Evolutionary and developmental perspective on annelid eye and nervous system: Insights from *Malacoceros fuliginosus*

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Abstract

Eye evolution is far from resolved and despite the considerable interest and decades of study, many questions remain on the evolutionary scenarios of eyes and photoreceptor cells. While examining their ultrastructure has been an important way for comparative studies, the high degree of variation of eye structures/complexities within species (and closely related species) calls for more detailed studies at the molecular level. As with many lophotrochozoan taxa, annelids also display simple to elaborate eye structures. Although general homology of the cerebral rhabdomeric eyes is assumed, this has not been firmly established leaving the scene in the annelid ancestor unanswered. To gain an understanding of the situation in a long pelagic annelid larva, we studied *Malacoceros fuliginosus*. The larvae possess multiple eyespots and therefore suitable for studies on how different eyespots develop and integrate into the nervous system. We used ultrastructure and gene expression studies to understand the eyespot structure and development. Our phylogenetic analysis of annelid r-opsins revealed the existence of two r-opsin paralogs - r-opsin1 and r-opsin3 within the two main annelid groups - sedentaria and errantia, whereas in basal branching annelids only a single r-opsin type is present. In comparison with the well-studied annelid *Platynereis dumerilii,* we find that the rhabdomeric eyes have several similarities in terms of spatial and temporal development, r-opsin expression dynamics and axonal connectivity. This suggests homology of the two rhabdomeric eyes and the more complex dorsal eyes in *P. dumerilii* is likely a case of augmentation of a simple eyespot. Apart from visual r-opsins, the eye PRCs in *M. fuliginosus* also expresses the newly classified opsin type, xenopsin. Inspection of the eye structure also revealed the existence of a prominent cilium in both rhabdomeric eyes. Additionally, we also identified a c-opsin in an extraocular cell type thereby making it the only species so far having both c-opsin and xenopsin. Taken together, our data provide insights into the eye organization of the annelid ancestor and adds information on how eye evolution is shaped by opsin gain and loss.

The second topic of interest is the nervous system development in the *M. fuliginosus* larva. The evolution of the bilaterian nervous system is a topic of long-standing debate inciting the need for studies at multiple levels along with broader species sampling. A major question is whether the centralized nervous system seen across taxa is derived from a common ancestor or independently originated multiple times. Characterization of the nervous system has been mainly done at the level of gene expression patterns along the major body axes, anterior-

posterior and dorsal-ventral. One aspect that has been overlooked particularly in lophotrochozoans is the development of pioneer neurons that give rise to the early neuronal scaffold. In *M. fuliginosus,* we identify at least three pioneer neurons that are responsible to form the complete early neuronal scaffold. While a posterior neuron pioneers the path for the ventral nerve cord, pair of neurons form the prototroch ring nerve and a ganglion cell near the apical organ with descending axons prefigures the central ganglia. Here we focused on the development of the posterior pioneer neuron and distinguish it from the rest of the neurons. It is one of the earliest cells to differentiate along with other ciliated cells of apical tuft and prototroch cells which are known to have mosaic development. The posterior neuron does not express the well-characterized proneural genes such as Ascl1, Olig, NeuroD, and Ngn and moreover, they even lack Prox1 and Elav which are represented by most other neurons. From a molecular perspective, the posterior pioneer neuron is indeed distinct from the rest of the neurons and may develop in a cell-autonomous manner.

List of papers

I. Suman Kumar, Sharat Tumu, Conrad Helm, Harald Hausen. (2019): The neuron pioneering the ventral nerve cord does not follow the common path of neurogenesis in the polychaete *Malacoceros fuliginosus*. Manuscript submitted.

II. Suman Kumar, Harald Hausen: Development and molecular characteristics of cerebral eyes in the sedentary polychaete *Malacoceros fuliginosus* – Insights into annelid eye evolution. Manuscript.

III. Clemens C. Döring*, Suman Kumar*, Sharat Tumu, Ioannis Kourtesis, Harald Hausen: Xenopsin in eyes of larval bryozoans and annelids. Manuscript.

    * Equal contribution.

List of abbreviations

CNS – central nervous system

dWISH – double whole mount in situ hybridization

dpf – days post fertilization

fpkm – fragments per kilobase million

FISH – fluorescent in situ hybridization

GPCR – G-protein coupled receptor

hpf – hours post fertilization

ISH – in situ hybridization

IHC – Immunohistochemistry

ipRGCs – intrinsically photosensitive retinal ganglion cells

mvPRC –microvilli of photoreceptor cell

mpf – minutes post fertilization

nuPRC – nucleus of photoreceptor cell

PRC – photoreceptor cell

PC – pigment cup cell

VGlut - vesicular glutamate transporter

VAChT - vesicular acetylcholine transporter

VNC - ventral nerve cord

Contents

1. Introduction

Part I: Evolution of eyes and photoreceptor cells

The evolution of photoreception has been one of the most intriguing topics in evolutionary biology. How natural selection has permitted the evolution of sophisticated eyes as known from vertebrates, insects, and cephalopods from a simple photoreceptor cell has been a puzzling question. Based on morphological studies of eyes and photoreceptors, it was proposed that photoreceptors may have evolved two times or multiple times in different lineages (EAKIN 1963; SALVINI-PLAWEN & MAYR 1977). However, later studies at the molecular level pointed more towards homology of the main cerebral eyes of various animals (KUMAR 2001; ARENDT 2003; VOPALENSKY & KOZMIK 2009). A conserved set of transcription factors act as key regulators of eye development and are found in all bilaterians and even in cnidarians (SUGA ET AL. 2010; GEHRING 2014).

Nevertheless, the organization, physiology, and function of eyes and other light-sensitive organs exhibit reasonable plasticity. Models studying eye evolution have predicted a very short geologic time for a complex eye to evolve (NILSSON & PELGER 1994). Fossil records of early arthropods have shown the presence of specialized compound eyes which suggests relatively rapid evolution of eyes (LEE ET AL. 2011). Even structurally constrained genomic regions of eye regulatory genes have undergone large sequence divergence in a short time (SWANSON & SCHWIMMER 2011). These findings suggest the ability of photosensory organs to diverge, become more complex or more simple at a fast pace. With the accumulation of data from studies on phylogenetically important organisms, it is possible to learn more about the intermediary and novel photosensory structures and gain a better understanding of the evolution of photoreceptor design and functionality.

1.1 Components of eyes

Many invertebrates have simple two-celled eyes (eyespot) composed of a photoreceptor cell (PRC) for light sensing and a shielding pigment cell for directionality (**Figure 1**). This arrangement is sufficient to provide basic functionality of phototaxis to either move towards (positive) or away (negative) from light. A more complex arrangement of PRCs as in arthropod and echinoderm compound eyes is capable of low-resolution image formation to navigate their surroundings. Eyes with retina and lens structures (focusing optics) like the

ones found in cephalopods and vertebrates are usually associated with high-resolution vision providing cues for recognizing prey and predator (NILSSON 2009; LAMB 2013; GARM & NILSSON 2014).

The most important component of eyes are PRCs, which are distinctive light sensory neurons that transduce light input to an electrical output. They usually have extensive cell membrane modifications and are broadly classified as ciliary (modified cilia) and rhabdomeric (modified microvilli) cell type (FAIN ET AL. 2010). These cell surface modifications substantially increase the surface area to accommodate a large number of light-sensing transmembrane proteins - opsins. The commonly found eye PRCs in vertebrates are ciliary type and in protostomes are rhabdomeric type. The two photoreceptor types also differ in their biochemical response: rhabdomeric photoreceptors use $G_\alpha q$ mediated G-protein signaling cascade to elicit a membrane depolarizing (influx of cations) response whereas the ciliary photoreceptors cause a membrane hyperpolarizing (efflux of cations) response using $G_\alpha t$ signaling cascade (FAIN ET AL. 2010).
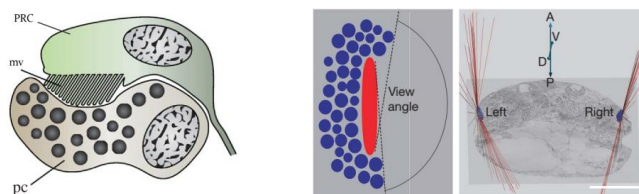
Figure 1: Two-celled eyespot and view angles. The structure of the pigment cup determines the angle of incident light as estimated here from electron microscopy reconstructions of *Platynereis dumerilii* larval eyespot. PRC – photoreceptor cell, mv – microvilli, pc – pigment cup cell. From (JÉKELY ET AL. 2008; NILSSON 2009).

1.2 Diversity of eyes

Eyes across bilaterians display enormous diversity in organization and function. Depending on the species lifestyle, eyes can become complex (and not limited to particular taxon) with the addition of more PRCs, pigment cells and lens structures (PURSCHKE ET AL. 2006; NILSSON 2009; RANDEL & JÉKELY 2016). Major protostome groups of annelids, mollusks, and arthropods all have diverse eye structures. They are usually composed of rhabdomeric PRCs but ciliary eyes are also not uncommon in several taxa.

Annelids have diverse eyes and many of them possess two or more pairs. Although the most common PRC types employed in the eyes are rhabdomeric, ciliary and phaosomal type are also found. The latter type has both cilia and microvilli projected into an intracellular vacuole which is thought to be a highly derived sensory innovation of annelids (PURSCHKE ET AL. 2006; DÖRING ET AL. 2013). Unusual eyes are not uncommon in annelids as shown in the Sabellid worms where the radiolar tentacles are reported to have compound ciliary eyes (BOK ET AL. 2016). These are non-cerebral eyes which mainly function as shadow detectors (BOK ET AL. 2017). Most arthropods have compound rhabdomeric eyes which are made up of several individual units of ommatidia (composed of lens elements and a rhabdome) (LAND & NILSSON 2002). Mollusks also display great diversity in eye structures, from simple eyespots of chitons to complex retinal eyes of cephalopods (SERB & EERNISSE 2008; VÖCKING ET AL. 2015). While most of these elaborate eyes are cephalic, some scallops have relatively well-developed non-cephalic eye structures composed of lens, reflective mirror, and retina capable of image formation (SPEISER & JOHNSEN 2008) (**Figure 2**). In animals closer to vertebrates, the protochordate, *Ciona intestinalis* has an ocellus made up of few ciliary PRCs and a shielding pigment cell whereas the amphioxus has both ciliary and rhabdomeric eyes (LAMB ET AL. 2007).
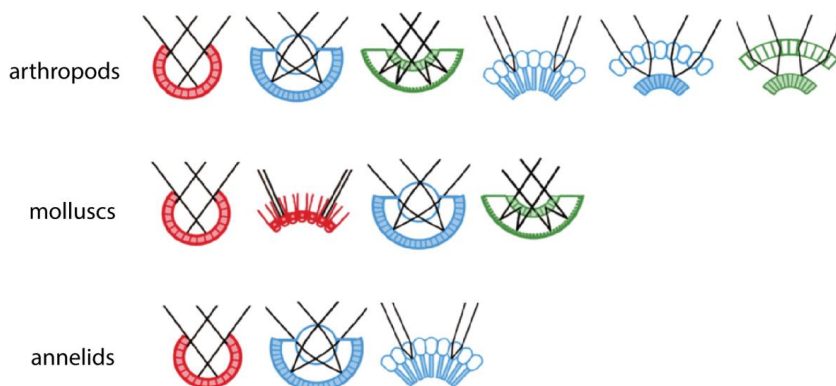
Figure 2: Eye types found in major protostomes. Both single-chambered and compound eyes are present in all three animal groups. Black lines indicate incident light. Colors indicate the mechanism of photoreception - red represents shadow detection; blue represents refraction; green represents reflection. From (ERCLIK ET AL. 2009).

