# Comprehensive characterization of glial cells in the urochordate *Ciona intestinalis*

Riccardo Esposito

Thesis for the degree of Philosophiae Doctor (PhD)
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*To my family*

## Scientific environment

The work presented in this thesis was conducted in the laboratory of Marios Chatzigeorgiou at Sars International Centre for Marine Molecular Biology, University of Bergen, Norway. The thesis is part of the PhD program of the Department of Biological Sciences of the University of Bergen. This work has been funded from the Sars core budget.

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And Forza Napoli sempre!

# Author contributions

I declare that I have written this thesis (including Manuscript I and II) myself with help from Marios Chatzigeorgiou in form of comments and formal corrections. I was involved in the design of the projects and performed most of the experiments.

Direct contribution of other authors to Manuscript I are the following:

Under my supervision, **Jordi Zwiggelaar** (bachelor student) collected data and performed preliminary analysis of the glutamate dynamics and glutamate chemogenetic experiments. He also collected, analyzed and plotted behavioral data. **Daniel Dondorp** contributed by obtaining final violin plots of the chemogenetic experiments. **Kushal Kolar** contributed by obtaining final PCA plots of the chemogenetic experiments.

Direct contribution of other authors to Manuscript II are the following:

**Daniel Dondorp** contributed by obtaining final plot for ATP cytoplasmic experiment and RNAseq plots.

**Marios Chatzigeorgiou** contributed to the live imaging experiments, ATP plots and filopodia quantifications.

# Abstract

There are two main classes of cells that are present in the nervous system: neurons and glia. Neurons are the signalling units of the nervous system and are responsible to transmit electrical and chemical signals to other cells, and their function heavily relies on specialized glial cells. Initially considered as the glue keeping neurons together in nervous tissues, decades of research have highlighted the importance of glial cells and the pivotal roles they have in the nervous system assembly and functions. Glial cells are involved in neurogenesis, neuronal differentiation and migration, axonal growing and myelination, synaptogenesis, regulation of the synaptic microenvironment controlling ions and neurotransmitter concentrations, synaptic pruning, structural and tropic support to neurons, and phagocytosis of cell debris or external pathogen in defence to neurons. Also, glia contributes to various neurological disorders such as autism, schizophrenia, and neurodegenerative diseases such as Alzheimer's, Parkinson's, and amyotrophic lateral sclerosis. Given the number of processes in which they are involved, glial cells have attracted the attention of researchers, trying to unravel many aspects of their biology that are still elusive. Glial cells among vertebrates and invertebrates species display commonalities in their molecular fingerprint and in performing analogous functions, raising questions about their evolutionary origin. Whether glial cells originate from a common ancestor and have evolved together with the nervous system or have appeared multiple times during evolution is still unclear. Neither is known to which extend glial cells had contributed to the evolution of the nervous system as a whole. To gain more information about glia history and have a more complete picture of the evolution of the nervous system it is therefore fundamental to study glial cells in unexplored organisms. Invertebrate organisms provide suitable experimental models to explore glia functions *in vivo* because of their simplified nervous system architectures and the availability of several molecular toolkits. In our current understanding, glial cells are present in the nervous system of nematodes, arthropods, annelids, mollusks, and ambulacrarians, while little is known about glial cells in the invertebrate chordates. The limited and sparse evidence about glial cell types in urochordates, the sister group of vertebrates, has led to the hypothesis that glia have been mostly lost in this group.

This thesis provides a comprehensive understanding of the glial cells present in the embryonic and larva CNS of the urochordate *Ciona*, exploring their molecular characteristic and investigating the roles they have in the development and functioning of the nervous system. This work shows that glial cells with specialize functions are present in the urochordate, the sister group of vertebrates, providing insight into the evolution of glial cells in the chordate lineage.

# List of Manuscripts

The following manuscripts are part of the thesis:

Manuscript I: Esposito R., Zwiggelaar J., Dondorp D., Kolar K., Chatzigeorgiou M. **Multi-level characterization of the support cells in the embryonic and larva CNS of the urochordate *Ciona*. (**in preparation)

Manuscript II: Esposito R., Dondorp D., Chatzigeorgiou M. **Ependymal cells contribute to nervous system remodelling during metamorphosis in *Ciona intestinalis.*** (in preparation)

During my training I also contributed to the publication:

Ambrosino, L., Vassalli, Q.A., D'Agostino, Y., Esposito, R., Cetrangolo, V., Caputi, L., Amoroso, A., Aniello, F., D'Aniello, S., Chatzigeorgiou, M., Chiusano, M.L., Locascio, A., 2019. **Functional conserved non-coding elements among tunicates and chordates**. Dev Biol 448, 101-110

# Contents

# 1. Introduction

### 1.1 Urochordates occupy a critical position in the chordate lineage

Urochordates are a large group of invertebrate marine animals characterized by the presence of a cellulose-containing tunic that covers their body (Nakashima et al., 2004). They are also commonly known as Tunicates. These animals are usually divided in three main groups: (1) Appendicularia, also known as larvaceans, (2) Thaliacea, which are pelagic animals, and (3) Ascidiacea, vulgarly known as sea squirts, which include diverse sessile solitary and colonial species (Braun et al., 2020; Stolfi and Brown, 2015; Swalla et al., 2000). Although Tunicates have always been considered to have only one common ancestor given their unique ability to synthesize cellulose independently, recent molecular phylogenetic analysis provided support for the monophyletic origin of Appendicularia and Thaliacea, but not for the Ascideacea (Delsuc et al., 2018; Swalla et al., 2000; Tsagkogeorga et al., 2009). Together with the two subphyla of Cephalochordata and Vertebrata, Urochordates constitute the phylum of Chordata (Fig.1). Based primarily on morphological evidence, Cephalochordates were considered the closed living organism of Vertebrate, with the Urochordates located in a basal position within the Chordate lineage (Beaster-Jones et al., 2008; Schubert et al., 2006). By implementing bioinformatics analysis on protein-coding gene sequences (both mitochondrial and nuclear), intron-exon boundaries, miRNAs, and other empirical evidence recent studies have proposed an inversion in the position of Cephalochordates and Urochordates, assigning a closer evolutionary relationship to Urochordates as the sister group of Vertebrates (Delsuc et al., 2006; Telford and Copley, 2011), and to the Cephalochordates a more basal position inside the lineage. Given their key phylogenetic position, Tunicates became a suitable model organism for evolutionary and developmental biologists aiming to uncover the mechanisms that drive chordate embryonic development and morphogenesis.

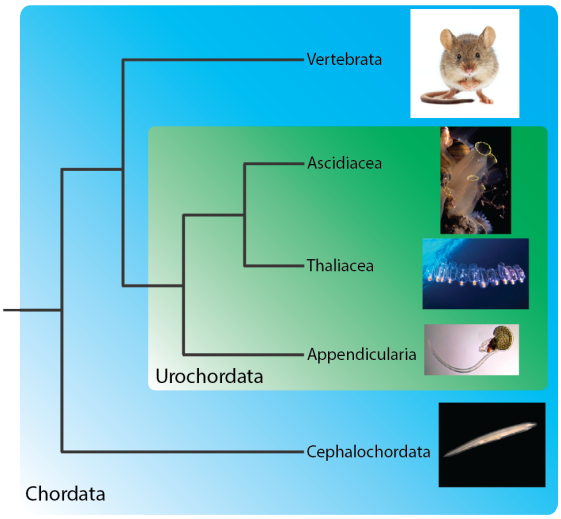


**Figure 1.** Simplified chordates phylogeny. Urochordates, the sister group of the vertebrates, are divided into three different classes: Appendicularia, Thaliacea and Ascidiacea.

### 1.2 *Ciona* genus: different species, same life cycle

Ascidians are invasive species in temperate waters. The genus *Ciona* is probably the more broadly studied one. For many years, researchers in Japan, North America, Northern and Southern Europe were under the impression that they were all working with the species *Ciona intestinalis*. Recently, a number of studies employing morphological and genomic analysis demonstrated that '*C. intestinalis*' is comprised of at least two species, *C. intestinalis* type A and *C. intestinalis* type B (Bouchemousse et al., 2016; Brunetti et al., 2015; Roux et al., 2013; Sato et al., 2012). Their distributions are very precise, with type A found in the Mediterranean, Pacific, and Southern Atlantic coast of Europe and type B found in the North Atlantic (Caputi et al., 2007; Nydam and Harrison, 2007, 2010), Norway included. In response to these findings, the tunicate community had to adjust the conventionally used nomenclature, since it has been shown that *Ciona intestinalis* belonged to the "Type B" species of Northern Europe, while Type A corresponds to *Ciona robusta* (Pennati et al., 2015). Further evidence suggests that *C. intestinalis* and *C. robusta* are two taxonomically and genetically distinct species, which are reproductively isolated

(Sato et al., 2014). Despite these differences, the two species exhibit an identical life cycle. Adult *Ciona* release simultaneously eggs and sperm into the surrounding seawater increasing the chances of fertilization (Satoh, 1994). Spawning occurs according to a light-dark cycle, and it happens with the first light of the morning (Svane and Havenhand, 1993). The developing *Ciona* embryo gives rise to a planktonic swimming larva. After settlement, the larva undergoes a process called metamorphosis in which a combination of environmental and endogenous signals drives the transformation of the body plan into a juvenile. This juvenile will give rise to the mature sessile adult animal within three months (Karaiskou et al., 2015; Sasakura et al., 2012). The adult body plan cannot justify the position of *Ciona* within the chordate lineage since it lacks the majority of chordate features besides the presence of pharyngeal slits that are shared by all chordates, even though they are not an exclusive characteristic of this lineage. In contrast, the larval stage provides hints regarding the simple chordate body plan. In fact, at this stage, it is possible to observe a centralized nervous system with a dorsal hollow nerve cord, the notochord and a post-anal tail that allow swimming (Holland et al., 2004).

### 1.3 Ascidian embryonic development

Ascidian embryos develop similarly to other chordates like amphibians and bony fishes (Ballard, 1981; Lane and Sheets, 2006), with the ectoderm deriving from the animal pole, endoderm from the vegetal pole and the mesoderm from the equator, with the advantage of having blastulae composed of only 64 cells rather than several thousand cells of other chordates (Lemaire et al., 2008). Moreover, ascidian display an invariant cleavage pattern, and its conserved fate map represent a valuable source to study cell lineages. The first description of an ascidian embryo's development dates back to the early twentieth century, when the American embryologist Edwin G. Conklin carried out research on *Styela canopus*. He was able to manipulate and trace single blastomeres during the development up to their final fate and describe the first complete cell lineage of an organism. Cleavage patterns, cell lineages, and final body

plan between distantly related ascidian such as *Ciona* and *Halocynthia* are significantly conserved (Hudson and Yasuo, 2008; Lemaire, 2009). Since the overall ontogeny of these organisms is very similar, cell lineage data obtained in one species often they are considered to be valid for the entire ascidian class. Ascidian embryogenesis has been considered a typical example of mosaic development (Conklin, 1905a). After fertilization, the eggs show an ooplastic segregation: cytoplasmic determinants start to localize in specific regions of the embryo and drive the specification of the early embryonic cell fate (Conklin, 1905b). Maternal determinants have been characterized for endoderm (Marikawa and Satoh, 1996), epidermis specification (Ishida and Satoh, 1998), gastrulation movements (Nishida, 1997), asymmetric cleavage (Nishikata et al., 1999), and muscle determinants (Kobayashi et al., 2002). Blastomeres are autonomously able to differentiate in the epidermis, muscle and endoderm without the involvement of any cell-cell signaling. On the other hand, the notochord, brain and pigment cells, are not able to differentiate when embryonic cells were dissociated, suggesting that these blastomeres require cell interactions (Bertrand et al., 2003; Kumano and Nishida, 2007; Lemaire, 2009; Nishida, 1997, 2005; Picco et al., 2007; Shi and Levine, 2008). The embryonic development is very easy to follow since cell divisions are bilaterally symmetrical. The complete development is very fast, indeed from the zygote to the swimming larva it takes about 18 hours at 18°C (Hotta et al., 2007)(Fig. 2). The first developmental stages are named according to the number of cells like 8-, 16-, 32-, 64- and 110- cell stages (Satoh, 2003). From the 8 cells stage is possible to follow four specific lineages, the cells two cells in the vegetal pole (A4.1 and B4.1) and the other two in the animal pole (a4.2 and b4.2). These blastomeres are restricted in fate early in development, from the beginning of gastrulation shortly before the 110-cell stage (Munro et al., 2006). The row of cells that will give rise to the neural plate starts forming before the completion of blastopore closure. Similarly, to vertebrates, ascidian CNS develops via neurulation, which begins with the formation of the neural plate and ends when the left and right epidermis overlying the neural tube fuse through a zipper mechanism to close the neural fold (Hashimoto and Munro, 2019; Hashimoto et al., 2015). Once the neural tube is completely closed, the tail becomes

elongated. This elongation process continues to form tailbud stages towards the larva stage where the animal is ready to hatch. The settlement and the metamorphosis of tadpole-like will follow and the juvenile will take 2 or 3 months to become an adult with reproductive capability, depending on the temperature of the environment (Marikawa et al., 1994).

