MEDICAL SCHOOL FACULDADE DE CIÊNCIAS MÉDICAS

UNIVERSIDADE NOVA DE LISBOA

GLIA IN INTERCELLULAR COMMUNICATION AT THE *DROSOPHILA* NEUROMUSCULAR JUNCTION

VICENTE DE SOUSA XAVIER

A dissertation submitted in partial fulfillment of the requirements for the Degree of Masters in Biomedical Research

Dissertação para obtenção do grau de Mestre em Investigação Biomédica

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Table of contents

1. Abstract 1

2. Resumo 2

3. Introduction 4

3.1. The nervous system 4

3.2. Neuronal growth and plasticity 5

3.3. *Drosophila* as a model organism 7

3.3.1.* Genetic tools in *Drosophila 7

3.3.2. Neuronal growth and plasticity at the *Drosophila* NMJ 8

3.4. Non-neuronal cells control neuron growth and function 10

3.4.1. Glial cells 11

3.4.1.1. Astrocytes 13

3.4.1.2. Cortex glia 16

3.4.1.3. Oligodendrocytes and ensheathing glia 16

3.4.1.4. Microglia 17

3.4.1.5. Schwann cells and wrapping glia 17

3.4.1.6. Subperineurial glia 18

3.4.1.7. Perineurial glia 20

3.4.1.8. Muscle cells 21

3.4.1.9. Tracheal and endothelial cells 22

3.5. Aims 24

4. Methods 25

4.1. Drosophila stocks and husbandry 25

4.2. Dissection and immunohistochemistry 25

4.3. Phosphatidylserine immunofluorescence assay 25

4.4. Stimulation protocols 25

4.5. Genetic cell ablation 26

4.6. Confocal imaging 26

4.7. Image analysis and calculations 26

4.8. Statistical analysis 26

5. Results 29

5.1. *In vivo* dynamics of bouton formation 29

5.2. Glial morphology at the NMJ 29

5.3. Wrapping glia 30

5.4. Subperineurial glia 31
5.5. Perineurial glia 37
5.6. Tracheal cells 39
5.7. Genetic ablation of glial subtypes 41
6. Discussion and conclusions 45
6.1. Glial morphology at the NMJ 45
6.2. Subperineurial glia 45
6.3. Perineurial glia 47
6.4. Tracheal cells 47
6.5. Genetic ablation of glial subtypes 47
6.6. Conclusions 48
7. Acknowledgements and funding 48
8. Bibliography 48

1. Abstract

Neuromuscular junctions (NMJs) are highly efficient synapses that despite being maintained throughout life, undergo both functional and structural remodelling. From *Drosophila* to mammals, glial cells invade the NMJ and interact with neurons, having roles in growth, maintenance and function. Peripheral nerves in *Drosophila* larvae are enwrapped by three glial subtypes, wrapping glia (WG), subperineurial glia (SPG) and perineurial glia (PG), and the two outermost layers (SPG and PG) are known to extend processes towards the nerve terminal, including synaptic boutons. Glia, together with muscle cells, clear cellular debris and immature boutons, formed following high neuronal activity, via the engulfment receptor Draper (MEGF10 in Mammalia). SPG are thought to induce bouton growth via glia-muscle Maverick (of the TGF-β, transforming growth factor β, family) signalling. PG contact synapses and grow along the nerve terminal, possibly in an activity-dependent fashion, but no functions other than blood-nerve barrier formation in adult flies have been identified. In adult NMJs, perisynaptic glia have functions such as glutamate recycling and trachea (the fly's oxygen distribution vessels) coupling to neurons. Despite these efforts to uncover glial function at the NMJ, questions remain such as: does each glial subtype have a specialized function? Is trachea-neuron coupling in larval NMJs performed by peripheral glia? Can glia regulate synaptic function, including plasticity, in response to activity?

We aimed to characterize intercellular interactions at the NMJ, in particular glial structure, dynamism and function at the 3rd instar *Drosophila* larval NMJ. To do this, we took advantage of *Drosophila* genetics and time-lapse live imaging following acute induction of synaptic structural plasticity. Live imaging revealed that SPG processes are dynamic and contact boutons during activity-driven bouton formation, being able to contract around mature boutons to promote budding of new pre-synaptic structures. PG are also dynamic and contact axonal branches and synaptic boutons. Co-labelling of PG and tracheal cells followed by 3D reconstruction revealed physical interactions between these 3 cell types, possibly reflecting a novel function of PG as mediators of neuron-trachea coupling. Tracheal cells also dynamically extend and retract and contact synaptic boutons, sometimes during bouton formation events, where trachea seem to guide the newly-formed bouton. Genetic ablation of WG, but not SPG, led to the accumulation of neuronal debris. As WG do not extend into the NMJ, we want to explore how WG contribute to debris clearance. Ablating SPG appears to affect the frequency of bouton formation.

Our data suggests that local signalling events take place in the contacts between glial, neuronal and tracheal processes. Our findings support the notion of specialized glial functions at the NMJ. Also, glial and/or tracheal presence may help determine the site of synaptic bouton formation. Finally, this work unravels an *in vivo* interplay between motor neurons, glia, muscle and trachea, highlighting the importance of intercellular communication for NMJ development and function. As non-neuronal cells are increasingly recognized as regulators of neuronal function, it becomes clear that understanding how

these different cell types interact will shed light onto the mechanisms that govern nervous system function and dysfunction, hopefully leading to new therapeutic approaches.

2. Resumo

As junções neuromusculares são sinapses altamente eficazes que, embora muitas vezes persistam durante toda a vida de um animal, exibem plasticidade, sofrendo mudanças funcionais e estruturais em resposta às necessidades de inervação do músculo. Desde invertebrados como a mosca da fruta, até humanos, células da glia invadem a junção neuromuscular e interagem com músculos e neurónios, regulando o desenvolvimento e função das sinapses. Os axónios periféricos em *Drosophila* são isolados do meio exterior por três camadas gliais: as *wrapping glia* (WG) contactam directamente os axónios enquanto as *subperineurial glia* (SPG) e as *perineurial glia* (PG) estabelecem a barreira hematoencefálica e interagem com os terminais pré-sinápticos. Juntamente com as células musculares, as células da glia fagocitam fragmentos de membrana neuronal e botões sinápticos recém-formados após actividade neuronal intensa e não destinados a maturar, através do receptor Draper, conservado entre mamíferos (MEGF10) e *Drosophila*. As SPG participam na sinaptogénese libertando Maverick, uma proteína da família TGF-β, que actua no músculo, levando a que este promova a formação de novos botões sinápticos. O desenvolvimento das PG é influenciado pelo crescimento neuronal e pensa-se que possam ser sensíveis a actividade sináptica, mas pouco se sabe sobre este subtipo glial. Em moscas adultas, um quarto subtipo glial, as *perisynaptic glia*, faz reciclagem de glutamato e interage simultaneamente com neurónios e com as traqueias, o sistema de distribuição de oxigénio em *Drosophila*. Apesar destes esforços para caracterizar o papel desempenhado pelas células da glia em junções neuromusculares, muitas questões permanecem por responder: cada subtipo é especializado em determinadas funções? Existem funções partilhadas? Como são estabelecidas as interacções entre neurónios e traqueias em larvas de *Drosophila*? São as células da glia capazes de regular funções e plasticidade sinápticas?

O objectivo deste estudo é caracterizar as interacções entre os diversos tipos celulares em junções neuromusculares larvares, com vista à identificação de funções gliais. Tirámos proveito de ferramentas genéticas em *Drosophila*, juntamente com microscopia confocal de fluorescência em tempo real e de protocolos de estimulação neuronal que induzem plasticidade sináptica estrutural para analisar a morfologia e dinâmica dos diferentes tipos celulares *in vivo*. Os nossos resultados indicam que as SPG contactam botões sinápticos através de protusões membranares dinâmicas e participam na formação de novos botões através de um mecanismo de contracção que promove a formação de estruturas pré-sinápticas e, possivelmente, sinalização local. A análise morfológica das PG revelou que são dinâmicas e contactam os neurónios, o músculo e as traqueias. Reconstruções 3D demonstram que protusões membranares das PG se colocam entre as traqueias e os botões, por vezes também os envolvendo e provavelmente reflectindo uma nova função das PG – mediar as interacções, incluindo trocas gasosas, entre botões sinápticos e traqueias.

As células que compõem as traqueias também são dinâmicas, constantemente explorando o ambiente extracelular através de finas extensões membranares. Estas contactam frequentemente botões, por vezes durante a génese e crescimento de novos botões, guiando-os. De modo a identificar novas funções gliais, eliminámos selectivamente cada subtipo e analisámos a morfologia neuronal e plasticidade sináptica estrutural. Quando na ausência de WG, mas não de SPG, fragmentos de membrana neuronal acumulam-se junto ao terminal axonal, e quando as SPG foram eliminadas a capacidade de formação de novos botões foi afectada.

Em suma, este trabalho revelou diversas interacções intercelulares *in vivo* em junções neuromusculares de *Drosophila*, onde diferentes subtipos gliais têm funções como promover o crescimento neuronal e mediar o contacto com as traqueias, assegurando o fornecimento de oxigénio. Perceber como as células não neuronais participam no desenvolvimento e funções neuronais permitirá uma melhor compreensão do sistema nervoso e possivelmente a criação de novas terapias que tenham como alvo estes mecanismos intercelulares.

3. Introduction

3.1. The nervous system

Since its appearance prior to the divergence of bilaterians and cnidaria, more than 700 million years ago, the nervous system has evolved into one of the most complex biological structures known to mankind[1]. Housing functions ranging from movement, vision and regulation of vital organs' functioning to memory and cognition, the nervous system is essential for complex animal life and behaviour. Thus, its development and function have been subjects of intense research as we try to unveil the biological basis for animal physiology and behaviour in health and disease.

It is classically divided into central and peripheral nervous system (CNS and PNS, respectively), with the PNS being responsible for carrying sensory information from the sensory organs to the CNS, where the information is integrated and processed before it is conveyed back through the PNS towards motor and visceral organs to generate motor and physiological responses to sensory stimuli.

Its functional units are neurons, electrically excitable cells that carry information coded in the form of electrical currents. Neurons are polarized cells and can be divided into three major compartments: dendrites, cellular processes where the information is received; the soma or cell body where the nucleus resides and information is integrated; and the axon, a thin, long process that carries electrical information in the form of action potentials (APs). Axons have specialized ramified endings (presynaptic terminals) capable of delivering information to other cells by releasing vesicles containing a neurotransmitter, forming a synapse. A pre-synaptic neuron can synapse onto a neuronal soma, dendrites, axon and axonal terminals, and this helps determine the response that presynaptic firing induces in the post-synaptic cell. Neurons exist in networks, called circuits, that are assembled during development and that perform specific functions[1].

Neurons do not come alone, as the brain and nerves contain several non-neuronal cell types such as glial cells and the endothelial cells that compose blood vessels. Glial cells were originally seen only as passive supporters but are emerging as important regulators of neuronal development and function. Additionally, besides neuron-neuron synapses, connections can be formed between neurons and other cell types, such as endocrine, gastric and muscle cells, to name a few. This multicellular nature of the neuronal environment has not been thoroughly explored but it is already clear that the interactions between these cell types and neurons are crucial for proper neuronal function.

The site of neuron-muscle contact is the neuromuscular junction (NMJ), where motor axon terminals innervate muscle fibres that possess post-synaptic specializations. In mammals, these peripheral excitatory synapses are cholinergic, meaning motor neurons (MNs) release acetylcholine, while CNS excitatory synapses usually use glutamate as a neurotransmitter. Each adult muscle fibre is innervated by a single motor neuron and the pre-synaptic specializations, called boutons, are rather big, allowing the study of individual neurons and synapses. These synapses must be highly efficient to assure proper communication between the nervous and the muscle systems, allowing for tightly coordinated motion and locomotion.

3.2. Neuronal growth and plasticity

Neurons are post-mitotic cells, meaning that, after they are 'born' as terminally differentiated cells, they stop dividing. Instead, they display an enormous ability to grow and change in accordance with the surrounding environment. During development, newly-born neurons start extending neurites, of which one will be an axon while the others will develop into dendrites, that must find and correctly connect to their synaptic targets, a step crucial for circuit establishment. In the axonal tip, a growth cone is formed: dynamic membrane protrusions such as filopodia and lamellipodia are extended and retracted, searching for external cues and guiding the axon towards its target. When the target is reached the synapses are established. Neurons must then adapt to their target tissue's innervation needs and the stimuli being received. Synapses can be added, strengthened, pruned and remodelled, and the intrinsic properties of a neuron, like excitability, can be altered, in a process known as neuronal plasticity.

Circuitry establishment during development is a balance between genetic determination and plastic, adaptive changes[2,3]. Neuronal targets express a variety of soluble and membrane-bound protein cues that allow specific innervation patterns to be reliably achieved. On the other hand, neuronal activity can shape circuits, providing a basis for neuronal adaptation in response to internal and external stimuli.

Neurons are able of adapting in (at least) three different ways: modifications at the post-synaptic end, the dendrites, can alter the weight of synaptic inputs at the individual synapse level; modulation of the intrinsic membrane properties changes the propagation of current, altering signal integration and AP firing probability; and regulation of synaptic vesicle release and content to achieve efficient information delivery to the post-synaptic cell[4]. Plasticity thus can occur by regulating the intrinsic properties of neurons, the synaptic levels of key proteins, and the number and size of synapses.

Synaptic plasticity can be roughly divided into functional and structural plasticity, even though these two forms of plasticity are usually interconnected. Functional plasticity is the change in the strength of a synapse, by means of pre- and post-synaptic machinery regulation. Presynaptically, vesicle release and quantal size, i.e. the amount of neurotransmitter in each vesicle, can be modulated by neuronal activity and several other factors. At the post-synapse, alteration of receptor number and function, along with numerous other downstream players, regulates target cell activation. A classic example of this is long-term potentiation or LTP: at glutamatergic synapses, glutamate binds to both NMDA and AMPA receptors (NMDAR and AMPAR), but at first only the Na^+-permeable AMPARs open, depolarizing the post-synaptic region. When the postsynaptic membrane is sufficiently depolarized (either from Na+ influx from AMPAR opening, from back-propagating action potentials or both) the Mg^{2+} ions blocking the NMDAR Na^+-and Ca^{2+}-permeable channel are dislodged and NMDARs open. The calcium influx then induces several downstream changes, including the regulation of AMPAR addition to the post-synaptic membrane and their activation via phosphorylation, inducing a long-lasting increase in post-synaptic depolarization upon pre-synaptic neurotransmitter release. NMDARs thus act as a coincidence detector of neurotransmitter release and post-synaptic depolarization, allowing for potentiation of repeatedly activated synapses and underlying some properties of plasticity such as associativity, cooperativity and competition. Long-term depression (LTD) works in the opposite direction, with AMPARs being removed from the synapse due to low presynaptic firing, making it more 'silent'[5].

Functional changes can also occur pre-synaptically as changes in vesicle release probability. This is achieved by regulating neurotransmitter release by changing the levels and function of the release machinery, a process regulated by activity-driven changes in intercellular calcium levels. Pre-synapses display LTP and LTD and are able to integrate activity patterns with retrograde signalling from the post-synapse, thus functionally matching their targets[6,7].

