UNCOVERING MOLECULAR REGULATORS OF GLIAL REGENERATION AND FUNCTION IN SCHMIDTEA MEDITERRANEA

by

BIDUSHI CHANDRA

(Under the Direction of Rachel Roberts-Galbraith, Ph.D.)

ABSTRACT

Glia are heterogenous non-neuronal cells prevalent throughout the nervous systems (central and peripheral) of most animal species. Glial cells play critical roles during development, physiology, and diseases/disorders (including regeneration) of the nervous system. Interestingly, the roles that glial cells play during nervous system regeneration are multifaceted depending on several intrinsic and extrinsic factors. Studies of glial biology, particularly in context of regeneration, are confined to few model organisms. Planarians are freshwater flatworms capable of robust regeneration of their nervous system. Recent discoveries of glial cells in planarians provide new avenues for studying how glial cells play a permissive role during nervous system regeneration. In this work, I establish foundational knowledge on planarian glial biology, including uncovering when glial cells regenerate, discovering what molecular regulators and signaling molecules are important for glial regeneration, and elucidating what functions glial cells play within the nervous system.

INDEX WORDS: Glia, Glial Cells, Planarian, Regeneration, Nervous System, Neurobiology, ETS-1, Behavior, *Schmidtea mediterranea*

UNCOVERING MOLECULAR REGULATORS OF GLIAL REGENERATION AND FUNCTION IN SCHMIDTEA MEDITERRANEA

by

BIDUSHI CHANDRA

Bachelor's of Science, University of Georgia, 2018

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements of the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GA

2023

UNCOVERING MOLECULAR REGULATORS OF GLIAL REGENERATION AND FUNCTION IN SCHMIDTEA MEDITERRANEA

by

BIDUSHI CHANDRA

Major Professor: Rachel H. Roberts-Galbraith

Committee: James D. Lauderdale
Shelley Hooks
Michael Tiemeyer

Electronic Version Approved:

Ron Walcott
Vice Provost for Graduate Education and Dean of the Graduate School
The University of Georgia
December 2023

DEDICATION

This dissertation is dedicated to my parents, who sacrificed so much to raise me. Thank you for taking me to all those museums, discovery centres, libraries, and for sitting with me at the dinner table to help me with my studies when I was younger. Most importantly, thank you for raising me to love learning and encouraging me to stay curious.

(I would also like to thank Naru, who graciously would chew all my terrible drafts.)

ACKNOWLEDGEMENTS

First and foremost, I'd like to thank my mentor, Dr. Rachel H. Roberts-Galbraith, who supported and challenged me to grow as a scientist and a person. To my lab mates, the worm squad (Jen Jenkins, Taylor Medlock-Lanier, Kendall Clay, Christina Endara Arnold, Skylar Settles, Macey Wilson), whose friendship, energy and passion for science made me love being in lab. To Dr. Jonathan Eggenschwiler, who took me on as an undergraduate researcher and opened the doors for me to explore developmental neuroscience. To my advisory committee, Drs. Jim Lauderdale, Shelley Hooks, and Michael Tiemeyer, who were indispensable during my PhD journey. I cannot express enough of my gratitude for each important impact these individuals have made on in my academic career.

TABLE OF CONTENTS

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Defining Mammalian Glia and Glial Diversity

Glial cells, also known as glia or neuroglia or gliocytes, are a population of non-neuronal cells that reside within the nervous system in close proximity to neurons. There is immense heterogeneity of glial cell types that have been characterized based on their morphology, function, localization, origin, and molecular profiles. In vertebrates, these cells outnumber or equal neurons in cell numbers depending on the species and region of the nervous system (Herculano-Houzel, 2014; Herculano-Houzel & Dos Santos, 2018; Von Bartheld et al., 2016). In mammals, there are four major glial types present in the central nervous system (CNS): astrocytes, oligodendrocytes, oligodendrocyte precursor cells (OPCs)/$NG2^{+}$ cells, and microglia (Allen & Lyons, 2018). In the mammalian peripheral nervous system (PNS), the major glial cells include Schwann cells, satellite glial cells, enteric glial cells, and olfactory ensheathing cells (Jessen & Mirsky, 2005; Kastriti & Adameyko, 2017). In invertebrates and other vertebrates (e.g. teleost), glial cells are categorized like their mammalian counterparts based on morphology, function and/or markers that they express. Reflecting this diversity and heterogeneity, glial cells play crucial but varied roles in nervous system development, physiology, and pathology.

Astrocytes are a major class of glial cells found in the vertebrate CNS. Depending on the species and region, and which neuroscientist you converse with, astrocytes can make up anywhere from 10-50% of the cell population in the CNS (Allen & Lyons, 2018; Von Bartheld et al., 2016). The term “astrocyte” was coined based on the cells’ star-like morphology, with highly branched processes (Miller & Raff, 1984; Zhou et al., 2019). Astrocytes are considered a highly

heterogenous cell population possessing different genetic profiles, morphologies, physiology, and regionalization (Bayraktar et al., 2015; Farmer & Murai, 2017; Minge et al., 2021; Yang & Jackson, 2019). Some astrocytes are known to have a highly stellate morphology with multiple processes that have been described as "bushy"(Zhou et al., 2019). These processes allow astrocytes to be in contact with multiple cells, and this association plays pivotal roles in astrocyte functions. For example, astrocyte processes associate with blood vessels at the blood brain barrier, closely contact different synapses to modulate synaptic functions at the tripartite synapse, facilitate in neurotransmitter recycling (e.g. glutamate) and ion homeostasis, and actively participate in synapse remodeling (Allen & Lyons, 2018; Khakh & Deneen, 2019; Khakh & Sofroniew, 2015; B. Zhou et al., 2019). Astrocytes also have been implicated in several other roles including providing structural scaffolds during CNS development and regeneration, participating in energy metabolism, delivering trophic factors, and reacting during injury or stress (Allen & Barres, 2009; Allen & Lyons, 2018; Faulkner et al., 2004; Khakh & Sofroniew, 2015). Several markers have been utilized to identify mammalian astrocytes, including *glial fibrillary acidic protein* (*gfap*), *S100 calcium binding protein β* (*S100β*), and transcription factor *sox9* (Jurga et al., 2021; Preston et al., 2019). However, it is important to note that common markers used to identify astrocytes are *not* universal across metazoans. Therefore, in non-mammalian animals, identification of astrocyte-like cells requires a combination of gene expression, morphology, and functional assessments. For detailed reviews on astrocyte heterogeneity and identifying mammalian astrocytes, see (Bayraktar et al., 2015; Jurga et al., 2021; Khakh & Deneen, 2019; Khakh & Sofroniew, 2015).

Oligodendrocytes are myelin-forming glial cells that are ubiquitously distributed throughout the vertebrate CNS (Bradl & Lassmann, 2010; Kuhn et al., 2019). Generated from

oligodendrocyte precursor cells (OPCs; also known as $NG2^+$ cells), these cells mainly function in insulation of neuronal axons by wrapping myelin sheath(s) around the axons (Zuchero & Barres, 2013). A single myelinating oligodendrocyte's processes have the ability to ensheathe multiple axons (Baumann & Pham-Dinh, 2001). In addition, there is a class of satellite oligodendrocytes that do not form myelin sheaths, but rather are perineural and regulate the microenvironment around neurons (Baumann & Pham-Dinh, 2001). Sophisticated studies with live imaging in transgenic zebrafish show that oligodendrocyte myelination of axons is a highly complex and tightly regulated process (Kirby et al., 2006). The myelin sheath helps facilitate rapid transmission of action potentials within axons by acting as an electrical insulator (Nave, 2010) and provides metabolic support by shuttling lactate to generate ATP (Bercury & Macklin, 2015; Fünfschilling et al., 2012). In some cases (mainly OPCs), oligodendrocytes play immunomodulatory roles as well (Arnett et al., 2001; Baerwald & Popko, 1998). For a detailed review of oligodendrocyte biology and functions, see (Bradl & Lassmann, 2010; Kuhn et al., 2019). New-born OPCs can be characterized by *DM-20* mRNA expression (Timsit et al., 1995). In more stable OPCs, the best characterized marker is *PDGFR-α* (Pringle & Richardson, 1993). Mature, differentiated oligodendrocytes are mainly identified with cell lineage specific markers *Olig1* and *Olig2* (Q. Zhou et al., 2000; Q. Zhou & Anderson, 2002).

Microglia represent a specialized population of immune cells within the CNS. Unlike astrocytes and oligodendrocytes, microglial cells are hematopoietically derived (Ginhoux et al., 2010) and migrate into the CNS from the embryonic yolk sac (Alliot et al., 1999). Microglia are distributed throughout the brain and spinal cord. Like astrocytes, microglia possess multiple cellular processes that extend from a small cell body and microglia can adopt distinct morphologies depending on activation state (Lawson et al., 1992; Stratoulias et al., 2019).

Although microglia are considered immune cells, they are active participants in several functions important for CNS development, physiology, and pathology. Some roles that microglial cells play include synapse modulation by pruning; synapse maturation and remodeling; phagocytosing cells and cellular components (e.g. myelin, axons); monitoring neuronal activity; and modulating inflammatory response by cytokine secretion during neuronal trauma or disease (Allen & Lyons, 2018; Bachiller et al., 2018; Fu et al., 2014). For a comprehensive overview of microglial cells, see (Lannes et al., 2017; Prinz et al., 2019).

In addition to the glial populations in the central nervous system, the vertebrate peripheral nervous system (PNS) also includes several categories of glial cells. The four main peripheral glial cell types are (1) myelinating and non-myelinating Schwann cells associated with peripheral nerves; (2) satellite glial cells found in the ganglia; (3) enteric glial cells located within the enteric nervous system of the gastrointestinal tract; and (4) olfactory ensheathing cells that reside in the primary olfactory system. Each type has primarily been characterized by morphology and anatomical location (Verkhratsky & Butt, 2013). Unlike CNS macroglia, PNS glial cells are neural crest derivatives (Jessen & Mirsky, 2005); in addition, all PNS glial cells require transcription factor *Sox10* in their development (Mollaaghababa & Pavan, 2003). Myelinating Schwann cells are the oligodendrocyte counterpart in the PNS and share several similar functional characteristics. However, Schwann cells only myelinate singular axons rather than multiple axons like oligodendrocytes (Rasband, 2016). Meanwhile, non-myelinating Schwann cells share several properties with astrocytes. For details on biology of Schwann cells, see (Kidd et al., 2013; Solovieva & Bronner, 2021). Satellite glial cells are glial cells that reside in sensory ganglia alongside Schwann cells; every individual sensory neuron in enclosed within a complementary sheath of satellite glial cells. Satellite glial cells have been implicated in pain

response (Andreeva et al., 2022). Enteric glial cells are heterogenous and reside throughout the digestive tract (Seguella & Gulbransen, 2021). Enteric glial cells play several critical roles, ranging from promoting barrier function within the epithelial cells to facilitating ionic balance (e.g. K^+ regulation) (Seguella & Gulbransen, 2021). For more details on enteric glial cell heterogeneity and functions, see (Baghdadi et al., 2022; Seguella & Gulbransen, 2021). Olfactory ensheating cells (OECs), located within the nasal cavity, are a unique class of glial cells that span both the CNS and PNS. These cells share morphological similarities to non-myelinating Schwann cells and functional similarities to astrocytes (Franklin & Barnett, 2000). OECs can both ensheath (*in vivo*) and myelinate axons (*in vitro*) (Franklin & Barnett, 2000). However, unlike glial cells derived from the CNS (neural tube) and PNS (neural crest), OECs are considered to originate from the olfactory placode alongside olfactory sensory neurons (Ramón-Cueto & Avila, 1998; Su & He, 2010). For a broad review of peripheral glial cells, their development and functions, see (Verkhratsky & Butt, 2013).

Glial Diversity Among Other Animal Species

Glial cells in nonmammalian vertebrates and invertebrates share several morphological, molecular, cellular location and/or functional similarities with their mammalian counterparts. Anamniotes do not possess stellate astrocytes. Instead, the major classes of glial cells present in fish include radial glia, glial cells of oligodendrocyte lineages, Müller glia (in retina), Schwann cells and microglia (Neely & Lyons, 2021). Unlike mammalian radial glia, zebrafish radial glial cells are capable of being both a transient progenitor to other glial cells, and taking on functions performed by mammalian astrocytes (Jurisch-Yaksi et al., 2020; Than-Trong & Bally-Cuif, 2015). For a comprehensive profile on zebrafish glial cells, see (Lyons & Talbot, 2015).

In *Drosophila*, glial cells can be divided into two wide categories: macroglia and MANF (mesencephalic astrocyte-derived neurotrophic factor) immunoreactive cells (MiCs) (Stratoulias & Heino, 2015; Walkowicz et al., 2017; Yildirim et al., 2019). *Drosophila* macroglial cells can be distinctively divided into 6 types: surface glia, cortex glia, astrocyte-like glia, ensheathing glia, midline glia, and PNS specific wrapping glia (Yildirim et al., 2019). Cortex glia, ensheathing glia, astrocyte-like glia, and midline glia share several conserved functional properties to their mammalian glial counterparts, including roles in migration and axon guidance, synapse remodeling, regulating ionic balance, providing tropic factors, and in regenerative response (Doherty et al., 2009; Freeman, 2015; Hartenstein, 2011; Ito et al., 1995; Yildirim et al., 2019). PNS specific wrapping glial cells share several functional similarities to mammalian olfactory ensheathing glia and non-myelinating Schwann cells, including roles in wrapping axons individually (Freeman, 2015; Yildirim et al., 2019). Furthermore, *Drosophila* surface glia function to wrap around both the CNS and PNS, functioning similar to both mammalian astrocytes and Schwann cells (Desalvo et al., 2011; DeSalvo et al., 2014). For a detailed comparison of *Drosophila* and mammalian glial cells, see (Freeman & Doherty, 2006; Yildirim et al., 2019).

Another popular invertebrate model for glial biology study is *Caenorhabditis elegans*. *C. elegans* glial cells are associated with sensory structures and can broadly be divided into two major categories: socket glia and sheath glia (Mizeracka & Heiman, 2015; Oikonomou & Shaham, 2011). *C. elegans* glial cells, while not directly analogous to specific mammalian glia, share several functional characteristics, including but not limited to establishment of neuronal structure, regulation of sensory ending size and morphology, and modulating neuronal activity at the synapses (Mizeracka & Heiman, 2015; Rapti et al., 2017; Shaham, 2006; Stout Jr. et al.,

2014; Yoshimura et al., 2008). It is important to note that unlike in other organisms, *C. elegans* glia are not necessary for neuronal survival but the loss of glia does result in defects in neuronal development, structures, and function (Bacaj et al., 2008; Sulston et al., 1983). For a review on glial diversity in other non-traditional model organisms (i.e. molluscs, including cephalopods; and annelids), refer to (Hartline, 2011; Le Marrec-Croq et al., 2013; Ortega & Olivares-Bañuelos, 2020).

Gliogenesis

During the formation of the nervous system in most invertebrate and vertebrate species, neurogenesis largely precedes gliogenesis (Bond et al., 2020, 2020; Freeman & Rowitch, 2013; Rusznák et al., 2016). Vertebrate macroglia (i.e. astrocytes and oligodendrocytes) are derived from neuroepithelial cells of the neural tube (Kessaris et al., 2008; Lago-Baldaia et al., 2020). Neuroepithelial precursor cells eventually give rise to radial glial cells, which are progenitors to neurons and glia, as well as intermediate progenitors. Radial glial cells divide asymmetrically to generate neurons directly or indirectly, and also guide migration of newborn neurons in the developing brain (Anthony et al., 2004; Campbell & Götz, 2002; Kriegstein & Alvarez-Buylla, 2009). Following generation of neurons, radial glial cells undergo a fate switch known as the "gliogenic switch" to generate glial cells post-embryonically (Kriegstein & Alvarez-Buylla, 2009). The timing of neurogenesis and gliogenesis is consecutive and temporally non-overlapping process in vertebrate neocortical development (Miller & Gauthier, 2007). Discrete populations of radial glial cells produce oligodendrocyte progenitors (OPCs) which give rise to oligodendrocytes (Li et al., 2021; Omoto et al., 2016). The remaining radial glial cells give rise to astrocytes (Campbell & Götz, 2002; Miller & Gauthier, 2007). The interplay of several secreted signals – notably Sonic Hedgehog (Shh), fibroblast growth factors (FGFs), Notch/Delta,

bone morphogenetic proteins (BMPs) and Wnts – serve as positional cues (Rowitch & Kriegstein, 2010). Additionally transcription factors like *Sox9*, *nuclear factor IA*, *Olig1*/*Olig2* are required for astrocyte and OPC production (Molofsky & Deneen, 2015; Zuchero & Barres, 2013). As previously mentioned, unlike astrocyte and oligodendrocytes, microglial cells originate from the mesoderm and are generated in the yolk sac during embryogenesis. Hematopoietic stem cells in the yolk sac become primitive macrophages that migrate into the CNS and become microglia (Ginhoux et al., 2010, 2013). This migration to the brain occurs prior to any major neurogenesis and gliogenesis, and formation of the blood-brain barrier (Bachiller et al., 2018; Ginhoux et al., 2010).

Drosophila CNS gliogenesis spatiotemporally occurs together with neurogenesis. Like vertebrates, most *Drosophila* neurons and glial cells arise from the neuroectoderm (except for meso-ectodermal derived midline glia) (Altenhein, 2015; Altenhein et al., 2016; Bossing & Technau, 1994; Ito et al., 1995; Omoto et al., 2016). Neural stem cells known as "neuroblasts" divide asymmetrically and give rise to ganglion mother cells (GMCs) (Altenhein et al., 2016; Doe, 1992; Omoto et al., 2016). Currently, there are 6 neural lineages that have been identified. In lineage type 1, neurons and glial cells can arise at the same time as siblings, but require Notch signaling (Ren et al., 2018). In lineage type 2, the ganglion mother cell can switch from generating neurons first to glial cells at a later time point (Boone & Doe, 2008; Jones, 2001). In rare cases (lineage 3), there are "glioblasts" that exclusively give rise to glial cells (Jones, 2001; Omoto et al., 2016). Transcription factor *glial cell missing* (*gcm*) is important for establishing most glial fate in *Drosophila* (Crews, 2019; Freeman et al., 2003; Jones et al., 1995; Sasse et al., 2015) . Additionally, interplay of Notch signaling and Hedgehog signaling dictates glial heterogeneity (Altenhein, 2015; Crews, 2019; Sasse et al., 2015).

Development of glial cells in *C. elegans* is unique as the glial lineages are pre-determined and stereotypical. While *C. elegans* glial cells do arise from a neuroepithelial precursor and certain glial cells (i.e. amphid sheath glia and cephalic sheath glia) have radial-glial like properties to give rise to neurons, the development of neurons and glial cells are not dependent on one another (Shaham, 2015).

In the mammalian PNS, by contrast, all glial cells originate from the neural crest. Analogous to the stepwise development seen in the CNS, neural crest cells (NCCs) first generate sensory neurons and then generate peripheral glial cells (Butler & Bronner, 2015; Jessen & Mirsky, 2005). The two glial cell types that are directly generated are Schwann cell precursors and satellite glial cells (Jacob, 2015; Jessen & Mirsky, 2005). Some Schwann cell precursors migrate into the peripheral nerves where they can further differentiate into myelinating Schwann cells, and non-myelinating Schwann cells (Petersen & Adameyko, 2017). Schwann cell precursor cells that arise from vagal and sacral NCCs can migrate into the enteric nervous system and give rise to enteric glial cells (Seguella & Gulbransen, 2021; Verkhratsky & Butt, 2013). Schwann cell precursors can also give rise to melanocytes, parasympathetic neurons, endoneural fibroblasts and mesenchymal stem cells (Jacob, 2015; Petersen & Adameyko, 2017). Concerted interplay of several transcriptional regulators and signaling molecules, including those encoded by *neuregulin*, *HDAC1/2*, *Pax3*, *Notch/Delta*, *FGFs*, and *Sox10*, impacts specific cell fates (Jacob, 2015; Jessen & Mirsky, 2005; Mollaaghababa & Pavan, 2003).

Glial Response to Injury

Perhaps one of the most intriguing roles that glial cells play is in response to trauma and injury. It has been classically known that when comparing vertebrate and invertebrate response

to repairing their CNS, invertebrates appear to have an advantage. Why? One of the major differentiating elements that separates the potential for PNS regeneration relative to CNS is glial cells (Brosius Lutz & Barres, 2014; Freeman & Rowitch, 2013). Any mammalian CNS injury or insult, including degeneration and disease, results in "reactive gliosis." Initial reactive gliosis is a non-specific response of several glial types, including astrocytes, microglia, and $NG2^{+}$ OPCs (Burda & Sofroniew, 2014; Escartin et al., 2021; Pekny & Nilsson, 2005; Sofroniew, 2009). In vertebrates, reactive glia can be identified by three primary properties: (1) upregulation of GFAP, (2) increased cellular hypertrophy, and (3) increased proliferation (Burda & Sofroniew, 2014; Escartin et al., 2021; Pekny et al., 2014; Pekny & Nilsson, 2005). Reactive gliosis is not an all-or-nothing response, but rather a continuous, graded, multi-stage response (Sofroniew, 2009; Verkhratsky & Butt, 2013). Mild to moderate reactive gliosis involves molecular and functional changes along with hypertrophy that resolves over time. These changes happen after mild trauma or at sites further away from a more severe injury (Burda & Sofroniew, 2014). Severe gliosis, on the other hand, can result in scar formation (Sofroniew, 2009). A "glial scar" is a structure of glial cells that forms around a lesion core that occurs after a major injury (i.e. spinal cord injury, traumatic brain injury, ischemic stroke, multiple sclerosis) (Adams & Gallo, 2018; Anderson et al., 2016; Faulkner et al., 2004; Rolls et al., 2009; Silver & Miller, 2004; Sofroniew, 2009). For extensive overviews on multicellular responses of glial cells during CNS damage, see (Burda & Sofroniew, 2014; Rolls et al., 2009; Silver & Miller, 2004).

Until recently, reactive gliosis has been viewed as an inhibitory force that prevent CNS regeneration. Reactive astrocytes and microglia have been characterize to be pro-neuroinflammation, with neurotoxin secretion that promotes neuronal and oligodendrocyte death (Liddelow et al., 2017). The glial scar was also proposed to be a physical barrier against axon

regeneration due to release of inhibitory proteins (i.e. chondroitin sulfate proteoglycans) that can hinder axon regrowth (Busch & Silver, 2007; Morgenstern et al., 2002). In contrast, after ischemic stroke, reactive astrocytes appear to play a protective role by promoting expression of neurotrophic factor (Hayakawa et al., 2016). Similarly, evidence indicates that a glial scar can be beneficial in stabilizing CNS tissue by sequestering damaged tissue damage away from healthy tissue (Faulkner et al., 2004; Myer, 2006). Glial scars may also promote axon regrowth in some cases (Anderson et al., 2016). The duality of reactive gliosis during CNS repair and regeneration has not been resolved. For more comprehensive current knowledge on complex roles of gliosis and glial scars, see (Adams & Gallo, 2018; Pekny et al., 2014; Silver & Miller, 2004).

Importantly, there is a large difference in regenerative capabilities between the vertebrate CNS and PNS; this dissimilarity has been attributed to the presence of distinct glial cells in the PNS (i.e. Schwann cells, satellite cells) (Brosius Lutz & Barres, 2014). In brief, during PNS axon injury, Schwann cells recruit macrophages to the site of injury, and together the cells quickly clear myelin debris (Brosius Lutz & Barres, 2014; Fissel & Farah, 2021; Martini et al., 2008; Tofaris et al., 2002). At the same time, Schwann cells also secrete several growth factors and increase production of pro-regenerative basal lamina (Brosius Lutz & Barres, 2014; Mehta et al., 1985). In addition, satellite glial cells also help promote axon regrowth (Avraham et al., 2020).

Similarly, specialized glial cells in other organisms are thought to play pro-regeneration roles. For example, zebrafish possess unique “bridging glia” that act as scaffolds for axon regrowth after spinal cord injury (Goldshmit et al., 2012; Mokalled et al., 2016). In the *Drosophila* CNS, acute injury results in a glial regenerative response in which ensheathing glial cells respond by undergoing compensatory proliferation, upregulating phagocytosis/engulfment properties, and helping to facilitate recovery of neuronal function (Hidalgo & Logan, 2017; Kato

et al., 2011; Losada-Perez et al., 2016). However, the phenomenon that axons can regenerate in the PNS and not in the CNS is still conserved in the *Drosophila* (Song et al., 2012, 2019).

Can glial cells promote regeneration in the *central* nervous system? Over decades, several animal models have been used to understand the role of glial activation, reactive gliosis and glial properties in regeneration. Depending on the species, type of injury, location of injury, duration of injury, and type of glial cell present at injury site, the glial response to injury in the central nervous system is complex (Burda & Sofroniew, 2014; Pekny et al., 2014). Unfortunately, studying what properties glial cells exhibit to promote or permit regeneration is often confounded by the model organism's biology and their lack of regenerative capacity within the CNS. To understand what makes glial cells pro-regenerative, there is a need for a model system that not only is capable of robust regeneration, but also possesses glial cells. I here argue that planarian flatworms are ideal for this experimental direction.

Planarians as a Model for Nervous System Regeneration

Non-parasitic freshwater flatworms, commonly referred to as planarians, have become emerging models for studying whole-body regeneration, including regeneration of the central nervous system. Planarians chiefly reside in lentic water and can range in size from 1 mm to 2 cm (depending on the species). Filed under the invertebrate phylum of Platyhelminthes (class: Turbellaria, order: Tricladida), planarians possess several complex organ systems, including a nervous system. For an overall coverage of the other organ types present in planarians, refer to (Newmark & Alvarado, 2002; Roberts-Galbraith & Newmark, 2015).

The planarian central nervous system (CNS) is located on the ventral side of the body. The CNS is composed of inverted U-shaped, bi-lobed cephalic ganglia (also referred to as the

brain) and two longitudinal ventral nerve cords (VNC) that run to the posterior of the body (Agata et al., 1998). Moreover, these two structures are not located in the same spatial plane: the brain sits dorsal to VNC (Agata et al., 1998). The two lobes of the brain are connected by a single(or multiple, depending on the species) anterior commissure (Agata et al., 1998; Fraguas et al., 2012; Hyman, 1951). The brain is organized with an outer layer of neuronal cell bodies and a neuropil, which is a process- and synapse- dense region devoid of cell bodies, within the interior core of the brain (Morita & Best, 1966). In addition to the cephalic ganglia, there are lateral projections known as brain branches that project outward from the cephalic ganglia into the periphery of the head and are associated with sensory functions including chemosensation (MacRae, 1967). Planarians also possess photoreceptor structures (e.g., eyespots and visual axons), which are located on the dorsal-most side of the planarian head and project into the cephalic ganglia (Carpenter et al., 1974; MacRae, 1964). Planarians also have a peripheral nervous system (PNS) (Ross et al., 2017) and a pharyngeal nervous system (PhNS) that innervate the remainder of the body (Baguñà & Ballester, 1978). The PNS has been primarily described to be composed of a subepidermal nerve plexus, a submuscular nerve plexus and a gastrodermal nerve plexus (Baguñà & Ballester, 1978). The sub-epidermal nerve plexus is described to be a diffuse network of singular processes under the basal membrane that runs in a transverse direction (Baguñà & Ballester, 1978). The sub-muscular plexus, on the other hand, is described as bundles of nerve fibers beneath the muscle body wall (Baguñà & Ballester, 1978; Ross et al., 2017). The gastrodermal nerve plexus is not as well characterized but is described as a single nerve net that surrounds the intestine (Baguñà & Ballester, 1978; Ross et al., 2017). In contrast, the pharyngeal nervous system is an independent nervous system within the pharynx, the feeding

organ of the planarian. It has been characterized as a circular array of nerve fibers (or nerve ring) that is housed beneath the outer muscular layer of the pharynx (Baguñà & Ballester, 1978).

The cellular composition of the planarian nervous system is complex. Studies in *Dugesia japonica* reported at least four distinct regions present in the brain that are defined by the expression of discrete *otd/Otx* family homeobox genes (Umesono et al., 1999). Planarian neurons have been described to be unipolar, bipolar and multipolar (which is a unique component among invertebrates) (Carpenter et al., 1974; Elvin & Koopowitz, 1994; Keenan et al., 1981; Morita & Best, 1966). Bipolar neurons have been found in the transverse commissure between the VNCs; sensory neurons have also been suggested to be bipolar (Carpenter et al., 1974; Keenan et al., 1981; Morita & Best, 1966). Additionally, there is a great diversity of neuronal subtypes present. Neuron types include cholinergic (Nishimura et al., 2010), GABAergic (Nishimura, et al., 2008), dopaminergic (Nishimura, et al., 2007), serotonergic (Nishimura, et al., 2007), octopaminergic (Nishimura, et al., 2008) and several peptidergic cell types (Collins et al., 2010). Each neuronal type is present with distinct regionalization and ratio relative to other neuronal cell types. Furthermore, several of these neuron types have been implicated in facilitating behavioral outcomes in planarians. Considerable advancements have been made within the field to uncover regulators that are crucial for the production and/or maintenance of neuronal subtypes. For a comprehensive list of transcription factors and other signaling factors currently known to specify neuronal cell populations, refer to (Roberts-Galbraith & Newmark, 2015; Ross et al., 2017).

Unlike many other vertebrates and invertebrates, planarians are capable of robust *de novo* regeneration of the nervous system within seven days without scarring. This feat is largely credited to the presence of mitotically active adult pluripotent stem cells referred to as ‘neoblasts’ that are distributed throughout the planarian body (Orii et al., 2005; Reddien &

Alvarado, 2004). Neoblasts are the sole source of new cells in planarians, during both cell turnover in intact planarians and regeneration (Baguñà et al., 1989; Eisenhoffer et al., 2008). Neoblast cells express *piwi-1*, which encodes a member of the PIWI/Argonaute family of proteins (Palakodeti et al., 2008; Reddien, 2005). Furthermore, bromodeoxyuridine labeling (BrdU) can be utilized to label neoblasts and their progeny (Eisenhoffer et al., 2008; Newmark & Sánchez Alvarado, 2000). More recent works have established the possibility of neoblast heterogeneity where in specialized neoblasts (i.e. 'sigma', 'gamma', and 'zeta') are poised to make certain lineages of cells (Molina & Cebrià, 2021; Molinaro & Pearson, 2016; van Wolfswinkel et al., 2014; Zhu & Pearson, 2016). For example, it has been hypothesized that sigma-Neoblasts give rise to neuronal cell populations (van Wolfswinkel et al., 2014)]. Using in silico lineage tracing, the sigma-Neoblasts cell population has also been further subcategorized to include a class of neoblast called nu-Neoblasts that give rise to most though not *all* neuronal cell populations (Molinaro & Pearson, 2016). Therefore, the presence of a *bona fide* neuronal stem cell population has yet been demonstrated in planarians. For a recent review on specialized neoblast progenitors and response to injury, see (Reddien, 2018).

Planarian nervous system regeneration can be broken down into 6 steps: (1) construction of an anterior blastema [0-12 hours post amputation (hpa)], (2) re-establishment of the anterior pole (12-24 hpa), (3) formation of a rudimentary brain (24 hpa), (4) neuronal pattern establishment, (5) assembly of a neural network and integration with existing tissue, and (6) functional recovery (≥5-7 days). In order to re-establish a head and fully functional nervous system, planarians must first form an anterior blastema, defined as an unpigmented area formed after wound closure in the first 24 hours in which newly born cells give rise to new tissue (Baguñà et al., 1989). Any wounding event can result in neoblast proliferation (Reddien, 2018;

Wenemoser et al., 2012; Wenemoser & Reddien, 2010; Wurtzel et al., 2015). In the event of a large tissue removal (in this case, a transverse amputation removing the head), neoblasts and post-mitotic neoblast progeny migrate to the wound site and give rise to daughter cells which form the blastema. In addition, the epidermis closes to cover the wound site (Kato et al., 2001; Ogawa et al., 2002; Scimone et al., 2022; Wenemoser et al., 2012). The blastema continues to grow due to the high level of local neoblast proliferation at the base of the blastema (Wenemoser & Reddien, 2010; Wurtzel et al., 2015). During this time, polarity cues, including those along the anterior-posterior axis, are re-expressed. Within 12 hpa, *notum*, which encodes a Wingless (Wnt) inhibitor, is expressed at the anterior wound site (Petersen & Reddien, 2011). *Notum* expression inhibits Wnt signaling; inhibition of *notum* via RNA interference (RNAi) can block head regeneration (C. P. Petersen & Reddien, 2011). In addition, other polarity cues that allow an anterior-permissive environment are also activated to inhibit or decrease Wnt signaling. This factors include activin antagonist *follistatin* (Roberts-Galbraith & Newmark, 2013) and *adenomatous polyposis coli* (*APC*) (Gurley et al., 2008). *Follistatin (fst)* works with *notum* to promote anterior identity (Roberts-Galbraith & Newmark, 2013). In addition, inhibition of *β-catenin-1* by RNAi can induce head regeneration and anteriorization of homeostatic tissue (Gurley et al., 2008; Iglesias et al., 2008; C. P. Petersen & Reddien, 2008). Other factors that work to promoting anterior regeneration also include homeobox gene *prep* (Felix & Aboobaker, 2010)*;* negative regulators of Hedgehog signaling pathway *patched (ptc)* and *suppressor of fused (sufu)* (Yazawa et al., 2009); and GPCR subunits *Gαq1* and *Gβ1*-4 (Jenkins & Roberts-Galbraith, 2023). In addition, several factors work to establish and maintain the *notum*$^{+}$ "anterior pole", which is a distinct region composed of small cell clusters at the anterior-most extremity. These factors include transcription factor *foxD* (Scimone et al., 2014); *zic-1* (Vásquez-Doorman

& Petersen, 2014), and *pbx* (Blassberg et al., 2013; Chen et al., 2013). The molecular network involved in anterior and posterior fate has only been partially deciphered. For a brief overview on current knowledge about anterior head regeneration in planarians, refer to (Cebrià et al., 2018; Owlarn & Bartscherer, 2016). In summary, the antagonistic suppression of Wnt signaling determines anterior-posterior polarity outcomes.

In addition to the anterior-posterior axis, the dorsal-ventral axis also plays a critical role in organizing components of the nervous system, including the ventral nerve cords and brain. The bone morphogenetic protein (BMP) signaling pathway is largely responsible for controlling the dorsal-ventral axis (Molina et al., 2007; Orii et al., 2005; Reddien et al., 2007). Silencing of BMP activity, via knocking down *bmp4*, promotes ventral identity (Gaviño & Reddien, 2011). Suppressing the inhibitor of bmp signaling, through knockdown of *anti-dorsalizing morphogenetic protein* (*admp*), results in dorsalization (Gaviño & Reddien, 2011). The third polarity axis, the medial-lateral axis, is also important for promoting several bilateral structures within the central nervous system. Reciprocal antagonistic activities of *slit* and *wnt5* regulate the midline domains (Cebrià et al., 2007; Gurley et al., 2008, 2010). *slit* is expressed medially between the VNC and inhibits lateral identity; *slit*(RNAi) results in ectopic neural structures at the midline (Cebrià et al., 2007). In contrast, *wnt5* is expressed lateral to the VNC and inhibits medial identity (Gurley et al., 2010). Previous reports show that *wnt5*(RNAi) animals exhibited lateral expansion of the nervous system (Adell et al., 2009; Gurley et al., 2010).

Following the formation of the blastema and establishment of the anterior pole, a rudimentary brain (or neural cluster) starts to form in the anterior blastema within 24-48 hpa (Cebrià et al., 2002; Ross et al., 2017). Several signaling factors facilitate pro-neural induction in the primordial brain. These factors include components of the epidermal growth factor (EGF)

receptor pathway, including the receptor *EGFR-3* and putative ligands *EGR-4* and *NGR-7* (Fraguas et al., 2014; Lei et al., 2016). Factors such as *F-Spondin* and *Low Density Lipoprotein Related Receptor* (*LDLRR*)*-1* and *-3* have also been implicated in being key signaling molecules required for regeneration (Roberts-Galbraith et al., 2016). In addition, Hedgehog signal transduction from ventral-medial neurons has been implicated in regulating the number of neural progenitors (Currie et al., 2016). Once a primordial brain is established, the neuronal pattern is established relatively soon after, anywhere from 48-72 hpa. Several transcription factors drive regeneration and maintenance of individual neuronal subtypes. For a summarized overview of regulators of neuronal cellular lineages, see (Roberts-Galbraith & Newmark, 2015). Between 72-120 hours, brain lobes are reconnected, lateral branches are formed, and the neural network formation is re-established (Agata & Umesono, 2008; Ross et al., 2017). By 7 days, brain morphology and function are considered to be fully recovered (Agata & Umesono, 2008). While pattern reformation is occurring, polarity cues continue to play critical roles in shaping boundaries. For example *nou-darake* (*ndk*), a FGFR-related gene, maintains the posterior spatial boundary of the head region. Knockdown of *ndk* results in posterior expansion of the head region (Cebrià et al., 2002). In conclusion, the planarian nervous system is a dynamic and plastic structure that is regulated by complex set of internal and external regulatory networks that is still actively being studied.

Introduction to planarian glial cells and open questions

The first evidence of a glia-like cell present in the CNS was derived from fine structural analysis of what were originally called “accessory cells” (Golubev, 1988; Lentz, 1967; Morita & Best, 1966). However, the argument for glial cell presence in planarians was often under scrutiny

due to the lack of molecular evidence. With more recent technological advancements and reinforced interest in uncovering molecular mechanisms implicated in nervous system regeneration using planarians, molecular markers identifying glial cells in planarians were serendipitously discovered (Roberts-Galbraith et al., 2016; Wang et al., 2016). Expression of markers *intermediate filament-1* (*if-1*; Roberts-Galbraith et al., 2016; Wang et al., 2016), *calamari* [*cali* (Wang et al., 2016)], and *estrella* (Roberts-Galbraith et al., 2016) was enriched within the CNS (both neuropil and VNCs) and closely associated with synapses. Importantly, these markers were not co-expressed with any neuronal markers (Roberts-Galbraith et al., 2016; Wang et al., 2016). Planarian *calamari* is a putative signaling protein with homology to vertebrate protocadherin, but lacks the cadherin domains (Wang et al., 2016). *calamari* is expressed primarily throughout the neuropil and VNC. In contrast, *estrella*, a putative signal peptide-encoding protein, is widely distributed throughout the ventral CNS (neuropil, VNC, brain branches), the dorsal PNS (near photoreceptors), in the peripheral nervous system (dorsal and ventral) and around the pharynx opening. *intermediate filament-1* (*if-1*) encodes for a cytoplasmic protein that is part of the intermediate filament family, and shares similarity to vertebrate *glial fibrillary acidic protein* in astrocytes, and neurofilament proteins in neurons (Roberts-Galbraith et al., 2016; Wang et al., 2016).

To determine if *if-1*$^{+}$ cells were *bona fide* glial cells, Roberts-Galbraith and colleagues (2016) sought to uncover whether *if-1* was co-expressed with genes involved in neurotransmitter uptake and recycling, a common and crucial function displayed by vertebrate astrocytes at the tripartite synapse (Cahoy et al., 2008; Schousboe et al., 1977). Indeed, *if-1* was highly co-expressed with *excitatory amino acid transporter* (*eaat*/*slc1a-5*), and *glutamine synthetase-1* (*gs-1*), two genes that are implicated in glutamate uptake and conversion to glutamate, respectively

(Roberts-Galbraith et al., 2016; Wang et al., 2016). *if-1* was also co-expressed with several other solute carrier family transporters implicated in neurotransmitter transport (e.g. *slc1a-3*, *slc6a-2*, *slc6a-8, slc7a-8*) (Roberts-Galbraith et al., 2016). In addition, Wang and colleagues (2016) showed that *if-1* was also co-expressed with several other transporters and common glial markers utilized in vertebrates and other invertebrates [e.g. GABA/cretine/taurine transporter *gat*, glucose transporter (*glut*), and a *transient receptor protein potential ion channel* (*trpm*)]. Likewise*, if-1* was not co-expressed with any neuronal transcription factors or channels associated with neuron function (i.e. voltage gated ion channels, calcium channels, sodium channels, and potassium channels), eliminating the possibility of *if-1*$^+$ cells being a neuronal cell population (Roberts-Galbraith et al., 2016; Wang et al., 2016). Moreover, expression of *if-1* and *estrella* was shown to downregulate in response to injury in planarians (Roberts-Galbraith et al., 2016). Furthermore, two published single cell transcriptome atlases showed that *if-1*$^+$ cells cluster separately from neuronal populations (Fincher et al., 2018; Plass et al., 2018). Interestingly, *if-1*$^+$ cells (as well as cells expressing other glial markers mentioned above) were grouped within the *cathepsin*$^+$ class (or "parenchymal" class in the atlas dataset by Plass et al.) due to high enrichment of a protease-encoding *cathepsin* (*ctsl2*) transcript (Fincher et al., 2018). The *cathepsin*$^+$ cluster also includes pigment cells and other novel uncharacterized cell populations, suggesting a class of cells that have primarily phagocytic activity (Fincher et al., 2018; Scimone et al., 2018). *if-1*$^+$ cells being specifically clustered in this population suggests that planarian glial cells may have phagocytic ability, a feature common to vertebrate and invertebrates glia (Fu et al., 2014; Konishi et al., 2022; Logan & Freeman, 2007; Morizawa et al., 2017; Raiders et al., 2021).

Taken together, there is strong evidence that suggests three important ideas: (1) planarians possess astrocyte-like glial cells, (2) markers utilized to identify the presence of glial

cells in planarians implicate *potential* (but not confirmed) roles that they may play in the nervous system, and lastly (3) downregulation of glial genes after injury suggests that planarian glial cells *do* react to injury. In other organisms, glial cells have been implicated in playing permissive and/or inhibitory roles during nervous system regeneration. More importantly, vertebrate glial cells respond to injury by *upregulating* gene expression, a contrast to what is seen in planarians. How and what makes glial cells undertake a permissive role during regeneration is not well characterized. The presence of glial cells in planarians raises several questions regarding the ability to robustly regenerate a nervous system without scarring and recover to full function. However, in order to establish planarians as a model system to study unique glial properties and glial interactions during successful nervous system regeneration, fundamental knowledge must be established first. I thus sought to discover: (1) when and how glial cells develop and regenerate in planarians and (2) what functional roles glial cells play in the planarian nervous system.

Dissertation Objectives

The current state of knowledge on planarian glial cells is at its beginning, allowing for several avenues of exploration. When do planarian glial cells regenerate? What is the relationship between neurons and glia during regeneration? Are glial cells required for regeneration of neurons? Are there multiple types of glial cells? How do glial cells change in response to injury? What genes are required for glial regeneration and maintenance in planarians? Do glial cells in planarians play conserved roles in neural architecture and physiology like in other organisms? In the following chapters, I provide insights to some of these fundamental questions and a scaffold for further investigation.

In order to establish planarians as a model for studying glial cells in a robust regeneration context, a blueprint of when and what to look for is important. In Chapter 2, I provide a detailed description of glial cell development and regeneration, and uncover the dependence that glial cells have on neurons during nervous system regeneration. Furthermore, I uncovered transcription factor encoding gene *ets-1* to be important for regeneration and maintenance of glial cells. Using *ets-1*(RNAi) to ablate glial cells, I also assessed multiple functional roles that glial cells play in the planarian nervous system during regeneration. My work opens several avenues of further research within planarian glial biology, including the potential for these cells to change state change in response to injury. In addition, because *ets-1* affects multiple *cathepsin*$^{+}$ cell types, uncovering genes with specificity to glial cell identity is a crucial next step. In Chapter 3, I identify additional molecular regulators important for glial regeneration or gene expression. Taken together, in the following chapters, I establish the timeline for when glial cell regeneration, elucidate key neuronal-glial interactions during regeneration and in physiology, and identify key molecular regulators that mediate glial cell maintenance and regeneration in planarians.

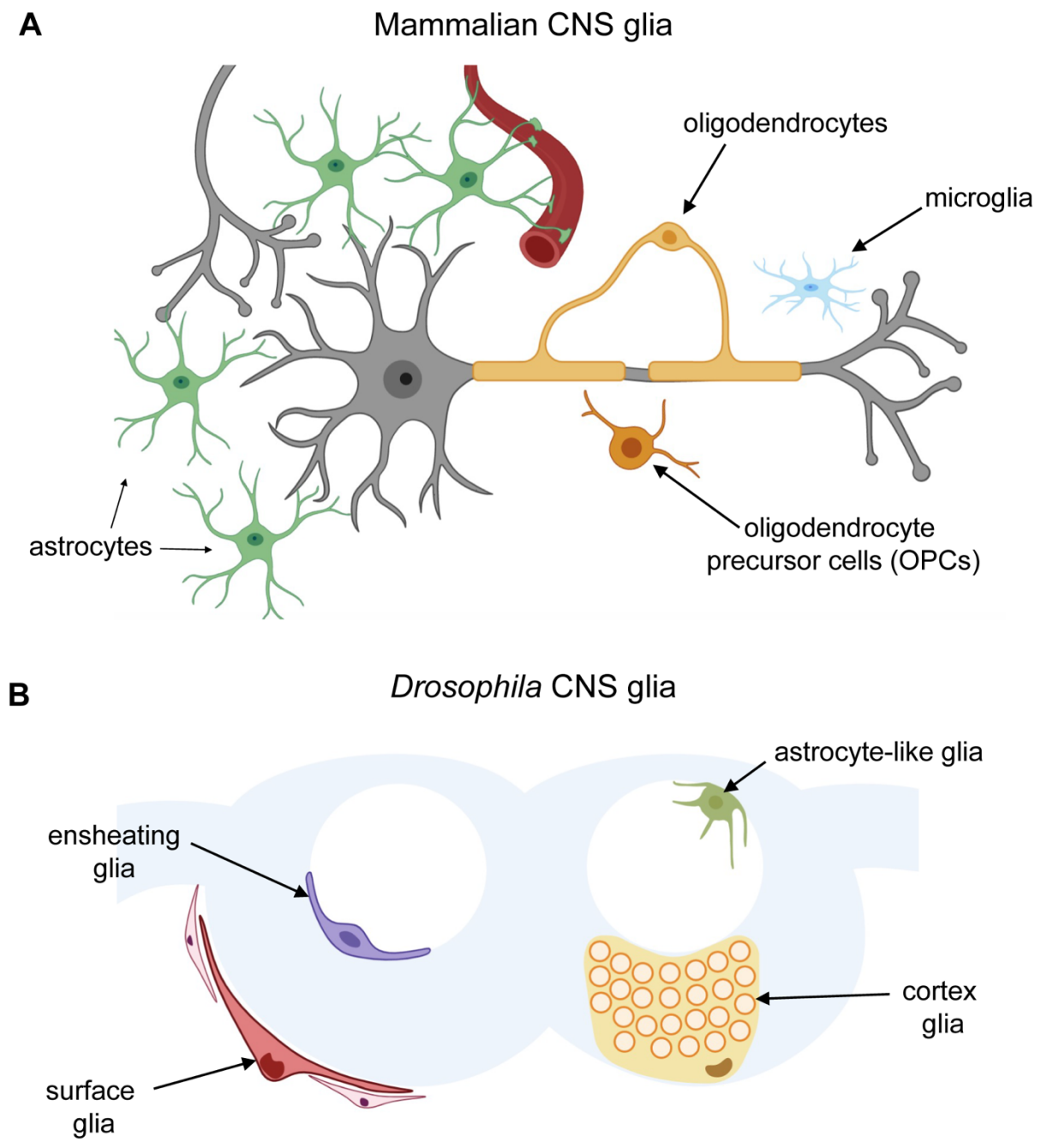


Figure 1.1 Glial cells show great diversity across animal species. (A) Mammalian CNS glial cells are comprised of 4 major glial cells: astrocytes, oligodendrocytes, oligodendrocyte precursor cells (OPCs) and microglia. (B) *Drosophila* CNS glial cells share remarkable morphological and/or functional characteristics to their mammalian glial counterparts. Some of

the major *Drosophila* CNS glial cells include astrocyte-like glial cells, cortex glia, surface glia, and ensheating glia. Images made with BioRender.