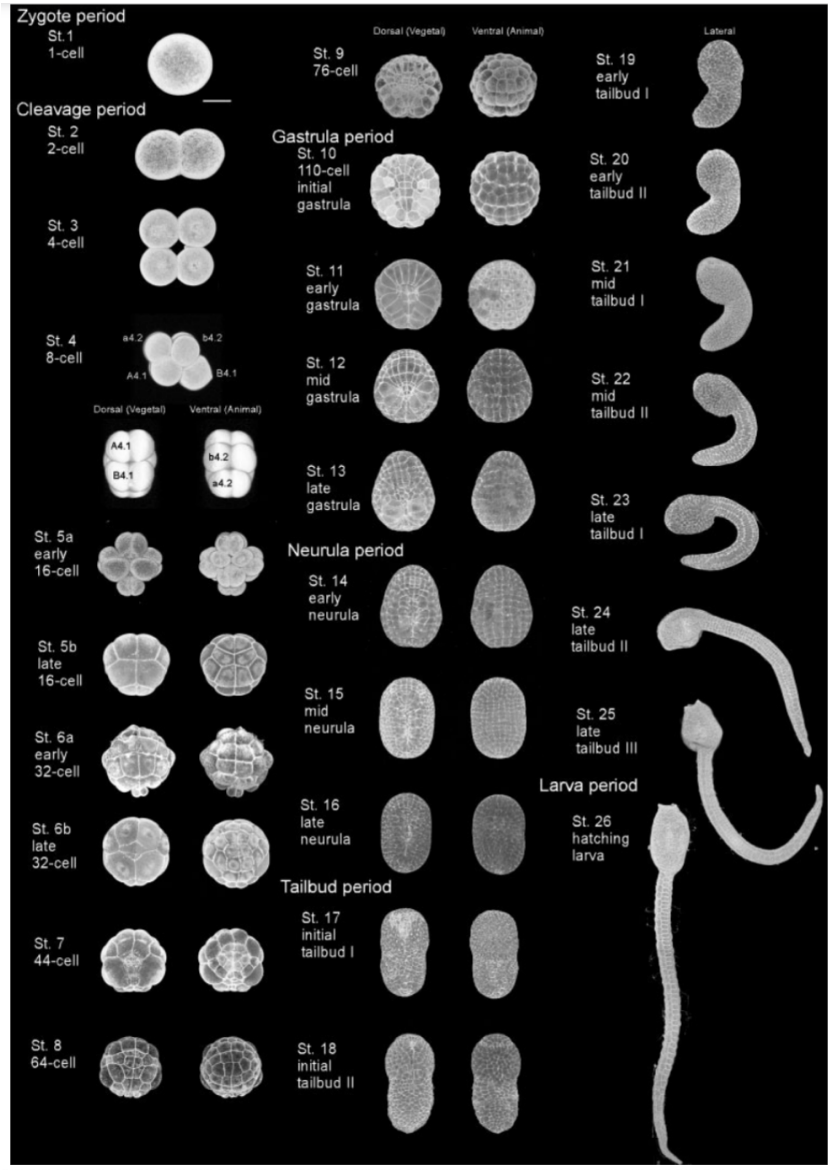


**Figure 2.** Three-dimensional reconstructed images of the *C. intestinalis* embryo. From (Hotta et al., 2007).

### 1.4 *Ciona intestinalis*: a suitable model system for biological research

During the last decade *Ciona* becomes a popular model for studying chordate development and molecular mechanisms behind cell differentiation and specification (Kourakis and Smith, 2015). *Ciona* has a very small and compact genome with very few genes duplicated, although several genes present in vertebrates and invertebrates have been lost. Moreover, most of the cis-regulatory elements are usually located in the proximity of the genes (Dehal et al., 2002; Holland and Gibson-Brown, 2003). The availability of the closely related species *Ciona savignyi* genome allowed comparison between complementary sequences. This analysis has facilitated the identification of conserved regulatory sequences, highly suitable for studying transcriptional regulation (Bertrand et al., 2003; Johnson et al., 2004; Squarzoni et al., 2011). *Ciona*'s embryonic development is very fast, and the cleavage pattern of the embryo is invariant (Satoh, 2003). Fate maps have been drawned to trace embryonic development (Conklin, 1905b; Lemaire, 2009; Whittaker, 1973). The embryos are transparent and small in size, allowing gene expression analysis via whole-month *in situ* hybridization (Satoh, 2001). Electroporation of *Ciona* eggs permits the generation of a large number of transgenic embryos and thus enables high-throughput functional screens and other types of experiments that utilize large numbers of constructs (Zeller, 2018). Microinjection of antisense morpholino oligonucleotides and dominant negative experiments to test gene functions have been widely employed (Stolfi and Christiaen, 2012). TALEN knock-down (Treen et al., 2014) and CRISPR/Cas9 (Sasaki et al., 2014; Stolfi et al., 2014) methods for genome editing have been successfully established in the community. With the publication of the whole *Ciona* larva CNS connectome (Ryan et al., 2016, 2017, 2018), *Ciona* become the second model organism after the nematode *C.elegans* to have a complete description of the neural network connections. The connectome represents an extremely valuable resource for the community helping *Ciona* to become a suitable model organism for neurobiological studies. In addition, the recent effort to quantify the larval behavioral repertoire (Rudolf et al., 2019), and to analyze its behavioral outputs in response to certain sensory cues (Kourakis et al., 2019; Salas et al., 2018) make *Ciona* a potentially great model for investigating how a simple brain can

perceive sensory stimuli, integrate them in the brain and generate a coordinated behavioral response. Importantly, as a simple chordate, *Ciona* can provide significant insight into the evolutionary origin of the vertebrate cell types. For this reason, single-cell transcriptome analysis has been performed and the transcriptomes of meta-cell data are available to the community (Cao et al., 2019; Sharma et al., 2019). Thus, all these features make *Ciona* a very suitable model in order to explore the establishment of typical basic chordate features and could potentially provide insight into vertebrate nervous system development and function.

### 1.5 *Ciona* nervous system

Ascidian eggs are a typical example of mosaic development. Indeed the differentiation of muscle, endoderm and epidermis cells depends entirely on maternal factors and does not require any inductive signal from the surrounding cells (Conklin, 1905a; Nishida, 2005; Nishida and Sawada, 2001). This autonomous cell specification happens to be partially present also in the differentiation of the nervous system cells. The *Ciona* CNS originate from the a-, b- and A-line blastomeres of the 8-cell stage embryo. The A-line blastomere is determined autonomously since no cell-cell communication is needed to differentiate (Minokawa et al., 2001). In contrast, the neural differentiation of the a-line blastomere needs an inductive signal coming from the blastomere of the vegetal site. Fibroblast growth factor (FGF) from the vegetal blastomere is responsible for activating two maternally expressed transcription factors, Ets and GATA, that in turn activate the transcription factor Otx which is essential for the specification of the anterior part of the CNS (Bertrand et al., 2003; Hudson and Lemaire, 2001; Khoueiry et al., 2010; Lemaire et al., 2002; Rothbacher et al., 2007). The same role in the induction and subsequent patterning of the CNS has been observed in vertebrates (Altmann and Brivanlou, 2001). The peripheral nervous system arises instead from both a- and b-line blastomeres. After gastrulation, rows of cells from the ectodermal layer give rise to the neural plate. *Ciona*'s embryonic neural plate is composed of 42 cells in total coming from the A-,

a- and b- lineages that provide 14, 24 and 4 cells respectively, organized in six rows along the A-P axis, and four bilateral pairs of columns (Gainous et al., 2015) (Fig.3). Rows I and II are derived from A-lineage while the rows III-VI come from the a-lineage. The formation of this structure is driven by the combination of three different signaling pathways. Nodal pathway is necessary for the specification of the lateral part of the a-lineage neural plate cells. FGF9/16/20 via activation of the Ras/MEK/ERK signaling pathway regulates the expression of Nodal in the b-line blastomere that then induces the lateral cell fates of adjacent A-line blastomeres (Hudson et al., 2003; Hudson and Yasuo, 2005). Delta2/Notch is required for both the second and fourth columns of cells in the later and earlier phases of this stage respectively. The neural plate's simple structure reflects a high degree of compartmentalization (Imai et al., 2009). The anterior rows V and VI will give rise to the anterior epidermis and palps (Abitua et al., 2015; Nishida, 1987), anterior rows III and IV generate parts of the sensory vesicle (Haupaix et al., 2014; Racioppi et al., 2014; Squarzoni et al., 2011) while the posterior row I and II will generate the posterior part of the CNS, such as the posterior sensory vesicle and the visceral ganglion (Christiaen et al., 2007; Nishida, 1987).

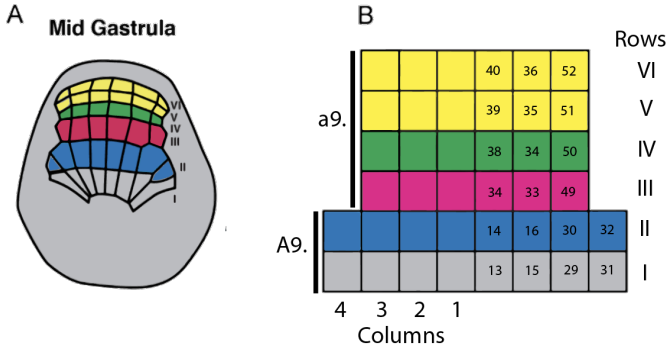


**Figure 3.** *Ciona* neural plate. **(A)** Schematic of a mid-gastrula stage embryo showing the organization of the 6-row neural plate. **(B)** Fates of the neural plate. Yellow cells give rise to anterior epidermis and pharynx/neurohypothesis precursors; Green cells generate anterior sensory vesicle; Pink cells, anterior sensory vesicle and pigmented cells; Blue posterior sensory vesicle and motor ganglion; Gray Tail nerve cord. Picture modified from (Gainous et al., 2015).

The ependymal cells in the sensory vesicle together with those present in the lateral and ventral rows of the tail arise from the row I and the medial lineages of row III with the transcription factor Myt1L repressing signals coming from the neighbor rows (Gainous et al., 2015). Myt1L is a nervous system specific transcription factor involved in proliferation, differentiation, and production of myelin in oligodendrocyte precursors in vertebrates (Armstrong et al., 1995; Kim et al., 1997; Kim and Hudson, 1992; Nielsen et al., 2004). During the neurulation process, neural plate cells roll up at the dorsal side of the body to form a tubular structure. The epidermal layer surrounding the neural plate contributes to this process via an actomyosin dependent mechanism which is regulated via Rho/ROCK signaling (Hashimoto and Munro, 2019; Hashimoto et al., 2015; Ogura et al., 2011). Nodal seems to be involved in this process as well since its perturbation results in neural tube closure failure (Mita and Fujiwara, 2007). The fully formed neural tube is very simple and is composed of only four rows of cells: a dorsal one, a ventral one, and two laterals. The ventral cells constitute the rudimentary floor plate, which acts as a signaling hub, receiving input from the underlying notochord (Shi et al., 2009). With the closure of the neural tube, the elongation phase of the animal begins and the separation of the tail from the trunk region takes place. During the elongation, the tail starts to bend while in the trunk the most important process is the pigmentation of the otolith and ocellus. Epidermal neurons elongate their cilia and palp formation is initiated by thickening and bulging of the anterior-most trunk epidermis (Hotta et al., 2007). The fully formed CNS of the tadpole *Ciona* larva is divided into the anterior sensory brain vesicle (BV) which contains the pigmented cells, connected by a neck to the motor ganglion (MG) within the larval trunk, and a caudal nerve cord (CNC) in the tail (Fig.4) (Nicol and Meinertzhagen, 1991).