Accompanying synaptic strength changes are structural modifications of the neuron, the basis of structural plasticity. Structural plasticity is the change in the morphology and organization of synaptic terminals in response to increased or decreased levels of activity. Synaptic boutons, which house active zones (AZs), the sites of synaptic vesicle release, can be added to existing pre-synaptic terminals. Bouton maturation then requires the acquisition of a post-synaptic specialization by the post-synaptic cell to form new synapse. Retraction or elimination of boutons can also occur, decreasing the strength of the pre-synaptic input. AZs themselves can be added or removed, providing a molecular basis for single synapse turnover. Vesicle release and docking can be modulated, where the distance between voltage-gated calcium channels (VGCCs) and vesicle docking sites can change therefore altering the SV-release efficiency. These presynaptic modifications occur in coordination with post-synaptic growth, and this coordination is thought to be mediated by retrograde messengers (e.g. endocannabinoids, nitric oxide or arachidonic acid, ligands from the TGF-β or BMP signalling, etc.[6,8,9]) and also by signals from surrounding cells, such as glial cells[7].

A widely studied aspect of post-synaptic structural changes in the mammalian brain is the morphological rearrangement of dendritic spines (post-synaptic specialization present in excitatory neurons). Newly formed spines are usually thin and filopodia-like but as they mature, they grow larger and can branch to give rise to two new spines. Spine growth is regulated by activity and functional changes such as LTP and LTD, which are accompanied by spine growth and shrinkage, respectively. Spine structural plasticity is crucial for learning and memory processes. Current memory theories posit that memories are encoded by specific sets of synapses and modulation of the synaptic strength allowing for learning and memory[10]. Hayashi-Takagi *et al.* showed in mice that during the learning of a new motor task, there were newly-formed spines induced by neuronal activity that allowed the learning and encoding of this new memory. Importantly, they showed that eliminating only those new spines leads to unlearning of the task. This shows that the formation and retraction of post-synaptic structures is required for memory formation and storage – crucial processes for learning[11].

Dysfunctions in spine plasticity underlie several cognitive and neuropathological disorders, from neuropsychiatric disorders as depression, schizophrenia and autism spectrum disorders (ASD), to neurodegenerative disorders such as Alzheimer's disease (AD)[12,13].

Neuronal plasticity is critical for animal development and adaptation to the environment throughout life[14], and thus understanding the mechanisms of plasticity is crucial for the development of therapies for nervous system dysfunctions that underlie diseases such as Alzheimer disease (AD) and amyotrophic lateral sclerosis (ALS). As studies with humans have technical and ethical constraints, several model systems have been developed to study neuronal function and plasticity, from cell culture to model organisms such as mice, the nematode *C. elegans* and the fruit fly *Drosophila melanogaster*. Non-neuronal cells that play roles in neuron development and function like glia and muscle, as well as many of the cellular processes and genes involved in neuronal plasticity are conserved between phyla. This makes simple model organisms such as *Drosophila* well suited to study how these different cell types are coordinated *in vivo*.

3.3. *Drosophila* as a model organism

Ever since James Hunt Morgan began working with banana fruit flies in 1909, *Drosophila melanogaster* has become a valuable tool, first providing insight into the mechanisms of inheritance – genetics – and later being used as a model of health and disease in countless fields, from cell signalling to behavioural neurobiology. Morgan chose the fly (here used to refer to *D. melanogaster*) as it is a simple organism with a short life cycle (taking only 10 days from egg laying to adulthood at 25ºC), high fecundity, and is easy and inexpensive to cultivate in large amounts[15]. Flies are kept in small vials or bottles with growth media on the bottom and are handled using either cold or CO_2 as anaesthesia.

Flies begin life as embryos, which hatch as larvae after a day of embryogenesis. The larval stage consists of three instars, the first two lasting for a day each and the third lasting for three days, with cuticle molts between instars, to allow the cuticle to adapt to the immense growth during these stages. When third instar larvae are ready to form a pupa, they crawl out of the food and find a dry place to pupariate. The pupal stage consists of 4 to 5 days of complete metamorphosis until an adult fly is ready to eclose from the pupal case (Fig.1A).

Drosophila's genome was the second to be fully sequenced in multicellular organisms and has undergone extensive mapping and curation. Although its genome is composed of only four chromosomes and is twenty times smaller than the human genome, around 75% of disease-related human genes have a fly homolog[16].

3.3.1. Genetic tools in *Drosophila*

A wide variety of genetic tools are available in flies, including those used in vertebrate model organisms such as gene knockdown by anti-sense RNA interference (RNAi) technology and others that are not yet available or feasible in more complex systems. Generating mutant and transgenic flies is relatively easy. The fly genome houses many transposable elements (TE), and a type of TE, P-elements, was introduced in flies by horizontal transmission from the closely-related *D. willistoni* species[17]. In 1982, Rubin and Spradling decided to engineer P-elements as a transgene delivery system by swapping the original transposase encoding gene with a gene of interest (GOI). This strategy allowed the creation of libraries of thousands of mutants and other genetic tools that are still used today.

Lethal mutations or other lines of interest can be kept over 'balancer chromosomes', which are marked chromosomes engineered to have multiple inversions thereby preventing recombination between the two chromosomes. Given that balancers have visible markers, this strategy allows the following of each chromosome and easy selection of the chromosome of interest without the need to sequence the progeny[18].

Later, a strategy to achieve tissue-specific gene expression was developed by inserting the yeast transcription factor GAL4 (composed of an activation, a middle and a DNA-binding domain) into either a P-element vector under control a chosen promoter, or directly in the fly genome under control of a native transcriptional enhancer[19] (enhancer trap). The upstream activating sequences (UAS), to which GAL4 binds to drive expression, were also cloned into P-element vectors, allowing any transgene to be placed under control of UAS and thus of Gal4, allowing tissue-specific expression by using tissue-specific Gal4 drivers. This binary expression system was then fine-tuned to eliminate variability in expression due to insertion site by adding to the vector sequences that promoted precise insertion of the vector in 'landing sites' that were created in the genome and later characterized[20,21].

The construction of enhancer traps with the Gal4 repressor Gal80, that blocks transcription by binding to the activation domain of Gal4 (GAD), led to refined Gal4-driven expression. Placing Gal80 under the control of the tubulin promotor allowed for ubiquitous repression[22,23], and temperature-sensitive variants of Gal80 ($Gal80^{ts}$)provide temporal control of expression[24]. Combined with the spatial control (Gal4), this created the possibility of having precise temporal and spatial control of gene expression.

Two additional binary systems have been established in flies that can be used alone or in combination with each other and with the UAS-Gal4 system. The LexA-LexAop system is based on the *E. coli* DNA damage response system [25,26], and the repressible Q-system is based on the regulatory genes from the *Neurospora crassa qa* gene cluster for quinic acid catabolism[27–29]. This offers exciting possibilities such as intersectional approaches and labelling and manipulation of up to three cell populations simultaneously, which is very useful when studying interactions between cell types[30,31].

An interesting technique to study the *in* vivo role of a certain cell or group/type of cells in a process is cellular ablation, by which the desired cells are selectively killed. Several methods exist such as UV laser ablation, antibody-mediated delivery of toxins and genetic cell ablation. Genetic cell ablation involves the expression of cell death genes or toxic proteins such as Diphteria toxin A chain[32,33] and Ricin A chain[34] that induce apoptosis[35] in the cell type where it is expressed. This tool was later applied to *Drosophila* using wild-type or a cold-sensitive mutant form of Ricin A chain that permitted temporal control over ablation[36].

Another useful tool is the genome-wide fosmid library of lines containing GFP-tagged genes along with regulatory sequences that have been inserted in a known place in the genome while leaving an intact wild-type copy. This is useful when assessing protein localization under endogenous control of expression[37], allowing GFP to be used as a proxy for protein localization.

Besides genetic tools, a variety of online databases exist that allow for easy searching of genes, proteins and interactions, etc., and a number of stock centers that maintain lines for research purposes[21].

All these tools and many others make the fly an excellent model for studying cellular and intercellular processes *in vivo*.

3.3.2. Neuronal growth and plasticity at the *Drosophila* NMJ

The *Drosophila* larval neuromuscular junction has been a widely-used model for studying neuronal development, function and plasticity ever since Jan and Jan examined its physiological properties in 1976[38,39], confirming at the time that glutamate is the main neurotransmitter released at this NMJ. Glutamate is the main excitatory neurotransmitter in the mammalian CNS and many aspects of glutamatergic synapses are conserved between mammals and flies, making the *Drosophila* NMJ a useful system to study glutamatergic signalling.

The nervous system of the fly is composed of a central brain (CB) and a ventral nerve cord (VNC) from which the peripheral nerves extend towards muscles, visceral and sensory organs. Development of the nervous system starts in the embryo, where most neurons are formed from neural stem cells – neuroblasts – that delaminate from the neuroectoderm, acquire both positional and gene expression identities and undergo specific cell division events to form roughly 70 motoneurons (MNs) and 400 interneurons per segment. These must then find their targets through growth cone steering mechanisms. Sensory neurons are the exception, originating from sensory organ precursors in the periphery[40,41]. Initially many muscles are ectopically innervated by

the 'wrong' MNs, and these connections are then eliminated by an activity-dependent process during late stages of embryogenesis, refining the initial genetically-determined patterns[42,43].

Thus, larvae hatch with correctly innervated muscles and the neurons must then grow to match the stunning 10-fold increase in neuronal size from first to third instar before being remodelled in the pupal stage (Fig. 1D). The adult nervous system is divided in the same way but is more complex. Many of the developmental processes such as neurogenesis and axon guidance show conservation between flies and mammals, from genetic pathways to morphology [44].

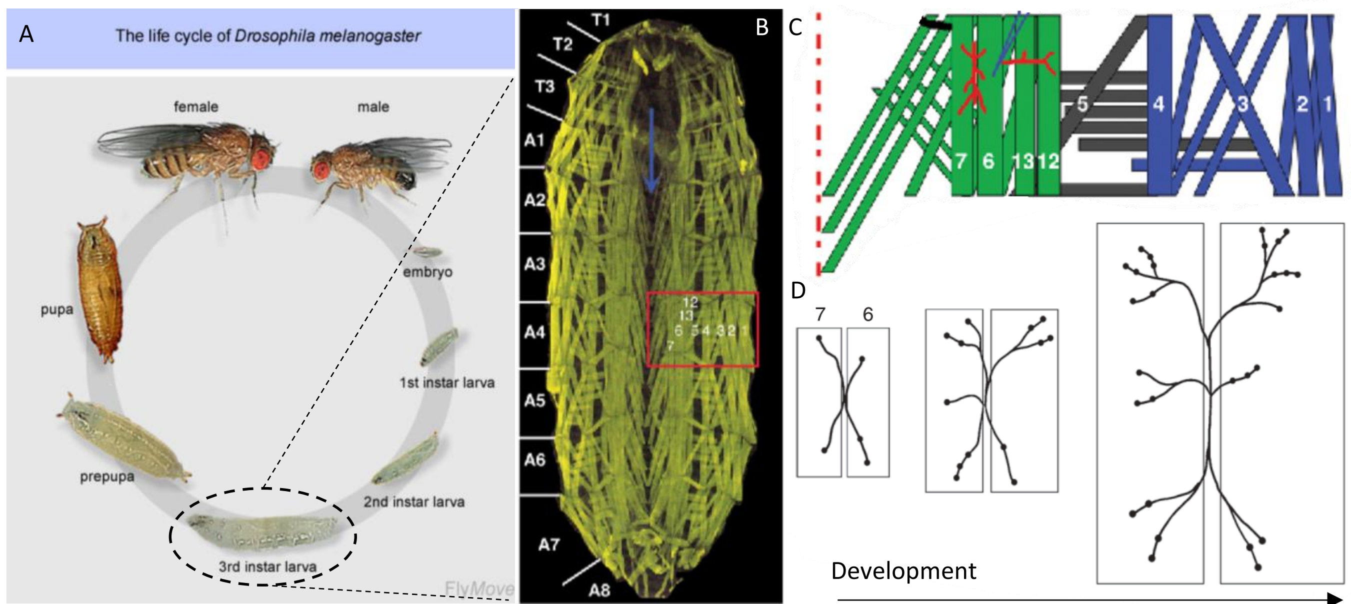


Figure 1 – The *Drosophila* 3rd instar larval NMJ as a model for neuronal growth and plasticity. A - Life cycle of *Drosophila melanogaster* (Adapted from FlyMove). B - Body wall musculature of a 3rd instar larva showing the segmented pattern from the first thoracic segment T1 to the last abdominal segment A8 and highlighting a single hemisegment set of muscle cells (red rectangle). Blue arrow points to the posterior end. C – Schematic of a single hemisegment musculature showing the motor neurons that innervate muscles 6, 7, 12 and 13 in red (B and C adapted from Zhang, B., & Stewart, B. Electrophysiological Recording from Drosophila Larval Body-Wall Muscles. *Cold Spring Harbor Protocols*, 2010). D – Depiction of the NMJ development from first to third instar at muscles 6/7 showing the growth and branching of the motor terminal in coordination with muscle growth (Adapted from Menon, K. *et al.* Development and plasticity of the Drosophila larval neuromuscular junction *Wiley interdisciplinary reviews. Developmental biology*. 2013).

Larvae are segmented into head, 3 thoracic and 8 abdominal segments (Fig. 1B). The abdominal segments A2 to A4 have been subject of more intense study due to their stereotypical morphology, with each hemisegment containing 30 identified muscles (each a single cell) arranged in a recognizable pattern and innervated by 32 identified MNs at the end of embryogenesis (Fig. 1C). There are three types of MNs: type I, II and III. Type I boutons are bigger than Types II and III, which are neuromodulatory, being octopaminergic (octopamine-releasing) and peptidergic (insulin; also releases glutamate). Type I boutons are divided into two subtypes – Ib (big) MNs usually innervate only one muscle, possessing big synaptic boutons (3—6 µM), exhibit sustained tonic firing, and are shorter and less branched than type Is (small) MNs, that have slightly smaller boutons (2—4 µM). A single MN of each type synapses onto a single muscle fibre, although many muscles are innervated by two, three and sometimes four different types of MNs[45,46]. The innervation patterns are morphologically and functionally similar between hemisegments and individuals, allowing repeated observations with minimal variability, which enables detection of subtle phenotypes through quantitative analyses. Together with the

accessibility for techniques such as dye labelling, electrophysiology, immunohistochemistry, electron microscopy (EM) and live imaging, the NMJ is highly suitable as a model of synaptic growth and plasticity[47].

The NMJ is composed of a terminal axonal arborization that contains boutons (both *en passant* and terminal), each housing up to 10 active zones aligned with postsynaptic receptors. The entire arbor is embedded in the muscle, and type I boutons are surrounded by post-synaptic membrane folds that make up the subsynaptic reticulum (SSR), whose function regarding neurotransmission, besides housing machinery such as glutamate receptors homologous to mammalian AMPARs, remains elusive.

The NMJ at muscle 6/7 (m6/7) (Fig. 1B-D) is the most extensively characterized. The two muscles are innervated by two Type I MNs, one Ib and one Is, whose terminals intertwine. Type Ib and Is boutons can be distinguished by size. Octopaminergic innervation is sometimes present and is also easily recognized. Using dye injections into each of the type I terminals and an antibody against horseradish peroxidase (HRP; that serendipitously stains neurons in *Drosophila*)[48,49], Lnenicka and Keshishian were the pioneers in the characterization of the morphology, physiology and synaptic plasticity of this synapse[50]. Terminal arbor size correlated well with muscle area, which is a good index for surface area since muscles are flat.