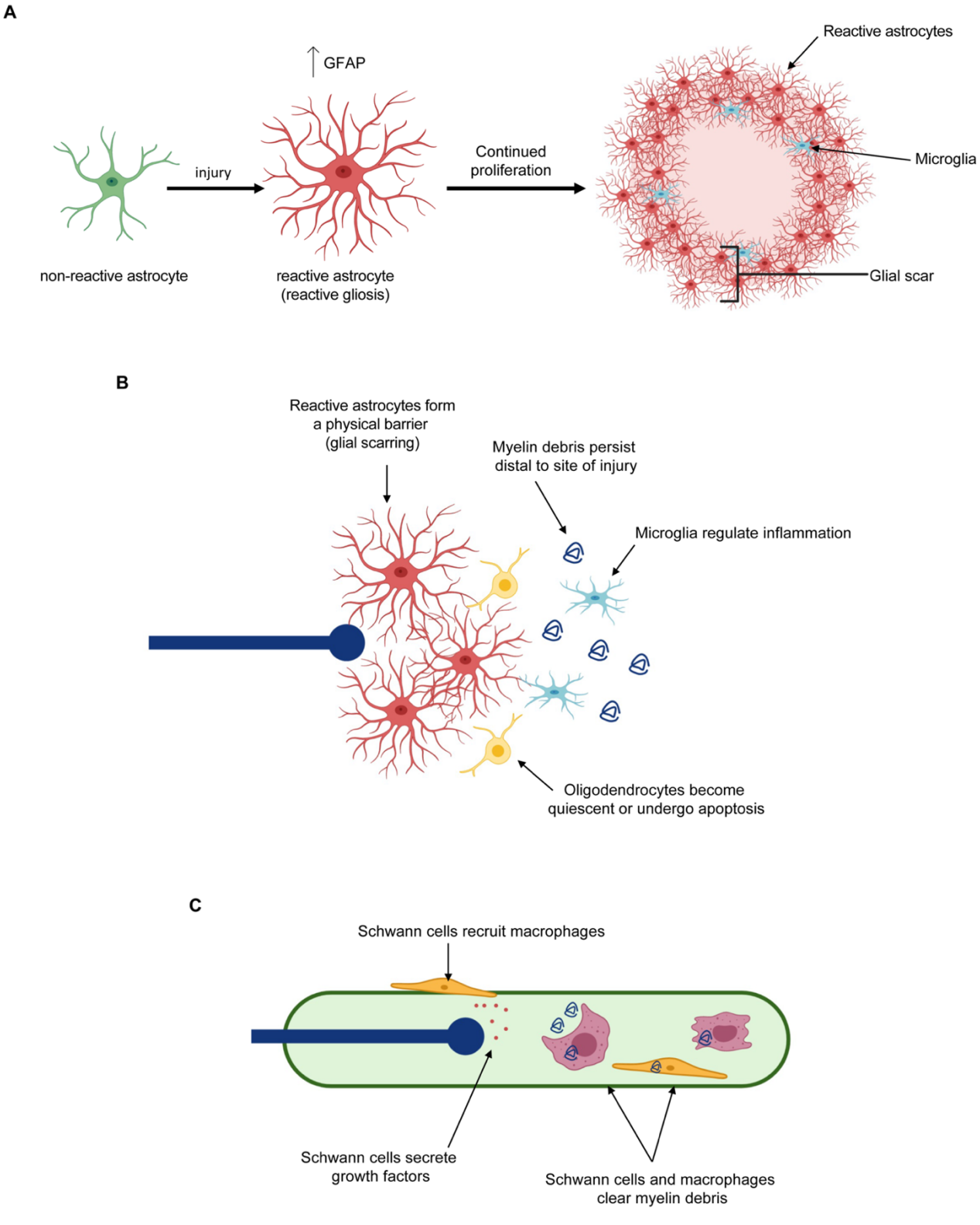

A
GFAP
injury
Continued
proliferation
Reactive astrocytes
Microglia
Glial scar
non-reactive astrocyte
reactive astrocyte
(reactive gliosis)
B
Reactive astrocytes form
a physical barrier
(glial scarring)
Myelin debris persist
distal to site of injury
Microglia regulate inflammation
Oligodendrocytes become
quiescent or undergo apoptosis
C
Schwann cells recruit macrophages
Schwann cells secrete
growth factors
Schwann cells and macrophages
clear myelin debris

Figure 1.2 Mammalian CNS and PNS injuries respond differently. (A) A simple schematic showing how reactive astrocytes form glial scars over time in a severe case of gliosis in the mammalian central nervous system (CNS). Any injury or insult can result in upregulation of glial fibrillary acidic protein (GFAP) which reflects a reactive astrocyte state. Continued proliferation of reactive astrocytes can lead to eventual physical barrier formation, which can sequester an area of damage. (B) In most cases, reactive gliosis can impede axon regrowth due to a physical barrier formed by reactive astrocytes and also the secretion of neurotoxins in the surrounding lesion area. (C) The mammalian peripheral nervous system (PNS) is capable of axon regeneration due to the presence of Schwann cells. These Schwann cells recruit macrophages to clear myelin debris and also secrete growth factors to promote axon regrowth. Figures made with BioRender

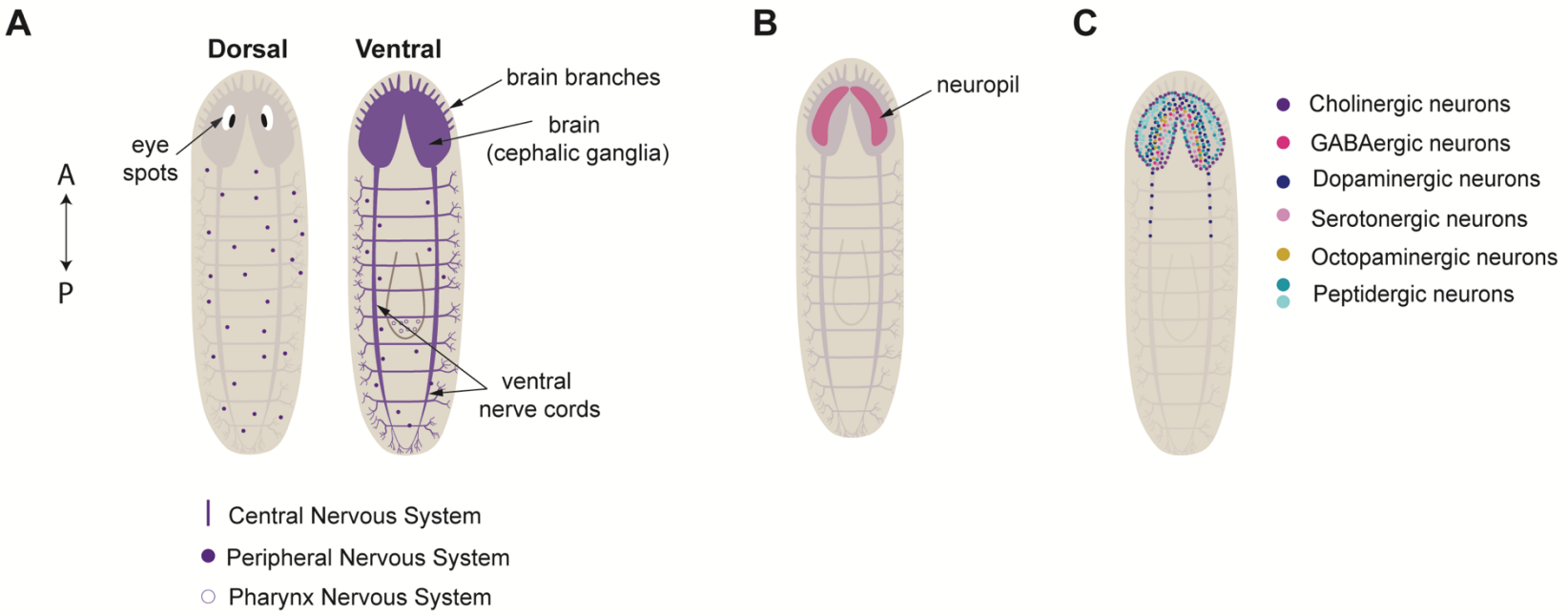


Figure 1.3 Planarians possess a complex nervous system. (A) A simple diagram showing the central, peripheral and pharyngeal nervous system on the dorsal and ventral side of planarians. The planarian CNS is composed of a bilobed brain connected at the anterior commissure, and two ventral nerve cords that run down the body. In addition, several sensory structures known as the brain branches, project out from the brain. On the dorsal side, photoreceptor neurons project axons into the brain on the ventral side. (B) A dense synapse- and dendrite-rich region resides between neuronal cell bodies and is known as the neuropil. (C) The planarian central nervous system is composed of multiple conserved neuronal subtypes with distinct spatial and regional patterning.

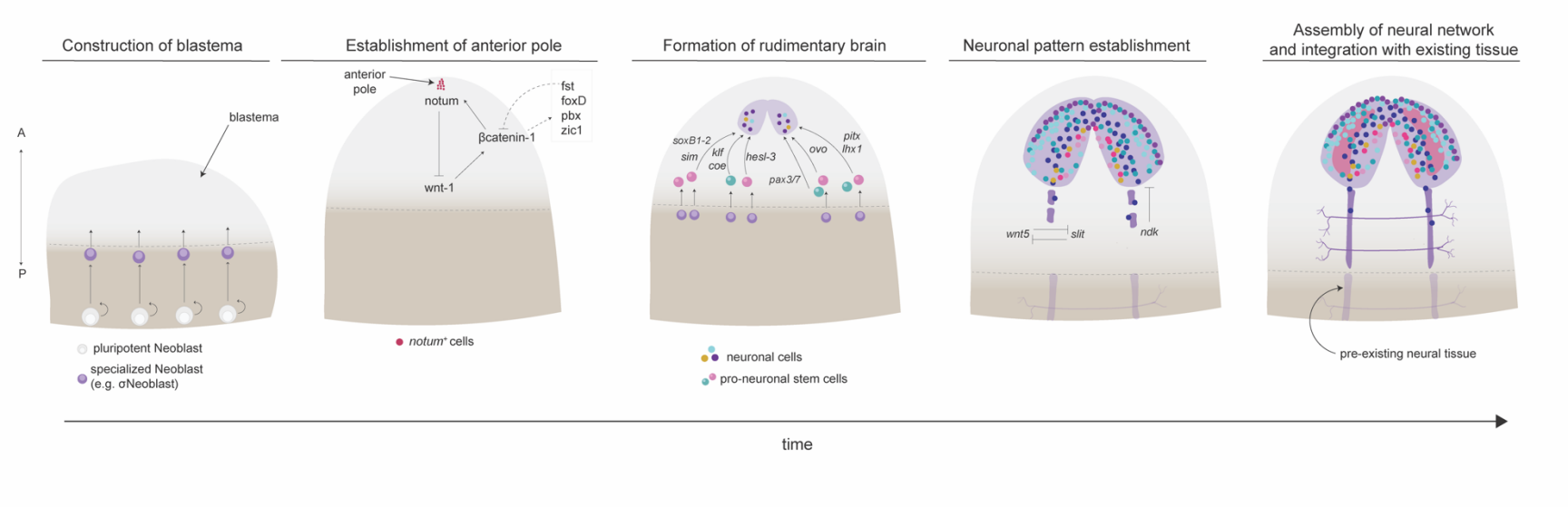


Figure 1.4 Anterior regeneration in planarians. A simple schematic showing how anterior regeneration occurs in planarians after injury. The first step is the formation of a blastema. Pluripotent stem cells called neoblasts proliferate and migrate to the area of injury. Over time, neoblasts become anterior-specific and specialize to be pro-neural. Following specification of neuronal cells and formation of a rudiment brain, neuronal patterning, network assembly and re-integration with existing CNS tissue is completed. Anterior (and brain) regeneration is completed by 7 days.

References

Adams, K. L., & Gallo, V. (2018). The diversity and disparity of the glial scar. *Nature Neuroscience*, *21*(1), 9–15. https://doi.org/10.1038/s41593-017-0033-9

Adell, T., Salò, E., Boutros, M., & Bartscherer, K. (2009). Smed-Evi/Wntless is required for β-catenin-dependent and-independent processes during planarian regeneration. *Development*, *136*(6), 905–910. https://doi.org/10.1242/dev.033761

Agata, K., Soejima, Y., Kato, K., Kobayashi, C., Umesono, Y., & Watanabe, K. (1998). Structure of the Planarian Central Nervous System (CNS) Revealed by Neuronal Cell Markers. *Zoological Science*, *15*(3), 433–440. https://doi.org/10.2108/zsj.15.433

Agata, K., & Umesono, Y. (2008). Brain regeneration from pluripotent stem cells in planarian. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *363*(1500), 2071–2078. https://doi.org/10.1098/rstb.2008.2260

Allen, N. J., & Barres, B. A. (2009). Glia—More than just brain glue. *Nature*, *457*(7230), 675–677. https://doi.org/10.1038/457675a

Allen, N. J., & Lyons, D. A. (2018). Glia as architects of central nervous system formation and function. *Science*, *362*(6411), 181–185. https://doi.org/10.1126/science.aat0473

Alliot, F., Godin, I., & Pessac, B. (1999). Microglia derive from progenitors, originating from the yolk sac, and which proliferate in the brain. *Developmental Brain Research*, *117*(2), 145–152. https://doi.org/10.1016/S0165-3806(99)00113-3

Altenhein, B. (2015). Glial cell progenitors in the *D rosophila* embryo: Gliogenesis in *Drosophila*. *Glia*, *63*(8), 1291–1302. https://doi.org/10.1002/glia.22820

Altenhein, B., Cattenoz, P. B., & Giangrande, A. (2016). The early life of a fly glial cell. *Wiley Interdisciplinary Reviews: Developmental Biology*, *5*(1), 67–84. https://doi.org/10.1002/wdev.200

Anderson, M. A., Burda, J. E., Ren, Y., Ao, Y., O'Shea, T. M., Kawaguchi, R., Coppola, G., Khakh, B. S., Deming, T. J., & Sofroniew, M. V. (2016). Astrocyte scar formation aids central nervous system axon regeneration. *Nature*, *532*(7598), 195–200. https://doi.org/10.1038/nature17623

Andreeva, D., Murashova, L., Burzak, N., & Dyachuk, V. (2022). Satellite Glial Cells: Morphology, functional heterogeneity, and role in pain. *Frontiers in Cellular Neuroscience*, *16*, 1019449. https://doi.org/10.3389/fncel.2022.1019449

Anthony, T. E., Klein, C., Fishell, G., & Heintz, N. (2004). Radial Glia Serve as Neuronal Progenitors in All Regions of the Central Nervous System. *Neuron*, *41*(6), 881–890. https://doi.org/10.1016/S0896-6273(04)00140-0

Arnett, H. A., Mason, J., Marino, M., Suzuki, K., Matsushima, G. K., & Ting, J. P.-Y. (2001). TNFα promotes proliferation of oligodendrocyte progenitors and remyelination. *Nature Neuroscience*, *4*(11), 1116–1122. https://doi.org/10.1038/nn738

Avraham, O., Deng, P.-Y., Jones, S., Kuruvilla, R., Semenkovich, C. F., Klyachko, V. A., & Cavalli, V. (2020). Satellite glial cells promote regenerative growth in sensory neurons. *Nature Communications*, *11*(1), 4891. https://doi.org/10.1038/s41467-020-18642-y

Bacaj, T., Tevlin, M., Lu, Y., & Shaham, S. (2008). Glia Are Essential for Sensory Organ Function in C. elegans. *Science*, *322*(5902), 744–747. https://doi.org/10.1126/science.1163074

Bachiller, S., Jiménez-Ferrer, I., Paulus, A., Yang, Y., Swanberg, M., Deierborg, T., & Boza-Serrano, A. (2018). Microglia in Neurological Diseases: A Road Map to Brain-Disease Dependent-Inflammatory Response. *Frontiers in Cellular Neuroscience*, *12*, 488. https://doi.org/10.3389/fncel.2018.00488

Baerwald, K. D., & Popko, B. (1998). Developing and mature oligodendrocytes respond differently to the immune cytokine interferon-gamma. *Journal of Neuroscience Research*, *52*(2), 230–239. https://doi.org/10.1002/(SICI)1097-4547(19980415)52:2<230::AID-JNR11>3.0.CO;2-B

Baghdadi, M. B., Ayyaz, A., Coquenlorge, S., Chu, B., Kumar, S., Streutker, C., Wrana, J. L., & Kim, T.-H. (2022). Enteric glial cell heterogeneity regulates intestinal stem cell niches. *Cell Stem Cell*, *29*(1), 86-100.e6. https://doi.org/10.1016/j.stem.2021.10.004

Baguñà, J., & Ballester, R. (1978). The nervous system in planarians: Peripheral and gastrodermal plexuses, pharynx innervation, and the relationship between central nervous system structure and the acoelomate organization: NERVOUS SYSTEM IN PLANARIANS. *Journal of Morphology*, *155*(2), 237–252. https://doi.org/10.1002/jmor.1051550208

Baguñà, J., Saló, E., & Auladell, C. (1989). Regeneration and pattern formation in planarians: III. Evidence that neoblasts are totipotent stem cells and the source of blastema cells. *Development*, *107*(1), 77–86. https://doi.org/10.1242/dev.107.1.77

Baumann, N., & Pham-Dinh, D. (2001). Biology of Oligodendrocyte and Myelin in the Mammalian Central Nervous System. *Physiological Reviews*, *81*(2), 871–927. https://doi.org/10.1152/physrev.2001.81.2.871

Bayraktar, O. A., Fuentealba, L. C., Alvarez-Buylla, A., & Rowitch, D. H. (2015). Astrocyte Development and Heterogeneity. *Cold Spring Harbor Perspectives in Biology*, *7*(1), a020362. https://doi.org/10.1101/cshperspect.a020362

Bercury, K. K., & Macklin, W. B. (2015). Dynamics and Mechanisms of CNS Myelination. *Developmental Cell*, *32*(4), 447–458. https://doi.org/10.1016/j.devcel.2015.01.016

Blassberg, R. A., Felix, D. A., Tejada-Romero, B., & Aboobaker, A. A. (2013). *PBX/extradenticle* is required to re-establish axial structures and polarity during planarian regeneration. *Development*, *140*(4), 730–739. https://doi.org/10.1242/dev.082982

Bond, A. M., Berg, D. A., Lee, S., Garcia-Epelboim, A. S., Adusumilli, V. S., Ming, G., & Song, H. (2020). Differential Timing and Coordination of Neurogenesis and Astrogenesis in Developing Mouse Hippocampal Subregions. *Brain Sciences*, *10*(12), 909. https://doi.org/10.3390/brainsci10120909

Boone, J. Q., & Doe, C. Q. (2008). Identification of *Drosophila* type II neuroblast lineages containing transit amplifying ganglion mother cells. *Developmental Neurobiology*, *68*(9), 1185–1195. https://doi.org/10.1002/dneu.20648

Bossing, T., & Technau, G. M. (1994). The fate of the CNS midline progenitors in Drosophila as revealed by a new method for single cell labelling. *Development (Cambridge, England)*, *120*(7), 1895–1906. https://doi.org/10.1242/dev.120.7.1895

Bradl, M., & Lassmann, H. (2010). Oligodendrocytes: Biology and pathology. *Acta Neuropathologica*, *119*(1), 37–53. https://doi.org/10.1007/s00401-009-0601-5

Brosius Lutz, A., & Barres, B. A. (2014). Contrasting the Glial Response to Axon Injury in the Central and Peripheral Nervous Systems. *Developmental Cell*, *28*(1), 7–17. https://doi.org/10.1016/j.devcel.2013.12.002

Burda, J. E., & Sofroniew, M. V. (2014). Reactive Gliosis and the Multicellular Response to CNS Damage and Disease. *Neuron*, *81*(2), 229–248. https://doi.org/10.1016/j.neuron.2013.12.034

Busch, S. A., & Silver, J. (2007). The role of extracellular matrix in CNS regeneration. *Current Opinion in Neurobiology*, *17*(1), 120–127. https://doi.org/10.1016/j.conb.2006.09.004

Butler, S. J., & Bronner, M. E. (2015). From classical to current: Analyzing peripheral nervous system and spinal cord lineage and fate. *Developmental Biology*, *398*(2), 135–146. https://doi.org/10.1016/j.ydbio.2014.09.033

Cahoy, J. D., Emery, B., Kaushal, A., Foo, L. C., Zamanian, J. L., Christopherson, K. S., Xing, Y., Lubischer, J. L., Krieg, P. A., Krupenko, S. A., Thompson, W. J., & Barres, B. A. (2008). A Transcriptome Database for Astrocytes, Neurons, and Oligodendrocytes: A New Resource for Understanding Brain Development and Function. *The Journal of Neuroscience*, *28*(1), 264–278. https://doi.org/10.1523/JNEUROSCI.4178-07.2008

Campbell, K., & Götz, M. (2002). Radial glia: Multi-purpose cells for vertebrate brain development. *Trends in Neurosciences*, *25*(5), 235–238. https://doi.org/10.1016/S0166-2236(02)02156-2

Carpenter, K. S., Morita, M., & Best, J. B. (1974). Ultrastructure of the photoreceptor of the planarian Dugesia dorotocephala: I. Normal eye. *Cell and Tissue Research*, *148*(2). https://doi.org/10.1007/BF00224579

Cebrià, F., Adell, T., & Saló, E. (2018). Rebuilding a planarian: From early signaling to final shape. *The International Journal of Developmental Biology*, *62*(6-7–8), 537–550. https://doi.org/10.1387/ijdb.180042es

Cebrià, F., Guo, T., Jopek, J., & Newmark, P. A. (2007). Regeneration and maintenance of the planarian midline is regulated by a slit orthologue. *Developmental Biology*, *307*(2), 394–406. https://doi.org/10.1016/j.ydbio.2007.05.006

Cebrià, F., Kobayashi, C., Umesono, Y., Nakazawa, M., Mineta, K., Ikeo, K., Gojobori, T., Itoh, M., Taira, M., Alvarado, A. S., & Agata, K. (2002). FGFR-related gene nou-darake restricts brain tissues to the head region of planarians. *Nature*, *419*(6907), 620–624. https://doi.org/10.1038/nature01042

Cebrià, F., Nakazawa, M., Mineta, K., Ikeo, K., Gojobori, T., & Agata, K. (2002). Dissecting planarian central nervous system regeneration by the expression of neural-specific genes. *Development, Growth and Differentiation*, *44*(2), 135–146. https://doi.org/10.1046/j.1440-169x.2002.00629.x

Chen, C.-C. G., Wang, I. E., & Reddien, P. W. (2013). Pbx is required for pole and eye regeneration in planarians. *Development*, *140*(4), 719–729. https://doi.org/10.1242/dev.083741

Collins, J. J., Hou, X., Romanova, E. V., Lambrus, B. G., Miller, C. M., Saberi, A., Sweedler, J. V., & Newmark, P. A. (2010). Genome-Wide Analyses Reveal a Role for Peptide Hormones in Planarian Germline Development. *PLoS Biology*, *8*(10), e1000509. https://doi.org/10.1371/journal.pbio.1000509

Crews, S. T. (2019). *Drosophila* Embryonic CNS Development: Neurogenesis, Gliogenesis, Cell Fate, and Differentiation. *Genetics*, *213*(4), 1111–1144. https://doi.org/10.1534/genetics.119.300974

Currie, K. W., Molinaro, A. M., & Pearson, B. J. (2016). Neuronal sources of hedgehog modulate neurogenesis in the adult planarian brain. *eLife*, *5*, e19735. https://doi.org/10.7554/eLife.19735

DeSalvo, M. K., Hindle, S. J., Rusan, Z. M., Orng, S., Eddison, M., Halliwill, K., & Bainton, R. J. (2014). The Drosophila surface glia transcriptome: Evolutionary conserved blood-brain barrier processes. *Frontiers in Neuroscience*, *8*. https://doi.org/10.3389/fnins.2014.00346

Desalvo, M. K., Mayer, N., Mayer, F., & Bainton, R. J. (2011). Physiologic and anatomic characterization of the brain surface glia barrier of *Drosophila*. *Glia*, *59*(9), 1322–1340. https://doi.org/10.1002/glia.21147

Doe, C. Q. (1992). Molecular markers for identified neuroblasts and ganglion mother cells in the *Drosophila* central nervous system. *Development*, *116*(4), 855–863. https://doi.org/10.1242/dev.116.4.855

Doherty, J., Logan, M. A., Tasdemir, O. E., & Freeman, M. R. (2009). Ensheathing Glia Function as Phagocytes in the Adult Drosophila Brain. *Journal of Neuroscience*, *29*(15), 4768–4781. https://doi.org/10.1523/JNEUROSCI.5951-08.2009

Eisenhoffer, G. T., Kang, H., & Alvarado, A. S. (2008). Molecular Analysis of Stem Cells and Their Descendants during Cell Turnover and Regeneration in the Planarian Schmidtea mediterranea. *Cell Stem Cell*, *3*(3), 327–339. https://doi.org/10.1016/j.stem.2008.07.002

Elvin, M., & Koopowitz, H. (1994). Neuroanatomy of the rhabdocoel flatwormMesostoma ehrenbergii (focke, 1836). I. Neuronal diversity in the brain. *The Journal of Comparative Neurology*, *343*(2), 319–331. https://doi.org/10.1002/cne.903430210

Escartin, C., Galea, E., Lakatos, A., O'Callaghan, J. P., Petzold, G. C., Serrano-Pozo, A., Steinhäuser, C., Volterra, A., Carmignoto, G., Agarwal, A., Allen, N. J., Araque, A.,

Barbeito, L., Barzilai, A., Bergles, D. E., Bonvento, G., Butt, A. M., Chen, W.-T., Cohen-Salmon, M., … Verkhratsky, A. (2021). Reactive astrocyte nomenclature, definitions, and future directions. *Nature Neuroscience*, *24*(3), 312–325. https://doi.org/10.1038/s41593-020-00783-4

Farmer, W. T., & Murai, K. (2017). Resolving Astrocyte Heterogeneity in the CNS. *Frontiers in Cellular Neuroscience*, *11*, 300. https://doi.org/10.3389/fncel.2017.00300

Faulkner, J. R., Herrmann, J. E., Woo, M. J., Tansey, K. E., Doan, N. B., & Sofroniew, M. V. (2004). Reactive Astrocytes Protect Tissue and Preserve Function after Spinal Cord Injury. *The Journal of Neuroscience*, *24*(9), 2143–2155. https://doi.org/10.1523/JNEUROSCI.3547-03.2004

Felix, D. A., & Aboobaker, A. A. (2010). The TALE Class Homeobox Gene Smed-prep Defines the Anterior Compartment for Head Regeneration. *PLoS Genetics*, *6*(4), e1000915. https://doi.org/10.1371/journal.pgen.1000915

Fincher, C. T., Wurtzel, O., de Hoog, T., Kravarik, K. M., & Reddien, P. W. (2018). Cell type transcriptome atlas for the planarian *Schmidtea mediterranea*. *Science*, *360*(6391), eaaq1736. https://doi.org/10.1126/science.aaq1736

Fissel, J. A., & Farah, M. H. (2021). The influence of BACE1 on macrophage recruitment and activity in the injured peripheral nerve. *Journal of Neuroinflammation*, *18*(1), 71. https://doi.org/10.1186/s12974-021-02121-2

Fraguas, S., Barberán, S., Ibarra, B., Stöger, L., & Cebri, F. (2012). Regeneration of neuronal cell types in Schmidtea mediterranea: An immunohistochemical and expression study. *The International Journal of Developmental Biology*, *56*(1-2–3), 143–153. https://doi.org/10.1387/ijdb.113428sf

Fraguas, S., Barberán, S., Iglesias, M., Rodríguez-Esteban, G., & Cebrià, F. (2014). Egr-4, a target of EGFR signaling, is required for the formation of the brain primordia and head regeneration in planarians. *Development*, *141*(9), 1835–1847. https://doi.org/10.1242/dev.101345

Franklin, R. J. M., & Barnett, S. C. (2000). Olfactory Ensheathing Cells and CNS Regeneration. *Neuron*, *28*(1), 15–18. https://doi.org/10.1016/S0896-6273(00)00080-5

Freeman, M. R. (2015). *Drosophila* Central Nervous System Glia. *Cold Spring Harbor Perspectives in Biology*, *7*(11), a020552. https://doi.org/10.1101/cshperspect.a020552

Freeman, M. R., Delrow, J., Kim, J., Johnson, E., & Doe, C. Q. (2003). Unwrapping Glial Biology. *Neuron*, *38*(4), 567–580. https://doi.org/10.1016/S0896-6273(03)00289-7

Freeman, M. R., & Doherty, J. (2006). Glial cell biology in Drosophila and vertebrates. *Trends in Neurosciences*, *29*(2), 82–90. https://doi.org/10.1016/j.tins.2005.12.002

Freeman, M. R., & Rowitch, D. H. (2013). Evolving Concepts of Gliogenesis: A Look Way Back and Ahead to the Next 25 Years. *Neuron*, *80*(3), 613–623. https://doi.org/10.1016/j.neuron.2013.10.034

Fu, R., Shen, Q., Xu, P., Luo, J. J., & Tang, Y. (2014). Phagocytosis of Microglia in the Central Nervous System Diseases. *Molecular Neurobiology*, *49*(3), 1422–1434. https://doi.org/10.1007/s12035-013-8620-6

Fünfschilling, U., Supplie, L. M., Mahad, D., Boretius, S., Saab, A. S., Edgar, J., Brinkmann, B. G., Kassmann, C. M., Tzvetanova, I. D., Möbius, W., Diaz, F., Meijer, D., Suter, U., Hamprecht, B., Sereda, M. W., Moraes, C. T., Frahm, J., Goebbels, S., & Nave, K.-A. (2012). Glycolytic oligodendrocytes maintain myelin and long-term axonal integrity. *Nature*, *485*(7399), 517–521. https://doi.org/10.1038/nature11007

Gaviño, M. A., & Reddien, P. W. (2011). A Bmp/Admp Regulatory Circuit Controls Maintenance and Regeneration of Dorsal-Ventral Polarity in Planarians. *Current Biology*, *21*(4), 294–299. https://doi.org/10.1016/j.cub.2011.01.017

Ginhoux, F., Greter, M., Leboeuf, M., Nandi, S., See, P., Gokhan, S., Mehler, M. F., Conway, S. J., Ng, L. G., Stanley, E. R., Samokhvalov, I. M., & Merad, M. (2010). Fate Mapping Analysis Reveals That Adult Microglia Derive from Primitive Macrophages. *Science*, *330*(6005), 841–845. https://doi.org/10.1126/science.1194637

Ginhoux, F., Lim, S., Hoeffel, G., Low, D., & Huber, T. (2013). Origin and differentiation of microglia. *Frontiers in Cellular Neuroscience*, *7*. https://doi.org/10.3389/fncel.2013.00045

Goldshmit, Y., Sztal, T. E., Jusuf, P. R., Hall, T. E., Nguyen-Chi, M., & Currie, P. D. (2012). Fgf-Dependent Glial Cell Bridges Facilitate Spinal Cord Regeneration in Zebrafish. *Journal of Neuroscience*, *32*(22), 7477–7492. https://doi.org/10.1523/JNEUROSCI.0758-12.2012

Golubev, A. (1988). Glia and neuroglia relationships in the central nervous system of the Turbellaria (Electron microscopic data). *Fortschritte Der Zoologie*, *36*, 185–190.

Gurley, K. A., Elliott, S. A., Simakov, O., Schmidt, H. A., Holstein, T. W., & Alvarado, A. S. (2010). Expression of secreted Wnt pathway components reveals unexpected complexity of the planarian amputation response. *Developmental Biology*, *347*(1), 24–39. https://doi.org/10.1016/j.ydbio.2010.08.007

Gurley, K. A., Rink, J. C., & Alvarado, A. S. (2008). Beta-Catenin Defines Head Versus Tail Identity During Planarian Regeneration and Homeostasis. *Science*, *319*(5861), 323–327. https://doi.org/10.1126/science.1150029

Hartenstein, V. (2011). Morphological diversity and development of glia in Drosophila. *Glia*, *59*(9), 1237–1252. https://doi.org/10.1002/glia.21162

Hartline, D. K. (2011). The evolutionary origins of glia. *Glia*, *59*(9), 1215–1236. https://doi.org/10.1002/glia.21149

Hayakawa, K., Esposito, E., Wang, X., Terasaki, Y., Liu, Y., Xing, C., Ji, X., & Lo, E. H. (2016). Transfer of mitochondria from astrocytes to neurons after stroke. *Nature*, *535*(7613), 551–555. https://doi.org/10.1038/nature18928

Herculano-Houzel, S. (2014). The glia/neuron ratio: How it varies uniformly across brain structures and species and what that means for brain physiology and evolution: The Glia/Neuron Ratio. *Glia*, *62*(9), 1377–1391. https://doi.org/10.1002/glia.22683

Herculano-Houzel, S., & Dos Santos, S. (2018). You Do Not Mess with the Glia. *Neuroglia*, *1*(1), 193–219. https://doi.org/10.3390/neuroglia1010014

Hidalgo, A., & Logan, A. (2017). Go and stop signals for glial regeneration. *Current Opinion in Neurobiology*, *47*, 182–187. https://doi.org/10.1016/j.conb.2017.10.011

Hyman, L. (1951). *The invertebrates: Platyhelminthes and Rhynchocoela*. McGraw-Hill.

Iglesias, M., Gomez-Skarmeta, J. L., Saló, E., & Adell, T. (2008). Silencing of *Smed* -β *catenin1* generates radial-like hypercephalized planarians. *Development*, *135*(7), 1215–1221. https://doi.org/10.1242/dev.020289

Ito, K., Urban, J., & Technau, G. M. (1995). Distribution, classification, and development ofDrosophila glial cells in the late embryonic and early larval ventral nerve cord. *Roux's Archives of Developmental Biology*, *204*(5), 284–307. https://doi.org/10.1007/BF02179499

Jacob, C. (2015). Transcriptional control of neural crest specification into peripheral glia: Control of Peripheral Glia Specification. *Glia*, *63*(11), 1883–1896. https://doi.org/10.1002/glia.22816

Jenkins, J. E., & Roberts-Galbraith, R. H. (2023). Heterotrimeric G proteins regulate planarian regeneration and behavior. *Genetics*, *223*(4), iyad019. https://doi.org/10.1093/genetics/iyad019

Jessen, K. R., & Mirsky, R. (2005). The origin and development of glial cells in peripheral nerves. *Nature Reviews Neuroscience*, *6*(9), 671–682. https://doi.org/10.1038/nrn1746

Jones, B. W. (2001). Glial cell development in the *Drosophila* embryo. *BioEssays*, *23*(10), 877–887. https://doi.org/10.1002/bies.1129

Jones, B. W., Fetter, R. D., Tear, G., & Goodman, C. S. (1995). glial cells missing: A genetic switch that controls glial versus neuronal fate. *Cell*, *82*(6), 1013–1023. https://doi.org/10.1016/0092-8674(95)90280-5

Jurga, A. M., Paleczna, M., Kadluczka, J., & Kuter, K. Z. (2021). Beyond the GFAP-Astrocyte Protein Markers in the Brain. *Biomolecules*, *11*(9), 1361. https://doi.org/10.3390/biom11091361

Jurisch-Yaksi, N., Yaksi, E., & Kizil, C. (2020). Radial glia in the zebrafish brain: Functional, structural, and physiological comparison with the mammalian glia. *Glia*, glia.23849. https://doi.org/10.1002/glia.23849

Kastriti, M. E., & Adameyko, I. (2017). Specification, plasticity and evolutionary origin of peripheral glial cells. *Current Opinion in Neurobiology*, *47*, 196–202. https://doi.org/10.1016/j.conb.2017.11.004

Kato, K., Forero, M. G., Fenton, J. C., & Hidalgo, A. (2011). The Glial Regenerative Response to Central Nervous System Injury Is Enabled by Pros-Notch and Pros-NFκB Feedback. *PLoS Biology*, *9*(8), e1001133. https://doi.org/10.1371/journal.pbio.1001133

Kato, K., Orii, H., Watanabe, K., & Agata, K. (2001). Dorsal and Ventral Positional Cues Required for the Onset of Planarian Regeneration May Reside in Differentiated Cells. *Developmental Biology*, *233*(1), 109–121. https://doi.org/10.1006/dbio.2001.0226

Keenan, C. L., Coss, R., & Koopowitz, H. (1981). Cytoarchitecture of primitive brains: Golgi studies in flatworms. *The Journal of Comparative Neurology*, *195*(4), 697–716. https://doi.org/10.1002/cne.901950412

Kessaris, N., Pringle, N., & Richardson, W. D. (2008). Specification of CNS glia from neural stem cells in the embryonic neuroepithelium. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *363*(1489), 71–85. https://doi.org/10.1098/rstb.2006.2013

Khakh, B. S., & Deneen, B. (2019). The Emerging Nature of Astrocyte Diversity. *Annual Review of Neuroscience*, *42*(1), 187–207. https://doi.org/10.1146/annurev-neuro-070918-050443

Khakh, B. S., & Sofroniew, M. V. (2015). Diversity of astrocyte functions and phenotypes in neural circuits. *Nature Neuroscience*, *18*(7), 942–952. https://doi.org/10.1038/nn.4043

Kidd, G. J., Ohno, N., & Trapp, B. D. (2013). Biology of Schwann cells. In *Handbook of Clinical Neurology* (Vol. 115, pp. 55–79). Elsevier. https://doi.org/10.1016/B978-0-444-52902-2.00005-9

Kirby, B. B., Takada, N., Latimer, A. J., Shin, J., Carney, T. J., Kelsh, R. N., & Appel, B. (2006). In vivo time-lapse imaging shows dynamic oligodendrocyte progenitor behavior during zebrafish development. *Nature Neuroscience*, *9*(12), 1506–1511. https://doi.org/10.1038/nn1803

Konishi, H., Koizumi, S., & Kiyama, H. (2022). Phagocytic astrocytes: Emerging from the shadows of microglia. *Glia*, *70*(6), 1009–1026. https://doi.org/10.1002/glia.24145

Kriegstein, A., & Alvarez-Buylla, A. (2009). The Glial Nature of Embryonic and Adult Neural Stem Cells. *Annual Review of Neuroscience*, *32*(1), 149–184. https://doi.org/10.1146/annurev.neuro.051508.135600

Krugelis MacRae, E. (1964). Observations on the fine structure of photoreceptor cells in the planarian Dugesia tigrina. *Journal of Ultrastructure Research*, *10*(3–4), 334–349. https://doi.org/10.1016/S0022-5320(64)80013-7

Kuhn, S., Gritti, L., Crooks, D., & Dombrowski, Y. (2019). Oligodendrocytes in Development, Myelin Generation and Beyond. *Cells*, *8*(11), 1424. https://doi.org/10.3390/cells8111424

Lago-Baldaia, I., Fernandes, V. M., & Ackerman, S. D. (2020). More Than Mortar: Glia as Architects of Nervous System Development and Disease. *Frontiers in Cell and Developmental Biology*, *8*, 611269. https://doi.org/10.3389/fcell.2020.611269

Lannes, N., Eppler, E., Etemad, S., Yotovski, P., & Filgueira, L. (2017). Microglia at center stage: A comprehensive review about the versatile and unique residential macrophages of the central nervous system. *Oncotarget*, *8*(69), 114393–114413. https://doi.org/10.18632/oncotarget.23106

Lawson, L. J., Perry, V. H., & Gordon, S. (1992). Turnover of resident microglia in the normal adult mouse brain. *Neuroscience*, *48*(2), 405–415. https://doi.org/10.1016/0306-4522(92)90500-2

Le Marrec-Croq, F., Drago, F., Vizioli, J., Sautière, P.-E., & Lefebvre, C. (2013). The Leech Nervous System: A Valuable Model to Study the Microglia Involvement in Regenerative

Processes. *Clinical and Developmental Immunology*, *2013*, 1–12. https://doi.org/10.1155/2013/274019

Lei, K., Thi-Kim Vu, H., Mohan, R. D., McKinney, S. A., Seidel, C. W., Alexander, R., Gotting, K., Workman, J. L., & Sánchez Alvarado, A. (2016). Egf Signaling Directs Neoblast Repopulation by Regulating Asymmetric Cell Division in Planarians. *Developmental Cell*, *38*(4), 413–429. https://doi.org/10.1016/j.devcel.2016.07.012

Lentz, T. L. (1967). Fine structure of nerve cells in a planarian. *Journal of Morphology*, *121*(4), 323–337. https://doi.org/10.1002/jmor.1051210406

Li, X., Liu, G., Yang, L., Li, Z., Zhang, Z., Xu, Z., Cai, Y., Du, H., Su, Z., Wang, Z., Duan, Y., Chen, H., Shang, Z., You, Y., Zhang, Q., He, M., Chen, B., & Yang, Z. (2021). Decoding Cortical Glial Cell Development. *Neuroscience Bulletin*, *37*(4), 440–460. https://doi.org/10.1007/s12264-021-00640-9

Liddelow, S. A., Guttenplan, K. A., Clarke, L. E., Bennett, F. C., Bohlen, C. J., Schirmer, L., Bennett, M. L., Münch, A. E., Chung, W.-S., Peterson, T. C., Wilton, D. K., Frouin, A., Napier, B. A., Panicker, N., Kumar, M., Buckwalter, M. S., Rowitch, D. H., Dawson, V. L., Dawson, T. M., … Barres, B. A. (2017). Neurotoxic reactive astrocytes are induced by activated microglia. *Nature*, *541*(7638), 481–487. https://doi.org/10.1038/nature21029

Logan, M. A., & Freeman, M. R. (2007). The scoop on the fly brain: Glial engulfment functions in Drosophila. *Neuron Glia Biology*, *3*(1), 1–3. https://doi.org/10.1017/S1740925X07000646

Losada-Perez, M., Harrison, N., & Hidalgo, A. (2016). Molecular mechanism of central nervous system repair by the Drosophila NG2 homologue kon-tiki. *Journal of Cell Biology*, *214*(5), 587–601. https://doi.org/10.1083/jcb.201603054

Lyons, D. A., & Talbot, W. S. (2015). Glial Cell Development and Function in Zebrafish. *Cold Spring Harbor Perspectives in Biology*, *7*(2), a020586. https://doi.org/10.1101/cshperspect.a020586

MacRae, E. K. (1967). The fine structure of sensory receptor processes in the auricular epithelium of the planarian, Dugesia tigrina. *Zeitschrift F□r Zellforschung Und Mikroskopische Anatomie*, *82*(4), 479–494. https://doi.org/10.1007/BF00337119

Martini, R., Fischer, S., López-Vales, R., & David, S. (2008). Interactions between Schwann cells and macrophages in injury and inherited demyelinating disease. *Glia*, *56*(14), 1566–1577. https://doi.org/10.1002/glia.20766

Mehta, H., Orphe, C., Todd, M. S., Cornbrooks, C. J., & Carey, D. J. (1985). Synthesis by Schwann cells of basal lamina and membrane-associated heparan sulfate proteoglycans. *The Journal of Cell Biology*, *101*(2), 660–666. https://doi.org/10.1083/jcb.101.2.660

Miller, F. D., & Gauthier, A. S. (2007). Timing Is Everything: Making Neurons versus Glia in the Developing Cortex. *Neuron*, *54*(3), 357–369. https://doi.org/10.1016/j.neuron.2007.04.019

Miller, R., & Raff, M. (1984). Fibrous and protoplasmic astrocytes are biochemically and developmentally distinct. *The Journal of Neuroscience*, *4*(2), 585–592. https://doi.org/10.1523/JNEUROSCI.04-02-00585.1984

Minge, D., Domingos, C., Unichenko, P., Behringer, C., Pauletti, A., Anders, S., Herde, M. K., Delekate, A., Gulakova, P., Schoch, S., Petzold, G. C., & Henneberger, C. (2021). Heterogeneity and Development of Fine Astrocyte Morphology Captured by Diffraction-Limited Microscopy. *Frontiers in Cellular Neuroscience*, *15*, 669280. https://doi.org/10.3389/fncel.2021.669280

Mizeracka, K., & Heiman, M. G. (2015). The many glia of a tiny nematode: Studying glial diversity using *Caenorhabditis elegans*: Glial diversity in *C. elegans*. *Wiley Interdisciplinary Reviews: Developmental Biology*, *4*(2), 151–160. https://doi.org/10.1002/wdev.171

Mokalled, M. H., Patra, C., Dickson, A. L., Endo, T., Stainier, D. Y. R., & Poss, K. D. (2016). Injury-induced ctgfa directs glial bridging and spinal cord regeneration in zebrafish. *Science*, *354*(6312), 630–634. https://doi.org/10.1126/science.aaf2679

Molina, M. D., & Cebrià, F. (2021). Decoding Stem Cells: An Overview on Planarian Stem Cell Heterogeneity and Lineage Progression. *Biomolecules*, *11*(10), 1532. https://doi.org/10.3390/biom11101532

Molina, M. D., Saló, E., & Cebrià, F. (2007). The BMP pathway is essential for re-specification and maintenance of the dorsoventral axis in regenerating and intact planarians. *Developmental Biology*, *311*(1), 79–94. https://doi.org/10.1016/j.ydbio.2007.08.019

Molinaro, A. M., & Pearson, B. J. (2016). In silico lineage tracing through single cell transcriptomics identifies a neural stem cell population in planarians. *Genome Biology*, *17*(1), 87. https://doi.org/10.1186/s13059-016-0937-9

Mollaaghababa, R., & Pavan, W. J. (2003). The importance of having your SOX on: Role of SOX10† in the development of neural crest-derived melanocytes and glia. *Oncogene*, *22*(20), 3024–3034. https://doi.org/10.1038/sj.onc.1206442

Molofsky, A. V., & Deneen, B. (2015). Astrocyte development: A Guide for the Perplexed: Mechanisms of Astrocyte development. *Glia*, *63*(8), 1320–1329. https://doi.org/10.1002/glia.22836

Morgenstern, D. A., Asher, R. A., & Fawcett, J. W. (2002). Chapter 22 Chondroitin sulphate proteoglycans in the CNS injury response. In *Progress in Brain Research* (Vol. 137, pp. 313–332). Elsevier. https://doi.org/10.1016/S0079-6123(02)37024-9

Morita, M., & Best, J. B. (1966). Electron microscopic studies of planaria. III. Some observations on the fine structure of planarian nervous tissue. *Journal of Experimental Zoology*, *161*(3), 391–411. https://doi.org/10.1002/jez.1401610307

Morizawa, Y. M., Hirayama, Y., Ohno, N., Shibata, S., Shigetomi, E., Sui, Y., Nabekura, J., Sato, K., Okajima, F., Takebayashi, H., Okano, H., & Koizumi, S. (2017). Reactive astrocytes function as phagocytes after brain ischemia via ABCA1-mediated pathway. *Nature Communications*, *8*(1), 28. https://doi.org/10.1038/s41467-017-00037-1

Myer, D. J. (2006). Essential protective roles of reactive astrocytes in traumatic brain injury. *Brain*, *129*(10), 2761–2772. https://doi.org/10.1093/brain/awl165

Nave, K.-A. (2010). Myelination and support of axonal integrity by glia. *Nature*, *468*(7321), 244–252. https://doi.org/10.1038/nature09614

Neely, S. A., & Lyons, D. A. (2021). Insights Into Central Nervous System Glial Cell Formation and Function From Zebrafish. *Frontiers in Cell and Developmental Biology*, *9*, 754606. https://doi.org/10.3389/fcell.2021.754606

Newmark, P. A., & Alvarado, A. S. (2002). Not your father's planarian: A classic model enters the era of functional genomics. *Nature Reviews Genetics*, *3*(3), 210–219. https://doi.org/10.1038/nrg759

Newmark, P. A., & Sánchez Alvarado, A. (2000). Bromodeoxyuridine Specifically Labels the Regenerative Stem Cells of Planarians. *Developmental Biology*, *220*(2), 142–153. https://doi.org/10.1006/dbio.2000.9645

Nishimura, K., Kitamura, Y., Inoue, T., Umesono, Y., Sano, S., Yoshimoto, K., Inden, M., Takata, K., Taniguchi, T., Shimohama, S., & Agata, K. (2007). Reconstruction of dopaminergic neural network and locomotion function in planarian regenerates. *Developmental Neurobiology*, *67*(8), 1059–1078. https://doi.org/10.1002/dneu.20377

Nishimura, K., Kitamura, Y., Inoue, T., Umesono, Y., Yoshimoto, K., Takeuchi, K., Taniguchi, T., & Agata, K. (2007). Identification and distribution of tryptophan hydroxylase (TPH)-positive neurons in the planarian Dugesia japonica. *Neuroscience Research*, *59*(1), 101–106. https://doi.org/10.1016/j.neures.2007.05.014

Nishimura, K., Kitamura, Y., Inoue, T., Umesono, Y., Yoshimoto, K., Taniguchi, T., & Agata, K. (2008). Characterization of tyramine β-hydroxylase in planarian Dugesia japonica: Cloning and expression. *Neurochemistry International*, *53*(6–8), 184–192. https://doi.org/10.1016/j.neuint.2008.09.006

Nishimura, K., Kitamura, Y., Taniguchi, T., & Agata, K. (2010). Analysis of motor function modulated by cholinergic neurons in planarian dugesia japonica. *Neuroscience*, *168*(1), 18–30. https://doi.org/10.1016/j.neuroscience.2010.03.038

Nishimura, K., Kitamura, Y., Umesono, Y., Takeuchi, K., Takata, K., Taniguchi, T., & Agata, K. (2008). Identification of glutamic acid decarboxylase gene and distribution of GABAergic nervous system in the planarian Dugesia japonica. *Neuroscience*, *153*(4), 1103–1114. https://doi.org/10.1016/j.neuroscience.2008.03.026

Ogawa, K., Ishihara, S., Saito, Y., Mineta, K., Nakazawa, M., Ikeo, K., Gojobori, T., Watanabe, K., & Agata, K. (2002). Induction of a noggin-Like Gene by Ectopic DV Interaction during Planarian Regeneration. *Developmental Biology*, *250*(1), 59–70. https://doi.org/10.1006/dbio.2002.0790

Oikonomou, G., & Shaham, S. (2011). The Glia of Caenorhabditis elegans. *Glia*, *59*(9), 1253–1263. https://doi.org/10.1002/glia.21084

Omoto, J. J., Lovick, J. K., & Hartenstein, V. (2016). Origins of glial cell populations in the insect nervous system. *Current Opinion in Insect Science*, *18*, 96–104. https://doi.org/10.1016/j.cois.2016.09.003

Orii, H., Sakurai, T., & Watanabe, K. (2005). Distribution of the stem cells (neoblasts) in the planarian Dugesia japonica. *Development Genes and Evolution*, *215*(3), 143–157. https://doi.org/10.1007/s00427-004-0460-y

Ortega, A., & Olivares-Bañuelos, T. N. (2020). Neurons and Glia Cells in Marine Invertebrates: An Update. *Frontiers in Neuroscience*, *14*, 121. https://doi.org/10.3389/fnins.2020.00121

Owlarn, S., & Bartscherer, K. (2016). Go ahead, grow a head! A planarian's guide to anterior regeneration: Planarian Anterior Regeneration. *Regeneration*, *3*(3), 139–155. https://doi.org/10.1002/reg2.56

Palakodeti, D., Smielewska, M., Lu, Y.-C., Yeo, G. W., & Graveley, B. R. (2008). The PIWI proteins SMEDWI-2 and SMEDWI-3 are required for stem cell function and piRNA expression in planarians. *RNA*, *14*(6), 1174–1186. https://doi.org/10.1261/rna.1085008

Pekny, M., & Nilsson, M. (2005). Astrocyte activation and reactive gliosis. *Glia*, *50*(4), 427–434. https://doi.org/10.1002/glia.20207

Pekny, M., Wilhelmsson, U., & Pekna, M. (2014). The dual role of astrocyte activation and reactive gliosis. *Neuroscience Letters*, *565*, 30–38. https://doi.org/10.1016/j.neulet.2013.12.071

Petersen, C. P., & Reddien, P. W. (2008). *Smed-* β *catenin-1* Is Required for Anteroposterior Blastema Polarity in Planarian Regeneration. *Science*, *319*(5861), 327–330. https://doi.org/10.1126/science.1149943

Petersen, C. P., & Reddien, P. W. (2011). Polarized *notum* Activation at Wounds Inhibits Wnt Function to Promote Planarian Head Regeneration. *Science*, *332*(6031), 852–855. https://doi.org/10.1126/science.1202143

Petersen, J., & Adameyko, I. (2017). Nerve-associated neural crest: Peripheral glial cells generate multiple fates in the body. *Current Opinion in Genetics & Development*, *45*, 10–14. https://doi.org/10.1016/j.gde.2017.02.006

Plass, M., Solana, J., Wolf, F. A., Ayoub, S., Misios, A., Glažar, P., Obermayer, B., Theis, F. J., Kocks, C., & Rajewsky, N. (2018). Cell type atlas and lineage tree of a whole complex animal by single-cell transcriptomics. *Science*, *360*(6391), eaaq1723. https://doi.org/10.1126/science.aaq1723

Preston, A. N., Cervasio, D. A., & Laughlin, S. T. (2019). Visualizing the brain's astrocytes. In *Methods in Enzymology* (Vol. 622, pp. 129–151). Elsevier. https://doi.org/10.1016/bs.mie.2019.02.006

Pringle, N. P., & Richardson, W. D. (1993). A singularity of PDGF alpha-receptor expression in the dorsoventral axis of the neural tube may define the origin of the oligodendrocyte lineage. *Development*, *117*(2), 525–533. https://doi.org/10.1242/dev.117.2.525

Prinz, M., Jung, S., & Priller, J. (2019). Microglia Biology: One Century of Evolving Concepts. *Cell*, *179*(2), 292–311. https://doi.org/10.1016/j.cell.2019.08.053

Raiders, S., Black, E. C., Bae, A., MacFarlane, S., Klein, M., Shaham, S., & Singhvi, A. (2021). Glia actively sculpt sensory neurons by controlled phagocytosis to tune animal behavior. *eLife*, *10*, e63532. https://doi.org/10.7554/eLife.63532

Ramón-Cueto, A., & Avila, J. (1998). Olfactory ensheathing glia: Properties and function. *Brain Research Bulletin*, *46*(3), 175–187. https://doi.org/10.1016/S0361-9230(97)00463-2

Rapti, G., Li, C., Shan, A., Lu, Y., & Shaham, S. (2017). Glia initiate brain assembly through noncanonical Chimaerin–Furin axon guidance in C. elegans. *Nature Neuroscience*, *20*(10), 1350–1360. https://doi.org/10.1038/nn.4630

Rasband, M. N. (2016). Glial Contributions to Neural Function and Disease. *Molecular & Cellular Proteomics*, *15*(2), 355–361. https://doi.org/10.1074/mcp.R115.053744

Reddien, P. W. (2005). SMEDWI-2 Is a PIWI-Like Protein That Regulates Planarian Stem Cells. *Science*, *310*(5752), 1327–1330. https://doi.org/10.1126/science.1116110

Reddien, P. W. (2018). The Cellular and Molecular Basis for Planarian Regeneration. *Cell*, *175*(2), 327–345. https://doi.org/10.1016/j.cell.2018.09.021

