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ABSTRACT

New perspectives on the origin of neurogenesis emerged with the identification of genes encoding post-synaptic proteins as well as many "neurogenic" regulators as the NK, Six, Pax, bHLH proteins in the Demosponge genome, a species that might differentiate sensory cells but no neurons. However, poriferans seem to miss some key regulators of the neurogenic circuitry as the Hox/paraHox and Otx-like gene families. Moreover as a general feature, many gene families encoding evolutionarily-conserved signaling proteins and transcription factors were submitted to a wave of gene duplication in the last common eumetazoan ancestor, after Porifera divergence. In contrast gene duplications in the last common bilaterian ancestor, Urbilateria, are limited, except for the bHLH Atonal-class. Hence Cnidaria share with Bilateria a large number of genetic tools. The expression and functional analyses currently available suggest a neurogenic function for numerous orthologs in developing or adult cnidarians where neurogenesis takes place continuously. As an example, in the *Hydra* polyp, the *Clytia* medusa and the *Acropora* coral, the *Gsx/cnox2* ParaHox gene likely supports neurogenesis. Also neurons and nematocytes (mechano-sensory cells) share in hydrozoans a common stem cell and several regulatory genes indicating that they can be considered as sister cells. Performed in anthozoan and medusozoan species, these studies should tell us more about the way(s) evolution hazards achieved the transition from epithelial to neuronal cell fate, and about the robustness of the genetic circuitry that allowed neuro-muscular transmission to arise and be maintained across evolution.

INTRODUCTION

Urbilateria and its older sisters Cnidaria and Ctenophora

In 1978, Ed Lewis in his seminal Nature paper (Lewis, 1978) predicted the evolutionary conservation of DNA-binding regulatory proteins that would control patterning along the anterior-posterior axis through cis-regulatory elements. Since then, the accumulation of molecular and genetic data indeed proved the wide conservation of the genetic networks regulating shared developmental processes among bilaterians, not only for the specification of the anterior to posterior axis but also the dorso-ventral axis, the head patterning and the eye

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specification (De Robertis, 2008). As anticipated, the main cellular differentiation processes in bilaterians also make use of evolutionarily-conserved genetic circuitries as those used for myogenesis (Yun and Wold, 1996), neurogenesis (Bertrand et al., 2002; Acampora et al., 2005; Denes et al., 2007; Tessmar-Raible et al., 2007), gametogenesis (Cox et al., 1998). Since 1991, orthologs of these bilaterian regulatory genes were identified not only in cnidarians (see below) but also in poriferans (Larroux et al., 2006; Larroux et al., 2008) and some could even be traced in choanoflagellates (King et al., 2008).

The zootype hypothesis proposed first that a same set of regulatory genes, namely homeobox genes, define the anterior to posterior (AP) axis in all animal species at an early and transient developmental stage (Slack et al., 1993). Subsequently the Urbilateria hypothesis proposed that, beside the AP axis, deuterostomes and protostomes also received from a common putative ancestor, named Urbilateria a genetic toolkit that specifies their dorso-ventral axis, including their neural tube (De Robertis, 2008). In the absence of extant Urbilaterian species, the Ctenophora and Cnidaria that diverged earlier in animal evolution but display anatomical polarities and differentiate a nervous system, are obvious candidates to test these hypotheses (Figure 1). In fact the initial expression analyses performed at the cellular level supported the hypothesis of a common origin for neurogenesis and also for the specification of the apical nervous system in cnidarians and anterior nervous system in bilaterians (Gauchat et al., 1998; Galliot and Miller, 2000). However this simple rule of the universal conservation of developmental genetic toolkits between animal phyla received some assault when it appeared that the zootype hypothesis could not be verified in cnidarians (Gauchat et al., 2000; Schierwater and Desalle, 2001; Chourrout et al., 2006; Kamm et al., 2006; Lee et al., 2006; Ryan et al., 2007; Chiori et al., 2009; Quiquand et al., 2009), and it is nowadays admitted that the specification of the embryonic AP axis by the Hox gene families only arose after Cnidaria divergence.

However what is true for the AP axis might not be true for the specification of the nervous systems. Alain Ghysen wrote about the *Origin and Evolution of the Nervous System*: "*The extreme variability of behaviors and survival strategies among triploblasts would be subordinate on the previous attainment by the urbilaterians of a high level of developmental stability in the building of elementary functional circuits. According to this view, the initial triploblast radiation may have been contingent upon reaching this highly evolved stage of neural development*" (Ghysen, 2003). In other words, the neurogenic circuitry was already established in a very stable way in Urbilateria (Arendt et al., 2008), suggesting that it might be possible to trace back some features of this ancestral nervous system in cnidarians that differentiate a rather sophisticated nervous system with numerous cellular and functional similarities to bilaterian ones. In bilaterians, homologous tasks such as differentiating nerve cells (Simionato et al., 2008) and mechanosensory organs (Ghysen, 2003), developing eyes (Pichaud and Desplan, 2002; Gehring, 2004), regionalizing the neural tube along the dorsoventral axis (Denes et al., 2007; Mieko Mizutani and Bier, 2008) or patterning the tripartite brain (Lichtneckert and Reichert, 2005) rely on a shared set of transcription factors. We propose here to review the current knowledge about the molecular mechanisms that support neurogenesis in cnidarians and discuss some scenario that led to this unique evolutionary transition.
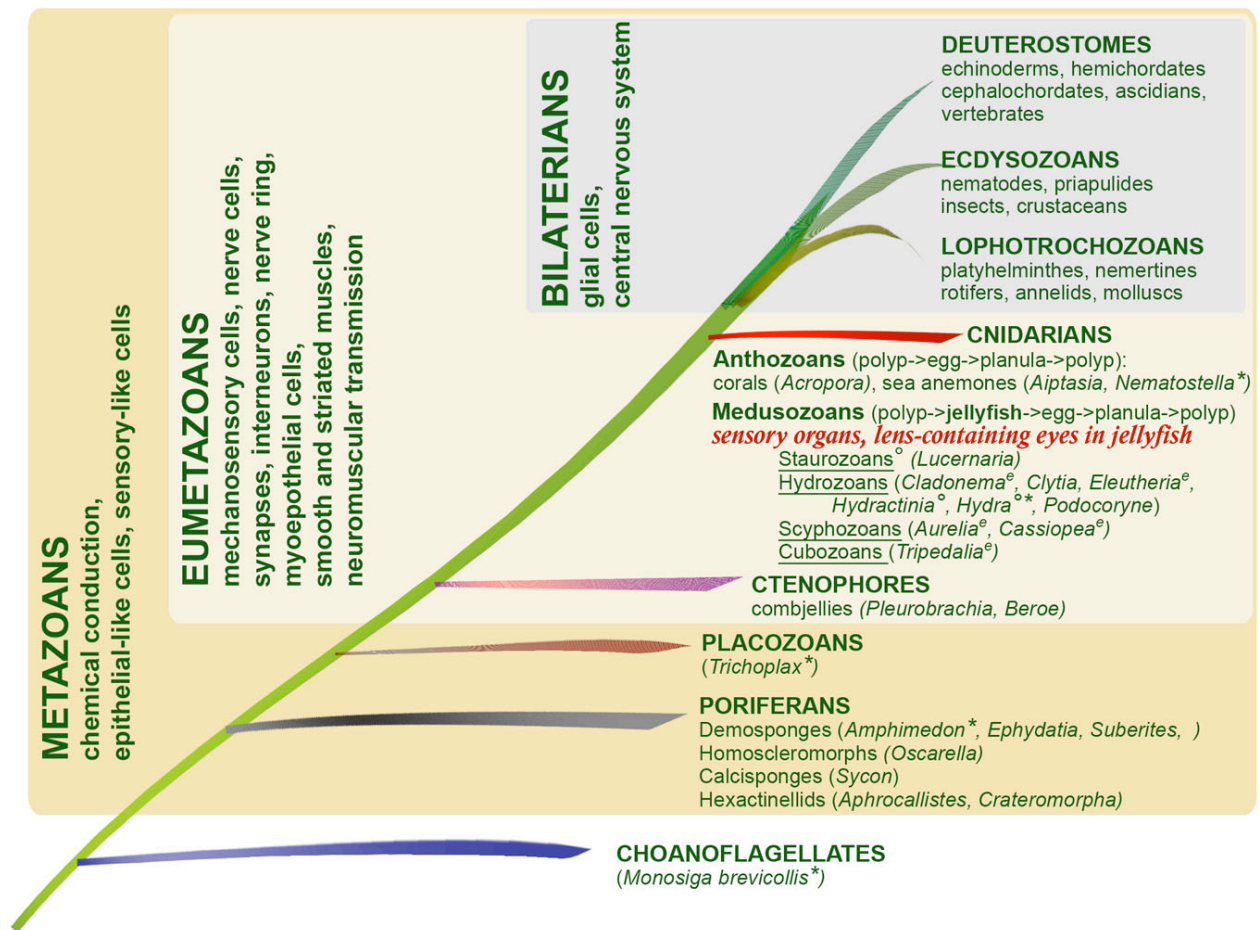
FIGURE 1: Origin of neurogenesis and progressive acquisition of a central nervous system along animal evolution.
The differentiation of cells with synaptic transmission can be traced back to the last common ancestor of eumetazoans, whereas the differentiation of sensory cells possibly emerged in the last common ancestor of choanoflagellates and metazoans; in Porifera choanocytes are proposed to correspond to sensory cells. Both Ctenophora and Cnidaria differentiate a nervous system; they diverged prior to Bilateria but their respective positions are controversial. Similarly the position of Placozoa in Metazoa is debated. * indicate species with sequenced genome; [e] species that differentiate eyes, [o] species that have lost the medusa stage.

The complex life cycle of cnidarians

Cnidaria is supposed to have diverged about 650 million years ago, preceding the Cambrian explosion, the period when ancestors to most extant bilaterian phyla arose from a common hypothetical ancestor named Urbilateria (Figure 1). Cnidarians are most often marine animals that commonly display a radial symmetry and are made up of two cell layers, the ectoderm and the endoderm, separated by an extracellular matrix named mesoglea (Bouillon, 1994b). However this "diploblastic" criterion is disputed as numerous cnidarian species actually differentiate "mesodermal" derivatives as striated muscle at one or the other stage of their life cycle (Seipel and Schmid, 2006). Cnidarian species cluster in two distinct classes (Bridge et al., 1995; Collins et al., 2006): the anthozoans that live exclusively as polyps (sea pens as *Renilla,* stony corals as *Acropora*, sea anemones as *Aiptasia, Anthopleura, Nematostella*) and the medusozoans that display a complex life cycle with a parental medusa stage and a sessile polyp stage.

Among those, the cubozoans (*Tripedalia cystophora)* and scyphozoans (*Aurelia aurita, Cassiopea xamachana)* predominantly live as medusae, whereas the hydrozoans (*Podocoryne*, *Clytia*, *Cladonema, Eleutheria)* usually follow a life cycle where they alternate between these two forms. However some hydrozoan species have lost the medusa stage as the marine *Hydractinia* and the freshwater *Hydra* polyps (Galliot and Schmid, 2002). Similarly the staurozoans that were only recently characterized as a group (Collins et al., 2006), live

exclusively as polyps. Cnidarian polyps are basically a tube with a single opening circled by a ring of tentacles, which has a mouth-anus function. Cnidarians together with ctenophores (combjellies) are the first phyla where movements are governed by a neuromuscular system, as exemplified by their active feeding behavior that requires coordinated movements of their tentacles (Westfall and Kinnamon, 1984; Westfall, 1996). Therefore, cnidarians and ctenophores provide appropriate model systems to trace back the first-evolved nervous systems (Anderson and Spencer, 1989). In contrast, poriferans (sponges), which diverged earlier during evolution and are capable of chemical conduction (Leys et al., 1999), do not display any cell types exhibiting synaptic conduction and usually feed by passive filtration.

Anatomy of the cnidarian nervous systems

The cnidarian neurons form nerve nets and nerve rings

In textbooks the organization of the cnidarian nervous system is described as a "diffuse nerve net" homogenously distributed along the polyps, which can be visualized by neuron-specific immunostaining. However in adults, this nerve net is certainly not homogenous as the distribution of neurons is not uniform, neither at the qualitative nor at the quantitative levels. For example, in *Hydra,* distinct subsets of neurons with specific spatial distribution could be identified (Grimmelikhuijzen et al.,

1989; Koizumi et al., 1990) and the nerve cell density is at least six fold higher in the head region than in the body column (Figure 2). Similarly in medusa the RFamide neurons are clearly more abundant in the manubrium and the tentacle bulbs (Grimmelikhuijzen and Spencer, 1984) (Figure 5). In addition to the nerve net, a dense anatomical structure, named the nerve ring was identified at the base of tentacles in some *Hydra* species (Koizumi et al., 1992), following the bell margin in jellyfish (Mackie, 2004), around the oral opening in *Nematostella* (Marlow et al., 2009). Nerve rings are considered as annular forms of central nervous system, involved in the coordination of behaviors (Grimmelikhuijzen and Westfall, 1995; Mackie, 2004; Garm et al., 2007; Koizumi, 2007).

Although the sensory systems and the behavioral repertoire are more elaborate in medusae than in polyps, the analysis of the differentiation of the nervous system is so far the most achieved in the *Hydra* polyp (Koizumi, 2002). Nonetheless the current emergence of new experimental cnidarian model systems (e.g. *Acropora, Nematostella, Clytia, Cladonema, Hydractinia, Cassiopea, Aurelia, Tripedalia,*) should soon complete the picture. In *Hydra*, neurons, which represent about 3% of the total cell number (David, 1973) are either sensory cells or ganglion neurons. Cell bodies of most sensory neurons are located within the ectodermal layer, their processes reaching the surface (Figure 4F), whereas the bipolar and multipolar ganglion neurons (Figure 4G-I), which are the most common type of neuronal cells, are spread in both cell layers, along the mesoglea and function as interneurons. In jellyfish sensory neurons can actually function as sensory-motoneurons, establishing bidirectional synapses with their target cells, namely myoepithelial cells and nematocytes (Anderson, 1985; Garm et al., 2006). In sea anemones, sensory neurons are associated with smooth muscle fibers, suggesting that they also behave as sensory-motoneurons (Grimmelikhuijzen et al., 1989). Synaptic transmission in cnidarians relies on fast neurotransmitters (glutamate, GABA, glycine) as well as slow ones (catecholamines, serotonine) and neuropeptides (see in Table 1). For a recent update about neurotransmission in cnidarian nervous systems, see (Kass-Simon and Pierobon, 2007).

The nematocytes (or cnidocytes) are phylum-specific mechanoreceptor cells

Beside neurons, cnidarians differentiate highly specialized mechanoreceptor cells that play a key role in the capture of preys and defense – see in (Bouillon, 1994a; Tardent, 1995). These phylum-specific stinging cells, named nematocytes (or cnidocytes, giving their name to the phylum), are abundant, representing 35% of the cells in *Hydra* (David, 1973). They display variable morphologies and functions; in anthozoans, spirocytes (Figure 3) are mechanosensory cells involved in adhesion to prey and non-prey (Kass-Simon and Scappaticci, 2002). Mature nematocytes are stimulated by chemicals or preys that contact their cnidocil, they then respond in nanoseconds by discharging the toxic content of a thick-wall capsule named nematocyst (Figure 4) (Nuchter et al., 2006). The nematocyst discharge immobilizes the prey by releasing large droplets of venom through an everting tubule (Tardent, 1995). The prey then releases the peptide glutathione, which induces the feeding response, i.e. tentacle bending and mouth opening (Loomis, 1955; Lenhoff et al., 1982; Shimizu, 2002).

Although electrical activity could be recorded in nematocytes (Anderson and McKay, 1987; Brinkmann et al., 1996), it is not clear how the information sensed by the cnidocil apparatus is transduced to target the discharge function. In fact, nematocyst discharge can occur in the absence of neuronal control indicating that nematocytes can behave as autonomous mechanoreceptor-effector units (Aerne et al., 1991). However ultrastructural studies showed the presence of two-cell as well as three-cell synaptic pathways in the tentacle epidermis of a sea anemone, including synaptic connections between nematocytes and surrounding neurons (Holtmann and Thurm, 2001; Westfall et al., 2002). This neuronal control is supposed to pace down the spontaneous firing activity of nematocytes.