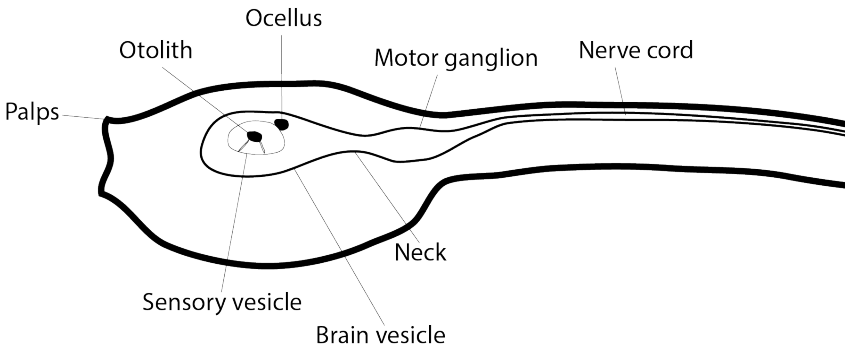


**Figure 4.** Drawing of the CNS of *Ciona* larva.

From the connectome we know that there are 330 cells, of which 177 neurons are asymmetrically positioned and connected. The remainder of the non-neuronal cells based on their EM derived morphology have been classified as ependymal cells. Moreover, some "ambiguous cells" or "accessory cells" with synaptic regions but lacking neuronal form have been previously described (Nicol and Meinertzhagen, 1991; Ryan et al., 2016). The CNS neurons can be divided into 50 different types and each of them has an average of 49 synapses with other cells, indicating that the neural network is still relatively complex even though it is a small one (Ryan et al., 2016). A number of sensory neurons which are located inside the brain vesicle together with relay neurons that form the posterior part of the optic vesicle extend their axons across the neck into the motor ganglion. Epidermal sensory neurons also project in the same posterior region, suggesting that the sensory vesicle may act as a processing center that can integrate sensory inputs and communicate with the motor ganglion (Horie et al., 2008). From the motor ganglion, motor neurons elongate and innervate posteriorly in the muscle tail. Massive analysis of nervous system-specific genes has been performed (Gibboney et al., 2020). The potential tripartite organization of the *Ciona* larva nervous system has been tested through the expression analysis of Otx, Pax2/5/8, Engrailed, and the Hox genes reflecting regional homology between tunicate and vertebrate CNS (Imai et al., 2002). In addition, the anterior part of the neural tube is considered homologous to the vertebrate anterior spinal cord (Imai et al., 2002). The presence of an MHB organizer similar to that found in vertebrates has been established through gene expression studies. In particular, FGF8/17/18, the

orthologue of the vertebrate FGF8, defines the posterior part of the sensory vesicle and the neck region by generating sequential patterns of regulatory gene expression, suggesting that the primitive MHB-like activity predates the vertebrate CNS (Imai et al., 2009). Finally, with the identification of rudimentary neural crest (Abitua et al., 2012), neurogenic placode (Abitua et al., 2015), and migrating neurons derived from neural crest cells (Stolfi et al., 2015), the *Ciona* nervous system has provided evidence for a deeper evolutionary origin of neuronal structures originally thought to be vertebrate specific. Despite all the established homology, except for ependymoglia cells little has been investigated regarding the presence of other supporting cells and their putative functions in the *Ciona* larva, although is well know the key role they play in the development and maintenance of both vertebrate and invertebrate nervous system.

### 1.6 Glial cells: from "support cells" to leading role in the nervous system

Even though the first studies on the morphology and electrophysiological properties of the grey and white matter in the human brain date back to the 16$^{th}$ and 18$^{th}$ centuries, biologists had relatively little evidence for the morphological diversity of cells in the nervous system. However, this picture started to change drastically in the early 19$^{th}$ century when new histological staining methods were combined with early forms of microscopy. One of the key cell types that this methodological revolution brought to the spotlight was glial cells (Fan and Agid, 2018; Garcia-Marin et al., 2007). The term "glia" derives from the greek γλία and γλοία which means glue. Indeed, one of the first definitions of glial cells from Rudolf Virchow in 1858 described glial cells as "*Connective substance which forms in the brain, in the spinal cord, and in the higher sensory nerves a sort of neural putty, in which the nervous system elements are embedded*" (Virchow et al., 1858). Some key early contributions in glial cell biology came from Camillo Golgi (Golgi, 1873, 1885) that first distinguished glial cells from the neurons because of the absence of axons, Rìo-Hortega who postulated the presence of microglial cells (del Río Hortega, 1920), and

Michael von Lenhossék (von Lenhossék, 1893) that introduced the term “astrocyte”. The real breakthrough in glial cell research is associated with the studies of Santiago Ramòn y Cajal (Ramon y Cajal, 1895, 1909, 1911, 1913a, b; Ramon y Cajal et al., 1888)**,** who based on primarily morphological evidence postulated different theories about the function of glial cells. Due to the increase of advanced electrophysiological and biochemical technologies applicable to neurons, and the unavailability of approaches to follow up with more detailed morphological observation, in the early 1900s glial cell research stagnated. This resulted in the formulation of nervous system models with a strong bias on the role and contribution of neurons. It was with the advent of electron microscopy in the 1950s (Peters and Palay, 1965) and the characterization of glial cell physiological properties (Coombs et al., 1955; Cornell-Bell et al., 1990) that scientists made substantial progress and explored the role of glial cells in the nervous system. In our current understanding of the nervous system, glial cells play a critical role in development from neuronal birth and migration, they contribute to axon guidance, synaptogenesis, and circuit assembly. In fully formed neural circuits, the role of glial cells in synaptic communication, pruning and plasticity, neuronal homeostasis, and network-level neuronal activity has been demonstrated (Allen and Lyons, 2018). Due to the variety of roles and functions that non-neuronal cells have, in the last decades researchers have been trying to characterize the presence of glial cells in several organisms among metazoans in order to obtain a complete understanding of the nervous system evolution. Currently, general glia knowledge is limited to specific phyla, and the evidence of functioning glial cells in many invertebrates is sparse. It is therefore important to study a diversity of organisms to expand our general understanding of glia. Morphological, genetic and functional characterization were used to infer glial identity and in the last decade have led to a conventional classification that can be used to catalog glial cells. In the next paragraphs, I will summarize the current understanding of the glial cells in vertebrates and in invertebrates.

### 1.6.1 Glial cells in vertebrates

It has been estimated that the ratio between glial cells and neurons in different mammals is around 3:1, while in the human brain this ratio is closer to 1:1 (Herculano-Houzel, 2014). Two different main groups of glial cells are present in the vertebrate nervous system: microglia and macroglia. The first group is composed of mesodermally derived macrophage cells that protect neurons during injury, infection or degenerative disease. Microglia are involved in clearing damaged neurons, synaptic pruning and modulation during development both in normal and pathological conditions, playing fundamental for maintaining the health of the nervous system (Bessis et al., 2007; Hong et al., 2016a; Hong et al., 2016b; Vasek et al., 2016). Macroglial cells are derived from the ectodermal layer of the embryo and are classified based on morphological, genetic and functional properties as astrocytes, oligodendrocytes, and Schwann cells. Astrocytes are the most abundant and the best studied type of cells in the adult brain. These highly heterogeneous cell types are usually morphologically described as cells with high structural plasticity possessing fine processes that contain large bundles of tightly packed intermediate filaments (Kettenmann and Ranson, 2005). Astrocytes link up to other glial cells and neurons with the surrounding capillaries providing nutrients as trophic support. Moreover, they are responsible for regulating the function of synapses by controlling the concentration of ions and neurotransmitters. Although glial cells do not generate action potentials, they have been found to participate in neuron-glial signaling processes (otherwise termed a tripartite synapse) (Kimelberg, 2010; Kimelberg and Nedergaard, 2010). In specific contexts, astrocytes act as nonprofessional phagocytes for corpse removal (Iram et al., 2016; Morizawa et al., 2017). Their involvement in neurodegenerative diseases and neurodevelopmental disorder has been well characterized (Butterworth, 2010; Felipo and Butterworth, 2002; Lennon et al., 2004; Li et al., 2011; Molofsky et al., 2012; Parpura and Haydon, 2008; Wingerchuk et al., 2007; Wyss-Coray et al., 2003). The molecular identification of astrocytes usually relies on the expression of the Glial fibrillary acidic protein (GFAP), although recent studies in rodents and humans highlighted subpopulation of astrocytes GFAP(-) (Kuegler et al., 2012; Zhang et al., 2016). Other markers such as S100β, FGFR3,

FABP7, BLBP, SOX9 and ALDH1 are not exclusive astrocyte-committed markers but they are expressed during different neurogenic stages (Adam et al., 2012; Rowitch and Kriegstein, 2010). AQP-4, GS, GLT-1, and GLAST-1 transporter are often used to study astrocyte differentiation (Kleiderman et al., 2016; Krencik and Ullian, 2013; Krencik and Zhang, 2011; Kuegler et al., 2010). It is for these reasons that a combination of marker genes, functional properties and morphological characters is needed to reliably identify astrocytes.

Oligodendrocytes and Schwann cells are small cells with relatively few processes, and they are responsible to form the myelin sheath that insulates axons and allows rapid conduction of signals. The myelin sheath is an extension of the oligodendrocyte and Schwann cell plasma membrane that protrudes from the cellular body and wraps concentrically the axons (Raine, 1984; Snaidero et al., 2014; Sobottka et al., 2011). It has been shown that myelin formation can be regulated by several factors such as signaling proteins in Schwann cells and calcium activity in oligodendrocytes (Baraban et al., 2018; Nave and Salzer, 2006). The myelination process has been fully described on the molecular level, and the cellular morphological mechanisms have been elucidated (Nawaz et al., 2015; Zuchero et al., 2015). Moreover, oligodendrocytes and Schwann cells provide neurons with trophic support (Nave, 2010; Saab et al., 2013; Simons and Nave, 2015). Oligodendrocytes are present in the central nervous system and share the same origin with other macroglia cells, while Schwann cells are present in the peripheral regions and arise from the neural crest lineage (Jessen and Mirsky, 1998). Several genes can be used to detect these cells trough out their development such as the transcription factors Olig1/2, SOX10, NG2 and Nkx2.2 (Kuhlbrodt et al., 1998; Qi et al., 2001; Zhou and Anderson, 2002; Zhou et al., 2000; Zhu et al., 2014). The importance of these cells in the pathology of demyelinating diseases is easily appreciated, as myelin loss directly affects nerve transmission (Fields, 2008; Saab et al., 2013).

Aside from these two main glial groups, there are the radial glial cells. So called because of their morphology, radial glial are one of the most versatile and intriguing glia types, and they contribute to several aspects of the nervous system development.

During embryonic stages, radial glial cells can differentiate in neurons by asymmetrical division or switch to generate mature specialized support cells (Götz and Huttner, 2005; Noctor et al., 2001; Noctor et al., 2004; Noctor et al., 2008). As neural precursor cells, the transition from the pluripotent state to differentiate cells is regulated by the exposure to a combination of environmental signals (Chambers et al., 2001; Del Bene et al., 2008; Malatesta et al., 2003; Shimizu et al., 2008), developmental timing (Anthony et al., 2004) and intrinsic signaling (Mizutani et al., 2007). A key factor in the specification of radial glial cells is the transcription factor Pax6 (Haubst et al., 2004; Heins et al., 2002). Mature radial glial cells can be recognized for their secretory trait and the ability to produce a glycoprotein named SCO-Spondin (Gobron et al., 2000; Gobron et al., 1996; Lehmann et al., 2001; Meiniel, 2007; Vera et al., 2013). Furthermore, radial glial cells close to differentiation share with astrocyte the expression of makers such as glutamate transporter GLAST, S100β, glutamine synthase (GS), BLBP, vimentin and tenascin (Gotz and Barde, 2005). Moreover, during nervous system development radial glial cells are fundamental as they act as a scaffold for neuronal migration and axon growing (Edmondson and Hatten, 1987; Noctor et al., 2001; Norris and Kalil, 1991). During these processes, different types of adhesion molecules are expressed in radial glial cells to ensure cell contact (Martinez-Garay, 2020; Valiente et al., 2011). In fish and amphibians, radial glia retain a radial morphology throughout their life and it has been suggested that they may carry out similar functions to astrocytes (Lyons and Talbot, 2014).

Another type of glial cells present in vertebrates are the ependymal cells. They are ciliated cells morphologically characterized from a cuboidal or columnar shape that separates the cerebrospinal fluid that fills cavities from the other tissue. Ependymoglial cells are involved in the production of cerebrospinal fluid and there is evidence in favor of the idea that they act as a reservoir for neurogenesis (Del Bigio, 1995, 2010; Jimenez et al., 2014; Johansson et al., 1999; Wozniak, 1999). During different times of their life, ependymoglia cells express several markers such as CD133, fibroblast growth factor FGF2, the calcium binding protein S100β and

glucose transporter (Coskun et al., 2008; Kobayashi et al., 1996; Kojima and Tator, 2000; Sarnat, 1998; Silva-Alvarez et al., 2005; Yu et al., 1995).

Glial cells are also present in the enteric nervous system, where contribute to the regulation of gastrointestinal motility, gut inflammation, epithelial barrier function and immunity (Grubisic and Gulbransen, 2017; Ochoa-Cortes et al., 2016; Sharkey, 2015). Finally, in ganglia of the peripheral nervous system, satellite glial cells enwraps the soma of sensory neuron (Pannese, 2010) and participate in sensory signals (Huang et al., 2013; Kim et al., 2016).

Neurons and glial cells, except for the microglial cells, all shared the same lineage that is summarized in Figure 5.