Complex camera-type eyes composed of a lens; retina and supporting structures are found in cubozoan cnidarians, cephalopod mollusks, and vertebrates. The existence of these similar

structures in different lineages is a classic example of convergent evolution (KOZMIK ET AL. 2008; YOSHIDA ET AL. 2015; PICCIANI ET AL. 2018). Such enormous diversity in eyes in terms of number and structure has therefore led to contradicting theories on how eyes and PRCs evolved (EAKIN 1963; SALVINI-PLAWEN & MAYR 1977).

1.3 Eye developmental genes and evolution

While the diversity of eyes probably suggests that a diverse set of genes may be employed, this is not the case as a conserved set of genes is known to be important for the development of cerebral eyes in most animal groups (ARENDT 2003; TREISMAN 2004; GEHRING 2014). These genes, however, are not specific to eyes as they are also expressed in other tissues and therefore may have been recruited repeatedly in different contexts during evolution (FERNALD 2006). Molecular analysis of eye development across the animal taxa has yielded convincing data of remarkable conservation of some of the transcription factors. The best-studied is Pax6: a member of the Pax family of transcription factors. Pax genes (Pax1-Pax9) are classified based on the presence of two DNA-binding domains (a homeodomain and a paired domain) and a co-factor binding domain (octapeptide domain) (BLAKE & ZIMAN 2014) – the presence of which allows them to regulate a large number of genes (KOZMIK 2008). Studies in cnidarians have also shown the involvement of Pax genes in eye development (SUGA ET AL. 2010).

Other deeply conserved genes involved in eye specification are Six, Eya, Otx, Rx and Mitf (VOPALENSKY & KOZMIK 2009). Moreover, ectopic expression of Pax6, Six1/2, Eya can all induce the formation of eyes and eye structures in *Drosophila* and vertebrates (BONINI ET AL. 1997; CHOW ET AL. 1999; WEASNER ET AL. 2007). Studies in *Drosophila* have shown the close association of eye genes to form a core network comprising of Pax–Six–Eya–Dach Network (PSEDN) (KOZMIK ET AL. 2007). First, the Pax6 paralogs, Ey and Toy specify the eye progenitors during larval development. Thereafter, Six, Eya and Dach expression is induced for the complete development of the *Drosophila* visual system (CZERNY ET AL. 1999). Some of the eye developmental genes are also involved in the specification of pigment cells and other supporting cells associated with the eye. In vertebrate lens development, Pax6, Prox1 and Sox1 are all involved in regulating the expression of lens structural proteins (CVEKL & ZHANG 2017).

Another important aspect regulating eye development is the expression levels or the potency levels of eye specification genes. It has been observed that eye specification genes can be

flexibly employed and certain members of the network can be upregulated or downregulated (a feature likely acquired in a lineage-specific manner) (DAVIS & REBAY 2017). Accordingly, in several species, gene perturbation of weakly expressed members of the network (including Pax6) does not seem to alter eye development (SALÓ ET AL. 2002). Despite the differences in eye organization, the developmental genes involved point towards a general homology of many eyes in bilateria. Furthermore, given the considerable diversification of eyes in different lineages, studies at multiple levels are needed to address questions on eye evolution.

1.4 Photosensing and phototransduction

Photosensing can be mediated by different classes of proteins such as opsins, cryptochromes, ion channels and adenylyl cyclases - out of which the common photosensors in animals are opsins and cryptochromes (PORTER 2016). And among these opsins are of interest as most animal light detection is based on opsins and their exclusivity to PRCs has made them a crucial component in the studies of eye evolution.

Opsins belong to the G-protein-coupled receptor (GPCR) family of transmembrane proteins and their defining feature is the presence of a conserved lysine residue in the active site of the seventh transmembrane that allows binding of light sensitive molecule - retinal, through a reversible covalent linkage. Upon light incidence, the retinal changes conformation from 11-cis to all-trans and stimulates specific G-protein signaling cascade depending on the opsin type. The heterotrimeric G proteins mediate GDP-GTP exchange using the Gα-subunit. The active Gα-GTP then interacts with downstream effectors for signal amplification (TERAKITA & NAGATA 2014). The well-studied are vertebrate c-opsins and invertebrate r-opsins. In ciliary PRCs, the all-trans retinal dissociates from the c-opsin (known as photobleaching) and is later regenerated in retinal pigment epithelium cells to the cis conformation. In contrast, the r-opsins are bistable pigments wherein the trans form can revert to the active conformation without dissociating by subsequent absorption of photon (FAIN ET AL. 2010). Regeneration of all-trans retinal is attributed to photoisomerases, a group of opsins consisting of retinochromes, RGR opsins and peropsins. RGR opsins are more exclusive to vertebrates as are retinochromes to invertebrates, with vertebrate relative ascidians having both RGR opsins and retinochromes (KUSAKABE ET AL. 2009).

1.5 Opsin evolution and classification

The active lysine residue in opsins is restricted to the seventh transmembrane domain across all animals. In one study, mutagenic analysis revealed that lysine residues in other locations are also capable of binding to retinal and activate downstream signaling cascade and yet only the lysine located in the seventh transmembrane domain is preferred which suggests strong regulatory constraint (DEVINE ET AL. 2013).

High-quality assemblies of genomes and transcriptomes have shown the presence of numerous opsins of different classes in several species. This indicates that opsin functionality extends far beyond basic vision and encompasses diverse and complex behavioral roles (COLBOURNE ET AL. 2011; FUTAHASHI ET AL. 2015; CRONIN & JOHNSEN 2016; PANTZARTZI ET AL. 2017). Different opsins are tuned to detect different wavelengths of light and accordingly species expressing multiple opsin paralogs in their eyes can have color vision as known in many arthropods and vertebrates (YOKOYAMA 1999; BRISCOE & CHITTKA 2001; FUTAHASHI ET AL. 2015).

An important aspect of opsin genes is their dramatic loss and duplication thereby shaping eye evolution accordingly in different animal lineages. Of particular interest is to study the functional consequences of gene duplication such as subfunctionalization and neofunctionalization. Opsins are well-characterized in arthropods where gene duplication, diversification, and subfunctionalization have been studied (COLBOURNE ET AL. 2011; HENZE ET AL. 2012; FRENTIU ET AL. 2015).

The molecular differences in opsins and their specific G-protein subtypes form a basis on which the opsins are classified (TERAKITA & NAGATA 2014). Resolving their phylogenetic relationships, however, has been slightly challenging mainly due to poor sampling and sequencing profiles. Recent expansion in genomic and transcriptomic data from across the animal taxa has made it possible to resolve the opsin phylogeny and interpret their evolution and diversification. In a study encompassing sequence data from 14 animal clades, it was proposed that the last common bilaterian ancestor already had 9 different types of opsins and the cnidarian-bilaterian ancestor had 4 different opsins (RAMIREZ ET AL. 2016) (**Figure 3**). The opsin distribution also indicated frequent duplications and gene losses in many taxonomic groups. Further, stronger support values for some loosely annotated opsins allowed to form new opsin groups such as bathyopsins, chaopsins and xenopsins (RAMIREZ ET AL. 2016) (**Figure 3**). Likewise, opsin gene structure analysis also supported the opsin subgroups and their distribution among taxa (VÖCKING ET AL. 2017).
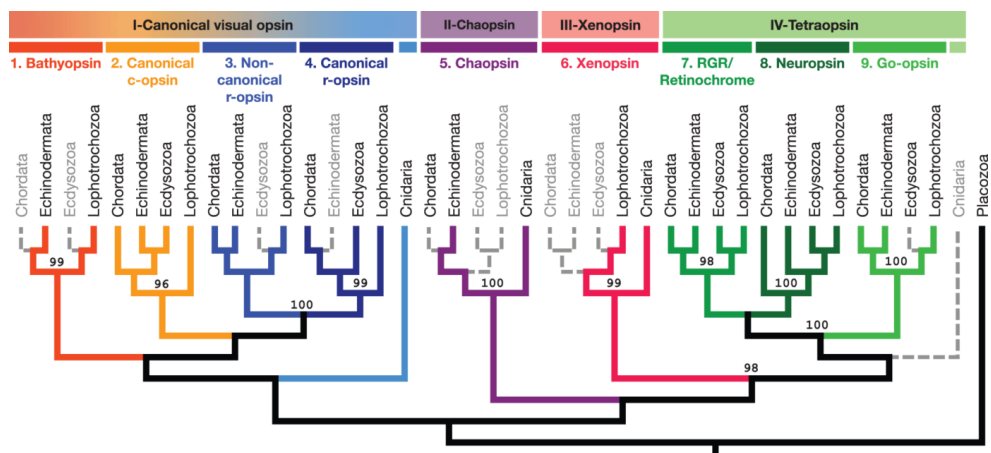
Figure 3: Phylogenetic distribution of opsins among the five major metazoan lineages (cnidarians, chordates, echinoderms, ecdysozoans and lophotrochozoans). The nine opsin types are grouped into four different classes. Solid lines indicate the presence of opsin in at least one species within the taxa. Dotted lines indicate the absence of opsin in particular taxa. From (RAMIREZ ET AL. 2016).

1.6 Evolution of photoreceptor cells

Earlier studies based on morphological analysis of eyes led to two popular views: diphyletic origin of PRCs (one line of rhabdomeric PRCs within protostomes and another line of ciliary PRCs in deuterostomes) (EAKIN 1963) and polyphyletic origin (40-65 independent lines) (SALVINI-PLAWEN & MAYR 1977). Molecular analysis has suggested that ciliary and rhabdomeric PRCs present in different taxa and different sensory organs including eyes are conserved lineages (TREISMAN 2004; GEHRING 2014; ARENDT ET AL. 2016). Although the same set of eye specification genes are involved in the development of both ciliary and rhabdomeric photoreceptors, some are more specific than others. It is hypothesized that Pax6 and Otx regulate r-opsin lineage, whereas Rx and Otx regulate c-opsin lineage (VOPALENSKY & KOZMIK 2009). These two cell types are also represented by conserved opsin type and transduction cascades with microvillar PRCs employing r-opsin use $G\alpha_q$ signaling and ciliary PRCs employing c-opsins use $G\alpha_i$ signaling (FAIN ET AL. 2010). Moreover, PRCs in independently evolved camera eyes of cephalopods and vertebrates share more than 70% of the genes in contrast to the accessory cell types which show least kinship (YOSHIDA ET AL. 2014).

In protostomes, microvillar PRCs expressing r-opsins are present in the main cerebral eyes whereas ciliary PRCs expressing c-opsins are very limited and are restricted to brain PRCs (ARENDT ET AL. 2004; VELARDE ET AL. 2005; BECKMANN ET AL. 2015). While the eyes mediate phototaxis, the ciliary brain PRCs functions in UV avoidance. Moreover, the ciliary PRCs make presynaptic contacts with the eye circuitry and overall this circuitry has been implicated in depth-sensing (VERASZTO ET AL. 2018). In contrast, vertebrates predominantly use ciliary PRCs employing c-opsins in the cerebral eyes and microvillar type PRCs are restricted to certain cell types of the retina (LAMB 2013). These retinal subtypes have biochemical responses similar to rhabdomeric cells and express melanopsin (opsin related to protostome r-opsins). Therefore, both ciliary and rhabdomeric PRCs do coexist in many taxa. Based on the expression of terminal selector genes, it was proposed that retinal ganglion, amacrine and horizontal cells of vertebrate retina are sister cell types of protostome rhabdomeric PRCs, while same is true for vertebrate ciliary PRCs (and bipolar cells) found in retina and pineal gland and invertebrate brain ciliary PRCs (ARENDT 2003; ARENDT ET AL. 2004, 2016).