Reddien, P. W., & Alvarado, A. S. (2004). FUNDAMENTALS OF PLANARIAN REGENERATION. *Annual Review of Cell and Developmental Biology*, *20*(1), 725–757. https://doi.org/10.1146/annurev.cellbio.20.010403.095114

Reddien, P. W., Bermange, A. L., Kicza, A. M., & Sánchez Alvarado, A. (2007). BMP signaling regulates the dorsal planarian midline and is needed for asymmetric regeneration. *Development*, *134*(22), 4043–4051. https://doi.org/10.1242/dev.007138

Ren, Q., Awasaki, T., Wang, Y.-C., Huang, Y.-F., & Lee, T. (2018). Lineage-guided Notch-dependent gliogenesis by *Drosophila* multi-potent progenitors. *Development*, dev.160127. https://doi.org/10.1242/dev.160127

Roberts-Galbraith, R. H., Brubacher, J. L., & Newmark, P. A. (2016). A functional genomics screen in planarians reveals regulators of whole-brain regeneration. *eLife*, *5*, e17002. https://doi.org/10.7554/eLife.17002

Roberts-Galbraith, R. H., & Newmark, P. A. (2013). Follistatin antagonizes Activin signaling and acts with Notum to direct planarian head regeneration. *Proceedings of the National Academy of Sciences*, *110*(4), 1363–1368. https://doi.org/10.1073/pnas.1214053110

Roberts-Galbraith, R. H., & Newmark, P. A. (2015). On the organ trail: Insights into organ regeneration in the planarian. *Current Opinion in Genetics & Development*, *32*, 37–46. https://doi.org/10.1016/j.gde.2015.01.009

Rolls, A., Shechter, R., & Schwartz, M. (2009). The bright side of the glial scar in CNS repair. *Nature Reviews Neuroscience*, *10*(3), 235–241. https://doi.org/10.1038/nrn2591

Ross, K. G., Currie, K. W., Pearson, B. J., & Zayas, R. M. (2017). Nervous system development and regeneration in freshwater planarians. *WIREs Developmental Biology*, *6*(3). https://doi.org/10.1002/wdev.266

Rowitch, D. H., & Kriegstein, A. R. (2010). Developmental genetics of vertebrate glial–cell specification. *Nature*, *468*(7321), 214–222. https://doi.org/10.1038/nature09611

Rusznák, Z., Henskens, W., Schofield, E., Kim, W. S., & Fu, Y. (2016). Adult Neurogenesis and Gliogenesis: Possible Mechanisms for Neurorestoration. *Experimental Neurobiology*, *25*(3), 103–112. https://doi.org/10.5607/en.2016.25.3.103

Sasse, S., Neuert, H., & Klämbt, C. (2015). Differentiation of *Drosophila* glial cells. *Wiley Interdisciplinary Reviews: Developmental Biology*, *4*(6), 623–636. https://doi.org/10.1002/wdev.198

Schousboe, A., Svenneby, G., & Hertz, L. (1977). UPTAKE AND METABOLISM OF GLUTAMATE IN ASTROCYTES CULTURED FROM DISSOCIATED MOUSE BRAIN HEMISPHERES. *Journal of Neurochemistry*, *29*(6), 999–1005. https://doi.org/10.1111/j.1471-4159.1977.tb06503.x

Scimone, M. L., Cloutier, J. K., Maybrun, C. L., & Reddien, P. W. (2022). The planarian wound epidermis gene equinox is required for blastema formation in regeneration. *Nature Communications*, *13*(1), 2726. https://doi.org/10.1038/s41467-022-30412-6

Scimone, M. L., Lapan, S. W., & Reddien, P. W. (2014). A forkhead Transcription Factor Is Wound-Induced at the Planarian Midline and Required for Anterior Pole Regeneration. *PLoS Genetics*, *10*(1), e1003999. https://doi.org/10.1371/journal.pgen.1003999

Scimone, M. L., Wurtzel, O., Malecek, K., Fincher, C. T., Oderberg, I. M., Kravarik, K. M., & Reddien, P. W. (2018). foxF-1 Controls Specification of Non-body Wall Muscle and Phagocytic Cells in Planarians. *Current Biology*, *28*(23), 3787-3801.e6. https://doi.org/10.1016/j.cub.2018.10.030

Seguella, L., & Gulbransen, B. D. (2021). Enteric glial biology, intercellular signalling and roles in gastrointestinal disease. *Nature Reviews Gastroenterology & Hepatology*, *18*(8), 571–587. https://doi.org/10.1038/s41575-021-00423-7

Shaham, S. (2006). Glia–neuron interactions in the nervous system of Caenorhabditis elegans. *Current Opinion in Neurobiology*, *16*(5), 522–528. https://doi.org/10.1016/j.conb.2006.08.001

Shaham, S. (2015). Glial Development and Function in the Nervous System of *Caenorhabditis elegans*. *Cold Spring Harbor Perspectives in Biology*, *7*(4), a020578. https://doi.org/10.1101/cshperspect.a020578

Silver, J., & Miller, J. H. (2004). Regeneration beyond the glial scar. *Nature Reviews Neuroscience*, *5*(2), 146–156. https://doi.org/10.1038/nrn1326

Sofroniew, M. V. (2009). Molecular dissection of reactive astrogliosis and glial scar formation. *Trends in Neurosciences*, *32*(12), 638–647. https://doi.org/10.1016/j.tins.2009.08.002

Solovieva, T., & Bronner, M. (2021). Schwann cell precursors: Where they come from and where they go. *Cells & Development*, *166*, 203686. https://doi.org/10.1016/j.cdev.2021.203686

Song, Y., Li, D., Farrelly, O., Miles, L., Li, F., Kim, S. E., Lo, T. Y., Wang, F., Li, T., Thompson-Peer, K. L., Gong, J., Murthy, S. E., Coste, B., Yakubovich, N., Patapoutian, A., Xiang, Y., Rompolas, P., Jan, L. Y., & Jan, Y. N. (2019). The Mechanosensitive Ion Channel Piezo Inhibits Axon Regeneration. *Neuron*, *102*(2), 373-389.e6. https://doi.org/10.1016/j.neuron.2019.01.050

Song, Y., Ori-McKenney, K. M., Zheng, Y., Han, C., Jan, L. Y., & Jan, Y. N. (2012). Regeneration of *Drosophila* sensory neuron axons and dendrites is regulated by the Akt pathway involving *Pten* and microRNA *bantam*. *Genes & Development*, *26*(14), 1612–1625. https://doi.org/10.1101/gad.193243.112

Stout Jr., R. F., Verkhratsky, A., & Parpura, V. (2014). Caenorhabditis elegans glia modulate neuronal activity and behavior. *Frontiers in Cellular Neuroscience*, *8*. https://doi.org/10.3389/fncel.2014.00067

Stratoulias, V., & Heino, T. I. (2015). MANF silencing, immunity induction or autophagy trigger an unusual cell type in metamorphosing Drosophila brain. *Cellular and Molecular Life Sciences*, *72*(10), 1989–2004. https://doi.org/10.1007/s00018-014-1789-7

Stratoulias, V., Venero, J. L., Tremblay, M., & Joseph, B. (2019). Microglial subtypes: Diversity within the microglial community. *The EMBO Journal*, *38*(17), e101997. https://doi.org/10.15252/embj.2019101997

Su, Z., & He, C. (2010). Olfactory ensheathing cells: Biology in neural development and regeneration. *Progress in Neurobiology*, *92*(4), 517–532. https://doi.org/10.1016/j.pneurobio.2010.08.008

Sulston, J. E., Schierenberg, E., White, J. G., & Thomson, J. N. (1983). The embryonic cell lineage of the nematode Caenorhabditis elegans. *Developmental Biology*, *100*(1), 64–119. https://doi.org/10.1016/0012-1606(83)90201-4

Than-Trong, E., & Bally-Cuif, L. (2015). Radial glia and neural progenitors in the adult zebrafish central nervous system: Adult Radial Glia in Zebrafish. *Glia*, *63*(8), 1406–1428. https://doi.org/10.1002/glia.22856

Timsit, S., Martinez, S., Allinquant, B., Peyron, F., Puelles, L., & Zalc, B. (1995). Oligodendrocytes originate in a restricted zone of the embryonic ventral neural tube defined by DM-20 mRNA expression. *The Journal of Neuroscience*, *15*(2), 1012–1024. https://doi.org/10.1523/JNEUROSCI.15-02-01012.1995

Tofaris, G. K., Patterson, P. H., Jessen, K. R., & Mirsky, R. (2002). Denervated Schwann Cells Attract Macrophages by Secretion of Leukemia Inhibitory Factor (LIF) and Monocyte Chemoattractant Protein-1 in a Process Regulated by Interleukin-6 and LIF. *The Journal of Neuroscience*, *22*(15), 6696–6703. https://doi.org/10.1523/JNEUROSCI.22-15-06696.2002

Umesono, Y., Watanabe, K., & Agata, K. (1999). Distinct structural domains in the planarian brain defined by the expression of evolutionarily conserved homeobox genes.

Development Genes and Evolution, *209*(1), 31–39. https://doi.org/10.1007/s004270050224

van Wolfswinkel, J. C., Wagner, D. E., & Reddien, P. W. (2014). Single-Cell Analysis Reveals Functionally Distinct Classes within the Planarian Stem Cell Compartment. *Cell Stem Cell*, *15*(3), 326–339. https://doi.org/10.1016/j.stem.2014.06.007

Vásquez-Doorman, C., & Petersen, C. P. (2014). Zic-1 Expression in Planarian Neoblasts after Injury Controls Anterior Pole Regeneration. *PLoS Genetics*, *10*(7), e1004452. https://doi.org/10.1371/journal.pgen.1004452

Verkhratsky, A., & Butt, A. (2013). *Glial Physiology and Pathophysiology* (1st ed.). Wiley. https://doi.org/10.1002/9781118402061

Von Bartheld, C. S., Bahney, J., & Herculano-Houzel, S. (2016). The search for true numbers of neurons and glial cells in the human brain: A review of 150 years of cell counting: Quantifying neurons and glia in human brain. *Journal of Comparative Neurology*, *524*(18), 3865–3895. https://doi.org/10.1002/cne.24040

Walkowicz, L., Kijak, E., Krzeptowski, W., Górska-Andrzejak, J., Stratoulias, V., Woznicka, O., Chwastek, E., Heino, T. I., & Pyza, E. M. (2017). Downregulation of DmMANF in Glial Cells Results in Neurodegeneration and Affects Sleep and Lifespan in Drosophila melanogaster. *Frontiers in Neuroscience*, *11*, 610. https://doi.org/10.3389/fnins.2017.00610

Wang, I. E., Lapan, S. W., Scimone, M. L., Clandinin, T. R., & Reddien, P. W. (2016). Hedgehog signaling regulates gene expression in planarian glia. *eLife*, *5*, e16996. https://doi.org/10.7554/eLife.16996

Wenemoser, D., Lapan, S. W., Wilkinson, A. W., Bell, G. W., & Reddien, P. W. (2012). A molecular wound response program associated with regeneration initiation in planarians. *Genes & Development*, *26*(9), 988–1002. https://doi.org/10.1101/gad.187377.112

Wenemoser, D., & Reddien, P. W. (2010). Planarian regeneration involves distinct stem cell responses to wounds and tissue absence. *Developmental Biology*, *344*(2), 979–991. https://doi.org/10.1016/j.ydbio.2010.06.017

Wurtzel, O., Cote, L. E., Poirier, A., Satija, R., Regev, A., & Reddien, P. W. (2015). A Generic and Cell-Type-Specific Wound Response Precedes Regeneration in Planarians. *Developmental Cell*, *35*(5), 632–645. https://doi.org/10.1016/j.devcel.2015.11.004

Yang, Y., & Jackson, R. (2019). Astrocyte identity: Evolutionary perspectives on astrocyte functions and heterogeneity. *Current Opinion in Neurobiology*, *56*, 40–46. https://doi.org/10.1016/j.conb.2018.11.006

Yazawa, S., Umesono, Y., Hayashi, T., Tarui, H., & Agata, K. (2009). Planarian Hedgehog/Patched establishes anterior–posterior polarity by regulating Wnt signaling. *Proceedings of the National Academy of Sciences*, *106*(52), 22329–22334. https://doi.org/10.1073/pnas.0907464106

Yildirim, K., Petri, J., Kottmeier, R., & Klämbt, C. (2019). Drosophila glia: Few cell types and many conserved functions. *Glia*, *67*(1), 5–26. https://doi.org/10.1002/glia.23459

Yoshimura, S., Murray, J. I., Lu, Y., Waterston, R. H., & Shaham, S. (2008). Mls-2 and vab-3 control glia development, hlh-17/Olig expression and glia-dependent neurite extension in C. elegans. *Development*, *135*(13), 2263–2275. https://doi.org/10.1242/dev.019547

Zhou, B., Zuo, Y., & Jiang, R. (2019). Astrocyte morphology: Diversity, plasticity, and role in neurological diseases. *CNS Neuroscience & Therapeutics*, *25*(6), 665–673. https://doi.org/10.1111/cns.13123

Zhou, Q., & Anderson, D. J. (2002). The bHLH Transcription Factors OLIG2 and OLIG1 Couple Neuronal and Glial Subtype Specification. *Cell*, *109*(1), 61–73. https://doi.org/10.1016/S0092-8674(02)00677-3

Zhou, Q., Wang, S., & Anderson, D. J. (2000). Identification of a Novel Family of Oligodendrocyte Lineage-Specific Basic Helix–Loop–Helix Transcription Factors. *Neuron*, *25*(2), 331–343. https://doi.org/10.1016/S0896-6273(00)80898-3

Zhu, S. J., & Pearson, B. J. (2016). (Neo)blast from the past: New insights into planarian stem cell lineages. *Current Opinion in Genetics & Development*, *40*, 74–80. https://doi.org/10.1016/j.gde.2016.06.007

Zuchero, J. B., & Barres, B. A. (2013). Intrinsic and extrinsic control of oligodendrocyte development. *Current Opinion in Neurobiology*, *23*(6), 914–920. https://doi.org/10.1016/j.conb.2013.06.005

CHAPTER II

ETS-1 TRANSCRIPTION FACTOR REGULATES GLIAL CELL REGENERATION AND FUNCTION IN PLANARIANS[1]

[1] Chandra, B., Voas, M.G., Davies, E.L., and Roberts-Galbraith, R.H. (2023). Ets-1 transcription factor regulates glial cell regeneration and function in planarians. *Development*, 150(18): dev201666. doi: https://doi.org/10.1242/dev.201666. Reprinted here with permission of publisher.

ABSTRACT

Glia play multifaceted roles in nervous systems in response to injury. Depending on the species, extent of injury, and glial cell type in question, glia can help or hinder the regeneration of neurons. Studying glia in the context of successful regeneration could reveal features of pro-regenerative glia that could be exploited for new human therapies. Planarian flatworms completely regenerate their nervous systems after injury—including glia—and thus provide a strong model system to explore glia in the context of regeneration. Here, we report that planarian glia regenerate after neurons and that neurons are required for correct glial numbers and localization during regeneration. We also identify the planarian transcription factor-encoding gene *ets-1* as a key regulator of glial cell maintenance and regeneration. Using *ets-1*(RNAi) to perturb glia, we show that glial loss is associated with altered neuronal gene expression and impeded animal movement, and impaired nervous system architecture – particularly within the neuropil. Importantly, our work reveals interrelationships of glia and neurons in the context of robust neural regeneration.

INTRODUCTION

Glial cells are a heterogenous group of non-neuronal cells within many animal nervous systems. Glial cells within the mammalian central nervous system (CNS) include astrocytes, oligodendrocytes, and microglia. Meanwhile, major glial types in the mammalian peripheral nervous system (PNS) include Schwann cells and satellite cells (Allen & Lyons, 2018; Jessen, 2004). Glial cells have been reported in most, but not all, bilaterian taxa studied (Hartline, 2011; Ortega & Olivares-Bañuelos, 2020). Molecularly, glial cells often express *glial fibrillary acidic protein* (GFAP), *glutamine synthetase* (GS), and/or *excitatory amino acid transporter* (EAAT), but these markers are not universal across species (Hartline, 2011). Functionally, glial cells play important and diverse roles in the development, maintenance, and activity of the nervous system across bilaterians. Glial roles include regulating neural cell numbers and migration; promoting axon guidance; maintaining ionic homeostasis; mediating neurotransmitter uptake; facilitating synapse architecture; and remodeling neural circuitry (Allen & Lyons, 2018; Jäkel & Dimou, 2017; Jessen, 2004; Oikonomou & Shaham, 2011; Shaham, 2015; Yildirim *et al.*, 2019).

In regeneration, glial cells play dynamic roles that depend on the species, location, extent, and duration of the injury. For instance, mammalian astrocytes and microglia respond to injury through "reactive gliosis," which can lead to formation of a "glial scar" (Adams & Gallo, 2018; Anderson *et al.*, 2016; Burda & Sofroniew, 2014; Escartin *et al.*, 2021; Gallo & Deneen, 2014; Pekny *et al.*, 2014). Glial scarring can promote neuronal survival, but can also limit axonal regeneration (Anderson *et al.*, 2016; Myer, 2006; Rolls *et al.*, 2009; Silver & Miller, 2004). In contrast, glial scarring does not occur in fish and insects due to the presence of bridging glia in the zebrafish spinal cord (Goldshmit *et al.*, 2012; Mokalled *et al.*, 2016), and phagocytic ensheathing glia in the adult *Drosophila* neuropil (Doherty *et al.*, 2009; Purice *et al.*, 2017). Glial

roles in the context of *successful* regeneration are only beginning to be uncovered, partly due to the limited regenerative capacity of many traditional model organisms (Alesci *et al.*, 2022; Silver *et al.*, 2015; Tanaka & Ferretti, 2009).

Freshwater flatworms called planarians undergo whole-body regeneration without scarring, including *de novo* regrowth and rewiring of the entire brain. Genes that include *intermediate filament* (IF-1), *calamari,* and *estrella* mark glia present in the planarian nervous system, opening an opportunity to identify glia and to explore glial biology in an organism with complete regenerative capacity for the first time (Roberts-Galbraith *et al.*, 2016; Wang *et al.*, 2016). Planarian glia express genes that encode proteins for neurotransmitter uptake and metabolism (*e.g. solute carrier 1a-5*/*EAAT*; *glutamine synthetase-1*), indicating an overlap in function with astrocytes in the mammalian CNS (Roberts-Galbraith *et al.*, 2016; Wang *et al.*, 2016). Previous work also determined that transcription factor *forkhead box protein factor-1* (Scimone *et al.*, 2018) and Hedgehog signaling from ventral-medial neurons impact glial gene expression in planarians (Currie *et al.*, 2016; Wang *et al.*, 2016), though the consequences of changes in glial gene expression are not known. Furthermore, single-cell sequencing atlases indicate that planarian glia share some gene expression features with additional cell types that express *cathepsin*, including pigment cells, parenchymal cells, and other uncharacterized cell types (Fincher *et al.*, 2018; Plass *et al.*, 2018). Transcriptional similarity between *cathepsin*$^{+}$ cell types has been interpreted as a lineage-based relationship, though this has not yet been experimentally shown. Many fundamental aspects of planarian glial biology remain unexplored, including how glia regenerate and what roles, if any, they play in the planarian nervous system during homeostasis and regeneration.

ETS transcription factors regulate gliogenesis in vertebrates and invertebrates (Kiyota *et al.*, 2007; Klämbt, 1993; Kola *et al.*, 1993). *Drosophila pointed*, which encodes an ETS transcription factor, is necessary and sufficient for longitudinal and midline glial differentiation (Klaes *et al.*, 1994). In *Xenopus*, *Ets-1* directly regulates radial glial formation and promotes neuron-glia interaction during embryogenesis (Kiyota *et al.*, 2007; Klämbt, 1993). Human and mouse *Ets-1* are expressed in cortical astrocytes and are involved in astrocyte differentiation (Amouyel *et al.*, 1988; Fleischman *et al.*, 1995). In planarians, previous studies indicate that *ets-1* plays roles in several cell types, including pigment cells, glial cells, and other uncharacterized *cathepsin*$^{+}$ cells (Dubey *et al.*, 2022; He *et al.*, 2017), but a definitive function for *ets-1* in glia has not been fully established.

In this study, we determined that the transcription factor Ets-1 promotes maintenance of existing glial cells in uninjured tissues and regeneration of new glial cells in the planarian *Schmidtea mediterranea*. Furthermore, using *ets-1*(RNAi) to perturb glia, we investigated potential roles for planarian glia for the first time. We determined that *ets-1*(RNAi) non-cell-autonomously impacts neuronal gene expression, neuropil size, and animal movement. Taken together, our work demonstrates that planarian *ets-1* plays a conserved and crucial role in glial cells during regeneration and tissue homeostasis in planarians. In parallel, this work explores spatiotemporal and functional relationships between planarian glial cells and neurons during regeneration and embryogenesis, revealing that planarian neurons promote glial regeneration as well.

RESULTS

Planarian glial cells arise after neurons.

In vertebrate and *Drosophila* development, neurogenesis precedes gliogenesis (Anthony *et al.*, 2004; Barnabé-Heider *et al.*, 2005; Bayraktar & Doe, 2013; Klämbt & Goodman, 1991; Viktorin *et al.*, 2011). We reasoned that understanding the sequence of neuronal and glial development and regeneration in planarians could help form testable hypotheses about glial cell specification and function. We first amputated planarians pre-pharyngeally and fixed animals at several time points to establish a timeline of neuronal and glial regeneration. Using a marker of cholinergic neurons, *choline acetyltransferase* (*ChAT*) (Nishimura et al., 2010), we observed re-establishment of neurons within a primordial brain around 3 days post-amputation (dpa) and clear brain organization at 5 dpa (Fig 2.1A, top). Our results were consistent with previous reports that new neurons are born beginning at 2 dpa (Supp. Fig 2.1A-B) (Cebrià, Nakazawa, et al., 2002; Inoue et al., 2004). The expression pattern of *ChAT* in the brain remained comparable from 5 dpa onward.

In contrast to *ChAT*, the earliest appearance of *estrella*$^{+}$ glial cells in the newly regenerated head occurred between 4-5 dpa (Fig 2.1A, middle) (Roberts-Galbraith *et al.*, 2016). Round *estrella*$^{+}$ cells initially re-appeared in small numbers; cell number increased over time and cells progressively adopted stellate morphology (Fig 2.1B). By 15 dpa, the distribution of *estrella*$^{+}$ cells appeared identical to that in uninjured planarians. We confirmed this timeline for glial cell regeneration using two additional glial markers: *intermediate filament-1* (*if-1*) (Roberts-Galbraith *et al.*, 2016; Wang *et al.*, 2016) and *calamari* (Wang *et al.*, 2016)(Fig 2.1A, bottom; Suppl. Fig 2.1A). *IF-1* is also downregulated after amputation in RNA-sequencing (RNA-seq) data and we confirmed that other glial markers were transiently downregulated at early time

points after injury (Fig 2.1C) (Roberts-Galbraith *et al.*, 2016). Taken together, our results show that the planarian glial cells respond to injury by changing gene expression and regenerate in new tissue after neurons.

Next, we asked whether the temporal order of cell birth holds true in embryogenesis. Development of the adult nervous system begins during Stage 5 (S5), with the expression of transcription factor-encoding genes with roles in neuronal subtype specification (Davies *et al.*, 2017). Genes required for differentiated neuron function, including terminal selector genes required for neuronal subtype maintenance and genes involved in synapsis and neurotransmission, show enriched expression during Stage 6 (S6), Stage 7 (S7), and Stage 8 (S8) (Davies *et al.*, 2017). Expression of the neuronal marker *pc-2* was detected by single embryo bulk RNA-seq as early as Stage 2 (S2) (Fig 2.2A). We did not detect robust expression of glial markers *if-1*, *calamari*, and *estrella* by bulk RNA-Seq prior to S7, apart from *EAAT*/*slc1a-5* (Fig 2.2A). However, single cell transcriptomic data from adult asexual planarians suggest that *EAAT* is also expressed in muscle and other *cathepsin*$^{+}$ cells (Fincher *et al.*, 2018; Plass *et al.*, 2018).

ISH was performed to examine spatiotemporal expression patterns for neuronal- and glial-enriched transcripts on staged planarian embryos and juveniles (1 week post-hatching; wph)]. By ISH, *ChAT*$^{+}$ neurons can be seen at S6 (Fig 2.2B) (Davies *et al.*, 2017). Neuronal markers *synaptotagmin* (*syt1-1*) and *pc-2* also appeared during S6 (Supp. Fig 2.1C). By the end of S7, neuronal markers highlighted well-developed brain and ventral nerve cord (VNC) structures that were nearly contiguous and extended to the posterior end of the embryo (Fig 2.2B, Supp. Fig 2.1C). Peripheral neurons were evident in both *ChAT* and *syt1-1* ISH during stage S6 and photoreceptor neurons were evident by S7 (Davies *et al.*, 2017)(Fig. 2.2C, Suppl. Fig 2.1C-D). In planarians, as in other organisms, neurogenesis initiated in the anterior with the formation of the

brain primordia, followed by VNC formation, which again showed early anterior bias (Fig 2.2B; S1C).

We next examined expression of glial markers during embryonic development. Markers specific for differentiated glial cells were expressed beginning in S6-S7 (Davies *et al.*, 2017) (Fig 2.2A). Expression of *calamari* in the CNS initiated first and was detected by WISH in the brain primordia and anterior domain of the developing VNC at mid-S6 (Fig 2.2B). *if-1* and *estrella* showed expression in the brain primordia and developing VNC in the anterior half of the embryo during S7 and in the posterior by S7.5 (Fig 2.2B). *estrella* was expressed near the mouth beginning at S7, around the eyes, and in putative PNS glial cells in S8 hatchlings (Fig 2.2B-C). All PNS glia and those around sensory structures like the eye appeared later than neurons. Expression of peripheral glial marker genes was heavily biased towards the ventral side of the embryos at all stages assayed (Fig 2.2B-C, Suppl. Fig 2.1C). Taken together, we concluded that gliogenesis occurs after neurogenesis during embryogenesis and adult regeneration, like the relative order of cell birth in other animal species.

Glial regeneration in nervous system depends on neurons.

In many organisms, the interactions between neurons and glia during development are dynamic and reciprocal. Neurons can act as a "blueprint" for glial cell development by regulating the migration, survival, and proliferation of glial cells (Allen & Lyons, 2018). The birth order we established for planarian glia and neurons led us to seek ways to test whether glia depend on neurons for birth or final location. *estrella*$^{+}$ glial cells are present throughout the CNS, near the photoreceptor neurons of the eyespots, and among the ciliated sensory neurons along the dorsal midline and lateral margins of the body (Fig 2.3, Suppl. Fig 2.2).

To investigate whether *estrella*$^{+}$ cell regeneration in the CNS depends on neurons, we knocked down regulators of neuronal regeneration, *coe* and *sim* (Cowles et al., 2013, 2014), and performed fluorescent *in situ* hybridization (FISH) to detect neuronal marker *ChAT* (magenta) and glial marker *estrella* (green). As previously reported, regenerating *coe*(RNAi) and *sim*(RNAi) animals have reduced expression of *ChAT* (Fig 2.3A-B). We saw significantly reduced *estrella*$^{+}$ cells in the brains of both *coe*(RNAi) and *sim*(RNAi) animals (Fig 2.3C). Next, we asked whether the expansion of the brain region would result in increased *estrella*$^{+}$ cells. Previous work showed that knockdown of *nou-darake* (*ndk*) causes posterior expansion of the brain (Fig 2.3D, E) (Cebrià, et al., 2002). We performed *ndk*(RNAi) to assess *estrella*$^{+}$ cells in the expanded area. Compared to control, we observed a significant increase in *estrella*$^{+}$ cells in the expanded brain tissue (Fig 2.3F). Our data suggests that CNS neurons could promote glial regeneration and/or localization to the brain.

To investigate whether *estrella*$^{+}$ cells depend on photoreceptors or pigment cells in the eye, we knocked down *ovo* to specifically reduce eyespot regeneration (Lapan & Reddien, 2012). As previously reported, *ovo*(RNAi) caused reduced expression of pooled photoreceptor neuron markers *sans*/*foxQ2*/*myoVIIA* (Fig 2.3G; Suppl. Fig 2.2A-B). We examined *estrella* expression in cells adjacent to the eyespots (Fig 2.3G-H) (Roberts-Galbraith *et al.*, 2016). We saw significant reduction of dorsal *estrella*$^{+}$ cells present in the head after *ovo*(RNAi) (Fig 2.3H). Our data suggest that the presence of photoreceptor neurons or pigment cup cells fosters localization of *estrella*$^{+}$ glial cells nearby.

We finally sought to determine if regeneration and maintenance of PNS glia depend on sensory neurons present along the medial and lateral dorsal surfaces. Transcription factor *soxb1-2* is specifically required for regeneration of many sensory neurons that function in these regions

(Ross *et al.*, 2018). We thus performed head amputations on *soxB1-2*(RNAi) animals, and examined *estrella* expression. As previously shown, *pkd2L-1/1L-2*$^{+}$ neurons decreased in number after *soxB1-2*(RNAi) in the medial stripes and the lateral margin of the body (Fig 2.3I; Suppl. Fig 2.2C-D)(Ross *et al.*, 2018). In both regenerated and non-regenerating tissue, we observed a decrease in *estrella*$^{+}$ cells in medial and lateral regions, however the differences were only statistically significant in the homeostatic tissue (Fig 2.3I; Suppl. Fig 2.2C-D). We do acknowledge that perduring *pkd*$^{+}$ and *soxb1-2*-independent neurons present in these locations may still permit glial localization. Additionally, *soxb1-2*(RNAi) also results in reduced brain size and we observed reduced CNS *estrella*$^{+}$ cells (Suppl. Fig 2.2E-G), reinforcing our previous observations that neurons promote regeneration of glial cells in the brain.

Taken together, our data show that perturbation of neurons within the CNS and PNS leads to reduction in local *estrella*$^{+}$ cells. Our results strongly suggest that population of the nervous system with glia during regeneration may be promoted by antecedent neuronal cell types.

Putative transcription factor ets-1 affects glial gene expression.

Beyond the impact of neurons on glial regeneration, we also wished to identify genes required cell-autonomously for glial regeneration. Work in several organisms established roles for ETS-family transcription factors in glial cell identity (Amouyel *et al.*, 1988; Fleischman *et al.*, 1995; Kiyota *et al.*, 2007; Klaes *et al.*, 1994; Klämbt, 1993). We utilized phylogenetic analysis to show that *S. mediterranea* Ets-1 is similar to *D. melanogaster* and *C. salei* Pointed (Chen *et al.*, 1992; Klämbt, 1993; Pribyl *et al.*, 1988) and mammalian and *Xenopus* Ets-1 (Suppl. 2.3) (Slupsky *et al.*, 1998; Stiegler *et al.*, 1990; Watson *et al.*, 1988).

Planarian *ets-1* is expressed widely throughout the mesenchyme (Fig 2.4A) and has been reported to play roles in specification and maintenance of pigment cells and other *cathepsin*$^+$ cell types (Dubey *et al.*, 2022; He *et al.*, 2017). Prior studies on planarian *ets-1* reported either no impact on *estrella* expression (He *et al.*, 2017) or decreased *calamari* expression in the head (Dubey *et al.*, 2022). To uncover the full impact of *ets-1* on glial cells, we optimized an RNAi paradigm by adjusting numbers and dosage of dsRNA feedings (Fig 2.4B, Suppl. Fig 2.4A). Following optimization, we performed *ets-1*(RNAi) and examined glia with multiple markers (Fig 2.4C-D). *ets-1*(RNAi) worms exhibited reduced *calamari* expression throughout the body compared to controls, in regenerated and uninjured tissue (Fig. 2.4C, Suppl. Fig 2.4F-G)(Wang *et al.*, 2016). Similarly, *ets-1*(RNAi) animals showed a reduction of *estrella* expression at 7dpa and during homeostasis (Fig 2.4D, Suppl. Fig 2.4H). We observed three distinct differences in *estrella* expression in *ets-1*(RNAi) animals. In both regenerating and uninjured animals, we observed decreased *estrella*$^+$ cell number in the newly regenerated head (Fig. 2.4D; Suppl. 2.4B-C); gaps in *estrella*$^+$ signal in the VNC (Fig. 2.4D; Suppl. Fig 2.4D-E, I-J); and reduction of peripheral *estrella*$^+$ cell number (Fig 2.4D, red arrowhead, L-N; Suppl. Fig 2.4K-L). Interestingly, the reduction of *calamari* expression was strongest in the VNC and more dramatic than the reduction of *estrella* (Fig 2.4C, Suppl Fig 2.4G). We further quantified relative expression of *if-1*, *estrella*, and *calamari* transcripts via RT-qPCR and observed significant reduction in all glial transcripts (59-72% reduction; Fig 2.4E) in *ets-1*(RNAi) animals. Taken together, our data indicate that *ets-1* promotes *calamari*$^+$ and *estrella*$^+$ cell maintenance in pre-existing tissue as well as during regeneration.

Our previous data showed that changes in *estrella* and *calamari* expression after *ets-1*(RNAi) were not identical. We investigated cell-specific effects of *ets-1*(RNAi) using FISH

(Fig 2.4F). As with ISH, we saw significantly reduced cell number using individual glial markers (Fig 2.4G-H). Looking at the overlap in gene expression, we saw that there were significantly fewer *estrella*$^{+}$/*calamari*$^{+}$ and *estrella*$^{-}$/*calamari*$^{+}$ cells in regenerating heads after *ets-1*(RNAi) (Fig 2.4I, K). However, there was no significant difference in rare *estrella*$^{+}$/*calamari*$^{-}$ cells (Fig 2.4J). Our data suggest that, in addition to impacting glial numbers overall, Ets-1 also influences gene expression in remaining glia, potentially reflecting additional roles in cell state or maturation. Overall, our data demonstrate a requirement for *ets-1* in glial cell maintenance, regeneration, and gene expression in planarians.

ets-1 affects multiple cathepsin^{+} *cell types.*

Continuous knockdown of *ets-1* eventually leads to animal death (Suppl. Fig 2.5)(Dubey *et al.*, 2022). This led us to ask how *ets-1* affects cell types beyond glia and pigment cells (Dubey *et al.*, 2022; He *et al.*, 2017). Single cell RNA sequencing (scRNA-seq) atlases cluster planarian pigment cells and glial cells with other cells that express *cathepsin* (Fincher *et al.*, 2018; Plass *et al.*, 2018)(Suppl. Fig 2.6A). We first confirmed previous reports that *ets-1* affects pigment cells in planarians (Fincher *et al.*, 2018; He *et al.*, 2017; Stubenhaus *et al.*, 2016)(Suppl. Fig 2.6B). After *ets-1*(RNAi), relative gene expression of pigment markers *pgbd-1* and *gst* were reduced by 12% and 28% relative to controls (Suppl. Fig 2.6B).

We also examined transcript levels for several genes expressed broadly in *cathepsin*$^{+}$ cells—*forkhead box factor-1* (*foxf-1*) (Scimone et al., 2018), *cathepsin F* (*ctsf*), and *low density lipoprotein receptor related-3* (*ldlrr-3*) (Roberts-Galbraith *et al.*, 2016). Compared to control, *ets-1*(RNAi) animals had significant reduction across all three *cathepsin*$^{+}$ cell markers (Suppl. Fig 2.6C). The *cathepsin*$^{+}$ cluster (also known as the parenchymal cluster) also includes eight

cellular subclasses or states that are uncharacterized, many of which express *ets-1* (Fincher *et al.*, 2018; Plass *et al.*, 2018)(Suppl. Fig 2.6A). We repeated RT-qPCR using eight additional genes that mark individual *cathepsin*$^+$ subclusters. After *ets-1*(RNAi), expression of these genes was significantly reduced (*aqp1, dd_5690,* and *dd_9*), significantly increased (*dd_1831),* or unchanged (*TTPA, dd_7593, cathepsin L2*/*ctsl2*, and *protein tyrosine phosphate receptor type ptprt*) (Suppl. Fig 2.6D-E). Our RT-qPCR data paints a complex picture of the roles for *ets-1* in *cathepsin*$^+$ cell types outside of pigment cells and glia, but we do not see universal downregulation of transcripts that fits neatly with the conclusion that *ets-1*(RNAi) affects all cells in the *cathepsin*$^+$ cluster in the same way. We also re-analyzed previously published RNA-seq data on *ets-1*(RNAi) animals that utilized different RNAi and amputation paradigms (Dubey *et al.*, 2022). We did not see consistent, significant downregulation of genes enriched in *cathepsin*$^+$ cell subtypes that would indicate a uniform loss of some or all *cathepsin*$^+$ cell types (Suppl. Fig 2.6F, Suppl. Table 2.4).

We conclude that *ets-1* knockdown induces differences in gene expression across multiple *cathepsin*$^+$ subclusters that depends on the specific target gene, cell subpopulation, and amputation site chosen. While our data strongly implicate *ets-1* in maintenance and regeneration of planarian glia and confirm the role of *ets-1* in pigment cells, roles for *ets-1* in regulating other specific subclusters of *cathepsin*$^+$ cell types will require further detailed study. Importantly, glial cells are the only *cathepsin*$^+$ cell type present specifically within the nervous system, which allowed us to use *ets-1(RNAi)* as a first step for perturbing glia and examining neuronal organization and animal behavior.

Reduction of ets-1 affects CNS neuron gene expression and organization.

Across metazoans, glial cells have been extensively implicated in neuronal development and physiology. For example *Drosophila* glia regulate neuronal proliferation and, consequently, neuronal numbers (Coutinho-Budd *et al.*, 2017; Ebens *et al.*, 1993; Plazaola-Sasieta *et al.*, 2019). Glial cells in *C. elegans* demarcate regions within the nervous system (*i.e.* nerve ring formation) before neuronal migration and consequently, also regulate neuron numbers (Rapti *et al.*, 2017; Yoshimura *et al.*, 2008). We showed that *ets-1*(RNAi) affects planarian glial gene expression and glial cell number. This discovery allowed us to investigate potential consequences of glial cell perturbation in planarians.

We first asked if perturbation of *ets-1* impacted gross morphology of the nervous system. We knocked down *ets-1* and examined expression of *ChAT* at 7dpa (Nishimura *et al.*, 2010)(Fig 2.5A). We quantified brain area relative to body area and saw a significant decrease in brain size after *ets-1*(RNAi) with no change to *ChAT* transcript levels (Fig 2.5B-C). Next, we examined whether specific neuronal subtypes were affected coincident with the loss of glia. We performed ISH on *ets-1*(RNAi) animals after regeneration and examined expression of neuronal markers *neuropeptide precursor-3* (*npp-3*; Collins *et al.*, 2010), *secreted peptide prohormone -12* (*spp12;* Ong *et al.*, 2016; Shimoyama *et al.*, 2016), *glutamic acid decarboxylase* (*gad*; Nishimura *et al.*, 2008), *tryptophan hydroxylase* (*tph*; Nishimura *et al.*, 2007), *tyrosine hydroxylase* (*th*; Fraguas *et al.*, 2012; Nishimura *et al.*, 2007), *tyramine beta-hydroxylase* (*tbh;* Nishimura et al., 2008), and *cintillo (Oviedo et al., 2003)*(Fig 2.5D-T; Suppl. Fig 2.7A-D). Cell numbers for each neuronal cell type in the CNS were unaffected by *ets-1*(RNAi) (Fig 2.5D-E, I-J, L-N, P, R-S; Suppl. Fig 2.7B, D). Interestingly, we noted that 62.5% of the regenerated *ets-1*(RNAi) animals had abnormal patterning of *gad*$^{+}$ cells, where the linear, arched organization of *gad*$^{+}$ cells seen in

control animals was lost in *ets-1*(RNAi) animals (Fig 2.5I). In all, we saw only a small increase in th^+ cells in the PNS in *ets-1*(RNAi) animals (Fig 2.5R). Further, we saw no changes to neuronal cell numbers or organization in uninjured animals after *ets-1* perturbation (Suppl. Fig 2.7E-K).

Despite no change in neuronal number, we observed that regenerated *ets-1*(RNAi) animals had significantly increased mRNA levels for *gad*, and to a lesser extent *th* and *tph* (Fig 2.5K, O, T). We also observed a significant increase in *th* expression within individual cells after *ets-1*(RNAi) (Fig 2.5P-Q), which could explain the increased global *th* transcript levels. Other neuronal transcripts, including neuropeptide-encoding mRNAs *npp-3* and *spp-12* were not affected by *ets-1*(RNAi) (Fig 2.5F, H). Based on our data, we conclude that loss of *ets-1* impacts several neuronal mRNAs but perturbs cell number for only rare subsets of neurons (e.g., PNS *TH+* cells) after regeneration.

Taken together, our data show that the reduction of *ets-1* results in: decreased brain size, altered organization of specific cell types (*gad*), anomalous neuronal gene expression, and no change in cell number for neuron types of the brain. After reduction in glial numbers, we did not see phenotypes like paralysis or death that would indicate widespread loss of neuronal function or failure to specify neurons during regeneration. Our data together indicate that inhibition of *ets-1*, likely due through loss of glial cells, impacts planarian neuronal gene expression and CNS organization during head regeneration.

Loss of ets-1 leads to changes in neural connectivity.

Glia also play important roles in axon guidance, axon fasciculation, and axonal targeting in other species (Hidalgo *et al.*, 1995; Mason & Sretavan, 1997; Poeck *et al.*, 2001). In

planarians, individual axon trajectories can be most clearly seen in the visual system. Furthermore, we noted that *estrella* can be seen in cells near the eyespots (Fig 2.3G) (Roberts-Galbraith *et al.*, 2016). To determine if glial cells play roles in photoreceptor axon trajectory in planarians, we performed *ets-1*(RNAi) to reduce glial cell number and stained with *anti*-Arrestin (Sakai *et al.*, 2000) (Fig 2.6A). We examined *ets-1*(RNAi) animals for defects in axon fasciculation (i.e., stray bundles or axons near optic chiasma or photoreceptor neurons, gaps in the optic chiasma) compared to controls (Fig 2.6B). We found that *ets-1*(RNAi) animals had significantly increased rates of these defects when all criteria were considered together (Fig 2.6A-B). When considered individually, each category of defect was enriched in *ets-1*(RNAi) animals by nonsignificant margins (Suppl. Fig 2.8A-D). We concluded that *ets-1* subtly affects axonal organization in the photoreceptor system.

Glial cells in other animal species play important roles in synapse organization and function (Eroglu & Barres, 2010; Lee & Chung, 2019; Neniskyte & Gross, 2017). As described, we observed decreased brain size after *ets-1*(RNAi) without decrease in neuronal cell numbers (Fig 2.5). We hypothesized that the decrease was due to the changes in the neuropil, a synapse- and process-rich structure in the interior of the planarian brain that is the primary location of most CNS glial cells. To determine if *ets-1* knockdown affected synapse density or organization in planarians, we stained 7dpa animals with *anti*-Synapsin (Fig 2.6C) (Ross *et al.*, 2015). We quantified medial brain gap (the space between the two brain lobes), average VNC gap length, average gap sizes, average width of the neuropil and average fluorescent intensity (Fig 2.6D-F; Suppl. Fig 2.8E-H). Regenerated *ets-1*(RNAi) worms had a significant decreased neuropil width compared to controls (Fig 2.6E). However, we saw no additional effects on organization or staining intensity in the synapses of regenerated *ets-1*(RNAi) worms (Fig 2.6C, F; Suppl Fig

2.8E-H). Reduction in neuropil size could explain our previous observation of a small brain size after *ets-1*(RNAi) without decrease in neuronal cell number (Fig 2.6B). We also asked whether synapse density or organization was affected in uninjured animals (Fig 2.6G-H). Interestingly, we observed significantly decreased average fluorescent intensity and increased neuropil width in intact *ets-1*(RNAi) animals compared to control (Suppl. Fig 2.8I-N), suggesting that *ets-1*(RNAi) also affects maintenance of synaptic density.

Taken together, our data suggests that perturbation of *ets-1*, possibly through loss of glial cells, results in defects in axon and synaptic organization during homeostasis and regeneration.

Perturbation of ets-1 results in locomotion defects.

We reasoned that reduction in glial cell number might, through the changes that we have detailed in prior sections, affect neuronal *function*. Impaired neuronal function would be reflected in planarian behavior, including response to light. Planarians normally exhibit negative phototactic behavior, preferring to move toward areas of low light (Fig 2.7A; Suppl. Fig 2.9; Supp Video 2.1; based on assays in (Paskin *et al.*, 2014; Zewde *et al.*, 2018)). *ets-1*(RNAi) animals consistently showed reduced ability to move into a dark space compared to control animals (Fig 2.7A-B; Supp Videos 2.1A-B). By 5 minutes, more than 50% of *ets-1*(RNAi) animals remained on the light side of the dish and by 10 minutes, 41.18% of *ets-1*(RNAi) animals failed to reach the dark side (Fig 2.7B; Supp Video 2.1B). In comparison, 75% of control worms reached the dark side within 3 minutes, and by 6 minutes, over 96% of control worms had reached the dark side (Fig 2.7B; Suppl Fig 2.9; Supp Video 2.1A). Interestingly, we noticed that *ets-1*(RNAi) worms often exhibited uncoordinated movements that included head lifts and inch-worming, a slow locomotion gait based on muscle contraction instead of cilia-

mediated movement (Suppl. Fig 2.9C, Supp Video 2.1B). When we quantified inch-worming behavior, 61.76% of *ets-1*(RNAi) animals exhibited inch-worming behavior as they initiated movement compared to control animals (9.38%) (Fig 2.7C). Even within an open field, 23.4% of *ets-1*(RNAi) animals exhibited inch-worming behavior at movement onset compared to 4.35 % of control animals (Fig 2.7D). We verified that photoreceptors, ciliated *soxb1-2*$^{+}$ neuronal numbers, and other neuronal numbers relevant to photophobic movement were unchanged in *ets-1*(RNAi) animals (Suppl. Fig 2.9D-J). Therefore, we conclude that *ets-1*(RNAi) impacts both the quality and outcome of planarian movement likely through glial roles in robust neuronal function or connectivity.

DISCUSSION

Planarians regenerate their nervous systems quickly and with high fidelity, making them an attractive model for studying glia during successful nervous system repair. In this work, we established timelines for development and regeneration of glial cells, demonstrating that glia arise after neurons and depend on neurons for their regenerative placement throughout the nervous system. We further show that transcription factor *ets-1* plays conserved roles in gliogenesis and glial maintenance in *Schmidtea mediterranea*. Our work further leveraged on our findings with *ets-1* to explore potential roles of glial cells in planarians for the first time. Our results indicate that *ets-1*(RNAi) causes altered gene expression in neuronal cell types, reduction in neuropil volume, and perturbed fluidity of animal movement. It is important to note that *ets-1* affects multiple *cathepsin*$^{+}$ cell types in planarians. Further work will be required to dissect the roles of Ets-1 in diverse cell types and to more specifically ablate glia to confirm and expand possible role(s) for glia in regulating neural physiology and behavior. Moreover, it will be

interesting to address how *ets-1* specifies glial fate and to explore the identity and function of Ets-1 targets in planarian glial cells.

The role of ets-1 in gliogenesis is conserved in planarians

Members of the *Ets* family have conserved roles in driving gliogenesis across metazoans. ETS transcription factors are important for both CNS and PNS gliogenesis (Hagedorn *et al.*, 2000). *Drosophila* Pointed, an ETS transcription factor expressed in glial cells, is required for differentiation of longitudinal and midline glial cells (Klaes et al., 1994; Klämbt, 1993). *Ets-1* is expressed in human cortex astrocytes and plays roles in astrocyte differentiation, proliferation, and regulation of genes involved in astrocyte signaling (Amouyel *et al.*, 1988; Fleischman *et al.*, 1995). ETS family transcription factors are also crucial for survival and proper regeneration of mammalian Schwann cells and zebrafish bridging glia after injury (Arthur-Farraj *et al.*, 2012; Klatt Shaw *et al.*, 2021; Nagarajan *et al.*, 2002; Parkinson *et al.*, 2002). Similarly, we found that planarian *ets-1* is required for glial regeneration and maintenance. *ets-1* knockdown reduces glial numbers, with distinct effects on individual glial markers.

Planarian *ets-1* also affects pigment cells (He *et al.*, 2017) and we showed that it regulates gene expression in other cell types in the planarian body, most of which are not well characterized. Interestingly, planarian glial cells cluster more closely with pigment cells than neurons in single-cell transcriptomic analyses (Fincher *et al.*, 2018). This finding argues against common progenitors for neurons and glia in planarians and suggests that glia might share a common progenitor with other phagocytic, *cathepsin*$^{+}$ cell types (*e.g.* pigment cells). Alternatively, *ets-1* could regulate cell state in a wide variety of phagocytic, *cathepsin*$^{+}$ cell types

that are not lineage-related but that cluster together due to similarities in gene expression that correspond to functional rather than lineage relationships.

Relationships between planarian glia and neurons

During CNS development in vertebrates and *Drosophila*, neurogenesis often precedes gliogenesis as stem cells produce neurons before switching programs to make glia (Campbell & Götz, 2002; Miller & Gauthier, 2007; Molofsky & Deneen, 2015). This sequence of events is shared across species with varying time scales. Here, we uncovered a similar "birth order" in planarian regeneration and development, in which neurons arise first and glia arise later. While vertebrates and *Drosophila* progenitors undergo a neurogenic-to-gliogenic switch during neural development, planarian neurons and glial cells are thought to arise from distinct lineages of pluripotent stem cells (Crews, 2019; Fincher *et al.*, 2018; Miller & Gauthier, 2007; Plass *et al.*, 2018; Roberts-Galbraith *et al.*, 2016; Wang *et al.*, 2016). Interestingly, though pluripotent stem cells give rise to all cells in the adult planarian body, this is the first observation to our knowledge of a "birth order" of cell types that contribute to a common tissue in planarians. Our observations raise new questions regarding why and how planarian stem cells produce glia and neurons with different timing after injury.

Potential roles for glial cells in the planarian nervous system

Glial cells fulfill diverse roles across animals including regulating neuronal cell numbers and migration, aiding axon guidance and growth, promoting neuronal differentiation, regulating synapse formation and pruning, regulating ion homeostasis, providing metabolic support, participating in sensory systems, and helping and/or impeding response to injury (Allen &

Lyons, 2018; Falk & Götz, 2017; Jäkel & Dimou, 2017; Oikonomou & Shaham, 2011; Ortega & Olivares-Bañuelos, 2020; Shaham, 2015; Yildirim *et al.*, 2019).

Our studies with Ets-1 allowed us to investigate potential roles for planarian glia for the first time. Our data indicate that planarian glia promote brain organization. We observed disorganized *gad*$^{+}$ cells after *ets-1* knockdown, suggesting that glial cells might play a role in proper patterning for specific neurons. In addition, we observed reduction of neuropil width after *ets-1*(RNAi) in regeneration and reduction in synapsin staining in the neuropil after *ets-1*(RNAi) in homeostasis. The neuropil is a process- and synapse-rich region devoid of neuronal cell bodies and is the primary location of glial cells. Spatial localization of planarian glia suggests possible roles in assisting in axon and synapse maturity, organization, or function. Further tools to study synaptic structure and activity (*i.e.*, calcium signaling) will be essential for investigation of glial effects on neurotransmission and synapse function. Conversely, our data do not support broad roles for planarian glial cells in regulating neuronal numbers or neuronal survival.

Glial expression of *glutamine synthetase* and *excitatory amino acid transporter* leads us to hypothesize that planarian glial cells regulate neurotransmitter uptake and recycling (Roberts-Galbraith *et al.*, 2016; Wang *et al.*, 2016). We also note that when *ets-1* was perturbed and glial cells are reduced, there was an increase in expression of several genes that encode neurotransmitter biosynthesis enzymes with no corresponding increase in neuronal number (e.g. *gad*). One possible explanation for this observation is that neurons may alter gene expression in response to persistence of extracellular neurotransmitters in the absence of recycling by glial cells (Araque *et al.*, 1999; Oliet *et al.*, 2001).