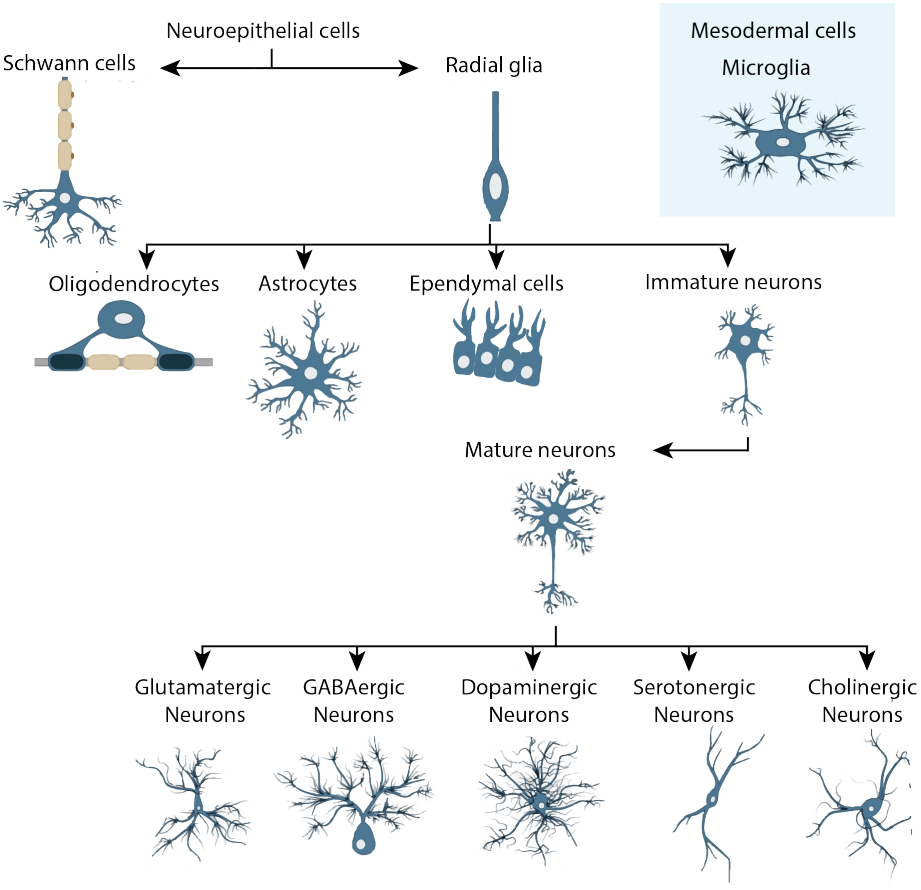

**Figure 5.** Simplified cell lineage that specifies for neurons and glial cells. Microglial cells are mesodermally derived cells, while neurons and all the other types of glial cells differentiate from the neuroepithelium. Images adapted from https://www.cellsignal.com/contents/science-cst-pathways-neuroscience/neuronal-and-glial-cell-markers/pathways-neuronal-and-glial-cell-markers

### 1.6.2 Glial cells in invertebrates

Invertebrate organisms offer several advantages for studying the nervous system. Due to their high experimental accessibility, they provide models for identifying and uniquely targeting specific cells population. Thanks to the toolkits that the scientific community has developed in the last decade, invertebrates represent a perfect reservoir of knowledge for understanding cell functions *in vivo*. Indeed, their contribution to understanding the fundamental proprieties of glial cells is remarkable. The first clear evidence of an interaction between neurons and glia comes from the studies in the squid *Loligo pealei*, in which myelinating cells in the peripheral nervous system were found to surround the giant axon. The hyperpolarization of a Schwann-like cell stimulates glutamate release from the nearby axons that act on the Schwann-like cell itself starting a series of signaling events between neurons and glia (Brown et al., 1991; Lieberman et al., 1989; Villegas et al., 1987). In the snail *Helix pomatia,* support cells are an integral component of the nervous system as their calcium changes affect neuronal calcium dynamics (Gommerat and Gola, 1994). Two different types of astrocyte-like glial cells were found in the segmental ganglion of the leech *Hirundo medicinalis*. Because of their large size and the connections with other cells through gap junctions, the electrophysiological profile of the giant glial cells has been highly characterized. Their ability to respond to neuronal signaling by generating calcium and potassium changes leads to a change in pH that affects neuronal excitability (Coggeshall, 1974; Deitmer et al., 1999; Deitmer and Schneider, 1995; Kuffler and Nicholls, 1966). Moreover, their responses to neurotransmitters such as glutamate or GABA have been examined (Deitmer and Rose, 1996; Deitmer et al., 1998; Schmidt and Deitmer, 1999).

Further insight into the importance of glial for neuronal development comes from insects. In *Manduca sexta*, the interaction between neurons and glia is necessary for driving the glia in the organization of the antenna lobe glomeruli and the olfactory receptor neurons (Rossler et al., 1999; Tolbert and Oland, 1990). In the grasshopper, the ablation of the segmented boundary glial cells results in an interaction failure with the intersegmental nerve, affecting the development of the nervous system wall (Rossler et al., 1999). In the honeybee drone, ions homeostasis in the retina is highly regulated by glial cells (Coles, 1989). Around 10% of cells present in the *D. melanogaster* nervous system have been identified as glia. The entire surface of the CNS is cover by cells with flat morphology called subperineural glia that form the blood-brain barrier (Schwabe et al., 2005). Perineural glia and wrapping glia cells are implicated in the neuromuscular junction (Stork et al., 2008), while cortex glia surround neuronal cell bodies in the neuronal cortex region. Moreover, astrocyte-like cells are present in the synaptic neuropil (Awasaki et al., 2008; Doherty et al., 2009). The gene expression profiles of the *D. melanogaster* glia are established, so specific markers are used to investigate the functions of specific cells via the use of binary expression systems (e.g. UAS-GAL4). Glia control axon guidance during the early assembly phases of neural circuits and provide trophic support to them (Bergmann et al., 2002; Jacobs, 2000; Poeck et al., 2001). Moreover, during metamorphosis glia are involved in synaptic growth and plasticity, axonal pruning (Awasaki and Ito, 2004), and can phagocyte dead neurons to clean up the CNS during development (Sonnenfeld and Jacobs, 1995), and in case of injury (MacDonald et al., 2006). *D. melanogaster* CNS glia are also involved in the clearance of the synaptic cleft from neurotransmitters. Indeed, both glutamate (EAAT) and GABA transporter (GAT) are express on their surface, together with enzymes for the neurotransmitters metabolism, such as glutamine synthetase (Freeman et al., 2003; Thimgan et al., 2006).

In worms, glial cells have been extensively characterized at both the morphological and functional levels. The nervous system of the nematode *Caenorhabditis elegans* has 50 glial-like cells which are associated with peripheral sensory structures (Shaham, 2005, 2006). All the cell interactions are well established, allowing for the detailed characterization of neuron-glia cooperation (Singhvi and Shaham, 2019).

Glia cells in *C. elegans* are fundamental for neuronal structure development. For example, the ablation of socket glia results in defective neuronal development (neuronal cilia cannot form/or have defective morphology). The perturbation of a subset of glia associated with cephalic sensory neurons results in deficient neuronal dendrite extension and connection to the axons in the brain (Yoshimura et al., 2008). Moreover, the perturbation of a subset of glia cells in adults sensory system results in difference in their chemotactic behavior and in neuronal activity changes (Bacaj et al., 2008).

### 1.6.3. Where do we stand in glial cells comprehension among Bilateria?

The presence of glial cells has been investigated in several animals among the Metazoa (Hartline, 2011). Given their close association to neurons and that they are involved in numerous functions related to the development and activity of the nervous systems, it is reasonable to wonder whether these cells had evolved in parallel or somehow had contributed to the evolution of the nervous system as a whole. A model describing the possible paths in the evolutionary emergence of glial cells in parallel with the evolution of the nervous system has been reported by Hartline in 2011 (Fig.6). From basiepithelial nervous systems (Fig.6A), epidermal supporting cells might have arisen (Fig.6B) and migrate to a deeper position in the body following the evolution of the nervous system (Fig.6B in yellow). In this way, internalized neurons might had been ensheathed by glial cells (Fig.6C), with (bottom section) or without (upper section) cytoplasmic penetration between axons. The internal nervous system resulted to be only surrounded by sheath cells (Fig.6D) or invaded by sheet-like interstitial glial cells (Fig.6E). Finally, sheath cells from stage D might had invaded the spaces between neurons, or interstitial glial cells from stage E might had expanded around the outside of the neurons to provide ensheathment (Fig.6F).

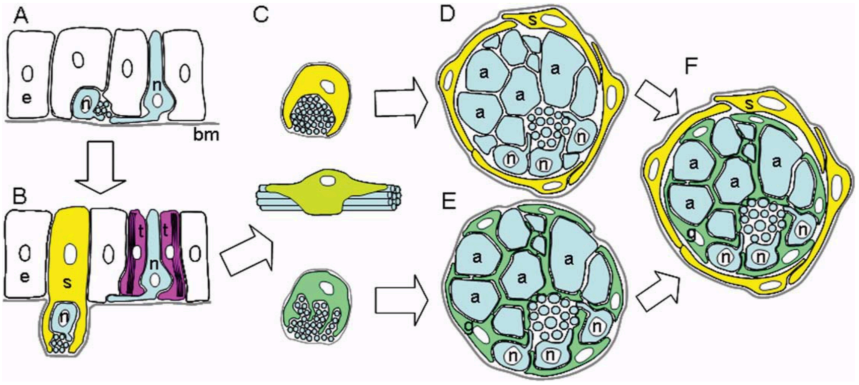


**Figure 6.** Schematic explaining putative paths that could have driven the emergence of glial cells. From (Hartline, 2011).

A clear examples towards the parallel evolution of nervous system and glia is represented by the support pigmented cells and their neuronal counterpart (Arendt, 2003; Arendt and Wittbrodt, 2001). Although old morphological based studies had proposed an independent evolution model for the photoreceptor cells, molecular and genetic approaches have confirmed a well conserved network of transcriptional factors that strongly support the idea that photoreceptors have evolved monophyletically (Erclik et al., 2008; Erclik et al., 2009). This neuron-support cell pair evolution observed in photoreceptor cells suggests that it is likely that some kind of glial cells might exist in close association with photoreceptor cells in well-developed nervous systems such as in Cnidaria. In fact, some epithelial cells in *Tripedalia cystophora* have been described as able to group some neuronal axons, suggesting this as an ancestral glia-like function (Garm et al., 2007). Unfortunately, the lack of functional and molecular analysis of these cells doesn't allow us to infer their glia identity and to be aware of the presence of glia in Cnidaria.

The molecular fingerprint of glial cells has provided considerable insight into their putative roles. In fact, different classes of genes are necessary in order to carry out specific functions. The expression of specific molecules has been used to distinguish glial cells from neurons and to assess glia functional proprieties in both vertebrates and invertebrates. Genes coding for proteins involved in providing trophic support and modulating levels of neurotransmitters at the synaptic cleft are characteristic glial

features. For example mammalian glia, as well as many other invertebrate glia, express neurotransmitter transporters together with enzymes involved in neurotransmitter metabolism (Halassa and Haydon, 2010; Stacey et al., 2010). Adhesion molecules are essential for ensuring glia-glia and glia-neuron interactions. Therefore, a combination of molecular and functional characteristics can then be used to define glial cells.

At present, we still miss the complete understanding of whether glial cells have appeared multiple times during evolution or whether they have been lost in some phyla. Also, to which extend it is possible to compare glial cells amongst Bilateria it is unclear. These open questions are the result of our lack of complete characterization of glia in many organisms. Glial cells have been reported in several marine invertebrates, even though their molecular identity and their functions in some phyla are still unknown (Ortega and Olivares-Banuelos, 2020). In marine arthropods, glial cells are involved in neurogenesis (Brenneis et al., 2013), osmoregulation (Harrison and Lane, 1981), nutrition of synaptic regions and axon ensheathment (Hamori and Horridge, 1966; Spencer and Linberg, 1986). In the annelids *Armandia brevis* and *Protodrilus sp,* glial cells have been reported as part of the light sensory organs (Hermans, 1969; Purschke, 1990), and in *Nereis diversicolor* glial can induce chemotaxis (Paemen et al., 1992). In mollusks, glial cells are involved in neurotransmitters uptake (Elekes, 1978), osmoregulation (Paemen et al., 1992) and transport of macromolecules and calcium homeostasis (Goldstein et al., 1982; Maggio et al., 1991). In Deuterostomes, outside vertebrates the comprehension of glia is limited. In Ambulacraria, evidence about the presence of radial glia as precursor of new glial cells and neurons, and their supporting role as scaffold for neuronal migration has been reported (Helm et al., 2017; Mashanov and Zueva, 2019; Mashanov et al., 2010, 2013, 2015). In the early brunches in the chordate lineage, the general understanding of glial cells is vague. In the amphioxus larvae it has been reported that several types of glia including ependymoglia, midline and axial glia are present for their similar morphology to oligodendrocytes (Lacalli, 2000; Lacalli and Kelly, 2002). Unfortunately, further molecular and functional characterization is still missing. It is only by expanding our morphological, molecular and functional

analysis of glia in phylogenetically key organisms that we will finally have answers and resolve the evolutionary history of glia cells.