In most cases, microvillar PRCs are accompanied by a single cilium or few cilia or are a mix of both (hybrid type PRCs). Although they are likely to be remnant structures, elaborate modifications in certain PRCs have indicated their significance. In the mollusk, *Leptochiton asellus,* the eye PRCs have distinct cilia and microvilli and expresses xenopsin and r-opsin. In other protostomes where only ciliary eye structures are present, they are known to express xenopsin, although in some studies they were earlier misclassified as c-opsins (PASSAMANECK ET AL. 2011; WANG ET AL. 2017; RAWLINSON ET AL. 2019). It is likely that xenopsins are important players in lophotrochozoan eye evolution in addition to canonical c-opsins and r-opsins. In annelids, most microvillar PRCs of cerebral eyes are usually accompanied by single remnant cilium (RANDEL ET AL. 2013; PURSCHKE & NOWAK 2015) which is unlike in mollusk and platyhelminth eyes where cilia are more prominent. More studies are needed to find the significance of such ciliary structures in lophotrochozoans.

1.7 Extraocular photoreceptors

Eye photoreceptors mainly serve to sense light for phototaxis, shadow response or vision. On the other hand, extraocular photoreceptors which are mainly unshielded cells which may serve several different non-visual functions pertaining to more complex behavior such as circadian photoentrainment and spawning. Although several descriptions exist, their evolution,

development and physiological roles are largely unknown. Many species have photoreceptors within their central nervous system and epidermal tissues (CRONIN & JOHNSEN 2016). In *Platynereis dumerilii*, ciliary photoreceptors expressing c-opsins are found in the brain within Rx expressing domain (ARENDT ET AL. 2004). In stomatopod crustaceans - animals known for their opsin diversity have brain photoreceptors expressing four different opsins (CRONIN & JOHNSEN 2016). In a chiton mollusc, anterior and posterior PRCs are present which have similar molecular characteristics as the main visual eye but without pigmented cells (VÖCKING ET AL. 2015).

1.8 Current knowledge of eye evolution in annelids

Eyes in annelids occur in different complexities in different taxa. The vast structural diversity of eyes has made them interesting subjects for studies on eye evolution. Ultrastructural data of eyes exist from several errant and sedentary annelids. The sedentary ones have very simple larval and adult eyes which are made up of few rhabdomeric PRCs and pigment cells. In contrast, many errant (free-living) annelids have well-developed complex adult eyes including accessory cells and lens-like structures (SUSCHENKO & PURSCHKE 2009) (**Figure 2**). Moreover, in difference to several other lophotrochozoan groups, annelids often possess more than one pair of eyes which can show up in different stages of development (BHAUD & CAZAUX 1987). In errant *P. dumerilii*, larval eye is very simple whereas the adult eyes are much more complex composed of numerous cells along with a structure resembling lens (RHODE 1992). The development of these eyes including the mechanism of larval phototaxis and eye circuitry has been investigated extensively (ARENDT ET AL. 2002; JÉKELY ET AL. 2008; RANDEL ET AL. 2014, 2015). Apart from this, *Capitella teleta* and *Helobdella robusta* are other species where the nervous system and eye development have been described (DÖRING ET AL. 2013; YAMAGUCHI & SEAVER 2013; MEYER ET AL. 2015). However, they possess only one pair of eyes and *Helobdella* is known to be a highly derived species. In terms of visual opsin expression, data exists only from *P. dumerilii* and *Capitella teleta* (RANDEL ET AL. 2013; NEAL ET AL. 2019). Investigations of eye structures in many errant annelids have suggested the common origin of these eyes (PURSCHKE & NOWAK 2015). However, it is not known how the eyes in sedentary and other basal annelids are related to the errant eyes, therefore leaving several open questions on the situation in annelid ancestor and other lophotrochozoans.

1.9 Eye circuitry and evolution

The hallmark of species behavior is the precise underlying neuronal connections. These microcircuits - a subset of otherwise complex circuitry, offers a more feasible level of circuitry studies. In lineages with diverse sensory structures, it is largely not known how the circuitry is organized and how it reflects their behavior. In most species, eyes are well integrated into the nervous system along with dedicated visual processing centers in the brain and in mammals they are even an extension of the forebrain (ELLIS 2016). Eye circuitries from some of the well-studied species known from vertebrates and arthropods are very complex involving a large number of cells structured in layers which ultimately relay visual information to the brain (SANES & ZIPURSKY 2010; LARDERET ET AL. 2017). However, in its very simplest form, as seen in box jellyfish larva, eyes can influence behavior without the involvement of a nervous system - a situation that is proposed to be ancestral (ARENDT ET AL. 2009). These PRCs bear locomotory cilia which are directly triggered by incident light (NILSSON 2009). The addition of shielding pigment granules provides directionality. Later diversification of this cell type may have given rise to two cells with specific tasks - a photoreceptor cell with projecting axons and a shielding pigment cell as proposed by 'division of labor' model of eye evolution (ARENDT ET AL. 2009).

Comparing the complex circuitry of taxa as distinct as vertebrates and flies and to interpret their evolution is a difficult task. Lophotrochozoans are interesting study subjects due to their phylogenetic position and simpler organization of neural elements in several representative species. This provides an opportunity to explore their circuitry development and evolution. It is best studied in the annelid larva of *P. dumerilii* where visual connectomes have been established for both larval and adult eyes from reconstructions of serial sections of electron microscopy data (RANDEL ET AL. 2014, 2015). While the larval eyespot has a very simple sensory-motor circuitry, the adult eye circuitry is slightly more complex and is related to the layered complexity seen in *Drosophila* and vertebrates. Therefore it would be interesting to study another annelid belonging to a closely related clade for a direct comparison with *Platynereis*. This would be informative for inferring the ancestral situation in annelids.

Part II: Nervous system development

Nervous systems are highly plastic and organized structures coordinating all behavioral aspects of animals. From simple nerve nets in cnidarians to highly complex mammalian brains, nervous systems display remarkable ability to evolve and adapt (**Figure 5**). The multitude of neuronal types, neurotransmitters and neuropeptides exemplifies the ability of the nervous system to process several sensory inputs. This vast diversity in neural structures across metazoa has fueled debates on how they evolved and what structures were present in the ancestor (LOWE ET AL. 2006; HOLLAND 2015; ARENDT 2018; MARTÍN-DURÁN ET AL. 2018).

The bilaterian central nervous system as represented by its major clades (lophotrochozoa, ecdysozoa and deuterostomes) is characterized by the presence of a central brain and a pair of longitudinal nerve bundles seen in majority of lophotrochozoans or a central tubular nervous system seen in chordates. Some of the clades have nervous systems with varying degrees of centralization including nerve net organization (LOWE ET AL. 2003, 2006; ARENDT 2018; MARTÍN-DURÁN ET AL. 2018). Nervous systems outside of bilaterians as in cnidarians also display different levels of neuronal condensation and in ctenophores the neuronal organization is rather diffuse and net-like (MACKIE 2004; MOROZ 2009) (**Figure 5**). Besides these, sponges and placozoans have neuron-like sensory cells which have a complement of synaptic proteins and ion channels (LIEBESKIND ET AL. 2017). Therefore tremendous diversity exists in various animal taxa thereby providing an opportunity to make inferences on homology, convergence and divergence.
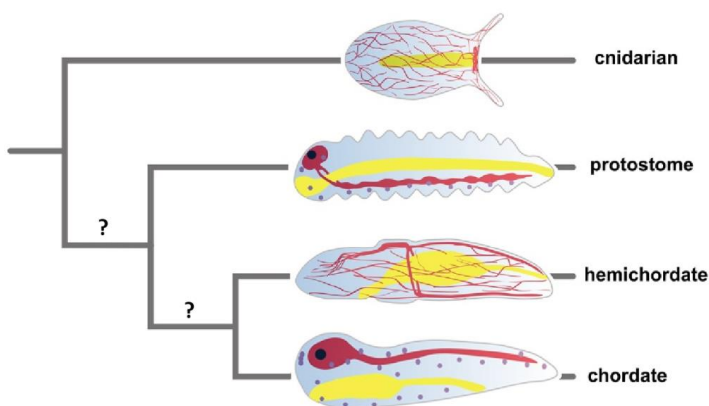
Figure 5: Generalized nervous system in metazoans. Nervous system (red) is more diffuse in cnidaria and hemichordate and centralized in majority of protostomes and chordates. Gut is indicated in yellow. (Adapted from (HOLLAND ET AL. 2013))

2. Nervous system evolution

In bilaterians, major signaling pathways such as BMP, Wnt, Notch and Hedgehog play important roles in body planning by establishing the major body axes and enabling neural patterning. Although several components of these pathways show a great degree of conservation, lineage specific adaptations are also frequent. A key aspect in the nerve cord/tube development is the specification of dorsoventral axis by BMP pathway components, with BMP signaling region promoting an epidermal fate and BMP inhibition promoting a neural fate (BIER & DE ROBERTIS 2015). BMP ligands and their inhibitors establish gradients along which specific genes are activated in specific domains (BIER & DE ROBERTIS 2015). The expression patterns of these genes are of interest from an evolutionary and comparative perspective and therefore several studies have heavily relied on these (LOWE ET AL. 2003, 2006; ARENDT 2018; MARTÍN-DURÁN ET AL. 2018).

Earlier comparative studies between vertebrates and invertebrates such as *Drosophila* and *Platynereis* showed the conservation of transcription factors expressed along the anterior-posterior axis in the form of otx, pax2/5/8 and gbx; and along the mediolateral or dorsoventral axis by nk2.1/2, nk6, pax6 and msx (HOLLAND ET AL. 2013; O'CONNELL 2013). But similar comparative studies in hemichordates and several other invertebrate clades showed the apparent lack of conservation of these transcription factors (mainly along the dorsoventral axis) and suggested independent convergent evolution of neural structures (LOWE ET AL. 2006; MARTÍN-DURÁN ET AL. 2018). Nonetheless, the evolution of nervous system is far from resolved and therefore more detailed comparisons are needed particularly in species that show deviations in patterning and nerve cord organization. Several questions remain about the gene regulatory networks and the cell types that are generated in different lineages and whether they have similar characteristics.

Another fundamental aspect of nervous system evolution is neural circuitry. As neural circuits define animal behavior and adaptation, comparative studies at this level will further the understanding of nervous system evolution. Expansion in connectome data from species across the animal taxa is allowing comparative studies and providing insights into circuit

evolution (VAN DEN HEUVEL ET AL. 2016). Neural circuits in complex systems have been mapped at very high resolution by combining electron microscopy with large-scale functional imaging and electrical recordings (BRIGGMAN ET AL. 2011; REAL ET AL. 2017). Adapting these techniques in simple invertebrate systems could be useful in identifying specific behaviors and their associated functional circuits.

2.1 Specification of neural elements

As neural elements are defining feature of animals, its specification and development is important from developmental and evolutionary perspective. Nervous system development is a multistep process involving various pathways and genetic modules acting out in a hierarchy. Despite the vast variations in bilaterian neural architecture, the process of early neurogenesis and the specification of different neural elements have many conserved features (HARTENSTEIN & STOLLEWERK 2015). Conserved signaling pathways are known to specify the major body axis, first along the antero-posterior axis and then along the dorso-ventral axis (HOLLAND 2000; BIER & DE ROBERTIS 2015) with strong influence on the development of neural structures. Therefore, steps of neural development offer features to be compared across the taxa and make evolutionary inferences.

A fundamental aspect of nervous system development which has been overlooked is the early axonal scaffold formation. Mainly reported from few studies in arthropods, nematodes and vertebrates, little is known in other taxa (CHÉDOTAL & RICHARDS 2010; STOLLEWERK 2016; HUTTER 2017). Therefore, study of neuronal scaffold formation in a major bilaterian superphylum as in lophotrochozoa could provide new understanding of nervous system evolution.