The NMJ axon terminals are plastic, growing in coordination with muscle cells (Fig. 1D) and being able to form new boutons in response to intense activity. Boutons can grow and shrink, and these remodeling events can occur between existing boutons or at the end of the arbor, either by symmetric division or asymmetrically budding off an existing bouton, as revealed by imaging intact larvae at different developmental stages[51]. Also, *de novo* bouton formation can occur from the terminal membrane[52]. New boutons initially lack a functional post-synapse, as shown by absence of the PSD95 (post-synaptic density protein of 95 kDa; a scaffolding protein) fly ortholog Discs large (Dlg) and are called ghost boutons. The opposite, named footprint, can also be found as Dlg staining without a pre-synaptic structure, and is thought to be synaptic remnants after a retraction event. It is possible to induce bouton growth and formation at the NMJ by stimulating MNs using either electrophysiology, optogenetics and hemolymph-like solutions containing high concentrations of Ca^{2+} and K^{+} ions[53]. By doing time-lapse imaging of the same NMJ in live preparations, our laboratory has recently found a mechanism for *de novo* bouton formation that shows the hallmarks of blebbing, a type of amoeboid cell migration favoured by low adhesion and high confinement environments. This mechanism involves rupture or detachment of the actin cortex beneath the cell membrane, allowing it to rapidly expand, forming a ghost bouton that can grow and mature or retract (Fernandes and Teodoro, *in preparation*). This mode of bouton formation was found to be activity-dependent and promoted by muscle contraction. While it remains unclear how this bouton growth is spatial- and temporally regulated, pre-synaptic calcium and local decreases in adhesion during contraction are good candidates and are currently being investigated in the lab.

3.4. Non-neuronal cells control neuron growth and function

Non-neuronal cells, especially glia, are evermore seen as key regulators of neuronal development and function throughout life, and dysfunctions of these cell types are often associated with neuronal disorders. Besides neurons and muscle cells, mammalian and *Drosophila* NMJs harbour other cell types such as peripheral glia that wrap the nerves and extend towards synapses, or trachea in *Drosophila* that supply oxygen and blood vessels in mammals that provide nutrients and oxygenation. All these cell types intimately contact each other and communicate to achieve

correct NMJ development and function. Their potential to influence neuronal functions such as plasticity is a matter of intense research that might lead to new therapies for nervous system pathologies.

3.4.1. Glial cells

The fact that the word glia derives from the ancient Greek γλία/γλοία (glía/gloía – glue) sums up the initial view of glial cells, after they were first discovered in 1856 by Rudolf Virchow, as mere connective tissue in the brain, providing passive support for neuronal function. Glia were thought as non-excitable cells, but although incapable of firing APs, glial cells possess a vast array of neurotransmitter receptors and ion channels[54]. In more recent years, the view on glia has radically changed with the discoveries that glia not only provide metabolic and structural support to neurons, but are clearly active participants in shaping neuronal function, including plasticity, and in the etiology of neuronal disorders such as AD, PD and ALS[55].

Glia evolved earlier than vertebrates (more than 500 million years ago), and glia-like cells can be found in Acoelomorpha, a primitive flatworm at the base of bilaterian phylogeny, as well as in most phyla, several of those possessing well-developed glia, such as Platyhelmintha, Mollusca, Annelida, Arthropoda and Chordata[56]. The conservation of glia and their functions allows the study of glial cell biology in invertebrate models such as *Drosophila*, taking advantage of all the available tools to dissect glial functions relevant for human health and disease[57,58].

Glia can be roughly divided into radial glia and myelinating glia. In the mammalian brain, three major glial subtypes are usually considered: astrocytes and microglia are radial glia, whereas oligodendrocytes myelinate axons. Other non-neuronal cells exist in the brain, such as ependymal cells, that secrete the cerebrospinal fluid (CSF), endothelial cells, composing the microvasculature of the brain and pericytes, associated with blood vessels. The *Drosophila* system houses three main glial cell types: cortex glia (CG) wrap around cell bodies in the cortex; astrocytes have cell bodies in the cortex but extend fine processes into the neuropil, contacting synapses and trachea in a way reminiscent of mammalian astrocytes; and ensheathing glia (EG) enclose the neuropil and wrap around axon tracts. Covering the entire CNS are subperineurial glia (SPG) that make pleated septate junctions, forming the blood-brain barrier (BBB). Above are perineurial glia (PG), that, together with macrophages, secrete a protective carbohydrate lamella around the whole nervous system. In the mammalian PNS, Schwann cells (SCs) wrap around large calibre axons and

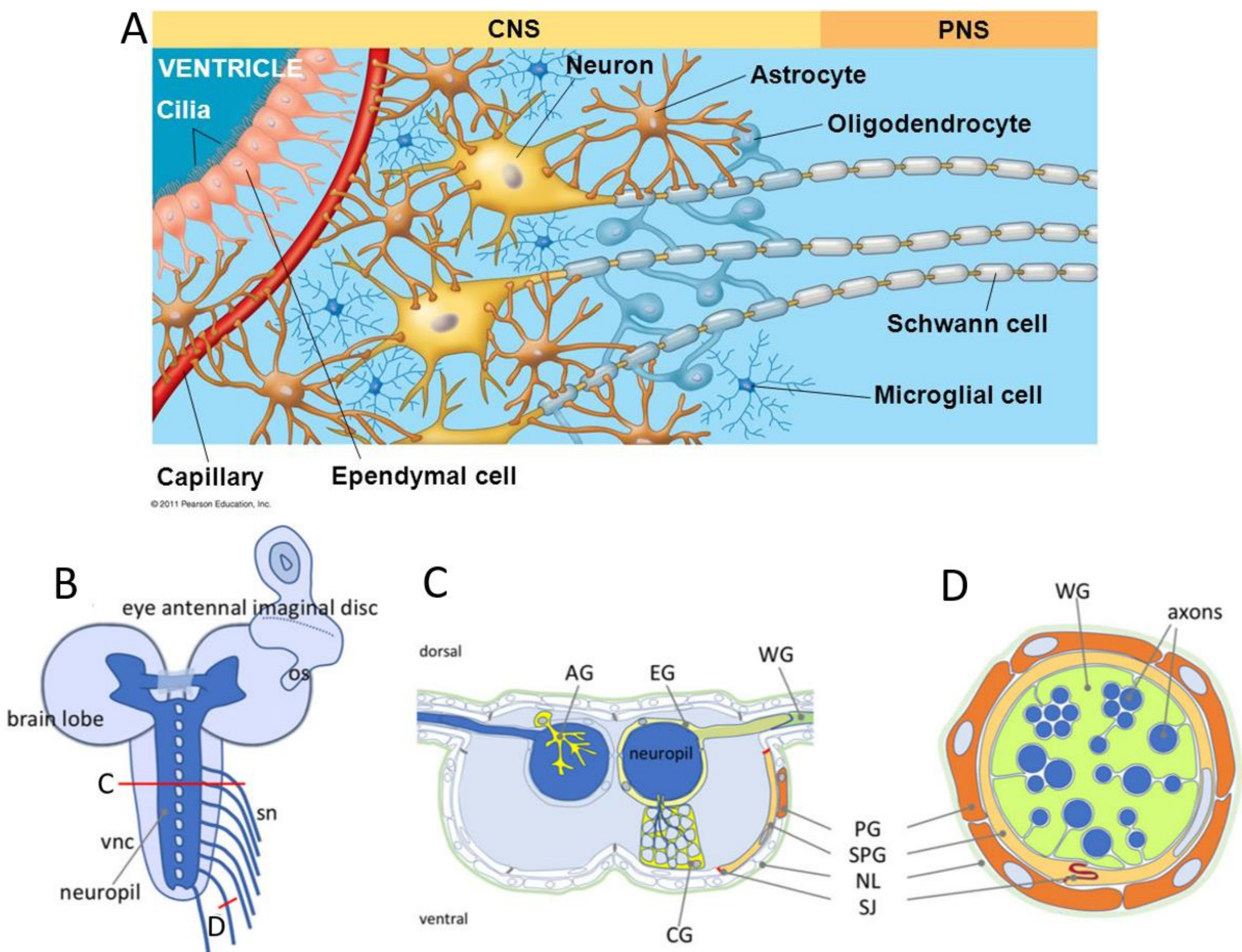


Figure 2 – Glial cells in mammals and *Drosophila*. A – Cartoon of mammalian glial subtypes in the CNS and PNS and their physical interactions with neurons, blood vessels and other glial subtypes. B – The CNS of *Drosophila* larvae is composed of a central brain with two lobes, and a ventral nerve cord (vnc), where segmental nerves (sn) exit the CNS. C – Cross section of a larval vnc. Astrocyte-like glia (AG) and ensheathing glia (EG) contact the neuropil, whereas cortex glia (CG) only interact with neuronal somas. Subperineurial and perineurial glial cells (SPG and PG) are surface glia that make up the blood-brain barrier as SPG establish septate junctions with each other. Surface glia are shared between the CNS and PNS, while wrapping glia (WG) is only found ensheathing peripheral nerves (B, C and D adapted from Yildirim, K. *et al.* Drosophila glia: Few cell types and many conserved functions. *GLIA* 2018).

can be found near synapses, while satellite cells cover the cell bodies in PNS ganglia. Thinner axons are wrapped together by non-myelinating SCs in Remak bundles. Both Remak fibres and myelinated axons are bundled together, enclosed by the endoneurium, a neural crest-derived tissue that secretes nerve supporting ECM, which is then covered by the perineurium, that forms the blood-nerve barrier. Perineurium-covered fascicles and blood vessels are once again packed inside the epineurium, composed of adipocytes and collagen, isolating the nerve from the outer environment. Peripheral nerves in *Drosophila* are ensheathed by wrapping glia, that insulate sensory and motor axons in a way that resembles mammalian Remak bundles. Subperineurial and perineurial glia then fully enclose the nerves, together establishing the blood-nerve barrier (BNB). In the adult fly NMJs, a recently identified glial subtype named perisynaptic glia associates with synaptic boutons and performs glutamate recycling as well as tracheal coupling.

Mammalian CNS macroglia (astrocytes and oligodendrocytes) originate from neuroepithelial progenitor cells (NPCs) in the embryonic neural tube and forebrain that differentiate into radial glia. These give rise to neurons and then suffer a 'gliogenic switch' that initiates the generation of astrocytes and oligodendrocyte precursor cells (OPCs) some of which will later mature into myelinating oligodendrocytes. Microglia are mesoderm-derived, being formed in the yolk sac during embryonic development where hematopoietic stem cells differentiate into primitive macrophages that then migrate to the CNS, becoming microglia. They are the immune cells of the CNS parenchyma, although there are other, bone-marrow derived, macrophages in the CNS.

Peripheral glia are born from neural crest cells, that generate sensory neurons of the dorsal root ganglia (DRG) and then differentiate into either SCs or satellite cells. Satellite cells remain in the ganglia, where they provide metabolic support and may contribute to integration of information, whereas SC precursors migrate along the nerves and mainly become myelinating SCs or non-myelinating SCs that wrap small calibre axons in Remak bundles, but can also give rise to melanocytes, parasympathetic neurons, endoneurial fibroblasts, or mesenchymal stem cells[59].

Drosophila glia are formed during embryogenesis, when midline glia differentiate from the mesectoderm and all other CNS glia arise from the neuroblasts that delaminate from the neuroectoderm. Most peripheral glia are also generated by neuroblasts except for a few that originate from sensory organ precursors. The numbers and positions of embryonic peripheral glia are known and after cell type determination these cells migrate along peripheral nerves to enwrap them.

While many of the glial developmental processes including signalling pathways for cell fate determination have been studied, how different glia types, neurons, muscle cells and blood vessels/trachea interact from development to adulthood remains unclear. Glia seem to participate and mediate much of this communication, and thus understanding glial development and function may reveal new ways in which they promote nervous system health.

3.4.1.1. Astrocytes

Astrocytes are the most numerous glia in the human brain. They are highly branched cells that dynamically extend and retract processes towards blood vessels, neuronal somas and synapses, and a single mouse astrocyte can contact ~100,000 synapses, from up to 600 dendrites, whereas in humans astrocytes are 2 to 2.5 times larger and can cover up to 2,000,000 synapses[60,61]. This close astrocyte-synapse association and the discovery that glia undergo elevations in internal $[Ca^{2+}]_i$ and can release neurotransmitters (gliotransmitters) in response to neuronal activity led to the concept of a tripartite synapse, where astrocytes in the CNS and perisynaptic SCs in the PNS

play structural, metabolic and neurotransmitter recycling support roles but are also actively involved in modulation of synaptic function and structure[62].

Two major types of astrocytes have been identified in rodents[63]. Fibrous astrocytes are mainly found in white matter and extend long, straight processes, contacting nodes of Ranvier[64]. They are known to express higher levels of glial fibrillary acidic protein (GFAP), an intermediate filament protein, and to associate with blood vessels, but their functions remain elusive[65]. Protoplasmic astrocytes occupy the gray matter and have branched, bushy morphologies. They extend endfeet that form the outmost layer of the blood-brain barrier (BBB), the glia limiting membrane, and regulate blood flow in an activity-dependent way[66]. Also, these cells can uptake glutamate at excitatory synapses, preventing glutamate spillover and isolating synapses from one another, and modulate synaptic function[65].

Drosophila astrocytes, also referred to as astrocyte-like glia (AG) closely resemble mammalian protoplasmic astrocytes at the morphological, molecular and function levels. They intimately contact synapses and tracheal cells, highlighting the conservation of tripartite synapses and neuron-vasculature coupling by glia.

Studies using culture of mouse retinal ganglion cells (RGCs) in serum-free medium have shown that in the absence of astrocytes, neurons are able to survive and extend neurites, but form few synapses. However, if these same RGCs are co-cultured with astrocytes or if astrocyte conditioned medium (ACM) is added, many synapses are formed, indicating that astrocytes must secrete synaptogenic factors. The lab of Ben Barres identified one of those factors - the ECM proteins thrombospondins (TSPs), which are expressed by protoplasmic astrocytes (TSP1 and -2), subventricular zone and white matter fibrous astrocytes (TSP4)[67,68]. Removing TSPs from ACM reduced the induction of synaptogenesis by ACM, and mice double mutant for TSP1 and -2 have fewer cortical excitatory synapses than wild-type (WT) animals. Immature astrocytes secrete TSPs during developmental synaptogenesis in the rodent cortex, promoting the formation of silent synapses, but this is downregulated in adulthood when injury can induce an increase in TSP expression that will induce new synapse formation, showing that astrocytes adapt to the neuronal environment[69].

The synaptogenic function of TSPs was found to be executed through interactions with a neuronal calcium channel subunit $\alpha 2\delta 1$ (Cacn$\alpha 2\delta 1$)[67]. Other secreted factors such as hevin and SPARC are known to induce or inhibit the induction of synaptogenesis, respectively, and may play a role after TSP-induce synapses form in maintaining or eliminating synapses[70].

Besides secreted factors, astrocyte-neuron contacts are essential for proper synapse formation, as neurons are unable to form synapses in the absence of astrocyte-neuron physical interactions. In cultured RGCs, astrocytic contacts cause neurons to become responsive to astrocyte-secreted factors that promote synaptogenesis. Contacts cause the adhesion molecule neurexin to relocate away from dendrites where they inhibit synapse formation[71]. This synaptogenic requirement of astrocyte contacts was also observed in cultured hippocampal neurons, where astrocytes activate integrin signalling via protein kinase C to facilitate excitatory synapse formation[72]. Also, homophilic γ-protocadherin adhesion between neuron and astrocytes induce glutamatergic and GABAergic synapse formation in mice spinal cords *in vivo*[73].

In addition to synapse formation, astrocytes also participate in synapse elimination. During a critical developmental period, mammalian astrocytes secrete TGF-β, promoting neuronal expression of a C1q, a complement system protein that opsonizes synapses for microglial engulfment[74,75]. Astrocytes can also directly remove synapses and neuronal debris through the

MEGF10 and MERTK phagocytic receptors[76]. MEGF10 is a receptor for C1q[77] and the mammalian ortholog of the cell corpse engulfment receptor CED-1 in *C. elegans*[78] and Draper in *Drosophila* that recognizes exposed phosphatidylserine in the membranes of apoptotic cells to mediate phagocytosis[79]. Draper is expressed at some point in most, if not all, glial cells in flies, and has been implicated in the clearance of injured axons, dying neurons in the CNS and neuronal debris at the NMJ. Astrocytes start expressing Draper at 6 hours after pupariation and selectively prune synapses during metamorphosis via both Draper-dependent and -independent mechanisms[80].