### 1.6.4. Glial cells in Urochordates

Our current understanding of glial cells in the Urochordates branch is limited to few studies. The only information we have come from the classes Appendicularia and the Ascidia. In *Oikopleura dioica* it is thought that support cells are generated after a wave of neurogenesis taking place during embryogenesis. 24 postmitotic support cells are reported to be present in the larva stage (Soviknes et al., 2005, 2007; Soviknes and Glover, 2007). However, a complete morphological, molecular and functional characterization of these cells is still missing. The general comprehension of glia in the Ascidian *Ciona* is slightly larger. In the cerebral ganglion of the sessile *Ciona* adult a few cells appear to retain some morphological glia-like features (Bullock et al., 1965) and even though no Schwann cells were spotted in the peripheral nerves, neurons seem to be surrounded by collagenous sheaths (Lane, 1972). A couple of molecular studies have suggested that *Ciona* larvae have ependymoglial cells in the inner cavity of the optic vesicle and in the neural tube (Takimoto et al., 2006; Takimoto et al., 2007) that give rise to a part of the adult nervous system (Horie et al., 2011). No full morphological description was made for the reported “ambiguous cells” or “accessory cells” found in synaptic regions (Ryan et al., 2016), and their genetic and functional characterization is still missing. Recent single cell transcriptional analysis confirmed the presence of ependymoglia and glia cells population in the *Ciona* larva (Cao et al., 2019), but their comprehensive investigation is still missing. It is then plausible to speculate that populations of glial cells with specific functions might be active components of the *Ciona* CNS, but experimental evidence in support of this theory are lacking. Given the accessibility to numerous toolkits available for cell specific functional in vivo characterization, *Ciona*’s simple nervous system with its unexplored glial cells provides a perfect model for the comprehension of basic functional glia processes. Moreover, obtaining

a complete understanding of glia cells in *Ciona* will help in filling the gap of information that precludes us to conclude about the evolution of glia cells in the Chordate lineage.

### 1.7. Live imaging and reporter gene tools available for functional analysis

*In vivo* functional analysis has become an extremely important approach in neuroscience, providing the ability to investigate cell behavior under physiological conditions. In the last decades, the use of modern genetically encoded indicators has helped researchers to follow neuronal activity changes in large populations of cells over long periods of time. For example, the genetically encoded calcium indicator (GECI) GCaMP (a fusion protein containing the calmodulin-binding domain from the myosin light chain kinase also called M13 peptide, the circularly permutated green fluorescent protein, and the calmodulin) became ordinary tools for imaging and measuring changes in calcium concentrations associated with neuronal activity (Fig.7A) (Akerboom et al., 2012; Chen et al., 2013; Tian et al., 2009). Similar tools allow to record and follow the presence of other types of molecules. For example, the intensity-based glutamate sensing fluorescent reporter iGluSnFR sensor allows researchers to visualize and quantify dynamic changes in glutamate levels, which is one of the most important signaling molecules in the nervous system (Marvin et al., 2013; Marvin et al., 2018). Constructed from the *E. coli* extracellular domain Glutamate/aspartate import solute-binding (GltI) and a circularly permutated GFP, iGluSnFR exhibits rapid kinetics that makes it possible to map excitatory synaptic activity in live animals (Fig.7B). Similarly, the single-wavelength genetically encoded fluorescent sensors iATPSnFRs is a promising tool for imaging ATP in the extracellular space and within cells (Lobas et al., 2019)(Fig.7C). With his circularly permuted superfolder GFP into the epsilon subunit of F0F1-ATPase from Bacillus PS3, this tool allows to follow live differences in ATP concentration. Other types of engineered tools, such as chemogenetics appear to be advantageous for manipulating cellular signal transduction in animals *in vivo*. Specifically, the chemogenetic tools

called Designer Receptors Exclusively Activated by Designer Drugs (DREADD) are used to recognize cellular circuits and to understand the basis of cellular interactions (Roth, 2016). hM3D and hM4D are G protein-coupled receptors that when activated by a chemical termed CNO (clozapine N-oxide)(Fig.7D), are able to interfere with second messengers, ion concentrations and influence kinase cascades. In this way, DREADDs are able to excite or silence cells in a spatiotemporally controlled manner (Armbruster et al., 2007). Genetically encoded tools are also suitable for performing cell specific perturbations. The DeAct-SpvB protein, for example, encodes for a mono(ADP-ribosyl) transferase domain that interferes with actin polarization, affecting cell structure and behavior (Harterink et al., 2017)(Fig.7E).

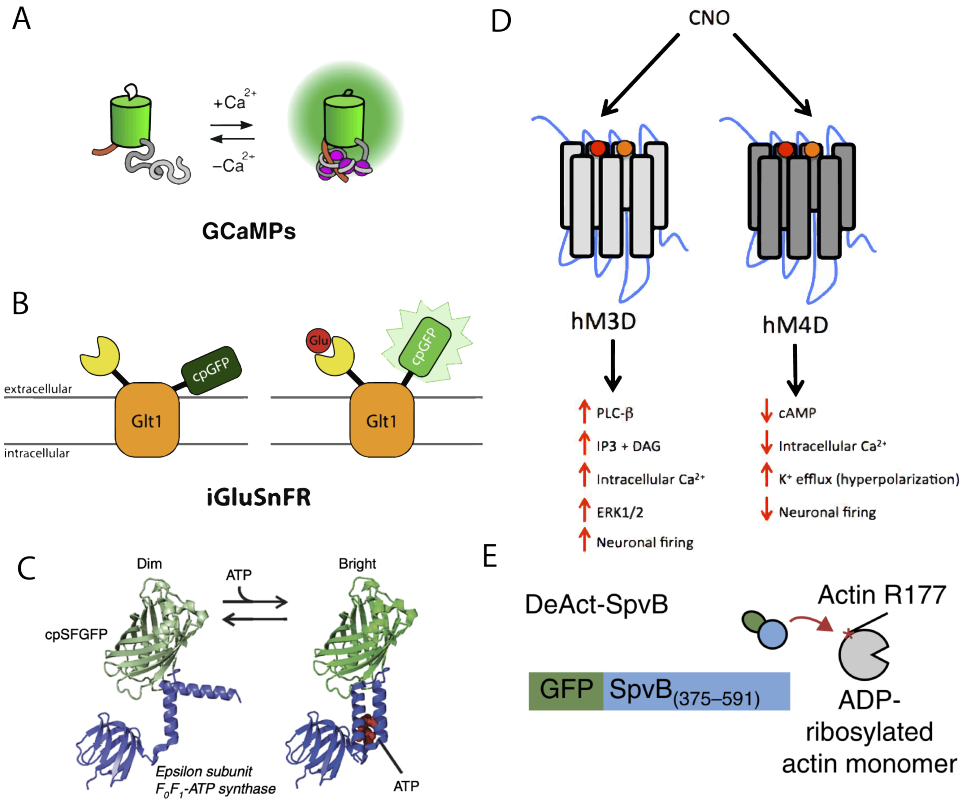


**Figure 7.** Graphic representation of modern genetical tools available for neuroscience research. **(A)** GCaMPs proteins are able to bind calcium ions with their calmodulin domain and activate the GFP. Image adapted from

(Pérez Koldenkova and Nagai, 2013). **(B)** In the iGluSnFR sensor, glutamate triggers the activation of the GFP domain. Image adapted from (Borghuis, 2019). **(C)** The single-wavelength genetically encoded fluorescent sensors iATPSnFRs. Image adapted from (Lobas et al., 2019). **(D)** Designer Receptors Exclusively Activated by Designer Drugs (DREADD). hM3D induce cellular excitation, while hM4D cellular inhibition. Image adapted from (Roth, 2016). **(E)** DeAct-SpvB tools allow cell specific actine manipulation, inducing actin disassembly. Image adapted from (Harterink et al., 2017).

# 2. Aim of the thesis

Despite the growing understanding of the importance of the glial cells in the development and functioning of the nervous system, the general knowledge about glia is still limited to few model organisms. To expand the knowledge on basic glia biology and to unravel insight on their evolutionary origin, it is fundamental to characterize glial cells in unexplored invertebrate species and to compare their functional and molecular identities among different taxa. It is plausible that the evolution of the different glial cell subtypes might have contributed to the evolution of the nervous system as a whole. Thus, characterizing the presence of glial cells in key phylogenetic organisms could provide new insights that will help to decipher the evolutionary trajectories that shaped nervous systems.

Little is known about the presence of glial cells in invertebrate chordates. With the work presented in this thesis, my colleagues and I provide new insight into invertebrate glial cells base on their molecular and functional characterization. In particular, this work aimed to unravel the glial cell populations present in the embryonic and larva central nervous system of the urochordate *Ciona*. This work is based on two different projects:

1) The identification of the glial cell populations presents in the *Ciona* nervous system from the early tailbud to the larva stages through the gene expression analysis of key orthologous glial markers present in the *Ciona* genome. In addition, by combining functional live imaging with modern genetic tools, we elucidate the contribution of glia in neuronal signaling at the larva stage and their involvement in behavioral and developmental processes.

2) *Ciona*'s life cycle is characterized by the transition from a tadpole larva state to a juvenile that gives rise to the sessile adult through a process called metamorphosis. Metamorphosis is associated with an apoptotic wave targeting most of the larval tissues, including the nervous system. Major remodeling of several tissues follows this wave of cell death. To date a substantial fraction of

the cellular and molecular mechanisms that are responsible for these processes is uncharacterized. Through functional live imaging and RNAseq analysis, we characterized the involvement of mobile ependymal cells in tissue remodeling and the clearance of dying neurons during metamorphosis.

# 3. Summary of the results

### 3.1 Multi-level characterization of the support cells in the embryonic and larva CNS of the urochordate *Ciona* (1st manuscript)

To investigate the glial cells present in the developing nervous system of *Ciona* embryos, we characterized the expression of a glycoprotein named SCO-Spondin. SCO-Spondin is the main components of the Reissner's fiber, the cerebrospinal fluid present in the central canal of the chordate dorsal nerve cord. It is known that vertebrate Reissner's fiber is secreted by a specific glial cell type, the radial glia. Radial glia have a remarkably diverse range of roles in CNS development and function. As multipotent progenitors, they can generate neurons and other support cells and are involved in several developmental processes like neuronal migration, synapse formation and maintenance. SCO-Spondin is expressed in the nervous system of *Ciona* embryos from the initial tailbud stage and it is present throughout the embryonic development including the larva stage. To characterize the molecular identity of SCO-Spondin(+) cells, we looked at the co-expression of specific marker genes. We demonstrated that subpopulations of SCO-Spondin(+) cells originate from a migratory cell lineage. These cells express Epithelial to Mesenchymal Transition (EMT). A subset of SCO-Spondin(+) cells during early tailbud stages express neurogenic markers, while later in development their subset display a neuronal signature. Moreover, we showed that part of the SCO-Spondin(+) cells expresses orthologous markers of astrocyte glial cells, suggesting that glial cells with specialized functions might be present in *Ciona* larvae. To further investigate this possibility, we characterize the molecular profile of these putative astrocyte-like cells and demonstrate that they express a combination of transporters and enzymes fundamental for the glutamate and GABA neurotransmitters metabolism. All these characteristics of the *Ciona* SCO-Spondin(+) cells resemble the vertebrate radial glial cells. Our findings argue against the idea that *Ciona* larvae have only a population of ependymoglia and show that amongst the population of glial cells a fraction displays an astrocyte-like molecular fingerprint.

After establishing that glial cells with a specific molecular profile are present in the *Ciona* embryonic nervous system, we performed a series of experiments aimed to study their functional roles. It is known that dynamic and bidirectional signals characterize the interaction between glia and neurons, and these relations can be studied by looking at the variation of intracellular calcium concentrations. Firstly, using the genetically encoded calcium indicator GCaMP6s and a machine learning analysis method based on time series clustering, we described the calcium dynamics of the glial cells at the larva stage and comprehensively demonstrate that glial cells display unique calcium dynamics that can be distinguished from those exhibited by neurons. Secondly, we show that glial cells functionally interact with neurons as the modulation of their activity via chemogenetic DREADDs generates differences in neuronal calcium dynamics. To further investigate glia and neuronal interaction, we explored to which extend glial cells expressing neurotransmitter recycling related genes present in the *Ciona* neural tube are involved in the glutamatergic signaling pathways. With the use of the intensity-based glutamate sensing fluorescent reporter iGluSnFR we followed variation in glutamate concentration and demonstrated the presence of stage-specific glutamate dynamics. Moreover, we described changes in their dynamics when the glutamatergic neurons activity is modulated, proving they are an active component of the glutamatergic circuits. The results of these two approaches demonstrated that *Ciona* glial cells are an active constituent of the signaling networks and emphasized the astrocyte-like identity of a subset of cells previously described as ependymal cells.