2.2 Neurogenesis

In general, the initial step in neural development is the induction of ectodermal cells to a neural cell fate. This is usually determined along the dorsal-ventral axis by concentration gradient of BMP - with the side having lowest concentration giving rise to the neuroectoderm. The so formed neuroectoderm starts proliferating by the activity of proliferation factors. Neurogenesis is the process wherein proliferating neural progenitors exit cycle, segregate from neuroepithelium and start to proceed towards a neuronal lineage. Each step of neurogenesis is spatially and temporally regulated which generates a diverse array of neuronal subtypes (FRITZSCH ET AL. 2015).

2.2.1 Neural genes in proliferation and differentiation

The initial impression from neural development across metazoans is the existence of conserved genetic elements and moreover a hierarchy in their deployment (HARTENSTEIN & STOLLEWERK 2015).

2.2.1.1 Sox genes in neuronal development

The Sox gene family of transcription factors is crucial for the development of early embryo as well as later adult stages. Based on sequence and structural homology they have been classified into several groups (A-H). Members belonging to groups B-E play an integral role in neurogenesis - from maintenance of neural stem cells to migration and differentiation and function by sequential binding to specific enhancer regions of neural genes (BERGSLAND ET AL. 2011). SoxB1 is one of the earliest expressed and also maternally derived Sox protein (GUTH & WEGNER 2008). Due to its transactivation domain, it mainly acts by activating other factors required for maintenance and proliferation of neuroectodermal stem cells. Sox2 (a member of SoxB1 group) was found to bind to the promoters of neuronal differentiation genes (SoxB2), proneural genes (Ngn, NeuroD) and other neurogenic genes which in turn make the cells receptive for neuronal differentiation (AMADOR-ARJONA ET AL. 2015). However, to exit the proliferation phase, SoxB1 has to be downregulated - a function that is performed by SoxB2 by directly interacting with the transrepressor domain of SoxB1. Thus, the activity of SoxB proteins is important for the balance between proliferation and differentiation (SANDBERG ET AL. 2005). Furthermore, downregulation of SoxB1 induces the expression of SoxC by proneural proteins which in turn induces the expression of additional neuronal genes that regulate cell cycle exit, neuronal migration and cell type specification by directly targeting their promoters (MU ET AL. 2012). SoxC acts transiently and once the cells proceed further into differentiation it gets downregulated (KAVYANIFAR ET AL. 2017).

2.2.1.2 Proneural genes and downstream targets

Proneural genes are transcription factors that regulate several aspects of neurogenesis, from progenitor maintenance to cell differentiation and morphogenesis. The well-studied are the basic Helix Loop Helix (bHLH) class of proteins, whose roles in neurogenesis is firmly established. Functional studies of different bHLH proteins, Ascl1, Atoh1, Olig and Neurogenins (Ngn) have shown that they capable of inducing the entire neuronal differentiation programs (VASCONCELOS & CASTRO 2014; GUILLEMOT & HASSAN 2017).

Moreover, proneural genes enforce spatiotemporal regulation by activating Notch ligands which in turn ensure only a subset of neurons is differentiating (Francisca 2014). Thus proneural genes can coordinate with other neuronal pathways to direct neuronal fate and subtype specification (BERTRAND ET AL. 2002).

During differentiation, proliferation genes are downregulated and neuronal subtype specifying genes are activated. Prox1 (prospero), a downstream target of proneural genes, is a homeodomain containing protein expressed in neural tissues. It controls cell lineage commitment by promoting cell-cycle exit and activating cell differentiation programs (LI & VAESSIN 2000). Role of Prox1 in regulating neuronal differentiation has been identified in all major bilaterian clades (HARTENSTEIN & STOLLEWERK 2015). Prox1 is pan-neural in *Drosophila*, whereas in vertebrates, it is confined to a subset of neuronal cells (LI & VAESSIN 2000; GALEEVA ET AL. 2007). Its mechanism is well characterized in *Drosophila*, where the expression of Prox1 is observed from neuroblast stage to final neurons. While the transcription levels remains constant is various cell stages, the translational level increases substantially in differentiating neurons (YANG ET AL. 2017). The key to increased translation is the transcription of a long isoform of Prox1 and subsequent stabilization by RNA binding proteins (RBPs) (YANG ET AL. 2017).

A characteristic feature of a neuronal cell is the ability to process RNA (mainly by alternative splicing) by multitude of RBPs, many of which are highly specific to neurons. RBPs harbor multiple conserved RNA binding motifs and can directly initiate several RNA modifications. Elav is one of the well-characterized RBP which is pan-neuronal in many species (DARNELL 2013). The functions of Elav in addition to promoting alternate splicing include increasing mRNA stability and translation, polyadenylation and mRNA localization (PASCALE ET AL. 2008; DARNELL 2013). The expression of Elav indicates post-mitotic differentiated state of a neuronal cell (PASCALE ET AL. 2008).

2.3 Pioneer neurons and their role in axonal scaffold formation

While the conserved genes and their modules are largely responsible for neurogenesis in most animals, the development and specification of the first neurite producing pioneering cells has mostly remained elusive. Due to their easy identification, pioneers were first described in arthropods wherein the early differentiating peripheral pioneer neurons create an axonal path for later appearing neurons of the central ganglia (Bate, 1976). Further work also attributed the characteristic axonal scaffold in arthropods to a conserved set of pioneer neurons (MEIER

ET AL. 1991; BIFFAR & STOLLEWERK 2015). Whether such early stereotypic patterns are present in other lineages, particularly the lophotrochozoans is not well known.

Much of the understanding of pioneer neurons and their involvement in the VNC development comes from studies in *Drosophila melanogaster* and *C. elegans*. Ablation experiments have demonstrated the importance of pioneer neurons in providing correct guiding paths for later outgrowing neurons (HIDALGO & BRAND 1997; HUTTER 2017). In *C. elegans*, the neurons which pioneer the VNC formation are specified by different transcription factors and are located in different regions (HUTTER 2003). While the neuron located in the anterior sends axon to the posterior direction guided by Wnt signaling, the two posterior neurons send axons in the opposite direction (HUTTER 2017; PARK & RONGO 2018). In lophotrochozoans, few studies have described the pioneer neurons involved in the formation of the nervous system. Studies in some annelids and molluscs have shown that peripheral sensory neurons form the central ganglia while VNC is pioneered by a posterior bifurcating neuron (MCDOUGALL ET AL. 2006; FISCHER ET AL. 2010; NEZLIN & VORONEZHSKAYA 2017).

Lophotrochozoans such as Molluscs, Nemerteans, Annelids and Platyhelminthes (despite their vast diversity in body plans) develop from characteristic spiral cleaving embryos (VAN DEN BIGGELAAR ET AL. 1997). In many annelids and molluscs, early development goes through a trochophore larval stage comprising of a prototroch ring and apical tuft. Development of such characteristic larval structures in these taxa is attributed to conserved and autonomously derived cell lines (COSTELLO 1945; VAN DEN BIGGELAAR ET AL. 1997; DOUG TISCHER1 2018). However, very little is known about the specification of neural elements and its dependence on regulative and autonomous development.

3. Study animal

Polychaetes are a very diverse group of marine animals with several thousand recognized species. Broader sampling has allowed resolving the annelid phylogenetic relationships and has enabled ancestral character reconstructions and comparative studies. Most species fall into two categories: errant forms which are usually free-living and sedentary forms which are usually tube dwelling filter feeders (WEIGERT ET AL. 2014). As polychaetes occupy a vast variety of habitats and have varied lifestyles, they are very suitable for studies of evolutionary innovation and conservation (FERRIER 2012).

3.1 *Malacoceros fuliginosus*

M. fuliginosus is a sedentary polychaete belonging to the group of Spionidae (**Figure 6,7**). It spends most of its time burrowed in temporary tubes constructed from sand. They are typical filter feeders and use long tentacles to capture food particles floating in water or from the sediment surface. They usually spawn synchronously releasing gametes into the water. The eggs are ellipsoid shaped with a major axis of 140 µm.

In the lab spawning can be induced any time of the year by placing individual worms in small bowls of filtered sea water for 1-2 h. After the eggs and sperm are released, they are fertilized in larger bowls and the staging begins from the time the gametes are combined. The embryo undergoes a typical spiral cleavage with the first cleavage starting at 40 mpf at 18 °C. Due to negligible yolk, the early nervous system development is easily tractable. This feature offers an unprecedented advantage over other annelids that have been more thoroughly investigated such as *P. dumerilii* and *Capitella teleta*.

The larva is a stereotypical trochophore consisting of an apical organ and locomotory structures - a prototroch and a telotroch ring. Being a planktotrophic larva, it has a relatively fast development. At 7-hpf a prototroch and apical tuft appears and the larvae is capable of swimming by 9 hpf. A telotroch is visible around 12 hpf. By 24 hpf, the larvae have a pair of eyes and exhibit phototaxis. Shortly after 24 hpf, chaetae start to develop. At 48 hpf stage, chaetae are elongated and they also possess three pairs of eyespots and exhibit stronger phototaxis. By this stage they are capable of feeding on phytoplankton such as *Chaetoceros calcitrans*. The early larvae display high synchronicity which reduces considerably in later stages of development as they continue to feed and grow. They have a long larval pelagic phase of up to 30 days before settling in sand sediment.

Not all molecular techniques were well-established in the *M. fuliginosus* larvae during the beginning of the project. The most applied techniques as in immunohistochemistry (IHC) and whole mount *in situ* (WISH) and double *in situ* RNA hybridization (dWISH) technique were subsequently optimized. Currently the eggs are not amenable for microinjections due to the very tough cuticle layer. Stage-wise larval transcriptome is available.