Synapse elimination by phagocytosis was found to be dependent on neuronal activity (unused synapses are selectively eliminated) and required for proper refinement of connectivity during development[76]. The expression of MEGF10 persists into adulthood, and astrocytes in the somatosensory cortex can prune excitatory and inhibitory inputs in 4 months-old mice. Interestingly, in the uninjured optic nerve head, astrocytes continuously engulf axonal evulsions that contain mitochondria, suggesting a role for astrocytic phagocytosis in the maintenance of neuronal fitness[81].

Given their extensive contacts with neurons and the already described functions, astrocytes are in a perfect position to regulate synaptic plasticity. Their fine processes contain ionotropic and metabotropic NT receptors, and activation of these receptors leads to increases in intracellular calcium, allowing astrocytes to sense neural activity. These calcium transients can occur slowly in the soma in response to intense neuronal activity or locally and fast in processes, where weaker activity can be detected and integrated in the large somatic calcium increases. Calcium can propagate between astrocytes through gap junctions and was found to induce a delayed increase in neuronal calcium. Two different mechanisms were proposed in 1994 by two independent laboratories: calcium waves spread to neurons through gap junctions[82], and astrocytic $[Ca^{2+}]_i$ rises leads to glutamate secretion that induces neuronal calcium influx through NMDARs[83]. Calcium transients in astrocytes are much slower than those required for neurotransmission, indicating they regulate neuronal function in a longer timescale.

Drosophila astrocytes also experience Ca^{2+} transients, although it is unclear whether these are spontaneous or activity-induced[84]. Induction of Ca^{2+} influx led to rapid paralysis, which was found to be mediated by fast endocytosis of GABA transporters in astrocytes, allowing inhibitory GABA to accumulate at synapses. This shows invertebrate astrocytes can also regulate neuronal activity and open doors for exploring glial control of neuronal activity in simple organisms.

Glia can act upon neurons by releasing gliotransmitters such as glutamate, ATP, adenosine and D-serine, that modulate synaptic transmission and plasticity. Both calcium-dependent and -independent mechanisms have been found to regulate gliotransmitter secretion[85] (e.g. Ca^{2+}-dependent release via exocytosis[86]). Single astrocytes release a multitude of gliotransmitters, each of them with the ability to act upon different targets, according to the type of circuit, localization and subtype of receptor expressed by neurons. Additionally, the propagation of calcium waves in astrocytes allows for the calcium increase triggered by one synapse to promote gliotransmission in distant synapses, as is the case with neuronal endocannabinoid-mediated astrocytic activation, that enhances synaptic function in a range of tens of microns away from the site of endocannabinoid release[87]. Another interesting example is the release of ATP by highly stimulated astrocytes in the CA1 region of the hippocampus, that is converted to adenosine and acts on A1 receptors to depress nearby synapses, showing astrocytes can mediate heterosynaptic communication[88]. These astrocytic properties allow them to regulate plasticity in several different ways according to the synaptic and circuit contexts.

Glial cells interact with each other, and astrocytes are no exception. For example, in the hippocampus, microglia secrete small amounts of ATP that activate astrocytes, promoting amplification of the signal through astrocytic ATP release, enhancing EPSPs[89].

3.4.1.2. Cortex glia

Cortex glia are the only glial cells in the *Drosophila* cortex, forming a honeycomb-like structure in which cell bodies and cortical axon tracts are enveloped[90]. They are in close contact with SPG and are thought to mediate the transfer of nutrients from the BBB to neuronal somas. Additionally, CG are likely involved in gas exchanges between the trachea that 'invade' the cortex and somata[80].

During development, CG together with SPG regulate neuroblast proliferation through insulin-like peptide signalling according to nutritional status, which in turn also regulates CG development[91,92].

Cortex glia seem to respond and regulate neuronal activity, as found in a screen that identified a temperature-sensitive mutant of the cortex glia-specific Na^{+}/Ca^{2+}, K^{+} exchanger Zydeco as responsible for seizures and hyperexcitability. Calcium transients in CG seem to act via Ca^{2+}/Calmodulin-dependent pathways to regulate neuronal excitability[93].

Draper is expressed in CG of the optic lobe from second instar until early pupal stages, being required for the removal of dead young neurons. Phosphatidylserine (PS) and two other Draper ligands expressed by apoptotic cells (or axons/boutons), Pretaporter and calcium-binding protein 1 (CaBP1) are thought to act as neuronal 'eat me' signals[94]. Usually on the inner leaflet of the cell membrane, PS exposure on the outer leaflet was shown to be both a cause and a consequence of dendrite degeneration in a phagocyte-dependent manner in *Drosophila* PNS sensory neurons[95] and to be a signal for axonal debris engulfment in explants of mice DRG neurons[96].

In summary, CG seem to share some of the properties and functions of other glial types while specifically acting upon neuronal somas.

3.4.1.3. Oligodendrocytes and ensheathing glia

Fast and efficient propagation of axon potentials requires insulation. In the mammalian CNS, this is performed by oligodendrocytes (OLs), whose branches wrap around axons and produce myelin, a lipid-rich substance with insulating properties. Changes in myelin coverage of neurons is emerging as an OL-based form of plasticity that by speeding up axonal propagation might be important for rapid behaviours such as escaping or predation and alterations of neuronal function through spike time-dependent plasticity[97].

Another function of OLs is metabolic support. Lactate provided by OLs is essential for neuron survival, as loss of the OL-enriched monocarboxylate transporter 1 (MCT1) causes neurodegeneration, with ALS patients and animal models having reduced levels of this transporter. Additionally, OLs can upregulate the levels of the glucose transporter GLUT1 in response to activation of oligodendroglial NMDARs by glutamate released by fast-spiking neurons[98]. This shows OLs can adapt to changes in axonal energy demands during activity, strengthening the view of glia as vital for neuronal maintenance and energetic homeostasis.

During development, OPCs require proper vascularization for nutrient and oxygen supply that fuels the energetically expensive membrane extension towards multiple axons. The cells can sense oxygen tension through hypoxia-inducible factor (HIF) dependent mechanisms. Hypoxia leads to stabilization of HIF1/2 complexes that activate the expression of hypoxia responsive element (HRE)-regulated genes. Constitutive HIF stabilization leads to developmental arrest

through autocrine Wnt signalling. On the other hand, OPC HIF1/2 are required for post-natal white matter angiogenesis[99], showing these cell types bidirectionally interact to correctly vascularize and myelinate the nervous system.

In the *Drosophila* CNS, ensheathing glia divide the neuropil into separate compartments and wrap around axonal tracts as soon as they enter the neuropil, having functions such as axonal growth promotion, fasciculation and guidance[100]. Being in such close contact with axons places EG in a perfect position to react to injuries. That is indeed the case, as EG phagocyte axonal debris after olfactory receptor neuron (ORN) injury through Draper[101].

Additionally, glutamate homeostasis in EG was found to contribute to control neuronal activity[102], further showing that, although very different in morphology, insulating glia in the mammalian and fly CNS seem to share roles other that ensheathment such as supporting growth and maintenance of axons.

3.4.1.4. Microglia

Microglia are the resident immune cells in the mammalian CNS, surveying their environment as they move their extensive branches while in resting state and becoming activated due to changes in neural homeostasis and neuronal injury. Activation states are diverse and are usually categorized into pro-inflammatory M1 and anti-inflammatory M2 phenotypes. The activation state determines whether microglia are cytotoxic or neuroprotective, which can contribute to injury and disease outcomes.

Being professional phagocytes, microglia play a role in programmed cell death and corpse removal of both neurons and glia during brain development[103]. Both during development and adulthood, microglial processes associate with synapses in an activity-dependent dynamic fashion[104]. They selectively prune weaker synapses during activity-dependent synaptic competition in post-natal development via the classical complement cascade, which involves binding of the complement protein C1q and C3 to cellular material, marking it for elimination[74,105]. Besides eliminating synapses, microglia have roles in synaptic function, maturation and connectivity that are also activity-dependent. Altogether, microglia promote correct neuronal development by remodelling synapses at the functional and structural levels, while monitoring the nervous system to readily respond to insults.

3.4.1.5. Schwann cells and wrapping glia

In the mammalian PNS, large calibre axons are insulated by several SCs, each wrapping multiple times around a determined axon segment, leaving nodes of Ranvier in between them. Thinner axons organized in Remak bundles are covered by non-myelinating SCs that share many resemblances with WG in *Drosophila*. Not much is known about WG due to the lack of specific Gal4 drivers. There are only three to four WG cells in each larval segmental nerve, which grow enormously to complete ensheath axons as the larva grows. Morphogenesis of WG depends on autocrine signalling via Vein (a neuregulin-1 homolog) activation of EGFR (epithelial growth factor receptor)[106]. Our laboratory recently identified the small GTPase Ral (Ras-like) as a regulator of WG growth during larval stages by promoting membrane addition via the exocyst complex[107].

In mammalian NMJs, the last SCs, called perisynaptic SCs (PSC), are non-myelinating and cap, but do not enclose completely, the axon terminal. These cells were first reported by Louis-Antoine Ranvier in 1878 as clusters of ‘arborization nuclei’ distinct from muscle fibre nuclei. Their identity and capping morphology have been studied extensively using electron microscopy (EM)[108] and

fluorescence microscopy in amphibian[109,110] and mammalian NMJs[111]. This further highlighted the tripartite nature of synapses, as glia establish interactions with both CNS and PNS synapses.

Around 3 to 5 PSCs exist per NMJ in amphibia and mammalians, and PSC number is positively correlated with endplate (post-synaptic specialization in vertebrate NMJs) size[112]. Although SCs are not essential for axon pathfinding [113,114], these cells appear and start extending processes at the NMJ soon after the establishment of the first neuron-muscle contact, after which the neuron grows along the pre-existing PSC processes, hinting for a role in synaptic growth, maturation and maintenance[115]. This role was further supported by PSC ablation experiments in frogs that, despite not having a short-term effect on neuronal structure and function, led to nerve retraction and weakening of remaining synapses. Furthermore, ablating PSCs during tadpole development prevents NMJ growth and addition and causes synaptic loss due to pre-synaptic terminals retracting[116].

Perisynaptic SCs are dynamic and can influence synaptic transmission and plasticity, and thus must act according to the synaptic environment by sensing synaptic activity. Frog PSCs extend calcium-channel containing membrane processes into the synaptic cleft, whereas in mammals these processes do not contact the cleft due to the presence of the ECM protein laminin 11[117]. In vertebrate PSCs, muscarinic metabotropic glutamate receptors and purinergic receptors are G protein-coupled receptors (GPCRs) that mediate the release of calcium from internal stores, allowing them to detect activity. Different activity patterns induce different calcium waveforms in PSCs, meaning PSCs can decode patterns of information[118]. This sensing is present from developmental stages, where glial sensing of synaptic strength may be involved in synaptic competition during NMJ development, as PSCs systematically show higher calcium responses to stronger synapses that are likely to be maintained[119]. These cells then act upon neurons by secreting neuromodulatory substances such as ATP, glutamate, prostaglandins and nitric oxide (NO)[118,120].

In an injury context, nerve degeneration leaves behind cellular debris that are cleared by PSCs[121], that then extend processes that serve as bridges for reinnervation by nearby uninjured motor neurons[122].

Dysfunctions in PSCs are associated with diseases such as Duchene muscular dystrophy and amyotrophic lateral sclerosis, and aging contributes to diminished PSC sprouting after injury[120,123] .

In summary, while wrapping glia and most SCs focus on insulating and metabolically support axons, PSCs are highly adaptable synaptic partners at the NMJ, promoting growth, function and remodelling of axonal terminals in both health and injury contexts. Interestingly, some of these active roles of glia are conserved in invertebrate NMJs, although played by different cell types such as subperineurial and perineurial glia in *Drosophila* larvae and perisynaptic glia in adults.

3.4.1.6. Subperineurial glia

Subperineurial glia cover the whole CNS and most of the PNS. They sit atop CG in the CNS and WG in the PNS, being formed during embryogenesis and growing but not dividing during larval stages to ensure complete axonal coverage. The establishment of pleated septate junctions (pSJs) between neighbour SPG cells makes a paracellular barrier to toxins and infectious agents while allowing controlled intracellular transport of nutrients. Many of the core SJ proteins and xenobiotic exporters of the BBB and BNB are conserved between SPG and mammalian glia[58].

The BNB terminates at the NMJ, near the site where the motor axon branches and forms boutons, but SPG extend processes beyond this point, contacting synaptic boutons and muscle cells. These

processes have a variety of morphologies, from blunt-ended to lamellipodia and filopodia-like. Some filopodial structures have a round ending and are sometimes referred to as gliobulbs. Subperineurial glia at the NMJ were found to function together with muscle cells to phagocyte neuronal membrane debris and ghost boutons. Shedding of pre-synaptic material occurs naturally at low levels but is increased by neuronal activity. The engulfment receptor Draper is required for clearance, as *draper* mutant NMJs accumulate debris and ghost boutons but have fewer mature type Ib boutons. Draper reduction by expressing RNAi against Draper using either a muscle driver (C57-Gal4) and the pan-glial driver Repo (*reversed polarity*)-Gal4 both produced NMJs with less type Ib boutons but NMJs whose glia lack Draper have excessive synaptic debris (although not as much as *draper* mutants) and normal numbers of ghost boutons, while muscle-specific Draper knock-down led to ghost bouton accumulation (again less than *draper* mutants) but wild-type levels of debris. Live imaging revealed that debris was present inside SPG processes and that detached ghost boutons either disintegrate into smaller pieces or are engulfed by muscle cells[124].

There are three isoforms of Draper, but only Draper-I and -III are expressed at larval NMJs. Expression of Draper-III in SPG only using Gliotactin-Gal4 (Gli-Gal4 also called rl82-Gal4) partially rescued the number of type Ib boutons in a *draper*$^{\Delta 5}$ mutant background, while neither expression of Draper-I in SPG nor Draper-I or -III in muscle was able to do so. The increase in ghost bouton number in *draper* mutants was fully rescued by Draper-I in SPG and Draper-III in muscle cells, and partially rescued by Draper-III in SPG and Draper-I post-synaptically in the muscle. Wild-type levels of debris were found at NMJs where Draper-I was overexpressed in SPG and Draper-III in the muscle also reduced debris levels in the *draper*$^{\Delta 5}$ background[124]. This suggests that glia preferentially remove debris but can also contribute to ghost bouton clearance, although it is unclear if by phagocytosis of the whole bouton or due to its decomposition into smaller membrane fragments, more likely due to size constraints. However, Gliotactin is only weakly expressed in 3rd instar SPG and is also expressed in trachea and other cells in the larva[125]. Draper-I and Draper-III seem to be more active in SPG and muscle, respectively, but many questions remain unanswered, *e.g.*: how do the differences in the isoforms relate to synaptic debris or ghost bouton preferential engulfment? How are debris and ghost bouton impairing neuronal growth? What is the 'eat-me' signal exposed by debris and unwanted ghost boutons and how its exposure is regulated? Overall, this study revealed a cooperative function of glia and muscle cells in clearing shed membrane debris and unwanted ghost boutons through an evolutionarily conserved phagocytic pathway.

Later, the same authors discovered that a SPG-secreted member of the TGF-β family, Maverick (Mav), induced retrograde muscle-neuron BMP signalling that promotes bouton growth (discussed in greater detail later), thus providing a means for muscle-glia coordination in guiding neuronal growth[126]. However, how Mav secretion is locally regulated and whether SPG can decode neuronal activity to fine tune remodelling has not been investigated. Nonetheless, these findings support the notion that glia and muscle cells interact to regulate neuronal plasticity.