Neurogenesis and nematogenesis in cnidarians

Neurogenesis and nematogenesis in the planula (swimming larva)

In developing hydrozoans, scyphozoans and anthozoans, nematogenesis and neurogenesis are initiated in late gastrula, as soon as the ectodermal and endodermal cell layers are established (Figure 2A). In hydrozoans and anthozoans, cells located in the endoderm, named interstitial stem cells in hydrozoans, give rise to nematoblasts and neuroblasts, which migrate towards the ectodermal layer (Figure 3). In *Podocoryne* larva, the nematocytes appear in the endoderm at 24 hours post-fertilization (hpf), homogenously distributed before migrating to the ectodermal layer, while a subset of larger nematocytes accumulates at the posterior end, the future oral pole (Groger and Schmid, 2001). At 24 hpf the first RFamide sensory neurons are detected in the mid-body region with neurites oriented along the anterior posterior axis. Few hours later, tyrosin-tubulin nerve cells are detected in the anterior region, with lateral neurites forming rings. Progressively novel tyrosin-tubulin neurons with lateral neurites differentiate towards the posterior pole forming repetitive units along the anterior posterior axis, while anterior and posterior connections also appear. This anterior to posterior development of the nervous system with repetitive units is highly reminiscent of the formation of the central nervous system in bilaterians (Groger and Schmid, 2001).

In the scyphozoan *Aurelia* planula where all neurons are ectodermal, the RFamide neurons differentiate first in the vicinity of the aboral pole and progressively form a dense graded plexus along the aboral half (Nakanishi et al., 2008). Similarly the *Acropora* planula develops asymmetrically, with the sensory nerve cells expressing RFamide, *Pax-C* or *Emx* that appear denser at the aboral pole but rare or absent from the oral pole (de Jong et al., 2006; Miller et al., 2000), and the ganglion and sensory neurons expressing *cnox-2Am* (*Gsx* ortholog) restricted to the ectoderm of the mid-body region (Hayward et al., 2001). Therefore the diffuse larval nerve net is already highly regionalized. In addition, some anthozoan planula as *Nematostella* develop at the anterior/aboral pole a transient sensory organ, named the apical tuft, which senses the signals that will induce settlement of the larva and its subsequent metamorphosis. The high density of sensory neurons at the aboral pole of the hydrozoan and scyphozoan planulae are supposed to play a role similar to the apical tuft. At the time of metamorphosis, part of the larval nervous system degenerates as observed in the hydrozoan and scyphozoan larvae where the aboral RFamide neurons disappear to reappear at the oral pole

of the polyp. Thus a complex reorganization of the nervous system is linked to the metamorphosis process, with complex migration patterns (Kroiher et al., 1990; Martin, 2000; Nakanishi et al., 2008). A similar process also probably occurs in metamorphosing anthozoans (de Jong et al., 2006).

Neurogenesis and nematogenesis in the adult polyp

The cnidarian polyps display an oral-aboral polarity, with differentiated tissues at the extremities but no sensory organs as recognized in medusae. In *Hydra* three distinct stem cells populations provide all cell types, the ectodermal epithelial cells, the endodermal epithelial cells and the interstitial cells, which are multipotent stem cells restricted to the ectoderm of the body column, continuously providing neurons, mechanoreceptor cells (nematocytes), gland cells and gametes when the animals follow the sexual cycle (Bode, 1996; Bosch, 2009). The epithelial stem cells divide every three to four days when the interstitial stem cells divide faster, once a day. Surprisingly enough, interstitial stem cells seem to be lacking in non-hydrozoan species, where it was proposed that neurons differentiate directly from epithelial cells. However cell lineage tracing analyses are required in non-hydrozoan model systems to clarify this question.

In *Hydra*, the nematocyte and neuronal differentiation pathways appear to share a common bipotent progenitor (Holstein and David, 1990; Miljkovic-Licina et al., 2007) before following distinct regulations: interstitial cells committed to the nematocyte lineage that are located in the ectodermal layer of the body column, undergo up to five synchronous cell cycle divisions, forming clusters of syncitial nematoblasts (Figure 4). Once they stop proliferating, the nematoblasts start differentiating their nematocyst vacuole, which can be of four distinct types (Holstein and Emschermann, 1995). Differentiated nematocytes then migrate to their definitive location, namely the tentacles, according to a process that relies on contact guidance from surrounding tentacles (Campbell and Marcum, 1980). In the tentacles, nematocytes are embedded within large epithelial cells named battery cells, each battery cell containing several nematocytes, themselves connected to sensory neurons by synapses. After discharge of their capsule, nematocytes are eliminated and replaced by new ones.
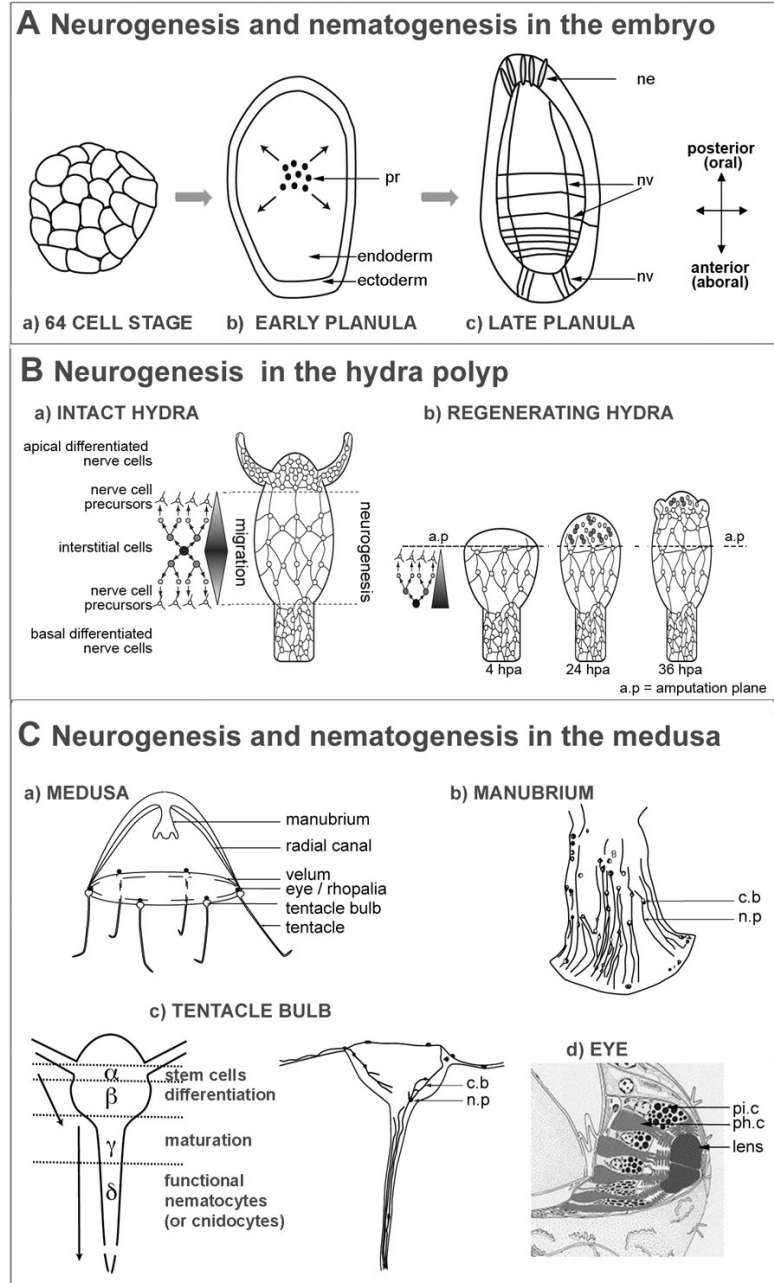

FIGURE 2: Schematic views of neurogenesis and nematogenesis during the cnidarian life cycle.

A) Neurogenesis in the developing *Podocoryne* hydrozoan. As for all medusozoan species with a medusa stage, the mature jellyfish release the gametes. At mid-gastrula stage (b) the precursors of nerve cells and nematocytes (pr) arise in the endoderm, rapidly differentiate and migrate to the ectoderm, forming a diffuse network throughout the swimming planula larva (c). At this stage the nerve cells (nv), detected here with an anti-tyrosine tubulin antibody, show laterally oriented neurites that form a ladder (Groger and Schmid, 2001). The anterior pole contains RFamide+ neurons (nv) and the posterior pole large mature nematocytes (ne). Upon metamorphosis, the larval anterior pole becomes the aboral region of the polyp (also named foot) and the larval posterior pole provides the oral region (also named head). B) In polyps the nerve net is much denser in oral and aboral regions than in the body column. In intact *Hydra* (a), neurogenesis takes place in the body column where interstitial stem cells provide neuronal progenitors that migrate and differentiate in the upper and lower regions of the body column. In head-regenerating *Hydra* (b), de novo neurogenesis takes place at the tip to reform in two days the apical nerve net. Progenitors are detected in the tip at 24 hpa and neurons after 32 hpa. C) In the adult medusa (a) neurogenesis takes place in three regions: the manubrium (b), the tentacle bulb (c) and the sensory organs, which may contain eyes (d) and statocysts. b) Closer view of a *Clytia* manubrium with the mouth opening directed to the bottom and the nerve net detected with the anti-RFamide antibody; cell bodies (c.b) and neuronal projections (n.p). c) Staggered nematogenesis in tentacle bulbs: stem cells located in the most proximal position (α) initiate nematocyte differentiation in less proximally (β), until nematocytes migrate distally in the maturation area (γ) and finally reach the tentacle when mature (δ) as shown by (Denker et al., 2008c). Tentacle bulbs are also the site of intense neurogenesis, as depicted on the right with RFamide nerve cells that project from the bulb to the tentacle. Neuronal precursors can also be found in the α zone as suggested by the *Gsx* expression in *Clytia* (see Figure 5). d) Drawing of a *Cladonema* eye after (Weber, 1981) with the tripartite lens, the ciliated photoreceptor cells (ph.c) and the pigment cells (pi.c).
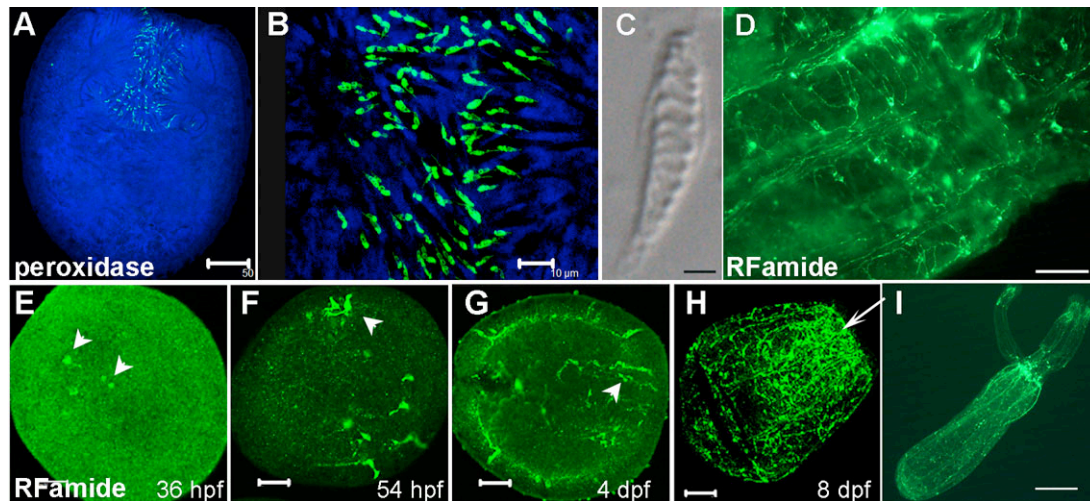
FIGURE 3: Nematogenesis and neurogenesis in the developing *Nematostella.*
A-C) Spirocytes (green) detected in *Nematostella* late planula thanks to their peroxidase activity. D-I) The RFamide sensory nerve net in juvenile (D, 31 days old) and developing *Nematostella.* Early neurons expressing the neuropeptide (arrowheads) appear in the endodermal layer (E), then migrate to the ectoderm (F, arrowheads) and form a net (G) in the mesoglea. In the newly metamorphosed polyp (H, here contracted), the RFamide nerve net is denser in the oral region (arrow) as observed in the fully metamorphosized polyp (I). Scale bars: 2 µm (C), 10 µm (B), 50 µm (A, E-H), 600 µm (I).

In contrast, the differentiation of nerve cells appears more direct: interstitial cells committed to this pathway are found predominantly along the body column, possibly in the head region but neither in the tentacles nor in the foot region. These progenitors go through S phase, get arrested in G2 until a signal will let them divide and terminally differentiate as a sensory or ganglion neuron (Schaller et al., 1989; Bode, 1996). Neuronal differentiation is more intense in the upper body column and peduncle region than in the central body column and mature neurons receive signals from the head and foot regions to migrate, explaining the higher neuronal densities recorded at the extremities. One striking finding was the high level of neuronal plasticity observed in adult *Hydra* polyps (Bode, 1992) with changes in neuropeptide phenotype according to the position of the neurons along the body column (Koizumi and Bode, 1986), but also transdifferentiation from ganglion to sensory neurons (Koizumi et al., 1988). This plasticity was also observed in the nematocyte lineage (Fujisawa et al., 1986).

Beside the highly dynamic adult homeostatic context, the regulation of neurogenesis can also be investigated in developmental contexts in *Hydra* as regeneration of the head and foot regions after bisection, asexual reproduction through budding when animals are well fed, reaggregation after tissue dissociation. After bisection, nematocytes and neurons disappear from the head regenerating tip and a wave of *de novo* neurogenesis occurs in the presumptive head region on the second day (Figures 2B, 4J-N), preceding the emergence of the regenerated head (Lentz, 1965; Yaross and Bode, 1978a; Venugopal and David, 1981; Koizumi et al., 1990; Miljkovic-Licina et al., 2007).

The nerve-free *Hydra* paradigm

In *Hydra*, neurogenesis can be disconnected from patterning by producing « nerve-free » polyps, which lack the interstitial lineage derivatives, namely nematocytes, sensory and ganglion neurons, and are thus named "nerve-free" or "epithelial" hydra. Such
animals can be obtained by different means : either chemically, upon colcemid, colchicine (Campbell, 1976) and hydroxyurea treatments (Yaross and Bode, 1978b), or genetically as in the *nf-1 Hydra magnipapillata* mutant

that completely lacks the interstitial lineage (Sugiyama and Fujisawa, 1978), or in the temperature sensitive *sf-1* mutant (Terada et al., 1988). It is also possible to maintain "pseudo-epithelial" hydra, which are depleted of all somatic interstitial lineage derivatives, but still contain stem cells restricted to the germ cell lineages (Nishimiya-Fujisawa and Sugiyama, 1995). As anticipated nerve-free animals completely lose their autonomous feeding behavior and can only be maintained by force-feeding (manual introduction of the food through the mouth opening with a pipette and subsequently washing of the gastric cavity). Nevertheless, epithelial hydra exhibit developmental patterning processes, like budding and regeneration (Marcum and Campbell, 1978a) although head regeneration in such hydra is significantly slower and less efficient (Miljkovic-Licina et al., 2007).