Additionally, planarian glia are likely to exhibit phagocytic properties, based on their classification as *cathepsin*$^{+}$ cells (Fincher *et al.*, 2018) and functional analyses (Scimone *et al.*,

2018). Vertebrate glia (notably astrocytes, microglia and Schwann cells), *Drosophila* glia, and *C. elegans* glia phagocytose apoptotic neurons and neurite debris during development and following neuronal injury (Aldskogius & Kozlova, 1998; Jessen & Mirsky, 2016; Jung & Chung, 2018; Logan & Freeman, 2007; Sulston *et al.*, 1983). However, new tool development will be necessary to assess the purpose of phagocytosis by planarian glia as well as additional roles for glia in modulating neurons.

Planarian glial cells in behavior and beyond

Our data indicate that loss of glial cells is associated with changes in planarian behavior, based on our finding that *ets-1*(RNAi) impacts the quality and speed of movement in planarians. Locomotion defects were more pronounced when *ets-1*(RNAi) animals initiated movement. We know little about whether planarian glia may impact movement locally at the neuromuscular junction (NMJ), or whether glia impact integration of sensory information, decision making, and initiation of movement through neurons in the CNS. In vertebrates, the NMJ is composed of a presynaptic motor neuron terminal, a post-synaptic muscle cell, and perisynaptic glial cells (typically Schwann cells) that cooperate for motor output (Reddy *et al.*, 2003). Further ultrastructural work could reveal whether planarian glia reside near synapses between neurons and/or between neurons and other cell types.

More work will also be required to determine the basis of movement defects in *ets-1*(RNAi) animals. One possibility is that dysregulation of neurotransmitter abundance in *ets-1*(RNAi) animals impacts negative phototaxis. In particular, gad^{+} GABAergic neurons and th^{+} dopaminergic neurons affect movement in planarians (Nishimura *et al.*, 2007; Nishimura *et al.*, 2008). Both *gad* and *th* mRNAs are dysregulated in *ets-1*(RNAi) animals. Further work will

explore whether neurotransmitter levels are impacted in the absence of glia and whether exogenous neurotransmitters or antagonists could rescue behavioral defects in *ets-1*(RNAi) animals.

In conclusion, a thorough characterization of planarian glia fills gaps in our understanding of glia in an underexplored, highly regenerative phylum. Future work may reveal fascinating new aspects of glial biology, and provide insight into glial evolution, development, and regeneration. Our work with planarian glia provides a valuable point of comparison and contrast with glial cells in other organisms, particularly in the areas of glial function and glial response to injury.

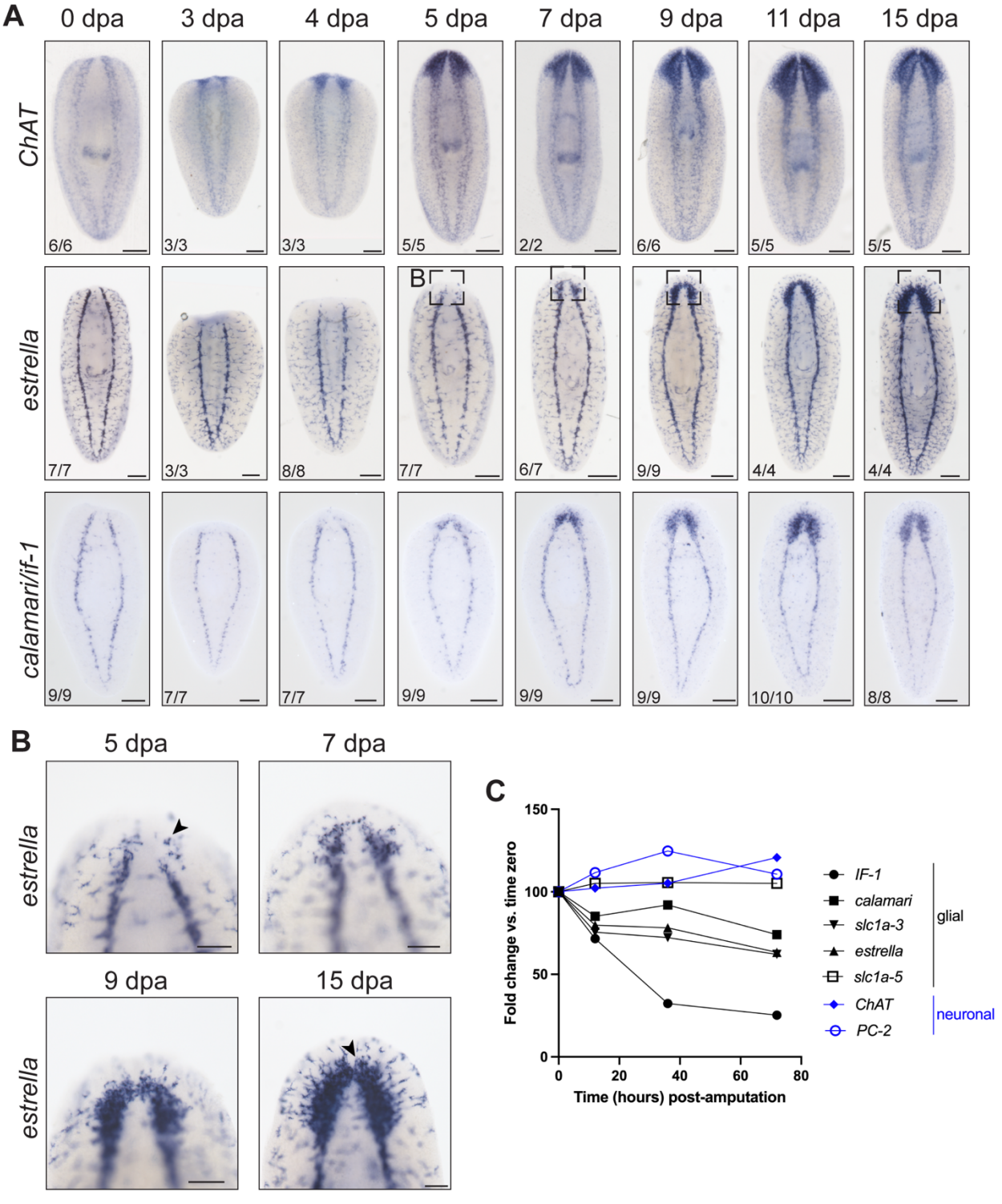

A
0 dpa
3 dpa
4 dpa
5 dpa
7 dpa
9 dpa
11 dpa
15 dpa
ChAT
estrella
calamari/if-1
6/6
3/3
3/3
5/5
2/2
6/6
5/5
5/5
7/7
3/3
8/8
7/7
6/7
9/9
4/4
4/4
9/9
7/7
7/7
9/9
9/9
9/9
10/10
8/8
B
5 dpa
7 dpa
9 dpa
15 dpa
estrella
estrella
C
Fold change vs. time zero
150
100
50
0
0
20
40
60
80
Time (hours) post-amputation
IF-1
calamari
slc1a-3
estrella
slc1a-5
ChAT
PC-2
glial
neuronal

Figure 2.1 Planarian glial cells regenerate after neurons.

(A) ISH regeneration timeline of neurons (top), marked by *choline acetyltransferase* (*ChAT*), and glial cells (bottom), marked by *estrella* or pooled *if-1*/*calamari*. (B) Inset of *estrella* expression in head blastema at 5, 7, 9, and 15 dpa. Arrowheads show round *estrella*$^{+}$ cells (5 dpa) that progress to stellate morphology (15 dpa). (C) RNA-seq of tail fragments regenerating head tissue illustrates glial and neuronal marker transcript levels at early time points post-amputation (adapted from Roberts-Galbraith *et al.*, 2016). Planarian glial markers are downregulated in the first 72 hours post amputation. Ventral view, anterior up. Scale bars: 200 μm; insets 100 μm.

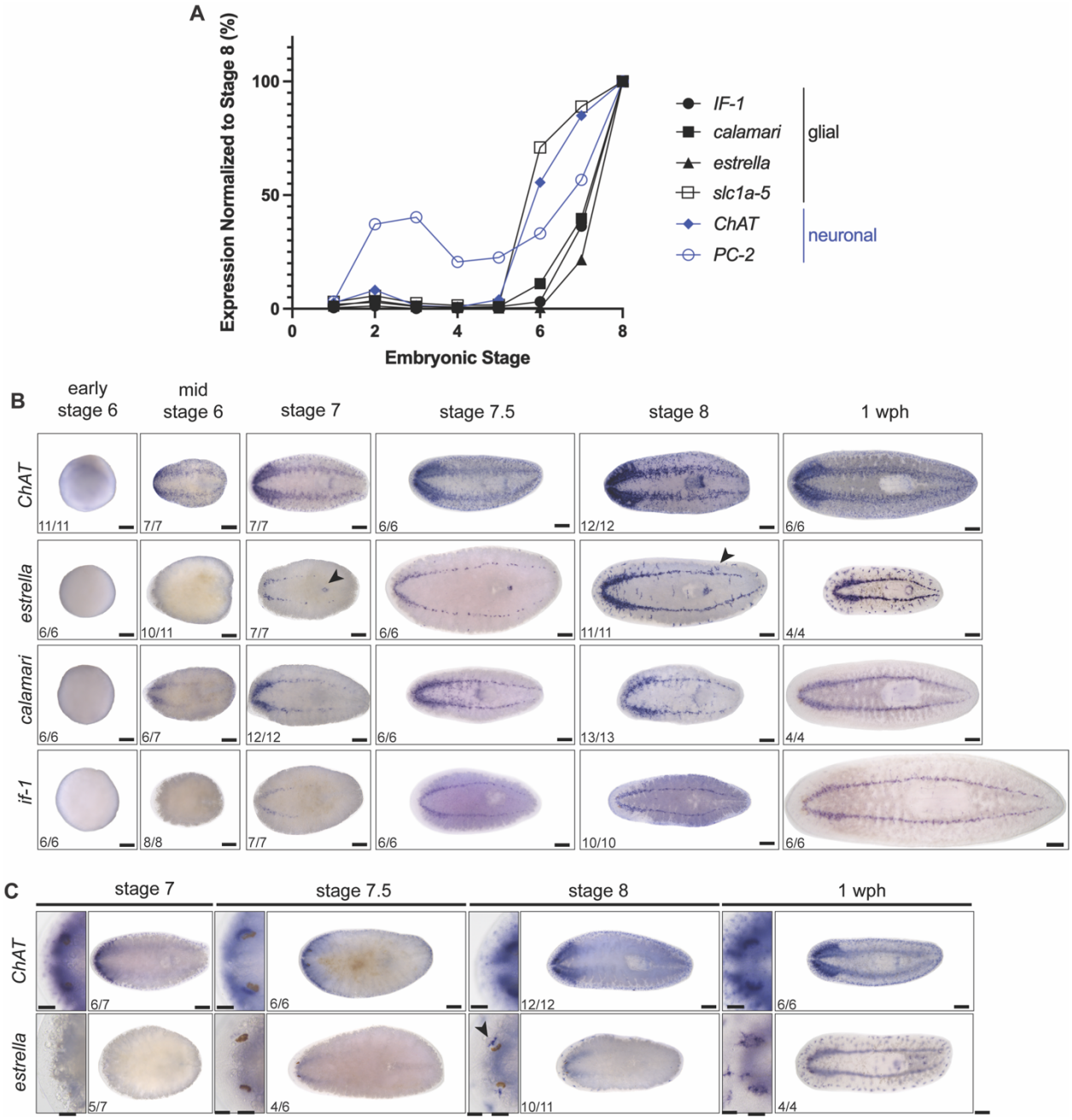


Figure 2.2 Planarian glial cells develop after neurons during embryogenesis.

(A) Single embryo RNA-sequencing shows expression of neuronal and glial markers (adapted from Davies *et al.*, 2017). (B) ISH of neuronal (*ChAT*) and glial markers (*if-1*, *calamari*, *estrella*) in planarian embryos (early S6, mid-S6, S7, S7.5, S8) and juveniles (1 week post-hatching), ventral views. Arrowheads show *estrella* expression in mouth (S7) and peripheral nervous system (S8). (C) ISH of *ChAT* and *estrella* on staged embryos, dorsal views. Insets show

expression near and within the eyespot. *estrella* expression near the eye appears at S8 (indicated by arrowhead); *ChAT* expression, in contrast, is seen as early as S7. Anterior left. Scale bars: 200 μm; eyes 50 μm.

Figure 3

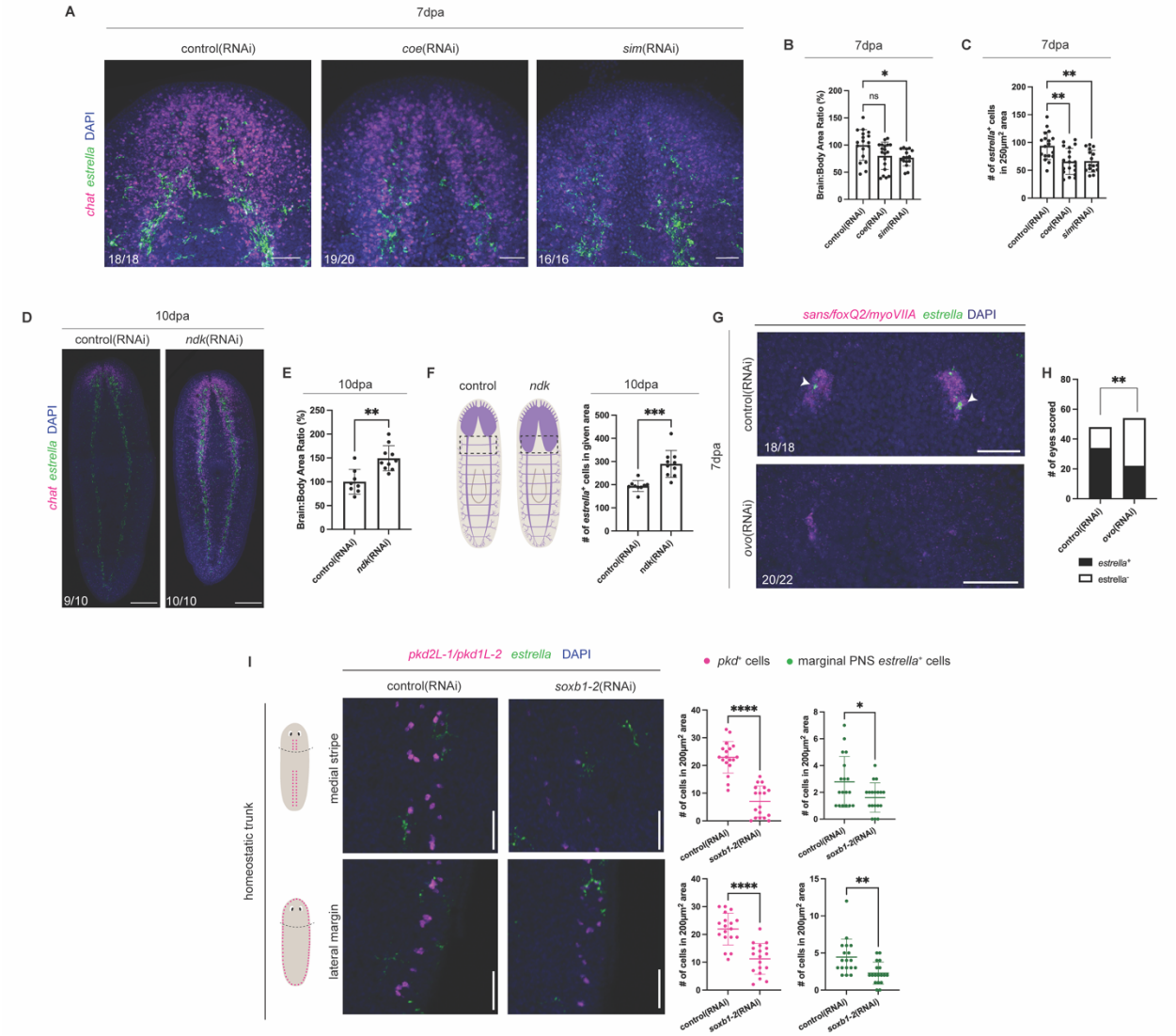


Figure 2.3 Presence of *estrella*$^+$ cells is neuron dependent.

(A) FISH of regenerated control, *coe*(RNAi) and *sim*(RNAi) animals detecting *ChAT* (neurons, magenta) and *estrella* (glia, green) transcripts and stained with DAPI (nuclei, blue). (B) Quantification of brain-to-body ratio (normalized so control is 100%). Unpaired t-test with Welch's correction. (C) Quantification of *estrella*$^+$ cells in head region in 250 μm^2 areas. Unpaired t-test with Welch's correction. (D) FISH of regenerated control and *ndk*(RNAi) animals detecting *ChAT* (magenta), *estrella* (green), and DAPI (blue). (E) Quantification of normalized brain-to-body ratio. Unpaired t-test with Welch's correction. (F) Quantification of *estrella*$^+$ cells in expanded posterior of the brain (depicted in illustration) in control and *ndk*(RNAi) animals. Unpaired t-test with Welch's correction. (G) FISH of regenerated control

and *ovo*(RNAi) animals detecting pooled *sans/foxQ2/myoVIIA* (photoreceptor neurons, magenta), *estrella* (green), and DAPI (blue). Arrowheads indicate *estrella*$^{+}$ cells in or near the eyespot. (H) Quantification of *estrella*$^{+}$ (black) or *estrella*$^{-}$ (white) eyespots after control or *ovo*(RNAi). Fisher's exact test. (I) FISH of control and *soxB1-2*(RNAi) animals detecting pooled *pkd2L-1*/*pkd1L-2* (sensory neurons, magenta), *estrella* (green), and DAPI (blue) in medial stripe and lateral margins in non-regenerated trunk tissue (dorsal view). Quantification of *pkd*$^{+}$ or *estrella*$^{+}$ cells in respective regions illustrated at the left. Unpaired t-test with Welch's correction (n=18). *p-value≤0.05, **p-value≤0.01, ***p-value≤0.001, ****p-value≤0.0001, ns=not significant. Scale bar: (A, G) 50 µm (D, I) 200µm.

Figure 4

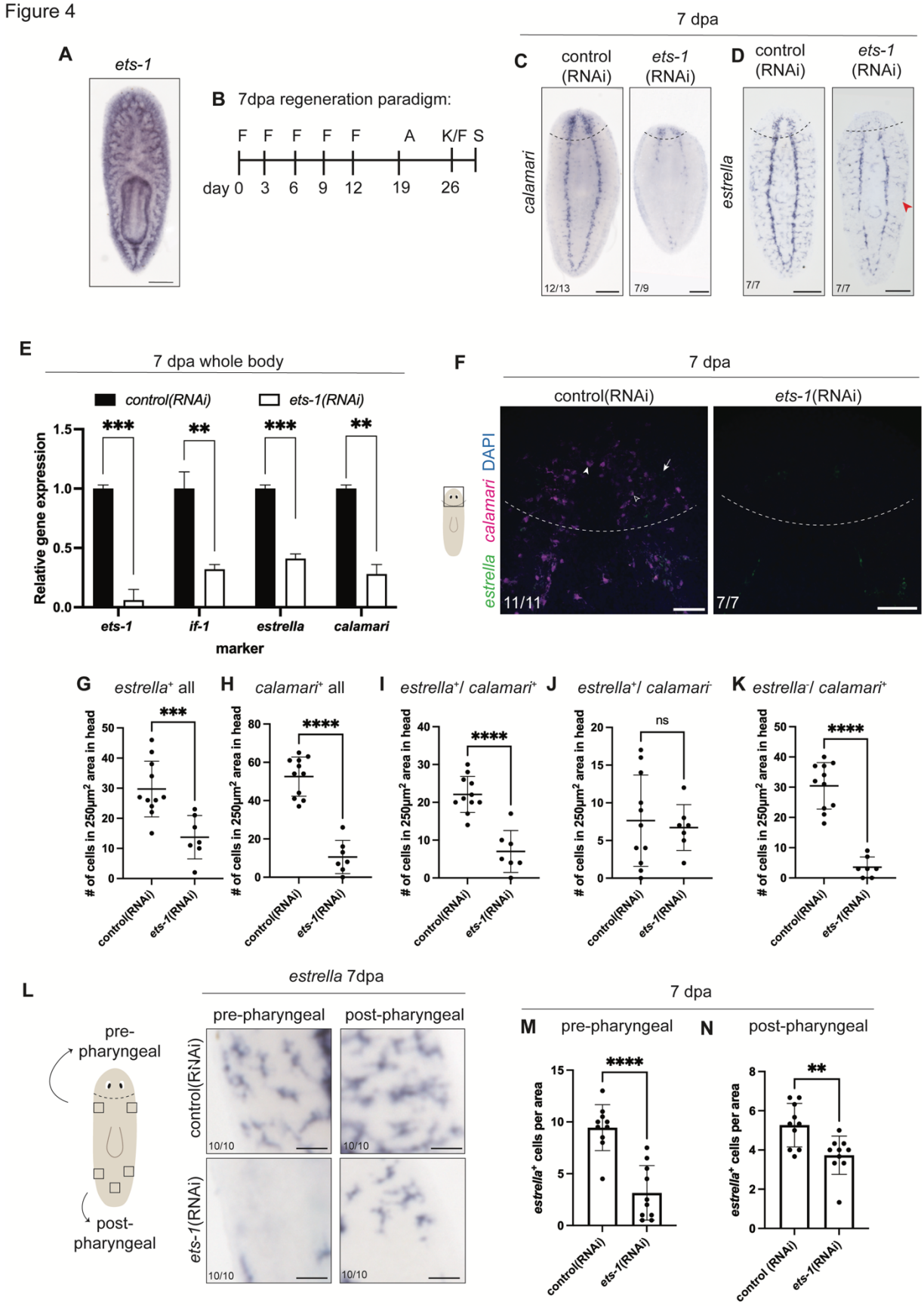

A
ets-1
B 7dpa regeneration paradigm:
F F F F F A K/F S
day 0 3 6 9 12 19 26
7 dpa
C control (RNAi) ets-1 (RNAi)
calamari
12/13
7/9
D control (RNAi) ets-1 (RNAi)
estrella
7/7
7/7
E
7 dpa whole body
control(RNAi) ets-1(RNAi)
Relative gene expression
***
**
***
**
ets-1 if-1 estrella calamari
marker
F
7 dpa
control(RNAi) ets-1(RNAi)
DAPI
calamari
estrella
11/11
7/7
G estrella+ all
H calamari+ all
I estrella+/ calamari+
J estrella+/ calamari-
K estrella-/ calamari+
# of cells in 250µm2 area in head
control(RNAi) ets-1(RNAi)
***
****
****
ns
****
L
estrella 7dpa
pre-pharyngeal post-pharyngeal
pre-pharyngeal
post-pharyngeal
control(RNAi)
ets-1(RNAi)
10/10
7 dpa
M pre-pharyngeal
N post-pharyngeal
estrella+ cells per area
****
**
control(RNAi) ets-1(RNAi)

Figure 2.4 *ets-1* affects glial gene expression.

(A) WISH of *ets-1* in uninjured, untreated animals. (B) RNAi feeding paradigm for *ets-1*(RNAi). Feeding (F), amputation (A), kill/fix (K/F) and staining (S) are indicated. (C-D) ISH of *ets-1*(RNAi) regenerated animals detecting *calamari* or *estrella* expression. (E) RT-qPCR used to detect levels of *ets-1, if-1, estrella,* and *calamari* transcripts after RNAi and regeneration. Unpaired t-test; error bar: SEM. (F) FISH with *calamari* (magenta), *estrella* (green), and DAPI (cell nuclei, blue) in newly regenerated heads in control and *ets-1*(RNAi) animals. Full arrowhead = *cali*$^{+}$/*estrella*$^{-}$; open arrowhead = *cali*$^{+}$/*estrella*$^{+}$; arrow = *cali*$^{-}$/*estrella*$^{+}$. (G-K) Quantification of glia markers in 250 μm^2 areas within head blastemas from F. Unpaired t-test with Welch's correction. (L-N) Illustration showing 200 μm^2 boxes drawn to quantify PNS *estrella*$^{+}$ cells. Insets and quantifications show reduced peripheral *estrella*$^{+}$ cells in *ets-1*(RNAi) animals (see also red arrowhead in D). Unpaired t-test with Welch's correction. Dashed lines: amputation sites. *p-value≤0.05, **p-value≤0.01, ***p-value≤0.001, ****p-value≤0.0001, ns=not significant. Scale bar: (A, C, D, F) 200 μm, (L) 50 μm.

Figure 5

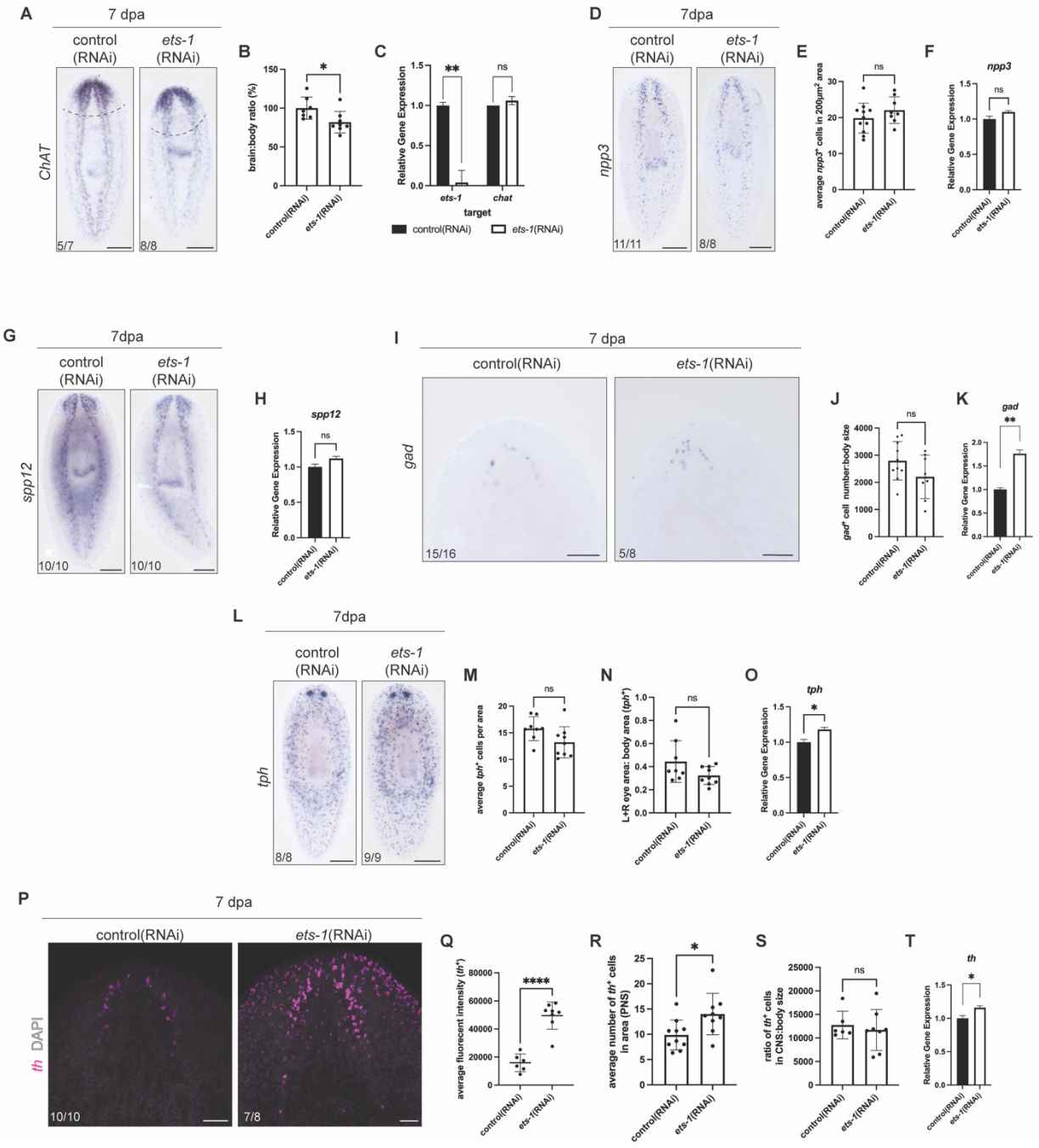


Figure 2.5 Knockdown of *ets-1* does not affect neuronal cell numbers.

(A) *ChAT* ISH of regenerated control and *ets-1*(RNAi) animals. Dashed line: amputation site. (B) Brain-to-body ratio quantification show reduced brain area for *ets-1*(RNAi) animals. Unpaired t-test with Welch's correction. (C) RT-qPCR was used to detect levels of *ets-1* and *ChAT*

transcripts in regenerated RNAi animals; the same cDNA samples were used for Fig. 5F, H, K, O, and T). Unpaired t-test; error bars: SEM. (D) 7 dpa control and *ets-1*(RNAi) animals subjected to ISH with *neuropeptide precursor*-3 (*npp3*). (E) *npp-3*$^{+}$ cells counted in 200 μm^2 areas throughout the body. Unpaired t-test with Welch's correction. (F) *npp-3* transcript levels detected with RT-qPCR in regenerated RNAi animals. Unpaired t-test, error bar: SEM. (G) ISH of 7 dpa control and *ets-1*(RNAi) animals with *secreted peptide prohorome*-12 (*spp12*) . (H) *spp-12* transcript levels detected with RT-qPCR in regenerated RNAi animals. Unpaired t-test, error bar: SEM. (I) ISH of control and *ets-1*(RNAi) animals with *glutamic acid decarboxylase* (*gad*). 62.5% of *ets-1*(RNAi) animals had disorganized *gad*$^{+}$ arch pattern. (J) Quantification of *gad*$^{+}$ cells, normalized to body size. Unpaired t-test with Welch's correction. (K) *gad* transcripts levels detected with RT-qPCR in regenerated RNAi animals. Unpaired t-test; error bar: SEM. (L) ISH of regenerated animals in control and *ets-1*(RNAi) animals with *tryptophan hydroxylase* (*tph*). (M-N) Quantification of *tph*$^{+}$ cells in specific areas throughout the body and within the eye compared to body size. Unpaired t-test with Welch's correction. (O) RT-qPCR detecting *tph* transcript levels in regenerated RNAi animals. Unpaired t-test, error bar: SEM. (P) 7 dpa control and *ets-1*(RNAi) animals subjected to FISH with *tyrosine hydroxylase* (*th,* magenta) and DAPI (grey). (Q) Average fluorescent intensity of *th* FISH was quantified for control and *ets-1*(RNAi) animals. Unpaired t-test with Welch's correction. (R) *th*$^{+}$ cells in the PNS were counted in 100 μm^2 areas. Unpaired t-test with Welch's correction. (S) Quantification of *th*$^{+}$ cells in the CNS was quantified in control and *ets-1*(RNAi) animals and normalized to body size. Unpaired t-test with Welch's correction. (T) RT-qPCR detecting *th* transcript levels in regenerated RNAi animals. Unpaired t-test, error bar: SEM. *p-value≤0.05, **p-value≤0.01, ****p-value≤0.0001, ns=not significant. Scale bars: (A,D,G,L) 200μm (I) 100μm (P) 50μm.

Figure 6

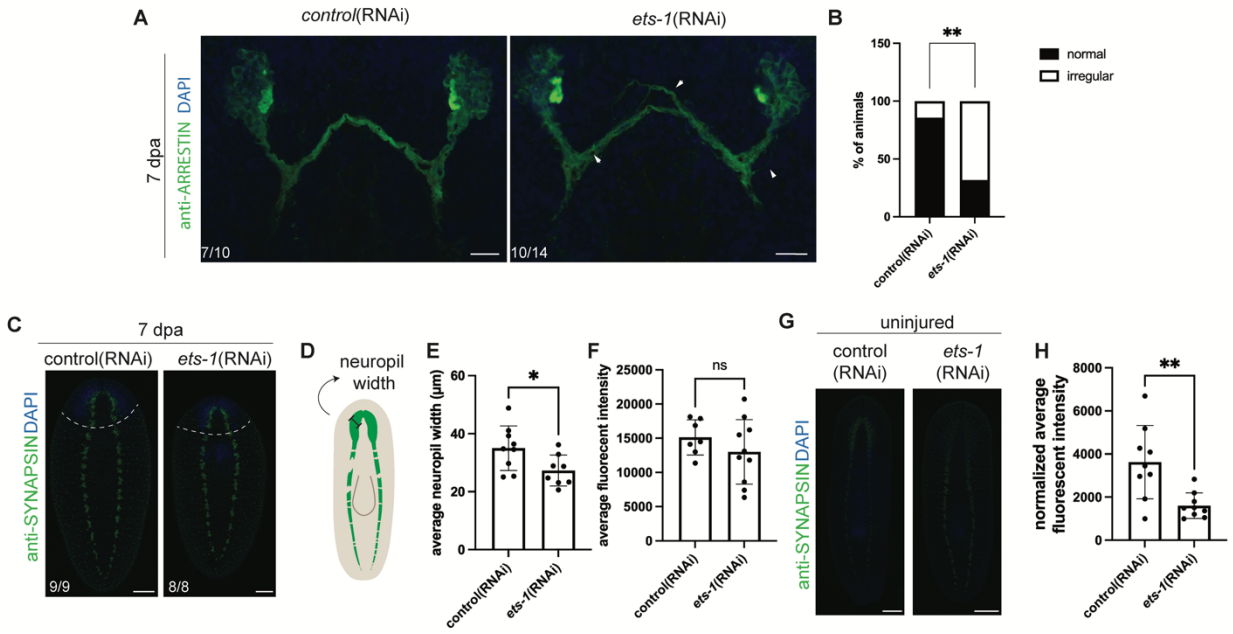


Figure 2.6 *ets-1* knockdown results in changes in neural architecture.

(A) 7 dpa regenerated control and *ets-1*(RNAi) animals were subjected to immunofluorescence (IF) against Arrestin (photoreceptor axons, green) with DAPI (blue). *ets-1*(RNAi) animals exhibited several defects in axon fasciculation (see arrowheads). (B) Percentage of control and *ets-1*(RNAi) animals exhibiting one or more defects in axon fasciculation. See methods for our criteria for "irregular" organization. Fisher's exact test. (C) IF with anti-Synapsin (synapses, green) and DAPI (blue) in regenerated control and *ets-1*(RNAi) animals. Dashed line: amputation site. (D) Illustration of neuropil width measurement. (E-F) Quantification of neuropil width and average fluorescent intensity. Unpaired t-test with Welch's correction. (G) IF with anti-Synapsin (green) and DAPI (blue) in uninjured control and *ets-1*(RNAi) animals. (H) Quantification of average fluorescent intensity. Unpaired t-test with Welch's correction. *p-value≤0.05, **p-value≤0.01, ns=not significant. Scale bars:(A) 20 µm (C) 100 µm (G) 200 µm.

Figure 7

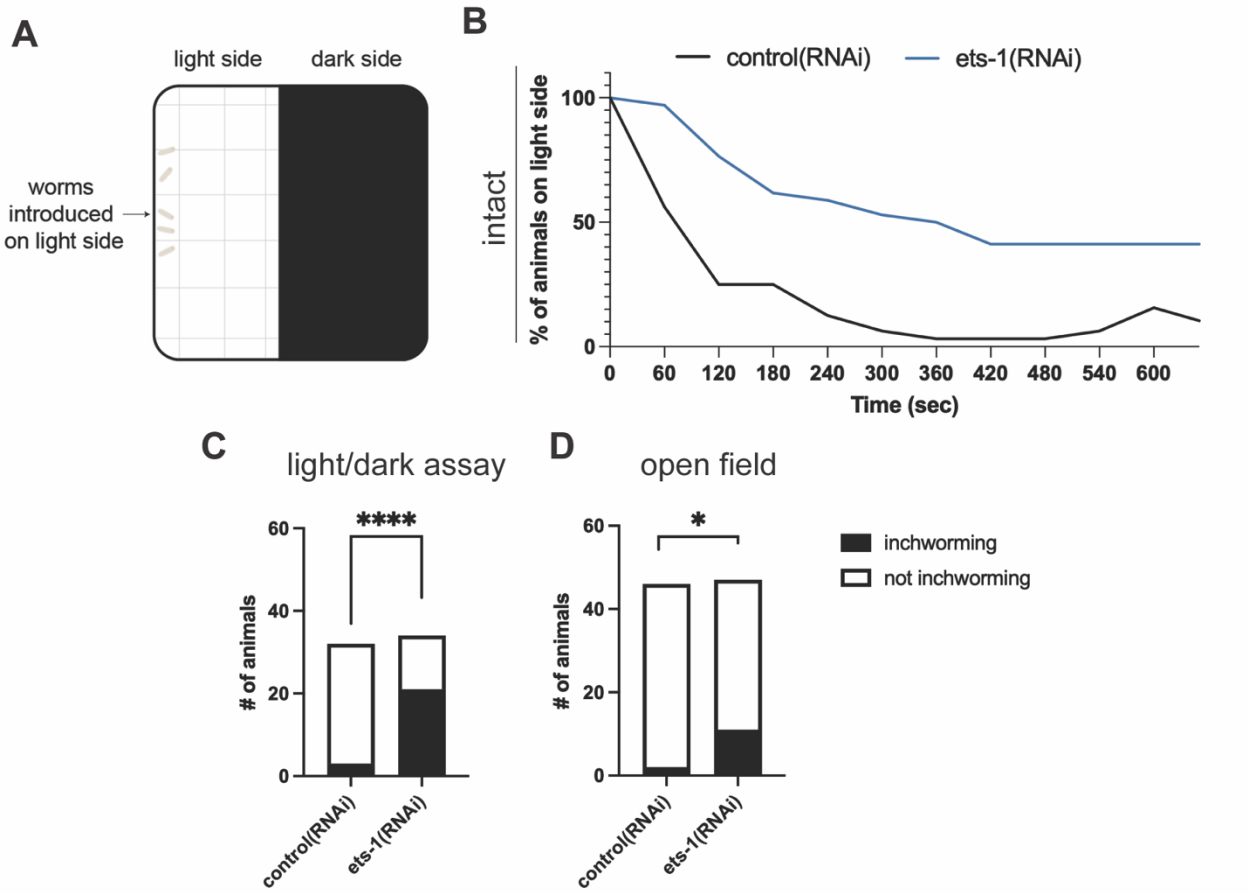
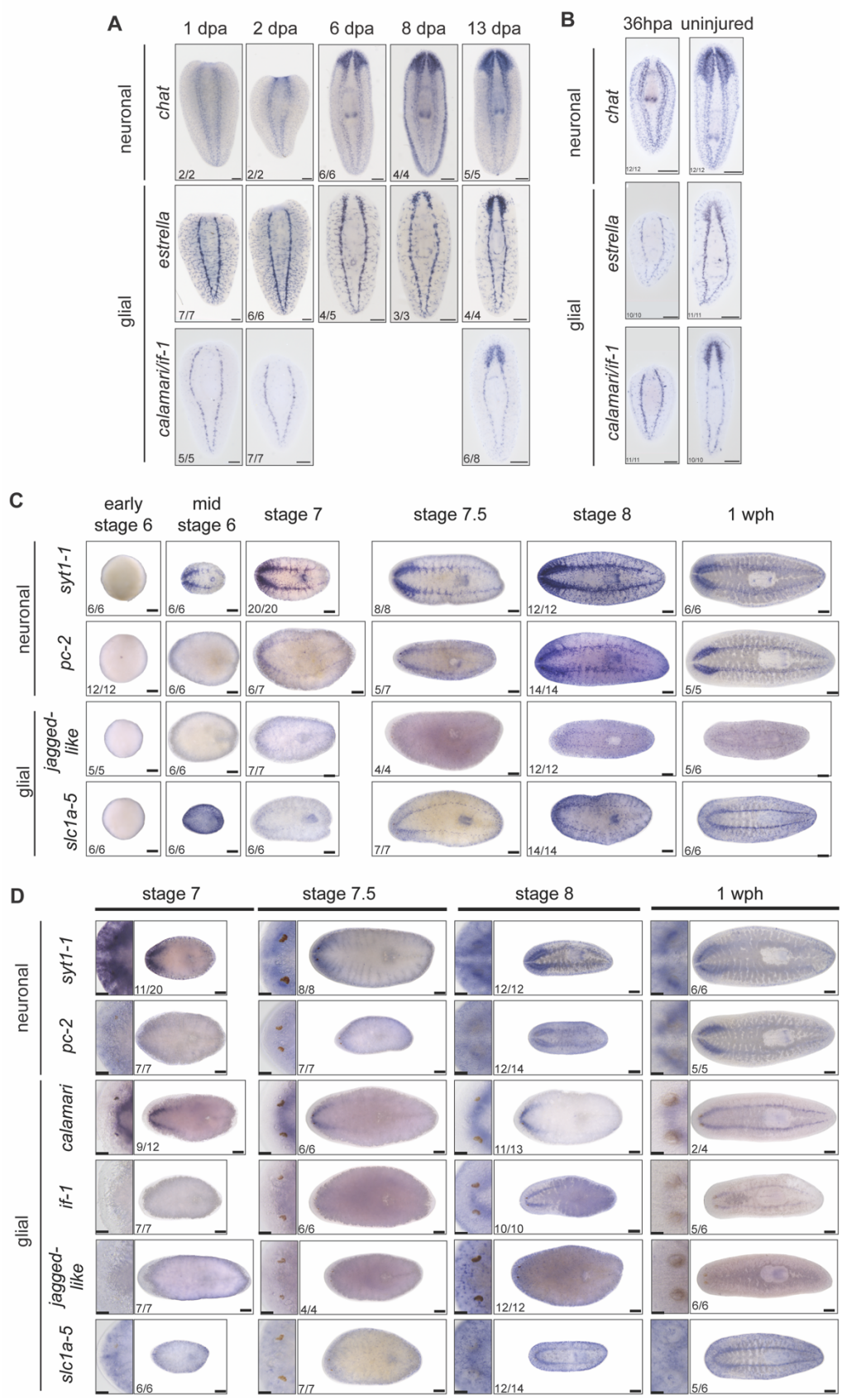


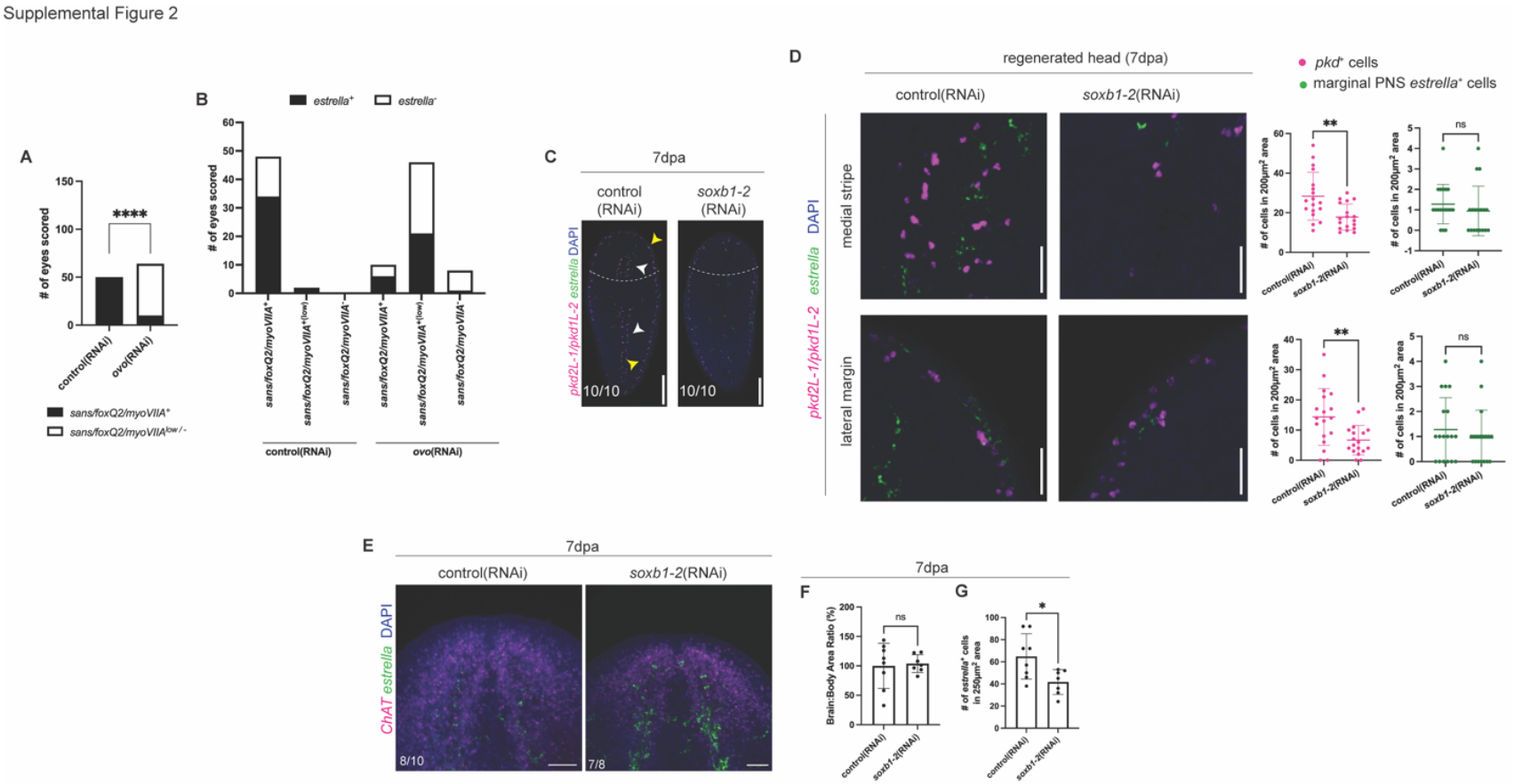
Figure 2.7 *ets-1* knockdown leads to changes in planarian behavior.

(A) Illustration of light/dark assay (Paskin *et al.*, 2014; Zewde *et al.*, 2018). (B) Graph shows percentage of intact animals on the light side (n=10-12 animals per replicate; 3 replicates, see also Fig. S9A). (C) Quantification of animals that exhibit inch-worming in the context of a light/dark assay. Fisher's exact test. (D) In an open field, *ets-1*(RNAi) still led to a significantly higher incidence of inch-worming behavior. Fisher's exact test. *p≤0.05, ****p≤0.0001.

Supplemental Figure 1



Supplemental Figure 2.1 Neuronal and glial gene expression during head regeneration.
(A) ISH regeneration timeline of neurons (*ChAT*) and glia (*estrella*, pooled *if-1*/*calamari*) in asexual planarians at 1, 2, 6, 8, and 13 days after head amputation (dpa). Ventral view, anterior up, from same time series as Fig 1A. (B) ISH of *ChAT* and glial markers 36 hours post head amputation (hpa) and in uninjured asexual planarians. (C) ISH of neuronal markers (*pc-2, syt1-1*) and glial markers (*slc1a-5/EAAT, jagged-like*) in planarian embryos (early S6, mid-S6, S7, S7.5, S8) and juveniles (1 week post-hatching). Anterior to left, ventral views. (D) ISH of neuronal markers *(pc-2, syt1-1)* and glial markers (*if-1*, *calamari*, *jagged-like*, *slc1a-5/eaat*) at indicated developmental stages. Dorsal views. Insets show expression in or near the eyes. Scale bars: whole animal images 200 μm; eyes 50 μm.

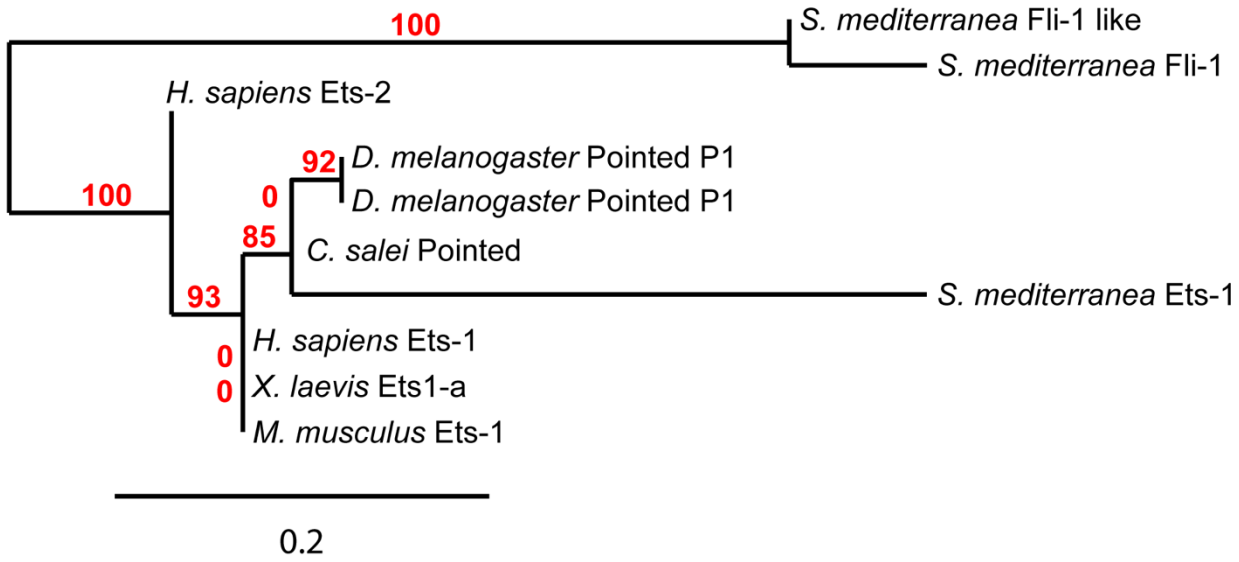


Supplemental Figure 2.2 Neural roles in glial regeneration.

(A) Quantification of photoreceptor neuron markers (pooled *sans*/*foxQ2*/*myoVIIA*) present (black) or reduced/absent (white) in control and *ovo*(RNAi) eyes. Left and right eyes were considered separately. Fisher's exact test. (B) Glia in *ovo*(RNAi) eyes separated by category based on *sans*/*foxQ2*/*myoVIIA* presence (+/low/-). (C) 7 dpa control and *soxB1-2*(RNAi) animals subjected to FISH detecting pooled *pkd2L-1*/*pkd1L-2* (magenta), *estrella* (green), and DAPI (blue). Arrowheads indicate where dorsal *estrella*$^{+}$ cells were

observed: medial stripe (white) and lateral margin (yellow). Dashed lines: amputation site. Whole body animal is shown from Figure 3I. Dorsal view. (D) Insets showing *estrella*$^{+}$ cells (green) and *pkd2L-1/1L-2*$^{+}$ cells (magenta) in regenerated head tissue in respective regions. Quantification of each cell type in given area is presented on the right. Unpaired t-test with Welch's correction. (E) 7 dpa control and *soxB1-2*(RNAi) animals subjected to FISH detecting *ChAT* (magenta) and *estrella* (green), and DAPI (blue). Unpaired t-test with Welch's correction. (F) Quantification of brain-to-body ratio for *soxb1-2*(RNAi) animals normalized to control. Unpaired t-test with Welch's correction. (G) Quantification of *estrella*$^{+}$ cells in regenerated heads in 200 μm^2 areas of control and *soxb1-2*(RNAi) animals. Unpaired t-test with Welch's correction. Anterior up. *p-value≤0.05, **p-value≤0.01, ns=not significant. Scale bar: (C) 200 μm (D,E) 50 μm.