Another glial-derived regulator of neuronal development was identified in a screen for targets of the transcription factor Repo, the Wnt-1 encoding gene *wingless* (*wg*). Wingless is also expressed and secreted by neurons, regulating terminal arbor growth, SSR expansion and glutamate receptor clustering at the post-synapse[127,128]. Subperineurial glia are the sole glial source of Wg, secreting it so it localizes near to all boutons. Glial-derived Wg also regulates GluR clustering and post-synaptic potentials, but in contrast to neuronal Wg, it increased the frequency of miniature excitatory junctional potentials (mEJPs)[128].

Altogether, there is compelling evidence that, as their mammalian counterparts, SPG actively participate in shaping the NMJ, dynamically infiltrating into the perisynaptic space and communicating with neurons and muscle cells to coordinate NMJ growth, maintenance and function. Pursuing the questions remaining after these recent discoveries will certainly shed light on conserved mechanisms of glial function.

3.4.1.7. Perineurial glia

The mammalian perineurium is composed of several perineurial cells, thin glial cells that partially overlap and form tight junctions with each other, acting as a barrier against toxins, infection and ionic flux. Originally thought to be fibroblasts and thus mesodermal in origin, they form a double basal lamina, express basement membrane-specific genes and enclose small axon bundles in nerve terminals, ruling out this possibility. The two other possible origins of PG are the neural crest and the neuroectoderm, which gives rise to the CNS. Studies on neural crest contributions to PNS glia revealed that endoneurial and Schwann, but not perineurial cells, are born in this tissue[129]. This leaves us with the neuroectoderm as the origin of PG, and in fact, in *Drosophila*, most PNS glia derive from neuroblasts[130]. Initial studies in frogs and chicks showed the neural crest was the major source of peripheral sheath cells but that a small proportion was neural tube-derived. In 1939, Jones hypothesized that the neural crest originates dorsal, or sensory, root sheath cells, while the neural tube is responsible for motor or ventral root sheath cells[131]. Sheath cells were found to be mostly SCs, except for a ventral population[132].

A study using in vivo time-lapse imaging in transgenic zebrafish expressing membrane-bound GFP controlled by *nkx2.2a* regulatory sequences – *Tg(nkx2.2a:megfp)* in lateral floor plate cells (spinal cord) revealed that, during motor axon outgrowth, these cells exited the CNS and migrated along growing axons. *nkx2.2a*$^{+}$ cells were always found peripheral to myelin basic protein positive (MBP^{+}) SCs, had zona occludins 1 (ZO1), a tight junction protein, a double basement and were morphologically similar to mammalian PG, confirming that perineurial glia in zebrafish originate in the spinal cord. Disruption of perineurial cell development and migration using a morpholino oligonucleotide (antisense RNA technology in zebrafish that blocks gene expression) against *nkx2.2a* (required for lateral floor plate development) led motor neurons, sometimes including cell bodies, to ectopically exit the spinal cord at irregularly spaced positions. These neurons were thinner and more branched, suggesting perineurial glia have a role in regulating motor neuron outgrowth location, in preventing neuronal cell bodies from exiting the CNS and in axon bundling. Additionally, SCs migration down motor nerves and myelination were defective, as SCs appeared isolated, disform and lacked MBP. Furthermore, *colorless* mutants (*cls*), which lack Sox10, a transcription factor necessary for SC development, have no SC wrapping of axons and display abnormal perineurial cell migration – uncoordinated and exhibiting excessively numerous and active filopodia and failing to enclose nerves, that showed signs of defasciculation. Later, these properties and functions were found to be conserved in rodent nkx2.2^{+} cells[133]. This provided evidence for the central origin of PG and their roles in neuronal as well as SC development through bidirectional communication[134].

Perineurial glia are also active during nerve regeneration, being the first cellular element to 'bridge' the gap between the proximal and distal ends after sciatic nerve severing in mice[135]. Further work by the Kucenas laboratory showed that perineurial glia also bridge neuronal injuries in zebrafish, phagocytosing debris alongside macrophages and allowing SCs and axons to extend distally during regeneration. Interestingly, SCs can promote attraction of PG to the injury site, again highlighting the bidirectionality of SC-PG interactions[136].

In *Drosophila*, PG make up the outermost layer of the nervous system, contacting SPG and the haemolymph and secreting the neural lamella. Although not completely covering SPG in larvae, they act as a cellular component of the BBB and BNB in adult flies. Contrary to other glial types, PG continue dividing until the beginning of pupariation, and have been found to originate other cell types such as WG, suggesting PG are a multipotent glial source during larval stages[58,137].

In the sensory nervous system, perineurial and subperineurial glia ensheath neuronal somas and extend processes towards dendrites, and contribute to normal dendritic morphology, integration of sensory stimuli and regeneration of injured dendrites, all of which were impaired in mutants where this somatodendritic glial wrapping is absent[138].

At the NMJ, PG extend beyond the BNB, covering branches, interdigitating with boutons and contacting muscle regions devoid of synapses. Like SPG, perineurial processes have a wide range of morphologies, and increasing either neuronal activity and NMJ size by rearing larvae at higher temperatures or using *highwire* mutants with bigger terminals but boutons with weaker activity causes higher frequency and size of PG processes and changes the distribution of morphologies from more blunt-ended to more complex, whereas reducing activity and growth has the opposite effect. This suggests PG grow in coordination with the terminal arborization and leaves open the question whether neuronal activity plays a role in PG development.

Hence, the perineurium appears not just as a blood-nerve barrier that covers the entire PNS but as an active player in regulating neuronal growth during PNS development and regeneration[139], and perineurial glia in *Drosophila*, although without no known role in NMJ growth and adaptation, act as mammalian perineurial cells in peripheral nerve organization.

3.4.1.8. Muscle cells

The muscle harbors the post-synaptic machinery, aligned with presynaptic AZs and subject to activity-dependent regulation. Trans-synaptic signaling mediates a route for coordinated growth of the pre- and post-synapse. This consists of both neuron to muscle (anterograde) and muscle to neuron (retrograde) signaling. Wingless (Wg), part of the Wnt family, and Glass bottom boat (Gbb), a member of the bone morphogenetic protein (BMP) and TGF-β families are the best characterized anterograde and retrograde signals at the NMJ[47].

Wg is synaptogenic in the rodent cerebellum[140], and at the *Drosophila* NMJ it is secreted by the MN and acts via pre- and post-synaptic targets. Wg activates the muscle receptor DFrizzled2 (DFz2) that is cleaved and translocated to the nucleus where it is required to promote SSR growth and glutamate receptor clustering[127,141]. Interestingly, Wg is released in extracellular HRP$^+$ vesicles characterized as exosomes[142,143]. Wg also signals autocrinally by interacting with components of the canonical Wnt pathway such as Dishevelled and the glycogen synthase kinase shaggy (GSK3), that locally stabilizes microtubules in loops through phosphorylation of the microtubule associated protein Futsch, a process though to be important for bouton stabilization[144].

Gbb is secreted from the muscle and acts on pre-synaptic TGF-β receptors Wishful thinking (Wit) and either Saxophone (Sax) or Thickveins (Tkv). This activates different signalling cascades that can regulate synaptic function and growth. One of those involves activation of the transcription factor Smad (Mothers against Dpp [Mad] in Drosophila) that is then imported to the nucleus where it activates BMP target genes. One of them is Trio, a guanine exchange factor (GEF) for the actin cytoskeleton-interacting small GTPase Rac1 and also known to regulate microtubule stability in terminal arbors, revealing a cross-talk between retrograde signalling and cytoskeleton rearrangements that promote growth[8,145,146]. Additionally, Wit activation can signal independently

of transcription through the protein LIM kinase 1 (Limk) to release its inhibition of Cofilin, an actin-severing protein that allows for actin turnover, a process found to precede bouton budding[147].

Synaptotagmin 4 (Syt4) is a member of the synaptotagmin vesicle membrane proteins of which Syt1 is the main Ca^{2+} sensor to promote synaptic vesicle fusion at pre-synaptic terminals. It is expressed in muscle cells, where it senses GluR-mediated Ca^{2+} influx and promotes local retrograde signalling events through $Syt4^{+}$ vesicle fusion that enhance function and induce ghost bouton formation[148]. Syt4 levels are regulated by activity and although the secreted factor(s) are yet to be identified, this pathway may provide a means to locally regulate bouton formation.

Overall, the muscle is able to receive and provide signals that influence synaptic bouton development, function and plasticity.

3.4.1.9. Tracheal and endothelial cells

Neuron function is very energy-demanding, as maintenance of the electrochemical gradient, neurotransmitter release, protein synthesis and signalling events all rely on a constant ATP supply. Mitochondria are found in most axonal terminals and perform oxidative phosphorylation as well as Ca^{2+} buffering. In boutons lacking mitochondria, glycolysis or ATP diffusion from mitochondria-containing boutons ensure the maintenance of energy availability. To power metabolism, neurons rely on nutrient and oxygen delivery from the circulatory system. In the mammalian brain, blood vessels must grow to match neuronal growth and prevent ischaemia/hypoxia. Major vessels are formed during embryogenesis but growth continues postnatally and is activity-dependent, as shown in a study by Whiteus et al. where perturbing neuronal activity patterns during a critical post-natal period led to angiogenic defects in mice[149].

The tracheal system is the respiratory system of flies, being composed by a network of vessels made up by tracheal cells that form an empty lumen where oxygen and other gases flow. In each embryonic segment, an epithelial sac of ~80 cells sprouts primary branches that later originate secondary branches, interconnecting with each other in a stereotypical pattern. Tertiary branch (tracheoles) formation is highly dependent on the target tissue oxygen needs. Tracheal branching is dependent on chemoattraction via interactions between the FGF Branchless (Bnl) and the FGF receptor Breathless (Btl). Breathless starts being expressed in the epithelial sac, followed by turning on and off Bnl expression and secretion in surrounding epithelial cells in a spatiotemporally-regulated manner to guide tracheal branch elongation. At the tip of trachea, the last cell is called terminal cell and does not form a lumen, instead, it probes the extracellular space in search of sources of Bnl, which target tissues such as muscle cells express during larval stages in response to oxygenation levels. Expression of Bnl in target tissues and of Btl in trachea is regulated by the stabilization of Similar (Sima), the fly homolog of hypoxia inducible factor α (HIFα) in these cells[150,151].

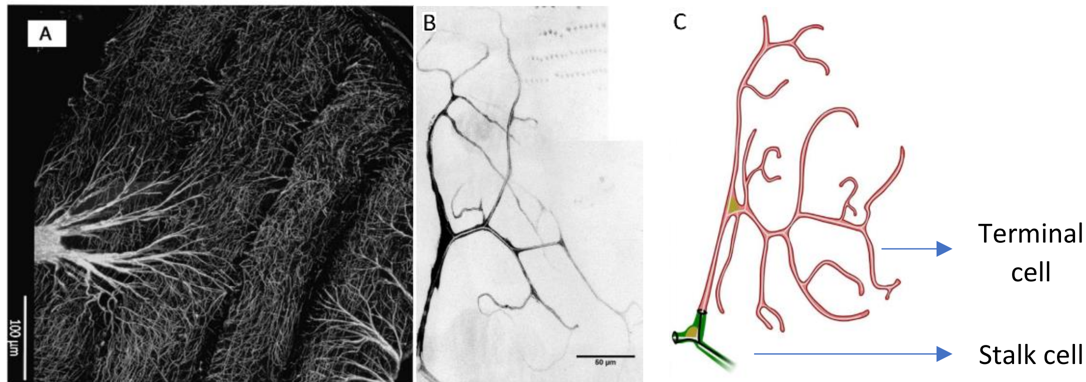


Figure 3 - **Tracheal vessels in *Drosophila*.** A – Confocal microscopy image of the trachea that oxygenate the dorsolongitudinal flight muscles of adult flies (Harrison, J.F. *et al.,* Developmental plasticity and stability in the tracheal networks supplying Drosophila flight muscle in response to rearing oxygen level. *Journal of Insect Physiology* 2017). B – Confocal image of a terminal cell in a 3rd instar larva. C – Schematic of a terminal cell (pink) connecting with a stalk cell (green) and forming SJs (black line). Nuclei in yellow and lumen in white. B and C adapted from Benedikt T. Best, Single-cell branching morphogenesis in the Drosophila trachea. *Developmental Biology* 2019.

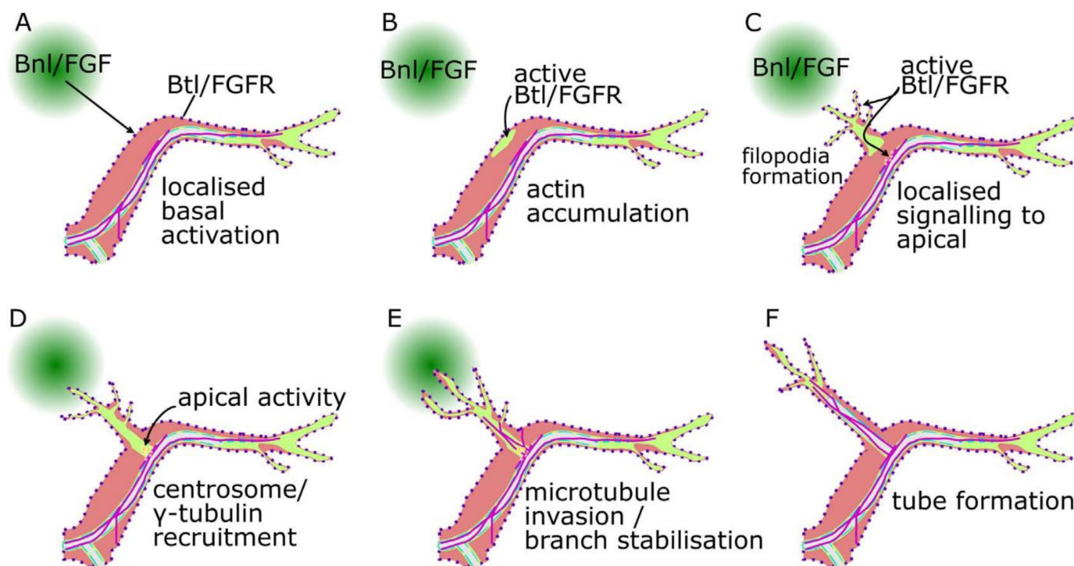


Figure 4 – Breathless/Branchless signalling in tracheal morphogenesis. Both during the first stages of stereotypical branching and later at the adaptive, oxygen-sensitive phase of tracheal branching, Bnl-Btl signalling mediates branch formation and elongation via chemoattraction. Branchless first activates Btl (A), leading to actin dynamics and filopodia formation (B and C). Microtubules are then recruited to the new branch (D and E) that then matures and forms a lumen (E; Benedikt T. Best, Single-cell branching morphogenesis in the Drosophila trachea. *Developmental Biology* 2019).

During embryonic and larval development, trachea that oxygenate the CB and VNC grow in close contact with glial cells as they surround and infiltrate the cortex and neuropil. Glia also seem to be near tracheal branches, indicating oxygenation and other tracheal factors may be important for glial development, as is the case with oligodendrocytes and endothelial cells in mammalian development. In the *Drosophila* embryo, the cerebral trachea (a primary branch) grows along neuropile glia, probably EG, which in larval stages enclose primary and secondary branches of the perineuropilar tracheal plexus and whose cells and membrane extensions accompany trachea and tracheoles that invade the neuropil. Besides Btl-Bnl signalling, a subset of neuropil glia named

midline glia secretes the guidance molecule Slit which acts on tracheal Robo 1 and 2 receptors to mediate attraction of a ganglionic tracheal branch towards the midline and inhibit crossing of the midline by trachea, respectively. Both when all glia are absent as in *glial cells missing* (*gcm*) mutants, or when only surface glia develop and neuropile and CG are ablated, tracheal morphogenesis is altered – excessive branching is observed[152]. This supports the hypothesis that glial cells restrict and guide tracheal growth, which together with a possible growth-supportive role of trachea on glia may represent a highly coordinated morphogenetic event conserved from flies to mammals.