The manipulation of such animals turned out to be very informative, showing that the differentiation of interstitial cells into nematocytes was not position dependent, whereas that of nerve cells was indeed position dependent, i.e. enhanced in the upper and lower parts of the body column (Yaross and Bode, 1978b). This position-dependent regulation of neurogenesis seems to be largely under the control of epithelial cells (Koizumi et al., 1990; Minobe et al., 1995). Together with experiments performed on chimeras formed between mophologically-distinct strains (Marcum and Campbell, 1978b), these data suggested that the interstitial lineage, and more specifically the neurons do not play a significant role in hydra morphogenesis (Fujisawa, 2003). However the situation is probably more complex as in the absence of nerve cells, the genetic circuitry is likely reprogrammed in the epithelial cells as already reported (Hornberger and Hassel, 1997). Moreover interstitial cells and their derivatives appear involved in the fine tuning of the morphogenetic processes driven by the epithelial cells, as for instance in the *reg-16* mutant where head regeneration that is strongly deficient, can be reestablished upon depletion of the interstitial lineage (Sugiyama and Wanek, 1993). Similarly the dramatic apoptosis of the neuronal and nematocyte lineages in head-regenerating tips immediately after mid-gastric amputation leads to the activation of the head regeneration program (Chera et al., 2009).
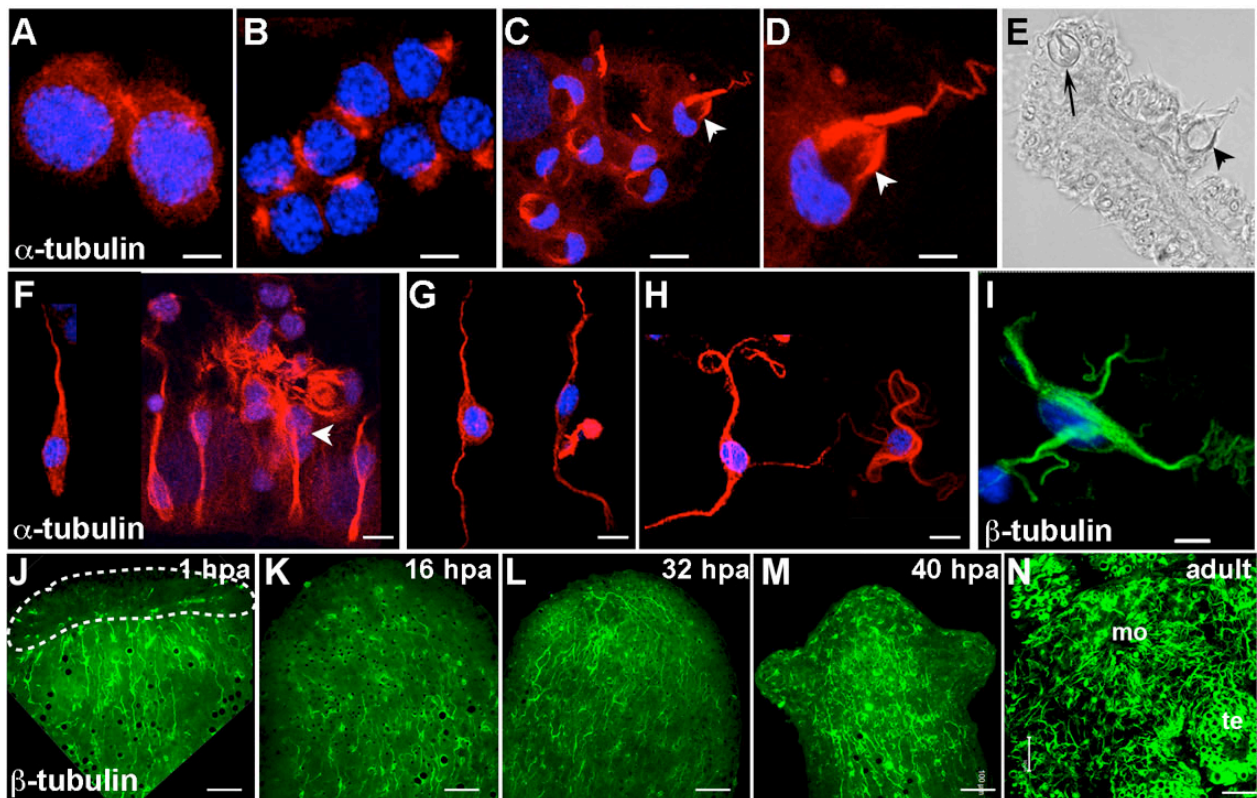
FIGURE 4: Nematogenesis and neurogenesis in the *Hydra* polyp.
A-E) In *Hydra* the interstitial stem cells (A) provide precursors (named nematoblasts, B) for the mature nematocytes. Those are characterized by a typical capsule, the nematocyst that discharges its content upon stimulation (arrowheads, C,D). E) Bright-field view of a tentacle with nematocytes either undischarged (arrow) or discharged (arrowhead) embedded in large epithelial battery cells. F-I) In *Hydra* the neurons detected here after tissue maceration can be sensory (F), bipolar (G) or multipolar (H,I) also named ganglion cells. In the ectoderm the sensory neurons are regularly distributed among epithelial and interstitial cells (F, arrowheads). The anti β-tubulin (I) more easily detects the ganglion than the sensory cells. J-N) *De novo* neurogenesis in head-regenerating *Hydra*. Following mid-gastric section, the tip of the head-regenerating half is immediately depleted of neurons (J, outline), progressively repopulated with neuronal progenitors (K) and mature neurons (L,M). However the apical nervous system at 40 hours post-amputation (hpa) is still less dense than in adult polyps (N, topview). mo: mouth opening; te: tentacles. Scale bars: 2 µm (A,B,D), 5 µm (C, F-I), 50 µm (J-N).

Neurogenesis and nematogenesis in the adult medusa

The manubrium and the tentacle bulbs

In the mature medusa, the manubrium and the tentacle bulbs are the sites of intense production of neurons and nematocytes as observed in the hydrozoan jellyfish (Figures 2C and 5). In contrast to *Hydra* polyps where all stages of nematogenesis and neurogenesis overlap along the body column, the expression analysis of neuronal and nematocyte markers coupled to *in vivo* cell labeling and morphological analyses revealed that the differentiation stages follow a proximo-distal gradient along the tentacle bulbs (Denker et al., 2008b) as depicted in Figure 2Cc. Moreover the tentacle bulb isolated from the medusa has the capacity to survive for several days in culture, opening the possibility for manipulations and functional studies.

The medusa-specific sensory organs: the ocellus, the camera eye and the rhopalia

Light sensing is widely spread in non-metazoan species but the clustering of photoreceptor cells to form sensory organs was a major innovation in animal evolution, an innovation that took place in the Cnidaria-Bilateria ancestor (Gehring, 2004). In cnidarians, both the medusa and the polyp can sense light in non-visual photosensitive structures (Santillo et al., 2006) but only the jellyfish can differentiate photoreception organs (Martin, 2002).These can be either simple ocelli as in *Aurelia* that are composed of photosensitive cells intermingled with pigment cells, or more complex as

camera eyes with a lens as observed in *Cladonema* (Figure 2Cd) and *Tripedalia*. The most complex eyes with a cornea, lens and ciliated photoreceptor cells forming retina are found in cubomedusae. In scyphozoan and cubozoan medusae, eyes associate with pressure sensing organs named *statocysts* to form complex sensory organs named rhopalia (Figure 5F-I), connected to the nerve ring (Garm et al., 2006). Therefore rhopalia were proposed to be part of the central nervous system. Behaviors can in fact be regulated through visual input, as the observed modulations of the swim pacemaker according to the light intensity in *Tripedalia* (Garm and Bielecki, 2008). Non-visual photosensitive structures also regulate animal behaviors (see in (Santillo et al., 2006)) as the pacemakers that regulate the periodic contractions of the *Hydra* body (Passano and McCullough, 1962; Passano and McCullough, 1963), the locomotion of eyeless pelagic species (Plickert and Schneider, 2004) or the triggering of spawning supported by the expression of opsins in gonads (Suga et al., 2008).

Beside adult eyes, pigmented photoreceptor cells were identified in the *Tripedalia* larva, which does not contain any nervous system (Nordstrom et al., 2003). These single cell ocelli appear quite original since they most probably function completely autonomously, sensing the light through their photoreceptors and regulating the animal behavior thanks to the motor-cilium they differentiate. Moreover these photoreceptors are rhabdomeric (microvilli) as observed in most invertebrates and not ciliated as in adult cnidarian eyes and vertebrates. It would be of interest to identify other cases of cnidarian larval eyes. In some species as

Cladonema, adult eyes can fully regenerate (Stierwald et al., 2004). Given the great variety of eye morphology, the question of a unique origin for all animal eyes or a repeatedly convergent evolution is a long-standing one (Arendt, 2003; Nilsson, 2004), still debated after the discovery of shared regulators of eye differentiation as the *Pax* and *Six* genes (Kozmik et al., 2003; Stierwald et al., 2004) and shared effectors as opsins (Suga et al., 2008; Kozmik et al., 2008).

Elements of the cnidarian neurogenic circuitry

1.- Growth factor signaling pathways in cnidarian neurogenesis

The deep conservation of the signaling machinery that support developmental processes in bilaterians came as a surprise in cnidarians, when components of the insulin-like (Steele, 2002), Wnts (Hobmayer et al., 2000; Wikramanayake et al., 2003; Kusserow et al., 2005; Teo et al., 2006; Momose and Houliston, 2007), Notch

(Kasbauer et al., 2007), VEGF (Seipel et al., 2004a), FGF (Matus et al., 2007b; Sudhop et al., 2004; Rentzsch et al., 2008) and Hedgehog (Matus et al., 2008) pathways were uncovered (Figure 6). Not only the ligands, receptors and intra-cellular components were identified but also the antagonists as the Dickkopf3 and Dickkopf1/2/4 Wnt-antagonists (Fedders et al., 2004; Guder et al., 2006b) and the Gremlin, Noggin and Follistatin BMP-antagonists (Matus et al., 2006; Rentzsch et al., 2006). This amazing conservation was actually confirmed by the even more surprising presence of these pathways in sponges (Nichols et al., 2006; Adamska et al., 2007) and partially in choanoflagellates (King et al., 2008).

However the experimental evidences concerning the contribution of these pathways to neurogenesis in cnidarians are still limited, although four of them are likely involved in neurogenesis (Table 1). The **FGF pathway** supports the differentiation of the apical sensory organ in *Nematostella* planula as demonstrated by loss-of-function assays (Rentzsch et al., 2008), but a similar role at the aboral pole of medusozoan planulae remains to be shown. **The canonical Wnt pathway** appears to
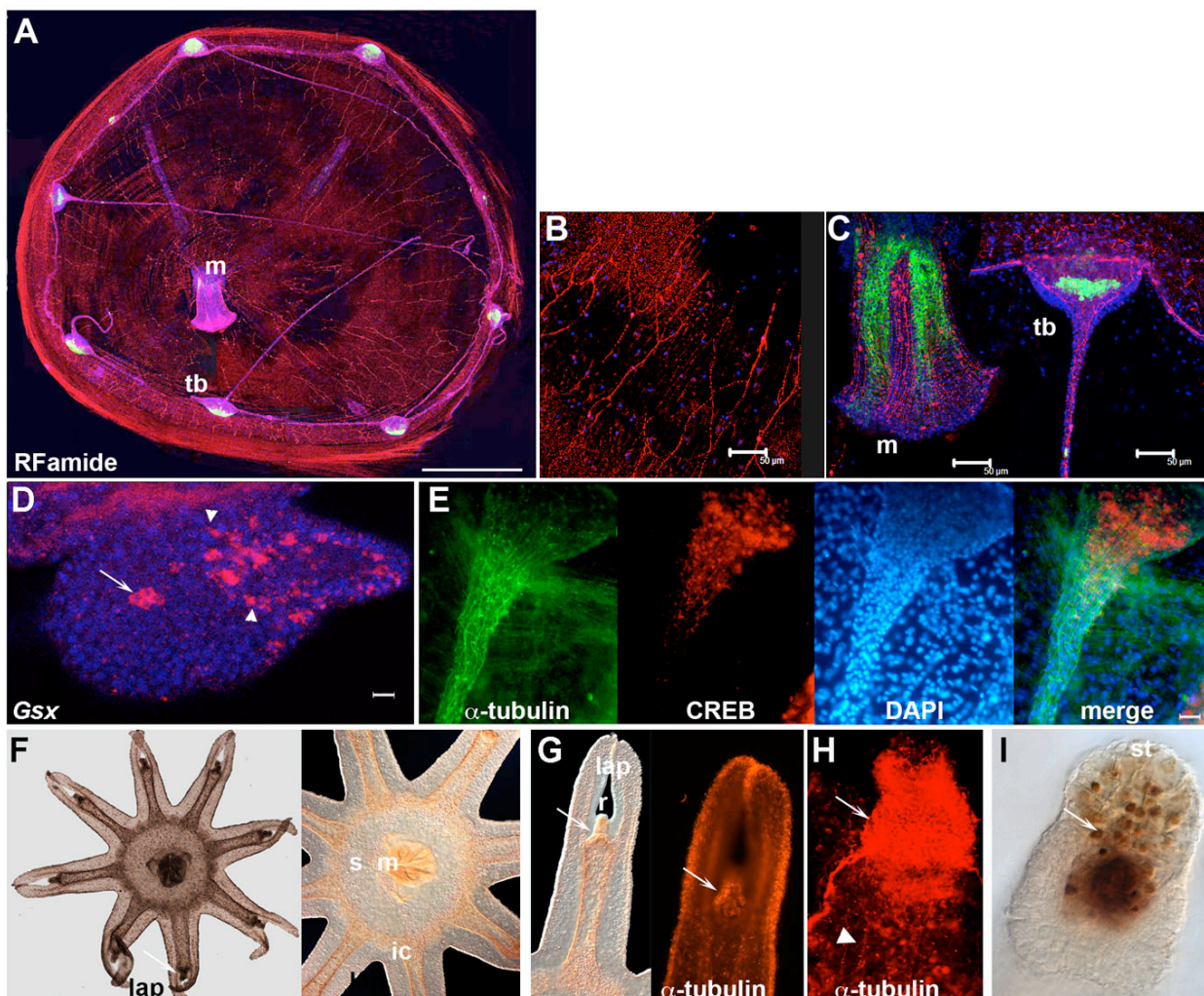

FIGURE 5: Neurogenesis in hydrozoan and scyphozoan medusae.
A-C) Bottom view of the *Clytia* medusa nervous system with the manubrium (m) and the tentacle bulbs (tb) containing numerous RFamide sensory neurons (purple-pink). Note the delicate nerve net in the velum (B) and the endogeneous bioluminescence in the tentacle bulbs (green, A,C). D) The proliferative (arrow) and differentiating (arrowheads) zones of the tentacle bulbs express the ParaHox gene *Gsx* (red; blue: DAPI staining). E) In the *Podocoryne* medusa, the differentiating neurons in the tentacle bulbs strongly express the CREB transcription factor detected with the anti-hydra CREB antibody (red). Scale bars: 500 μm (A), 50 μm (B,C) 10 μm (D,E). F-I) Rhopalia in the immature *Aurelia* medusa (ephyra). Most of the adult features can be already observed: the mouth (m), the developing stomach (s), three types of radial canals: adradial, perradial and interradial (ic) and the rhopalia (r; arrows), each of them guarded by a pair of lappets that contain a diffuse nerve net (F, G). At the base of the rhopalium, a stratified epithelium includes columnar ciliated cells with basal axons and cells with intra-epithelial flagella stained with α-tubulin (G, H, arrowhead). In the center of the rhopalium the elongated ovoidal lithostyle (I) contains the photoreception organs named ocelli that contain pigmented cells, and a terminal statocyst (st) that senses gravitation.