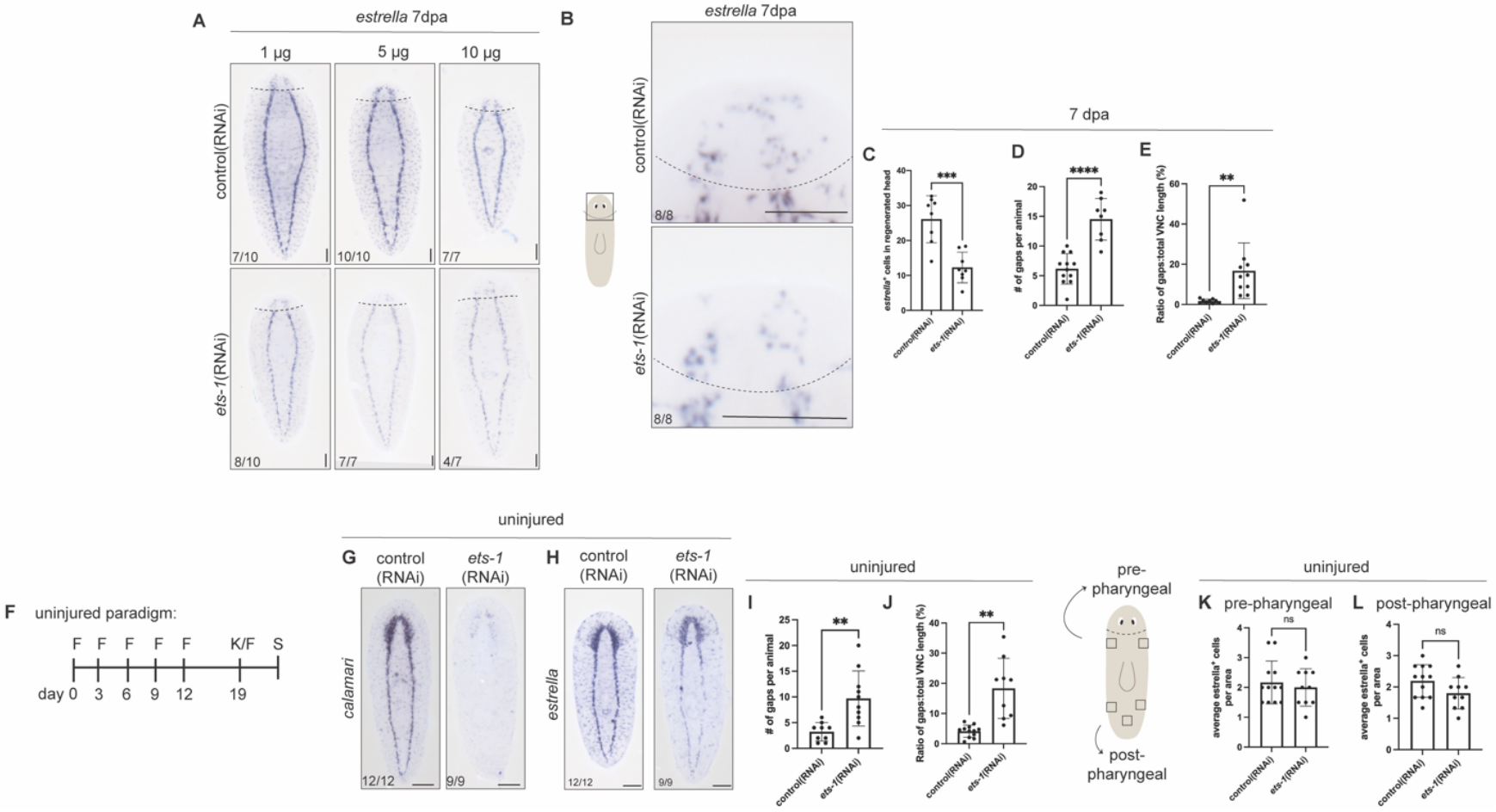
Supplemental Figure 3



Supplemental Figure 2.3 Ets-1 protein sequence is highly conserved.

Phylogenetic tree of Ets-1 protein sequence of 6 species based on longest open reading frame. Analysis shows the relationship between planarian Ets-1 and Ets-1 protein sequences in other species. The outgroup is planarian Fli-1 and Fli-1-like proteins, two proteins that possess an ETS domain, but have no established role in glial cells. Red text denotes the percentage of support for each node.

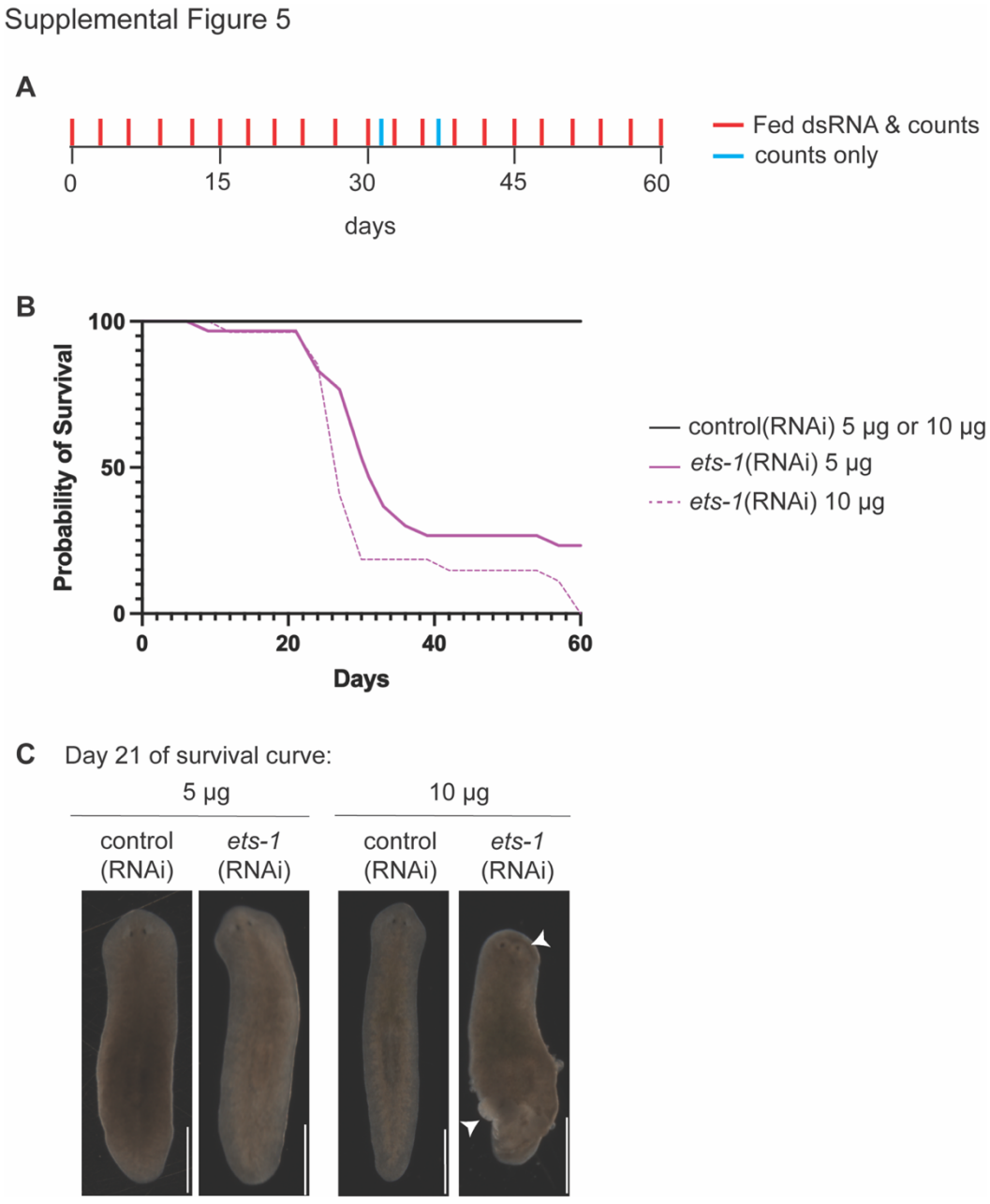
Supplemental Figure 4



Supplemental Figure 2.4 Ets-1 knockdown phenotype on glial markers varies depending on dosage.

(A) Animals were fed 1 µg, 5 µg or 10 µg *in vitro*-transcribed dsRNA targeting *ets-1* for 5 feedings 3 days apart. Animals were amputated, fixed at 7 dpa, and subjected to ISH with *estrella*. 5 µg and 10 µg regimens yielded more robust reduction of *estrella*$^{+}$ cells

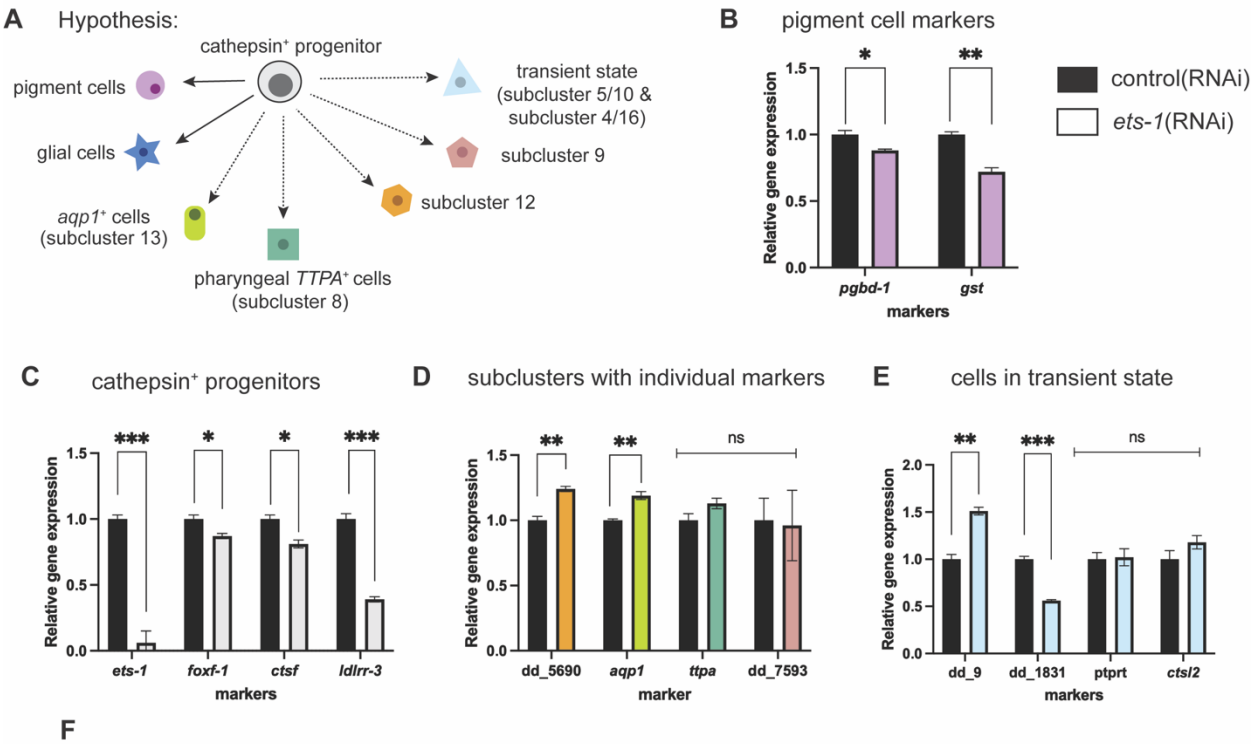
in regenerated heads in *ets-1*(RNAi) animals. (B) *ets-1*(RNAi) resulted in fewer *estrella*$^{+}$ cells present in 7 dpa regenerated heads compared to control. (C) Quantification of *estrella*$^{+}$ cells in regenerated head blastemas in control and *ets-1*(RNAi) animals. Regenerated *ets-1*(RNAi) animals have (D) more gaps and (E) longer stretches of VNC gaps compared to control. Dashed lines: amputation site. (F) Uninjured RNAi paradigm. (G-H) Uninjured control and *ets-1*(RNAi) animals were subjected to ISH with glial markers *calamari* and *estrella*. (I-J) Quantification of number of gaps, and percentage of VNC with gaps in uninjured control and *ets-1*(RNAi) animals. (K-L) Quantification of *estrella*$^{+}$ cells in the PNS in uninjured control and *ets-1*(RNAi) animals. Unpaired T-test with Welch's correction. **p-value≤0.01, ***p-value≤0.001, ****p-value≤0.0001, ns=not significant (unpaired t-test with Welch's correction). Scale bar: 200 μm.



Supplemental Figure 2.5 *Ets-1* knockdown leads to eventual animal death.

(A-B) Animal cohorts were subjected to a 60-day dsRNA-feeding regimen, with feeding every 3 days. Red: Days with feeding and animal counts. Blue: days with animal counts only. Survival curve depicting the relative percentage of surviving animals at different dsRNA feeding doses after long term RNAi; N=30 animals each. (C) Live images of control and *ets-1*(RNAi) animals at day 21 (post 8th feeding). *ets-1*(RNAi) animals at 10 µg dsRNA exhibited lesions (white arrowheads) in the tail and around eyes that led to eventual lysis. Scale bar: 200 µm.

Supplemental Figure 6



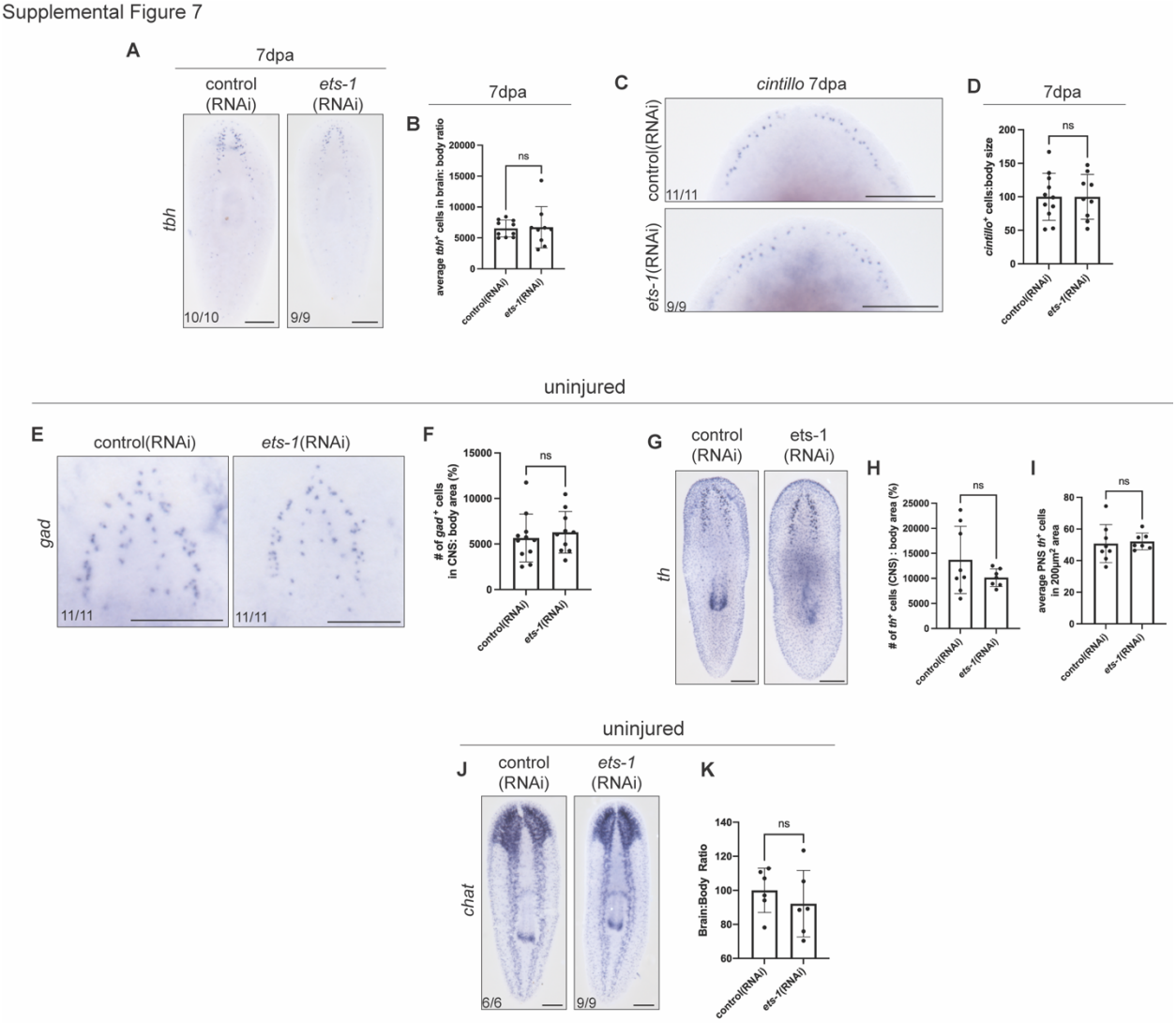
F

cell cluster	7 dpa (head) qPCR	5 dpa (sagittal) RNA-seq from Dubey *et al.* 2022	homeostasis RNA-seq from Dubey *et al.* 2022
cathepsin⁺ progenitor	↓	↓	↓
pigment cells	↓	↓↑	↑ (n.s.)
glial cells	↓	↓↑ (n.s.)	↓↑ (n.s.)
aqp1⁺ cells (subcluster 13)	↑	↓↑ (n.s.)	↓↑ (n.s.)
pharyngeal *TTPA*⁺ cells (subcluster 8)	↑ (n.s.)	↓↑	↓↑
subcluster 12	↑	↓↑ (n.s.)	↓↑ (n.s.)
subcluster 9	↓ (n.s.)	↓	↓↑ (n.s.)
transient state (subcluster 5/10 & subcluster 4/16)	↓↑	↓	↓↑ (n.s.)

Supplemental Figure 2.6 *ets-1* affects gene expression in individual *cathepsin*⁺ cell types in distinct ways.

(A) Graphical illustration of *cathepsin*⁺ cell subclusters identified in single cell transcriptomic atlases (Fincher *et al.*, 2018; Plass *et al.*, 2018). Clusters are color-coded as follows: grey =

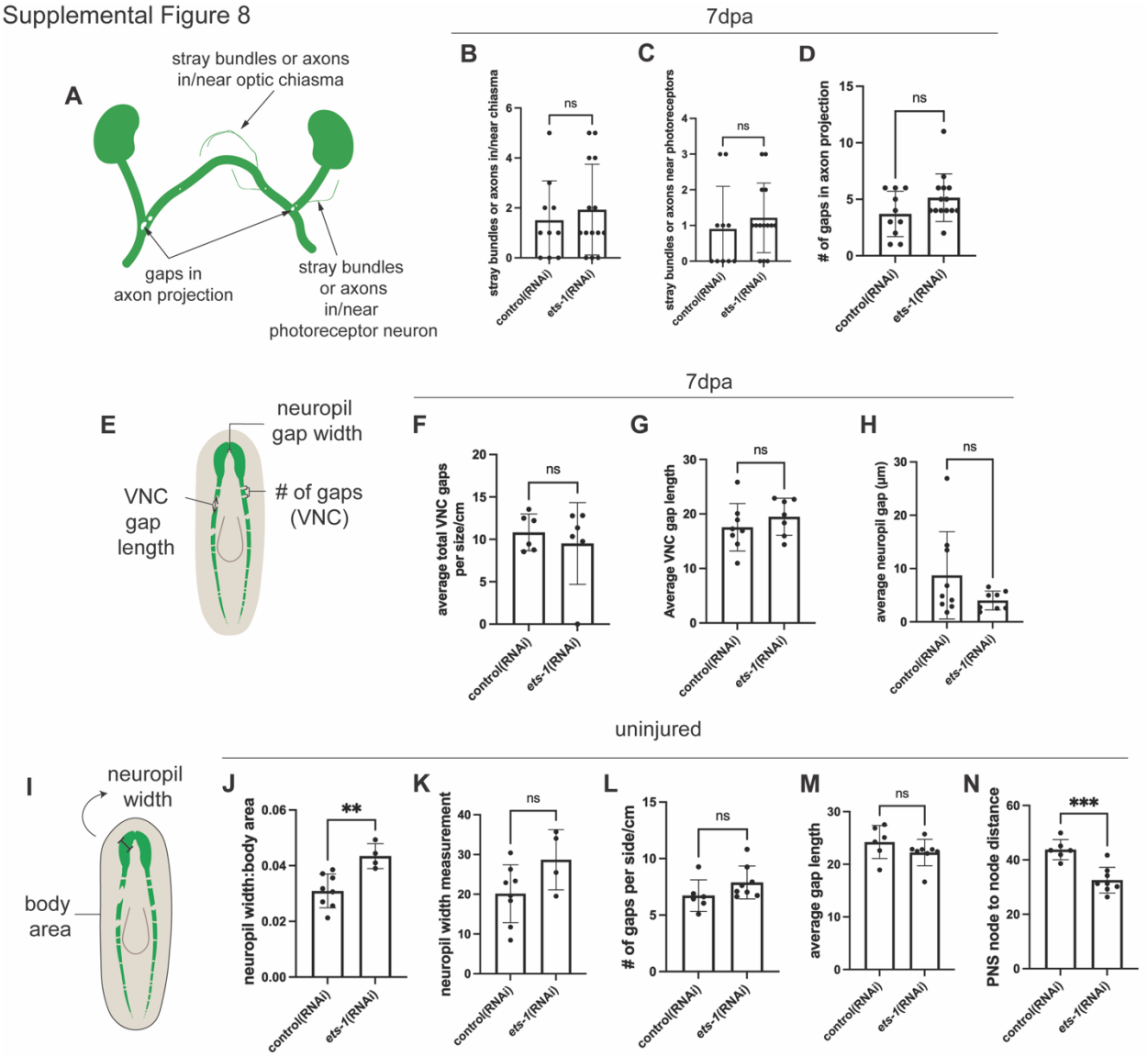
cathepsin^{+} progenitor cells, purple = pigment cells, dark blue = glial cells, light green = *aqp1*$^{+}$ cells, green = *TTPA*$^{+}$ cells, orange = subcluster 12, pink = subcluster 9, light blue = subclusters 5/10 and subcluster 4/16. Lineage relationships are drawn as currently hypothesized. (B-E) RT-qPCR was used to detect levels of *ets-1* and other markers of *cathepsin*$^{+}$ subclusters after RNAi with color coding as in A. Details of each marker are provided in Table S3. *p≤0.05, **p-value≤0.01, ***p-value≤0.001, ****p-value≤0.0001, ns=not significant (Unpaired t-test, error bars: SEM). (F) Summary table of trends seen after *ets-1*(RNAi) in each *cathepsin*$^{+}$ cell type from qPCR data and published RNA-seq data (Dubey *et al.*, 2022). Details of each gene analyzed under each subcluster are provided in Table S4.



Supplemental Figure 2.7 *ets-1* knockdown does not affect neuronal cell number.

(A) 7 dpa control and *ets-1*(RNAi) animals were subjected to ISH with *tyramine beta-hydroxylase* (*tbh*) riboprobe. (B) Quantification of *tbh*$^{+}$ cells in brain (compared to body size). (C) Regenerated control and *ets-1*(RNAi) animals subjected to ISH with sensory neuron marker *cintillo*. (D) Quantification of *cintillo*$^{+}$ cells compared to body size in control and *ets-1*(RNAi) animals. (E) Uninjured control and *ets-1*(RNAi) were subjected to ISH with *gad* riboprobe. (F) Quantification of *gad*$^{+}$ cell numbers in the CNS compared to body size in uninjured control and *ets-1*(RNAi) animals. (G) ISH of uninjured control and *ets-1*(RNAi) animals against riboprobe marker *th*. (H-I) Quantification of *th*$^{+}$ cells in CNS and PNS normalized to body size or specific area, respectively, in *ets-1*(RNAi) animals compared to control. (J) ISH of uninjured control and

ets-1(RNAi) animals with riboprobe against *ChAT*. (K) Quantification of brain-to-body ratio (normalized to control). Unpaired t-test with Welch's correction. ns=not significant. Scale bar: 200 μm.

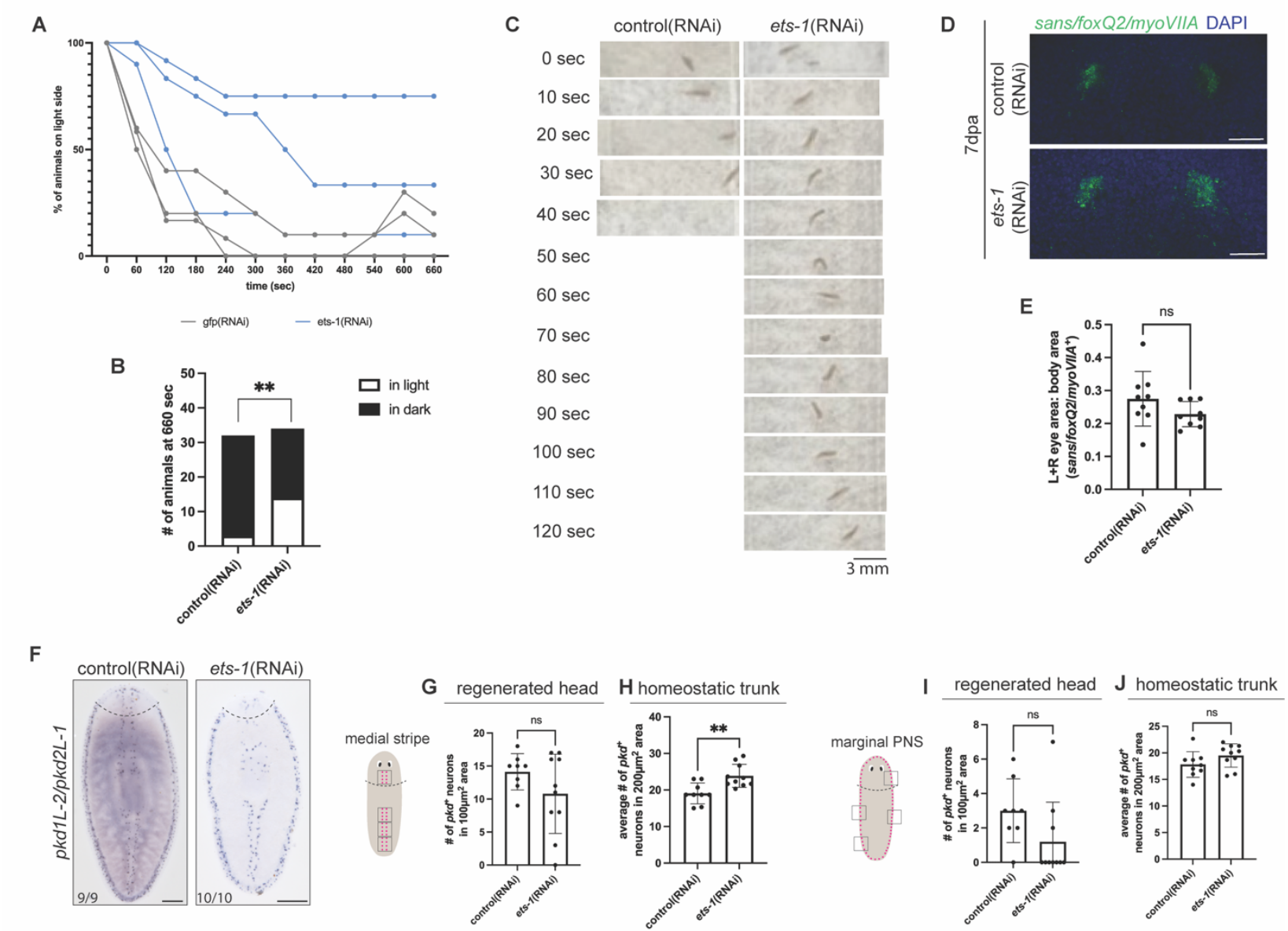


Supplemental Figure 2.8 *ets-1* knockdown affects neural architecture.

(A) Graphical illustration of criteria quantified individually in anti-Arrestin immunofluorescence images: stray bundles or axons in/near optic chiasm; stray bundles or axons in/near photoreceptor neuron; and gaps in axon projection. (B-D) Quantification of stray bundles or axons in or near the optic chiasm, near photoreceptors (left and right), and gaps in axon projections in control and *ets-1*(RNAi) animals. Each point is an individual animal. Aggregated data are in Fig. 6B. (E) Graphical illustration of criteria that were quantified from anti-Synapsin immunofluorescence images: gap length, number of gaps, and neuropil gap length. (F-H) Quantification of total gaps in VNC per cm, average gap size in VNC (µm), and average gap

length between neuropils (μm) for regenerated control and *ets-1*(RNAi) animals. (I) Graphical illustration of additional criterion (beyond those shown in Fig. S8E) that was quantified from anti-Synapsin immunofluorescence images in uninjured animals: neuropil width (compared to body area). (J-N) Quantification of neuropil width compared to body area, average neuropil width measurement, number of total gaps in VNC per cm, average gap size in VNC (μm), and average PNS node-to-node distance for uninjured control and *ets-1*(RNAi) animals. Unpaired t-test with Welch's correction. **p-value≤0.01, *** p-value≤0.001, ns = not significant.

Supplemental Figure 9



Supplemental Figure 2.9 Individual replicates of *ets-1*(RNAi) animals exhibited changes in phototaxis behavior.
(A) Graph showing percentage of animals from individual replicates of control and *ets-1*(RNAi) animals that remained on the light side during filming across 660 seconds. Three replicates were completed (n=10-12 per replicate; aggregated data are in Fig.7B). (B) Quantification of the number of control and *ets-1*(RNAi) animals what were in the light or dark side of the dish at 660 seconds. Fisher's exact test. (C) Image stills of control and *ets-1*(RNAi) animals from Supplemental Video 1A-B. (D) Control and *ets-1*(RNAi) regenerated animals were subjected to FISH with pooled photoreceptor neuron markers *sans*/*foxQ2*/*myoVIIA* (green) and DAPI (blue). (E) Quantification of photoreceptor neuron marker area (normalized to body size) in *ets-1*(RNAi) animals compared to control. (F) Control and *ets-1*(RNAi) regenerated animals were subjected to ISH with pooled sensory neuron marker *pkd1L-2/2L-1*. (G-H) Quantification of *pkd1L-2/2L-1*$^{+}$ cells in the medial stripe in the regenerated head and homeostatic trunk. (I-J) Quantification of *pkd1L-2/2L-1*$^{+}$ cells in the marginal PNS in regenerated heads and homeostatic trunks. Unpaired t-test with Welch's correction. **p-value≤0.01, ns = not significant. Scale bar (B) 50 μm, (D) 200 μm.

Supplemental Video 2.1 *ets-1*(RNAi) animals have changes in negative phototaxis behavior. (A) Control animals before head amputation in light/dark assay, 20x playback speed. (B) *ets-1*(RNAi) animals before head amputation in light/dark assay, 20x playback speed. Video only shows the light side of the dishes.

Supplemental Video 2.2 ets-1(RNAi) animals exhibit locomotion defects.
(A) Control animals before head amputation with no stimulus, 20x playback speed. (B) *ets-1*(RNAi) animals before head amputation with no stimulus, 20x playback speed.

Supplemental Table 2.1 List of cloning primers.
Supplemental Table 2.2 List of protein sequences for phylogeny.
Supplemental Table 2.3 List of RT-qPCR primers.
Supplemental Table 2.4 Metadata of qPCR and RNA-seq of *ets-1*(RNAi) across multiple gene expression across in *cathepsin*$^+$ cells (Dubey *et al., 2022;* Fincher *et al.*, 2018; Plass *et al.*, 2018)**.**

METHODS

Animal Husbandry

A clonal line of diploid, asexual *Schmidtea mediterranea* (CIW4 strain) was maintained at 18-22°C in the dark. Animals were kept in Ziploc® (9 cup) reusable containers in 1X Montjuïc salts as previously described (Cebrià & Newmark, 2005). Organic, pureed calf or beef liver (White Oak Pastures, Georgia) was used to feed animals once per week. Animals were starved for a minimum of 1 week prior to use in experiments. For sexual *Schmidtea mediterranea* husbandry and embryo staging, outbred cohorts of sexually reproducing planarians descended from animals collected in Sardinia by Dr. Maria Pala (1999) and Drs. Longhua Guo and Alejandro Sánchez Alvarado (2015) were reared in 1X Montjuïc salts (Cebrià & Newmark, 2005) at 20˚C in constant darkness. Sexually mature animals were housed at low density and fed homogenized beef liver (White Oak

Pastures, Georgia) twice per week. To promote fertility, breeding adult sexual *Schmidtea* stocks were replaced every 3 months with young adults (6-8 weeks post-hatching) or adult regenerates (6-8 weeks post-amputation). Egg capsules were collected daily, soaked in 10% bleach for 3 minutes, rinsed 4-6 times, and stored in 1X Montjuïc water in a 20˚C incubator.

Identification of genes and cloning

Planarian homologs of genes of interest were identified from PlanMine 3.0 (Rozanski *et al.*, 2019) based on homology and from scRNA-seq data (Fincher *et al.*, 2018). Primers shown in Suppl. Table 2.1 were designed using Primer3 (Rozen & Skaletsky, 1999) to PCR-amplify 500-750 bp segments of genes of interest from asexual *S. mediterranea* cDNA synthesized with an iScript kit (Bio-Rad)(Collins *et al.*, 2010). Each PCR product was ligated into Eam1105I-digested pJC53.2 vector for use in RNAi and ISH experiments using standard molecular biology protocols (Collins *et al.*, 2010).

Protein alignment and Phylogenetic Analysis

Protein alignment and phylogenetic analysis were performed as described in (Jenkins & Roberts-Galbraith, 2022). Briefly, the longest open reading frame for each sequence was identified using web-based translation tool Expert Protein Analysis System [ExPASy, https://www.expasy.org/; (Gasteiger, 2003)] or NCBI Open Reading Frame Finder (https://www.ncbi.nlm.nih.gov/orffinder/). Protein sequences of Ets-1 from other species were aligned to reference sequences (Suppl. Table 2.2). Phylogeny was performed using www.phylogeny.fr (Dereeper *et al.*, 2008) using MUSCLE for sequence alignment with the "A

la Carte" option (Edgar, 2004) and PhyML for phylogenetic tree construction (Guindon *et al.*, 2010).

RNAi experiments

For RNAi experiments, animals (10-12 worms; 3-4 mm in size) were kept in 60 mm Petri dishes, washed after feeding and supplemented with 1:1000 gentamicin sulfate (50 mg/mL stock, [Gemini Bio-Products]) throughout the experiment. dsRNA was synthesized using standard molecular biology techniques (Collins *et al.*, 2010). dsRNA matching *Aequorea victoria green fluorescent protein* (*GFP*) was used for negative control feedings. For *ets-1*(RNAi) paradigms, animals were fed 5-10 µg dsRNA mixed with 25-30 µL of 3:1 beef liver:1X Montjüic salts mixture and 2 µL McCormick® green food dye. Feedings were completed every 3 days for a total of 5-6 feedings. For regeneration experiments, animals were amputated pre-pharyngeally seven days after the last feeding and fixed according to different downstream protocols. For *ovo*(RNAi) and *soxb1-2*(RNAi), animals were fed 5 µg dsRNA every 4-5 days for a total of 4 feedings. For *coe*(RNAi) and *sim*(RNAi), animals were fed 5 µg dsRNA every 3 days for 5 feedings. Amputation and fixation were performed as previously described. For *ndk*(RNAi) paradigms, animals were fed 5 µg dsRNA every 3 days for 3 feedings. Amputation was performed as before and animals were fixed 10 days after regeneration. For long-term *ets-1*(RNAi) experiments, 30 animals per RNAi condition were fed 5 µg or 10 µg dsRNA every 3 days for a period of 60 days. The number of surviving animals was quantified as detailed in Fig S5. Survival curves were plotted using GraphPad Prism9.

in situ hybridization (ISH) and immunofluorescence (IF)

Animals utilized for ISH were treated with 7.5% N-acetyl-L-cysteine (NAC) in Phosphate Buffered Saline (PBS: 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 20 mM KH_2PO_4, pH 7.4), and fixed in 4% formaldehyde (in PBSTx: PBS + 0.3% Triton-X 100). Riboprobes for ISH on asexual planarians were generated using PCR-amplified products from pJC53.2 vectors with T7 primers (orientations provided in Suppl. Table 2.1). Antisense probes were synthesized with digoxigenin-11-UTP (Roche) or fluorescein-12-UTP (Roche) using standard molecular protocols (Collins *et al.*, 2010). ISH experiments were performed on asexual animals as previously described (King & Newmark, 2013). Some samples were processed in an Insitu Pro (Intavis) hybridization robot. Probes were detected using anti-digoxigenin with Fab fragments (Sigma-Aldrich) conjugated to alkaline phosphatase (Roche) for colorimetric ISH or horse-radish peroxidase (Roche) for fluorescent ISH (FISH). For colorimetric ISH, 5-Bromo-4-chloro-3-indolyl phosphate (BCIP, [Roche]) and nitro blue tetrazolium chloride (NBT, [Roche]) in alkaline phosphatase (AP) buffer were used for signal development. Animals were mounted in 80% glycerol. Samples were imaged with an Axiocam 506 color camera mounted on Zeiss Axio Zoom V.16 microscope using ZEN 2.3 pro software.

For FISH, probes were detected using anti-digoxigenin and anti-fluorescein Fab fragments conjugated to horseradish peroxidase (Roche). 1:500 fluor-tyramide (TAMRA or FAM), 1:1000 4-IPBA, and 0.003% H_2O_2 in Tyramide Signal Amplification (TSA) buffer (2 M NaCl; 0.1 M Boric acid, pH 8.5) was used for signal development for 45 minutes. Double FISH samples were incubated in Sodium Azide solution (100 mM sodium azide [Fisher Scientific] in PBS + 0.3% TritonX-100 (PBSTx)) for 45 minutes to inactivate peroxidase activity before secondary signal development. FISH samples were mounted in VectaShield® Antifade Mounting Medium and

imaged using a Zeiss LSM 880 Confocal microscope with an upright AXIO Imager Z2 and ZEN Black 2.3 SPI software. Whole animals were imaged using a Plan-Neofluar 10X/0.3 objective and smaller fields were imaged with Plan-Apochromat 20X/0.8 objective (no immersion). For each channel, the cut-off between signal and background was determined using the fluorescent intensity range indicator. Images shown are representative.

Embryo staging was performed according to guidelines set forward previously (Davies *et al.*, 2017). The collection date was considered 1 day post-egg capsule deposition (dped). Early spherical Stage 6 (S6) embryos were fixed at 6-7 dped; elongating mid-stage 6 (mid-S6) embryos were fixed at 8 dped; Stage 7 (S7) embryos were fixed at 10 dped; Stage 7.5 (S7.5) embryos were fixed at 12 dped; and Stage 8 (S8) embryos were fixed at 14-15 dped using the protocol described in (Davies *et al.*, 2017) for colorimetric ISH with the following modifications: Embryos were removed from egg capsules immediately prior to fixation. S6 and S6.5 embryos were fixed for 6 hours to overnight in 4% paraformaldehyde in PBSTx (PBS+0.5% Triton X-100) at room temperature. S7, S7.5 and S8 embryos were treated with 5% NAC in PBS (S7: 2 minutes; S8: 4 minutes), immediately followed by fixation in 4% paraformaldehyde in PBSTx (PBS+0.5% Triton X-100) for 45 min (S8) or 2 hours (S7, S7.5) at room temperature. Colorimetric WISH images were acquired on a Zeiss Axio Zoom V16 equipped with a Axiocam 305 color camera. For image processing, a polygonal lasso tool was used to extract images of embryos from the original TIFFs; these were transferred to a white background along with the scale bar. Brightness and contrast were adjusted on colorimetric images to facilitate visualization of the colorimetric ISH signal.

Briefly, for immunofluorescence experiments, asexual planarians were treated in 2% HCl, fixed for 15 minutes in 4% formaldehyde in PBSTx (PBS+0.3% TritonX-100), and then bleached in 6% H_2O_2 in PBSTx overnight. Animals were blocked in 1% Bovine Serum Albumin (BSA) in PBSTx overnight at 4°C. The primary antibodies used were anti-Synapsin (1:100, 3C11 concentrate; Developmental Studies Hybridoma Bank) and anti-Arrestin (1:1000, cat #016-arrestin-01, LagenLabs). Secondary antibodies were used at a dilution of 1:500-1:1000 (goat anti-mouse Alexa Fluor 488 and goat-anti-rabbit Alexa Fluor 488, respectively [Invitogen]) (Ross *et al.*, 2015; Sakai *et al.*, 2000). Samples were mounted in VectaShield® Antifade Mounting Medium and imaged using a Zeiss LSM 880 Confocal microscope with ZEN Black software. Whole animals were imaged using 10X/0.3 objective, specific regions were imaged with 20X/0.8 objective (no immersion).

Analysis of RNA sequencing data

Analysis for glial gene expression during early regeneration of asexual planarians and development of sexual planarians was performed using previously published RNA-sequencing data (Davies *et al.*, 2017; Roberts-Galbraith *et al.*, 2016) and plotted using GraphPad Prism9. Re-analysis of *ets-1*(RNAi) gene expression across different *cathepsin*$^+$ subclusters was performed using previously published RNA-sequencing data (Dubey *et al.*, 2022) and expression profiles provided in previous transcriptome data analysis (Fincher *et al.,* 2018; Plass *et al.*, 2018).

Real-time quantitative PCR (RT-qPCR)

Total RNA was isolated from RNAi-treated animals (7 dpa, whole worms) using Trizol (Invitrogen) and the manufacturer's protocol. 1 µg of RNA was reverse transcribed into cDNA using an iScript cDNA Synthesis Kit (BioRad) as per manufacturer's protocol. RT-qPCR was completed using Applied Biosystems QuantStudio3 Real-Time PCR system and GoTaq qPCR Master Mix with SYBR Green (Promega). All primers used for RT-qPCR are shown in Table S3. All measurements were performed in biological and technical triplicates; RNAi and RNA purification were performed from 3 individual Petri dishes (10-12 worms each, biological triplicates) for each RNAi condition, and then we completed three identical qPCR reactions per sample/primer pair (technical triplicates). Overall transcript normalization was accomplished using *beta-tubulin* mRNA within each sample. Statistical analyses were performed using GraphPad Prism9; details of each statistical test are provided in the figure legends.

Quantification of ventral nerve cord gaps, brain-to-body ratio, cell numbers and neural structures

Quantification of VNC gaps (*estrella* staining): For quantification of ventral nerve cord (VNC) gaps in *estrella* expression, FIJI (Schindelin *et al.*, 2012) was utilized to measure overall VNC length per animal by summing the length of the left and right VNC. The number of true gaps (defined by absence of *estrella* expression in VNC) was counted manually across animal body length. The length of each true gap was summed per animal and averaged. Then, a ratio of gap length to total VNC length per animal was determined using the sum of all gap lengths relative to the VNC length.

Quantification of cell numbers: For quantification of *estrella*$^+$ cells in *ets-1*(RNAi) head regenerates, cells were counted manually in the new blastema for each animal and averaged. For quantification of *estrella*$^+$ cells in the trunk pieces in *ets-1*(RNAi), *estrella*$^+$ cells were counted for 5 separate pre- and post-pharyngeal in 100μm^2 area (intact) or 200 μm^2 areas (regenerated) per animal and averaged, as previously described (Stelman *et al.*, 2021). For quantification of *estrella*$^+$*, calamari*$^+$*, estrella*$^+$/*calamari*$^+$, *estrella*$^+$/*calamari*$^-$, *estrella*$^-$/*calamari*$^+$, *gad*$^+$, and *th*$^+$ cells in *ets-1*(RNAi) animals; *estrella*$^+$ and *pkd2L-1*/*pkd1L-2*$^+$ cells in *soxb1-2(*RNAi) animals; and *estrella*$^+$ in *coe*(RNAi), *sim*(RNAi), *ndk*(RNAi) animals: FISH images were quantified in Bitplane IMARIS 9.9 (Oxford Instruments) using spots or colocalization modules. Cells were counted in the new head blastema or specified regions for each animal within 250 μm^2 or 200 μm^2 areas, respectively. For quantification of *estrella*$^+$ cells in and near the eye, z-stack images were analyzed using ImarisViewer 9.9.1 (Oxford Instruments) and then we manually quantified the presence or absence of *estrella*$^+$ cells adjacent to eyespots (left and right) based on three-dimensional placement for each animal per condition. For ISH samples, quantification of individual markers is as follows: quantification of *gad*$^+$ cells from colorimetric ISH: cell numbers were manually counted in head blastemas; for quantification of *npp3*$^+$ and *tph*$^+$ cells in *ets-1*(RNAi) animals: cell counts were averaged for 5 non-overlapping 200 μm^2 boxes, or 6 non-overlapping 100 μm^2 boxes, respectively; for quantification of *tbh*$^+$: cell numbers were quantified in the brain lobe and VNC and compared to body size; for quantification of *pkd*$^+$ cells in *ets-1*(RNAi) animals: cells were quantified in 100 μm^2 boxes (head) or averaged from 3 boxes of 200 μm^2 area (trunk) in the medial stripe and marginal PNS regions.

Quantification of brain or eye size: Brain-to-body ratios were determined by tracing the *ChAT*$^+$ expression in the brain using FIJI and comparing brain area to body area, as previously described

(Roberts-Galbraith *et al.*, 2016; Schindelin *et al.*, 2012). In these experiments, we normalize ratios so that controls are set at 100%. Similarly, area of *tph*$^{+}$ or *foxQ2*/*myoVIIA*/*sans*$^{+}$ expression in the eyes was traced using FIJI (summation of left and right eye area) and compared to body size.

Quantifications of neural structures: For quantification of neural structures in anti-Synapsin and anti-Arrestin immunofluorescence images, identities of samples were masked and randomized for quantification. Traits were quantified from maximum intensity projection images (area) or single planes (intensity). For anti-Arrestin, samples were quantified for number of stray bundles or axons near the photoreceptors, stray bundles or axons in/near optic chiasma, stray bundles or axons near the neuropil, gaps in axon trajectory. We set parameters to describe axonal organization as follows: if the gap number was less than 4 *and* the total number of stray projections was less than 2 *and* if fraying in midline was 0, then axonal organization was within the range of control(RNAi) animals and was considered "normal". If one or multiple of the parameters were not satisfied for an animal, it was counted as an irregular phenotype. For anti-Synapsin, samples were quantified for neuropil width (brightest parts and including fainter edges), brain gaps, and VNC gaps using FIJI. Brain width and brain gap criteria were measured in triplicate and averaged for each individual animal. For quantification of VNC gaps, the number of gaps on the left and right side was averaged and then divided by the total length of the animal (in mm) to get the average gap length per mm. To calculate the average gap length, measurements of each gap per individual sample were taken and then averaged per animal. The samples were then unmasked, and measurements were averaged for each RNAi condition. All statistical analyses were conducted using GraphPad Prism9; details of each statistical test are shown in the figure legends.

Behavioral Assays

Prior to light/dark behavioral assays, RNAi animals were placed in VWR Square Petri Dishes (Electron Microscopy Sciences,13x13 mm) after the last dsRNA feeding to acclimate to the dishes for two days. For the light/dark assay, new Petri dishes were half covered with black electrical tape (Duck Brand®) on the lid and the corresponding side of the petri dish. Then 10-12 animals per RNAi condition were introduced at the edge or corner of the light side of the petri dish and recorded on an iPhone® 11 (Apple) from a height of approximately 17 centimeters from the top of the dish for approximately 10 minutes. The behavioral test was repeated in biological triplicate for each experimental condition. To quantify, the number of animals remaining on the light side was recorded at 60 second intervals. 0 seconds indicates when animals were introduced to the dish. Then, the percentage of animals (over three biological replicates) for each RNAi condition residing in the light side was calculated for each time point and plotted using GraphPad Prism9. For publication, videos were sped to 20X using Adobe Photoshop; stills from videos were taken in 10 second increments using iMovie.

Acknowledgements:

We are grateful for assistance from University of Georgia Biology Microscopy Core and Dr. K.M. Kandasamy. We thank Kendall Clay, Jennifer Jenkins, and Taylor Medlock-Lanier for thoughtful feedback on this work and manuscript. anti-Synapsin antibody was obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa. We are grateful to Dr. Alejandro Sánchez Alvarado and Shane Merryman (Stowers Institute) for planarians provided during our laboratory start-up. We are

grateful to the farmers at White Oak Pastures (Bluffton, GA) for raising healthy cattle that we used for our beef liver. This research was supported by the Alfred P. Sloan Foundation (RRG), the McKnight Foundation (RRG), the University of Georgia Department of Cellular Biology, and the Center for Cancer Research, National Cancer Institute, National Institutes of Health Intramural Research Program project number ZIABC0120009 (ED). The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products or organizations imply endorsement by the US Government.