Figure 6: Early larva and juvenile of *Malacoceros fuliginosus*
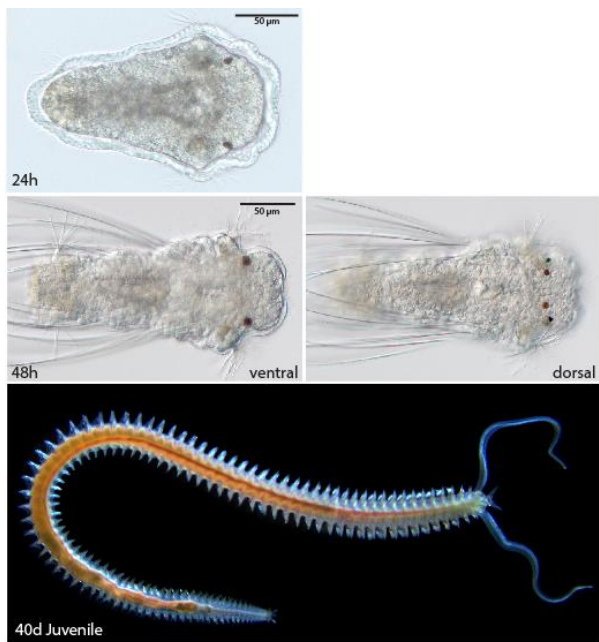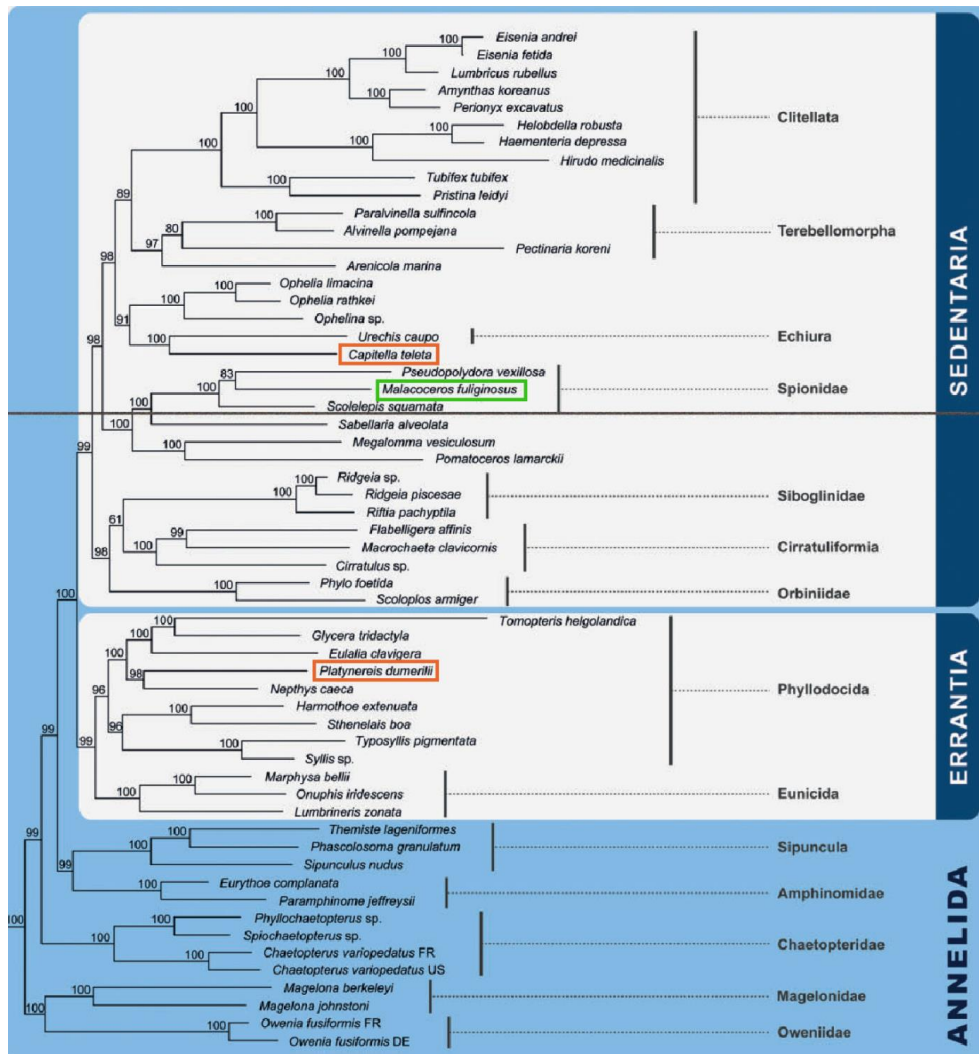
Figure 7: Annelid phylogeny. Sedentaria and Errantia represent the two major groups within Annelids. *Malacoceros fuliginosus* (highlighted in green) represents the sedentaria group. The other well-studied species (highlighted in red), *Capitella teleta* and *Platynereis dumerilii* represent sedentaria and errantia respectively. From (WEIGERT ET AL. 2014).

4. Aims of the project

One part of the thesis deals with the early nervous system development in *M. fuliginosus* (Paper I) and the other part is concerned with the molecular and ultrastructural characterization of eye development including its connectivity in the sedentary annelid, *M. fuliginosus* (Papers II and III).

Early nervous system development in the annelid, *M. fuliginosus*

Nervous system centralization, an organization featuring a central brain and longitudinal nerve cords or neural tube is seen across bilaterians. Alternatively the nervous system can be less centralized with varying degrees of centralization including diffuse nerve nets. Whether these structures are comparable and whether they are homologous or not is intensely debated. Characterization of the nervous systems at the level of general neuroarchitecture and patterning along major body axes form the basis for comparative studies in nervous system evolution. However, the axonal framework for such patterning is laid out by early developing pioneer neurons which are understudied in lophotrochozoans. Studies in arthropods and vertebrates have attributed the characteristic early neuronal scaffold to a small number of pioneer neurons. Whether the different lophotrochozoan pioneer neurons are related and whether they maintain positional information, how they are specified and how they develop are the main questions of this study. Therefore, here the aim was to investigate the early nervous system development in the annelid *M. fuliginosus,* focusing on pioneer neurons that initiate the CNS scaffold and how they correlate with overall neurogenesis.

Photoreceptor development and function in the annelid, *M. fuliginosus*

Previous work in *P. dumerilii* has provided thorough data on eye development, phototactic behavior and its circuitry (JÉKELY ET AL. 2008; FISCHER ET AL. 2010; RANDEL ET AL. 2014, 2015). The larvae of *M. fuliginosus* have multiple eyes and how they are related to other annelid eyes is not known. While *M. fuliginosus* is a sedentary polychaete with three pairs of eyes, *P. dumerilii* is an errant polychaete with two pairs of eyes belonging to the other major annelid branch. The larval eyespot of *P. dumerilii* has a simple sensory-motor connection for early phototaxis whereas the adult eyes has cerebral connection – how does the connectivity differ in *M. fuliginosus,* what type of connectivity represents the feature of the annelid ancestor and how many eyes were present are the main questions that were investigated. In terms of visual r-opsins, two paralogs have been identified in *P. dumerilii* eyes, whereas only

one is known in *Capitella teleta* (another sedentary annelid) (RANDEL ET AL. 2013; NEAL ET AL. 2019). As there are two r-opsin paralogs in the sedentary *M. fuliginosus,* it would therefore be informative to know how they compare to *P. dumerilii*. Besides *P. dumerilii* and few studies in *Capitella teleta,* and apart from ultrastructural information, very limited molecular data of the eyes exists from other annelids. Therefore, the eye developmental genes were investigated to address relationship of the eyes with other annelids and also extending to other lophotrochozoans in general.

Distribution and evolutionary significance of Xenopsin in lophotrochozoan eyes

Opsins usually localize to specific subcellular spaces as it is known for c-opsins (within cilia) and r-opsins (within microvilli). But for many of the opsins it is not known where they localize and what function they serve. In *M. fuliginosus,* of the several opsin sequences recovered from the transcriptome, xenopsin was one of them. In recent opsin phylogenetic analysis new opsin clades were identified, of which xenopsins became a focus group because of their wide occurrence in Lophotrochozoa. Previous study in the mollusc, *Leptochiton asellus* showed that xenopsin and r-opsin are coexpressed in the main cerebral eye PRCs having both cilia and microvilli. It showed that ciliary structures in protostomes are likely to have xenopsin and not c-opsin thereby providing a new perspective on PRC evolution (VÖCKING ET AL. 2017). The aim here was to study the evolution of this opsin type, to analyze the evolutionary significance of its expression in *M. fuliginosus* larva and data retrieved from the bryozoan *Tricellaria inopinata,* which also has a similar opsin type.

5. Summary of results

The neuron pioneering the ventral nerve cord does not follow the common path of neurogenesis in the polychaete Malacoceros fuliginosus. (Paper I)

In this study the development of pioneer neurons was characterized in relation to general neurogenesis in the larvae of *M. fuliginosus.* Using immunostaining against acetylated-tubulin and *in situ* hybridization for key neurogenic genes pioneer neurons were identified that forms the CNS scaffold and other neurons that are patterned along the animal body. The nervous system first develops from both the anterior and posterior sides. As early as 9 hpf, sensory neurons start to appear – one close to the apical tuft and one in the posterior. Only the cell in the posterior starts to send out bifurcating neurites growing anteriorly (at around 10 hpf) while

also acquiring a curved morphology with sensory cilia projecting to the exterior on the ventral side. This posterior cell functions as a pioneer neuron as the neurites originating from it prefigures the future VNC.

In the anterior, pioneer neurons are visible at around 14 hpf; first, a pair of sensory cells lying adjacent to the ventral prototroch cells send out neurites on either side to create a nerve ring connecting all the prototroch cells; then, at 16 hpf, a single ganglion cell adjacent to the apical organ sends out a descending axon. In later stages more sensory cells appear in pairs alongside the ganglion cells. Neurites from these cells crisscross along the same path of the primary descending axon and creates the primary neural plexus or the larval brain.

Next, the development of the nervous system was studied using commonly used neuronal markers, serotonin and FMRFamide. While FMRFamide signal is first detected at 14 hpf in a single cell near the apical tuft, serotonergic signal appears only after 21 hpf in a pair of cells again in proximity to the apical tuft cell. At 24 hpf, both serotoninergic and FMRFamidergic cells still represent only a small subset of neuronal cells mostly restricted to the anterior and not the trunk and posterior regions. By 48 hpf, however, much more neurons in the brain and now including the trunk are identified by serotonin and FMRFamide immunostainings.

In examination of the neurogenic genes during different stages of development, almost all neurons follow a common developmental path (SoxB1-SoxC-Prox1-Elav-Syt1) with the exception of the posterior pioneer neuron. The gene expression in the anterior pioneer neurons could not be tracked as they develop in later stages making it difficult to unambiguously correlate data from *in situ* hybridization to the many cells identified by immunohistochemistry. The first neurogenic genes identified in the posterior pioneer were the synaptic genes. Moreover, the differentiation of the posterior cell occurs while all of the surrounding cells are still expressing the proliferative marker, SoxB. None of the other common proneural genes such as Ascl1, NeuroD, Ngn and Olig are expressed in the posterior pioneer. This shows that the VNC establishing posterior pioneer neuron does not adhere to the developmental path that most other neurons follow.

Development and molecular characteristics of cerebral eyes in the sedentary polychaete *Malacoceros fuliginosus* – Insights into annelid eye evolution. (Paper II)

Here, the aim was to characterize the photoreceptor cell (PRC) development and opsin expression in the eyespots of *M. fuliginosus*. Additionally, behavioral tests were performed to

get insights into the eye circuitry and function of the larval eyes. This was a comparative study with *P. dumerilii,* where eye development and circuitry has been studied in detail with the further aim to get insights into the organization of eyes and their neural projection in the annelid ancestor. The larvae of *M. fuliginosus* have multiple eyespots, two of which are typical rhabdomeric eyespots comparable to the eyes of *P. dumerilii.* The third ciliary eyespot is not considered further due to absence of molecular markers and comparable eyespot in other investigated annelids.

As is common in many annelids, *M. fuliginosus* larva first develops a pair of pigmented mid-ventral eyespots (rhabdomeric) around 19 hpf. Thereafter, by 42 hpf two more pairs (one rhabdomeric and one ciliary) appear on the dorsal side. Note that the PRCs develop much earlier than the pigment cup cells. Due to early differentiation, the development of the first ventral eye PRC could be tracked with tubulin antibody staining. This PRC has a well-delineated axon projecting to the brain in comparison to the simple sensory-motor type connection of the *P. dumerilii* eyespot. However, before the axon is projecting to the brain, it bends towards the extensions coming from the adjacent prototroch suggesting a sensory-motor interaction as in *P. dumerilii*.

The opsin phylogeny revealed that the annelid canonical r-opsins split occurred before the emergence of annelid subgroups of Errant and Sedentaria. Next, the expression of r-opsin1 and r-opsin3 was characterized in the rhabdomeric eyespots. The ventral eyespot has three PRCs which are sequentially added ventral to the first PRC. While all three PRCs express r-opsin3, only the second PRC coexpresses r-opsin1/3. In the subsequent stages, the expression of r-opsin1 increases significantly and surpasses the combined expression of r-opsin3 (based on fpkm values). Moreover, at 48 hpf onwards, the expression of r-opsin1 is observed in the axon projecting to the brain. In the dorsal eyespot, a single r-opsin1/3 coexpressing PRC is present. It has characteristics similar to the second PRC of the ventral eyes since the opsin expression follows similar dynamics and the axon projecting to the brain expresses r-opsin1.