SIGNALING	Anthozoans	Medusozoans	References
FGF (FGFa1, FGFRa, FGFa2)	*Nv-pl:* apical tuft **(lof)**	?	(Rentzsch et al., 2008)
Wnt3	*Nv:* 11 Wnt families, neurogenesis ?	*Hv, Hm (Wnt3):* apical organizer, terminal differentiation of nematoblast *Hs:* i-cells differentiation, apical organizer	(Kusserow et al., 2005) (Hobmayer et al., 2000; Guder et al., 2006a; Khalturin et al., 2007) (Muller et al., 2007; Teo et al., 2006)
Dkk1/2/4	?	*Hm:* expressed in body column, gland cells, putative neurogenic	(Guder et al., 2006b)
Dkk3	?	*Hm:* differentiating nematocytes *Ch-med:* TeBu differentiating nematocytes	(Fedders et al., 2004) (Denker et al., 2008b)
BMP2/4	*Am* larva: oral region *Nv-ga:* asymmetric expression, *Nv-pl:* apical tuft	*Hm:* neurogenesis ?? *Pc* larva: aboral, neurogenesis ?	(Hayward et al., 2002; Finnerty et al., 2004); (Reber-Muller et al., 2006);
BMP5/8	*Nv-pl:* pan-endodermal, apical tuft	*Hv: (2x)* endoderm peduncle & tentacle zone, neurogenesis	(Matus et al., 2006) (Reinhardt et al., 2004)
chordin	*Nv-ga:* blastopore, asymmetric ectodermal oral-aboral expression	*Hv:* endoderm, up-regulated during organizer formation	(Matus et al., 2006; Rentzsch et al., 2006) (Rentzsch et al., 2007)
follistatin	*Nv-pl:* circumoral, neurogenesis ?	?	(Matus et al., 2006)
gremlin, GDF5	*Nv-ga:* asymmetric endo. oral-aboral, *Nv-pl:* apical tuft	?	(Matus et al., 2006; Rentzsch et al., 2006)
noggin	*Nv-pl (Noggin1):* endo. facing the apical tuft; pharyngeal asymmetrical	?	(Matus et al., 2006)
Jun kinase	?	*Hv:* differentiating nematocytes	(Philipp et al., 2005)
RSK 2	?	*Hv:* i-cells, neurons, nematoblasts, epithelial cells apical organizer **(lof)**	(Kaloulis et al., 2004; Chera et al., 2006; Chera et al., 2007)
Notch	*Nv-pl:* similar to Musashi	*Hm:* nematocyte differentiation	(Marlow et al., 2009), (Kasbauer et al., 2007; Khalturin et al., 2007)
Hedgehog	*Nv-pl (Int1, Int2, Int3)* neural precursors	?	(Matus et al., 2008)
Opsins	?	*Cr* (20x): eyes, gonads	(Suga et al., 2008)
PEPTIDES			
GLWamides (NP)	*(I-VIII) Af:* Hym-54, Hym-370 -> muscle contraction	*Hm:* Hym-53, -54, -248, -249, -331, -338, -370 peptides in sensory neurons -> muscle contraction *He-pl:* sensory neurons stimulating migration & metamorphosis **(lof)**	(Grimmelikhuijzen et al., 2002; Hansen et al., 2002; Takahashi et al., 2003), (Leitz et al., 1994; Gajewski et al., 1996; Plickert et al., 2003; Katsukura et al., 2003; Katsukura et al., 2004)
KAamide (NP)	*Ae:* inhibits muscle contraction	?	(Grimmelikhuijzen et al., 2002)
KVamides (NP)	?	*Hm:* Hym-176 -> muscle contraction	(Yum et al., 1998; Hansen et al., 2002)
PWamide (EP)	?	*Hm:* Hym-33H inhibits neural differentiation	(Takahashi et al., 1997; Takahashi et al., 2009; Lentz, 1965)
RFamides (NP)	*(I, II, III) Ae:* endo. neurons *Ps:* slow muscle contraction *Rk:* ecto. & endo. sensory neurons, pharyngeal nerve net	*(A, B, C) Hm, Hv:* ecto. Sensory neurons *He-pl:* sensory neurons inhibiting migration & metamorphosis	(Grimmelikhuijzen et al., 2002; Hansen et al., 2002; Pernet et al., 2004), (Katsukura et al., 2003; Katsukura et al., 2004)
RGamides (NP)	?	*Hm:* Hym-355, positively regulates neural differentiation	(Takahashi et al., 2000; Hansen et al., 2002)
RIamide (NP)	*(I,II) Ae:* inhibit muscle contraction	?	(Grimmelikhuijzen et al., 2002)
RNamides (NP)	*(I, II) Ae:* antagonistic action on longitudinal and circular muscles	?	(Grimmelikhuijzen et al., 2002)
RPamides (NP)	*(I-V) Ae:* tentacle contractions		(Grimmelikhuijzen et al., 2002)
RWamides (NP)	*(I, II) Ab, Ps:* slow contraction of endo. muscles; *Cp:* sphincter contraction	?	(Grimmelikhuijzen et al., 2002)
ANTP-class Homeoproteins			
Emx	*Am:* aboral larval sensory neurons	*Hs:* neurogenic ? *Hydra:* ?	(de Jong et al., 2006) (Mokady et al., 1998)
Not	?	*Hv:* tentacle root sensory neurons	this work
Msx	*Am:* oral larval ectoderm (neurons ?)	*Hv:* body column neurons *Pc:* progenitor maintenance	(de Jong et al., 2006), (Miljkovic-Licina et al., 2004), (Galle et al., 2005)
Gsx / Anthox2 / cnox-2 (ParaHox)	*Am-pl (cnox-2):* ecto. bipolar and multipolar neurons except aboral *Nv-pl (Anthox2):* ecto. neural precursors, oral region *Nv-po (Anthox2):* ecto. neural precursors in body column, pharynx, tentacles	*Hv:* apical neurogenesis **(lof)**, nematogenesis along body *Pc-pl:* endo., aboral; *Pc-po:* + pattern ? *Ch-pl:* endo. progenitors ? *Ch-med:* ecto. TeBus progenitors, neurons *Ed-pl, Ed-po:* ecto., oral *Ed-med:* endo, early buds **(lof)** *Hs:* ecto. body column (antibody)	(Hayward et al., 2001; de Jong et al., 2006), (Finnerty et al., 2003), (Miljkovic-Licina et al., 2007), (Yanze et al., 2001), (Chiori et al., 2009; Quiquand et al., 2009) (Jakob and Schierwater, 2007) (Cartwright et al., 2006)
Pdx/Xlox (ParaHox)	*Nv-pl, Nv-po (Xlox/Cdx):* endo. ventral midline stripes	*Ch-pl:* endo. progenitors ?	(Ryan et al., 2007) (Quiquand et al., 2009), this work
Cdx (ParaHox)	?	*Ch-pl:* ecto. oral/aboral *Ch-med:* TeBu. nematogenesis *Ed-po:* ecto, aboral	(Chiori et al., 2009) (Jakob and Schierwater, 2007)
PG-1 (Hox)	*Nv-pl* 2x *(Ax6):* endo. pharyngeal ring *(Ax6a):* body wall *Nv-po (Ax6):* oral, mouth opening; endo. base and tips of tentacles	*Ch-med (Hox1):* mechano-sensory cells in statocysts *Ed-pl (Cnox5):* ecto., aboral *Ed-po (Cnox5):* ecto/endo, oral/aboral **(lof)** *Pc-pl (Cnox1):* aboral ecto/endo *Hv (cnox-1):* endo/ecto.hypostome	(Finnerty et al., 2004; Ryan et al., 2007) (Chiori et al., 2009) (Kamm et al., 2006; Jakob and Schierwater, 2007) (Yanze et al., 2001) (Gauchat et al., 2000)
PG-2 (Hox)	*Nv-pl (Ax7):* body-wall endoderm *Nv-po (Ax7):* pair of mesenteries *Nv-pl (Ax8a):* pharyngeal endo. *Nv-pl (Ax8b):* ventral midline *Nv-po (Ax8a-Ax8b):* ventral pair mesenteries, endo. tentacle base	no ortholog ?	(Finnerty et al., 2004; Matus et al., 2006; Ryan et al., 2007)
PG-9 like (Hox)	*Nv-pl (Ax1):* ecto. apical tuft *Nv-pl (Ax1a):* endo. asymmetric body wall *Nv-po (Ax1a):* ventral mesenteries, endo. tentacle base	*Ch-pl (Hox9-14A, -B):* oral pole *Ch-med (Hox9-14A):* ecto. TeBu, ecto. manubrium *Ch-pl (Hox9-14C):* ecto. aboral *Ed-med (Cnox-1):* ecto. oral ring **(lof)** *Ed-med (Cnox-3):* ecto. oral ring **(lof)** *Hv (Cnox-3):* ecto. hypostome	(Finnerty et al., 2004; Ryan et al., 2007) (Chiori et al., 2009) (Kamm et al., 2006; Jakob and Schierwater, 2007) (Gauchat et al., 2000)
orphan Hox-like	no ortholog ?	*Pc-pl (cnox2):* ecto/endo aboral *Pc-po (cnox2):* apical tip *Pc-med (cnox2):* endo. gastrovascular	(Masuda-Nakagawa et al., 2000)

PRD-class Homeoproteins			
prdl-a	?	*Hv:* apical neurons and precursors, organizer during head formation	(Gauchat et al., 1998; Miljkovic-Licina et al., 2004)
prdl-b	?	*Hv:* body neural cells, proliferating nematoblasts	(Gauchat et al., 2004; Miljkovic-Licina et al., 2004)
homeobrain	*Nv*-pl: oral neural-like cells; *Nv*-po: restricted to tentacles	?	(Marlow et al., 2009)
Gsc	*Nv*-pl: endo. pharyngeal, apical tuft, asymmetric directive axis	*Hm:* apical sensory neurons	(Pang et al., 2004; Matus et al., 2006) (Broun et al., 1999)
Otp	*Nv*-po: ecto. oral nerve ring	?	(Marlow et al., 2009)
Rx	*Nv:* neural subsets	?	(Matus et al., 2007a)
Repo	*Nv:* nerve ring	?	(Marlow et al., 2009)
Otx	*Nv* (3x): nerve ring ? *Am* (2x) nerve ring ?	*Hm, Pc:* cell migration (budding) but not detected in nervous system	(de Jong et al., 2006; Mazza et al., 2007), (Muller et al., 1999; Smith et al., 1999)
Pax-A/C (pox neuro)	*Nv:* putative neural, spirocyte precursors and neural cell types *Am:* neural cell types	?	(Matus et al., 2007a) (Miller et al., 2000; Plaza et al., 2003)
Pax-B (Pax2/5/8)	*Nv:* patterning of the nerve ring ?	*Tc* med: rhopalia *Pc*-pl, med: neurogenesis	(Matus et al., 2007a), (Kozmik et al., 2003), (Groger et al., 2000)
Pax-D (Pax3/7)	*Am:* embryonic stripes neurogenesis ? *Nv*-pl (*PaxD1*): stripe, i-cells ? *Nv*-po (*PaxD3*): tentacle ecto.	no ortholog	(de Jong et al., 2006; Matus et al., 2007a)
SIN-class Homeoproteins			
Six1/2, Six3/6, Six4/5	?	*Pc*-med, *Cr*-med: neurogenesis	(Stierwald et al., 2004)
Six1/2, Six3/6	?	*Cr*-med: eye	(Stierwald et al., 2004)
Six3/6	?	*Cr*-med:nematogenesis	(Stierwald et al., 2004)
b HLH TFs			
Achaete-scute (A type)	?	*Hm:* late nematogenesis, sensory neurons *Pc*-pl: neural precursors, *Pc*-med: neural & muscle precursors, nematocytes	(Grens et al., 1995; Hayakawa et al., 2004; Lindgens et al., 2004), (Muller et al., 2003; Seipel et al., 2004c)
COE (collier-type)	*Nv*-pl: apical tuft	?	(Pang et al., 2004)
HMG TFs			
SoxB	*NvSox2-pl:* ecto. neural-like cells *NvSox2-po:* restricted to tentacles	?	(Magie et al., 2005)
	NvSoxB1-pl: apical tuft, pharyngeal *AmSoxB1-pl:* presumptive ecto.	?	(Magie et al., 2005)
	NvSoxB2-pl: ecto. aboral half *AmSoxBa-pl:* ecto. aboral half	?	(Magie et al., 2005) (Shinzato et al., 2008)
SoxC	*Am-pl:* ecto. sensory neurons *Nv*-pl: ecto, sensory neurons *Nv*-po: developing tentacles	?	(Shinzato et al., 2008)
MADS-box TFs			
FoxB (Fkh3)	*Nv*-pl: pharyngeal ecto. *Nv*-po: oral ring	*Ch*-pl: nematogenesis, endo. ganglion cells*; Ch*-med: ecto. TeBu, statocysts	(Magie et al., 2005) (Chevalier et al., 2006)
FoxD1	*Nv*-pl: broad aboral domain *Nv*-po: base of tentacles	?	(Magie et al., 2005)
Mef2	*Nv:* ecto. Precursors, nematocytes, neurons	*Pc*-po: apex, *Pc*-med: manubrium	(Martindale et al., 2004) (Spring et al., 2002)
SRF	?	*Hv, He:* undifferentiated i-cells	(Hoffmann and Kroiher, 2001)
Zinc-containing TFs			
COUP-TF (nuclear receptors)	*Am:* 10x orphan, neurogenesis ?	*Hv:* differentiating nematoblasts; body column neurons	(Grasso et al., 2001), (Gauchat et al., 2004)
RXR (nuclear receptors)	?	*Tc:* putative regulator of crystallin genes	(Kostrouch et al., 1998)
Zic	?	*Hv:* early dividing nematoblasts	(Lindgens et al., 2004)
Gli	*Nv*-pl, *Nv*-po (*Gli3*): endo. body wall	*Hv:* ubiquitous expression	(Matus et al., 2008) (MM-L, BG, unpublished)
GCM (Glial cells missing)	*Nv*-pl: oral ecto. scattered cells *Nv*-po: endo. oral nerve ring	?	(Marlow et al., 2009)
Various TFs			
C/EBP (bZIP TF)	?	*Pc*-pl: ecto. aboral; *Pc*-po: endo. bud *Pc*-med: muscles, tentacle, TeBu,	(Seipel et al., 2004b)
CREB (bZIP TF)	?	*Hv:* i-cells, neurons & nematoblasts, epithelial cells apical organizer **(lof)**	(Kaloulis et al., 2004; Chera et al., 2007); (LG, SC, BG, unpublished)
Mafl (bZIP TF)	?	*Pc*-pl: endo. aboral; *Pc*-po: endo. bud *Pc*-med: muscles, tentacle, TeBu,	(Seipel et al., 2004b)
CBFb (Runx TF)	*Nv*-po: ecto. tentacle, nematocytes	?	(Sullivan et al., 2008)
Runx (Runx TF)	*Nv*-po: ecto. tentacle, nematocytes	?	(Sullivan et al., 2008)
Smad	?	*Hv,* ubiquitously expressed, stronger in i-cells, nematocyte differentiation	(Hobmayer et al., 2001)
NEURAL stem cell markers			
ELAV1 (RNA-binding protein)	*Nv*-pl: scattered ecto. neural precursors *Nv*-po: ecto/endo, body wall, tentacles	?	(Marlow et al., 2009)
Musashi (Msi) (RNA-binding protein)	*Nv*-pl: ecto. oral, tentacle bud *Nv*-po: ecto, pharyngeal, tentacles	?	(Marlow et al., 2009)
CHROMATIN regulators			
Polycomb related	?	*Hy-AEP, Hm:* HyEED i-cells, nematoblasts **(gof)**	(Genikhovich et al., 2006; Khalturin et al., 2007)
CBP/p300	?	*Hv:* i-cells, neurons & nematoblasts, epithelial cells apical organizer **(lof)**	(LG, SC, BG, unpublished)

Table 1: Putative regulators of cnidarian neurogenesis identified in analyses performed at the cellular expression level and/or functional level. **Abbreviations:** Ax: anthox (anthozoan Hox/ParaHox gene); Cx: cnox (Hox/ParaHox gene); ecto.: ectodermal, endo.: endodermal; EP: epitheliopeptide; ga: gastrula; **gof:** gain of function assay; i-cells: interstitial cells; **lof:** loss of function assay; med: medusa; NP: neuropeptide; pl: planula; po: polyp; TeBu: tentacle bulb; x: copy number for a given gene family. **Species code:** *Ab: Anthopleura ballii; Ae: A. elegantissima; Af: A. fuscoviridis; Am: Acropora millipora; Ch: Clytia hemispherica; Cp: Calliactis parasitica; Cr: Cladonema radiata; Hy-AEP: Hydra sexual strain; He: Hydractinia equinata; Hm: Hydra magnipapillata; Hs: Hydractinia symbiolongicarpus; Hv: Hydra vulgaris; Nv: Nematostella vectensis; Pc: Podocoryne carnea; Ps: Protanthea simplex; Rk: Renilla koellikeri; Tc: Tripedalia cystophora.*

2.- The transcription factors in cnidarian neurogenesis

In bilaterians, homeoproteins in combination with the bHLH proteins bring a major contribution to neurogenesis during development and adulthood (Guillemot, 2007). According to the sequence of their homeodomain, homeogenes fall into classes, which do have cnidarian representatives (Galliot et al., 1999; Holland and Takahashi, 2005; Ryan et al., 2006). Genes from the ANTP, PRD, SIN, POU and LIM classes perform neurogenic tasks in bilaterians, but in cnidarians, expression and in few cases functional data are only available for the ANTP, PRD and SIN gene families. We will review here what is currently known about the neurogenic function of those gene families in Cnidaria.