Trachea-glia interactions were recently found to be important for neuronal development at the NMJ. Mutants for *Trachealess* (*Trh*), a gene required for cell fate determination in trachea development, have defective trachea and consequently poor oxygenation at the organismal level. At the NMJ, this led to the formation of satellite boutons, 'flower-like' structures made of several small boutons that emerge from a single bigger bouton, and this was found to be dependent on the pre-synaptic action of glia-derived Wg. Wingless expression is upregulated during hypoxia as a consequence of Sima stabilization, and acts via neuronal microtubules to promote satellite bouton formation, which is thought to maintain normal synaptic activity levels required to maintain coordinated locomotion in hypoxic conditions[153]. Thus neuron-glia-trachea communication is essential for NMJ development and adaptation to environmental conditions such as oxygenation.

Similarly to astrocytic coupling of blood capillaries and neurons in mammals, in adult flies, perisynaptic glia in dorsal lateral flight muscles physically interact with tracheal cells and are thought to mediate and regulate gas exchanges, although very little is known about how they perform these functions and whether larval glia can also interact with trachea. Understanding how these glial cells 'grab on' to trachea to promote adequate oxygen supply to neurons at the NMJ and other tissues will certainly be important to fully unveil the mechanisms of intercellular communication that allow coordinated growth of NMJs in accordance with activity levels and growth factor, nutrient and oxygen availability.

In summary, neurons, glia and other cell types such as endothelial/tracheal and muscle cells are all part of a network of intercellular signalling that controls coordinated development and adaptation in several different contexts, and from flies to mammals. Interestingly, many functions, signalling pathways and developmental processes are conserved despite the lack of direct homology between some of these cell types. This reinforces the need to study these interactions given the clear importance of intercellular communication for nervous system development and function and, ultimately, animal life.

3.5. Aims

In this study we aimed to thoroughly characterize intercellular interactions at the NMJ of *Drosophila* larvae to better understand and discover new ways in which glia, trachea and muscle cells participate in neuronal growth and plasticity. Specifically, we aimed to understand:

- The function of each glial subtype and assess if they have shared or overlapping functions
- If local signalling occurs at glial extensions into the perisynaptic space
- If and how are trachea coupled to neurons in larval NMJs
- The dynamics of glial and tracheal interactions with each other and with neurons
- The role of perineurial glia membrane protrusions

4. Methods

4.1. Drosophila stocks and husbandry

Flies were kept at 25ºC unless stated otherwise, in agar-yeast-cornmeal medium with dry yeast. Larvae were reared either in agar-yeast-cornmeal vials or agar-apple juice petri dishes supplemented with yeast paste (dry yeast + water) at 25ºC unless stated. Fly handling was done under CO_2 anaesthesia. Table 1 lists the stocks used in this study and their origins.

4.2. Dissection and immunohistochemistry

Third instar larvae were dissected according to previously published protocols. Briefly, larvae were selected, rinsed and placed in a petri dish containing a silicone elastomer (SYLGARD™ 184 Silicone Elastomer, The Dow Chemical Company, USA) that allowed using thin metal pins (Austerlitz Minutiens Insect Pins, Entomoravia, Czech Republic) or tissue glue (Gluture Topical Tissue Adhesive, Zoetis, USA) to immobilize the larvae. First the head and tail of the larvae are pinned in place, then, using precision scissors, an incision is made on the posterior-most region of the dorsal cuticle. A second cut along the antero-posterior axis opens the larva dorsally and, following removal of the internal organs, the ventral abdominal body wall musculature is visible. Four extra pins are placed to stretch the 'flaps' created by the cuts, opening the larva while keeping it alive. Larvae were dissected immersed in HL3.1, a hemolymph-like solution that preserves larval vitality (in mM: 70 NaCl, 5 KCl, 0.1 $CaCl_2$, 4 $MgCl_2$, 10 $NaHCO_3$, 5 Trehalose, 115 Sucrose, 5 HEPES-NaOH, pH 7.3-7.4). For fixed imaging, larvae were fixed 20 minutes at room temperature (RT) in 4% paraformaldehyde in PBS, with 2 mM Ca^{2+} for better preservation of small structures (integrin and cadherin adhesion molecules are calcium-dependent). Then the pins were removed, and larvae placed on an Eppendorf tube for immunohistochemistry. Three 15 min. washes in PBT (PBS + 0.3% Triton X-100) were followed by a 30 min. to 1h blocking incubation with agitation at RT with 5% NGS (normal goat serum) in PBT, to minimize unspecific antibody binding. Primary antibodies and the concentrations they were used in are listed in Table 2. Primary antibody incubation was done in 5% NGS in PBT, at 4ºC with agitation, overnight. Three washing and one blocking step were again performed prior to incubation with a secondary antibody (listed in Table 3) for 2h at RT in the dark with agitation, and 3 more washes were done after. Larvae were transferred to 50% glycerol before being immersed in a viscous mounting medium (1,4-diazabicyclo[2.2.2]octane – DABCO, Sigma Aldrich, USA), mounted in a microscope slide, sealed with nail polish and stored at 4ºC in the dark until imaging.

For live imaging applications, larvae were dissected on a slide with Sylgard bedding using either smaller pins or tissue glue, and a coverslip held in place by sticky tape kept the larvae immersed in HL3.1 during imaging.

4.3. Phosphatidylserine immunofluorescence assay

To detect exposed phosphatidylserine (PS), a kit by RayBio® was used (RayBio® Annexin V-Cy3 Apoptosis Detection Kit), in which the PS-binding protein Annexin V is conjugated with the Cy3 red fluorophore. Live dissected larvae were incubated for 5 min in Annexin V-Cy3 in binding buffer (1:100) provided by the kit, at RT in the dark, prior to fixation and IHC or imaging

4.4. Stimulation protocols

For stimulation protocols, larvae were first semi-dissected – head and tail pins plus initial incisions - to allow solutions to bathe the body wall muscles. Then the tail pin was placed closer to the head pin, so the larva is not stretched, allowing for activity- induced contraction and preventing tissue tear. Larvae were then immersed in a depolarizing solution based on HL3.1[154] containing high levels of K^+ (90 mM) and Ca^{2+} (1.5 mM, the concentration of the other constituents is

adjusted to maintain osmolarity) named High K^+ , alternating between HL3.1 and High K^+ in a pulsed manner. For fixed imaging a short stimulation was used[155] (SStim), in which three two-minute High K^+ pulses with two 15´ rest periods in between were applied, followed by a 30´ final rest period to finish the dissection (re-stretching the larvae) before fixation. For live imaging, both SStim and single 10- or 16-min. High K^+ pulse protocols were used, named massed stimulation[52] (MStim, pseudo-massed in the original report).

4.5. Genetic cell ablation

Cell ablation was performed by expressing Ricin A under the control of UAS promoter. The line $TubGal80ts; \frac{UAS\text{–}Ricin}{TM6B,\ Tb}$ was crossed to the desired cell type-specific Gal4 line in laying pots at 29ºC to allow Gal4 binding to UAS sequences and larvae were selected against Tubby (Tb).

4.6. Confocal imaging

Fixed samples were imaged on a Zeiss LSM710 scanning confocal microscope with a 63X oil immersion lens. Live samples were imaged on a spinning disk microscope composed of a Nikon Eclipse Ti coupled with an Andor Revolution spinning disk system and an EMCCD (electron multiplier charge-coupled device) Andor iXon^{EM}+ camera, using a 60X oil immersion objective.

4.7. Image analysis and calculations

Image analysis was performed using Fiji/Image J (National Institutes of Health, Bethesda, USA) – for 2D morphology assessment and quantification – and Imaris (Bitplane Inc, Switzerland), for 3D morphological analysis, using the Surfaces tool and an xTension (Surface Surface Contact Area by Matthew Gastinger) for surface-surface contact area estimation. All Z-projections are maximum intensity projections. Images were processed in the following way: bilinear interpolation to increase the resolution two-fold and background subtraction with a rolling ball algorithm (radius: 50px).

Identification of new activity-dependent boutons was based on the presence of a pre-synaptic structure not surrounded by the post-synaptic marker Discs large.

Calculation of the area and perimeter of the glial 'cavity' in Fig. 10 was performed in Casio online calculator (available at https://keisan.casio.com/exec/system/1223289167) using the formulas for ellipse area and circumference: $Area = ab\pi$; $Perimeter = 4aE(k)$ where a and b are the bigger and smaller radius of the ellipse, respectively, k is eccentricity $k = \sqrt{1-(\frac{b}{a})^2}$ and $E(k)$ is the complete elliptic integral of the second kind.

4.8. Statistical analysis

Statistical analysis and graph generation were perform in GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA). For debris and ghost bouton analysis, normality of the sample distribution was tested using the D'Agostino & Pearson omnibus normality test. As the distributions of both ghost bouton number and debris score were not normal, we used the Kruskal Wallis test followed by the Dunn's multiple comparisons test to correct for multiple comparisons.

Table 1 – *Drosophila* stocks used in this study

Genotype	Source
w^{1118}	Bloomington Drosophila Stock Center (BDRC) Stock 3605
Sco/CyO; DVGlut-lexA, lexAOp-mCherry/TMBb	Gift from César Mendes (Baek et al., 2013)[156]
nSyb-GAL4	BDSC 19183
Nrv2-GAL4	BDSC 6800
Repo-GAL4	BDSC 7415
moody-GAL4	Stork et al., 2008[157]
Bsg-GAL4 (PG-GAL4)	Drosophila Genomics and Genetic Resources, Kyoto Stock Center 105188
FlyFos nrv2	Vienna Drosophila Resource Center 318289 (Sarov el al. 2016)[37]
OK6-Gal4	BDSC 64199
w^{1118}*; ; Mdr65-Gal4*	BDSC 50472
10XQUAS-6XGFP, UAS-tdTomato/CyO; NSyb-QF2/TM6b, Tb	BDSC 66479
Shn-GFP	BDSC 42671
btl-Gal4, UAS-Actin-GFP/CyO	BDSC 8807
UAS-mCD4-GFP	BDSC 35836
UAS-mCD8-GFP	BDSC 32184
UAS-tagBFP	BDSC 56807

UAS-LifeActRuby	BDSC 58362
UAS-myr-RFP	BDSC 7118
TubGal80ts/CyO; UAS-Ricin/TM6b, Tb	Gift from César Mendes
Sqh^{AX3}/Y; ; Sqh-GFP	BDSC 57144

Table 2 – Primary antibodies used in this study

Primary Antibodies	**Concentration used**	**Source**
Mouse anti-Discs large (4F3)	1:250	Developmental Studies Hybridoma Bank (DSHB), deposited by Goodman, C..
Mouse anti-Draper (5D14)	1:500	DSHB, deposited by Logan, M..
Rabbit anti-GFP	1:10,000	

Table 3 – Secondary antibodies used in this study

Secondary Antibodies	**Concentration used**	**Source**
Goat anti-HRP Cy3 (conjugated)	1:500 (from a 50% dilution in glycerol according to manufacturer's instructions)	Jackson Immunoresearch
Donkey anti-Mouse Alexa Fluor 488		
Donkey anti-Mouse Alexa Fluor 647		
Donkey anti-Rabbit Alexa Fluor 488		

5. Results

To unravel glial and tracheal functions at the NMJ during neuronal development and plasticity, we took advantage of *Drosophila* genetics and stereotypical neuronal development by using 3rd instar larvae, which have already formed and matured synaptic connections in the PNS as a result of larval development but that have not yet entered the pupal remodelling stage. Thus, our model NMJ is highly consistent between segments and individuals while remaining plastic and able to form new boutons in response to acute synaptic activity-inducing protocols.

5.1. *In vivo* dynamics of bouton formation

Bouton formation can occur in two different ways as described by previous work in the lab: it can happen very fast in an explosive fashion, or in a slower and sustained way. Both can happen in both unstimulated and stimulated conditions, and ghost boutons can be identified by the morphology and lack of post-synapse in fixed samples and their dynamics analysed in live imaging movies (Fig. 5). While 'explosive' events are always preceded by a wave of muscle contraction, in slow and sustained events muscle contraction is weaker and sometimes absent.

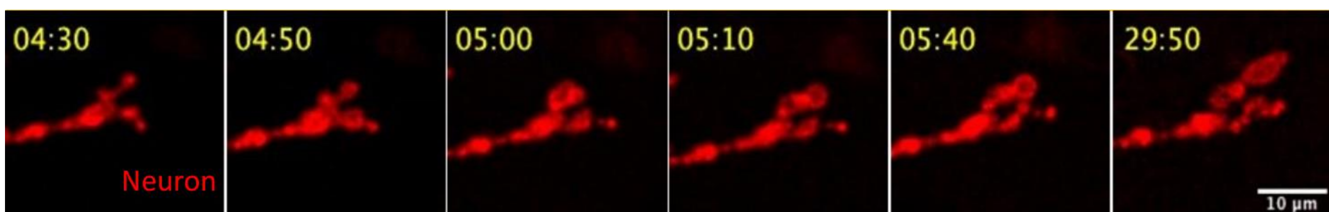


Figure 5 – Live dynamics of synaptic bouton formation. New boutons quickly form in a muscle 6/7 of a 3rd instar larva after a massed stimulation protocol of a single 16' High K^+ pulse and continue growing slowly. Genotype: *moody-Gal4/+; DVGlut-LexA, LexAOp-mCherry/UAS-CD8-GFP*. Video available as supplementary material.

5.2. Glial morphology at the NMJ

To uncover glial functions at the 3rd instar larval NMJ, we first analysed glial morphology at muscles 6/7 by doing confocal imaging with all glia labelled with membrane-tethered GFP under control of the pan-glial driver Repo (Repo-Gal4; UAS-mCD8-GFP) and neurons stained with an αHRP antibody. This revealed the presence of a variety of glial processes at the NMJ as expected (Fig. 6A), and these protrusions were found to contact the axon, synaptic boutons (Figs. 6A and 6B), muscle areas devoid of innervation (Fig. 6D, unlabelled) and trachea, whose lumen (due to chitin) has blue autofluorescence when excited by a 405 nm laser (Fig. 6A, 2nd leftmost panel). Analysis of single z-planes of 0.5 µm confirmed the apposition between glial membranes and trachea/boutons (Figs. 6B and 6C). Neuronal debris is continuously shed at the NMJ, accumulating after neuronal activity, and in our preparations HRP^+ neuronal membrane debris was found in many NMJs even in the absence of stimulation, and in some instances, glial membranes contacted debris (Fig. 6B).