REFERENCES

Adams, K. L., & Gallo, V. (2018). The diversity and disparity of the glial scar. *Nature Neuroscience*, *21*(1), 9–15. https://doi.org/10.1038/s41593-017-0033-9

Aldskogius, H., & Kozlova, E. N. (1998). Central neuron–glial and glial–glial interactions following axon injury. *Progress in Neurobiology*, *55*(1), 1–26. https://doi.org/10.1016/S0301-0082(97)00093-2

Alesci, A., Pergolizzi, S., Lo Cascio, P., Fumia, A., & Lauriano, E. R. (2022). Neuronal regeneration: Vertebrates comparative overview and new perspectives for neurodegenerative diseases. *Acta Zoologica*, *103*(2), 129–140. https://doi.org/10.1111/azo.12397

Allen, N. J., & Lyons, D. A. (2018). Glia as architects of central nervous system formation and function. *Science*, *362*(6411), 181–185. https://doi.org/10.1126/science.aat0473

Amouyel, P., Gégonne, A., Delacourte, A., Défossez, A., & Stéhelin, D. (1988). Expression of ETS proto-oncogenes in astrocytes in human cortex. *Brain Research*, *447*(1), 149–153. https://doi.org/10.1016/0006-8993(88)90976-6

Anderson, M. A., Burda, J. E., Ren, Y., Ao, Y., O'Shea, T. M., Kawaguchi, R., Coppola, G., Khakh, B. S., Deming, T. J., & Sofroniew, M. V. (2016). Astrocyte scar formation aids central nervous system axon regeneration. *Nature*, *532*(7598), 195–200. https://doi.org/10.1038/nature17623

Anthony, T. E., Klein, C., Fishell, G., & Heintz, N. (2004). Radial Glia Serve as Neuronal Progenitors in All Regions of the Central Nervous System. *Neuron*, *41*(6), 881–890. https://doi.org/10.1016/S0896-6273(04)00140-0

Araque, A., Parpura, V., Sanzgiri, R. P., & Haydon, P. G. (1999). Tripartite synapses: Glia, the unacknowledged partner. *Trends in Neurosciences*, *22*(5), 208–215. https://doi.org/10.1016/S0166-2236(98)01349-6

Arthur-Farraj, P. J., Latouche, M., Wilton, D. K., Quintes, S., Chabrol, E., Banerjee, A., Woodhoo, A., Jenkins, B., Rahman, M., Turmaine, M., Wicher, G. K., Mitter, R., Greensmith, L., Behrens, A., Raivich, G., Mirsky, R., & Jessen, K. R. (2012). C-Jun Reprograms Schwann Cells of Injured Nerves to Generate a Repair Cell Essential for Regeneration. *Neuron*, *75*(4), 633–647. https://doi.org/10.1016/j.neuron.2012.06.021

Barnabé-Heider, F., Wasylnka, J. A., Fernandes, K. J. L., Porsche, C., Sendtner, M., Kaplan, D. R., & Miller, F. D. (2005). Evidence that Embryonic Neurons Regulate the Onset of Cortical Gliogenesis via Cardiotrophin-1. *Neuron*, *48*(2), 253–265. https://doi.org/10.1016/j.neuron.2005.08.037

Bayraktar, O. A., & Doe, C. Q. (2013). Combinatorial temporal patterning in progenitors expands neural diversity. *Nature*, *498*(7455), 449–455. https://doi.org/10.1038/nature12266

Burda, J. E., & Sofroniew, M. V. (2014). Reactive Gliosis and the Multicellular Response to CNS Damage and Disease. *Neuron*, *81*(2), 229–248. https://doi.org/10.1016/j.neuron.2013.12.034

Campbell, K., & Götz, M. (2002). Radial glia: Multi-purpose cells for vertebrate brain development. *Trends in Neurosciences*, *25*(5), 235–238. https://doi.org/10.1016/S0166-2236(02)02156-2

Cebrià, F., Kobayashi, C., Umesono, Y., Nakazawa, M., Mineta, K., Ikeo, K., Gojobori, T., Itoh, M., Taira, M., Alvarado, A. S., & Agata, K. (2002). FGFR-related gene nou-darake

restricts brain tissues to the head region of planarians. *Nature*, *419*(6907), 620–624. https://doi.org/10.1038/nature01042

Cebrià, F., Nakazawa, M., Mineta, K., Ikeo, K., Gojobori, T., & Agata, K. (2002). Dissecting planarian central nervous system regeneration by the expression of neural-specific genes. *Development, Growth and Differentiation*, *44*(2), 135–146. https://doi.org/10.1046/j.1440-169x.2002.00629.x

Cebrià, F., & Newmark, P. A. (2005). Planarian homologs of netrin and netrin receptor are required for proper regeneration of the central nervous system and the maintenance of nervous system architecture. *Development*, *132*(16), 3691–3703. https://doi.org/10.1242/dev.01941

Chen, T., Bunting, M., Karim, F. D., & Thummel, C. S. (1992). Isolation and characterization of five Drosophila genes that encode an ets-related DNA binding domain. *Developmental Biology*, *151*(1), 176–191. https://doi.org/10.1016/0012-1606(92)90225-6

Collins, J. J., Hou, X., Romanova, E. V., Lambrus, B. G., Miller, C. M., Saberi, A., Sweedler, J. V., & Newmark, P. A. (2010). Genome-Wide Analyses Reveal a Role for Peptide Hormones in Planarian Germline Development. *PLoS Biology*, *8*(10), e1000509. https://doi.org/10.1371/journal.pbio.1000509

Coutinho-Budd, J. C., Sheehan, A. E., & Freeman, M. R. (2017). The secreted neurotrophin Spätzle 3 promotes glial morphogenesis and supports neuronal survival and function. *Genes & Development*, *31*(20), 2023–2038. https://doi.org/10.1101/gad.305888.117

Cowles, M. W., Brown, D. D. R., Nisperos, S. V., Stanley, B. N., Pearson, B. J., & Zayas, R. M. (2013). Genome-wide analysis of the bHLH gene family in planarians identifies factors

required for adult neurogenesis and neuronal regeneration. *Development*, *140*(23), 4691–4702. https://doi.org/10.1242/dev.098616

Cowles, M. W., Omuro, K. C., Stanley, B. N., Quintanilla, C. G., & Zayas, R. M. (2014). COE Loss-of-Function Analysis Reveals a Genetic Program Underlying Maintenance and Regeneration of the Nervous System in Planarians. *PLoS Genetics*, *10*(10), e1004746. https://doi.org/10.1371/journal.pgen.1004746

Crews, S. T. (2019). *Drosophila* Embryonic CNS Development: Neurogenesis, Gliogenesis, Cell Fate, and Differentiation. *Genetics*, *213*(4), 1111–1144. https://doi.org/10.1534/genetics.119.300974

Currie, K. W., Molinaro, A. M., & Pearson, B. J. (2016). Neuronal sources of hedgehog modulate neurogenesis in the adult planarian brain. *ELife*, *5*, e19735. https://doi.org/10.7554/eLife.19735

Davies, E. L., Lei, K., Seidel, C. W., Kroesen, A. E., McKinney, S. A., Guo, L., Robb, S. M., Ross, E. J., Gotting, K., & Alvarado, A. S. (2017). Embryonic origin of adult stem cells required for tissue homeostasis and regeneration. *ELife*, *6*, e21052. https://doi.org/10.7554/eLife.21052

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J.-F., Guindon, S., Lefort, V., Lescot, M., Claverie, J.-M., & Gascuel, O. (2008). Phylogeny.fr: Robust phylogenetic analysis for the non-specialist. *Nucleic Acids Research*, *36*(Web Server), W465–W469. https://doi.org/10.1093/nar/gkn180

Doherty, J., Logan, M. A., Tasdemir, O. E., & Freeman, M. R. (2009). Ensheathing Glia Function as Phagocytes in the Adult Drosophila Brain. *Journal of Neuroscience*, *29*(15), 4768–4781. https://doi.org/10.1523/JNEUROSCI.5951-08.2009

Dubey, V. K., Sarkar, S. R., Lakshmanan, V., Dalmeida, R., Gulyani, A., & Palakodeti, D. (2022). Smed - ETS-1 regulates cathepsin+ cell function and epidermal lineage landscape via basement membrane remodeling. *Journal of Cell Science*, jcs.259900. https://doi.org/10.1242/jcs.259900

Ebens, A. J., Garren, H., Cheyette, B. N. R., & Zipursky, S. L. (1993). The Drosophila anachronism locus: A glycoprotein secreted by glia inhibits neuroblast proliferation. *Cell*, *74*(1), 15–27. https://doi.org/10.1016/0092-8674(93)90291-W

Edgar, R. C. (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, *32*(5), 1792–1797. https://doi.org/10.1093/nar/gkh340

Eroglu, C., & Barres, B. A. (2010). Regulation of synaptic connectivity by glia. *Nature*, *468*(7321), 223–231. https://doi.org/10.1038/nature09612

Escartin, C., Galea, E., Lakatos, A., O'Callaghan, J. P., Petzold, G. C., Serrano-Pozo, A., Steinhäuser, C., Volterra, A., Carmignoto, G., Agarwal, A., Allen, N. J., Araque, A., Barbeito, L., Barzilai, A., Bergles, D. E., Bonvento, G., Butt, A. M., Chen, W.-T., Cohen-Salmon, M., … Verkhratsky, A. (2021). Reactive astrocyte nomenclature, definitions, and future directions. *Nature Neuroscience*, *24*(3), 312–325. https://doi.org/10.1038/s41593-020-00783-4

Falk, S., & Götz, M. (2017). Glial control of neurogenesis. *Current Opinion in Neurobiology*, *47*, 188–195. https://doi.org/10.1016/j.conb.2017.10.025

Fincher, C. T., Wurtzel, O., de Hoog, T., Kravarik, K. M., & Reddien, P. W. (2018). Cell type transcriptome atlas for the planarian *Schmidtea mediterranea*. *Science*, *360*(6391), eaaq1736. https://doi.org/10.1126/science.aaq1736

Fleischman, L. F., Holtzclaw, L., Russell, J. T., Mavrothalassitis, G., & Fisher, R. J. (1995). ets-1 in astrocytes: Expression and transmitter-evoked phosphorylation. *Molecular and Cellular Biology*, *15*(2), 925–931. https://doi.org/10.1128/MCB.15.2.925

Fraguas, S., Barberán, S., Ibarra, B., Stöger, L., & Cebri, F. (2012). Regeneration of neuronal cell types in Schmidtea mediterranea: An immunohistochemical and expression study. *The International Journal of Developmental Biology*, *56*(1-2–3), 143–153. https://doi.org/10.1387/ijdb.113428sf

Gallo, V., & Deneen, B. (2014). Glial Development: The Crossroads of Regeneration and Repair in the CNS. *Neuron*, *83*(2), 283–308. https://doi.org/10.1016/j.neuron.2014.06.010

Gasteiger, E. (2003). ExPASy: The proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Research*, *31*(13), 3784–3788. https://doi.org/10.1093/nar/gkg563

Goldshmit, Y., Sztal, T. E., Jusuf, P. R., Hall, T. E., Nguyen-Chi, M., & Currie, P. D. (2012). Fgf-Dependent Glial Cell Bridges Facilitate Spinal Cord Regeneration in Zebrafish. *Journal of Neuroscience*, *32*(22), 7477–7492. https://doi.org/10.1523/JNEUROSCI.0758-12.2012

Guindon, S., Dufayard, J.-F., Lefort, V., Anisimova, M., Hordijk, W., & Gascuel, O. (2010). New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0. *Systematic Biology*, *59*(3), 307–321. https://doi.org/10.1093/sysbio/syq010

Hagedorn, L., Paratore, C., Brugnoli, G., Baert, J.-L., Mercader, N., Suter, U., & Sommer, L. (2000). The Ets Domain Transcription Factor Erm Distinguishes Rat Satellite Glia from Schwann Cells and Is Regulated in Satellite Cells by Neuregulin Signaling. *Developmental Biology*, *219*(1), 44–58. https://doi.org/10.1006/dbio.1999.9595

Hartline, D. K. (2011). The evolutionary origins of glia. *Glia*, *59*(9), 1215–1236. https://doi.org/10.1002/glia.21149

He, X., Lindsay-Mosher, N., Li, Y., Molinaro, A. M., Pellettieri, J., & Pearson, B. J. (2017). FOX and ETS family transcription factors regulate the pigment cell lineage in planarians. *Development*, *144*(24), 4540–4551. https://doi.org/10.1242/dev.156349

Hidalgo, A., Urban, J., & Brand, A. H. (1995). Targeted ablation of glia disrupts axon tract formation in the Drosophila CNS. *Development*, *121*(11), 3703–3712. https://doi.org/10.1242/dev.121.11.3703

Inoue, T., Kumamoto, H., Okamoto, K., Umesono, Y., Sakai, M., Alvarado, A. S., & Agata, K. (2004). Morphological and Functional Recovery of the Planarian Photosensing System during Head Regeneration. *Zoological Science*, *21*(3), 275–283. https://doi.org/10.2108/zsj.21.275

Jäkel, S., & Dimou, L. (2017). Glial Cells and Their Function in the Adult Brain: A Journey through the History of Their Ablation. *Frontiers in Cellular Neuroscience*, *11*. https://doi.org/10.3389/fncel.2017.00024

Jenkins, J. E., & Roberts-Galbraith, R. H. (2022). *Heterotrimeric G proteins regulate planarian regeneration and behavior* [Preprint]. Developmental Biology. https://doi.org/10.1101/2022.03.07.483311

Jessen, K. R. (2004). Glial cells. *The International Journal of Biochemistry & Cell Biology*, *36*(10), 1861–1867. https://doi.org/10.1016/j.biocel.2004.02.023

Jessen, K. R., & Mirsky, R. (2016). The repair Schwann cell and its function in regenerating nerves: Repair Schwann cell and its function in regenerating nerves. *The Journal of Physiology*, *594*(13), 3521–3531. https://doi.org/10.1113/JP270874

Jung, Y.-J., & Chung, W.-S. (2018). Phagocytic Roles of Glial Cells in Healthy and Diseased Brains. *Biomolecules & Therapeutics*, *26*(4), 350–357. https://doi.org/10.4062/biomolther.2017.133

King, R. S., & Newmark, P. A. (2013). In situ hybridization protocol for enhanced detection of gene expression in the planarian Schmidtea mediterranea. *BMC Developmental Biology*, *13*(1), 8. https://doi.org/10.1186/1471-213X-13-8

Kiyota, T., Kato, A., & Kato, Y. (2007). Ets-1 Regulates Radial Glia Formation During Vertebrate Embryogenesis. *Organogenesis*, *3*(2), 93–101. https://doi.org/10.4161/org.3.2.5171

Klaes, A., Menne, T., Stollewerk, A., Scholz, H., & Klämbt, C. (1994). The Ets transcription factors encoded by the Drosophila gene pointed direct glial cell differentiation in the embryonic CNS. *Cell*, *78*(1), 149–160. https://doi.org/10.1016/0092-8674(94)90581-9

Klämbt, C. (1993). The Drosophila gene pointed encodes two ETS-like proteins which are involved in the development of the midline glial cells. *Development*, *117*(1), 163–176. https://doi.org/10.1242/dev.117.1.163

Klämbt, C., & Goodman, C. S. (1991). The diversity and pattern of glia during axon pathway formation in the drosophila embryo: GLIA IN DROSOPHILA EMBRYO. *Glia*, *4*(2), 205–213. https://doi.org/10.1002/glia.440040212

Klatt Shaw, D., Saraswathy, V. M., Zhou, L., McAdow, A. R., Burris, B., Butka, E., Morris, S. A., Dietmann, S., & Mokalled, M. H. (2021). Localized EMT reprograms glial progenitors to promote spinal cord repair. *Developmental Cell*, *56*(5), 613-626.e7. https://doi.org/10.1016/j.devcel.2021.01.017

Kola, I., Brookes, S., Green, A. R., Garber, R., Tymms, M., Papas, T. S., & Seth, A. (1993). The Ets1 transcription factor is widely expressed during murine embryo development and is associated with mesodermal cells involved in morphogenetic processes such as organ formation. *Proceedings of the National Academy of Sciences*, *90*(16), 7588–7592. https://doi.org/10.1073/pnas.90.16.7588

Lambrus, B. G., Cochet-Escartin, O., Gao, J., Newmark, P. A., Collins, E.-M. S., & Collins, J. J. (2015). Tryptophan hydroxylase Is Required for Eye Melanogenesis in the Planarian Schmidtea mediterranea. *PLOS ONE*, *10*(5), e0127074. https://doi.org/10.1371/journal.pone.0127074

Lapan, S. W., & Reddien, P. W. (2012). Transcriptome Analysis of the Planarian Eye Identifies ovo as a Specific Regulator of Eye Regeneration. *Cell Reports*, *2*(2), 294–307. https://doi.org/10.1016/j.celrep.2012.06.018

Lee, E., & Chung, W.-S. (2019). Glial Control of Synapse Number in Healthy and Diseased Brain. *Frontiers in Cellular Neuroscience*, *13*, 42. https://doi.org/10.3389/fncel.2019.00042

Logan, M. A., & Freeman, M. R. (2007). The scoop on the fly brain: Glial engulfment functions in Drosophila. *Neuron Glia Biology*, *3*(1), 1–3. https://doi.org/10.1017/S1740925X07000646

Mason, C. A., & Sretavan, D. W. (1997). Glia, neurons, and axon pathfinding during optic chiasm development. *Current Opinion in Neurobiology*, *7*(5), 647–653. https://doi.org/10.1016/S0959-4388(97)80084-0

Miller, F. D., & Gauthier, A. S. (2007). Timing Is Everything: Making Neurons versus Glia in the Developing Cortex. *Neuron*, *54*(3), 357–369. https://doi.org/10.1016/j.neuron.2007.04.019

Mokalled, M. H., Patra, C., Dickson, A. L., Endo, T., Stainier, D. Y. R., & Poss, K. D. (2016). Injury-induced ctgfa directs glial bridging and spinal cord regeneration in zebrafish. *Science*, *354*(6312), 630–634. https://doi.org/10.1126/science.aaf2679

Molofsky, A. V., & Deneen, B. (2015). Astrocyte development: A Guide for the Perplexed: Mechanisms of Astrocyte development. *Glia*, *63*(8), 1320–1329. https://doi.org/10.1002/glia.22836

Myer, D. J. (2006). Essential protective roles of reactive astrocytes in traumatic brain injury. *Brain*, *129*(10), 2761–2772. https://doi.org/10.1093/brain/awl165

Nagarajan, R., Le, N., Mahoney, H., Araki, T., & Milbrandt, J. (2002). Deciphering peripheral nerve myelination by using Schwann cell expression profiling. *Proceedings of the National Academy of Sciences*, *99*(13), 8998–9003. https://doi.org/10.1073/pnas.132080999

Neniskyte, U., & Gross, C. T. (2017). Errant gardeners: Glial-cell-dependent synaptic pruning and neurodevelopmental disorders. *Nature Reviews Neuroscience*, *18*(11), 658–670. https://doi.org/10.1038/nrn.2017.110

Nishimura, K., Kitamura, Y., Inoue, T., Umesono, Y., Sano, S., Yoshimoto, K., Inden, M., Takata, K., Taniguchi, T., Shimohama, S., & Agata, K. (2007). Reconstruction of dopaminergic neural network and locomotion function in planarian regenerates. *Developmental Neurobiology*, *67*(8), 1059–1078. https://doi.org/10.1002/dneu.20377

Nishimura, K., Kitamura, Y., Inoue, T., Umesono, Y., Yoshimoto, K., Takeuchi, K., Taniguchi, T., & Agata, K. (2007). Identification and distribution of tryptophan hydroxylase (TPH)-positive neurons in the planarian Dugesia japonica. *Neuroscience Research*, *59*(1), 101–106. https://doi.org/10.1016/j.neures.2007.05.014

Nishimura, K., Kitamura, Y., Inoue, T., Umesono, Y., Yoshimoto, K., Taniguchi, T., & Agata, K. (2008). Characterization of tyramine β-hydroxylase in planarian Dugesia japonica: Cloning and expression. *Neurochemistry International*, *53*(6–8), 184–192. https://doi.org/10.1016/j.neuint.2008.09.006

Nishimura, K., Kitamura, Y., Taniguchi, T., & Agata, K. (2010). Analysis of motor function modulated by cholinergic neurons in planarian dugesia japonica. *Neuroscience*, *168*(1), 18–30. https://doi.org/10.1016/j.neuroscience.2010.03.038

Nishimura, K., Kitamura, Y., Umesono, Y., Takeuchi, K., Takata, K., Taniguchi, T., & Agata, K. (2008). Identification of glutamic acid decarboxylase gene and distribution of GABAergic nervous system in the planarian Dugesia japonica. *Neuroscience*, *153*(4), 1103–1114. https://doi.org/10.1016/j.neuroscience.2008.03.026

Oikonomou, G., & Shaham, S. (2011). The Glia of Caenorhabditis elegans. *Glia*, *59*(9), 1253–1263. https://doi.org/10.1002/glia.21084

Oliet, S. H. R., Piet, R., & Poulain, D. A. (2001). Control of Glutamate Clearance and Synaptic Efficacy by Glial Coverage of Neurons. *Science*, *292*(5518), 923–926. https://doi.org/10.1126/science.1059162

Ong, T.-H., Romanova, E. V., Roberts-Galbraith, R. H., Yang, N., Zimmerman, T. A., Collins, J. J., Lee, J. E., Kelleher, N. L., Newmark, P. A., & Sweedler, J. V. (2016). Mass Spectrometry Imaging and Identification of Peptides Associated with Cephalic Ganglia

Regeneration in Schmidtea mediterranea. *Journal of Biological Chemistry*, *291*(15), 8109–8120. https://doi.org/10.1074/jbc.M115.709196

Ortega, A., & Olivares-Bañuelos, T. N. (2020). Neurons and Glia Cells in Marine Invertebrates: An Update. *Frontiers in Neuroscience*, *14*, 121. https://doi.org/10.3389/fnins.2020.00121

Oviedo, N. J., Newmark, P. A., & Sánchez Alvarado, A. (2003). Allometric scaling and proportion regulation in the freshwater planarian *Schmidtea mediterranea*: Scale and Proportion in Planarians. *Developmental Dynamics*, *226*(2), 326–333. https://doi.org/10.1002/dvdy.10228

Parkinson, D. B., Langner, K., Namini, S. S., Jessen, K. R., & Mirsky, R. (2002). β-Neuregulin and Autocrine Mediated Survival of Schwann Cells Requires Activity of Ets Family Transcription Factors. *Molecular and Cellular Neuroscience*, *20*(1), 154–167. https://doi.org/10.1006/mcne.2002.1109

Paskin, T. R., Jellies, J., Bacher, J., & Beane, W. S. (2014). Planarian Phototactic Assay Reveals Differential Behavioral Responses Based on Wavelength. *PLoS ONE*, *9*(12), e114708. https://doi.org/10.1371/journal.pone.0114708

Pekny, M., Wilhelmsson, U., & Pekna, M. (2014). The dual role of astrocyte activation and reactive gliosis. *Neuroscience Letters*, *565*, 30–38. https://doi.org/10.1016/j.neulet.2013.12.071

Plass, M., Solana, J., Wolf, F. A., Ayoub, S., Misios, A., Glažar, P., Obermayer, B., Theis, F. J., Kocks, C., & Rajewsky, N. (2018). Cell type atlas and lineage tree of a whole complex animal by single-cell transcriptomics. *Science*, *360*(6391), eaaq1723. https://doi.org/10.1126/science.aaq1723

Plazaola-Sasieta, H., Zhu, Q., Gaitán-Peñas, H., Rios, M., Estévez, R., & Morey, M. (2019). Drosophila ClC-a is required in glia of the stem cell niche for proper neurogenesis and wiring of neural circuits. *Glia*, *67*(12), 2374–2398. https://doi.org/10.1002/glia.23691

Poeck, B., Fischer, S., Gunning, D., Zipursky, S. L., & Salecker, I. (2001). Glial Cells Mediate Target Layer Selection of Retinal Axons in the Developing Visual System of Drosophila. *Neuron*, *29*(1), 99–113. https://doi.org/10.1016/S0896-6273(01)00183-0

Pribyl, L. J., Watson, D. K., McWilliams, M. J., Ascione, R., & Papas, T. S. (1988). The Drosophila ets-2 gene: Molecular structure, chromosomal localization, and developmental expression. *Developmental Biology*, *127*(1), 45–53. https://doi.org/10.1016/0012-1606(88)90187-X

Purice, M. D., Ray, A., Münzel, E. J., Pope, B. J., Park, D. J., Speese, S. D., & Logan, M. A. (2017). A novel Drosophila injury model reveals severed axons are cleared through a Draper/MMP-1 signaling cascade. *ELife*, *6*, e23611. https://doi.org/10.7554/eLife.23611

Rapti, G., Li, C., Shan, A., Lu, Y., & Shaham, S. (2017). Glia initiate brain assembly through noncanonical Chimaerin–Furin axon guidance in C. elegans. *Nature Neuroscience*, *20*(10), 1350–1360. https://doi.org/10.1038/nn.4630

Reddy, L. V., Koirala, S., Sugiura, Y., Herrera, A. A., & Ko, C.-P. (2003). Glial Cells Maintain Synaptic Structure and Function and Promote Development of the Neuromuscular Junction In Vivo. *Neuron*, *40*(3), 563–580. https://doi.org/10.1016/S0896-6273(03)00682-2

Roberts-Galbraith, R. H., Brubacher, J. L., & Newmark, P. A. (2016). A functional genomics screen in planarians reveals regulators of whole-brain regeneration. *ELife*, *5*, e17002. https://doi.org/10.7554/eLife.17002

Rolls, A., Shechter, R., & Schwartz, M. (2009). The bright side of the glial scar in CNS repair. *Nature Reviews Neuroscience*, *10*(3), 235–241. https://doi.org/10.1038/nrn2591

Ross, K. G., Molinaro, A. M., Romero, C., Dockter, B., Cable, K. L., Gonzalez, K., Zhang, S., Collins, E.-M. S., Pearson, B. J., & Zayas, R. M. (2018). SoxB1 Activity Regulates Sensory Neuron Regeneration, Maintenance, and Function in Planarians. *Developmental Cell*, *47*(3), 331-347.e5. https://doi.org/10.1016/j.devcel.2018.10.014

Ross, K. G., Omuro, K. C., Taylor, M. R., Munday, R. K., Hubert, A., King, R. S., & Zayas, R. M. (2015). Novel monoclonal antibodies to study tissue regeneration in planarians. *BMC Developmental Biology*, *15*(1), 2. https://doi.org/10.1186/s12861-014-0050-9

Rozanski, A., Moon, H., Brandl, H., Martín-Durán, J. M., Grohme, M. A., Hüttner, K., Bartscherer, K., Henry, I., & Rink, J. C. (2019). PlanMine 3.0—Improvements to a mineable resource of flatworm biology and biodiversity. *Nucleic Acids Research*, *47*(D1), D812–D820. https://doi.org/10.1093/nar/gky1070

Rozen, S., & Skaletsky, H. (1999). Primer3 on the WWW for General Users and for Biologist Programmers. In S. Misener & S. A. Krawetz, *Bioinformatics Methods and Protocols* (Vol. 132, pp. 365–386). Humana Press. https://doi.org/10.1385/1-59259-192-2:365

Sakai, F., Agata, K., Orii, H., & Watanabe, K. (2000). Organization and Regeneration Ability of Spontaneous Supernumerary Eyes in Planarians—Eye Regeneration Field and Pathway Selection by Optic Nerves—. *Zoological Science*, *17*(3), 375–381. https://doi.org/10.2108/jsz.17.375

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., & Cardona, A. (2012). Fiji: An open-source platform for

biological-image analysis. *Nature Methods*, *9*(7), 676–682. https://doi.org/10.1038/nmeth.2019

Scimone, M. L., Wurtzel, O., Malecek, K., Fincher, C. T., Oderberg, I. M., Kravarik, K. M., & Reddien, P. W. (2018). FoxF-1 Controls Specification of Non-body Wall Muscle and Phagocytic Cells in Planarians. *Current Biology*, *28*(23), 3787-3801.e6. https://doi.org/10.1016/j.cub.2018.10.030

Shaham, S. (2015). Glial Development and Function in the Nervous System of *Caenorhabditis elegans*. *Cold Spring Harbor Perspectives in Biology*, *7*(4), a020578. https://doi.org/10.1101/cshperspect.a020578

Shimoyama, S., Inoue, T., Kashima, M., & Agata, K. (2016). Multiple Neuropeptide-Coding Genes Involved in Planarian Pharynx Extension. *Zoological Science*, *33*(3), 311. https://doi.org/10.2108/zs150170

Silver, J., & Miller, J. H. (2004). Regeneration beyond the glial scar. *Nature Reviews Neuroscience*, *5*(2), 146–156. https://doi.org/10.1038/nrn1326

Silver, J., Schwab, M. E., & Popovich, P. G. (2015). Central Nervous System Regenerative Failure: Role of Oligodendrocytes, Astrocytes, and Microglia. *Cold Spring Harbor Perspectives in Biology*, *7*(3), a020602. https://doi.org/10.1101/cshperspect.a020602

Slupsky, C. M., Gentile, L. N., Donaldson, L. W., Mackereth, C. D., Seidel, J. J., Graves, B. J., & McIntosh, L. P. (1998). Structure of the Ets-1 pointed domain and mitogen-activated protein kinase phosphorylation site. *Proceedings of the National Academy of Sciences*, *95*(21), 12129–12134. https://doi.org/10.1073/pnas.95.21.12129

Stelman, C. R., Smith, B. M., Chandra, B., & Roberts-Galbraith, R. H. (2021). CBP/p300 homologs CBP2 and CBP3 play distinct roles in planarian stem cell function. *Developmental Biology*, *473*, 130–143. https://doi.org/10.1016/j.ydbio.2021.02.004

Stiegler, P., Wolff, C.-M., Baltzinger, M., Hirtzlin, J., Senan, F., Meyer, D., Ghysdael, J., Stéhelin, D., Befort, N., & Remy, P. (1990). Characterization of *Xenopus laevis* cDNA clones of the c- *ets-1* proto-oncogene. *Nucleic Acids Research*, *18*(17), 5298–5298. https://doi.org/10.1093/nar/18.17.5298

Stubenhaus, B. M., Dustin, J. P., Neverett, E. R., Beaudry, M. S., Nadeau, L. E., Burk-McCoy, E., He, X., Pearson, B. J., & Pellettieri, J. (2016). Light-induced depigmentation in planarians models the pathophysiology of acute porphyrias. *ELife*, *5*, e14175. https://doi.org/10.7554/eLife.14175

Sulston, J. E., Schierenberg, E., White, J. G., & Thomson, J. N. (1983). The embryonic cell lineage of the nematode Caenorhabditis elegans. *Developmental Biology*, *100*(1), 64–119. https://doi.org/10.1016/0012-1606(83)90201-4

Tanaka, E. M., & Ferretti, P. (2009). Considering the evolution of regeneration in the central nervous system. *Nature Reviews Neuroscience*, *10*(10), 713–723. https://doi.org/10.1038/nrn2707

Viktorin, G., Riebli, N., Popkova, A., Giangrande, A., & Reichert, H. (2011). Multipotent neural stem cells generate glial cells of the central complex through transit amplifying intermediate progenitors in Drosophila brain development. *Developmental Biology*, *356*(2), 553–565. https://doi.org/10.1016/j.ydbio.2011.06.013

Wang, I. E., Lapan, S. W., Scimone, M. L., Clandinin, T. R., & Reddien, P. W. (2016). Hedgehog signaling regulates gene expression in planarian glia. *ELife*, *5*, e16996. https://doi.org/10.7554/eLife.16996

Watson, D. K., McWilliams, M. J., Lapis, P., Lautenberger, J. A., Schweinfest, C. W., & Papas, T. S. (1988). Mammalian ets-1 and ets-2 genes encode highly conserved proteins. *Proceedings of the National Academy of Sciences*, *85*(21), 7862–7866. https://doi.org/10.1073/pnas.85.21.7862

Yildirim, K., Petri, J., Kottmeier, R., & Klämbt, C. (2019). Drosophila glia: Few cell types and many conserved functions. *Glia*, *67*(1), 5–26. https://doi.org/10.1002/glia.23459

Yoshimura, S., Murray, J. I., Lu, Y., Waterston, R. H., & Shaham, S. (2008). Mls-2 and vab-3 control glia development, hlh-17/Olig expression and glia-dependent neurite extension in C. elegans. *Development*, *135*(13), 2263–2275. https://doi.org/10.1242/dev.019547

Zewde, A. M., Yu, F., Nayak, S., Tallarida, C., Reitz, A. B., Kirby, L. G., & Rawls, S. M. (2018). PLDT (planarian light/dark test): An invertebrate assay to quantify defensive responding and study anxiety-like effects. *Journal of Neuroscience Methods*, *293*, 284–288. https://doi.org/10.1016/j.jneumeth.2017.10.010

Chapter III

INVESTIGATING MOLECULAR REGULATORS OF PLANARIAN GLIAL REGENERATION AND HOMEOSTASIS[1]

[1] Chandra, B., and Roberts-Galbraith, R.H. (2024). To be submitted to *Development.*

Abstract

Glial cells – a heterogenous population of non-neuronal cells – play an integral roles in nervous system regeneration. Glial cells can play either pro- or anti-regenerative roles in nervous system regeneration depending on several contexts (e.g. species, region, duration of injury, and presence of specialized glial cells). In addition to assisting in neuron regeneration, glial cells themselves must regenerate and/or proliferate after injury. Mechanisms of glial regeneration are currently understudied due to limitations in traditional model organisms' capacity to regenerate. Planarians (freshwater flatworms) are unique invertebrates capable of regenerating a fully functional nervous system *de novo*. The recent discovery of astrocyte-like glial cells in planarians provides a rare opportunity to study how glial cells regenerate, and how they interact with neurons in the context of regeneration. Investigating molecular regulators using the planarian system can help us elucidate the factors that drive glial regeneration after injury. In this study, we uncover 8 molecular regulators of planarian glial regeneration. In addition, we show that some of these regulators may alter glial cell state. More importantly, uncovering novel glial regulators will allow us to specifically ablate glial cell populations to study roles that planarian glia play in regeneration and animal physiology.

Introduction

The capacity to regenerate is widely distributed across Metazoans but the extent of regeneration varies by species. While most organisms can regenerate specific organs and structures, very few have the capacity to regenerate the entire nervous system. A critical mediator of an organism's ability to regenerate neural tissue comes down the presence of pro-regenerative glial cells (Freeman & Rowitch, 2013). Glial cells are heterogenous non-neuronal cells that participate in critical processes in development, homeostasis, and pathology of the nervous system. Glial cells can play either pro-regenerative or anti-regenerative roles in central nervous system (CNS) regeneration, depending on the species, severity of injury, and the regional composition of glia around the injury site. Studies of glial biology are mostly confined to few model organisms (nematodes, *Drosophila*, zebrafish, rodents, and humans). However, none of these model organisms are adept at limitless central nervous system regeneration. Thus, uncovering the properties of glial cells that allow for pro-regeneration responses could reveal avenues for therapeutic development.

For successful regeneration, glial cells play two essential roles: 1) repairing and preserving surviving neurons and 2) helping new cells restore damaged tissue (Adams & Gallo, 2018; Gallo & Deneen, 2014). In addition to assisting in regeneration of new neurons, glial cells themselves must regenerate within the missing tissue. One of the key gaps in knowledge in the field is how glial cells regenerate following injury. Currently, studies in *Drosophila* have shown that spontaneous proliferation of glial cells and re-wrapping of axons is heavily regulated by feedback loops of Prospero and Notch following injury (Kato et al., 2011). Adult gliogenesis in vertebrates is often limited to specific regions and sources (e.g. olfactory bulb; subventricular zone) (Rusznák et al., 2016). Thus, what dictates the glial regeneration response and how it can

be manipulated for pro-regeneration is a critical, yet understudied realm within regenerative neuroscience.

Planarians are freshwater flatworms capable of full body regeneration, including robust regeneration of their nervous system *without* scarring. Planarians are bilaterians and possess a complex neural anatomy, including the newly discovered presence of glial cells (Roberts-Galbraith et al., 2016; Wang et al., 2016). However, due to their recent discovery, knowledge on planarian glial cells is relatively sparse. Single cell transcriptome (scRNA-seq) atlases have uncovered genes enriched in every cell population in the planarian body, including glial cells (Fincher et al., 2018; Plass et al., 2018). Previous work showed that *forkhead box protein factor-1* (*foxf-1*) and ETS transcription factor *ets-1*, as well as Hedgehog signaling from ventral-medial neurons influence gene expression in glial cells (Chandra et al., 2023; Dubey et al., 2022; Scimone et al., 2018; Wang et al., 2016). However, fundamental regulators of planarian glial cell fate remain to be uncovered. Moreover, how these regulators cooperate together in regulating glial cell state remains elusive.

In this study, we report identification of 10 genes important for glial regeneration in planarians. We discovered that several transcription factors, including MYB family proto-oncogene (*myb),* BATF basic leucine zipper ATF-like (*atf-like-1*), early growth response protein 3 (*egr-3*) and T-cell factor transcription factor-7 (*tcf-7*), are important for generation of new *cali*$^{+}$ glial cells in regenerating tissues. Additionally, we uncovered that transcription factor *foxf-1* and *ets-1* work cooperatively to influence glial cell state. Finally, we found that Notch signaling plays a role in glial regeneration, potentially working along with *foxf-1* and *ets-1*. Taken together, our work reveals key regulators in glial cell regeneration and identity in planarians.

Methods and Materials

Animal Husbandry

Asexual *Schmidtea mediterranea* (CIW4 strain) were maintained as previously described in (Cebrià & Newmark, 2005; Chandra et al., 2023). In brief, *S. mediterranea* worms were housed in 1x Montjuïc salts at 18-20□C. Worms were fed organic pureed calf/beef liver per week. Prior to conducting experiments, worms were set aside in a separate container and starved for at least one week.

Identification of genes and cloning

Genes of interest were identified in PlanMine 3.0 (Rozanski et al., 2019) based on homology or from single cell RNA-seq data (Fincher et al., 2018; Plass et al., 2018). Primers were designed using BatchPrimer 3.0 to amplify 500-750 bp segments of genes of interest which were cloned from asexual *S. mediterranea* cDNA (as per protocol in (Collins et al., 2010)). Each PCR product was amplified and then ligated into Eam11051-digested pJC53.2 vector using standard molecular biology protocols (Collins et al., 2010). See Table 3.1 for list of primers. Genes that were screen at first pass with *calamari* are highlighted in blue.

in vitro dsRNA synthesis and RNA interference (RNAi) experiments

For RNAi experiments, dsRNA was synthesized *in vitro* using standard molecular biology techniques as previously described (Chandra et al., 2023; Collins et al., 2010). dsRNA matching *Aequereo victoria green fluorescent protein* (*GFP)* was used as a negative control for RNAi feedings. dsRNA matching *S. mediterranea piwi-2* homolog was used as a positive control (Reddien, 2005). Animals were fed 5 µg dsRNA mixed with green food dye and pureed calf/beef

liver. Animals were fed 5 times, 3-4 days apart, amputated pre-pharyngeally one week after the last feeding, and killed/fixed 7 days post amputation (7 dpa). For each experiment, 10-12 animals (ranging from 3-4 mm in size) are housed in a 60 x 15 mm Petri dishes, washed after each feeding, and treated with 1:1000 gentamicin sulfate (50 mg/mL stock, Gemini Bio-Products).

in situ hybridization (ISH)

For *in situ* hybridization, animals were treated and fixed as per protocol in (Chandra et al., 2023; King & Newmark, 2013). Briefly, animals were treated in 7.5% N-acetyl-L-cysteine in 1X phosphate-buffered saline (PBS) and then fixed in 4% formaldehyde in PBSTx (PBS + 0.3% Triton-X 100). Riboprobes for *in situ* experiments were made from amplicons from pJC53.2 vectors generated with primers that match the T7 site in the vector backbone (see Table 3.1 for details on orientations). Antisense probes were synthesized using digoxigenin-11-UTP (Roche) or fluorescein-12-UTP (Roche) using standard molecular techniques (Collins et al., 2010). ISH experiments were performed as aforementioned (Chandra et al., 2023; King & Newmark, 2013). Some ISH experiments were performed in an InsituPro VSi hybridization robot (Intavis, Germany). Probes were detected with anti-digoxigenin and anti-fluorescence Fab fragments (Sigma-Aldrich) conjugated to alkaline phosphatase (Roche) or horse-radish peroxidase (Roche) for colorimetric and fluorescent *in situ* hybridization, respectively. Colorimetric and fluorescent *in situ* were performed as previously described (Chandra et al., 2023; King & Newmark, 2013). Briefly, for colorimetric *in situ*, probes were detected with 5-bromo-4-chloro-3-indolyl phosphate (Roche) and nitro blue tetrazolium chloride (Roche) in alkaline phosphatase buffer. Samples were cleared and mounted in 80% glycerol solution. For fluorescent *in situ*, samples were incubated in fluor-tyramide (TAMRA/FAM; 1:500), 4-Iodophenylboronic acid (1:1000)

and 0.003% H_2O_2 in Tyramide Signal Amplification buffer for 15-30 minutes. For double fluorescence *in situ* hybridization, samples were incubated in 0.1M sodium azide solution for 45 minutes to inactivate peroxidase activity prior to second signal development. Samples were mounted in VectaShield Antifade Mounting Medium (Vector Laboratories). Samples were imaged using an Axiocam 506 color camera mounted on Zeiss Axio Zoom V.16 microscope with the Zen 2.3 Pro software for colorimetric *in situ*. For fluorescent *in situ* experiments, images were obtained on a Zeiss LSM 880 confocal microscope with an upright AXIO Imager Z2 and ZEN Black 2.3 SPI software. For whole animals, images were taken with a Plan-Neofluar 10X/0.3 objective, and for smaller and higher magnification fields, a Plan-Apochromat 20X/0.8 objective was used. For each channel, the signal and background cutoff point were determined using the fluorescence intensity range indicator. All images shown are representative.

Real-time quantitative PCR (RT-qPCR)

Real-time quantitative PCR was performed as previously described (Chandra et al., 2023). Briefly, RNA was isolated from RNAi-treated animals (7 dpa, whole worms) using Trizol (Invitrogen) and the manufacturer's protocol. 1 µg of total RNA was reverse transcribed into cDNA using iScript cDNA Synthesis kit (BioRad) using the manufacturer's protocol. RT-qPCR was performed using Applied Biosystems QuantStudio3 Real-Time PCR system and GoTaq qPCR Master Mix with SYBR Green (Promega). qPCR primers are listed in Table 3.2, and used as previously published (Chandra et al., 2023). All measurements were performed in biological and technical triplicates. Overall transcript levels were normalized using *β-tubulin* mRNA within each sample. Statistical analyses were performed in GraphPad Prism9; details of statistical tests are provided in respective figure legends.

Results & Discussion

Uncovering genes required for glial regeneration.

To uncover genes required for glial cell regeneration in planarians, we first devised a list of transcripts with expression enriched in glial cells (Fincher et al., 2018). We also identified transcripts enriched in *cathepsin*$^+$ progenitors, which are proposed to be precursors for glial cells (Fincher et al., 2018). We identified 197 genes of interest, of which 83 genes were enriched in *cathepsin*$^+$ progenitors only, 52 were enriched in glial cells only, and 14 genes were enriched in both *cathepsin*$^+$ and glial cells. We also included 48 candidate genes that were identified as homologs to transcription factors and regulators of glial cell biology in other organisms (Fig 3.1A). Of 192 candidate genes, we successfully cloned 170. We next sought to examine the expression patterns of these genes. Genes enriched in the glial subcluster ranged in expression patterns from the central nervous system only (i.e. *IF-1, dd_2982, ANPEP, DPP6, CACNA1S, dd_2516, dd_1862, dd_2123, dd_2293*), or enrichment in both the central nervous system and the parenchyma (i.e. *dd_18612, dd_5019, SLC13a-1*, *PXDN*, *BHMT*) (Fig 3.1B). A few were expressed in similar patterns consistent with the digestive or excretory systems (Fig 3.1B, top). Transcripts enriched in the *cathepsin*$^+$ cluster were expressed in patterns similar to the expression pattern seen for planarian stem cells (i.e. *ETS-1*, *TLL-1*, *PTPRJ*) or in the digestive system (i.e. *ZFP64*, *DBI*, *PSAP*) (Fig 3.1B, bottom left). Genes enriched in both glial and *cathepsin*$^+$ clusters were found to be expressed in the central nervous system and/or a parenchyma by ISH (i.e. *jagged-like*) (Fig 3.1B, bottom middle). We also identified planarian homologs of miscellaneous genes encoding transcription factors or glial regulators in other organism, such as *glial maturation factor β* (*GMFβ*) (Fig 3.1B, bottom right).

We next asked whether each candidate gene was important for regeneration of glial cells in planarians. To assess regeneration, we amputated RNAi animals pre-pharyngeally (anterior to pharynx) and let them regenerate their heads. Following head regeneration, we processed the animals and performed colorimetric *in situ* with a glial marker. Initially, we performed a pilot screen with the broad glial marker *estrella* (Roberts-Galbraith et al., 2016). However, we found that *estrella* was not a sensitive enough marker to pick up all expected hits. Therefore, we decided to use *calamari* and then follow up with *estrella* as the secondary identifier. In addition, we also recognized that knockdown of *cathepsin*$^{+}$ genes (including glial genes) required further optimization from the traditional feeding paradigm (Chandra et al., 2023; Scimone et al., 2018). We opted for more feedings within a small timeframe to robustly knockdown our genes of interest (Fig 3.2B).

Following multiple optimizations, we performed an RNAi screen of 56 genes using the glial marker *calamari* (Wang et al., 2016)(Fig 3.2A, C-D, Supp. Fig 3.1). From our first pass of the RNAi screen, we identified 10 candidates for further studies: *MYB proto-oncogene* (*myb)*, *BATF basic leucine zipper ATF-like (atf-like-1)*, *ets-1*, *dd_919*, *defender against cell death 1* (*dad1*/*slc6a-8*), *calsynthenin 2* (*clstn2*), *early growth response protein 3* (*egr-3*), *T-cell factor transcription factor-7 (tcf7), protein tyrosine phosphatase receptor type J (ptprJ)*, and *dd_1866* (Fig 3.2C-D; Suppl. Fig 3.1-3.2). Compared to control, knockdown of *myb* resulted in animals showing decreased expression of *calamari* in the trunk, "gaps" in staining of the ventral nerve cord, and a fused regenerated brain (Fig 3.2C). Similarly, knockdown of *clstn2* and *ptprJ* resulted in decreased *calamari* expression in the regenerated head and subtle loss of expression in the ventral nerve cords compared to control animals (Fig 3.2C). In addition, knockdown of *atf-like-1* resulted in decreased expression of *calamari* in the regenerated head, compared to controls

(Fig 3.2C). On the other end of the spectrum, knockdown of genes like *clstn2*, *tcf7*, and *dd_1866* resulted in a fused brain phenotype compared to control animals without an overall decrease in *calamari* expression (Fig 3.2C). In two cases, *dd_919*(RNAi) and *dad1/slc6a-8*(RNAi), animals lysed suggesting broader roles in regeneration or tissue maintenance rather than a specific role in glial regeneration (Fig 3.2C; Suppl. Fig 3.2). Knockdown of *ets-1* resulted in loss of *calamari* expression in both the newly regenerated head tissue and the homeostatic trunk tissue (Fig 3.2D), which we characterized in a prior manuscript (Chandra et al., 2023).

Next, we asked if some of the hits from our screen affected other glial markers as well or was uniquely affecting *calamari* expression. We repeated our RNAi paradigm on 3 of our candidates (*myb*, *atf-like-1*, and *clstn2*), this time performing *in situ* hybridization with glial marker *estrella*. Knockdown of *myb* resulted in reduced *estrella* expression in both regenerated head and throughout the ventral nerve cord compared to control (Fig 3.3). Likewise, *atf-like-1*(RNAi) resulted in reduced *estrella* expression only in the regenerating head compared to control (Fig 3.3). In contrast, we found that knockdown of *clstn2* does not affect *estrella* expression compared to control (Fig 3.3). Taken together, our data suggests that while *myb* and *atf-like-1* are important for glial cells regeneration, *clstn2* may be only required *calamari* expression. In addition, it is interesting to note that, with the exception of *ets-1,* none of the putative transcriptional regulators we uncovered in our screen have been characterized for functions in glial biology in other organisms. We also want to make a note that many genes that we screened may not be essential for glial maintenance or regeneration but might still play roles in glial function, which could be explored in further experiments (e.g. behavioral assays). In addition, it is possible that loss of a gene may result in more *calamari* expression, which cannot be accurately discernible by *in situ* hybridization and may require quantitative methods.

Furthermore, as noted above, some genes might impact *estrella* expression but not *calamari* expression, and vice versa.

Transcription factor *foxf-1* impacts glial gene expression.

Previous work showed that transcription factor encoding gene *forkhead F1*(*foxf-1*) is important for *calamari* expression in planarians (Scimone et al., 2018). However, our previous work showed that individual glial markers behave differently after some gene perturbations (Chandra et al., 2023). We also proposed that these different gene expression trends might represent glial cell state. To uncover whether *foxf-1* is important for glial maintenance, regeneration or potential cell state, we knocked down *foxf-1* using the same paradigm as performed in the RNAi screen (Fig 3.2B). Like previous reports, we observed robust decrease in *calamari* expression in *foxf-1*(RNAi) animals compared to control (Fig 3.4A, top). We also observed a modest decrease in *estrella* expression in the regenerated head tissue and in homeostatic trunks, particularly in the PNS, following *foxf-1*(RNAi) (Fig 3.4A, bottom). Our observations in *foxf-1*(RNAi) animals were similar to results from *ets-1*(RNAi) (Chandra et al., 2023). However, unlike *ets-1*(RNAi), we did not see the same degree of reduced *estrella* expression throughout the whole body of *foxf-1*(RNAi) animals. Based on previous work with *ets-1*(RNAi) showing that glial cells may have different states (either after injury or other context), we hypothesized that *foxf-1* worked similarly in regulating *calamari* and *estrella* expression. We repeated the *foxf-1*(RNAi) paradigm and performed fluorescent *in situ* hybridization to observe the overlap in *calamari* and *estrella* gene expression. Interestingly, we observed different ratios of *cali*$^{+}$/*estrella*$^{+}$ cells in *foxf-1*(RNAi) animals compared to control

animals (Fig 3.4B). This led us to conclude that *foxf-1* may play a role in regulate glial gene expression or cell state rather than regeneration alone.

We next wanted to understand the relationship between *foxf-1* and *ets-1,* as both transcription factor-encoding genes play roles in glial cell regeneration and both mRNAs are enriched in the *cathepsin*$^{+}$ progenitor cluster (Fincher et al., 2018). Furthermore, we know from previous work that *ets-1*(RNAi) results in a modest, yet significant decrease in *foxf-1* mRNA levels (Chandra et al., 2023). However, we do not know whether the relationship of *foxf-1* and *ets-1* is unilateral or cooperative. To investigate this, we repeated our knockdown of *foxf-1* and then performed colorimetric *in situ* against *ets-1* probe. We observed a modest decrease in *ets-1* expression following regeneration. Our results suggests that *ets-1* and *foxf-1* may have a cooperative relationship in regulating each other. Meanwhile, a previous study suggests a linear relationship between *foxf-1* and *ets-1* (He et al., 2017). Therefore, further work is required to uncover the exact relationship between the two transcription factors in planarians.

Notch signaling plays a role in altering glial gene expression.

Across Metazoa, Notch signaling drives glial fate in both the central *and* peripheral nervous systems during development and regeneration (Losada-Perez et al., 2016; Patten et al., 2006). In addition, recent work implicates Notch signaling in mediating quiescent and reactive astrocyte morphology (Acaz-Fonseca et al., 2019). Thus, we set out to ask whether Notch played a conserved role in gliogenesis in planarians as well. We identified 2 putative *notch* mRNA expressed within the *cathepsin*$^{+}$ progenitor cluster and glia clusters based on publicly available single cell transcriptome data: *notch1* (dd_12732) and *notch2* (dd_7067) (Elliott, 2016; Fincher et al., 2018). *notch2* mRNA is enriched in the *cathepsin*$^{+}$ progenitor cluster while *notch1* mRNA

is enriched in the glia cluster only. We designed two non-overlapping dsRNA constructs matching *notch2* (henceforth *notch2-1* and *notch2-2*). We repeated our knockdown paradigm with each *notch2* dsRNA construct individually and then together. We then performed *in situ* hybridization against broad glial marker estrella on the regenerated worms. Individual knockdown of *notch2* construct 1, *notch*2-1, resulted in no obvious difference in *estrella* expression compared to control. In contrast, knockdown with the second *notch2* construct, *notch2-2,* yielded a more robust reduction in *estrella* expression compared to control in the regenerated head tissue. Furthermore, knockdown of both constructs together (*notch2*-1/2) resulted in a striking decrease in *estrella*$^{+}$ signal in the newly regenerated head tissue (Fig 3.5A). This need for more aggressive RNAi paradigm for *notch* is consistent with prior reports (Elliott, 2016). We did not see any discernable differences in the maintained posterior tissue.

In addition to *notch1*, we also looked at putative homologs of other components of Notch signaling identified within the *cathepsin*$^{+}$ and glia subclusters (e.g. *Delta, jagged*) (Fincher et al., 2018). We identified putative Delta-related genes *delta-1* and *delta-like-4* (*dll4*), and *jagged-like*. While both *dll4* and *jagged-like* are enriched in the *cathepsin*$^{+}$ progenitor cluster and in the glia cluster, *delta1* is only enriched in the *cathepsin*$^{+}$ cluster. We repeated our knockdown paradigm for each of these genes and stained with glial marker *calamari* and *estrella* (Supp. Fig 3.1, Supp. Fig 3.3). We did not see any discernable changes in *calamari* expression in the knockdown animals compared to control, reinforcing our previous observation that components of the Notch signaling pathway does not affect *calamari* expression in planarians. However, we *also* did not see any changes in *estrella* expression either after *dll4*(RNAi) and *jagged-like*(RNAi) compared to control (Supp. Fig 3.3).

Taken together our data suggest that components of the *Notch* signaling pathway, particularly *notch2*, are required for *if-1* and *estrella* gene expression in regenerated head tissue but not for *calamari* expression and is therefore not required for maintenance of existing glia. Furthermore, we hypothesize that Notch signaling may play an important role in specifying glial cell state along with *foxf-1* and *ets-1*.

phred-1 is required for glial regeneration and maintenance.