After establishing the bidirectional communication between glia and neurons, we studied the possible involvement of glial cells in modulating animal behavior. By combining larva behavioral imaging with chemogenetic tools, we demonstrated that glial cells in *Ciona* retain a neuromodulatory function, as differences in the larva behavior and posture were observed when we chemogenetically modulated glia activity.

Glial cell involvement in nervous system development is well established in both vertebrates and invertebrates. Indeed, glia dysfunctions cause neurodevelopmental

disorders and neurodegenerative diseases. We tested the contribution of the *Ciona* glia in the development of the nervous system by perturbing their cellular structure using the DeAct-SpvB protein, which by interfering with actin polarization affects cellular behavior and functions during development. The perturbation of the *Ciona* glial cells produces neural tube closure defect due to nervous system development failure, confirming their fundamental role for the CNS. Moreover, an intriguing absence of pigmented cells in a significant number of *Ciona* larvae suggested that glial cells might be involved in the differentiation process of pigmented cells.

### 3.2 Ependymal cells contribute to nervous system remodeling during metamorphosis in *Ciona intestinalis* (2nd manuscript)

The ependymoglia cells in the inner cavity of the nervous system have been for a long time the only glial cells recognized in the *Ciona* larva. Their characterization has mostly focused on their contribution to the formation of the adult nervous system, the cerebral ganglion. It is well established that during metamorphosis the central nervous system cells, except for a subset of glutamatergic and cholinergic motor neurons, and other larva tissues are killed by a programmed apoptotic wave and dead cells are somehow eliminated before the formation of the juvenile and adult tissues. The ependymal cells have been identified as the Neuronal Stem Cells (NSCs) source that gives rise to the adult nervous system. Little is known about the mechanisms that underlying the remove of the apoptosed neuronal tissue. Glial cells in both vertebrates and invertebrates are known to be the main players of these processes, with their engulfment mechanisms found to be fundamental for the development and maintenance of the nervous system in both normal and pathological conditions. In this work, we investigated the role of the ependymal cells during metamorphosis, focusing on the CRALBP(+) cells located in the brain vesicle and the visceral ganglion. We performed time-lapse live imaging in *Ciona* larvae undergoing metamorphosis and characterize their cellular behavior. To recognize the onset of the tail regression process, we expressed in the larva nervous system different genetically

encoded cell death indicators. A significant increase in their expression indicative of an apoptotic wave in the nervous system coincided with a change in the cellular behavior of ependymal cells which switched from immotile epithelial cells to motile mesenchymal-like cells. As motile cells they surveyed the entire central nervous system using protruding filopodia structures to sense and migrate towards apoptosing neurons. While the overlap in signal between apoptosing neurons and ependymoglial cells increased, we observed a reduction in the non-overlapping signal from dying neurons, as the dead cells were progressively eliminated by ependymal cells, possibly via a phagocytosis based mechanism. We also identified a small number of ependymal cells occupying the anterior nerve cord, which acted as pivot points that facilitated the regression of the neuronal axons and cell bodies located along the tail part of the nerve cord towards the animal trunk. Finally, at the end of the tail regression process, we found that ependymal cells encircled the pigmented cells in the sensory vesicle and dislodged them from their original position, triggering their migration towards the posterior part of the animal at the early stage of the body axis rotation. To investigate the presence of putative molecular cues that may have signaled to ependymal cells the onset of neuronal apoptosis, we monitored and quantified ATP concentration in the ependymal cells and neurons at the larva stage and during the tail regression stage. We saw that at the onset and during metamorphosis both neuronal cytosolic and ependymal cells plasma membrane ATP levels rose relative to pre-metamorphosis recordings, implicating ATP as a putative cue that could trigger the activation of the ependymoglial cells.

Changes in cell state and behavior are often associated with changes in calcium signaling patterns. We monitored calcium activity in ependymal cells during their stationary state at the larva stage and during the motile state in the tail regression stage and demonstrated that the calcium dynamics at the later stage are arguably more dynamic and more complex when compared to the larva stage.

Finally, we wondered whether the shift from the static state to the highly motile behavior observed in the ependymal cells during the tail regression could be mediated by particular changes in their gene expression profile. Also, if the proposed

engulfment mechanism could be supported by the expression of particular genes defining a specific cellular molecular fingerprint consistent with motility, phagocytosis and other related cellular processes. To answer these questions, we performed bulk RNAseq analysis on MACS sorted ependymal cells from the larva and the tail regression stage. We identified 8636 genes that are significantly differentially expressed in the two stages, with 4266 genes being upregulated and 4370 genes being downregulated in the larva compare to the tail regression stage. Gene Ontology Term analysis (GO) identified 418 terms that are overrepresented among the downregulated genes and 330 terms that are upregulated during metamorphosis. This analysis highlighted the upregulation of genes belonging to specific biological processes such as calcium ion transport, cell migration, cell polarity, phagocytosis and inflammatory response during the late larval stage just prior to the onset of tail regression. Our findings indicate that this late larva stage is the timepoint at which the ependymal cells acquire the molecular requirements that determine the behaviors we observed in the tail regression stage. Among the upregulated genes during the late metamorphosis stage, we found mainly terms related to cell homeostatis but also cell proliferation, chromatin remodeling and stem cell maintenance. These findings confirm the previously proposed role of CRALBP(+) cells as putative NSCs that give rise to the adult nervous system.

# 4. Discussion

The motivation behind the work presented in this thesis arose from previous analysis on the origin of glia, and from the belief that glial cells have been mostly lost in urochordates (Hartline, 2011). In this review, the author emphasized that expanding our knowledge about glia in several unexplored organisms is necessary to provide a clear picture of the evolution and function of glia. Insight about the origin of glia comes from the presence of glia-like cells in Xenoacelomorpha (Bery et al., 2010), the sister group of all the Bilateria. These findings raised two different possibilities: (1) glial cells must have arisen independently multiple times during history as an example of convergent evolution, or (2) they arose a few times or even only once as plesiomorphic characters and then have been lost in several organisms. While it is easier to explain that the increase of nervous system complexity could have played a fundamental role in the advent of new cell types that perform support functions to neurons, it is more complicated to find a logical explanation to justify their loss. One simple possibility is that the absence of glia in an extant organism could result from never having had that specific cell type in the ancestral line, or because there was no longer a need of a cell type with specific functions. Unfortunately, these explanations cannot be used to justify the absence of glial cells in invertebrate chordates. In fact, urochordates are the sister group of vertebrates (Delsuc et al., 2006), in which glial cells have been extensively characterized on the molecular, functional and morphological level, ruling out the absence of glia in the urochordates ancestral line. In addition, the presence of putative glial cells have been described in cephalochordates (Lacalli, 2000; Lacalli and Kelly, 2002) and in appendicularians (Soviknes et al., 2005, 2007; Soviknes and Glover, 2007). Finally, several studies investigating the homologies between urochordates and vertebrates in the context of the nervous system (Abitua et al., 2012; Ikuta et al., 2004; Imai et al., 2002; Imai et al., 2009; Keys et al., 2005; Meinertzhagen and Okamura, 2001; Negron-Pineiro et al., 2020; Nishino, 2018; Ryan et al., 2017; Satoh, 2003; Wada et al., 1998), argue against the possibility that glial cells are functionally redundant in urochordate nervous systems. Moreover, processes involving glial cells such as neurogenesis,

neuronal migration, axon growing and pruning, neuronal signaling and synaptic maintenance haven't been fully understood yet in urochordates.

Why glial cells should have mostly been lost in urochordates? This question has been the driving force for my work, and I tried to answer it through the course of my PhD by exploring glia in the urochordate *Ciona*. When I started working on this topic, the evidence available on glia in *Ciona* embryos were limited to the ependymoglial cell population lining the optic vesicle, the visceral ganglion and the dorsal neural tube at the larva stage (Etani and Nishikata, 2002; Kusakabe et al., 2009; Takimoto et al., 2006; Takimoto et al., 2007; Tsuda et al., 2003). However, the work describing them acting as stem-cell like cells for the formation of the adult nervous system (Horie et al., 2011) proposed that these cells belonged to a heterogeneous population of cells, opening at the possibility that more than one glia type could be present in the *Ciona* nervous system. This hypothesis was recently confirmed via the single-cell transcriptomic analysis of *Ciona* embryos, in which metadata of "glial cells" and "ependymal cells" populations was published (Cao et al., 2019). Before these data were generated, one way I had to pursue this idea was to test the expression of known glia marker genes and explore the molecular identities of the cells expressing them. Next, to confirm their cellular identity as glial cells, I examined their contribution to modulating neuronal activity and behavior as well as their involvement in developmental processes (1st manuscript). After establishing that the *Ciona* larva ependymal cells are a heterogeneous population, with some of them resembling astrocyte-like cells, we explored the possibility that glia could be taking part in the morphogenetic processes defining the metamorphosis (2nd manuscript). The results of these studies and the strategy we used to approach these problems are discussed in the next paragraphs, pointing out some general questions that might have arisen from it.

### 4.1 Identification of glial-like cells (1st manuscript)

Three different methodological approaches have been used to characterize a specific cell population. The early way used to recognize cell diversity in a nervous tissue

relied on morphology (Bullock et al., 1965; Ramon y Cajal, 1895, 1909, 1911, 1913a, b; Ramon y Cajal et al., 1888), and consolidated morphological features have been outlined to identify glial cells among invertebrates. Glia are generally in close association with neuronal cell bodies, axons and the synaptic cleft with fine processes branching and invading nerve bundles, leading to a characteristic irregular cell shape. Also, glial cells can have a flat or cuboidal shape, arranged around the periphery or central canal of the central nervous structure to generate ensheathments. Alternatively, they can form a barrier between blood vessels and neurons (Pentreath, 1989). However, invertebrate's glia can differ in their morphology across different organisms, thus morphology cannot be the only criterion for assessing cell type identity. With the advent of modern technologies, other criteria to identify glial cells rely on the analysis of their molecular signature. Indeed, molecular properties turn out to be an important resource to generate a list of molecular markers that can help to distinguish glia from neurons. Some of the established markers, include genes associated with neurotransmitter recycling such as the excitatory amino acid transporters (EAATs) as well as GABA related transporters, Glutamine synthetase (GS) and GABA transaminase (Anlauf and Derouiche, 2013; Freeman et al., 2003; Magi et al., 2019; Nieoullon, 2009; Niva et al., 2008; Soustelle et al., 2002; Stacey et al., 2010). Cytoskeletal structural components of glia such as Vimentin, Tenasin and the Glial fibrillary acidic protein (GFAP) are also popular glial markers (Cahoy et al., 2008; Eng et al., 2000). Furthermore, neural cell adhesion molecules are present as orthologs in several organisms among Bilateria and can be used to identify support cells (Halassa and Haydon, 2010; Hillen et al., 2018; Silies and Klämbt, 2010; Togashi et al., 2009). The third approach to identify glia is based on the study of their functions. Glia functions include structural and trophic support, homeostasis, neuronal insulation through myelin formation, modulation of neuronal activity and phagocytosis (of apoptotic cellular debris), uptake and release of neurotransmitters, circuit regulation (Huxtable et al., 2010; Kriegstein and Alvarez-Buylla, 2009; Kuffler, 1967; Pentreath, 1989; Zhang, 2001). In addition, they take part in developmental processes such as neurogenesis, neuronal network assembly and function, neuronal migration and axon growing (Allen and Lyons, 2018; Falk and

Gotz, 2017). Glial cells in diverse taxa perform analogous tasks (Barres, 2008; Bullock and Horridge, 1965), with some examples coming from invertebrate marine organisms such as echinoderms (Flammang and Jangoux, 1993; Mashanov and Zueva, 2019; Mashanov et al., 2013), mollusks (Elekes, 1978; Goldstein et al., 1982; Maggio et al., 1991; Willmer, 1978), arthropods (Brenneis et al., 2013; Chaigneau et al., 1991; Hamori and Horridge, 1966) and annelids (Ortega and Olivares-Banuelos, 2020; Paemen et al., 1992; Wells et al., 1972).