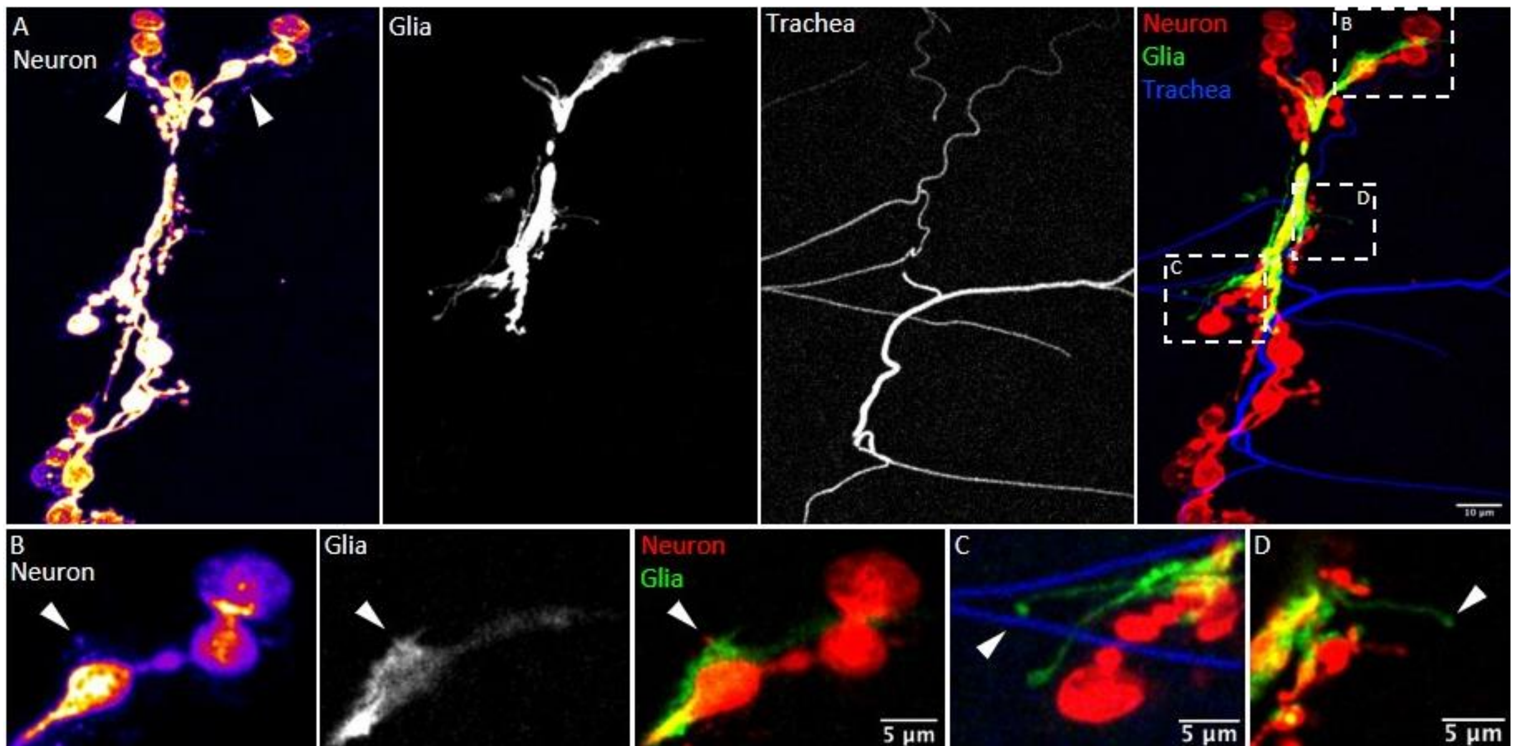


Figure 6 – Glia extend processes towards multiple cell types at NMJs. Pan-glial labelling in *Repo-Gal4; UAS-CD4-GFP* 3rd instar larvae (muscle 6/7) after a spaced stimulation protocol of three 2' High K^+ pulses with 10' rest in between. Neurons stained with α-HRP Cy3. Tracheal lumen autofluorescence in blue. A - Z projection showing glia extending membranes along the terminal axonal arbor, contacting synaptic boutons (B), celular debris (arrows in A and B), tracheal cells (arrow in C) and the muscle (arrow in D). B—D are single z-planes of 0.5 µm.

In order to assess the individual contribution of glial subtypes at the NMJ, subtype-specific drivers were combined with fluorescent reporters and the presence of each glial subtype was analysed in both stimulated and unstimulated samples.

5.3. Wrapping glia

Previous studies indicate that WG membranes do not extend into the NMJ, terminating at the site where the MNs become embedded in the muscle. Accordingly, most of the NMJs imaged lacked WG membranes, labelled with GFP under the control of the native Na^+/K^+ ATPase *nervana 2* (*nrv2*) regulatory sequences (FlyFos nrv2), except for a single instance in which a thin process with a bulbous end contacted the muscle (Fig. 7). Besides WG, FlyFos nrv2 showed faint labelling of tracheal vessels, which we noted to extend into the NMJ area.

Our observations are consistent with the described role of WG as specialized axonal insulators, not directly affecting NMJ function while remaining crucial for axon potential propagation, which drives muscle contraction and locomotion.

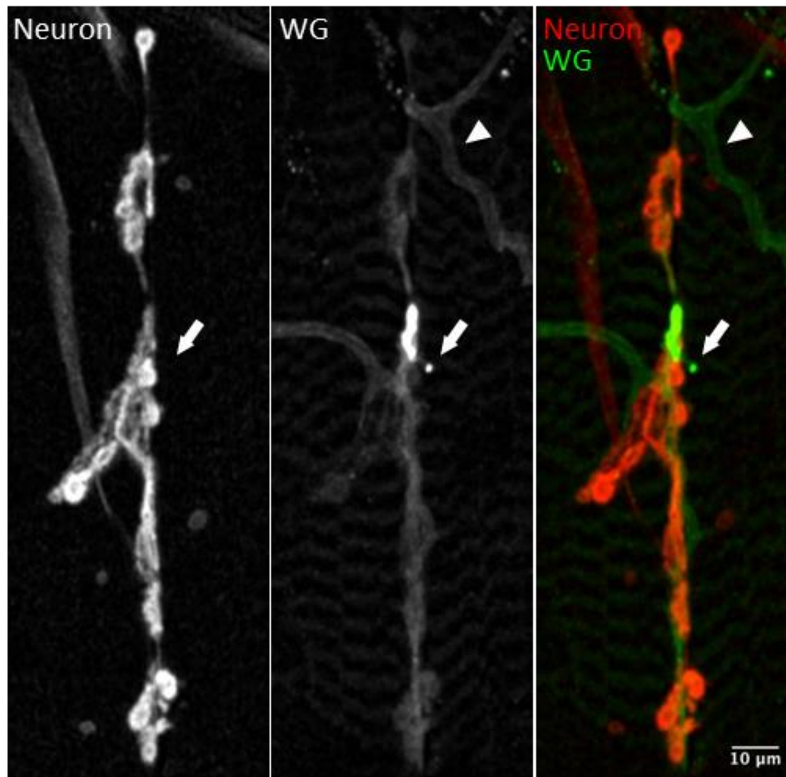


Figure 7 – A wrapping glia process invading the NMJ. A small, round WG protrusion (arrow) contacting the muscle at a 6/7 NMJ after a short stimulation protocol. Note the faint labelling in trachea (arrowheads). Genotype: *OK6-Gal4, UAS-myr-RFP/+; FlyFos nrv2/+*.

5.4. Subperineurial glia

Analysis of SPG morphology in fixed preparations, by using the moody-Gal4 driver, described as SPG-specific[158], combined with a fluorescent membrane-bound reporter, corroborated previous descriptions of SPG morphology at the NMJ[124]. However, in moody-Gal4; UAS-mCD8-GFP larvae, GFP was also found around tracheal lumen, leaving us with the question of whether moody was SPG-specific. Since *moody* encodes two G-coupled protein receptors required for SJ assembly and tracheal cells establish SJs, it makes sense that Moody is also expressed in tracheal cells. Fortunately, it is possible to distinguish between trachea and SPG in moody-Gal4; UAS-mCD8-GFP larvae due to the blue fluorescent lumen, and also more specific drivers are available such as *Multi drug resistance 65*, a xenobiotic transporter of the BBB conserved between flies and humans[159].

Glial membranes covered the initial segment of the terminal arborization and then branched into thinner processes with variable endings such as bulb- and filopodial-like (arrows in Fig. 8). Bulbous processes were sometimes seen touching, but not covering, both mature and ghost boutons (Fig. 9D). SPG morphology was confirmed in live preparations, and time-lapse imaging revealed that sometimes their processes were in contact with boutons from which other boutons emerged. We quantified glial presence and physical contact at the NMJ by performing a 3D reconstruction using the surface tool and then the Imaris XTension for surface-surface contact analysis (Fig. 8). The mean values for neuron and glia surface area, volume and contact area are summarized in Fig. 8D, and we found that, on average, SPG cover 14 ± 6.3 % of the terminal axon arborization.

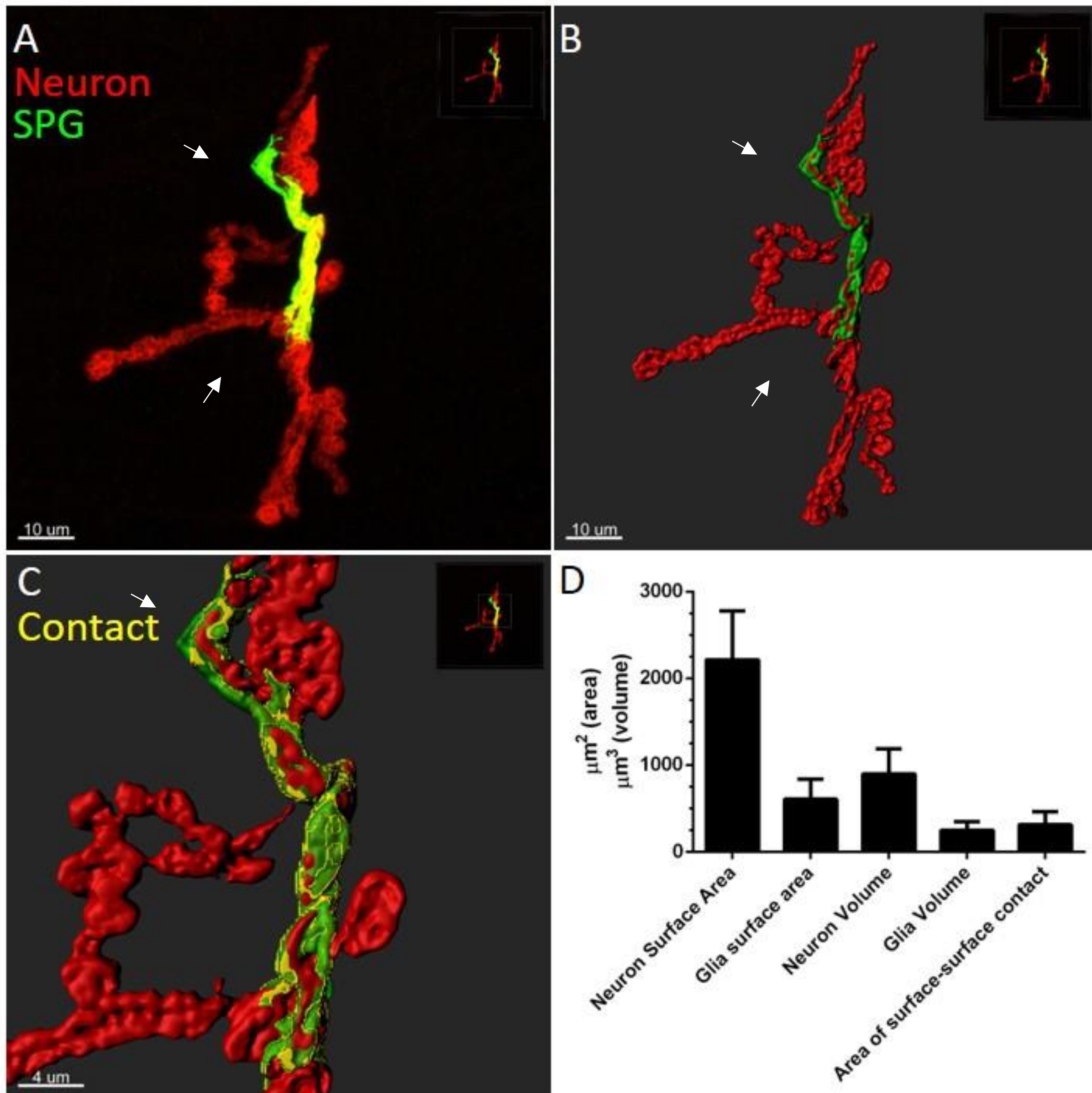


Figure 8 – Subperineurial glia intimately contact the axonal terminal. SPG processes cover the main branch and extend filopodial processes towards synaptic boutons (A, arrows). B – 3D reconstruction of the neuronal and glial cells. C – Closer view of the glial cell in B, showing in yellow the area of contact between the two cells. D – Quantification of neuronal and glial surface area, volume and contact area. n=7 NMJs. Unstimulated preparations. Genotype: *UAS-CD4-GFP/+; Mdr65-Gal4/+*.

In one event after a massed stimulation protocol, a 'gliobulb' was found in close contact with a bouton before and during the fast budding of new boutons from the initial one (Fig. 9). Although some muscle contraction occurred at the time of budding, it was not of the same magnitude as usually observed (not quantified), this suggested that the glial contact was promoting bouton formation and not a simple coincidence. The gliobulb remained in place even though the initial and new boutons went out of the focal plane after 13 minutes of imaging, which was reminiscent of the muscle engulfment of ghost boutons described by Fuentes-Medel *et al.*[124] (Fig. 9E). This glial process branched from the main arbor at a different site than the initial bouton, not covering