In addition to *notch1* and *notch2*, we identified another gene with enriched expression within the glial cluster as well as in muscle clusters – *phred-1* (Fincher et al., 2018). *phred-1* is a *neurexin-1* homolog and previous work determined that *phred-1* is required for regeneration of muscle fibers within the pharynx (Adler & Sánchez Alvarado, 2017). Previous work also showed that Neurexin-1 protein in vertebrate astrocytes plays an integral role during synapse assembly (Trotter et al., 2020). In addition, neuroligin-neurexin adhesion mechanisms between astrocytes and neurons are an indispensable regulators influencing astrocyte morphology (Tan & Eroglu, 2021). Therefore, we found Smed *phred-1* a compelling candidate for further investigation. We repeated our RNAi paradigm to knock down *phred1* and stained with glial markers *calamari* and *estrella*. Compared to control animals, we observed mild reduction of *calamari* expression in the regenerated head in *phred1*(RNAi) animals (Fig 3.6A, top). In contrast, we detected decreased *estrella* expression in the regenerated head as well as in the homeostatic trunk, specifically in peripheral *estrella*$^{+}$ cells (Fig 3.6A, bottom). Interestingly, we noted that we observed a disorganized *calamari* expression in the regenerated head but not disorganized *estrella* expression. We also performed real time quantitative PCR (RT-qPCR) on control and *phred1*(RNAi) animals using three distinct glial markers: *if-1*, *calamari*, and *estrella*. We

observed a significant decrease in *if-1* and *estrella* expression after *phred1*(RNAi) (Fig 3.6B). We also noted a significant increase in *calamari* expression levels, which was not initially observable by *in situ* (Fig 3.6B). Our data suggest a potential role of *phred-1* in either 1) regulating glial maturation, and/or 2) playing a role in planarian glia state, possibly via the interplay of *calamari* and *estrella* expression over different time points.

Conclusion

In summary, freshwater flatworms capable of robust regeneration of their nervous system allow for a unique perspective into glial cell regeneration. In this work, we identify molecular regulators required for glial cell regeneration in planarians. Our results indicate that knockdown of transcription factor-encoding genes such as *myb* and *atf-like-1* results in loss of glial cells during regeneration, and/or results in defects in brain morphology, respectively. Furthermore, we also uncover that transcription factors *foxf-1* and *ets-1* may work cooperatively to dictate glial cell states. Additionally, we find that Notch signaling also plays a critical and conserved role in generation of new glial cells. Further work will be required in uncovering the specificity of each molecular regulators in glial cell fate. Moreover, it will be important in addressing how these regulators work in networks to specify glial identity and function in planarians.

Acknowledgements

We are grateful to members of the Roberts-Galbraith lab for thoughtful feedback on this work. We also want to thank to University of Georgia Biology Microscopy Core for providing us with training and assistance on the LSM 880 confocal microscopy. We are thankful to Dr. Alejandro Sanchez-Alvarado and Shane Merryman (Stowers Institute) for providing planarian stocks

during our laboratory start up. We would like the farmers at the White Oak Pastures (Bluffton, Georgia, USA) for providing us with healthy calf/beef liver for our planarians.

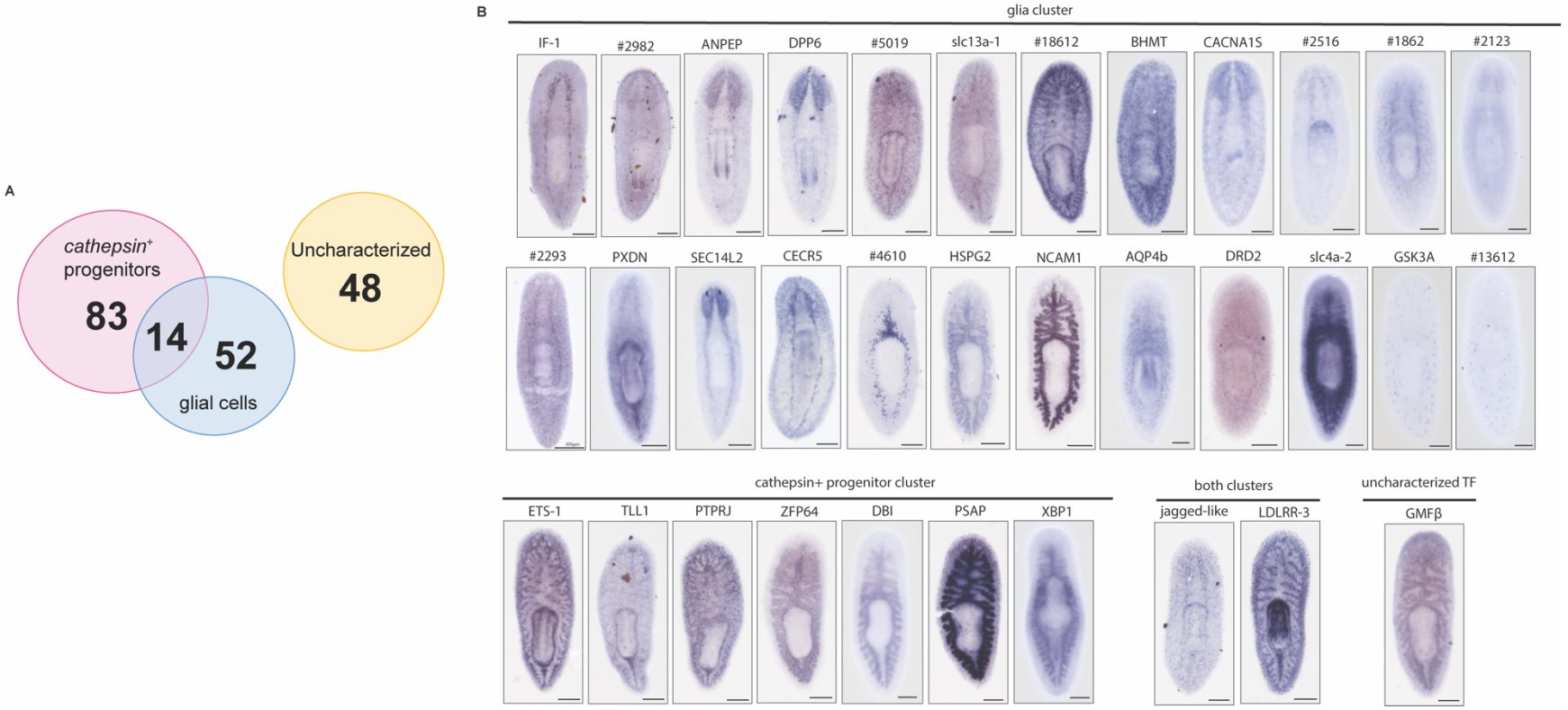


Figure 3.1 Investigating candidate genes for glial regeneration. (A) Venn diagram illustrating the number of genes pooled from each subcluster from transcriptome atlas for RNAi screen (Fincher et al., 2018). Total number of genes identified: 192 genes; *cathepsin*$^{+}$ progenitors (pink): 83 genes; glial cell subcluster (blue): 52 genes; enriched in both *cathepsin*$^{+}$ progenitor and glial clusters: 14 genes; miscellaneous transcription factors and signaling factors identified by homology (yellow): 48 genes. (B) Expression pattern of candidate genes identified for an RNAi screen separated by the cluster in which the gene expression is enriched in. Scale bar: 200 µm.

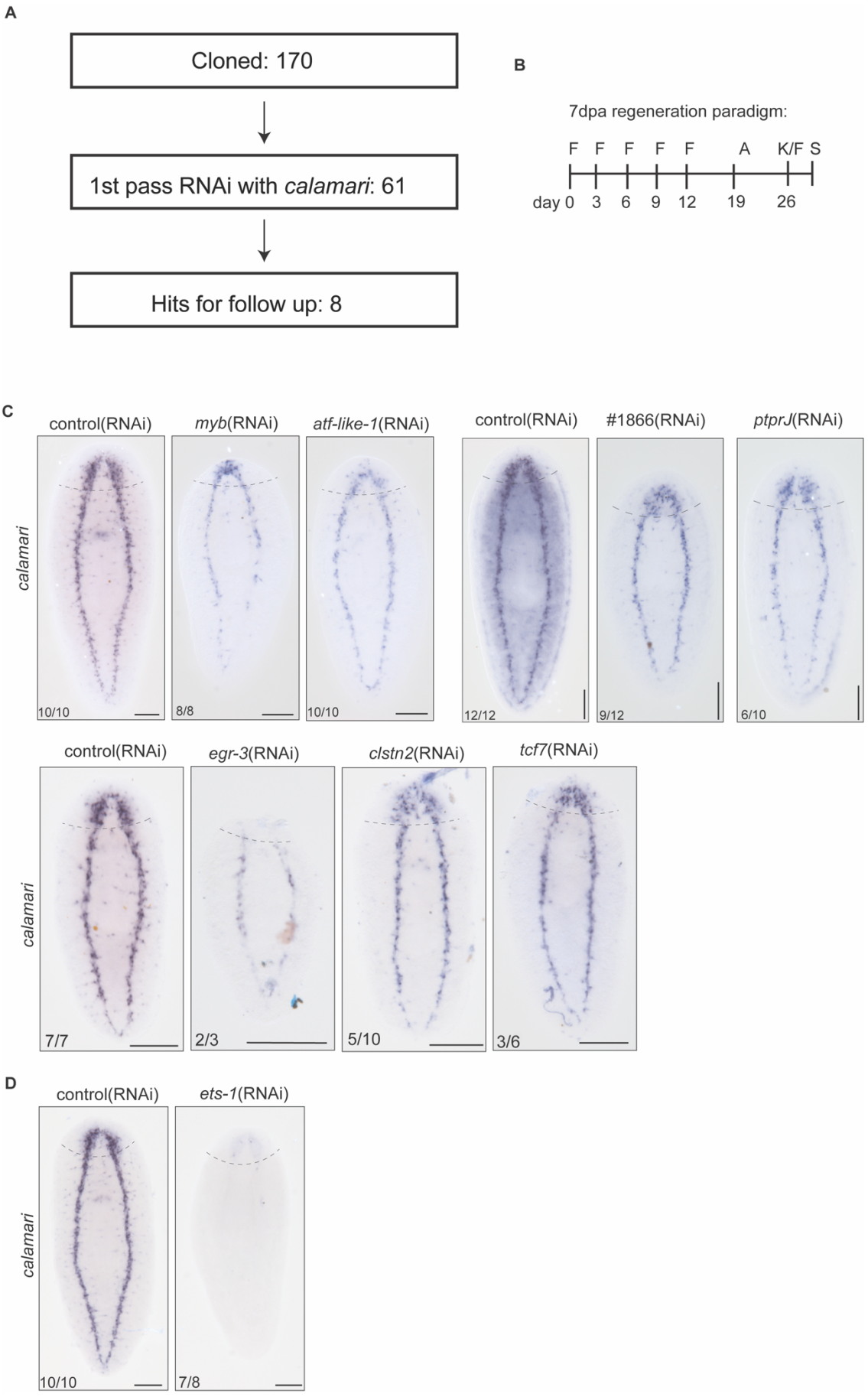

A
Cloned: 170
1st pass RNAi with calamari: 61
Hits for follow up: 8
B
7dpa regeneration paradigm:
F F F F F A K/F S
day 0 3 6 9 12 19 26
C
control(RNAi)
myb(RNAi)
atf-like-1(RNAi)
control(RNAi)
#1866(RNAi)
ptprJ(RNAi)
calamari
10/10
8/8
10/10
12/12
9/12
6/10
control(RNAi)
egr-3(RNAi)
clstn2(RNAi)
tcf7(RNAi)
calamari
7/7
2/3
5/10
3/6
D
control(RNAi)
ets-1(RNAi)
calamari
10/10
7/8

Figure 3.2 A large scale RNAi screen uncovers 10 candidate genes of glial regeneration (A) Simple schematic showing genes that were cloned (170 genes), genes went through a first pass RNAi screen with glial marker *calamari* (56 genes); and how many hits were obtained from the first screen (8 genes). (B) RNAi feeding paradigm. Animals were fed for a total of 5 feedings 3-4 days apart. Animals were amputated pre-pharyngeally (removing head tissue) one week after last feeding, and then killed/fixed one week afterwards for downstream experiments. F = feeding; A = amputation; K/F = kill/fix; S = staining. (C-D) We performed *in situ* hybridization with glial marker *calamari* to uncover genes of interest that may be important for glial regeneration. We uncovered 8 potential candidates for follow up studies: *myb*, *atf-like-1*, *egr-3*, *clstn-2*, *tcf7*, *dd_1866*, *ptprJ,* and *ets-1*. Knockdown of *dd_919* (not pictured) and *dad1* (see Fig 3.S2) resulted in lysing. For details on genes, see Table 3.1. Dashed line = amputation site. Scale bar: 200 μm.

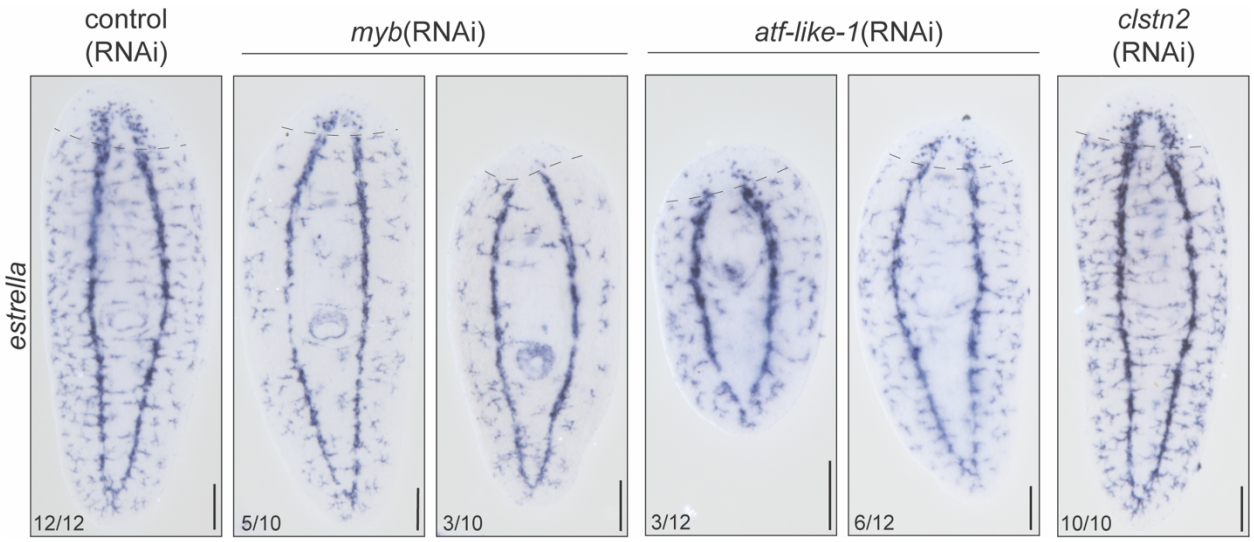


Figure 3.3 Hits from screen also affect other glial markers. We repeated our RNAi paradigm on 3 of our hits and performed *in situ* hybridization with glial marker *estrella* to whether these genes were required for all glial gene expression. Knockdown of *myb and atf-like-1* resulted in reduced *estrella* expression in regenerating head tissue, suggesting that these transcription encoding genes affect all glial cells in planarians. Meanwhile, knockdown of *clstn2* does not affect *estrella* expression, suggesting specificity to affecting *calamari*. Dashed line = amputation site. Scale bar: 200 μm.

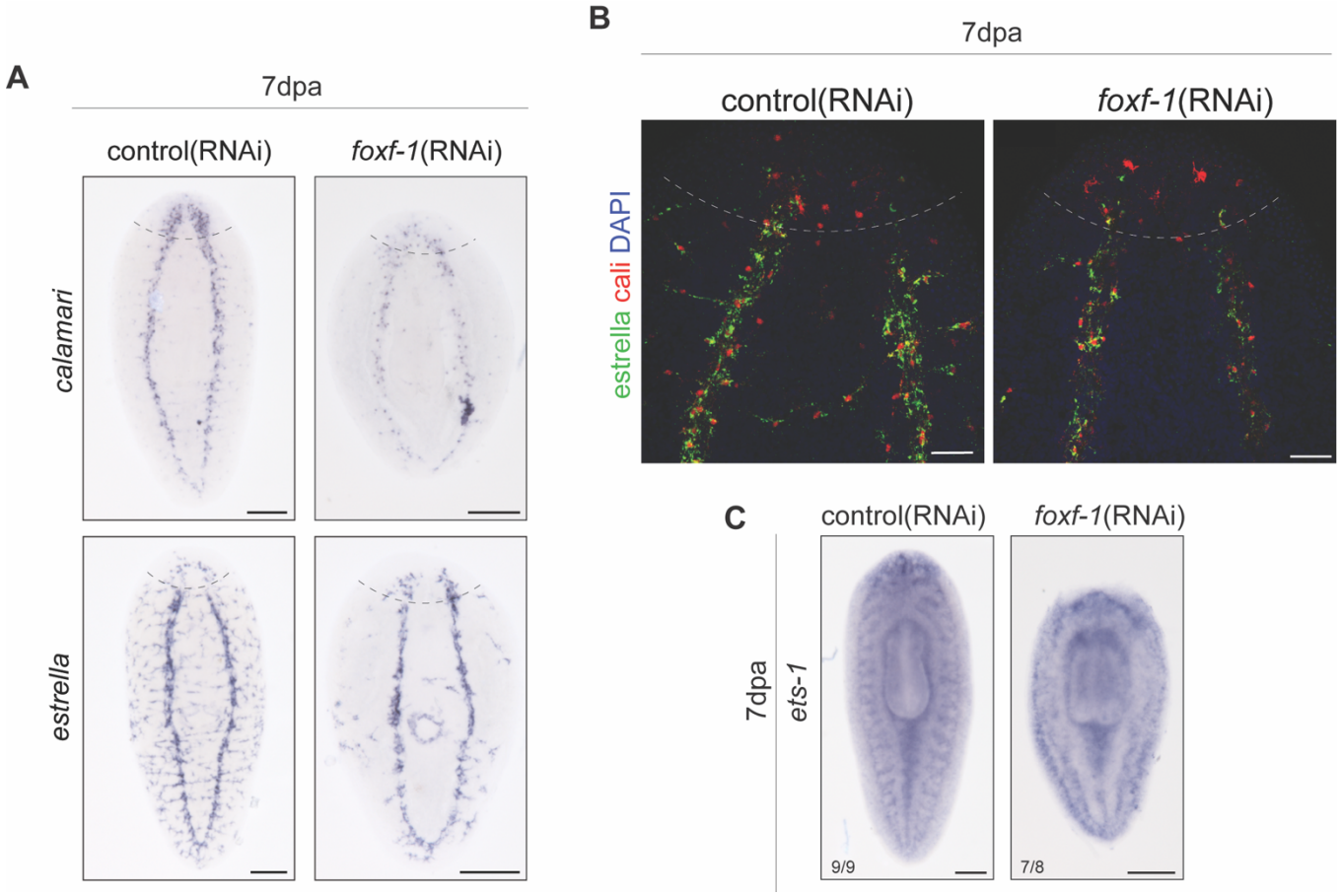


Figure 3.4 Transcription factor *foxf-1* is important for glial gene expression. (A) We performed *foxf-1*(RNAi) and then *in situ* hybridization on regenerated animals with glial markers *calamari* (top) and *estrella* (bottom). As previously reported, *foxf-1*(RNAi) affects glial gene expression in both regenerated head as well as in homeostatic trunk tissue (Scimone et al., 2018). (B) Control and *foxf-1*(RNAi) animals were subjected to double fluorescent *in situ* hybridization of *calamari* (red) and *estrella* (green), and stained with DAPI (cell nuclei, blue) in newly regenerated heads. (C) *in situ* hybridization of control and *foxf-1*(RNAi) regenerated animals with probe for *ets-1*. Knockdown of *foxf-1* subtly affects *ets-1* gene expression. Dashed line = amputation site. Scale bar (A, C) 200 µm; (B) 50 µm.

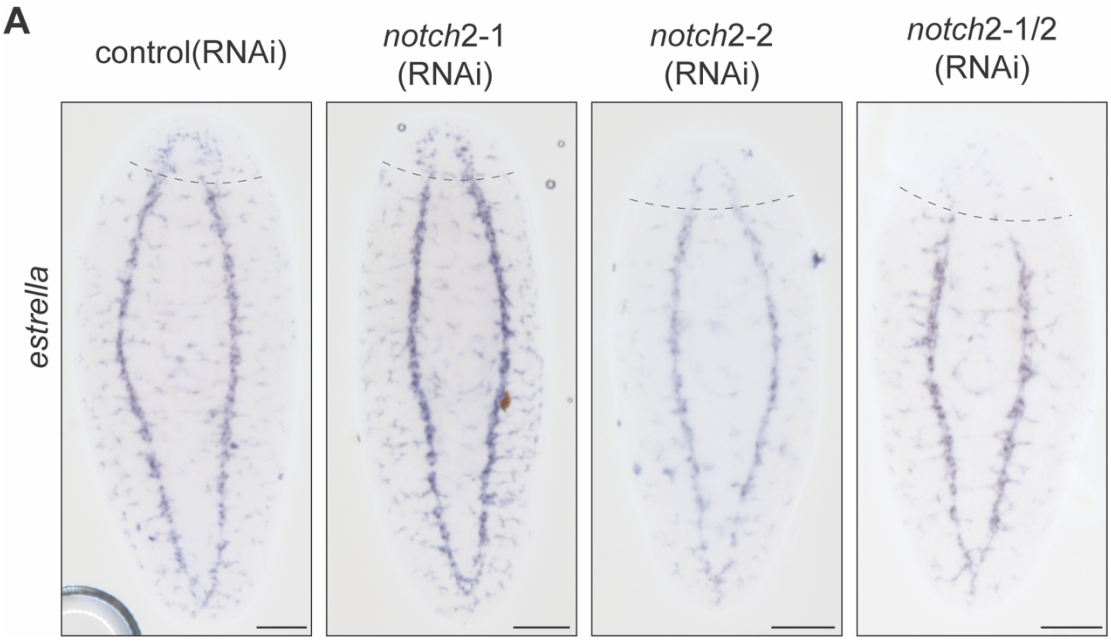


Fig 3.5 *notch2*(RNAi) affects *estrella* expression. (A) Knockdown of *notch2* constructs 1 and 2 and double knockdown with both *notch2* constructs (*notch2-1/2*) results in reduction of *estrella*$^{+}$ cells in regenerated head, compared to control. Dashed line = amputation site. Scale bar: 200 µm.

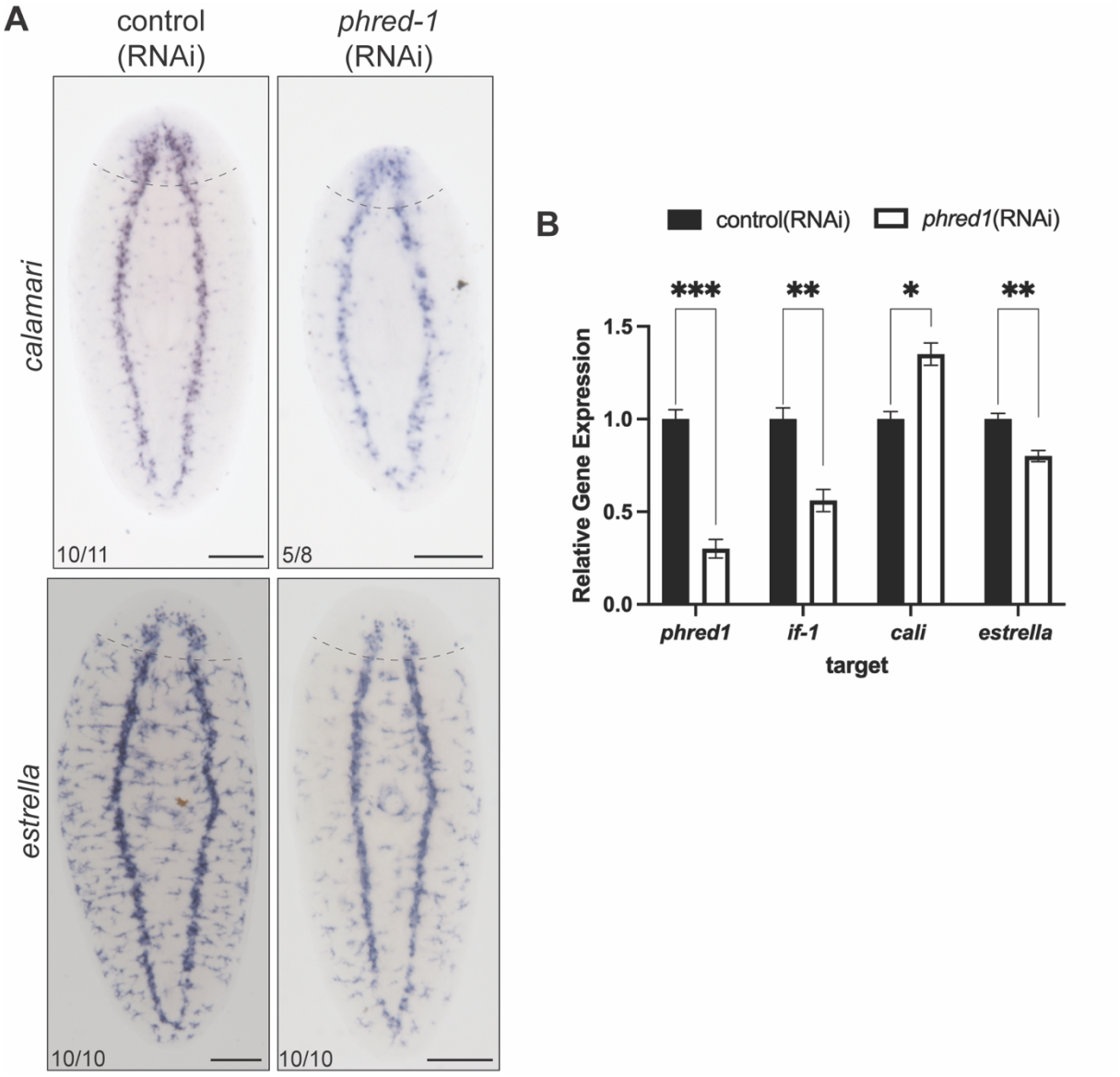
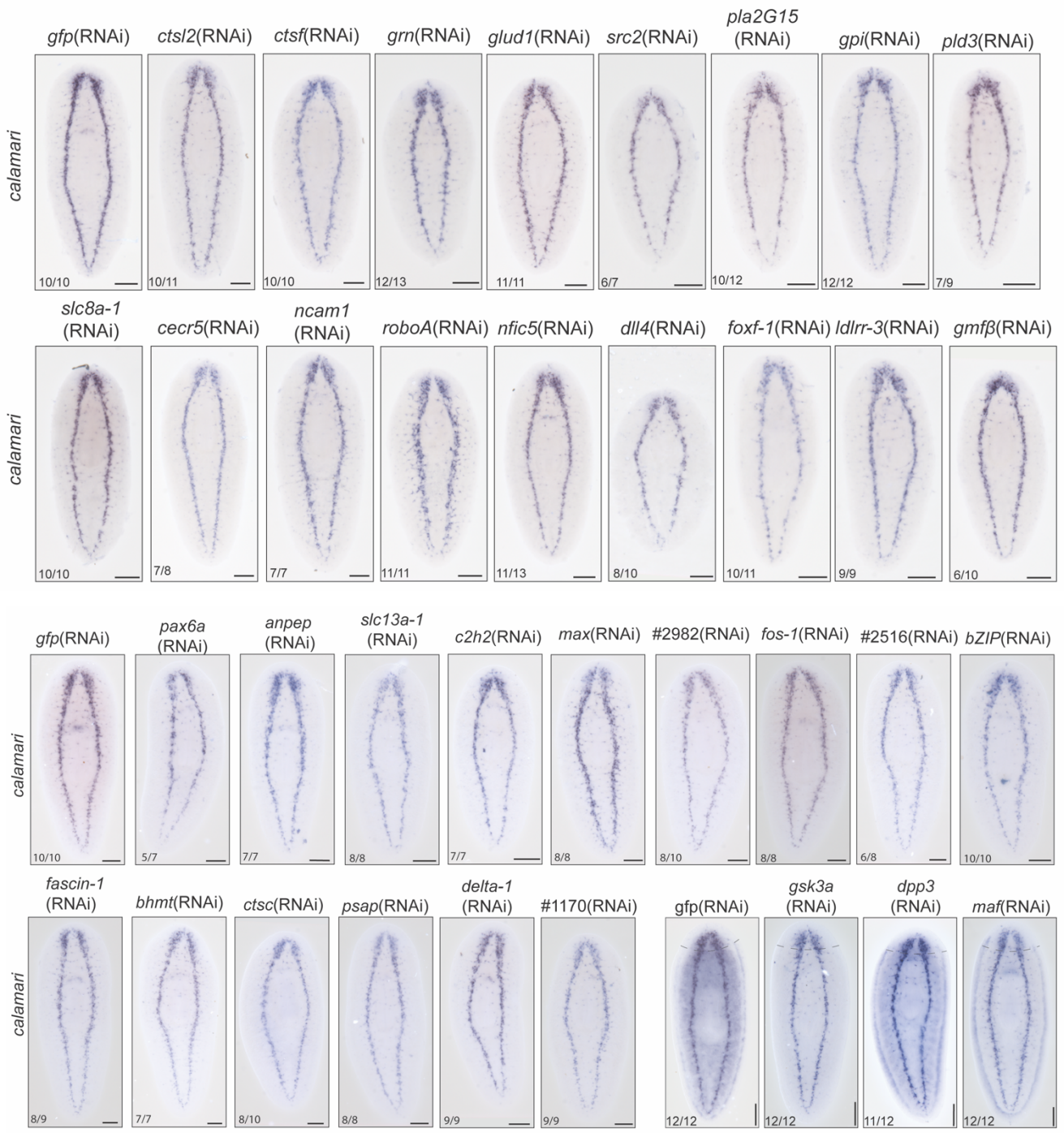


Figure 3.6 Knockdown of neurexin homolog *phred-1* results in altered glial gene expression. **(**A) *phred-1*(RNAi) animals were subjected to *in situ* hybridization with glial markers *calamari* (top) and *estrella* (bottom). Compared to control, *calamari* expression in *phred-1*(RNAi) animals was disorganized. In contrast, we observed reduced *estrella*$^{+}$ cells throughout *phred-1*(RNAi) animals. (B) We performed RT-qPCR on whole body control and *phred-1*(RNAi) animals to detect levels of *phred-1*, *if-1*, *estrella* and *calamari* transcripts after RNAi and regeneration. Unpaired t-test with Welch's correction. Data are mean ± s.e.m. *P≤0.05, **P≤0.01, ***P≤0.001Scale bar: 200 µm.


gfp(RNAi)
ctsl2(RNAi)
ctsf(RNAi)
grn(RNAi)
glud1(RNAi)
src2(RNAi)
pla2G15 (RNAi)
gpi(RNAi)
pld3(RNAi)
calamari
10/10
10/11
10/10
12/13
11/11
6/7
10/12
12/12
7/9
slc8a-1 (RNAi)
cecr5(RNAi)
ncam1 (RNAi)
roboA(RNAi)
nfic5(RNAi)
dll4(RNAi)
foxf-1(RNAi)
ldlrr-3(RNAi)
gmfβ(RNAi)
calamari
10/10
7/8
7/7
11/11
11/13
8/10
10/11
9/9
6/10
gfp(RNAi)
pax6a (RNAi)
anpep (RNAi)
slc13a-1 (RNAi)
c2h2(RNAi)
max(RNAi)
#2982(RNAi)
fos-1(RNAi)
#2516(RNAi)
bZIP(RNAi)
calamari
10/10
5/7
7/7
8/8
7/7
8/8
8/10
8/8
6/8
10/10
fascin-1 (RNAi)
bhmt(RNAi)
ctsc(RNAi)
psap(RNAi)
delta-1 (RNAi)
#1170(RNAi)
gfp(RNAi)
gsk3a (RNAi)
dpp3 (RNAi)
maf(RNAi)
calamari
8/9
7/7
8/10
8/8
9/9
9/9
12/12
12/12
11/12
12/12

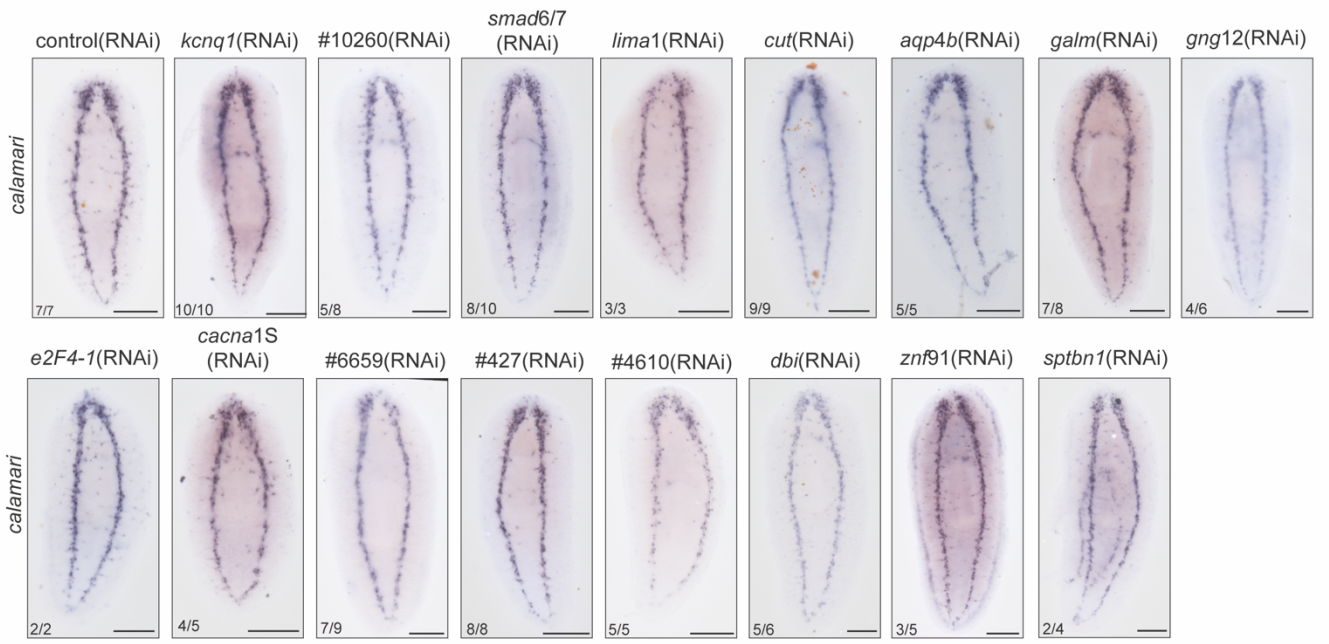


Figure 3. S1. Large scale RNAi to uncover regulators of glial regeneration. RNAi animals were subjected to *in situ* hybridization with glial marker *calamari* as a first pass of the RNAi screen. Controls are same as in Figure 3.1. For details on genes, see Table 3.1. Scale bar: 200 µm.

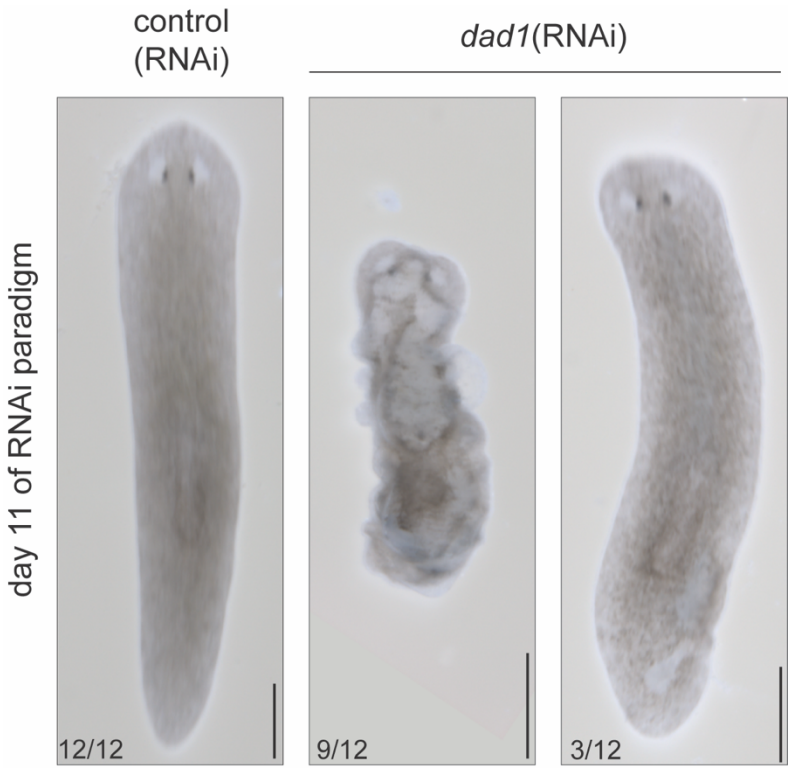


Figure 3. S2. *dad1*(RNAi) results in animal lysing. Live images of control and *dad1*(RNAi) animals at day 11 of RNAi paradigm (one day prior to 5th feeding). For details on gene, see Table 3.1. Scale bar: 500 µm.

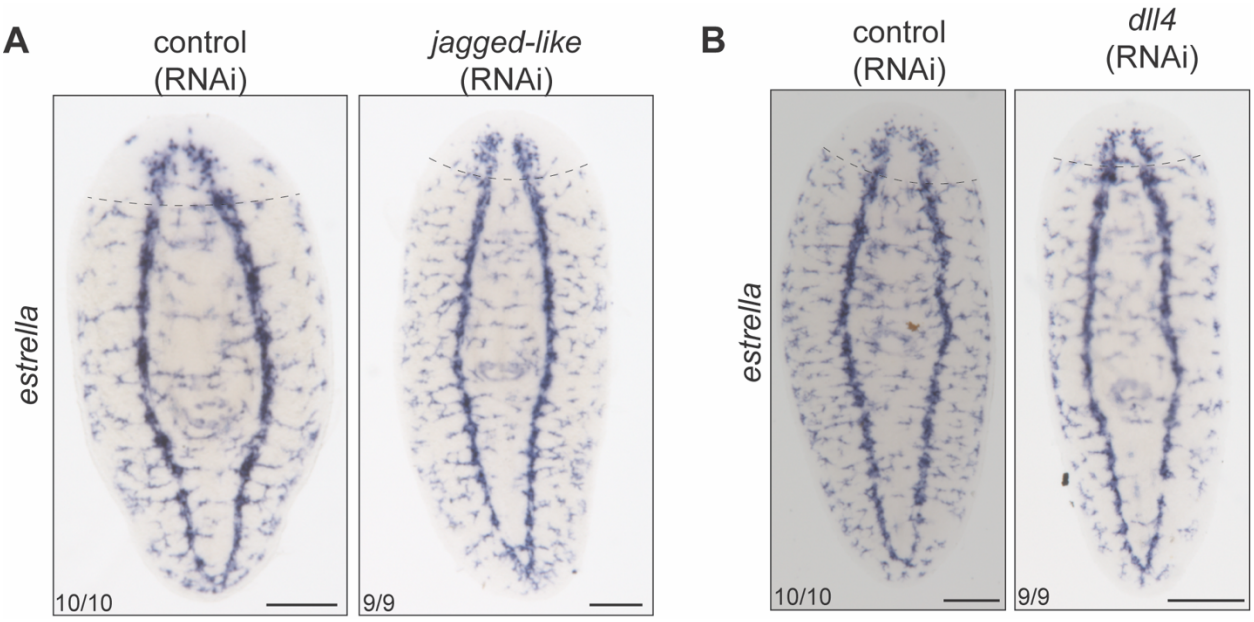


Figure 3. S3. Notch signaling ligands *jagged-like* and *dll-4* are not required for estrella gene expression. (A-B) *in situ* hybridization of *jagged-like*(RNAi) and *dll4*(RNAi) , compared to control, with broad glial marker *estrella* resulted in no difference in expression level in regenerated head or pre-existing trunk tissue. Scale bar: 200 μm.

Table 3.1 List of genes cloned for RNAi screen

Dresden contig	Homolog	Fwd Primer	Rev Primer	Orientation	Cluster
dd_Smed_v6_6910_0_1	Forkhead box f1 (foxf-1)	GGCAGACCTGGTAAAGGACA	GCAACAGCTGCTACAGTGGA	SP6	cathepsin+ progenitor
dd_Smed_v6_12472_0_1	Delta-like 4 (dll4)	AACCGATCCCAGAAGTTTCC	TCAACGTCAGTGTTGGCTTT	SP6	cathepsin+ progenitor; glia
dd_Smed_v6_1054_0_1	Low density lipoprotein receptor related 3 (ldlrr-3)	GAAAATGGAAAGTCCCAGCA	TCGCATCACGTGACAATACA	T3	cathepsin+ progenitor
dd_Smed_v6_456_0_1	Cathepsin F (CTSF)	TCCATGGCTAGAACCGAAAC	CAGAGCACCAATTTGCCATA	SP6	cathepsin+ progenitor; glia
dd_Smed_v6_2092_0_1	v-ets erythroblastosis virus E26 oncogene homolog 1 (ETS1)	ACTGCCGAATTATTGCGTTC	CCGGTAAATCGGGTTTCTTT	SP6	cathepsin+ progenitor
dd_Smed_v6_1398_0_1	Glucose-6-phosphate isomerase (GPI)	CAAGGATTCGCTTTCAATGAT	ATTACCCATGACCCCATTCA	SP6	cathepsin+ progenitor; glia
dd_Smed_v6_953_0_1	src-2	AAATGGGGTCGTGGATATGA	ATCAGCATGCCAACAATCAA	SP6	cathepsin+ progenitor
dd_Smed_v6_7422_0_1	Neural cell adhesion molecule 1 (ncam1)	TGGTCCAGTAACATGCCAAA	TTCTTGCGGCAAATTTCTCT	SP6	glia
dd_Smed_v6_12312_0_1	Nuclear factor 1C (CCAAT-binding transcription factor) (NFIC)5	TGTTATGCGATTTTGCTTGC	CACGTAATTATGGCGAGCATC	SP6	cathepsin+ progenitor
dd_Smed_v6_2804_0_1	Glia maturation factor, β	TATCGTCGAAGGAGGTTTGC	TCATAGCCGGCGAATAAAAA	T3	cathepsin+ progenitor
dd_Smed_v6_8494_0_1	roboA	ATGTGCCACCTGTGATTGAA	TCAAATGGTGAATGGGATCA	T3	glia

dd_Smed_v6_3674_0_1	Cat eye syndrome chromosome region, candidate 5 (CECR5)	GGGTTCGAGCTCTTGCATTA	CGCAAGGGTACAGGAAAGAA	T3	cathepsin+ progenitor
dd_Smed_v6_3303_0_1	Slc8a-1	TCCAATATTTTTCCCCAGCA	CAGAGCACCAATTTGCCATA	T3	cathepsin+ progenitor
dd_Smed_v6_1306_0_1	Phospholipase A2, group XV (PLA2G15)	TTCACCCCAAATGATTGGTC	GAGAGCACCCGAAATAGCAG	T3	cathepsin+ progenitor
dd_Smed_v6_932_0_1	Glutamate dehydrogenase 1 (GLUD1)	CCTGAGCCGATTTTGCTAAC	TCATTTCGACCTCCTTTTGG	SP6	cathepsin+ progenitor
dd_Smed_v6_915_0_1	Granulin (GRN)	TTCCGATCGAATGTCACTTG	TTTGTGCGACCAGTTCTGAC	T3	cathepsin+ progenitor
dd_Smed_v6_175_0_1	Cathepsin L2 (CTSL2)	TTCATCTGCTTCAGGTGTCG	GCATGAACTCTGGCGTACTG	T3	cathepsin+ progenitor
dd_Smed_v6_1032_0_1	CREB3	TTCAAACATTTCTCCGCAATC	CGTTCTAGCCGTCTGCTTTC	T3	
dd_Smed_v6_20_0_1	Glutathione S transferase-1 (GST-1)	CCAATAAACAACCCCAATGC	GGGTTCAGCTCTGAAAGACG	T3	cathepsin+ progenitor; glia
dd_Smed_v6_7067_0_1	Notch2 construct 1	TAATCAATGGACCGGGAAAA	TAACCCAACAAACCCCTGAA		cathepsin+ progenitor
dd_Smed_v6_7067_0_1	Notch2 construct 2	GTTTTGATGGAGGCGATTGT	CCCCTAAAACAACCAAGCAA		
dd_Smed_v6_696_0_1	Chloride channel accessory 4 (clca4)	CGATTGATGAAAACCCTATCG	TGATCTTGCCATCGATTTGA	T3	cathepsin+ progenitor; glia
dd_Smed_v6_1581_0_1	Low density lipoprotein receptor related 1 (ldlrr-1)	GCCTCACAATAGCACTTTCG	GTAAATATTGATAATATATTTAAT		cathepsin+ progenitor
dd_Smed_v6_4849_0_1	Mitogen activated protein kinase kinase kinase 5 (MAP3K5)	TTGTTTTTCCCGTCCAACTC	AACCGAAACTCCAAACATCG	SP6	cathepsin+ progenitor
dd_Smed_v6_12264_0_1	Klf4	CCAATAGCAGTAAGCCAATG	ACGATTCGGAATGTTTTCTT	SP6	cathepsin+ progenitor; glia
dd_Smed_v6_8802_0_1	Src-4	TCAGCGAATCTCCCTGACTT	AACGTTCCACTGCTCTTGCT	SP6	

dd_Smed_v6_5127_0_1	Hunchback-like	CGAAAAGGAAATCGACGAAA	AGGAGCTGGCTGATGATTGT	T3	cathepsin+ progenitor
dd_Smed_v6_415_0_1	X-box binding protein (XBP1)	TGGGAATGGTATGGGAAAAA	TGGCTGACTTTGGCTAAAAA	SP6	cathepsin+ progenitor
dd_Smed_v6_4789_0_1	Phred-1	CGGCCTCATGTATCACACAC	GCACATCTGGGATCCACTTT	SP6	cathepsin+ progenitor
dd_Smed_v6_10221_0_1	Jagged-like	GCGTTGATGGATTTTCAGGT	CGGATATACATTCGAGCCAGA	SP6	cathepsin+ progenitor; glia
dd_Smed_v6_8720_0_1	Zfp-1	GCCAGTTTTATTTCACCAGGA	AGCGCATGCCTCTGTAGATT		
dd_Smed_v6_4539_0_1	CREB1	TGATGACTGTAGCCCAACGA	CGCACTCGTCATCGTAGAAA	SP6	
dd_Smed_v6_5996_0_1	src-3	TGCCCGTATCCGATTAAAAA	CGGATTTGGCATCCTGTAAC	SP6	cathepsin+ progenitor
dd_Smed_v6_5749_0_1	Jun-1	TGAATCGACCTCTTCCATCC	TGCTTCATGAATGACAGTCG	T3	cathepsin+ progenitor
dd_Smed_v6_10007_0_1	Lmx1a	AAGAGCCCCACACAACAAAG	TCCGGTGGATTGTCTGATTT	T3	
dd_Smed_v6_28567_0_1	Ptf-3	GACGAAGCTCCACCGGTATGT	TTTCGGTCAATCCACAATCA	T3	
dd_Smed_v6_347_0_1	Y box binding protein 2 (YBX2)	TGTGTTTGCTTGGGTAACGA	GCACAACTGTCGCTTCTTGA	SP6	cathepsin+ progenitor
dd_Smed_v6_703_0_1	Deoxyhypusine dehydrozylase/monooxygenase (DOHH)	GATGGCCGTTATCCTTGGTA	TCATGCGGAAATTGTCTCAC	SP6	cathepsin+ progenitor; glia
dd_Smed_v6_5875_0_1	bZIP	TGAAAAGTGGGTGCAAAATG	TCGACAGACATCCCGGTAAT	T3	
dd_Smed_v6_12732_0_1	Notch 1	AGTTGCAAAGCCTGCTCATT	ACAAGTACCGCCGTTTTCAC	T3	
dd_Smed_v6_5177_0_1	ZFP64 zinc finger protein (ZFP64)/	ATCGAAGCAATTGGAAATGG	AAGGTCGTTCTGGATGATGG	SP6	cathepsin+ progenitor
dd_Smed_v6_12068_0_1	POU6F1	CAAAGTTGTGGAATCGAAGG	TCTTCCTTTTTGGGAAATCTTG	T3	cathepsin+ progenitor; glia

dd_Smed_v6_4685_0_1	CREB2	AGTCGCAAACCTGGAACAAC	TGCAAGCTTTCTGGGAAAAT	SP6	
dd_Smed_v6_6470_0_1	Alanyl (membrane) aminopeptidase (ANPEP)	TCAGGATGGCTGGGTTTTAG	ATTGCTTTTGGTCGCATAGG		
dd_Smed_v6_3410_0_1	Max	CTCAATATGGCTCGGGAAAA	GTTTGTGCTGCCCGATAAAT	SP6	
dd_Smed_v6_17726_0_1	Pax6a	GGCAGCAAACCAAGAGTAGC	CTGCTACAGCCGACAATGAA	SP6	
dd_Smed_v6_642_0_1	Cathepsin C (CTSC)	GCGATCTTACTGGCACGATT	CGAGAGCGACAACACAAGTC	T3	cathepsin+ progenitor
dd_Smed_v6_5476_0_1	Slc13a-1	TTCCTCGGAAGTCTCATCGT	ACAGCTCCGCAAAACTCATT	T3	glia
dd_Smed_v6_9183_0_1	Dipeptidyl-peptidase 6 (DPP6)	AACCGTGAGCAAAATGAAGC	ATCCACCATAGGACCATCCA		glia
dd_Smed_v6_11513_0_1	Sec14-like 2	TGGCGCCTAATCAAGAAAAT	TCGGCAGCACTTTAGGATCT	T3	glia
dd_Smed_v6_1866_0_1		AGTGCGAGTCAACATTTCTG	TGTCAAGTTTCACAACGGTA		cathepsin+ progenitor
dd_Smed_v6_89_0_1	Delta-1	GTCAAATGCCCGAAATCTGT	CCATTTACACATGCGACGAC	SP6	cathepsin+ progenitor
dd_Smed_v6_2982_0_1		CGCAGTTGTGCACCTGTAAT	AGGCATCGCTTTGATCACTC	T3	glia
dd_Smed_v6_718_0_1	Tubulin, alpha 1b (TUBA1B)	AAGCTCTTGCTGCCTTGATG	TTACGTCCGCAACAAAACAG	SP6	cathepsin+ progenitor
dd_Smed_v6_2351_0_1	Chtochrome p450, family 2, subfamily B, polypeptide 6 (CYP2B6)	CGAATTCGTACGGCTATGGT	ACTGCCAGTACTTGGGGATG	T3	glia
dd_Smed_v6_1783_0_1	Protein tyrosine phosphatase receptor type J (PTPRJ)	GCGTATAACCTTGCGGGTAA	CTCCCCAACGAAAGTTGTGT	SP6	cathepsin+ progenitor
dd_Smed_v6_996_0_1	Fascin 1	CCCGATAAGACGGCAGAATA	GGA CCT GTG GAT GAA TTG CT	T3	cathepsin+ progenitor
dd_Smed_v6_23655_0_1	Glycogen synthase kinase 3 alpha (GSK3A)	ATTTCTGCGTCAGCCAAGTT	TGACATCCGGAAAGTTGTGA	T3	glia