In the work presented in the 1st manuscript, we started our investigation using the candidate gene approach, proposing that putative glia-like cells in *Ciona* would express some of the established glia marker genes. We looked at the expression profile of specific glia marker genes conserved in the *Ciona* genome through the technique of whole mount *in situ* hybridization. A limitation of this approach comes from the fact that gene function might not be conserved among lineages. The first gene we consider was the Glial fibrillary acidic protein (GFAP) that belongs to the cytoplasmic intermediate filaments (IF) protein family type III together with Vimentin. This gene is well conserved among vertebrates (Kalman, 1998; Kalman and Pritz, 2001; Kalman et al., 1993; Shehab et al., 1989; Yanes et al., 1990) and invertebrates (Cardone and Roots, 1990; Riehl and Schlue, 1998), even though is absent in *C.elegans* (Stout et al., 2014) and *D. melanogaster* (Doherty et al., 2009). As already mentioned in 1.6.1, GFAP is widely used to identify astrocyte-like cells, although around 50% of astrocyte in the mice brain are estimated to not express GFAP (Cahoy et al., 2008; Kuegler et al., 2012; Lovatt et al., 2007; Zhang et al., 2016). Ascidians genomes encode four orthologs of the vertebrate GFAP (Wang et al., 2002) and its immunoreactivity was tested in *Styela plicata* in the context of nervous system regeneration (Medina et al., 2015). We tested expression territories of GFAP in *Ciona* embryos and found its presence in the tail epidermis starting from the early tailbud to the larva stage. An interesting question arose from this result is how a gene can acquire a new function or how it can be expressed in new tissue. The advent of new binding sites in the regulatory elements, or a new splicing variant, mutation and domain shuffling in the coding sequence are the main candidates to explain this phenomenon (Lynch and Wagner, 2008; Prud'homme et al., 2007). Nevertheless, the

absence of GFAP(+) cells in the embryonic *Ciona* CNS means that we cannot use this gene as a useful marker in our case.

Previous biochemical analysis revealed the presence in *Ciona intestinalis* of orthologous glycoproteins of the bovine Reissner's fiber (Lichtenfeld et al., 1999), which is a secretory product of radial glia cells in the roof plate of adult vertebrates. Among the proteins that constitute this extracellular matrix, the thrombospondin type I protein SCO-Spondin is indicated as one of the most abundant (Gobron et al., 2000; Gobron et al., 1996; Lehmann et al., 2001), and has been associated with neuronal survival and differentiation, cell adhesion modulation, cell migration (Huh et al., 2009; Meiniel, 2007; Vera et al., 2013). It has been shown that SCO-Spondin knockout in zebrafish shows a defect in the morphogenesis of the body axis (Cantaut-Belarif et al., 2018). Moreover, the presence of SCO-Spondin among chordates (Gobron et al., 1999) ambulacraria (Helm et al., 2017; Mashanov et al., 2009; Viehweg et al., 1998) and insects (Lichtenfeld et al., 1998) indicates an early evolution of this gene, allowing speculation about the origin of the radial glia cells in last common ancestor of deuterostomes and protostomes (Helm et al., 2017). We studied the temporal and spatial expression of SCO-Spondin in *Ciona* embryos starting from the early tailbud to the larva stage, which allowed us to identify previously unexplored glia-like cells in the *Ciona* CNS. The co-expression analysis of known marker genes provided information about the identity of the SCO-Spondin cells. They partly originate from the neural plate border, a cell lineage that gives rise to migratory cells (Stolfi et al., 2015). The SCO-Spondin (+) cells express EMT and cell adhesion molecules and at later developmental stages neurogenesis markers. From the mid-late tailbud stage, some SCO-Spondin cells express either a combination of NPCs and neuronal markers or neurotransmitter modulator genes associated with astrocyte-like cells. Finally, they partly co-express some of the established ependymal markers, supporting the heterogeneous identity of the ependymal cell population. Our findings show that, based on their developmental and molecular characteristics, at least part of the SCO-Spondin(+) cells resemble the vertebrate radial glia. Ultrastructural analysis revealing their morphology is missing and could play a fundamental role in establishing the presence of radial glia-like cells

in urochordates. Potentially, the use of cell specific RNAseq to study their stage specific gene expression profiles combined with single cell ATAC seq (Assay for Transposase-Accessible Chromatin with highthroughput sequencing) will elucidate their involvement in neurogenesis and could help to identify putative transcription factors and the gene regulatory network (GRN) controlling their differentiation. Some candidate genes are Hox10 and Pax3/7. Interestingly, preliminary results from a Pax3/7 knock-out in *Oikopleura dioika* suggest a role for this transcription factor in glial cell specification (D. Chourrout lab, personal communication). Moreover, RNAseq is suitable to identify the transcriptional states of these cells as they differentiate, clarifying their neural precursor cells (NPCs) trait and the presence of putative glial intermediate cell states (ICSs) that are typically observed in heterogeneous and dynamic cell populations (MacLean et al., 2018; Martinez-Cerdeno and Noctor, 2018). So far, neuro-gliogenesis models based on radial glial cells have been described only in vertebrates and echinoderms (Alvarez-Buylla et al., 2001; Gotz and Barde, 2005; Götz and Huttner, 2005; Heins et al., 2002; Kriegstein and Gotz, 2003; Mashanov et al., 2010, 2013, 2015) and our data lead the path for new findings in this direction. The results of the candidate gene approach left us with three main conclusions: (1) that radial glia-like cells might be present in *Ciona*, (2) that *Ciona* ependymoglia cells are a heterogeneous cell population and (3) that glial cells with specialized functions could be present in the *Ciona* larva.

To comprehensively characterize these cells, we pursued the study of their functional properties and introduced for the first time the use of modern advanced molecular tools in *Ciona* that are available now for the community. The tools that we have developed in this study are reported in paragraph 1.7. To guide their expression in a cell specific way, we relied on the regulatory sequences (promoters) of our marker genes. Unfortunately, we didn't succeed in isolating the cis-regulatory element of SCO-Spondin. Instead we decided to use the regulatory elements of genes that we have shown to co-express with SCO-spondin (e.g. Ci-Nut). Our calcium imaging analysis follows a series of recent calcium imaging studies in the developing *Ciona* embryos (Abdul-Wajid et al., 2015; Akahoshi et al., 2017; Hackley et al., 2013) and larvae (Okawa et al., 2020). Compared to these studies our study is the most

exhaustive in terms of calcium dynamics across different cell types in the nervous system. By using a substantial number of gene regulatory elements, we observed a surprising diversity of calcium activity patterns given the small size of the larval nervous system. Future studies should be directed towards elucidating how different cell populations in the larval CNS use the available calcium signaling machinery in diverse ways in order to carry out their signaling functions. We established that *Ciona* glia display unique calcium dynamics that differ from neuronal calcium activity pattenrs, and we demonstrated that glial cells are integrated with the underlying neuronal circuits and influence the signaling networks. To provide evidence for the functional interaction of glia with neurons, we introduced the use of chemogenetics in *Ciona* that were best suited for our purpose. In fact, optogenetics tools are not recommended for this type of analysis due to the non-selectivity of their cations transporters that can lead to intracellular acidification or to a large increase of calcium that could affect downstream signaling cascade and affect the modulation of the glia functions (Beppu et al., 2014; Wang et al., 2013).

Next, we explored the involvement of the astrocyte-like cells in the glutamate circuit, by performing live glutamate imaging through the extracellular iGluSnFR sensor. This tool has been used in mouse (Barnes et al., 2020; Xie et al., 2016), *D. melanogaster* (Richter et al., 2018; Stork et al., 2014) and *C.elegans* (Marvin et al., 2013; Thapliyal et al., 2018) to study glia-neuronal interactions and we introduced it for the first time in *Ciona*. We observed local bursts of fluorescence in the astrocyte-like cells during *Ciona* development, suggesting a role for glutamate in development. By combining glutamate imaging with the chemogenetic DREADDs, we demonstrated their interaction with the glutamatergic neurons. These results are in line with our calcium analysis, demonstrating that the *Ciona* glia are active components of the larval neuronal circuits and contribute to neuronal signaling. To further investigate glutamate involvement in development, RNA interference or CRISPR/Cas9 could be used to knockdown or knockout EAAT GLT-1 glutamate transporter and study how glutamate dynamics are influenced (Nishiyama and Fujiwara, 2008; Stolfi et al., 2014). In mice, glial glutamate transporter expression changes during development and increases concurrently with the development of

neuronal networks and synaptogenesis (Furuta et al., 1997; Kugler and Schleyer, 2004; Ullensvang et al., 1997). Also, GLT-1 knockout shows selective neurodegeneration with spontaneous epileptic seizures and premature cell death due to the high concentration of glutamate in the extracellular space, known as excitotoxicity (Aida et al., 2015; Tanaka et al., 1997). Loss of the glia EAAT transporter expression in *D. melanogaster* larvae shows severe locomotor dysfunction (Stacey et al., 2010), and knockdown through RNA interference shows in the adults neuronal degeneration and behavioral deficits (Rival et al., 2006; Rival et al., 2004). In *C.elegans*, GLT-1 inactivation affects repetitive behavior (Katz et al., 2019).

Given the multiple lines of evidence on the contribution of glia in modulating behavioral output (Chen et al., 2015; Ma et al., 2016; Raiders et al., 2020; Seugnet et al., 2011; Stout et al., 2014), we studied the swimming behavior of the *Ciona* larvae in combination with the use of the chemogenetic DREADDs. We demonstrated that activation or silencing of the ependymal cells and the astrocyte-like glial cells produces effects on animal speed and postures, indicating that *Ciona* glia are involved in the modulation of different swimming behavior features.

Finally, the role of glia in development is well established from embryo to adults (Jakel and Dimou, 2017; Jessen, 2004; Reemst et al., 2016; Shaham, 2005, 2006; Stork et al., 2008; Stork et al., 2014) in neurogenesis, scaffolding for neuronal migration and axon growth, influence in circuit development, and control of synaptic microenvironments (Edmondson and Hatten, 1987; Kim et al., 2017; Rakic, 1972). We demonstrated that *Ciona* glia perturbation has an effect on the larva nervous system development. For this experiment, instead of opting for a targeted gene approach such as CRISPR/Cas9, RNA interference or morpholino, we chose to use DeAct-SpvB, a tool that interferes with actin polarization, perturbing cellular structure and motility (Harterink et al., 2017). In this way, we were able to target specific genetically defined cell populations with the use of suitable gene regulatory sequences and understand their contribution during nervous system development.

These data provide robust evidence that *Ciona* nervous system has specialized glial cells that resemble vertebrate glia. We showed a high degree of similarity in terms of molecular fingerprint, role in the signaling networks and contribution to the nervous system development. We provided evidence that specialized glia types are conserved in the urochordate lineage, contributing to the understanding of glia evolution in chordates. Understanding the evolutionary origin of glial cells and their roles in unexplored species will further inform our understanding of these systems, aiding in the development of models for experimental investigation and validation. We demonstrated that *Ciona* is a suitable model to study glia biology at the same level as other invertebrate model species. In the future, we hope to extend our analysis to other tunicate models e.g. *Oikopleura dioica,* in which our preliminary analysis of the nervous system based on gene expression analysis supported the presence of well-developed glia types. However, for a complete understanding of how glia evolved in invertebrate chordate, a comprehensive description of glia in cephalochordates is also needed.

