequently specifically activate these neurons to see whether their activation induces muscle contraction in *Nematostella*. In complement, we could also use a transgenic line expressing the halorhodposin under the *NvPrdm14d* promoter. By contrast to the channelrhodopsin, the halorhodopsin induces a hyperpolarization suppressing any action potential, hence inhibiting neuronal activity (Mattingly et al., 2018; Zhang et al., 2019). The use of halorhodopsin would allow the inhibition of neurons derived from *NvPrdm14d*$^{+}$ NPCs and the determination of whether it affects muscle contraction. *Nematostella* is a suitable model organism to perform optogenetics as it is a small and transparent animal, hence the light stimulus can be directly applied to the animals without requesting any surgery.

Together, studying this supplemental set of transgenic and mutant lines would allow us to confirm or reject the hypothesis of a potential motoneuron identity of some *NvPrdm14d*::GFP$^{+}$ neurons.

The characterization of the *NvPrdm14d::GFP* line reported in **Paper I** suggested that the terminal selector gene *NvPOU4* may regulate part of the terminal differentiation of *NvPrdm14d*::GFP$^{+}$ neurons (**Figure 6 & 9, Paper I**). Since a mutant line for *NvPOU4* has been previously generated (Tournière et al., 2020), it would be interesting to cross those mutants with the *NvPrdm14d::GFP* line and determine to what extend the loss of *NvPOU4* affects the development of *NvPrdm14d*::GFP$^{+}$ neurons. Indeed, *NvPOU4* is expressed in a large portion of *Nematostella* nervous system, including ectodermal and mesendodermal neurons, as well as cnidocytes. However, a role for this gene in the terminal differentiation could only be shown for cnidocytes (Tournière et al., 2020). Compared to the *NvElav1::mOrange* line used to screen for a phenotype caused by the loss of *NvPOU4* in neurons, the *NvPrdm14d::GFP* line only labels a small portion of mesendodermal neurons. Thus, this line is a good candidate to test whether *NvPOU4* also regulates the terminal differentiation of neurons, and more specifically of mesendodermal neurons. Furthermore, if a defect in the development of *NvPrdm14d*::GFP$^{+}$ neurons is observed in *NvPOU4* mutants, it would indicate that there

are at least some common regulators of terminal differentiation in both ectodermal and mesendodermal neurogenesis.

Lastly, the analysis of the *NvPrdm14d*::GFP$^{+}$ transcriptome provided a panel a genes potentially involved in mesendodermal neurogenesis. Performing double fluorescent *in situ* hybridization (DFISH) of these genes together with *NvPrdm14d* would determine whether they are expressed at the same time than *NvPrdm14d*. Combining these genes with other genes known to play a role at different steps of neural development would also inform about their respective role. Similarly, these genes could be combined with each other. Together, it would provide a time course of the expression of genes potentially involved in the *NvPrdm14d*$^{+}$ mesendodermal neuronal lineage.

Within this panel of genes, there is one of particular interest: *NvAtonal/neuroD*. Indeed, the ISH for this gene suggested that it might be exclusively expressed in a subset of mesendodermal *NvPrdm14d*$^{+}$ cells (**Figure 9 & S5, Paper I**). It would, therefore, be very interesting to further investigate the role of *NvAtonal/neuroD* by DFISH, nuclear EdU staining, and the generation of both reporter and mutant lines.

### 5.2-Description and functional characterization of *NvPrdm6d* and *NvPrdm13b*

The temporal and spatial expression of *NvPrdm6d* and *NvPrdm13b* provided in **Paper II** offers a basis for further investigations of the function of these genes in *Nematostella* cnidogenesis. Indeed, a deeper characterization is required to strongly associate these genes in such process.

First, the expression analysis of both genes could be completed through DFISH. Performing such assay would allow the comparison of the respective expression of these two genes with other genes involved in neurogenesis, such as the early neurogenic marker *NvSoxB(2)* expressed in NPCs (Richards and Rentzsch, 2014; Richards and Rentzsch, 2015), the differentiated neurons marker *NvElav1* (Nakanishi et al., 2012), or genes involved in differentiating and differentiated cnidocytes, such as *NvDcx*

(doublecortin; J. Kraus and F. Rentzsch, unpublished), *NvNCol3* (Zenkert et al., 2011) and *NvPOU4* (Tournière et al., 2020). This would allow the determination of the expression timing of *NvPrdm6d* and *NvPrdm13b* during neurogenesis as well as indicating at what steps both genes are required. Additionally, the comparison of the expression of these two genes combined by DFISH would also inform about whether they act at the same time and possibly, in the same cells. Moreover, the combination of FISH and nuclear EdU staining would tell whether these genes are involved in proliferating or post-mitotic cells.

In addition, the generation of transgenic reporter lines for each of these two genes would provide more insights about the identity of the cells produced by either *NvPrdm6d* or *NvPrdm13b* expressing cells. Indeed, the expression of a membrane-tethered fluorescent protein under the respective regulatory region of these genes would allow the direct observation of the location, diversity, and morphology of the *NvPrdm6d*$^{+}$ or *NvPrdm13b*$^{+}$ cells. This would, hopefully, confirm the expression of these genes in the cnidocyte lineages. Crossing these lines with the existing *NvNCol3*::mOrange2 transgenic reporter line (Sunagar et al., 2018) would permit the estimation of the cnidocyte proportion specified by these genes. Moreover, these transgenic lines could be intercrossed to check whether both genes specify two distinct cnidocyte subpopulations or whether some overlap exist. Furthermore, these transgenic lines could be used to respectively sort *NvPrdm6d*$^{+}$ and *NvPrdm13b*$^{+}$ cells for performing light as well as electron microscopy, specifically on these cells. This would allow the verification of whether these genes are indeed involved in the development of, respectively, spirocytes and mastigophores. If this is the case, generating the transcriptome of both cell populations would provide a very informative resource.

Finally, the generation of CRISPR/Cas9-mediated mutant lines for *NvPrdm6d* and *NvPrdm13b* would allow us to functionally test their respective role in cnidogenesis. Indeed, if mutants exhibit a reduced number of cnidocytes, it will confirm their role in cnidogenesis. It would be very intriguing to check whether each mutant lacks a specific type of cnidocytes, but this expectation would depend on previous observations made with the experimental design described above. Another phenotype in line with a role for

these genes in cnidogenesis would be a lack of mature cnidocytes. Indeed, cnidocytes could still be generated but the cnidocysts could fail to mature as described in the *NvPOU4* mutants (Tournière et al., 2020). This eventuality can be tested by specifically labelling the cnidocysts with a *NvNCol3* antibody (Zenkert et al., 2011) or with a DAPI/EDTA staining as described by (Babonis and Martindale, 2017; Szczepanek et al., 2002). For both genes, we would expect mutants to develop properly until primary polyp. However, if *NvPrdm6d* is indeed involved in the development of spirocytes that are mainly found on the tentacles, we would expect these mutants to die shortly after, due to the lack of the feeding ability. By contrast, we would expect *NvPrdm13b* mutants to feed and grow properly if this gene is indeed involved in the development of mastigophores as they are mainly found on the body column.

Altogether, these future directions would provide new insights about the development of cnidocytes, a cnidarian-specific novel cell type, whose development is currently poorly documented in anthozoans.

# ANNEXES

**Manuscript of paper I:** *NvPrdm14d* identifies a population of non-ectodermal neural progenitor cells in *Nematostella vectensis* (in preparation)

**Manuscript of paper II:** The expression patterns of *NvPrdm6d* and *NvPrdm13b* suggest roles in cnidogenesis in the cnidarian *Nematostella vectensis* (in preparation)

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