dd_Smed_v6_10446_0_1	bZIP/Maf	GAA GAT CGA CCA AGG AAC GA	TAA TTT CGA TTG GCG GAA AC	SP6	cathepsin+ progenitor; glia
dd_Smed_v6_241_0_1	Prosaposin (PSAP)	CGAGTGTGACCTATGCGAGA	TCAAAGTGGAACGAGCAAGA	T3	cathepsin+ progenitor
dd_Smed_v6_919_0_1		ACGGCAAAGAAACCG TTATG	TTTCGAGAGCATCGTGTTTG	SP6	cathepsin+ progenitor; glia
dd_Smed_v6_2789_0_1	Fos-1	AAACAACTCGAAGCGGAAG A	TCCATTGAGTTGCGAGTCTG	SP6	
dd_Smed_v6_2109_0_1	Defender against cell death 1 (DAD1)/slc6a-8	GCATCGGATTATGCCAAAAC	TCAGCAAATGCTCTT TCAGG		cathepsin+ progenitor; glia
dd_Smed_v6_3847_0_1	Atf-like-1			T3	
dd_Smed_v6_652_0_1		AAAAACGTTGGATGGACTGC	TCCATGGCTAGAACCGAAAC	SP6	cathepsin+ progenitor
dd_Smed_v6_2516_0_1		CTGCGAACCCTGAAGAAAGA	TCGAAGCCATGAAAAATAAA AA	T3	glia
dd_Smed_v6_1399_0_1	bZIP	AAAGCGGACGGTTATTTTCA	CAAAAGGTAAGCCAAAGTCC A	T3	
dd_Smed_v6_1170_0_1	Cat eye syndrome chromosome region, candidate 5 (CECR5)	TGGTGAACCAACAGTTCCAA	ATCCAGTTCTGATGCCCTTG		glia
dd_Smed_v6_3238_0_1	DAB 2 interacting protein (DAB21P)	TTTCAGCAGACTCCTCAGCA	CGATGGAAATTCGTGATGGT	T3	cathepsin+ progenitor
dd_Smed_v6_3443_0_1	C2H2 (ZnF)	AATGCTCCGTTGCAGAGAGT	GGCGAACTTGATAGGCTGAG	SP6	
dd_Smed_v6_873_0_1	Betaine-homocysteine S-methyltransferase (BHMT)	TTATGTATCGGACCCGAAGG	AATTTTCGCACCAGAAGCAT	T3	glia
dd_Smed_v6_9273_0_1	Early growth response-2 (egr-2) / KROX20	GATTAACG CT CGCATAAA	TCAGACCGAGCGAATTTCTT	T3	cathepsin+ progenitor
dd_Smed_v6_19755_0_1	Dopamine receptor D2 (drd2)	TATGGAGAGTGGCCTTGGAT	GATCCTCGGACAGTGATTCC	SP6	glia
dd_Smed_v6_1967_0_1	Tolloid-like 1	ACCGGTTTGTCATCCCAATA	CACTCCCCTTTGGACTTGAA	SP6	cathepsin+ progenitor

dd_Smed_v6_2307_0_1	Tolloid-like 2	TCTTGTAAAGACACGAACCCAAT	TGATACTGCCATGCCTGAAA	T3	glia
dd_Smed_v6_4381_0_1	myb	TTGGTTAGCAATCGGAAAGG	TTATTGGCCAACGAAACTCC	SP6	cathepsin+ progenitor
dd_Smed_v6_1518_0_1	Calsyntenin 2 (CLSTN2)	TCCGCAAGAACAGACATCAA	GACCCAGAAAAATGCCTTGA	T3	glia
dd_Smed_v6_12410_0_1	Early growth response 3 (egr-3)	CGT CGG GAT GAA TTG AAA AG	TTG TCT GAA ATG TCG CAA CC	SP6	
dd_Smed_v6_160_0_1	Diazepam binding inhibitor (GABA receptor modulator, acyl-CoA binding protein) (DBI)	ATGCAGAATCAGTCGGCTTT	GTTGCATGCCACAGTTCATT	T3	cathepsin+ progenitor
dd_Smed_v6_6015_0_1	SPDEF	AACCATTGAGGTTTGGCAAG	GGGGAAAGGGATTGGAATAA	SP6	
dd_Smed_v6_8152_0_1	TCF7	AAA CGG AGC GTT AGG GTT TT	GTT GTC GAA GAT GCG ACT GA	T3	
dd_Smed_v6_8899_0_1	Calcium channel, voltage-dependent L type, alpha 1S subunit (CACNA1S)	GCGAATAATACGCCACCAGT	CCGTAATGATATCCCCAGCA	T3	glia
dd_Smed_v6_994_0_1	Pantothenate kinase 4 (PANK4)	GGGTTGAAGGTCACGACAGT	CCCACCTCCACTTCCAATAA	T3	glia
dd_Smed_v6_5019_0_1		TCAATATGCCCTGTGATGGA	TGGTATCAACGCAGAGTTCG	T3	glia
dd_Smed_v6_4610_0_1		AAGGAGTAAATGGCGCGTTA	TATCACGGAACACTCCCACA	SP6	glia
dd_Smed_v6_1122_0_1	Spectrin, beta, non-erythrocytic 1 (SPTBN1)	GAATCGTCACAACTCGCTCA	CGAGTTTTCCCATTCGTG TT	T3	cathepsin+ progenitor; glia
dd_Smed_v6_8606_0_1	PREP	TCAATGAGTGGCTGCACTTC	TGAATGAGGATTCGGAGGTC	SP6	
dd_Smed_v6_427_0_1	granulin (GRN)	AAATGGATCTCGATCAAATGG	CGATGCTCGGGTTGATAAAT	T3	glia
dd_Smed_v6_6659_0_1	C2H2 (ZnF)	GCC GAA CAG CAA CAC AGT AA	GTG TTT GGT TCA CCG GAA CT	T3	

dd_Smed_v6_1694_0_1	HNF4	CTCAATCCGGCATTCTCCTA	CGACCGCCTTATTACTCCAA	SP6	cathepsin+ progenitor
dd_Smed_v6_7964_0_1	Actinin, alpha 2 (ACTN2)	CATCCACCAGTCATGGAAAA	AACCCCATACATGGGAATGA	T3	glia
dd_Smed_v6_67_0_1	Granulin (GRN)	TGGGGGTTATCTCCGATACA	ATGAACGACTTCCTCGCATT	SP6	cathepsin+ progenitor
dd_Smed_v6_12627_0_1		CATTTCATGAAGCCAATTTGA	ATCGGCATAATCCACCACAT	SP6	glia
dd_Smed_v6_692_0_1		TTGCGCATAAACTCAACCAA	TTTTTCAATGGGAGTAAGTCAGC	SP6	glia
dd_Smed_v6_4367_0_1	4-aminobutyrate aminotransferase (ABAT)	CGAATCAATGGCAAACTGAA	TGGTAGAGTGGCTGGTCGAT	SP6	glia
dd_Smed_v6_3130_0_1	E2F4-1	CCA TTC GTC GAG GAA ACT GT	CTC CAG TTT GTC CGT CAA CA	T3	
dd_Smed_v6_9739_0_1	Potassium channel, subfamily T, member 1 (KCNT1)	ACACAAAAAGGGTCGCTACG	CGTTTGTTGGTTGTCCATTG	SP6	glia
dd_Smed_v6_2749_0_1	bHLH	AAT CGT TGC AAT GTG CCT TT	TCC CTG CAA AGA CGA AGT TT	SP6	
dd_Smed_v6_32090_0_1		GGCATCCGGATTGATACAGT	ATCTGGAAGTCCTGGCTCTG	T3	glia
dd_Smed_v6_12027_0_1	slc7a-8	ACCGGTGGATTATTGAGGTG	CCACAATGGACATGTTCGAC	SP6	glia
dd_Smed_v6_7324_0_1	homeobox	GACACGAAATCCAGC CAAAT	CGGATAAGGTTT GTCGCAAT	SP6	cathepsin+ progenitor
dd_Smed_v6_2282_0_1	Carboxypeptidase A4 (CPA4)	GCACACAATTGGTTTACAAT GC	CGGGCACGATATAAATACGG	T3	glia
dd_Smed_v6_6597_0_1	slc23a-1	TACGGGGATCAAACGTCAAT	CTGTCCACTGGGAAGGGTTA	T3	glia
dd_Smed_v6_8465_0_1	PHTF2	CCCAATAAACCGAATGGATG	CATGTCTTTCGACAGCCTGA	T3	
dd_Smed_v6_7171_0_1		TTGGACAGGATTCGGAAATT A	CTCCCCAGAGGTTTCAGGAT	SP6	glia
dd_Smed_v6_6612_0_1	Metastasis suppressor 1-like (MTSS1L)	AGCGATTCCTTTACCGGTTT	TTCCAAAGGGCGATTTACAG	T3	glia

dd_Smed_v6_2237_0_1		ATTGCTGCTAAAACGGGTTG	AGCAACTTCCGACCGTTAAA	SP6	glia
dd_Smed_v6_298_0_1		AGTGGTCGCTGAATTTTTGG	TTCGAATATGCGACCTTGAA	Sp6	glia
dd_Smed_v6_25197_0_1	CUT	CATGAGCTCTTCGACCAACA	GCAAAAATTGCCTGTGGAAT	T3	
dd_Smed_v6_74_0_1		CATTCGGGTTGACATCCTCT	GTTGAAAATACGCCGGACAT	SP6	glia
dd_Smed_v6_7905_0_1	Slc4a-2	GATGGATACCTCGGTGCAAT	TAGGCCATGGTGAAAGTTCC	T3	glia
dd_Smed_v6_4878_0_1	Jun-like-1	CCG ATT CGC TGG AAA TTA AA	GAT GAG CAT TTT GGC CAT TT	SP6	
dd_Smed_v6_9401_0_1	slc23a-2	ACAATGCCAACCACGATACA	CACTTGAAACGACTTGAAAG ATTG	T3	glia
dd_Smed_v6_10714_0_1		CTTTGGCAAGATCACCACCT	TTCGGTCCTCGATATTGGTC	T3	glia
dd_Smed_v6_3678_0_1	LIM domain binding 1 (LDB1)	CAGGTCAGCAGCAAAAATCA	GTG TGG ACC ACT CAT GTT CG	T3	cathepsin+ progenitor
dd_Smed_v6_5615_0_1	Fms-related tyrosine kinase 1 (FLT1)	CAA ACC CTC GGG GAA ATT AT	CGG TGA TTT CAA CAC GAA TG	T3	cathepsin+ progenitor
dd_Smed_v6_1882_0_1	slc42a-1	TGATGTTCAAGGTGCTACGC	CCAATTCGGCGCTTATTTTA	SP6	glia
dd_Smed_v6_3106_0_1	Cytochrome P450, family 3, subfamily A, polypeptide 4 (CYP3A4)	CAGTTTCCAGTTGGATG	TCAGTTTGATGGGGACAACA	SP6	glia
dd_Smed_v6_7830_0_1	Calmodulin-like 5 (CALML5)	GATGGTGAAGACCACCGAAT	CGTATTCCAGCATCAACTGG	T3	glia
dd_Smed_v6_35892_0_1	Pax6b-like	ATGGACGACCACTTCCTGAC	AATGATGTCCGGCTTCTTTG	T3	
dd_Smed_v6_11135_0_1	slc22a-3	GCCATTGACTGTGGGATTTC	TGACTTCAACACCCCTCCTC	SP6	glia
dd_Smed_v6_6958_0_1	Laeverin (AQPEP)	ATTAATTGTGGCGCCTATCG	GCCCACTCGTTGCATATTTT	T3	glia
dd_Smed_v6_9140_0_1	LEF1	AAGGTGATGCCACACATGAA	GAGAGCACGTCACAGTTCCA	SP6	

dd_Smed_v6_1947_0_1	Galactose mutarotase (aldose 1-epimerase) (GALM)	GGGGAGTGGGGTATGAAAAT	TCATGATTCATCCGGGAAAT	T3	glia
dd_Smed_v6_10387_0_1	Potassium voltage-gated channel, KQT-like subfamily, member 1 (KCNQ1)	TGCGTTGTCTCGATTAGTGC	AACGGTATACGCTCGTTTGG	SP6	glia
dd_Smed_v6_7664_0_1	Znf91	CCT GGC GAC ATA TCC AAA GT	TGT TCC ACA CAT TCG TTC GT	T3	
dd_Smed_v6_537_0_1	LIM domain and actin binding 1 (lIMA1)	CACATAGTCGGCCTGTGAAA	GGTTGATGTAAA CGGCATTG	T3	cathepsin+ progenitor
dd_Smed_v6_7877_0_1	CUT	AATGAACCGGAAGCATATCG	TCGCCTGTGATGTCTTCTTG	SP6	
dd_Smed_v6_3514_0_1	slc1a-3	CCGTGAAATGCAACAATCAA	AATTGCCTGACTCCGAAATG	T3	glia
dd_Smed_v6_3140_0_1	mlx	GTTTGCTCTGC CATAATGA	CACCCAGTGAATGATTGTCG	T3	
dd_Smed_v6_7938_0_1	MITFL-1	ACCAGCAAAAGCCTTCTTCA	CGGACCTAACCGACGATTTA	SP6	
dd_Smed_v6_9186_0_1	Smad6/7	CTGACTCACTGTGGCAGGAA	AATTTCGCATCGACAAGGAC	SP6	
dd_Smed_v6_1224_0_1	Aquaporin 4 (AQP4)b	GGCGTACGTTTCGTCGTATT	GGGTTTCCCAAGGATTGAGT	T3	glia
dd_Smed_v6_11975_0_1	Six6	ATGGCCATTTCAGTTTGCTC	CGACCTCGAAGT TTTTCAGC	T3	cathepsin+ progenitor; glia
dd_Smed_v6_2113_0_1	slc3a-1	TCAATATGCCCTGTGATGGA	TGGTATCAACGCAGAGTTCG	SP6	glia
dd_Smed_v6_3620_0_1	Transient receptor potential cation channel, subfamily M, member 5 (TRPM5)	GTTGGTTTACTGGCGTCGAT	GGTGCAGAAGGAAACCGTAA	T3	glia
dd_Smed_v6_10260_0_1	C2H2 (ZnF)	ATATTGCGGTCGTCGTTTTC	GGATGGATT CTGCATCGACT	SP6	
dd_Smed_v6_2883_0_1	Guanine nucleotide binding protein (G protein), gamma 12 (GNG12)				cathepsin+ progenitor

dd_Smed_v6_762_0_1	GLI pathogenesis-related 2 (GLIPR2)	ATTTCCCGAAAAATCGTCCT	AGTGACAGCCAGCAGGTCTT	T3	cathepsin+ progenitor
dd_Smed_v6_1106_0_1	slc1a-5	TTTCGGTTTCAGGTTTTTGA	CGACAATCGCAAATGCTAAA	T3	glia
dd_Smed_v6_4795_0_1	NFX	TGAGTTGCAAATCCCATCAA	GCCCTGGTGACAGATCCTAA	T3	
dd_Smed_v6_7204_0_1	slc7a-6	TTTTGCCTGATCCTTGCTTT	TAAACAAAACGGCAACATCG	SP6	glia
dd_Smed_v6_3603_0_1	Peroxidasin homolog (PXDN)	CGGCCTCATGTATCACACAC	GCACATCTGGGATCCACTTT	T3	glia
dd_Smed_v6_18278_0_1	Kallikrein B, plasma (Fletcher factor)1 (KLKB1)	CGGAAATCAAGGTGGTTCAG	TATTCTCCACTTCCGGATCG	SP6	glia
dd_Smed_v6_8276_0_1	Endothelin converting enzyme 2 (ECE2)	TGGTTGCATCAAATTCTCCA	CCGCAGACAAACTGACTGAA	T3	
dd_Smed_v6_4278_0_1	Sterol O-acyltransferase 1 (SOAT1)	TCGGCTATTGTTGTGTTGGA	GGCAAAATTCGAGACGACAT	T3	cathepsin+ progenitor
dd_Smed_v6_1536_0_1	FKBP2	TCCGAAGAATCGAAATCCAC	TGAATGCCTAGATCCCATCC	T3	cathepsin+ progenitor
dd_Smed_v6_921_0_1	xin actin-binding repeat containing 2 (XIRP2)	TCAACAGCGCCCATATTGTA	GCCCATTAGAATGTCGAGGA	T3	cathepsin+ progenitor
dd_Smed_v6_13795_0_1		GTGGCTCACAATCAAAATGC	TCACCACAATAAACACAACTCAA	SP6	cathepsin+ progenitor
dd_Smed_v6_905_0_1	Staphylococcal nuclease and tudor domain containing 1 (SND1)	AAAGCCGTTGGTGTTCATTC	TAGGTAAACGCCTTGCTGCT	SP6	cathepsin+ progenitor
dd_Smed_v6_2228_0_1	APOBEC1 complementation factor (A1CF)	CAAGCGACAACGCAGATTTA	CGTTGAGCGATTGTTGAGAA	SP6	cathepsin+ progenitor
dd_Smed_v6_1206_0_1	Hematopoietic prostaglandin D synthase (HPGDS) / gst	TCGCGTGTTCATGATCAGTAT	AAAAATGCACAAATCAACAAAA	SP6	cathepsin+ progenitor
dd_Smed_v6_7316_0_1	MITFL-2	CGATGGATTTGGCATTTTCT	GGGGTGTTATTGGCTGAAGA	SP6	
dd_Smed_v6_8747_0_1		CGGTATCTGAAGACCGGAAA	TCCGAGATGACAATGATGGA	T3	cathepsin+ progenitor

dd_Smed_v6_10337_0_1	Translocase of outer mitochondrial membrane 70 homolog A (TOMM70A)	TACAGACCCATCCGAACTCC	CCTTGAGATACGTGGCGAAT	SP6	cathepsin+ progenitor
dd_Smed_v6_678_0_1	Heat shock protein 90kDa alpha (cytosolic), class A member 1 (HSP90AA1)	TGACACTGGAATTGGGATGA	GCTTGTCCTCACCTTCTTCG	T3	cathepsin+ progenitor
dd_Smed_v6_102_0_1		ATCCGTGATTGCGAATGAAT	CTTGGACATTGGGCTGATTT	T3	cathepsin+ progenitor
dd_Smed_v6_1732_0_1		TGTGCCAATGTTGAAGGAAA	ATGCATGCCCAGTCACTCTT	SP6	cathepsin+ progenitor
dd_Smed_v6_3257_0_1		GCTGCAAACAATGCTGAAAA	AAGTAAGCGAGTCCGCTCAA	T3	cathepsin+ progenitor
dd_Smed_v6_7738_0_1	MITFL-3	CGAAGAACCCGATATTGGAA	TGCGTTGGACTACTCGACAG	T3	
dd_Smed_v6_758_0_1		TCATTGCGAGCTATGTTTGC	TTGAAGTCAAAGGCATGCTG		cathepsin+ progenitor
dd_Smed_v6_1537_0_1	Protein disulfide isomerase family A, member 5 (PDIA5)	TCGCATTTATGACGAATCCA	CAAATGTGTTTCGGTTGTCG	T3	cathepsin+ progenitor
dd_Smed_v6_8468_0_1		TCGGATGCTCAAGAGTGATG	CGGAAAATTGGCTGGAAATA	SP6	
dd_Smed_v6_4831_0_1		GTCGCGAAAGAAAAATCGAG	GCAGGAGATCGTTGGTGAAT	SP6	cathepsin+ progenitor
dd_Smed_v6_2002_0_1	Coproporphyrinogen oxidase (CPOX)	AGATGAAAGCGCGATTGAGT	ATTTGGTGCCTCGGTCATAG	T3	cathepsin+ progenitor
dd_Smed_v6_6736_0_1	Tenascin C (TNC)	AAACGGGGAGCAATGTGTAG	ATCCCATCCCCTTGTAAACC	T3	cathepsin+ progenitor
dd_Smed_v6_2019_0_1	Cholinergic receptor, nicotinic, alpha 10 (neuronal) (CHRNA10)	TCAAGCAATGGTGAATGGAA	CGAGGAATACGCGATCTAGC	T3	cathepsin+ progenitor
dd_Smed_v6_2421_0_1	Topoisomerase (DNA) 1 (TOP1)	GTCGAGGAAATCATCCGAAA	TCGTCTGTGCTTGAATCGAC	T3	cathepsin+ progenitor
dd_Smed_v6_220_0_1	Calreticulin-1	TTGGTCCCGATATTTGTGGT	TTCGGCCTGTTTTGTAGCTT	T3	cathepsin+ progenitor

dd_Smed_v6_2206_0_1		GCGTTGCAGAAGAATTGACA	AATTACGAGTTGGCGGAAAG	SP6	cathepsin+ progenitor

Table 3.2. qPCR primers

Dresden contig	**Homolog**	**Fwd Primer**	**Rev Primer**
dd_Smed_v6_4789_0_1;	Phred-1	TGTAAAAACGGTGGCTTGTG	TTGAGCACTTTCGTTCGTTG
dd_Smed_v6_83_0_1; EG418138.1	beta-tubulin (control)	TGGCTGCTTGTGATCCAAGA	AAATTGCCGCAACAGTCAAATA
dd_Smed_v6_1792_0_1	estrella	GTGCGGTCTCCAAACTGAAT	CTCGCAGCTAACACGTTGTC
dd_Smed_v6_9961_0_1	calamari (cali)	GCTCCTCGTACAGCTCCATC	TCGACTGTTGCAGATCTTGG
dd_Smed_v6_12254_0_1	intermediate filament 1 (if-1)	TCAAAATCAGCAGCGTATGG	ATCCTGCCAATTTTTCGTTG

References

Acaz-Fonseca, E., Ortiz-Rodriguez, A., Azcoitia, I., Garcia-Segura, L. M., & Arevalo, M.-A. (2019). Notch signaling in astrocytes mediates their morphological response to an inflammatory challenge. *Cell Death Discovery*, *5*(1), 85. https://doi.org/10.1038/s41420-019-0166-6

Adams, K. L., & Gallo, V. (2018). The diversity and disparity of the glial scar. *Nature Neuroscience*, *21*(1), 9–15. https://doi.org/10.1038/s41593-017-0033-9

Adler, C. E., & Sánchez Alvarado, A. (2017). PHRED-1 is a divergent neurexin-1 homolog that organizes muscle fibers and patterns organs during regeneration. *Developmental Biology*, *427*(1), 165–175. https://doi.org/10.1016/j.ydbio.2017.04.012

Cebrià, F., & Newmark, P. A. (2005). Planarian homologs of netrin and netrin receptor are required for proper regeneration of the central nervous system and the maintenance of nervous system architecture. *Development*, *132*(16), 3691–3703. https://doi.org/10.1242/dev.01941

Chandra, B., Voas, M. G., Davies, E. L., & Roberts-Galbraith, R. H. (2023). Ets-1 transcription factor regulates glial cell regeneration and function in planarians. *Development*, dev.201666. https://doi.org/10.1242/dev.201666

Collins, J. J., Hou, X., Romanova, E. V., Lambrus, B. G., Miller, C. M., Saberi, A., Sweedler, J. V., & Newmark, P. A. (2010). Genome-Wide Analyses Reveal a Role for Peptide Hormones in Planarian Germline Development. *PLoS Biology*, *8*(10), e1000509. https://doi.org/10.1371/journal.pbio.1000509

Dubey, V. K., Sarkar, S. R., Lakshmanan, V., Dalmeida, R., Gulyani, A., & Palakodeti, D. (2022). Smed - ETS-1 regulates cathepsin+ cell function and epidermal lineage landscape

via basement membrane remodeling. *Journal of Cell Science*, jcs.259900. https://doi.org/10.1242/jcs.259900

Elliott, S. A. (2016). *Studies of conserved cell-cell signaling pathways in the planarian, schmidtea mediterranea* [University of Utah School of Medicine]. https://collections.lib.utah.edu/ark:/87278/s622633p

Fincher, C. T., Wurtzel, O., de Hoog, T., Kravarik, K. M., & Reddien, P. W. (2018). Cell type transcriptome atlas for the planarian *Schmidtea mediterranea*. *Science*, *360*(6391), eaaq1736. https://doi.org/10.1126/science.aaq1736

Freeman, M. R., & Rowitch, D. H. (2013). Evolving Concepts of Gliogenesis: A Look Way Back and Ahead to the Next 25 Years. *Neuron*, *80*(3), 613–623. https://doi.org/10.1016/j.neuron.2013.10.034

Gallo, V., & Deneen, B. (2014). Glial Development: The Crossroads of Regeneration and Repair in the CNS. *Neuron*, *83*(2), 283–308. https://doi.org/10.1016/j.neuron.2014.06.010

He, X., Lindsay-Mosher, N., Li, Y., Molinaro, A. M., Pellettieri, J., & Pearson, B. J. (2017). FOX and ETS family transcription factors regulate the pigment cell lineage in planarians. *Development*, *144*(24), 4540–4551. https://doi.org/10.1242/dev.156349

Kato, K., Forero, M. G., Fenton, J. C., & Hidalgo, A. (2011). The Glial Regenerative Response to Central Nervous System Injury Is Enabled by Pros-Notch and Pros-NFκB Feedback. *PLoS Biology*, *9*(8), e1001133. https://doi.org/10.1371/journal.pbio.1001133

King, R. S., & Newmark, P. A. (2013). In situ hybridization protocol for enhanced detection of gene expression in the planarian Schmidtea mediterranea. *BMC Developmental Biology*, *13*(1), 8. https://doi.org/10.1186/1471-213X-13-8

Losada-Perez, M., Harrison, N., & Hidalgo, A. (2016). Molecular mechanism of central nervous system repair by the Drosophila NG2 homologue kon-tiki. *Journal of Cell Biology*, *214*(5), 587–601. https://doi.org/10.1083/jcb.201603054

Patten, B. A., Sardi, S. P., Koirala, S., Nakafuku, M., & Corfas, G. (2006). Notch1 Signaling Regulates Radial Glia Differentiation through Multiple Transcriptional Mechanisms. *The Journal of Neuroscience*, *26*(12), 3102–3108. https://doi.org/10.1523/JNEUROSCI.4829-05.2006

Plass, M., Solana, J., Wolf, F. A., Ayoub, S., Misios, A., Glažar, P., Obermayer, B., Theis, F. J., Kocks, C., & Rajewsky, N. (2018). Cell type atlas and lineage tree of a whole complex animal by single-cell transcriptomics. *Science*, *360*(6391), eaaq1723. https://doi.org/10.1126/science.aaq1723

Reddien, P. W. (2005). SMEDWI-2 Is a PIWI-Like Protein That Regulates Planarian Stem Cells. *Science*, *310*(5752), 1327–1330. https://doi.org/10.1126/science.1116110

Roberts-Galbraith, R. H., Brubacher, J. L., & Newmark, P. A. (2016). A functional genomics screen in planarians reveals regulators of whole-brain regeneration. *eLife*, *5*, e17002. https://doi.org/10.7554/eLife.17002

Rozanski, A., Moon, H., Brandl, H., Martín-Durán, J. M., Grohme, M. A., Hüttner, K., Bartscherer, K., Henry, I., & Rink, J. C. (2019). PlanMine 3.0—Improvements to a mineable resource of flatworm biology and biodiversity. *Nucleic Acids Research*, *47*(D1), D812–D820. https://doi.org/10.1093/nar/gky1070

Rusznák, Z., Henskens, W., Schofield, E., Kim, W. S., & Fu, Y. (2016). Adult Neurogenesis and Gliogenesis: Possible Mechanisms for Neurorestoration. *Experimental Neurobiology*, *25*(3), 103–112. https://doi.org/10.5607/en.2016.25.3.103

Scimone, M. L., Wurtzel, O., Malecek, K., Fincher, C. T., Oderberg, I. M., Kravarik, K. M., & Reddien, P. W. (2018). foxF-1 Controls Specification of Non-body Wall Muscle and Phagocytic Cells in Planarians. *Current Biology*, *28*(23), 3787-3801.e6. https://doi.org/10.1016/j.cub.2018.10.030

Tan, C. X., & Eroglu, C. (2021). Cell adhesion molecules regulating astrocyte–neuron interactions. *Current Opinion in Neurobiology*, *69*, 170–177. https://doi.org/10.1016/j.conb.2021.03.015

Trotter, J. H., Dargaei, Z., Sclip, A., Essayan-Perez, S., Liakath-Ali, K., Raju, K., Nabet, A., Liu, X., Wöhr, M., & Südhof, T. C. (2020). *Compartment-Specific Neurexin Nanodomains Orchestrate Tripartite Synapse Assembly* [Preprint]. Neuroscience. https://doi.org/10.1101/2020.08.21.262097

Wang, I. E., Lapan, S. W., Scimone, M. L., Clandinin, T. R., & Reddien, P. W. (2016). Hedgehog signaling regulates gene expression in planarian glia. *eLife*, *5*, e16996. https://doi.org/10.7554/eLife.16996

CHAPTER IV

DISCUSSION

The term ‘glia’ (Greek for glue), coined by Rudolf Virchow in 1856 (‘Nervenkitt’ or nerve cement in German), has carried many connotations over the centuries (Fan & Agid, 2018; Freeman & Doherty, 2006; Freeman & Rowitch, 2013; Somjen, 1988; Virchow, 1856). Work in the last few decades challenged the idea of glia as mere support cells. Perhaps the most important revelation that has arisen from the reinvigorated interest in glial cells is how crucial glia are to development, physiology, and diseases of the nervous system. At present, study of glial biology is limited to a few model organisms (i.e. nematodes, *Drosophila*, zebrafish, and mouse) and to *in vitro* studies of human cells or organoids. Limited study systems pose a few challenges, especially in the context of glial roles in regeneration and the evo-devo aspect of glial origin and development.

Planarians are an exemplary model organism for the study of glia in regeneration in that they are capable of regenerating virtually any anatomical feature *de novo*, including a complex nervous system. With the development of new molecular tools, using planarians to study regeneration has become more commonplace. With new methods, from robust RNA interference (RNAi) screens to single cell RNA sequencing, we have not only uncovered the presence of glial cells in planarians but also identified genes enriched in expression in glial cells (Fincher et al., 2018; King & Newmark, 2013; Plass et al., 2018; Roberts-Galbraith et al., 2016; Rouhana et al., 2013; Shibata & Agata, 2018; Wang et al., 2016). However, the underlying mechanisms of glial cell development and regeneration, as well as how glial cells respond in the context of nervous

system injury in planarians remained elusive. The objective of my thesis was to investigate molecular regulators important for planarian glial regeneration, so we can understand glial regeneration and, with this knowledge, specifically ablate the glial population. With the absence of glial cells, we would have the ability to assess functional roles of glia in planarian physiology and nervous system regeneration. My work provided an essential scaffold for many new ideas for the future of planarian glial biology, as discussed below.

Planarian glial cells develop and regenerate neurons and may require neurons for cues.

In chapter 2, I established the glial regeneration timeline in planarians. I showed that glial cells arise after neurons in both asexual and sexual *S. mediterranea*. Asexual planarian neurons arise as early as 2 days post amputation (dpa) and form a fully bi-lobed brain structure by 5 dpa. In contrast, glial cells *start* appearing in the regenerated head tissue at 5 dpa. In vertebrates and *Drosophila*, a similar neural development timeline exists, starting with neurogenesis before switching molecular programs to make glial cells (Altenhein, 2015; Bond et al., 2020; Delaunay et al., 2008; Fu et al., 2021; Holguera & Desplan, 2018; Miller & Gauthier, 2007). While vertebrate and *Drosophila* neurons and glial cells arise subsequently from the same precursor or lineage origin that explains this molecular switch, planarian glial cells are hypothesized to form from a separate "lineage" and to be molecularly distinct from neurons according to single cell transcriptome atlases (Fincher et al., 2018; Plass et al., 2018). Therefore, the conserved relative timing of neuronal and glial cell birth in planarians, despite the cells arising from different cell populations, is particularly fascinating.

The birth order raised the question of whether glia rely on neurons for regeneration. As discussed in Chapter 2, I next uncovered that the presence of neurons is important for glial cell

regeneration within a specific tissue. This result reinforces the concept of neuronal dependence during regeneration, raising more questions in the context of planarian nervous system regeneration (Kumar & Brockes, 2012). What cues, if any, do planarian neurons send to signal correct spatiotemporal regeneration and localization of glial cells? Do existing glial cells, in reciprocity, provide cues for neuronal regeneration in planarians? Is there a specific ratio of neurons to glial cells and what phenotypes arise if there is an imbalance of this ratio? In addition, performing techniques such as BrdU labeling (Newmark & Sánchez Alvarado, 2000) and TUNEL assays (Pellettieri et al., 2010; Stubenhaus & Pellettieri, 2018) will provide additional information on 1) how a glial cell is born and 2) the lifecycle and retention of the glial cell population within planarians.

Planarian glial cells share several but not all conserved functional roles in the nervous system.

In Chapter 2, I utilized *ets-1*(RNAi) to ablate glial cells to uncover potential roles that glial cells play within the nervous system. I revealed several potential functions for glial cells within the planarian nervous system. They 1) assist in proper neuron spatial organization, 2) adjust synapse volume, and 3) assist in axon connections. However, detailed analysis, using correlative light and electron microcopy (CLEM) (De Boer et al., 2015), to visualize synapses may allow detailed exploration into how glial cells direct the formation and refinement of synaptic plasticity and axon connectivity during regeneration.

Glial cells in other organisms are active communicators at synaptic clefts, where they not only communicate with neurons but also surrounding glial cells (Araque et al., 1999; Farhy-Tselnicker & Allen, 2018; Halassa et al., 2007). This communication is often facilitated using gliotransmitters (e.g. ATP, glutamate, D-serine) and received by GPCRs (Petrelli & Bezzi, 2016;

Ubink et al., 2003; Walter et al., 2004; Zhang & Haydon, 2005). Do planarian glial cells communicate to neurons and other glial cells via gliotransmission, and if so, what are these gliotransmitters and their target? Previous work showed that planarians possess several conserved secreted '*neuro'*-peptides and GPCRs (Collins et al., 2010; Jenkins & Roberts-Galbraith, 2023). Of fascination are the neuropeptide Y (NPY) family members, as this neuropeptide family has been implicated in astrocyte communication in other organisms (Ubink et al., 2003). In addition, several planarian neuropeptides (e.g. *mpl-1*, *npy-1*, *npy-2*, *npy-7*, *ppl-1*, *spp-14*, *spp-15*, and *spp-17*) also show remarkable regional similarity in expression pattern to glial cells (Collins et al., 2010). Therefore, uncovering whether any planarian neuropeptide-encoding genes are coexpressed in glial cells would be a pivotal next step in investigating glial communication.

Furthermore, the presence of glial cells in planarians is primarily identified by usage of molecular markers *intermediate filament-1* (*if-1*), *calamari*, and *estrella* (Roberts-Galbraith et al., 2016; Wang et al., 2016). While *if-1* is a putative cytoskeleton protein, *calamari* and *estrella* both encode proteins with putative signal peptide (Roberts-Galbraith et al., 2016; Wang et al., 2016). What do these glial factors (i.e. calamari, estrella) themselves do? Are these proteins important for facilitating neuron-glia communication or for dictating terminal fate of a glial identity? Therefore, assessing functional roles (i.e. morphology, structural connectivity, uptake of neurotransmitters, etc.) following knockdown of additional glial genes may address their functional roles within glial cells.

Several molecular regulators are required for glial maintenance and regeneration.

In chapter 2, I showed that knockdown of *ets-1* affects glial regeneration and homeostasis. However, a limitation of this study is that *ets-1* plays a pleiotropic roles in planarians. Knockdown of *ets-1* affects multiple *cathepsin*$^+$ cell populations, including pigment and glial cells (Dubey et al., 2022; He et al., 2017). Likewise, another study and work in my Chapter 3 show that transcription factor *foxf-1*, in addition to affecting glial cells, also affects other *cathepsin*$^+$ cells (He et al., 2017; Scimone et al., 2018). Therefore, it is important to identify downstream targets of *ets-1* and *foxf-1* that are specific for glial cell identity in planarians. In chapter 3, I also uncovered several novel molecular regulators that affect *calamari* gene expression (i.e. *notch2*, *atf-like-1*, *myb*) during homeostasis and/or regeneration. Uncovering whether these regulators are specific to glial cell identity is a crucial next step. In addition, understanding how these regulators work cooperatively or within regulatory networks will strengthen our understanding of glial biology within planarians. Performing methods such as co-immunoprecipitation or yeast 2-hybrid would help us uncover whether these transcription factors directly interact with each other to regulate glial gene expression. In addition, utilizing techniques such as ChIP-seq or DAP-seq in planarians is a pivotal next step in examining where these transcription factors bind and how they drive glial cell identity and/or function (Bartlett et al., 2017).

During my work uncovering regulators of glial regeneration, my work showed that individual glial markers can respond differently to injury or gene perturbation. This raises a potential challenges when screening for genes that affect glial cells using a singular marker. Because I performed a first pass of my RNAi screen using glial marker *calamari*, it is possible that some genes in my screen do not affect *calamari* but do affect other glial markers such as *estrella* and/or *if-1*. While I performed a first pass screen with *calamari* to identify potential

candidates and then followed up with a second pass with *estrella*, the reciprocal (e.g. *estrella* first, *calamari* second) will also be informative. It would be an exciting discovery to find regulators that only affect one glial marker, which could address potential glial cell states.

We want to use planarians as a model system to study glial roles in regeneration because: 1) they can regenerate without scarring and 2) glial markers are downregulated following injury (Roberts-Galbraith et al., 2016). Therefore, uncovering what molecular components allow for altered "state" of planarian glial cells is an integral next step. In essence, can we make a hyperactive glial cell within planarians? Will hyper-activation interfere with planarian regeneration, and recapitulate vertebrate glial regenerative response? Uncovering molecular regulators that play an important part in downregulating glial gene expression within the first 3 days of regeneration may provide the platform for future studies.

The broader implication of planarian glial biology, an evolutionary-development perspective.

Characterizing glial cells in emerging model organisms presents unique challenges. Molecular markers have often been used to identify cell types and have become a prevalent tool for cell identification in the 'omics era. However no genes are universally expressed among all glial types or in glia across all species. Thus, identification of glial cells in unexplored species remains challenging. Previous works showed the importance of ETS transcription factors in both glial development and regeneration in vertebrates (Hagedorn *et al.*, 2000; Kiyota *et al.*, 2007; Parkinson *et al.*, 2002). My discovery that *ets-1*, a transcription factor-encoding gene with conserved roles in gliogenesis, also plays an important role in glial cell regeneration in planarians suggests that *ets-1* or downstream targets of *ets-1* could be potential candidates for molecular markers that could be used to identify novel glial cells in other animal models.

Furthermore, the characterization of glial cells in planarians provides a unique perspective on the origin and evolution of glial cells. In nematodes, glial cells arise side by side with neurons. In vertebrates and *Drosophila* central nervous systems, glial cells arise from the same lineage as neurons (Altenhein, 2015; Delaunay et al., 2008; Freeman & Doherty, 2006). Meanwhile, vertebrate peripheral glial cells originate from the neural crest (which also gives rise to pigment cells) (Jessen & Mirsky, 2005; Kastriti & Adameyko, 2017; Petersen & Adameyko, 2017). Planarian glial cells sit at a crossroad: they are not derived from the same lineage as neurons (in fact, they are clustered more closely with pigment cells) yet they are present in the central nervous system. The presence of glial cells in one of the earliest bilaterians raises many questions: in what species did glial cells originally arise, and when did they become specialized and interconnected with neurons? Studying planarian glial cells may provide a fresh lens to view the evolutionary history of glia.

Conclusion

In summary, through the body of work described within this dissertation, I established foundational knowledge on planarian glial cells that will act as a framework for further studies and in establishing planarians as a new model system for studying glial biology. Specifically, I pioneered the first comprehensive exploration into glial function in planarians, finding that glial cells serve as architects in CNS formation and function. I also uncovered several molecular regulators of glial identity and showed that planarian glial cells have different cell states. This dissertation also addresses some of the challenges of investigating glial cells in a non-traditional model system. My work helps expand many avenues that can be pursued in order to establish planarians as a complex yet rewarding model system to study glial biology.

References

Altenhein, B. (2015). Glial cell progenitors in the *D rosophila* embryo: Gliogenesis in *Drosophila*. *Glia*, *63*(8), 1291–1302. https://doi.org/10.1002/glia.22820

Araque, A., Parpura, V., Sanzgiri, R. P., & Haydon, P. G. (1999). Tripartite synapses: Glia, the unacknowledged partner. *Trends in Neurosciences*, *22*(5), 208–215. https://doi.org/10.1016/S0166-2236(98)01349-6

Bartlett, A., O'Malley, R. C., Huang, S. C., Galli, M., Nery, J. R., Gallavotti, A., & Ecker, J. R. (2017). Mapping genome-wide transcription-factor binding sites using DAP-seq. *Nature Protocols*, *12*(8), 1659–1672. https://doi.org/10.1038/nprot.2017.055

Bond, A. M., Berg, D. A., Lee, S., Garcia-Epelboim, A. S., Adusumilli, V. S., Ming, G., & Song, H. (2020). Differential Timing and Coordination of Neurogenesis and Astrogenesis in Developing Mouse Hippocampal Subregions. *Brain Sciences*, *10*(12), 909. https://doi.org/10.3390/brainsci10120909

Collins, J. J., Hou, X., Romanova, E. V., Lambrus, B. G., Miller, C. M., Saberi, A., Sweedler, J. V., & Newmark, P. A. (2010). Genome-Wide Analyses Reveal a Role for Peptide Hormones in Planarian Germline Development. *PLoS Biology*, *8*(10), e1000509. https://doi.org/10.1371/journal.pbio.1000509

De Boer, P., Hoogenboom, J. P., & Giepmans, B. N. G. (2015). Correlated light and electron microscopy: Ultrastructure lights up! *Nature Methods*, *12*(6), 503–513. https://doi.org/10.1038/nmeth.3400

Delaunay, D., Heydon, K., Cumano, A., Schwab, M. H., Thomas, J.-L., Suter, U., Nave, K.-A., Zalc, B., & Spassky, N. (2008). Early Neuronal and Glial Fate Restriction of Embryonic

Neural Stem Cells. *Journal of Neuroscience*, *28*(10), 2551–2562. https://doi.org/10.1523/JNEUROSCI.5497-07.2008

Dubey, V. K., Sarkar, S. R., Lakshmanan, V., Dalmeida, R., Gulyani, A., & Palakodeti, D. (2022). Smed - ETS-1 regulates cathepsin+ cell function and epidermal lineage landscape via basement membrane remodeling. *Journal of Cell Science*, jcs.259900. https://doi.org/10.1242/jcs.259900

Fan, X., & Agid, Y. (2018). At the Origin of the History of Glia. *Neuroscience*, *385*, 255–271. https://doi.org/10.1016/j.neuroscience.2018.05.050

Farhy-Tselnicker, I., & Allen, N. J. (2018). Astrocytes, neurons, synapses: A tripartite view on cortical circuit development. *Neural Development*, *13*(1), 7. https://doi.org/10.1186/s13064-018-0104-y

Fincher, C. T., Wurtzel, O., de Hoog, T., Kravarik, K. M., & Reddien, P. W. (2018). Cell type transcriptome atlas for the planarian *Schmidtea mediterranea*. *Science*, *360*(6391), eaaq1736. https://doi.org/10.1126/science.aaq1736

Freeman, M. R., & Doherty, J. (2006). Glial cell biology in Drosophila and vertebrates. *Trends in Neurosciences*, *29*(2), 82–90. https://doi.org/10.1016/j.tins.2005.12.002

Freeman, M. R., & Rowitch, D. H. (2013). Evolving Concepts of Gliogenesis: A Look Way Back and Ahead to the Next 25 Years. *Neuron*, *80*(3), 613–623. https://doi.org/10.1016/j.neuron.2013.10.034

Fu, Y., Yang, M., Yu, H., Wang, Y., Wu, X., Yong, J., Mao, Y., Cui, Y., Fan, X., Wen, L., Qiao, J., & Tang, F. (2021). Heterogeneity of glial progenitor cells during the neurogenesis-to-gliogenesis switch in the developing human cerebral cortex. *Cell Reports*, *34*(9), 108788. https://doi.org/10.1016/j.celrep.2021.108788

Hagedorn, L., Paratore, C., Brugnoli, G., Baert, J.-L., Mercader, N., Suter, U., & Sommer, L. (2000). The Ets Domain Transcription Factor Erm Distinguishes Rat Satellite Glia from Schwann Cells and Is Regulated in Satellite Cells by Neuregulin Signaling. *Developmental Biology*, *219*(1), 44–58. https://doi.org/10.1006/dbio.1999.9595

Halassa, M. M., Fellin, T., & Haydon, P. G. (2007). The tripartite synapse: Roles for gliotransmission in health and disease. *Trends in Molecular Medicine*, *13*(2), 54–63. https://doi.org/10.1016/j.molmed.2006.12.005

He, X., Lindsay-Mosher, N., Li, Y., Molinaro, A. M., Pellettieri, J., & Pearson, B. J. (2017). FOX and ETS family transcription factors regulate the pigment cell lineage in planarians. *Development*, *144*(24), 4540–4551. https://doi.org/10.1242/dev.156349

Holguera, I., & Desplan, C. (2018). Neuronal specification in space and time. *Science*, *362*(6411), 176–180. https://doi.org/10.1126/science.aas9435

Jenkins, J. E., & Roberts-Galbraith, R. H. (2023). Heterotrimeric G proteins regulate planarian regeneration and behavior. *Genetics*, *223*(4), iyad019. https://doi.org/10.1093/genetics/iyad019

Jessen, K. R., & Mirsky, R. (2005). The origin and development of glial cells in peripheral nerves. *Nature Reviews Neuroscience*, *6*(9), 671–682. https://doi.org/10.1038/nrn1746

Kastriti, M. E., & Adameyko, I. (2017). Specification, plasticity and evolutionary origin of peripheral glial cells. *Current Opinion in Neurobiology*, *47*, 196–202. https://doi.org/10.1016/j.conb.2017.11.004

King, R. S., & Newmark, P. A. (2013). In situ hybridization protocol for enhanced detection of gene expression in the planarian Schmidtea mediterranea. *BMC Developmental Biology*, *13*(1), 8. https://doi.org/10.1186/1471-213X-13-8

Kiyota, T., Kato, A., & Kato, Y. (2007). Ets-1 Regulates Radial Glia Formation During Vertebrate Embryogenesis. *Organogenesis*, *3*(2), 93–101. https://doi.org/10.4161/org.3.2.5171

Kumar, A., & Brockes, J. P. (2012). Nerve dependence in tissue, organ, and appendage regeneration. *Trends in Neurosciences*, *35*(11), 691–699. https://doi.org/10.1016/j.tins.2012.08.003

Miller, F. D., & Gauthier, A. S. (2007). Timing Is Everything: Making Neurons versus Glia in the Developing Cortex. *Neuron*, *54*(3), 357–369. https://doi.org/10.1016/j.neuron.2007.04.019

Newmark, P. A., & Sánchez Alvarado, A. (2000). Bromodeoxyuridine Specifically Labels the Regenerative Stem Cells of Planarians. *Developmental Biology*, *220*(2), 142–153. https://doi.org/10.1006/dbio.2000.9645

Parkinson, D. B., Langner, K., Namini, S. S., Jessen, K. R., & Mirsky, R. (2002). β-Neuregulin and Autocrine Mediated Survival of Schwann Cells Requires Activity of Ets Family Transcription Factors. *Molecular and Cellular Neuroscience*, *20*(1), 154–167. https://doi.org/10.1006/mcne.2002.1109

Pellettieri, J., Fitzgerald, P., Watanabe, S., Mancuso, J., Green, D. R., & Sánchez Alvarado, A. (2010). Cell death and tissue remodeling in planarian regeneration. *Developmental Biology*, *338*(1), 76–85. https://doi.org/10.1016/j.ydbio.2009.09.015

Petersen, J., & Adameyko, I. (2017). Nerve-associated neural crest: Peripheral glial cells generate multiple fates in the body. *Current Opinion in Genetics & Development*, *45*, 10–14. https://doi.org/10.1016/j.gde.2017.02.006

Petrelli, F., & Bezzi, P. (2016). Novel insights into gliotransmitters. *Current Opinion in Pharmacology*, *26*, 138–145. https://doi.org/10.1016/j.coph.2015.11.010

Plass, M., Solana, J., Wolf, F. A., Ayoub, S., Misios, A., Glažar, P., Obermayer, B., Theis, F. J., Kocks, C., & Rajewsky, N. (2018). Cell type atlas and lineage tree of a whole complex animal by single-cell transcriptomics. *Science*, *360*(6391), eaaq1723. https://doi.org/10.1126/science.aaq1723

Roberts-Galbraith, R. H., Brubacher, J. L., & Newmark, P. A. (2016). A functional genomics screen in planarians reveals regulators of whole-brain regeneration. *eLife*, *5*, e17002. https://doi.org/10.7554/eLife.17002

Rouhana, L., Weiss, J. A., Forsthoefel, D. J., Lee, H., King, R. S., Inoue, T., Shibata, N., Agata, K., & Newmark, P. A. (2013). RNA interference by feeding in vitro-synthesized double-stranded RNA to planarians: Methodology and dynamics: Planarian RNAi by Feeding in Vitro-Synthesized dsRNA. *Developmental Dynamics*, *242*(6), 718–730. https://doi.org/10.1002/dvdy.23950

Scimone, M. L., Wurtzel, O., Malecek, K., Fincher, C. T., Oderberg, I. M., Kravarik, K. M., & Reddien, P. W. (2018). foxF-1 Controls Specification of Non-body Wall Muscle and Phagocytic Cells in Planarians. *Current Biology*, *28*(23), 3787-3801.e6. https://doi.org/10.1016/j.cub.2018.10.030

Shibata, N., & Agata, K. (2018). RNA Interference in Planarians: Feeding and Injection of Synthetic dsRNA. In J. C. Rink (Ed.), *Planarian Regeneration* (Vol. 1774, pp. 455–466). Springer New York. https://doi.org/10.1007/978-1-4939-7802-1_18

Somjen, G. G. (1988). Nervenkitt: Notes on the history of the concept of neuroglia. *Glia*, *1*(1), 2–9. https://doi.org/10.1002/glia.440010103

Stubenhaus, B., & Pellettieri, J. (2018). Detection of Apoptotic Cells in Planarians by Whole-Mount TUNEL. In J. C. Rink (Ed.), *Planarian Regeneration* (Vol. 1774, pp. 435–444). Springer New York. https://doi.org/10.1007/978-1-4939-7802-1_16

Ubink, R., Calza, L., & Hökfelt, T. (2003). 'Neuro'-peptides in glia: Focus on NPY and galanin. *Trends in Neurosciences*, *26*(11), 604–609. https://doi.org/10.1016/j.tins.2003.09.003

Virchow, R. (1856). *Gesammelte A bhantllungen zur Wissenschaftlichen Medicin.* 1024.

Walter, L., Dinh, T., & Stella, N. (2004). ATP Induces a Rapid and Pronounced Increase in 2-Arachidonoylglycerol Production by Astrocytes, a Response Limited by Monoacylglycerol Lipase. *The Journal of Neuroscience*, *24*(37), 8068–8074. https://doi.org/10.1523/JNEUROSCI.2419-04.2004

Wang, I. E., Lapan, S. W., Scimone, M. L., Clandinin, T. R., & Reddien, P. W. (2016). Hedgehog signaling regulates gene expression in planarian glia. *eLife*, *5*, e16996. https://doi.org/10.7554/eLife.16996

Zhang, Q., & Haydon, P. G. (2005). Roles for gliotransmission in the nervous system. *Journal of Neural Transmission*, *112*(1), 121–125. https://doi.org/10.1007/s00702-004-0119-x