The neurogenic function of the non-HOX (NK-like) ANTP-class homeogenes

The ANTP-class of homeogenes contains numerous gene families that distribute into two sub-classes : the non-Hox (also named NK-type) and the *Hox/paraHox* families (Gauchat et al., 2000 ; Holland, 2001). The non-Hox families are highly conserved from cnidarians to bilaterians and thus form well defined sister groups (Gauchat et al., 2000; Schierwater and Desalle, 2001; Chourrout et al., 2006; Kamm et al., 2006; Quiquand et al., 2009). We will consider here only those that are putative regulators of the cnidarian nervous systems.

Divergent roles for the *empty spiracle / emx* gene family in hydrozoans and anthozoans: The *emx* genes are involved in forebrain formation in vertebrates with a special emphasis on the cytoarchitecture of the cerebral cortex (Cecchi et al., 2000), whereas mutation of the *Drosophila* homologue, *Ems,* eliminates the deutero- and tritocerebrum (Hirth et al., 1995). In the hydrozoan *Hydractinia*, the *Emx* homologue is expressed in endodermal epithelial cells of the hypostome (head region) and up-regulated in posterior regions of the planula larva experimentally converted to anterior fate (Mokady et al., 1998). However in the coral *Acropora*, *Emx* is expressed in sensory neurons of the aboral half of the larva until their density drastically decreases at the time of metamorphosis (de Jong et al., 2006). Therefore *Emx* might belong to the ancestral neurogenic genetic circuitry but more studies, particularly in developing medusozoans, are needed to strengthen this conclusion.

The *Hydra Not* ortholog, a marker for apical sensory neurons: *Not* homeobox genes are involved in neurogenesis in bilaterians: in *Drosophila*, the Not-like *90Bre* gene participates in the differentiation of the neuroblasts of the posterior brain (Dessain and McGinnis, 1993), in Xenopus *Xnot-2* promotes notochord formation (Gont et al., 1996), in chicken *Cnot1* and *Cnot2* are expressed in the early neurectoderm (Stein et al., 1996), in zebrafish the Not-related *floating head* gene is required for neurogenesis of the epiphysis (Masai et al., 1997). In *Hydra*, the *cnot* gene is expressed in sensory neurons at the root of the tentacles (Figure 7A). During head formation, either budding or regeneration, *cnot* transcripts start to be expressed in a limited number of neuronal cells at the place where tentacle rudiments will emerge. Hence the *Hydra cnot* gene appears to be restricted to the differentiation of a limited subset of neurons.

The *msh/msx* gene, a candidate regulator of neurogenesis: The *msh/msx* homeogene family is highly conserved from Porifera to bilaterians (Larroux et al., 2007). Beside the homeodomain, *msx* genes also encode some Groucho-interacting domains that are conserved in *Nematostella* but not in hydrozoans (Takahashi et al., 2008). In *Drosophila*, the *msh* gene is involved in both dorsoventral patterning and neurogenesis, specifying neuroblasts in the dorsal neuroectoderm. In the leech, *Le-msx* transcripts are present in embryonic stem cells, and subsequently restricted to the neural tissue. In amphioxus, *msx* is expressed in dorsal cells of the neural tube, similarly to the *msx3* expression pattern observed in mice embryos. From these results, it was proposed that the *msh/msx* genes specify the differentiation of the dorso/lateral neural tube in an evolutionarily conserved manner (Cornell and Ohlen, 2000). In the coral *Acropora, msx3-Am* is expressed in the ectoderm of the oral region but no cell-type specificity was noted (de Jong et al., 2006). In *Hydra Msx* is expressed exclusively in neurons along the body column (Figure 7A), forming a nerve net in the central region of the polyp (Miljkovic-Licina et al., 2004). In contrast in the jellyfish *Podocoryne, msx* appears involved in the maintenance of progenitors during medusa budding and transdifferentiation (Galle et al., 2005). As above more studies are required to conclude about a conserved neurogenic fonction for *msx* in cnidarians.

The *EHG* gene family appears missing in cnidarians: The evolutionarily-conserved neurogenic function of homeogenes was first reported with the engrailed homeoprotein in arthropods, annelids and chordates (Patel et al., 1989). In vertebrates, two engrailed-related genes (*en-1* and *en-2*) specify the cerebellar territory (Wassef and Joyner, 1997), whereas in *Drosophila*, *engrailed* exhibits a dual function, during segmentation and neurogenesis, the latter one being considered as ancestral (Gibert, 2002). However, a cnidarian *engrailed* ortholog was not identified so far, suggesting that this gene family arose later during evolution or was lost in cnidarians, implying thus that it was not essential at the origins of neurogenesis.

The ParaHox and Hox-like cnidarian genes

The *Hox/ParaHox* gene families, which are highly conserved among bilaterians, exhibit a much lower level of conservation from cnidarians to bilaterians that the ANTP non-Hox families (Gauchat et al., 2000; Schierwater and Desalle, 2001; Chourrout et al., 2006; Kamm et al., 2006; Quiquand et al., 2009). Recent analyses showed that the three ParaHox families (*cnox2/Anthox2/Gsx, Pdx/Xlox, Cdx/Cnox4-Ed)* exhibit a much higher level of conservation than the Hox-like ones, which belong to paralogous groups (PG) in only two cases (*Anthox6/Cnox1/PG1, Anthox7/Anthox8/PG2*), whereas the others display limited conserved features (*Anthox1/cnox-3/PG9*) or are highly derived (Chiori et al., 2009; Quiquand et al., 2009). It was proposed that the absence of Hox/ParaHox genes in poriferans together with the higher level of conservation of the ParaHox genes would correlate with cellular innovations that took place in the last common Cnidaria Bilateria ancestor (CBA). However, the expression of these *Hox/ParaHox* genes appears tightly regulated during developmental processes in hydrozoans and anthozoans, suggesting that they act as developmental genes. *Hox* genes likely participate in the development or the maintenace of the cnidarian nervous system as the *Clytia Hox1* (PG1) in statocysts, the *Nematostella Anthox1* (PG9-like) in the apical tuft, or the *Eleutheria Cnox-3* (PG9-like) in the oral ring (see in Table 1). However cellular and functional

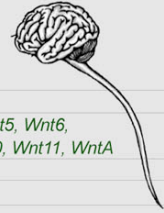|	PORIFERA	CNIDARIA	URBILATERIA	VERTEBRATES
	epithelial-like cells, sensory-like cells, chemical conduction, polarity, filtrate-feeders	myoepithelial cells, smooth / striated muscle cells, tissue layers, oral-aboral polarity, active behaviors (carnivorous), camera eye, rhopalia	AP + DV axes, mouth, anus, head, central nervous system, peripheric nervous sys., glial cells	forebrain, neural crests, inner ear, myelin sheath
SIGNALING		Nerve ring	?	
Notch	Notch / delta	Notch°/ delta	Notch / delta	
Wnt	Wnt7/8-like	**Wnt1, Wnt2, Wnt3**$^{P\text{-}A}$**, Wnt4, Wnt5, Wnt6,** Wnt7, Wnt8, **Wnt10, Wnt11, WntA**	Wnt1, Wnt2, Wnt3, Wnt4, Wnt5, Wnt6, Wnt7, Wnt8, **Wnt9 (?),** Wnt10, Wnt11, WntA	
Wnt antagonists	?	**Dkk1/2/4**^{N}**, Dkk3°**	Dkk1/2/4, Dkk3	
BMP	GDNF-like	**BMP2/4, BMP5/8**	GDNF-like	
BMP-antagonists		**chordin, gremlin, follistatin**		
FGF	?	FGF-D*, orphans	**FGF-A,** FGF-D, **FGF-H**	
FGF antagonist	?	**sprouty**	sprouty	
Hedgehog	Hedgling	Hedgling, **Hedgehog**	~~Hedgling~~, Hedgehog	
patched	patched	patched	patched	
Jak/Stat	Jak/Stat	Jak/Stat	Jak/Stat	
TRANSCRIPTION FACTORS (TFs) — Homeodomain TFs				
NK-type	NK2-4, NK5-7, Tlx, Hex, Msx, BarH, Barx	NK3, NK4, 2, NK5, 6, 7, Tlx, Hex, Msx, Barx, **Hlx, Lbx, Not*, Emx*, Dlx, ro, NK1,**	Msx, Not, Emx, Dlx, BarH, Barx, Hlx, **Dbx,** Lbx, Hex, Tlx, NK3, NK4, NK2, NK5, NK6, NK7	
EHGBox	**not detected**	**Gbx, Mnx**	Gbx, Mnx, **en**	
ParaHox	**not detected**	**Gsx***°**, Pdx, Cdx**	Gsx, Pdx, Cdx	
Hox	**not detected**	**PG1, PG2, Hox-like, Mox, Evx**	PG1, PG2, PG3, **PG4,** PG9, Mox, evx	
paired-like (Q50)	arx, Hbn, rx, OG-2	arx (prdl-b, alx), hbn/prdl-a, rx, ~~OG-2~~, **repo, ceh-10, Alx3/4, unc4, Otp, OG12, Dux, rax**	arx, hbn, rx, OG-2, repo, ceh-10, Alx3/4, unc4, Otp, OG12, Dux, **Arix, Prx**	
Otx-like (K50)	**not detected**	**Otx, Gsc, Ptx, Dmbx**	Otx, Gsc, Ptx, Dmbx	
Pax (S50)	PaxB (2/5/8)	PaxB(2/5/8), **PaxA/C** (pox neuro), PaxD(3/7)	Pax2/5/8, pox neuro, Pax3/7, **Pax1/9, Pax4/6**	
Six-class HP	Six-1/2	Six-1/2, **Six-3/6, Six-4/5**	Six-1/2, Six-3/6, Six-4/5	
POU-class HP	POU I, POU VI, POU II-IV	POU I, POU II-**III**, POU IV, POU VI	POU I, **POU II,** POU III, POU IV, POU VI	
LIM-class HP	Lim3, lin11, islet	Lim3, lin11, islet, **Lmx, Apt, Lhx6/7**	Lim3, lin11, Lmx, islet, apt, Lhx6/7	
TALE-class HP	Irx, TALE	Irx, **Pbx, Tgif, Meis**	Irx, Pbx, Tgif, Meis	
bHLH — *Twist-class*	**not detected**	**Mesp, Hand, Paraxis, SCL, Twist, PTFb**	Mesp, **MyoR,** Hand, Paraxis, SCL, **NSCL,** Twist, PTFb, **PTFa**	
Atonal-class	Net-like,	Net, **Atonal-like/Atl1*, Atl2, orphans1,2,3,4**	Net, Atl, **Neurogenin, NeuroD, Beta3, Olig, Mist, Delilah**	
Achaete-Scute	Asc-like	**ASCa***°**, ASCb**	ASCa, ASCb	
Nuclear Receptors	HNF4	**COUP-TF***°**, RXR**$^{€}$**,** other families ?	Type II: HNF4(2A), RXR(2B), TR2/4(2C), Tll(2D), COUP-TF(2E); Type I: Rev-erb, THR, RAR, Ftzf	
C2H2 Zinc finger	?	**Zic**°**, Gli, snail,** other families ?	Zic/Odd, Gli, KLF, SP, Wilms' tumour, Huckebein, Snail, Ovo, Spalt, Blimp-1, Fez	
bZIP	CREBZF, Jun, Xbp1, ATF2^{T}, CREB^{T}, CREM^{T}, NFE2^{T},	**PAR, Oasis-b, ATF6,** CREB*°**,** Xbp1, **S-Maf,** NFE2, Jun, **ATF4, ATF3,** ATF2,	PAR, **E4BP4,** Oasis, Oasis-b, ATF6, CREB, Xbp1, S-Maf, **L-Maf,** NFE2, **Bach,** Jun, ATF4, Fos, ATF3, BATF, C/EBP, **c/EBP-g,**	
SOX/TCF	SoxB, SoxC, SoxF, TCF	SoxB, SoxC, SoxE, SoxF, TCF^{A}	**SoxA,** SoxB, SoxC, **SoxD,** SoxE, SoxF, **SoxG, SoxH, SoxI, SoxJ,** TCF	
FOX	FoxD, proFox, FoxG, FoxL2, Fox1, FoxP, FoxJ	**FoxA, B, A/B, C,** D,**E,** ~~FoxG,~~ **FoxK,** ~~FoxJ,~~ FoxL2, **FoxN2/3, FoxO,** ~~FoxP,~~ **FoxQ2**	FoxA, B, A/B, C, D, E, **FoxF,** FoxG, J, K, **FoxL1,** FoxL2, **FoxN1/4,** FoxP, Q2,	
MADS box	Mef2	Mef2, **SRF°**	type I (Srf), type II (Mef2)	
ETS box	Ets	Ets-domain gene families ?	Ets-domain gene families ?	
T-box	Bra, Tbx1, Tbx4/5, Tbx2/3^{T}	Bra1^{A}, Tbx4/5, Tbx2/3, Tbx1/10, **Tbx15, Tbx20**	Bra, **Tbr,** Tbx4/5, 2/3, 1/10, 15/18/22, 20, **Tbx6**	
Runx/CBFbeta	Runx, CBFbeta	Runx, CBFbeta	Runx, CBFbeta	

FIGURE 6: Early diversification of the regulatory gene families involved in neurogenesis in bilaterians.
The candidate regulatory genes of the cnidarian nervous system are underlined and the cell lineage is indicated when expression and/or functional data are available: neurogenesis (*), nematogenesis (°), eye differentiation (€). The signaling pathways and transcription factors present in poriferans and/or placozoans (*Trichoplax* with gene names followed by a T when not detected in Porifera) represent gene families that emerged in the last common ancestor of metazoans or even earlier. The gene families that emerged later, either in eumetazoans or in urbilaterians are written bold in the respective columns; the gene families possibly lost in cnidarians or in urbilaterians are stricken. Note that, except for the Atonal-class, the wave of duplications from which arose all modern gene families occurred in the last common ancestor of cnidarians and bilaterians. For references see the text. The cnidarian apical pole surrounding the mouth opening can be considered as a primitive head as the nerve net is denser in that region and forms in some species the nerve ring. In urbilaterians the neuronal cell populations diversified and a new cell type appeared: the glial cells (Reichenbach and Pannicke, 2008). These cell types became highly connected, organized in hierarchical networks, forming the central and peripheral nervous systems. The acquisition of a myelin sheath is a vertebrate innovation that allowed a faster speed for synaptic transmission in large size animals (Zalc et al., 2008).

analyses are required to confirm this statement. Evidences for a neurogenic function were only obtained for the *cnox2/Anthox2/Gsx* paraHox gene.

Gsx, a regulator of nematogenic and neurogenic precursors: *Gsx* genes belong to the ParaHox gene cluster (Brooke et al., 1998) and in phylogenetic analyses group together with the *Pdx/PG2/PG3* gene families (Quiquand et al., 2009). In *Drosophila* embryos, the *Gsx* ortholog named *ind* is expressed as a longitudinal band in the intermediate neuroectoderm where it promotes activation of proneural genes in the specific set of neuroblasts (Weiss et al., 1998). The *cnox-2/Gsx* gene family is currently the most widely studied in cnidarians (Schierwater et al., 2002; Finnerty et al., 2003) and its regulation has been documented in *Hydra* (Schummer et

al., 1992; Gauchat et al., 2000; Miljkovic-Licina et al., 2007), *Hydractinia* (Cartwright et al., 1999), *Podocoryne* (Yanze et al., 2001), *Clytia* (Chiori et al., 2009; Quiquand et al., 2009); *Acropora* (Hayward et al., 2001; de Jong et al., 2006) and *Nematostella* (Finnerty et al., 2003). In anthozoans, *cnox-2 Am* expressing cells display a neuronal morphology and are restricted to the oral pole of the larva; in the developing *Nematostella* planula (swimming larva), *Gsx* is expressed in the future head region. In the *Podocoryne* and *Clytia* larvae, early zygotic *Gsx* transcripts are initially localised in the anterior endoderm before extending towards the posterior pole, i.e. the future head region.