### 4.2 Metamorphosis and phagocytic activity of glial cells (2nd manuscript)

Metamorphosis is a developmental process that takes place at the transition of the larval stage to the juvenile/adult and includes morphological, physiological, biochemical, histological and behavioral changes that affect several tissues at different levels (Buchholz et al., 2006; Shi, 2000). This process occurs in diverse branches of the animal kingdom, from amphibians (Brown and Cai, 2007), Teleost (McMenamin and Parichy, 2013), cephalochordates (Paris et al., 2008), tunicates (Karaiskou et al., 2015), echinoderms (Williamson, 1992), arthropods (Hall and Martin-Vega, 2019; Rolff et al., 2019), mollusks (Bonar, 1976) and cnidaria (Müller and Leitz, 2002). Metamorphosis has fascinated researchers for centuries and remains an attractive experimental model for studying mechanisms of post-embryonic development. The origin of metamorphosis is still controversial (Laudet, 2011; Truman and Riddiford, 1999; Zhang et al., 2018), and even though it is

morphologically diverse among the animal kingdom, there are some common principles. First, the larvae and the adult often live in different environments or at least do not share the same resources (Hamilton, 2008; Jackson et al., 2002). Examples come from the amphibians, where the tadpole is aquatic whereas the frog is terrestrial, or the tunicate planktonic larvae with their benthic adults (Karaiskou et al., 2015; Vitt and Caldwell, 2014). Second, the onset of metamorphosis is usually triggered in close connection to the environment, as environmental cues play a crucial role in initiating the transformation of the larvae to adults (Denver et al., 2002; Gilbert, 2012) Third, metamorphosis can be a radical transformation that induces a complete reorganization of the animal body plan or can cause minor structural changes (Shi, 2000). In both cases, programmed cell death plays fundamental for remodeling and sculpting the larva tissues (Galluzzi et al., 2018; Lee and Park, 2020; Tettamanti and Casartelli, 2019). Amphibians and insects have been widely investigated and have provided insight into the molecular mechanisms mediating metamorphosis. In anurans, metamorphosis is mainly triggered by the thyroid hormones (TH) (Dodd and Dodd, 1976; Shi, 2000; Tata, 1998), and it has been reported that exogenous TH prematurely induces metamorphosis, while inhibition of endogenous TH production blocks metamorphosis, resulting in the growth of giant tadpoles (Gobdon et al., 1943; Tata, 2006). TH levels modulate different processes in the different tissues (Furlow and Neff, 2006; Heimeier et al., 2010) and are responsible for the enhanced degeneration of tail tissues during the tail regression. Indeed, TH-responsive cell death occurs through autolysis via lysosomal and non-lysosomal hydrolases, a process known as the suicide mechanism. These cells can also release hydrolytic enzymes to the extracellular milieu, triggering the death of the surrounding cells through a murder mechanism (Ishizuya-Oka et al., 2010; Nakai et al., 2017; Yaoita, 2019). In the last phase of tail regression, intraepithelial macrophage-like cells get activated in the degenerating larval epithelium, and together with larval immune cells remove and digest tissue debris (Ishizuya-Oka et al., 2010; Izutsu, 2009; Kerr et al., 1974; Nishikawa et al., 1998). TH levels are also fundamental to regulate the remodeling of the nervous system, promoting larval-specific neural circuit reconstruction for the formation of the adult neural networks

(Estabel et al., 2003). TH regulated metamorphosis is an ancient Bilateria feature, and have been reported also in amphioxus (Paris et al., 2008), flounder (Inui and Miwa, 1985) and sea urchin (Chino et al., 1994), and thyroid hormone receptor genes have been found annelids, mollusks and flatworms (Laudet, 2011). The role of TH in ascidians metamorphosis has not been highly characterized, even though TH synthesis at the larva stage seems to be required for the onset of the process (Patricolo et al., 2001; Patricolo et al., 1981). Ascidians larvae undergo metamorphosis after adhesion to a substrate using the anterior palps, from which sensory neurons project axons toward posterior nervous system regions (Ryan et al., 2018; Takamura, 1998). Mitogen-activated protein kinase (MAPK) levels increase in the nervous system during the swimming period of the *Ciona* larvae as well as during the adhesion phase (Chambon et al., 2002; Chambon et al., 2007; Tarallo and Sordino, 2004). MAPK has been identified as the pivotal factor for the tail regression triggering pathway, as MAPK chemical inhibition has a direct effect the tail tissue's apoptotic wave (Chambon et al., 2007). Also, the β1-adrenergic receptor in the palps, brain vesicle, and the dorsal nerve cord has been identified as a further link between the anterior and posterior part of the animal that could explain the onset of apoptosis in the tip of the tail (Kimura et al., 2003). These observations, together with neuropeptide based studies (Coniglio et al., 1998; Hozumi et al., 2020; Kimura et al., 2003; Zega et al., 2005), confirmed that the nervous system plays a key role in initiating ascidian metamorphosis. As one can readily imagine, extraordinary plasticity of all tadpole tissues is required to rearrange all body structures. Most of the tail cell types die by programmed cell death during the tail regression event (Chambon et al., 2002; Jeffery, 2002; Tarallo and Sordino, 2004), and because many cells are eliminated it is reasonable to wonder which are the mechanisms and the players that are responsible for this process. In a normal course of events, in the last step of programmed apoptotic cell death, professional and non-professional macrophage cells deal with the clearance of dead cells (deCathelineau and Henson, 2003), but no evidence about the removal of tissue debris has been reported during metamorphosis in *Ciona*. In the work presented in the second manuscript, we studied the behavior of the nervous system cells during the metamorphosis of *Ciona* and identified in the ependymal cells

the actuator that mediates the elimination of the dying neurons during the tail regression. We reported that during *Ciona* metamorphosis, at the onset of the neuronal cell death the ependymal cells transition from a static epithelial-like state and become motile cells that crawl around the larval trank, extending filopodia and migrating towards phosphatidylserine (PS) expressing neurons to remove apoptotic debris by endocytosis. Temporal similarity can be drawn with the tail regression process in amphibians, where the activation of non-professional macrophage cells follows the programmed cell death of the tail tissue. We reported a similar sequence of events, with ependymal cells changing behavior to accomplish their function after the occurrence of neuronal cell death. Additionally, we reported that the switch in the behavior of the ependymal cell is followed by an increase in calcium activity during the motile phagocytosing phase. This finding is in line with studies describing how changes in cell behavior and cell state are often associated with changes in calcium signaling patterns (Hunter et al., 2014; McKinney and Kulesa, 2011; Wood, 2012).

To our knowledge, phagocytosis in *Ciona* has been so far investigated only in the contest of innate immune memory in the haemocyte cell population in adult organisms (Melillo et al., 2019; Vizzini et al., 2018). The phagocytosis of apoptotic cells has been described as a recurring process in botryllid ascidians, where a cyclical generation of new zooids by budding occurs during the asexual reproduction, and old zooids are removed (Manni et al., 2007). The takeover of the old zooids tissues is characterized by the extensive programmed cell death and the periodical participation of professional and non-professional phagocytes cells that act removing dying cells (Ballarin et al., 2008; Lauzon et al., 1992; Lauzon et al., 1993; Tiozzo et al., 2006). Phagocytes are attracted by dying cells that expose phosphatidylserine (PS) on the outer layer of the plasma membrane, a typical “eat-me” signal (Cima et al., 2003; Cima et al., 2010; Nagata et al., 2016). As in mammals, the CD36 receptor on the phagocyte surface is the key factor that mediates the recognition of dying cells (Cima et al., 2003; Ren et al., 1995). Finally, it has been reported that phagocytes can undergo phagocytosis-induced apoptosis and can be ingested by other phagocytes (Franchi et al., 2016), similarly to what is observed in mammals (Frankenberg et al., 2008; Kirschnek et al., 2005). In *Botryllus schlosseri*, phagocytosis has been also

investigated in the contest of foreign cells and particles, where the engulfment mechanism coincides with a transient rise in cytosolic calcium concentration (Ballarin, 2008; Ballarin et al., 1997).

We identified ATP as a possible cue that activates and attracts ependymal cells towards neurons. Our results suggest scenarios that resemble microglia cells, the vertebrate immune-competent cells of the central nervous system. Resting microglial cells are characterized by a ramified morphology with motile processes that constantly monitor their surroundings, and become activated into a motile state in response to brain injury, dying neurons and infection (Jacobowitz et al., 2012; Kreutzberg, 1996; Nimmerjahn et al., 2005). ATP is a key regulator of the microglia behavior and dynamics, as ATP sources attract and trigger the extension of microglial processes (Davalos et al., 2005; Dissing-Olesen et al., 2014). In addition, interruption of the ATP signal decreases microglia basal mobility (Davalos et al., 2005), while the increase of ATP enhances it (Fontainhas et al., 2011).

The ability of specific cells in changing behavior and functions is an ordinary event. The most common example is the epithelial–mesenchymal transition (EMT), a process that occurs during early embryonic development and injury. Dynamic changes in gene expression and cellular organization lead to a switch from a static epithelial to a motile mesenchymal identity (Hay, 1995). EMT has been extensively studied due to its involvement in the formation of metastasis (Baum et al., 2008; Serrano-Gomez et al., 2016; Seton-Rogers, 2016; Ye and Weinberg, 2015). In *Drosophila*, a reverse process named mesenchymal-epithelial transition (MET) is responsible for the formation of the blood-brain barrier, where subperineural glial cells (SPG) of mesenchymal origin need to switch from a motile to a stationary state to form an epithelium that envelops the CNS (Schwabe et al., 2017). A further example is provided by astrogliosis, where astrocyte cells are responsible to fill the space resulting from neuronal loss during CNS structural lesions (Eddleston and Mucke, 1993; Sofroniew and Vinters, 2010). In this context, our RNAseq analysis showed a clear difference in the transcriptome identity of the ependymal cells that explains the two different observed behavioral states. Moreover, the gene ontology

analysis allowed identifying candidate genes that can be used as starting points to characterize and explain processes such as cell migration, structural remodeling and cell interaction.

The glial phagocytic activity has been explored in numerous vertebrate and invertebrate models, and the remarkable molecular similarities across species indicate deep evolutionary conservation, and perhaps origins, of this critical glial function (Raiders et al., 2021). In the whole *Drosophila* development, about 50% of the neurons die and are removed by ectodermal derived glial cells (Bangs and White, 2000; Buss et al., 2006; Kurant et al., 2008). During embryogenesis, dying neurons are eliminated from the cortex glial cells through phagocytosis (McLaughlin et al., 2019; Nakano et al., 2019). In the early metamorphosis phases, axons and dendrites of the larval nervous system are pruned (Awasaki and Ito, 2004; Truman, 1990; Truman, 1993), and astrocyte-like cells are reported as the main non-professional macrophage cells driving this process (Hakim et al., 2014; Tasdemir-Yilmaz and Freeman, 2014). Finally, ensheathing glia is the third glial type that during metamorphosis and in the adult nervous system act as phagocytes of apoptotic neurons (Doherty et al., 2009; Hilu-Dadia et al., 2018). The phagocytic activity of the glial cells is mediated at the molecular level by the expression of two receptors, the Draper receptor (Drpr) (Hilu-Dadia et al., 2018), and the six-Microns-Under receptor (SIMU) (Freeman et al., 2003; Kurant et al., 2008). Studies on mutant embryos have demonstrated that SIMU is required for recognition and engulfment of apoptotic neurons, whereas Draper was thought to be mainly fundamental for their degradation (Freeman et al., 2003; Kurant et al., 2008; Shklyar et al., 2014). However, recent studies have shown that Draper is necessary for both engulfment and degradation of dying neurons in the first phases of metamorphosis (Hilu-Dadia et al., 2018; Tasdemir-Yilmaz and Freeman, 2014). A combination of phosphatidylserine (PS) exposure on the outer layer of the plasma membrane and increase of caspase activity are crucial for the recognition and the engulfment of embryonic neurons by glia (Shklyar et al., 2013; Tung et al., 2013). The Drpr signaling cascade is one of the most well characterized intracellular cascades in *Drosophila* and is known that Drpr mediated phagocytosis is coupled with calcium release from the endoplasmic

reticulum that leads to an extracellular calcium influx (Cuttell et al., 2008; Etchegaray et al., 2012; Ziegenfuss et al., 2008). Drpr is also expressed in epidermal cells during metamorphosis, which can remove degenerating dendrites and axons of peripheral neurons (Han et al., 2014). Finally, the *Drosophila* genome also encodes for CD36 homologous genes, and Croquemort (Crq) engulfing receptor has been identified as important for the phagosome maturation (Guillou et al., 2016; Meehan et al., 2016). The expression of these receptors is directly controlled by key transcription factors regulator of the glial cells fate such as the transcription factors Glial Cells Missing (GCM/GCM2) and Reversed Polarity (Repo) (Shklyar et al., 2014).

*C.elegans* does not have professional phagocytes, and neuronal debris after injury are removed by hypodermal cells, body wall muscles, gonadal sheath cells, and intestinal cells (Zhou et al., 2001; Zhou et al., 2004). A homologue of the *Drosophila* Draper receptor, the cell death abnormal receptor 1 (CED-1), have been identified as the activators of the engulfment signaling pathways (Chiu et al., 2018; Ellis et al., 1991). Strikingly, glial engulfment had not been described in this animal model for a long time. Recent findings reported that the sheath glial cells in the amphids, the *C.elegans* sensory organ, are responsible to prune the thermosensory neurons endings by engulfment, similarly to the vertebrate astrocyte and microglial cells, with CED-10 receptor orchestrating the process (Raiders et al., 2020). The phagocytosis machinery resulted in being enriched with orthologous *Drosophila* and mammals proteins involved in analogous processes, underscoring the conservation of these molecules across species (Mangahas and Zhou, 2005; Raiders et al., 2020; Wang and Yang, 2016).

Our RNAseq analysis provides the molecular bases to characterize the role of the ependymal cells as non-professional phagocytes cells during metamorphosis. Through the GO Term analysis, we generate lists of candidate genes involved in the inflammatory response, endosomal transport, phagosome acidification and phagocytosis activity that will allow the characterization of the molecular actuator of the glial engulfment mechanism in *Ciona.* Our work contributes in the understanding

of the roles of glial cells in urochordates, highlighting they accomplish similar function of known vertebrates and invertebrates glia.

# 5. Conclusion

The results of this work provide new insight into the understanding of the *Ciona* embryonic and larva nervous system, establishing the presence of specialized glial cell populations that actively contribute to neuronal and developmental processes. In an evolutionary context, we demonstrate that urochordates have glial cells that appear to be similar to vertebrate glia on the molecular and functional level, providing new insight into the evolution of glial cells among deuterostomes.

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