Next we characterized the photoreceptor cells in terms of their neurotransmitter type. Using fluorescent double *in situ* hybridizations we found that the *M. fuliginosus* ventral eye at 24 hpf expresses both cholinergic and glutamatergic markers (VAChT and VGluT respectively). In general, both VGluT and VAChT have broad expression domains in the brain and near the apical organ but coexpression is observed only in the first PRC. In the 2-cell stage of the ventral eye (shortly after 24 hpf), VAChT is restricted only to the first developed PRC and the same was observed in the 3-cell stage (48 hpf). While expression of VAChT gets

progressively weaker, VGluT is strongly expressed in all three PRCs of the ventral eye and also in the dorsal eye PRC.

The common eye developmental genes were then characterized by fluorescent double *in situ* hybridizations. As early as 14 hpf, Pax6, Prox1, Otx, Six1/2 are co-expressed with r-opsin3 in the first ventral PRC. The expression of Pax6 is broader specifying multiple cells in the eye region and the CNS. Prox1 also specifies multiple cells in the developing eye including several cells along the CNS and trunk region. Otx and Six1/2 are strongly expressed in the PRCs from early stage and continue to do so in later stages as well. On the other hand, Eya was only found weakly expressed in the early stage but not detected in the later stages while Dacshund could not be detected in any of the stages observed.

Xenopsin in eyes of larval bryozoans and annelids. (Paper III)

It was previously identified in a mollusc larval eye, that they express both r-opsin and xenopsin (VÖCKING ET AL. 2017). Ultrastructural data showed that these photoreceptors have well-differentiated microvilli and cilia, thereby providing an unprecedented perspective on opsin evolution; with r-opsin localizing in the microvilli and xenopsin likely localizing in the cilia (VÖCKING ET AL. 2017).

In a collaborative study we followed up on the previous work by identifying xenopsins in other protostome clades; bryozoans and annelids. My work was in the annelid, *M. fuliginosus*. Although xenopsins are present in many of the protostome clades, they were misclassified as c-opsins in previous studies. Therefore to further support our phylogenetic analysis, the gene structure of potential xenopsins was determined (by genome walking) for both *Tricellaria inopinata* and *M. fuliginosus*. Additional sequences from publicly available genomic data of several other species were also surveyed. This provided us with intron-exon boundaries which revealed conservation of two such boundaries in most of the sequences that fall under xenopsins.

Next, the cellular expression, the localization of xenopsin and the ultrastructure of the eyes was determined in both species. The larvae of *T. inopinata* have one median eye and a pair of lateral eyes, all of which have ciliary structure. *In situ* hybridization revealed strong expression of Tin-xenopsin in all eyes. Antibodies which were custom-made to Tin-xenopsin specifically stain all eyespots. In the annelid, *M. fuliginosus*, the larvae have 3 pairs of eyespots. The lateral epidermal eyespot is strictly ciliary whereas the other two eyespots are typical rhabdomeric eyespots having extensive microvilli along with single cilium. Only the

microvillar photoreceptors show weak expression of Mfu-xenopsin in addition to strong r-opsin3 expression. Custom-made antibodies to Mfu-r-opsin3 clearly localized the r-opsin to the microvilli but the Mfu-xenopsin antibodies did not yield any specific staining. Additionally, we recovered c-opsin sequence from *M. fuliginosus,* making it the only known species having both xenopsin and c-opsin. This finding certainly provides new perspectives for opsin and eye evolution in bilaterians.

6. Discussion

6.1 Nervous system evolution

The neural genes employed in the process of neurogenesis follow a hierarchy which is remarkably conserved across the animal taxa. However, neural elements that are established are quite diverse with varying degrees of centralization within different evolutionary lineages. A fundamental question in evolutionary biology is how these diverse neural elements are related and whether they can be traced to a common ancestor or not. Although comparative studies in different taxa have provided crucial insights, several questions remain as to whether the nervous system centralization has a common origin or independently evolved multiple times evoking the need for more data. One aspect that has received little attention particularly in lophotrochozoans is the formation of early neural scaffold. Despite the crucial role of pioneer neurons in the formation of early neural scaffold, their evolution and development is largely unknown. Here we studied the development of the axonal scaffold formation in the annelid, *M. fuliginosus* with respect to general neurogenesis and uncovered that VNC forming pioneer cell follows a distinct mode of development and is probably largely depending on cell autonomous specification.

6.1.1   General neurogenesis in *M. fuliginosus*

Several lines of studies have suggested the conservation of early neurogenic steps across metazoa (HARTENSTEIN & STOLLEWERK 2015). In our present study we found that most neurons in the anterior and trunk are likely to follow the general path SoxB1-SoxC-Prox1-Elav-Syt1.

SoxB gene which usually starts out as maternally inherited transcript is well-documented across metazoa for its role in neuroectoderm specification (GUTH & WEGNER 2008; NERIEC & DESPLAN 2014). In *M. fuliginosus,* SoxB1 is expressed throughout the ectoderm in early stages of development but its expression becomes more restricted in later stages as gaps appear in different regions indicating zones of differentiation. Rightly so, double labeling of SoxB1 with differentiation marker, Prox1, we observed expression of Prox1 only within these zones which indicates dedicated regions of differentiation. By 18 hpf, SoxB1 is mostly restricted to the anterior and progressively reduces in later larval stages (6-7 dpf). This suggests that the addition of neurons probably reduces from late larva onwards.

Studies in echinoderms and vertebrates have shown that SoxC is required for commitment of all neural progenitors (CHEATLE JARVELA ET AL. 2016; GARNER ET AL. 2016; JACOB ET AL. 2018) and moreover Brn1/2/4 (a vertebrate subtype specification transcription factor) is expressed downstream of SoxC in the sea urchin (GARNER ET AL. 2016). We found that SoxC is mainly restricted to the anterior during the early stages (until 12 hpf) and extends towards trunk in later stages (from 14 hpf). Subset of SoxC cells lying close to the differentiation zone then coexpress Prox1. Whether the posterior neurons develop from SoxC lineage is not clear as only weak transient expression was observed after 12 hpf. However, expression of Brn1/2/4 is already seen in the posterior region from 10 hpf (in addition to a pair of cells in the anterior and the trunk) while SoxC expression is still restricted to the anterior. We also did not observe co-expression of SoxC and Brn1/2/4 in the stages analyzed. Therefore, the posterior subset of neurons flanking the posterior pioneer cell are likely independent of SoxC which suggest its importance only for neurogenesis in the anterior and trunk domains but not the posterior domain.

The differentiation marker, Prox1, starts in *M. fuliginosus* in the anterior and soon is expressed in pairs of cells along the trunk and posterior by 12 hpf. In the posterior (including the pair of cells in the anterior and trunk), subset of cells coexpress Prox1 and Brn1/2/4 suggesting that Prox1 is expressed in different neuronal lineages. Expression of post-mitotic neuronal gene, Elav, first appears simultaneously near the apical organ in the anterior and in a pair of cells in the posterior at 14 hpf coexpressing with Prox1. The common proneural genes, Ascl1, NeuroD, Ngn and Olig also start expressing in the anterior first. Shortly after the posterior pioneer sends out neurites, Ngn expression was observed in few adjacent cells which are likely the follower neurons. In the annelids *P. dumerilii* and *Capitella teleta,* the progression of neural genes in general is from anterior to posterior (SIMIONATO ET AL. 2008; SUR ET AL. 2017) and is consistent with that of *M. fuliginosus.* Moreover, although the progression of most neural genes is from anterior towards posterior, specification of certain posterior subtypes in *M. fuliginosus* could be independent of anterior gene regulatory networks.

6.2 Importance of pioneer neurons in nervous system evolution

Pioneer neurons generating the first axonal tracts of different neural elements are largely described from vertebrates and ecdysozoans (CHÉDOTAL & RICHARDS 2010; STOLLEWERK 2016; HUTTER 2017). In arthropods, easy identification of pioneer neurons has largely

contributed to the understanding of their development (EHRHARDT ET AL. 2014). The development of the characteristic axonal scaffold in arthropods is attributed to a conserved set of pioneer neurons (MEIER ET AL. 1991; BIFFAR & STOLLEWERK 2015; EVANS 2016). In all vertebrates, the pioneer neurons arise from comparable regions and are known to be responsible for conserved stereotypic patterns (CHÉDOTAL & RICHARDS 2010).

Several factors distinguish pioneer neurons from other neurons: from their precise positional cues and distinct molecular profiles to unique pathfinding abilities (STOLLEWERK 2016; HUTTER 2017). The positioning of neurons is usually an important factor for optimal wiring and therefore evolution is largely influenced by this principle. However, studies in *C. elegans* have shown that certain neurons, particularly the pioneer neurons, distinctly deviate from this principle. This possibly reflects a developmental constraint for pioneer neurons (CHEN ET AL. 2006).

Comparative studies of *Drosophila* and *Tribolium* have revealed the existence of conserved pioneer neurons which retain positional information despite small variations in their molecular profiles (BIFFAR & STOLLEWERK 2015). This suggests that molecular changes only reflect evolutionary divergence and do not necessarily affect the function of pioneer neurons and highlights their importance in insect nervous system development. However, a significant gap exists in our understanding of such pioneer neurons within the many taxa of lophotrochozoans. Therefore, close comparative characterization of pioneer neurons will be informative and crucial in tracing evolutionary paths of nervous systems.

6.2.1 Pioneer neurons in Lophotrochozoa

Lack of markers or difficulty in their identification has eluded the study of pioneer neurons in many lophotrochozoans. However, few studies have reported the development of central ganglia from peripheral sensory neurons originating from the lateral sides of the episphere (VORONEZHSKAYA & IVASHKIN 2010; NEZLIN & VORONEZHSKAYA 2017). In *M. fuliginosus*, we do observe multiple peripheral sensory neurons sending in neurites but not before a pioneer tract has been created at the base of the apical organ by a single non-sensory pioneer ganglion cell located adjacent to it. Detailed studies in *Capitella teleta* have also shown the central origin of the nervous system (MEYER ET AL. 2015).

The development of the VNC is attributed to neurons originating in the posterior. While in some annelids a single posterior neuron is involved as in the errant annelid *P. dumerilii* and

sedentary annelid *Pomatoceros lamarcki*; molluscs and nemerteans may have a pair of neurons in the posterior pioneering the VNC neurites (RAINERI 1995; NEZLIN & VORONEZHSKAYA 2003; MCDOUGALL ET AL. 2006; FISCHER ET AL. 2010; VON DÖHREN 2016). In *M. fuliginosus,* a single posterior neuron gives rise to the characteristic pair of VNC neurites running along the ventral side. The posterior neuron along with the cerebral ganglion cell and the prototroch nerve cells create the early scaffold, the structure of which appears similar to the ones described in other lophotrochozoans (REDL ET AL. 2014; YURCHENKO ET AL. 2018).

In lophotrochozoans, the first neurons usually described are based on FMRFamide and serotonin immunostainings (BRINKMANN & WANNINGER 2008; VORONEZHSKAYA & IVASHKIN 2010). Although the use of such conserved neuronal markers has largely helped nervous system descriptions in many invertebrates, they only represent a subset of neurons. Depending on the species, the onset of their expression and the proportion of neurons they represent are highly variable. It was shown that in comparable developmental stages of sea star and sea urchin, the number of serotonergic neurons is significantly different (CHEATLE JARVELA ET AL. 2016).

In some annelid species the posterior cell expresses serotonin; molluscs have either serotonin or FMRFamide, whereas few others express neither (MCDOUGALL ET AL. 2006; BRINKMANN & WANNINGER 2008; FISCHER ET AL. 2010). In *Capitella teleta,* the nervous system development has been described in sufficient detail; however, there is no suggestion of a posterior pioneer neuron which can be attributed to a secondary loss (MEYER ET AL. 2015). In *M. fuliginosus* none of the pioneer neurons express serotonin or FMRFamide (at least during the early developmental stages). We observed that FMRFamide starts expressing in a small domain at the base of the apical tuft cell from 12 hpf, but the onset of serotonin expression is around 21 hpf in the central neural plexus - prior to which several other neurons are already present both in the anterior and posterior. Moreover, phenotypic plasticity of neurons is well documented in vertebrates where developmental stage or subtle changes in the microenvironment can change the expression of neurotransmitter type (IACOVITTI ET AL. 1987; DEMARQUE & SPITZER 2011). Another important point to consider is that the pioneer neurons are most clearly discernable during the early stages of development. In species with yolky embryos or numerous cilia, even the existence of pioneer neuron could be overlooked.