In the *Hydra* adult polyp, *cnox-2* is expressed in the head region and along the body column in a subset of

neuronal cells in the apical region (Figure 7A) and in dividing interstitial cells and clusters of nematoblasts in the body column (Figure 7B). During head regeneration, these two types of *cnox-2* expression are submitted to opposite regulations: in head-regenerating tips induction of *cnox-2* expression is observed from 24 hours post-amputation (hpa), first in proliferating neuronal cells then in *de novo* differentiated neurons, whereas *cnox2* expression in nematoblasts vanishes soon after amputation (Miljkovic-Licina et al., 2007). Hence the two *cnox-2* expressing cell populations respond differently to the signals propagated during head regeneration. Interestingly, the regulation detected in the neuronal apical cells during head formation in *Hydra* correlates well with that observed during larval development in *Podocoryne, Clytia, Acropora* and *Nematostella*. Moreover in *cnox-2(RNAi)* knocked-down *Hydra*, the apical nerve net is not maintained in adult polyps and head regeneration is significantly delayed (Figure 7C), suggesting a contribution of *cnox-2* progenitors and/or neurons in the head patterning process (Miljkovic-Licina et al., 2007). In the *Clytia* medusa, *Gsx* is expressed in the tentacle bulbs, proximally in clustered interstitial cells and more distally in neurons (Figure 5D) as reported by (Chiori et al., 2009). The same group also reported about a *Cdx* ortholog expressed in differentiating nematoblasts in the tentacle bulb. These data definitely support a role for the cnidarian ParaHox genes in the regulation of the nervous system.

The PRD-class genes as regulators of nematogenesis, neurogenesis and eye differentiation

The PAIRED-class (PRD-class) gene families distribute into three main sub-classes : the *paired-like* genes, the *Otx*-related genes and the *Pax* genes (Galliot et al., 1999). Most PRD-class gene families carry out neurogenic functions in bilaterians. Twenty bilaterian PRD-class gene families do have representatives in cnidarians (Galliot et al., 1999; Ryan et al., 2006) whereas the sponge *Amphimedon* genome contains eight *paired-like* genes and a single *Pax* gene but no *Otx*-related genes (Larroux et al., 2008). In developing and adult cnidarians, most *paired-like* and *Pax* gene families exhibit regulations that suggest a specific role during neurogenesis.

The aristaless-like paired-like gene, *prdl-a* is expressed as a regulator of neurogenesis. In intact *Hydra* polyps, *Prdl-a* is predominantly expressed in neuronal precursors and sensory neurons located in the most apical region (Figure 7A), being overexpressed in multiheaded mutants (Gauchat et al., 1998). However, during the early stages of head regeneration and budding, *prdl-a* is transiently expressed in a distinct cell lineage, the endodermal myoepithelial cells located in the presumptive head region. This transient wave of endodermal expression occurs concomittantly with the raise in organiser activity detected in the regenerating stump by transplantation experiments (MacWilliams, 1983). Subsequently *prdl-a* is reexpressed in the differentiating neurons of the presumptive head region (Gauchat et al., 1998; Miljkovic-Licina et al., 2004). This biphasic mode of expression observed during patterning processes is highly reminiscent of that displayed by the vertebrate paired-like genes *Hesx1/Rpx, Otx2* and *Gsc* during early mouse development (Thomas and Beddington, 1996; Rhinn et al., 1998): these genes that support early head patterning in the embryo, are expressed as two successive waves, a first one in the

anterior visceral endoderm / hypoblast that induces a second one in the sus-jacent neurectoderm of the rostral region (Foley and Stern, 2001). This similarity suggested an ancient commitment of « neurogenic » paired-like genes in apical/anterior nervous system patterning (Galliot and Miller, 2000).

The aristaless-like paired-like gene *prdl-b*: In contrast to *prdl-a*, *prdl-b* is expressed in proliferating nematoblasts and in a subset of neurons in the gastric region but is not expressed during patterning processes (Gauchat et al., 2004; Miljkovic-Licina et al., 2004). These data suggest that some cnidarian paired-like genes like *prdl-a* already exhibit two separate functions with distinct regulations, one during maintenance of apical neurogenesis in homeostatic conditions and another during patterning processes, whereas others as *prdl-b* would be restricted to neuronal differentiation in homeostatic conditions.

***Goosecoid (Gsc)* in *Hydra* apical sensory neurons:** The *Hydra goosecoid* homolog, *CnGsc*, is expressed in the adult polyp in sensory neurons of the hypostome but also in endodermal epithelial cells at the base of tentacles and along the body column (Broun et al., 1999). During patterning processes, *CnGsc* is first repressed in the regenerating stump or growing bud, and reexpressed at later stages in the presumptive head region, suggesting that it is not involved in the organizer activity that drives head regeneration. However, when expressed in *Xenopus* embryos, *CnGsc* exhibits organiser activity (Broun et al., 1999). A single *Gsc* ortholog is present in *Nematostella* (Ryan et al., 2006) but its regulation and function are currently unknown.

The *Rx* and *repo*-related genes: In *Nematostella* *NvRx1* the ortholog of the Retinal homeobox gene *Rx* that is expressed upstream of *Pax6* during eye development in vertebrates, is expressed in scattered ectodermal neuronal-like cells in planula and polyps, suggesting a role in the specification of a neuronal subset (Matus et al., 2007a). Similarly, *NvRepo* the ortholog of the glial-specific paired-like gene *Repo* is expressed at the oral nerve ring in planula and polyps (Marlow et al., 2009).

The cnidarian *Otx*-related genes are expressed as putative regulators of morphogenetic movements and of the nerve ring. Among PRD-class genes, *Otx/Otd* orthologs were identified in *Podocoryne, Hydra* and *Nematostella* however their neurogenic function is currently doubtful. In *Podocoryne* and *Hydra Otx* genes are likely involved in cell migration, like that observed during the budding process (Muller et al., 1999; Smith et al., 1999). However in the *Hydra* polyp *Otx* is also expressed in the neurogenic zones, i.e. in the tentacle zone and along the body column, but was not detected in neurons (Smith et al., 1999). In *Podocoryne, Otx* expression is actually initiated during the budding process and maintained in the striated muscle cells of the medusa but was not detected during planula development (Muller et al., 1999).

In anthozoans two *Otx* genes were identified in the coral *Acropora* and three clustered ones in the *Nematostella* although with unclear phylogenetic relationships between these paralogs (de Jong et al., 2006; Mazza et al., 2007). The three *Nematostella Otx* genes show a very similar biphasic expression pattern, with a first wave during gastrulation, restricted to the earliest involuting endoderm that rapidly occupies the aboral region, and a later wave at the oral pole, detected as an endodermal pharyngeal

ring surrounding the presumptive mouth and in the first developing tentacles (Mazza et al., 2007). In *Acropora*, the two *Otx* genes exhibit distinct regulations, with *OtxA* predominantly ectodermal, also detected as a ring at the oral pole and along the body column in scattered cells, and *OtxB,* endodermal throughout development (de Jong et al., 2006). Thus a common trait for the *Otx* anthozoan genes would be their expression at a place and at a time when the nerve ring forms. If confirmed, this would suggest that the *Otx* function in apical/anterior neuronal patterning emerged in the CBA.

The *Pax* genes as regulators of neurogenesis and eye differentiation in cnidarians: In bilaterians the *Pax* gene families that likely derive from five urbilaterian ancestors, play a critical role in neurogenesis, eye development as well as myogenesis, segmentation and organogenesis. The identification of a single *Pax* gene in Porifera and of three *Pax* families in Cnidaria proved that *Pax* genes were submitted to an early wave of gene duplications likely after the divergence of Porifera (Hoshiyama et al., 2007; Matus et al., 2007a; Larroux et al., 2008). The anthozoans express four *Pax* gene families that represent these three ancestral families: *Pax-A* and *Pax-C* for *Pox neuro, Pax-B* for *Pax2/5/8,* and *Pax-D* for *Pax3/7* (Catmull et al., 1998; Miller et al., 2000). By contrast hydrozoans have lost the *Pax3/7* ortholog and only express *Pax-A* as *Pox neuro* ortholog and *Pax-B* related to *Pax2/5/8*. Therefore a definitive *Pax4/6* ortholog has not been found in cnidarians. However these cnidarian Pax proteins bind the consensus Paired-response elements with broader specificity than the mammalian ones, likely allowing more flexibility (Miller et al., 2000; Sun et al., 2001; Plaza et al., 2003).

The expression data suggests that *Pax* genes are already involved in neurogenesis in cnidarians as in *Nematostella*, the *PaxA/C* genes (*pox-neuro* related) are expressed in putative neuronal / spirocyte precursors and neural cell types (Matus et al., 2007a) similarly to the *Acropora PaxC* gene (Miller et al., 2000). The *Nematostella PaxB* is expressed in scattered ectodermal cells and around the oral region suggesting a role in the patterning of the nerve ring (Matus et al., 2007a). The *PaxD* genes are present as a single copy in *Acropora* but four in *Nematostella* where expression of only two could be detected. In both species the *PaxD* genes are expressed as stripes around the circumference of the embryo. In *Acropora* the *PaxDam* domain is first aboral and then oral after settlement (de Jong et al., 2006); in *Nematostella* the *NvPaxD1* domain corresponds to the upper body column region where interstitial stem cells generate specific cells for the oral region and the *NvPaxD3* domain is restricted to the tentacles (Matus et al., 2007a). Further studies should confirm the neurogenic function of *Pax* genes in anthozoans.

In medusozoans, the studies mostly focused on the role of *Pax* genes on eye differentiation in homologous (medusa) and heterologous (*Drosophila)* contexts. Interestingly the adult jellyfish *Tripedalia* expresses *Pax-B* exclusively in the rhopalia (Kozmik et al., 2003). In cell culture *PaxB* efficiently transactivates the *Tripedalia* crystallin promoters and the *Drosophila* rh6 rhodopsin; moreover *PaxB* can partially rescue the *spa* (*Pax-2*) phenotype and induce ectopic eye formation in *Drosophila* (Kozmik et al., 2003). These data suggest that cnidarian *Pax* genes can already regulate eye differentiation and possibly in scattered photosensing cells in cnidarian species that do not differentiate eyes.

The coral *PaxB* and *PaxD* genes cannot induce by themselves eye formation when expressed in *Drosophila* imaginal discs but can achieve such task when chimeric (Plaza et al., 2003). In the jellyfish *Podocoryne* that does not differentiate eyes, *Pax-B* is expressed in the early steps of neuronal cell differentiation (Groger et al., 2000). These data strongly speak for a neurogenic function for *Pax* genes that arose in the CBA, and further studies in *Trichoplax* (Hadrys et al., 2005) and Porifera (Larroux et al., 2006) should trace the ancestral function of *Pax* genes in cell specification. However the expression of *Pax* genes is clearly not restricted to the nervous system in cnidarians as *NvPaxC* appears to be expressed also in gland cells, *NvPaxB* at the endodermal/ectodermal boundary of the pharynx, and *Pc Pax-B* in the entocodon during medusa formation.

The *Six* genes and the eye differentiation in jellyfish: In contrast to the *Pax* genes, the *Six* gene families were already established in the CBA and remained highly conserved along cnidarian and bilaterian evolutions. A single *Six* gene was identified in Porifera, related to the *Six1/2-so* family (Hoshiyama et al., 2007) whereas three distinct families were identified in cnidarians: *Six1/2, Six3/6* and *Six4/5*, suggesting a wave of gene duplication that took place between Porifera and eumetazoans. In two hydrozoan medusae, *Podocoryne* (no eyes) and *Cladonema* (with eyes) these three families are likely involved in neurogenesis being expressed along the manubrium, in the nerve ring and/or the tentacle bulbs (Stierwald et al., 2004). Moreover the *Six1/2* and *Six3/6* genes are expressed in the eye cup, at low and high levels respectively (Stierwald et al., 2004). During eye regeneration *Six1/2* and *Six3/6* but not *Six4/5* are up-regulated very early, *Six1/2* preceding *Six3/6* suggesting that *Six1/2* is acting rather upstream in the cascade directing eye formation but is probably not required for eye maintenance. In the scyphozoan jellyfish *Aurelia* the *Six1/2* ortholog is also expressed in the rhopalia (Bebenek et al., 2004). Finally given the genetic interactions that occur between the *Pax* and *Six* genes during eye specification (but also for muscle specification and kidney differentiation) in bilaterians, one can speculate that this interaction was already at work in cnidarians (Hoshiyama et al., 2007). Hence several key components of the genetic circuitry driving eye specification in bilaterians are already available and properly regulated in cnidarians. However the same question remains disputed (Fernald, 2004; Kozmik et al., 2008): How to explain that the same gene regulatory network supports eye differentiation in a large variety of phyla ? Does it reflect a common origin for all the eyes across the phyla or rather a reiterated recruitment of the same regulatory network in distinct contexts ?

The bHLH genes as candidate regulators of neurogenesis and myogenesis in hydrozoans

As for many gene classes involved in developmental processes, the complement of basic Helix-loop-Helix (*bHLH*) genes was already established when Cnidaria arose and remained strikingly stable over the evolution (Simionato et al., 2007). The *bHLH* genes were initially characterized in genetic analyses as proneural genes, i.e. directing the ectodermal cells towards a neuronal fate : In *Drosophila*, the *achaete* and *scute* genes exhibit a proneural function in sensory organ formation (Jan and Jan, 1994), and the vertebrate orthologs play a similar proneural function during development (Bertrand et al., 2002). Surprisingly a sponge *bHLH* gene was recently shown to display proneuronal properties when expressed

in *Xenopus* or *Drosophila* (Richards et al., 2008). Hence pieces of the metazoan neurogenic circuitry predated the emergence of a nervous system.

The type A *achaete-scute* ortholog appears restricted to the nervous system in hydrozoans. The *Achaete-Scute (ASH)* genes distribute in two distinct classes, A and B. The A class is represented by four genes in *Drosophila* and *C. elegans*, two in mouse whereas the class B is absent in *Drosophila* but present in mouse, *C. elegans* and *Podocoryne* indicating an ancient duplication event (Ledent et al., 2002; Seipel et al., 2004c). In *Hydra* the type A ortholog, *CnASH*, is expressed in clusters of differentiating nematoblasts (Grens et al., 1995; Lindgens et al., 2004) and in sensory neurons at the base of tentacles (Hayakawa et al., 2004). When ectopically expressed in *Drosophila* instar larvae, *CnASH* led to the formation of ectopic sensory organs similarly to the *Drosophila* cognate genes when ectopically expressed; moreover a partial rescue was noted when *CnASH* was expressed in *achaete/scute* double mutants (Grens et al., 1995). In the jellyfish *Podocoryne carnea*, two Achaete/Scute genes were analyzed: the first one, *Ash1,* also related to class A, consistently showed an expression in differentiating nematocytes (Muller et al., 2003) whereas the second, *Ash2,* related to the class B, is likely involved in the differentiation of secretory cells (Seipel et al., 2004c). These data suggest that the neurogenic function of type A *ASH* genes is ancestral and conserved from cnidarians to bilaterians.

The Atonal-like gene *Atl1* is a candidate proneural gene in the jellyfish *Podocoryne*. In the developing jellyfish *Podocoryne,* an *Atonal-like* gene (*Atl1*) was found expressed in endodermal neuronal precursors, and in adulthood, in mechanosensory cells and neuronal precursors located in the tentacle bulbs and the manubrium. Moreover, when *in vitro* transdifferentiation is induced, *Atl1* is upregulated in proliferating neuronal precursors arising from adult striated muscle cells (Seipel et al., 2004c). Interestingly this study also mentions that during medusa budding, the striated muscle precursors in the entocodon expressed *Atl1*, highlighting the fact that neurogenesis and myogenesis that are supposed to share a common origin, indeed make use of common regulators.

The putative neurogenic function of the nuclear receptors RXR and COUP-TF

Nuclear receptors (NRs) are ligand-dependent transcription factors activated by steroid hormones and non-steroid molecules such as retinoic acid, thyroid hormone and vitamin D (Moras and Gronemeyer, 1998). However, some of the NRs are considered as "orphan", i.e. lack a well-identified ligand (Benoit et al., 2006). In cnidarians, a variety of nuclear receptors were characterized including a unique *COUP-TF* gene in *Hydra* but six in *Acropora,* a *FTZ-F1* gene in *Nematostella* and a *RXR* gene in *Nematostella* and *Tripedalia* (Kostrouch et al., 1998). Therefore, the NRs gene families diversified very early during evolution, before divergence of Cnidaria (Figure 6).

A putative function in eye differentiation for the nuclear receptor RXR: As in vertebrates, the *Tripedalia* RXR ortholog might also regulate the expression of the crystallin genes as it is predominantly expressed at the medusa stages and it specifically recognizes in vitro direct repeats identified in the crystallin gene promoter of this cubozoan jellyfish (Kostrouch et al., 1998). Moreover, similarly to its vertebrate cognates, the *Tripedalia* RXR transcription factor is potentially regulated by retinoic acid as it binds the 9-cis retinoic acid as a ligand. Further studies in hydrozoan and scyphozoan jellyfish should establish whether the RXR function is a common trait in cnidarian eye differentiation.

The neurogenic and nematogenic function of the nuclear receptor COUP-TF: In all bilaterian species the orphan *COUP-TF* genes were clearly associated with neurogenesis (Park et al., 2003). In mice, *COUP-TFI* disruption results in multiple defects of the central nervous system (Qiu et al., 1997) and together with *Pax6* and *Emx2*, it acts as an early intrinsic factor for early regionalisation of the neocortex (Zhou et al., 2001). Hence *COUP-TF* genes, which in most contexts behave as potent negative transcriptional regulators (Achatz et al., 1997), bring a major contribution to both neurogenesis and the CNS patterning during the embryonic life, as well as in neurophysiology of the adult nervous system (Pereira et al., 2000; Cooney et al., 2001). According to these data, neurogenesis is considered as the ancestral developmental function of *COUP-TF* genes whereas the vertebrate *COUP-TFII* gene seems to be devoted to mesenchymal-epithelial interactions during organogenesis (Pereira et al., 1999). In *Hydra*, *hyCOUP-TF* is expressed in few interstitial cells, in proliferating and differentiating nematoblasts, as well as in neurons of the body column (Gauchat et al., 2004). In the nematocyte pathway *hyCOUP-TF* actually seems to be turned on later than *prdl-b*, at a time when nematoblasts enter the differentiation phase (Fig. 4). In the neuronal cell lineage, *hyCOUP-TF* expressing cells correspond to a subset of small bipolar neurons. When animals were rendered "nerve-free", *hyCOUP-TF* expressing cells disappeared in few days. During budding and regeneration, *hyCOUP-TF* expression vanished in regions where either apical or basal differentiation occured (Gauchat et al., 2004). Moreover the *Hydra* hyCOUP-TF expressed in mammalian cell cultures can repress the transactivation induced by the RAR:RXR nuclear receptors. In summary, the *Hydra* hyCOUP-TF is supposed to promote the differentiation of both nematocytes and neurons, reflecting hence an ancestral neurogenic function for the *COUP-TF* NR family.

Various transcription factor families involved in neurogenesis

The neurogenic function of the C2H2 zinc-finger transcription factors, Zic, Gli: The Zic and Gli transcription factors form two highly related evolutionarily-conserved families that interact with each other and bind their target sequences thanks to their C2H2 zinc finger domains (Aruga et al., 2006). In vertebrates, ascidians and nematode, *Zic* genes exert multiple functions during neuronal development including early neural patterning in vertebrates as evidenced by the region-specific morphogenetic alterations of the central nervous system induced upon inactivation of the different *Zic* genes in mice (Aruga, 2004; Merzdorf, 2007). However, they also specify mesodermal derivatives as in amphioxus and ascidians (Gostling and Shimeld, 2003) and the *Drosophila Zic* ortholog *odd-paired* is a segmentation gene not involved in neuronal differentiation. In *Hydra* a *HyZic* gene is expressed in the nematocyte lineage where it is turned on during the first synchronous divisions of nematoblasts and off at the final

runs of division, before differentiation of mature nematocytes occurs (Lindgens et al., 2004). In nerve-free animals *Hyzic* expression is rapidly turned off supporting the hypothesis that *Hyzic* function is restricted to the early stages of nematocyte differentiation; moreover in *cnox-2(RNAi)* hydra *Hyzic* expression is abolished (Figure 7D), suggesting that *Hyzic* is directly or indirectly regulated by the *Gsx* ortholog (Miljkovic-Licina et al., 2007). Further studies in anthozoan and medusozoan species should confirm and refine the neurogenic function of *Zic* genes, i.e. restricted or not to nematocyte differentiation, and involved or not in patterning of the cnidarian nervous system.

The Sox/TCF transcription factors in cnidarians: The *Sox* genes encode High Mobility Group (HMG) transcription factors that are already present in choanoflagellates and sponges (King et al., 2008; Larroux et al., 2008) and diversified early in metazoan evolution with representatives of the groups B, C and F in sponges, B, C, E and F in cnidarians and ctenophores (Magie et al., 2005; Jager et al., 2006; Jager et al., 2008; Shinzato et al., 2008). In bilaterians *Sox* genes are involved in germ cell specification, mesendodermal patterning, neural induction, development of the central and peripheral nervous systems and organogenesis (Guth and Wegner, 2008). In *Nematostella, Acropora* and *Clytia*, 14, 6 and 10 *Sox* genes respectively were identified (Figure 6); among those, the expression patterns of the *Acropora* and *Nematostella SoxC* orthologs suggest some role in the specification of the ectodermal sensory neurons during development (Table 1). *Nematostella* and *SoxB2* genes in ectodermal neuronal-like cells in developing *Nematostella* suggest some ancestral neurogenic function. Similarly the expression of the *SoxB* and *SoxE* genes in the neuro-sensory structures of the adult ctenophore combjelly *Pleurobrachia* evoke some role in the maintenance of the nervous system (Jager et al., 2008). Consequently some *Sox* genes might have been recruited at the time of the emergence of the nervous system.

The Fox and MADS box transcription factors: The *Fox* genes encode transcription factors that bind DNA thanks to their winged-helix domain and are involved in the development of the nervous system in deuterostomes (Mazet and Shimeld, 2002; Mazet et al., 2005). Among the 20 families identified in bilaterians, at least 6 families already diversified in sponges whereas 9 new families appeared in cnidarians and only 3 in urbilaterians (Magie et al., 2005; Larroux et al., 2006; Chevalier et al., 2006; Larroux et al., 2008). In *Clytia* two of these gene families already diversified, the *FoxB* gene being expressed in numerous places where neurogenesis takes place including in the sensory organs named statocysts that develop along the bell rim (Chevalier et al., 2006). As FoxB genes are implicated in neurogenesis in bilaterians, these data suggest an evolutionarily-conserved function in neurogenesis for some of the *Fox* families. In *Hydra*, *Budhead*, a fork head/HNF3 ortholog rather appears involved in apical specification (Martinez et al., 1997). Concerning the MADS box transcription factors, the expression of the *Nematostella Mef2* gene is consistent with a role in the differentiation of ectodermal cell types including nematocytes and neurons (Martindale et al., 2004) whereas the *Hydra SRF* ortholog possibly plays a similar function in the interstitial cell precursors and nematoblasts (Hoffmann and Kroiher, 2001).

The basic leucin zipper (b-ZIP) CREB transcription factor. The bZIP transcription factors, defined by the presence of a basic domain followed by a leucine zipper domain involved in DNA-binding and dimerization respectively, form a large class of transcription factors that can be traced in fungi, plants and animals. In bilaterians, this class is formed of 19 families, 13 of them being already expressed in cnidarians (Figure 4). Phylogenetic analyses including cnidarian sequences indeed concluded that an early wave of gene duplications took place in the last common-CBA (Amoutzias et al., 2007). In bilaterians, the CREB transcription factor that is targetted by a wide variety of stimulus, regulates multiple developmental and physiological processes including neuron survival and neuron degeneration (Mantamadiotis et al., 2002), nervous development, learning and memory (Lonze and Ginty, 2002). In *Drosophila*, *Aplysia*, rats and mice, CREB-dependent transcription is required for synaptic plasticity and learning and memory processes, more specifically for the transition from short-term to long-term memory, suggesting that CREB is an universal modulator of memory in bilaterians (Barco et al., 2006). In *Hydra*, the CREB transcription factor was initially identified as a key regulator of the early stage of head regeneration (Galliot et al., 1995; Kaloulis et al., 2004). However, the CREB protein was also detected at strong levels in proliferating progenitors, including progenitors for the nematocyte and neuronal cell lineages, as well as mature nematocytes, ganglion and bipolar sensory neurons (Chera et al., 2007). In hydrozoan medusae CREB might play a similar role, being strongly expressed in differentiating neurons in the tentacle bulbs (Figure 5E, here *Podocoryne*). Future work should tell us more about the various functions of CREB in cnidarian nervous systems.

The *Runx* and *CBPβ* genes in *Nematostella* neurogenesis: The DNA-binding of the Runx transcription factors is enhanced upon heterodimerization, specially with *CBPβ* when co-expressed. These two gene families appears to form a rather stable old couple, already present in Porifera and Cnidaria, which did not diversify prior to the emergence of bilaterians (Sullivan et al., 2008). In the adult *Nematostella*, *Runx* and *CBPβ* are expressed in putative neurons and neural precursors in the tentacles, in scattered ectodermal cells along the body column and in case of *CBPβ*, also in the mouth and upper pharynx. Detailed histological analyses suggest that these two genes are often co-expressed and participate in the differentiation and maintenance of the apical nervous system (Sullivan et al., 2008).

3.- What role for the phylum-specific genes in neurogenesis and nematogenesis?

Two types of molecules are considered as putative phylum-specific actors, first the bioactive peptides, neuropeptides or epitheliopeptides that are often evolutionarily-conserved but most probably play phylum-specific roles in differentiation and developmental processes, and second the phylum-specific genes. Recently a peptide-gated Na-channel was found expressed at the root of *Hydra* tentacles, suggesting that fast transmission through neuropeptides already existed in ancient nervous systems (Golubovic et al., 2007). The
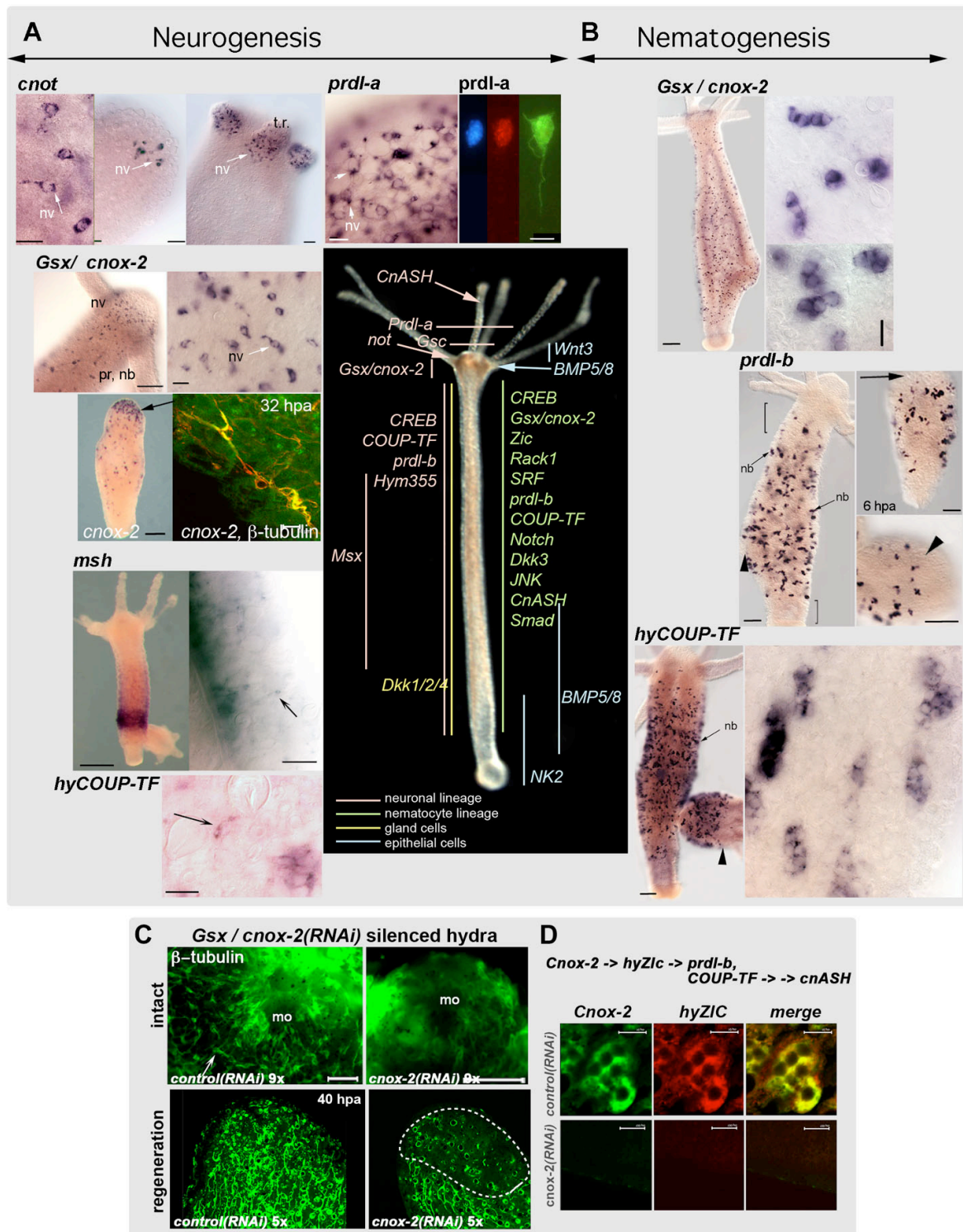
FIGURE 7 : Regulatory genes involved in neurogenesis in bilaterians likely support neurogenesis and nematogenesis in *Hydra* polyps.
A) MARKERS of NEUROGENESIS: ***Cnot)*** Neurons (nv) located at the root of tentacles in the adult polyp (left) and in the spots where tentacle rudiments (t.r.) emerge during budding (middle) and head regeneration (right, here at 52 hpa), express the ANTP-class homeogene *cnot*. ***Prdl-a)*** Sensory neurons and their progenitors located at the apical pole (top view) express the paired-like homeogenes *prdl-a* (white arrows). Right panel: macerated head tissues stained with Hoechst (blue), anti-prdla (red) and anti α-tubulin (green). ***Gsx/cnox-2)*** Progenitors and apical neurons (nv, white arrow) located at the base of the head region express the ParaHox homeogene *Gsx/cnox-2*. During head regeneration, *cnox-2* is up-regulated in proliferating precursors and neurons that differentiate in the regenerating tip, shown here at 32 hpa (arrow). Right panel: Apical neurons co-expressing *cnox-2* transcripts (green) and β-tubulin (red). ***Msx)*** Neurons of the body column express the ANTP-class homeobox *msx* gene. *Msx+* neurons are denser in the budding zone and restricted to the ectodermal layer (black arrow). ***HyCOUP-TF, prdl-b)*** A subset of sensory neurons in the body column express *Hy-COUP-TF* and the paired-like homeogene *prdl-b* (not shown). B) MARKERS of NEMATOGENESIS: *Gsx/cnox-2, hyCOUP-TF* and *prdl-b* are expressed in synchronously dividing nematoblasts (nb, thin black arrows) along the body column. Both *hyCOUP-TF* and *prdl-b* genes are repressed in the adult apical and basal regions (brackets) but also in the presumptive head region during budding (arrowheads) and head-regeneration (large arrow). These genes are expressed at distinct stages along the nematocyte pathway, with *Gsx/cnox2* transcripts detected in precursors and *hyCOUP-TF* expressed in nematoblast clusters that start differentiating. Scale bars: 100 μm and 10 μm. For references see in the text. C) Silencing of *Gsx/cnox-2* through RNAi leads to alterations of neurogenesis after repeated exposures to dsRNAs: In intact *Hydra* the apical nerve net is no longer visible (upper panels); after amputation (lower panels) the *de-novo* neurogenesis normally observed in head-regenerating tips (left, here at 40 hpa) is drastically reduced (outline, right). mo: mouth opening. Scale bars: 50 μm. D) Putative epistatic relationships in *Hydra* nematogenesis deduced from studies performed by (Lindgens et al., 2004; Miljkovic-Licina et al., 2007). *Gsx/cnox-2* regulates directly or indirectly *HyZIC* expression in proliferating nematoblasts: note the complete disappearance of *HyZIC* transcripts (red) in *cnox-2(RNAi)* silenced cells (green). Scale bar: 10 μm.

neurogenic function of peptides is well known in cnidarians. Neuropeptides can modulate muscle activity (Grimmelikhuijzen et al., 1996) as hym-176 that triggers contraction of the ectodermal myoepithelial cells in *Hydra* (Yum et al., 1998). In fact the (eyeless) *Hydractinia* planulae exhibit dramatically increased phototaxis when exposed to RFamide indicating that RFamides, expressed by the neurosecretory cells in all cnidarian species, whatever the stage of the life cycle, can modulate the non-visual behavior (Plickert and Schneider, 2004). The authors propose that the RFamide cells act as interneurons between photosensing cells and myoepithelial cells.