As molecular profiles of pioneer neurons is currently not known, detailed analysis of this cell type is therefore necessary to make comparative studies and elucidate their evolution. The

presence of a posterior pioneer neuron in both errant and sedentary annelids suggests that this may be ancestral for annelids or at least for the clade containing Errantia and Sedentaria.

6.2.2 Distinctive development of the posterior pioneer neuron.

Early development in many spiral cleaving animals such as lophotrochozoans is largely influenced by cell autonomous (non-regulative) mechanism (RABINOWITZ & LAMBERT 2010; HENRY 2014). This contributes to the early establishment of non-homogeneous cells having pre-determined developmental potential (RABINOWITZ & LAMBERT 2010). According to the recent lineage tracing data from *P. dumerilii* episphere, the appearance of bilateral founder cells marks the regulative phase of development (VOPALENSKY ET AL. 2018). Although these cell pairs may originate from different cell lineages, they may acquire the same fate according to their later positions. Prior to this stage, the ciliated cells of prototroch and apical tuft are already in place suggesting that such cells have autonomous (non-regulative) mode of development (DOUG TISCHER1 2018). Several classical blastomere isolation techniques in annelids and molluscs have shown differentiation of prototroch or ciliated cells by autonomous specification (WILSON 1904; COSTELLO 1945; VAN DEN BIGGELAAR ET AL. 1997). Therefore, early differentiating cells in many lophotrochozoans are likely to be predetermined by their maternal content and not influenced much by external factors.

Similarly in *M. fuliginosus,* as early as 7 hpf, the first differentiated cells of the prototroch and apical tuft are visible. Soon thereafter at 8 hpf, a single posterior neuron is visible which at 9 hpf has dense tubulin staining. This cell subsequently develops a distinct morphology along with extending sensory cilia which is akin to the anterior counterparts. The early differentiation of the posterior cell is unusual as during this stage, the cells adjacent to the posterior neuron are still expressing SoxB1. And moreover the expression of proneural and differentiation factors is yet to begin. According to the neural gene expression profiles and in correlation with immunohistochemical data, none of the investigated genes involved in general neurogenesis including Mfu-SoxB1, Mfu-SoxC, Mfu-Prox1 and Mfu-Elav are expressed in the posterior pioneer neuron. However, synaptic markers, Mfu-Synaptotamin1 and Mfu-Rab3 are the first genes we observed in the posterior cell at around 12 hpf suggesting that its differentiation is independent of common neural genes. These features make the posterior pioneer a peculiar neuron in terms of its development.

In *Capitella teleta*, it is reported that the brain is autonomously specified in contrast to the VNC. Isolation of the first-quartet of micromeres resulted in the formation of the head along

with the brain, whereas the formation of the VNC required the induction of 2d micromere from 2Q macromeres (CARRILLO-BALTODANO & MEYER 2017). Notably though, presence of a posterior pioneer cell is not reported in *Capitella teleta* and the VNC likely develops entirely from anterior domain.

In *M. fuliginosus*, besides the ciliated cells, the first ventral eye PRC also differentiates early at around 12 hpf in the lateral position. However, by this stage the eye developmental genes are already being expressed including Otx, Six1/2, Pax6 and Prox1. As shown in the lineage tracing study in *P. dumerilii*, the onset of Otx expression signifies the appearance of bilateral founder cells which are conditionally specified (DOUG TISCHER1 2018). Additionally, blastomere deletion techniques in *Capitella teleta* have reported the ability of multiple blastomeres to give rise to eye structures in the absence of the primary eye generating blastomere (YAMAGUCHI ET AL. 2016). Therefore similar to the the ciliated cells of prototroch and apical tuft, the posterior pioneer possibly develops in a cell autonomous manner in difference to the early bilaterally developing sensory cells such as PRCs which are conditionally specified.

While it is accepted that the neurites laid out by pioneers are retained in later stages, it is not clear whether the pioneer neurons itself persist. In our case, we were unable to track the development of posterior pioneer cell after 24 hpf. The lack of synaptotagmin1 expression in the posterior region from 48 hpf and the considerable morphological changes indicate that the posterior pioneer neuron is indeed a transient neuron. Studies in arthropods have shown that pioneers of the peripheral nervous system undergo apoptosis (KUTSCH & BENTLEY 1987). Whether the anterior pioneers in *M. fuliginosus* also undergo apoptosis or adopts a different fate to allow integration into the nervous system is not known; as these neurons appear slightly later in development making it difficult to identify gene expression in single cell resolution.

6.3 Conclusions and future perspectives

Comparing distantly related extant taxa is not easy and as more variations are seen in gene expression patterns and gene regulatory networks in various animal lineages, it is increasingly becoming evident that deeper understanding of the nervous system development and evolution requires comparative analysis at multiple levels. Studies in different animal groups have attributed the establishment of conserved and characteristic neuronal scaffold to early pioneer neurons which therefore represents an important comparative feature. The absence of

common neural genes in pioneer neurons and their distinctive localization agrees with previous studies that they are indeed different from most other neurons (CHEN ET AL. 2006). General neuronal subtype specification involves highly regulative mechanisms acting temporally and spatially to generate precise number and neuronal type, but this is not a prerequisite for early individual pioneers whose main role is pathfinding for later appearing neurons.

Further developmental and molecular studies of pioneer neurons in lophotrochozoans should provide more insights into their role in nervous system development and evolution. Moreover, in addition to molecular characterization, cell lineage studies could be more important for identifying comparable pioneer cell types.

7. Eye and opsin evolution within annelids and other lophotrochozoans

7.1 Opsin and PRC evolution in lophotrochozoans

Several transcriptome based studies have reported the existence of numerous opsin types across different species of both protostomes and deuterostomes and likely correlates with the vast diversity of eyes and light sensory extraocular structures (COLBOURNE ET AL. 2011; FUTAHASHI ET AL. 2015; PANTZARTZI ET AL. 2017). Recent broad sampled opsin phylogenies have yielded well resolved groups providing new perspectives on their evolution and offering insights into evolutionary mechanisms such as gene duplications and losses which is a characteristic of opsin genes (BENTLEY ET AL. 2016; RAMIREZ ET AL. 2016; BOK ET AL. 2017; VÖCKING ET AL. 2017). In lophotrochozoans the main visual opsins are known to be r-opsins and in the newer phylogenetic classification invertebrate visual r-opsins were categorized as canonical r-opsins as opposed to several other less studied r-opsins or non-canonical r-opsins which form a sister group (RAMIREZ ET AL. 2016; VÖCKING ET AL. 2017). In addition, newer opsin clades were revealed of which xenopsins became a focus group because of their recently discovered wide occurrence throughout Lophotrochozoa (RAMIREZ ET AL. 2016; VÖCKING ET AL. 2017; RAWLINSON ET AL. 2019). The gene structure analysis revealed conserved exon-intron arrangement thereby confirming xenopsins as a distinct opsin type and further supporting the tree topology (VÖCKING ET AL. 2017).

In terms of eyes, although rhabdomeric eyes are common in lophotrochozoans, there is growing evidence that in many clades ciliary and hybrid eyes (microvillar and ciliary) are

widespread (Vöcking et al. 2017; von Döhren & Bartolomaeus 2018). Molluscs are known for their vastly diverse eye structures and in many species ciliary eye structures exist (Serb & Eernisse 2008). The well-known is the scallop eyes where the retinal layers are composed of alternating ciliary and rhabdomeric PRCs (Serb & Eernisse 2008). Similar diversity also exists in free-living flatworms as both rhabdomeric and ciliary eyes are present (Lanfranchi et al. 1981; Sopott-Ehlers 1991). In the polyclad flatworm, *Pseudoceros canadensis*, the larva and adult have different eye structures. While the larval epidermal and cerebral eyes have ciliary PRCs (or combination of both ciliary and microvilli), they are entirely rhabdomeric in the adults (Eakin & Brandenburger 1981). In nemerteans, presence of cilia along with microvilli is seemingly a common feature. The proportion of cilia depends on the species; from one cilium per rhabdomeric PRC in *Lineus viridis* to several cilia in *Lineus ruber* (von Döhren & Bartolomaeus 2007, 2018). Studies of these diverse eyes are therefore important to trace evolution of eyes in bilateria.

7.2 Evolution of c-opsins and xenopsins

In the first study on cellular expression of xenopsins it was found being co-expressed with r-opsin in the eyespots of *Leptochiton asellus*, where the PRCs have both microvillar and ciliary structures. While the r-opsin was shown to localize to the microvilli, xenopsin localization to cilia was not validated (but the expression of a subset of ciliary transporters was reported) (Vöcking et al. 2017). In the bryozoan, *T. inopinata*, the larval eyes have an entirely ciliary structure and the Tin-xenopsin antibodies clearly localizes to the cilia. In the flatworm, *Maritigrella crozieri,* it was recently shown that the ciliary eyespot expresses xenopsin and is capable of phototransduction through $G\alpha_{i/o}$ signaling (Rawlinson et al. 2019). These points out two things. First, xenopsins are indeed able to enter cilia and function as visual opsin. Second, ciliary PRCs are more prevalent in lophotrochozoans than previously assumed and likely express xenopsin. From the recent phylogenomic studies, it is now clear that opsin type present in *Terebratalia* and scallop, whose eyes also contain ciliary PRCs is indeed xenopsin and not c-opsin as it was initially thought (Passamaneck et al. 2011; Wang et al. 2017).

In annelids, xenopsins are scarcely represented thus far which is unlike in molluscs. The only known finding is in *Owenia fusiformis* and *M. fuliginosus.* This seems to agree with the fact that xenopsin localizes within cilia and most prevalent PRC structures in annelid eyes are microvillar (Purschke et al. 2006). Intriguingly, in all of the surveyed transcriptomes and genomes (including other lophotrochozoans), it appeared that xenopsin and c-opsin are

mutually exclusive (VÖCKING ET AL. 2017). In annelids, c-opsin was only known from *P. dumerilii* as the opsin type of the brain PRCs (ARENDT ET AL. 2004), however, xenopsin is absent and so are distinct cilia in the cerebral eyes. In *M. fuliginosus,* both c-opsin and xenopsin are expressed which is indeed surprising and so far the only animal having this status. Moreover, Mfu-c-opsin is not associated with brain or the eyes since its expression is outside the head region and probably represents a cell type distinct from that of *P. dumerilii*. However, further investigation of this cell type is important to understand its relationship to brain PRCs of *P. dumerilii* and of ecdysozoans.

Whether all ciliary mollusc eyes express xenopsin and whether the hybrid eyes in flatworms coexpress r-opsin and xenopsin remains to be identified. Since canonical c-opsins are weakly represented in invertebrates and are only confined to the brain PRCs of some ecdysozoans and the annelid *P. dumerilii* (ARENDT ET AL. 2004; VELARDE ET AL. 2005; BECKMANN ET AL. 2015), it is conceivable that the ciliary structures of lophotrochozoan cerebral eyes are likely to possess only xenopsin. Moreover, their presence also in hybrid PRCs as in the case of *Leptochiton asellus* and *M. fuliginosus* suggests either co-option into these structures or they are evolutionary linked to a mixed PRC originating in the bilaterian ancestor.

7.3 Variations in eye developmental genes in annelids

Although the structural diversity is vast, the metazoan eyes are represented by a conserved set of genes. The strong conservation of these genes in different animal taxa agrees with the assumption of general homology. In our comparison of the common eye developmental genes in ventral and dorsal rhabdomeric eyes of *M. fuliginosus* and *P. dumerilii*, we find several similarities. The well-characterized eye genes such as Pax6, Six1/2 and Otx are all strongly expressed within the PRCs from very early to later development stages in both ventral and dorsal eyes. Moreover, in *M. fuliginosus*, Pax6 specifies larval eye precursors and also continues to be expressed in the fully differentiated ventral and dorsal eyes which is in difference to *P. dumerilii* where the developing dorsal eyes do not express Pax6 after the precursors have been specified by it (Arendt 2002). Other important eye genes such as Eya and Dach (which are part of the eye gene network) are either weakly expressed in early stages or show no expression within the PRCs. In Drosophila, both Eya and Dach are potent regulators of eye development shown by its level of expression and interacting partners (Davis 2017).This indicates possible lineage specific divergence and also reiterates the differences in potency of these genes in different lineages (Davis 2017).

7.4 Diversification of cerebral eyes in annelids.

In many of the annelid clades, two pairs of eyes are present. Typically ventral eyes appear first and then dorsal eyes which are usually referred to as larval and adult eyes respectively. But in many annelid species, the restriction to certain life stages is not clear since eyes appearing during the larval stages are often retained in the adult stage. It is also accepted that larval eyes are usually two-celled (one PRC and one pigment cell) and adult eyes are multicellular (PURSCHKE & NOWAK 2015) but this is not the case in *M. fuliginosus* as the second appearing dorsal eyes is two-celled while the first appearing ventral eyes have at least three PRCs and two pigment cells. Furthermore, in *M. fuliginosus*, the ventral eye is retained in the adult stage along with the pair of dorsal eyes, which appear a little later, but still in the larval phase. To prevent confusion by unclear terms, we hereby avoid the terms larval and adult eyes and refer *M. fuliginosus* eyes as ventral and dorsal eyes. Although the third pair of lateral epidermal eyes appear during the larval stage, it is not considered further due to the absence of molecular markers to follow their development. Moreover, in difference to the other two rhabdomeric eyes, the epidermal eyes have entirely ciliary structure and whether they are related to any other lophotrochozoan eyes is not known.

7.4.1   Diversification of r-opsins in annelids.

For most species of lophotrochozoans, only one canonical r-opsin gene has been discovered except for annelids and arthropods (COLBOURNE ET AL. 2011; RANDEL ET AL. 2013; FUTAHASHI ET AL. 2015). According to our r-opsin tree, the r-opsin split occurred early in annelid evolution before the divergence of the two main annelid groups – Sedentaria and Errantia. The two paralogs (ropsin1 and r-opsin3) have been identified in the errant polychaete *P. dumerilii* and are expressed in both the larval eyespot and the adult eyes (RANDEL ET AL. 2013). A similar pattern of expression is observed in both rhabdomeric eyespots of *M. fuliginosus* – while all PRCs of the ventral and dorsal eye express r-opsin3, r-opsin1 is only found co-expressing with r-opsin3 in the second PRC and the dorsal eye PRC. Moreover, r-opsin3 expression precedes r-opsin1in both ventral and dorsal eye PRCs which suggests the existence of similar regulation in the two eyes. It is also likely r-opsin1 arose due to tandem duplication as it is known to be the common mechanism of opsin gene family expansion (RENNISON ET AL. 2012; LIN ET AL. 2017). In the early branching annelids such as *Owenia fusiformis,* only one canonical r-opsin is known. *Helobdella sp.* and *Capitella teleta*

also have one canonical r-opsin which groups together with r-opsin3 which likely indicates secondary loss of r-opsin1.

7.4.2 The first eye is well-conserved in annelids

In close comparison of the ventral eyes in *M. fuliginosus* and *P. dumerilii,* we observe that they have several common features. The order in which the PRCs develop and the r-opsin1/3 expression dynamics suggest a common origin of these eyes. Thus it appears that despite the diversity of eyes in terms of number and organization, the first developing eye in most annelids is fairly simple and appears to be well-conserved. It is usually a few celled inverse pigment cup eyes as corroborated by ultrastructural data from several annelids (PURSCHKE ET AL. 2006). It is also likely that the first eye develops in a ventral position, similar to the eyespots in *M. fuliginosus* and *P. dumerilii*. In terms of opsin expression, r-opsin3 is common in the first eye. Moreover, the first r-opsin3+ PRC can mediate early phototaxis in both *M. fuliginosus* and *P. dumerilii* (JÉKELY ET AL. 2008). These data point out the importance of the first r-opsin3+ eye for early phototaxis and probably a feature of the annelid ancestor.

7.4.3 Is the second eye ancestral?

Many annelids possess a second eye appearing in a dorsal position. How these dorsal eyes are related is not known as they display much more complexity than the simple ventral eyes particularly in the errant species. Based on ultrastructural studies, the dorsal eyes of errant annelids are inferred to have a single origin (PURSCHKE & NOWAK 2015). Also referred to as adult eyes in *P. dumerilii* and other errant annelids, they evidently reflect an active lifestyle. Despite the differences in size and complexity the dorsal eyes of *M. fuliginosus* and *P. dumerilii* are comparable in many regards. The development of these eyes occurs in a similar position from the dorsal epidermis using the same complement of eye genes as the ventral eye. They first express r-opsin3 before co-expressing with r-opsin1 and then the expression of r-opsin1 increases and later on extends into the axon projecting to the brain (RANDEL ET AL. 2013, 2015). These features suggest that the dorsal eyes of *M. fuliginosus* and *P. dumerilii* are likely homologous.

The existence of similar eyes in two major annelid clades suggests that the ancestor of Errantia and Sedentaria already had two pairs of eyes - one ventral pair mediating early phototaxis and one dorsal pair mediating later phototaxis or even complementing the ventral eye if it persisted in later stages. Given that r-opsin1/3 co-expressing PRCs (PRC[(r1/r3)]) are

present in both ventral and dorsal eyes along with identical opsin expression dynamics suggests that the r-opsin duplication event likely preceded eye duplication event. In basal annelids, the number of eyes, what stage they appear and which position varies (WILSON 1982; IRVINE ET AL. 1999; HELM ET AL. 2016) and only a single r-opsin is known. But the identity of these eyes in terms of structure, opsin expression, and their ontogeny is not known. Therefore the situation in the annelid ancestor eyes is still hard to reconstruct.

7.5 Development and evolution of annelid eye circuitry

In *M. fuliginosus,* the first ventral eye PRC develops very early along with other bilateral sensory neurons which emerge at the periphery and together they start to send axons towards the central nerve plexus. Even before the pigment cup appears, a well delineated axon from this PRC is already reaching the brain. By the time the pigment cup develops at around 19 hpf, the primary neuronal scaffold of *M. fuliginosus* larvae is established and the larvae display phototaxis soon after.

In comparison, the first PRC in *P. dumerilii* projects an axon directly to the adjacent prototroch cells to make a cholinergic synapse and is already able to mediate phototaxis upon illumination (sensory-motor function) (JÉKELY ET AL. 2008). Only later does this axon turn to contact the secondary optic neuropil and then form synapses with motor neurons (Randel 2014). However, the phototactic ability of the ventral eyespot is only during the early stages as the large dorsal eye quickly takes over this functionality. Therefore the ventral eyespot is a sensory-motor eyes and is suggested that in later stages it either modulates phototaxis or mediates a distinct sensory-motor response (RANDEL ET AL. 2015).

We observed that in *M. fuliginosus* the perikaryon of the first PRC in the ventral eye is located close to the prototroch cells and the axon emerging passes the basal extensions of prototroch cells before it bends and projects towards the developing brain. Thus, a direct synaptic contact to the prototroch seems possible. Adding to this is the presence of both cholinergic (VAChT) and glutamatergic markers (VGluT) in the first PRC. Interestingly, only the first PRC of the ventral eye co-expresses two neurotransmitter markers, whereas the second and third PRCs only express VGluT. Further studies are needed to determine the significance of neurotransmitter co-expression in this PRC – whether they influence two target regions, prototroch (cholinergic) and brain (glutamatergic). The second PRC[(r1/r3)] in *M. fuliginosus* also directs its axon towards the brain similar to the first PRC and whether they have the same targets is not known. In *P. dumerilii,* the second PRC[(r1/r3)] projects to the

primary optic neuropil in the brain (RANDEL ET AL. 2015). Further characterization of the *P. dumerilii* ventral eye PRCs in terms of neurotransmitter type is needed for better comparison.

The dorsal eyes are glutamatergic in both species. In *P. dumerilii,* the dorsal eye PRCs[(r1/r3)] project to the primary optic neuropil similar to the ventral eye PRC[(r1/3)] (RANDEL ET AL. 2015). In *M. fuliginosus,* it is possible that a similar situation exists as the r-opsin1 mRNA staining reveals the axons emerging from the ventral and dorsal eye PRC[(r1/r3)] are seen converging towards the same region of the brain. Therefore the second PRC[(r1/r3)] may very well have similar targets in both species. Therefore the circuits of the ventral and dorsal eyes may be conserved in the two species.

When we look at the situation in the annelids outside of errantia-sedentaria, the position and number of eyes are variable and in some cases also appear much later during larval stages. In the case of *Owenia fusiformis,* the eyes develop after 14 dpf (HELM ET AL. 2016) *Magelona sp.* has two pairs of eyespots only during the larval stages wherein they lack a true prototroch ring (WILSON 1982). Is it cerebral connectivity from the start in these species? It has been proposed that the larvae of Urbilateria (the last common ancestor of bilateria) was ciliated and possessed simple two-celled eyespot mediating sensory-motor function (ARENDT ET AL. 2002). As the first PRC in both *M. fuliginosus* and *P. dumerilii* are cholinergic, existence of such a simplified sensory-motor PRC in the ancestor is likely and the situation in basal annelids is probably more derived.

7.6 Conclusions and future perspectives

Species separated by short evolutionary scale like the Errant-Sedentaria clades of annelids are ideal for studies on sensory structure evolution and species specific adaptations. The two rhabdomeric eyes in the sedentary *M. fuliginosus* and errant *P. dumerilii* display several similarities and likely share a common origin. Despite the considerable complexity of the multicellular dorsal eye in *P. dumerilii,* the basic two-celled eyespot structure appears to have augmented in such errant annelids by the addition of more sensory cells, pigment cells and accessory lens-like structures. The similar temporal expression dynamics of r-opsin1/3 and the subsequent expansion of r-opsin1 expression and localization in *M. fuliginosus* and *P. dumerilii* suggest the subfunctionalization of the two opsins. Additional characterization of these opsins, particularly r-opsin1 will be necessary to elucidate its function. To further understand the eyes and its connectivity in the annelid ancestor - whether it had a sensory-motor or cerebral connection or both; additional data is required from other established

species such as *Capitella teleta* and from basal branching annelids such as *Owenia* and *Magelona* starting with the neurotransmitter type of the eyes. In lophotrochozoans, the complete eye circuitry is only known from *P. dumerilii*; therefore, eye circuitry at the level of synaptic contacts will allow more detailed comparisons and provide further insights into their evolution.

Growing evidence has shown the importance of xenopsin in many protostome groups as the opsin representative of ciliary structures in cerebral eyes. While protostome r-opsins can be found co-expressed with xenopsin, protostome c-opsins always represent a distinct non-ocular cell type as in brain PRCs. The presence of both xenopsin and c-opsin (again in distinct cell types) in *M. fuliginosus* is a unique case so far as it is the only known species having both these opsins. Further characterization of xenopsin is needed to shed light on its molecular cascade and function, particularly in cells that co-express r-opsins.

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Papers I, II, III

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