Beside physiological activity, neuropeptides can trigger or enhance neuronal differentiation as hym-355 (Takahashi et al., 2000), or head activator that also affects cell proliferation and head patterning processes (Schaller et al., 1989; Hobmayer et al., 1997). Similarly the neuropeptides LW-amides play a pivotal role in developmental processes as larval metamorphosis in *Hydra*ctinia (Plickert et al., 2003). Interestingly neuronal differentiation is also under the control of epithelial cells as among peptides that were identified in the systematic *Hydra* peptide project (Fujisawa, 2008), the epitheliopeptides belonging to the LPW family can inhibit nerve cell differentiation (Koizumi, 2002). In bilaterians, peptides are best known as neurotransmitters or hormonal regulators involved in physiological processes (Boonen et al., 2009); hence homologous functions in cell differentiation or developmental processes remain to be deciphered. As nematogenesis is a cnidarian-specific process, genes that trigger nematocyte differentiation are frequently phylum-specific. A microarray analysis identified 51 genes as nematocyte-specific, most of them encoding putative secreted proteins expressed at distinct stages of the pathway (Hwang et al., 2007). Out of these 82% do not have bilaterian orthologs implying that beside conserved regulatory genes, nematocyte differentiation makes use of a large proportion of genes, which were not retained in bilaterians and whose origin could not be traced back so far.

A tentative integrative view of the early evolution of neurogenesis

Common molecular tools for the specification of the cnidarian and bilaterian nervous systems

Several criteria support a common origin for neurogenesis in cnidarians, ctenophores and bilaterians: first the presence in non-bilaterian phyla of gene families orthologous to those that encode transcription factors with neurogenic functions shared by protostomes and deuterostomes, second their consistent cellular expression patterns in the cnidarian nervous system or during its differentiation, third the loss and gain of function assays that affect the maintenance or the differentiation of the cnidarian nervous system (even though only few gene families were tested so far) and fourth the heterologous assays that proved that the cnidarian genes can affect neurogenesis when expressed in bilaterian developmental contexts. The functional dissection of the genetic cascades regulating the differentiation of the nervous system in cnidarians and ctenophores was only recently launched but the expression analyses currently available indicate that the ANTP-class (*not, msx, Gsx*), PRD-class (*Pax, paired-like, Rx, repo, gsc, six*), bHLH-class (*Achaete scute, atonal*), HMG-group (*Sox2, SoxB2, SoxB, SoxE*), winged-helix

group (*FoxB*), MADS-box class (*Mef2*), zinc fingers (*zic*), nuclear receptors (*COUP-TF, RXR), Runx/CBPβ* and bZIP (*CREB*) transcription factors likely regulate neuronal differentiation since early eumetazoan evolution (for references see above), similarly to the *bHLH* family members in myogenesis (Muller et al., 2003; Seipel et al., 2004c). The reiterated and independent recruitment of orthologous genes and signaling pathways to perform similar functions in various phyla cannot be excluded and is actually discussed concerning eye evolution (Kozmik et al., 2008; Suga et al., 2008), but does not represent a parsimonious scenario.

What roles for the "neurogenic" genes in Porifera, a non-neuronal phylum?

The evolutionarily-conserved regulatory genes expressed in nematocytes and neuronal cells were in most cases identified in poriferans: the genes for the ANTP, Pax, POU, LIM-HD, Sox, nuclear receptor, Fox (forkhead), T-box, Mef2, Ets and bHLH transcription factors emerged and in many instances already diversified prior to the Porifera/eumetazoan split (Larroux et al., 2006; Jager et al., 2006; Richards et al., 2008). Similarly the main signaling pathways Wnt, TGF-β, RTK, Notch, Hedgehog, and Jak-Stat as well as the adhesion molecules are present in sponges (Nichols et al., 2006). Moreover the modulated expression of the bHLH (Richards et al., 2008) and NK-type (Gazave et al., 2008) genes during embryogenesis suggest a possible role in cell differentiation and region specification. This surprising finding according to which the origin of the neuronal genetic circuitry predated the occurence of nerve cell differentiation, is intriguing. In fact the choanocytes were proposed to exert some sensory function, and as such might represent a proto-neuronal cell type (Gazave et al., 2008). Therefore, these gene families, which likely constitute the hallmark of metazoans, might already be committed to a "proto-neuronal" function. Alternatively, they might specify cell fate independently of their ability to differentiate mechanoreceptor and/or nerve cells. Two types of arguments can be proposed to explain the absence of neurogenic "success" for the poriferan "neurogenic" genes: firstly the absence of some essential neurogenic genes explaining that the genetic circuitry cannot be mounted properly; among those missing genes the *ParaHox/Hox-like* genes were never identified in Poriferans and we saw that *Gsx/cnox-2* plays an essential role in the regulation of neuronal precursors in *Hydra* as well as in bilaterians, secondly the absence of some target structures, i.e. myofibers, might make the organization of a rudimentary genetic circuitry useless. However one cannot rule out the possibility that these genes represent a genuine neurogenic program originally acquired by the metazoan ancestor and secondarily lost in poriferans.

The early diversification of the regulatory gene families in eumetazoans and the emergence of neurogenesis in the Cnidaria-Bilateria ancestor

Interestingly most of these putative neurogenic gene families that encode transcription factors underwent an early wave of amplification in the last common Cnidaria Bilateria ancestor (Figure 6). This event likely preceded the emergence of the nervous system as evidenced by the few representatives present in sponges versus the large number of cnidarian-bilaterian orthologous families belonging to the Homeobox, bHLH, bZIP, Wnt classes. Moreover the comparative analysis of the transcription

factor classes between one poriferan and two cnidarian genomes confirmed that several classes that exhibit an evolutionarily-conserved role in neurogenesis in bilaterians have emerged after the divergence of Porifera. This is the case of the *Hox/ParaHox, Otx-like, Atonal/Twist* gene families that are obvious candidates for having conducted the emergence of the nervous system. As the bilaterian orthologs also regulate neurogenic functions, we speculate that the combination of transcription factors that drove neurogenesis in the Cnidaria-Bilateria ancestor was iteratively used along evolution. In a limited number of cases, these "eumetazoan" families underwent a secondary wave of duplications after the divergence of cnidarians, in urbilaterians, suggesting that the duplication of genes previously recruited for neurogenesis, led to the complexification of the neuronal structures thanks to paralogous genes.

Symbiosis might have contributed to the emergence of sophisticated sensing tools

Our current knowledge about the molecular tools supporting the various cellular differentiation pathways in distinct animal phyla tremendously increased over the past ten years with genomic sequencing, extended phylogenetic analyses of gene classes, cellular expression and functional analyses. This vast bag of informations allowed us to see emerging principles for understanding cell type specification. Detlev Arendt recently proposed three common principles that applied all along animal evolution to homologous cell types and sister cells (Arendt, 2008), 1) *the multifunctionality of ancestral cells* as the myoepithelial cnidarian cells that carry out epithelial functions but also muscular contraction and electric conduction (Mackie and Passano, 1968), 2) *the progressive segregation of the ancestral functions* in more specialized cell types that abandon some of the ancestral functions by silencing the corresponding genetic functions, to carry out a more limited number of functions, 3) *the divergence of some functions* thanks to the duplication of the molecular tools such as the amplification of gene families within a given class.

The current set of data concerning neurogenesis in non-bilaterian species certainly obey these principles of linear diversification of cell types but the analysis of the origin and early evolution of neurogenesis also requires to take into account decisive processes that might constitute a fourth principle: *the incorporation of foreign genetic material*, either through horizontal gene transfer or through symbiosis. Few cases are currently documented but these certainly deserve attention. The analysis of the respective behaviors of the different cellular contingents in *Hydra* actually led to the proposal that the venom capsule (named nematocyst or cnidocyst) in nematocytes would result from a symbiogenetic process (Shostak, 1993). More recently Denker et al. showed that the horizontal transfer of a bacterial gene encoding a subunit of bacterial poly-γ-glutamate (PGA) synthase in the genome of the cnidarian ancestor might have been decisive for the specification of the nematocyte weapon, the cnidocyst (Denker et al., 2008a). In fact only the receptor part of this highly sophisticated cell, the cnidocil, might be retained along evolution, sharing typical features with other mechano-sensory cells (Holstein and Hausmann, 1988), while structures homologous to the nematocyst capsule were not identified in other animal phyla so far, indicating that phylum-specific innovations

might also stand alone, even when they contributed to the sustained evolutionary success of the phylum where they arose.

The second case where the importation of foreign material might have been decisive, concerns the specification of the eyes that combine photoreceptor and pigment cells since their origin (Gehring, 2004). As photoreception is widely distributed among living organisms, including in bacteria, Walter Gehring proposed that photoreception in the cnidarian ancestor might result from a series of symbiotic transfers, a cyanobacteria into a red algae, a red algae into a dinoflagellate, the transformation of the chloroplast into an eye inside the dinoflagellate and finally the transfer of the dinoglagellate into a cnidarian. A long way to go before seeing, but that certainly highlights the potency of such mechanisms to bring novelties in organisms that were less constrained than most bilaterian species.

What was the proto-neuronal cell from which nerve cells evolved ?

If we assume that sharing molecular signatures signify a common history, then the nerve and muscle cells should be considered as sister cells in cnidarians. In fact members of the *Six, Pax, bHLH, Sox, Pou* gene classes are involved in both neurogenesis and myogenesis in bilaterians. In the *Podocoryne* jellyfish where both differentiation pathways were monitored during medusa budding and induced transdifferentiation, the analysis of the *Six, C/EBP, MafL, Atonal like 1, Achaete-scute 2* genes (Table 1) as well as the observation of the transient expression of neuronal markers during myogenesis (Seipel et al., 2004b; Seipel et al., 2004c; Stierwald et al., 2004) suggested that muscle cells and nerve cells derive from common myoepithelial cells. These molecular data actually fit with the three steps model proposed by George Mackie for the origin of neuromuscular transmission (Mackie, 1970) whereby muscle cells and nerve cells would have diverged from myoepithelial cells (see Figure 5a in Arendt, 2008). The scenario is as follows: Starting from a primordial myoepithelium capable of "neuroid" conduction, the protomyocytes progressively detached from the basal side of the myoepithelium to sink into the interior; at the second step protoneurons evolve from the myoepithelium to connect the myocytes to the outside forming a group of electrically interconnected cells; at the third step, neurosensory cells and neurons evolved from the protoneurons, developing long processes that connected them to each other and to the myocytes by chemical polarized junctions. This scenario that is largely driven by the electrophysiological properties of the different cell types, is coherent and attractive. However it predicts that the origin of the neurosensory cells followed the emergence of protoneurons. In fact sensory-like cells appear to have predated by far the origin of the nervous system, already present in early metazoans as poriferans where they seem to use a proto-neural program, the Notch/Delta and bHLH pathway to differentiate (Richards et al., 2008). These results suggest that the program leading to the emergence of neurogenesis was multilayered, a pre-program being already available in the different cell types of the last common metazoan ancestor, therefore the differentiation of neurons could have arisen from myoepithelia as well as from sensory cells.

CONCLUSIONS & PERSPECTIVES

Most if not all the pieces of the puzzle that regulates the nervous system in bilaterians are present in cnidarians, but given the almost complete absence of functional analysis, the question of how these different pieces interact in cnidarians remains completely open. There are nevertheless clear differences between neurogenesis in cnidarians and bilaterians as for instance the origin of the neuronal precursors during early development: in cnidarians those were identified in the endoderm, from where they migrate towards the ectodermal layer, showing hence a major difference with bilaterians where partitioning of the ectoderm into neural and non-neural portions during early embryogenesis is the first event. However, this cnidarian specificty might be revisited as such migration was not observed in developing scyphozoans (Nakanishi et al., 2008). The fact that in some hydrozoan species the destruction or removal of the interstitial stem cells results in the elimination of the sensory-motor, ganglion and nematocyte cell lineages but not of the ectodermal sensory cells would be consistent with this ectodermal neurogenic potential (Martin and Thomas, 1981; Thomas, 1987).

Also some genes that were expected to be universal regulators of neurogenesis appear missing; the best example is *engrailed* that is apparently not expressed in cnidarian genomes. Also some key genes for brain patterning in protostomes and deuterostomes appear to be submitted to looser constraints in cnidarians, as *Otx* that might support cell migration in developmental processes in hydrozoans, but be required for the formation of the nerve ring in *Nematostella*. Also some genes provide inconsistent expression patterns between different cnidarian species, making difficult to identify the common themes among these developmental variations. Sampling more taxa in Cnidaria and Ctenophora will help unravel the core genetic mechanisms that allowed the emergence of neurogenesis and neuromuscular transmission.

The identification of epistatic interactions between cnidarian regulatory genes is just embryonic. However, at least two candidate gene regulatory networks might be shared by cnidarians and bilaterians: one involved in neurogenesis along the axis, including the three ANTP-class genes *msx*, *Gsx/cnox-2* and *NK2,* possibly regulated by the BMP pathway (Mieko Mizutani and Bier, 2008) and the second one, involved in eye specification with the *Pax, Six, Dachsund* and *eya* genes (Stierwald et al., 2004). Future approaches that combine high throughput genomic analyses and functional analyses in homologous and heterologous contexts should characterize the regulatory elements that drive neuronal cell differentiation in both cnidarians and bilaterians, establish a genetic hierarchy and subsequently deduce the level of conservation of the neurogenic circuitry across evolution. Ultimately such core robust genetic circuit could be implemented in non-neuronal species to induce neurogenesis and possibly modify the behavior of target cells as for instance ciliated cells (Jekely et al., 2008). Parallel efforts could induce muscles, photoreceptors and one can dream of sponges walking towards the light ! Still these spectacular experiments would definitively prove that a given circuitry is indeed efficient but would leave unsolved the question of their ancestral recruitment in natural evolution.

There is a growing consensus about the absence of homology between the oral-aboral cnidarian axis and the bilaterian axes (see above) and we believe that the emergence of novel cell types likely preceded the shared organization of a body axis between cnidarians and bilaterians. However the question of the mechanisms driving the emergence of novel cell types was much less investigated than those driving axis patterning, certainly for technical reasons. Therefore there is clearly much to be learnt about the molecular mechanisms that drove and maintained cellular novelties. Those were complexified between cnidarians and bilaterians, but the core processes were already at work in cnidarians. Also if the urbilaterian axis was not established in cnidarians yet, the organization of the oral pole might share some common rules with the anterior patterning in bilaterians and it would be of utmost interest to trace the phylogenetic relationships between the processes that allow the development of annular nerve rings in cnidarians and the central nervous system in bilaterians. Finally given the variety of the developmental contexts that can be tested beside sexual development in cnidarians, these approaches will certainly also highlight the intricate relationships between neurogenesis and patterning processes.

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