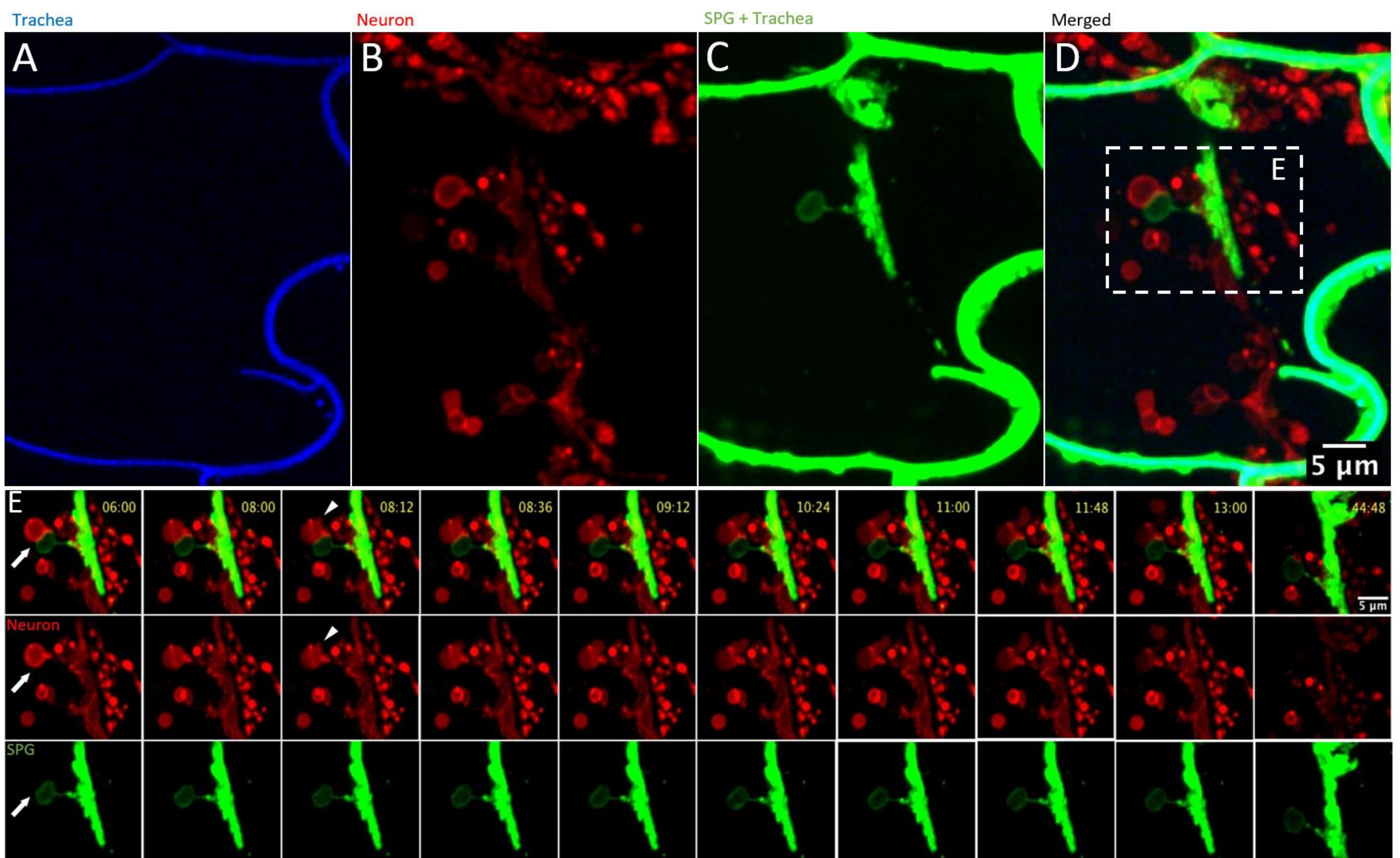


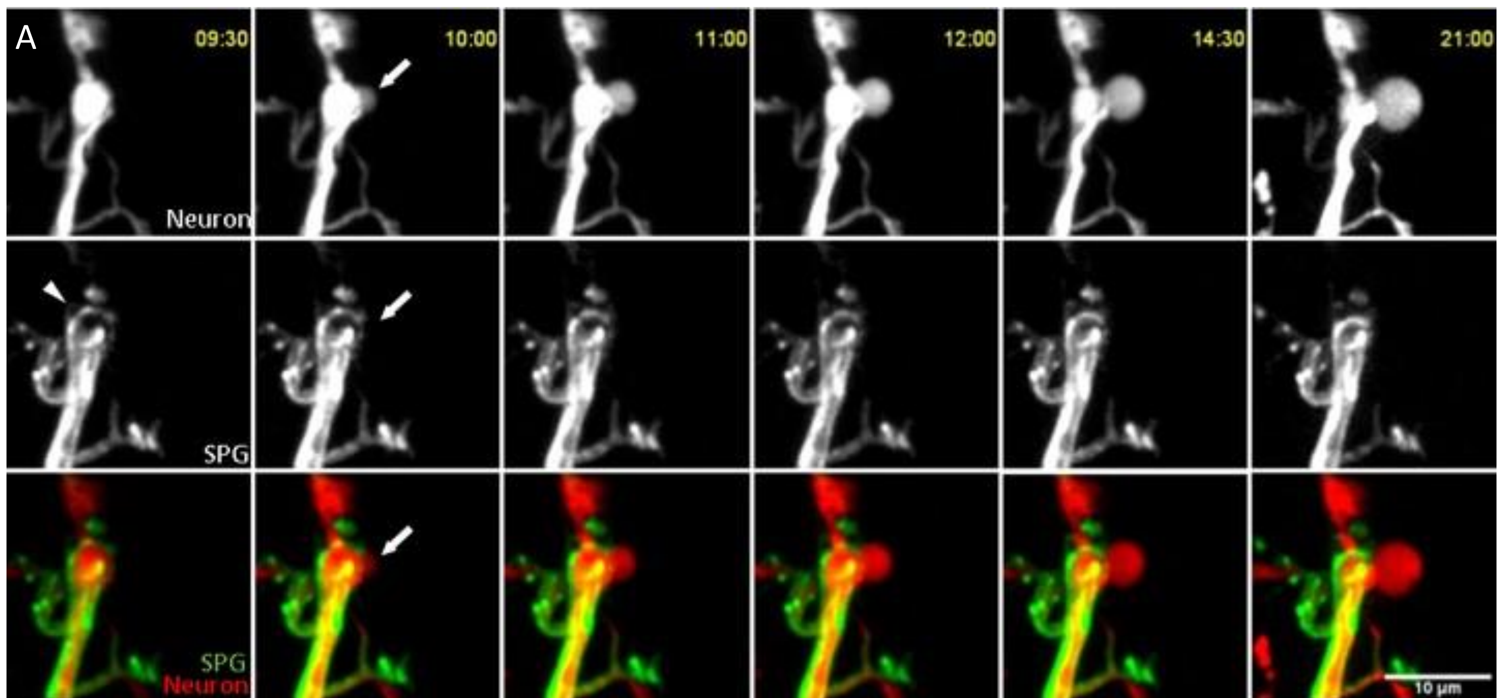
Figure 9 – SPG 'gliobulb' contacting a growing bouton after stimulation. The glial process can be distinguished from GFP+ trachea by absence of blue autofluorescence (A,C and D) and coverage of the axon (B-D) E – Stills (maximum intensity projections) from time-lapse live confocal imaging of a 3rd instar 6/7 NMJ showing a new bouton (arrowheads) forming from a SPG-contacted existing bouton (arrows) after a massed stimulation protocol. The glial process persists long after the event ended. Genotype: *moody-Gal4/+; DVGlut-LexA, LexAOp-mCherry/UAS-CD8-GFP*. Video available as supplementary material.

the branch that connected the bouton to the arbor and contacted the neuron on the opposite side of where the bouton budded. Little change in glial shape was observed, indicating that an increase in confinement due to 'pushing' was unlikely to be involved in promoting this event of bouton formation. Instead, local signalling may have happened between bouton and gliobulb.

In another event in an unstimulated preparation, a glial membrane partially wrapped around a synaptic bouton which housed a slow and sustained bouton formation event. The glial process (arrowhead in Fig. 10A) contracted around the bouton as it was forming, as visible by a decrease in the area and perimeter of the 'cavity' circumscribed by the glial membrane (Fig.10B, compare the first and last time-points in Fig. 10A). This event happened in the absence of noticeable

muscle contraction, suggesting that glia contributed to an increase in confinement and promoted bouton formation through a ‘squeezing’ mechanism.

Using a functional GFP-tagged version of the light chain NMII (Sqh-GFP, *Spaghetti squash* encodes NMII) expressed under the native promoter in a *Sqh* mutant background (*SqhAX3*, lethal, lethality rescued by expression of Sqh-GFP), we observed an event in which a non-neuronal source of NMII appears to contract around the site in an axonal branch where a chain of boutons formed in an explosive manner after a stimulation protocol (Fig. 11). While it is unclear whether the source of this NMII pool is tracheal or glial (note the shape of a nearby NMII pool in Fig. 11 that indicates the presence of a lumen) this finding further supports our claims that cells other than the muscle are able to promote bouton formation through contraction.



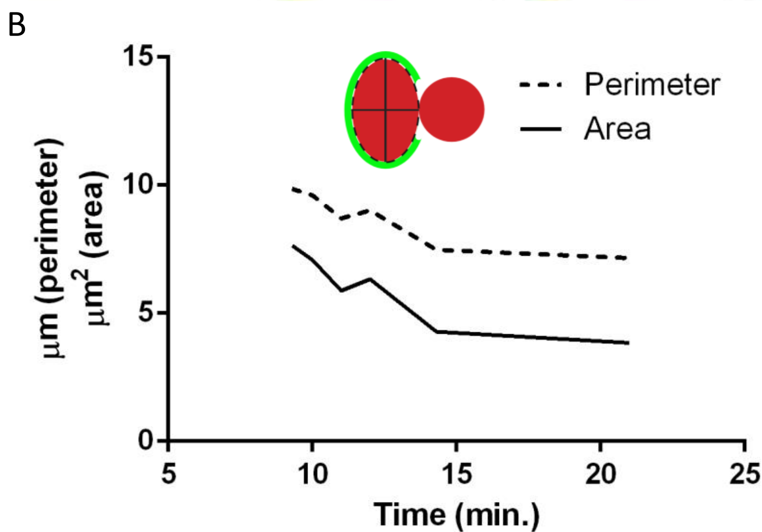


Figure 10 - Subperineurial glia may promote bouton formation through contraction. A – A thin glial membrane wrapped around a synaptic bouton (arrowheads) contracts during a bouton formation event (arrows) in the absence of stimulation. Genotype: *10XQUAS-6XGFP, UAS-tdTomato/+; Mdr65-Gal4/NSyb-QF2*. Video available as supplementary material. B – Both the area (defined by full lines in scheme) and perimeter (dashed line) of the ‘cavity’ formed by the SPG membrane decrease during the formation and expansion of the new bouton.

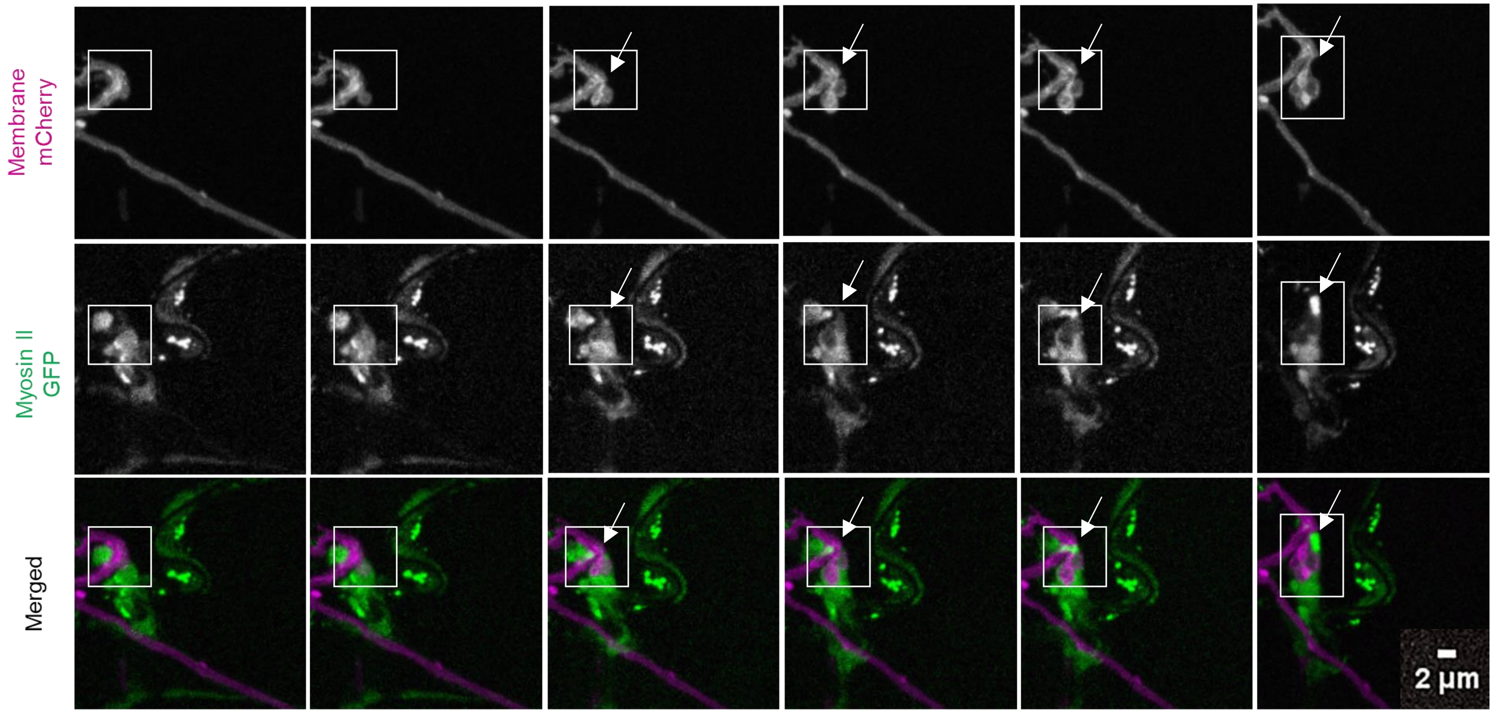


Figure 11 – Dynamics of a non-neuronal pool of non-muscle Myosin II during a bouton formation event. A group of boutons forms from a branch in a fast, explosive manner after stimulation (boxes). At the same time, a non-neuronal source of NMII seems to contract around where the boutons budded (arrows). Genotype: *SqhAX3/Y; DVGlut-LexA,LexAOp-mCherry/SqhGFP*. Video available in supplementary data. Images and video courtesy of Andreia Fernandes.

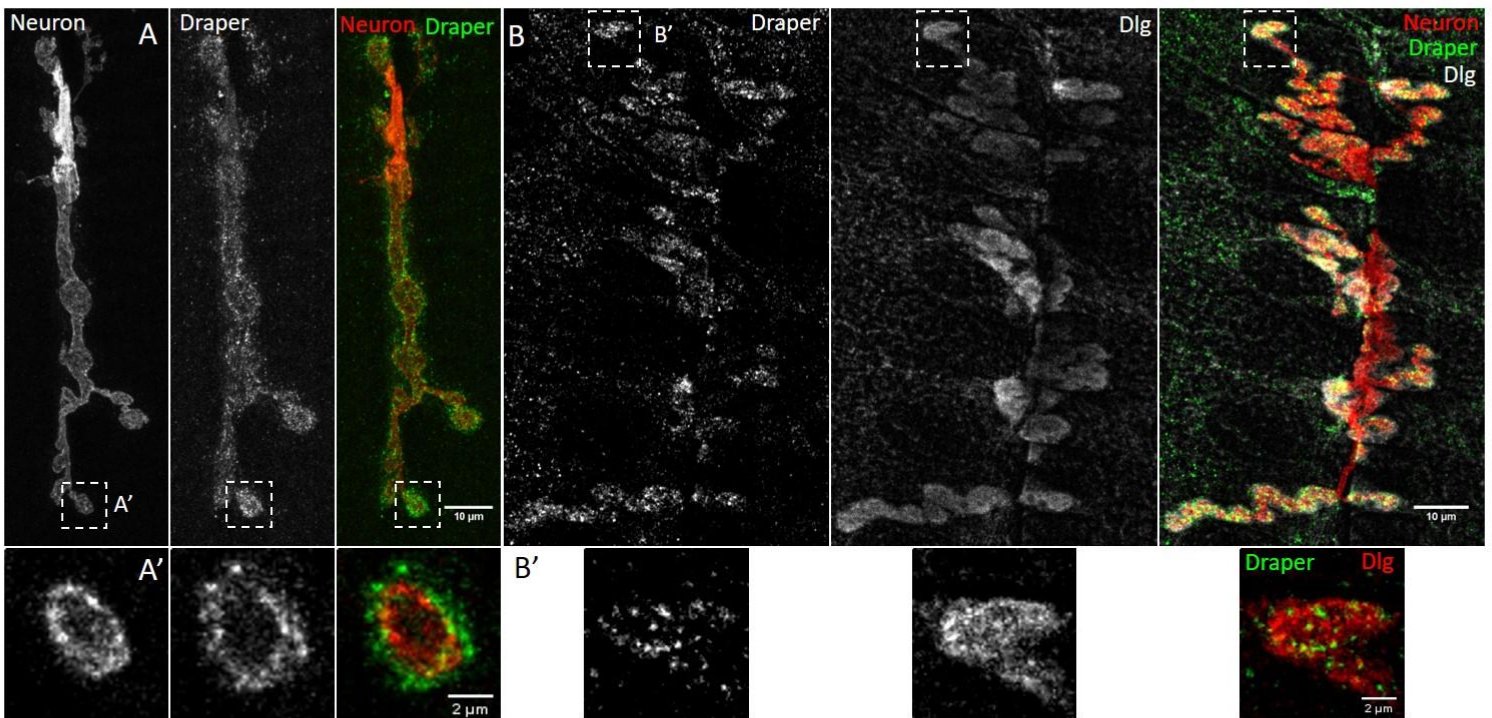


Figure 12 – Draper localizes to the post-synapse and surrounds all boutons. A – Draper labelled by anti-Draper antibody (5D14) surrounds all synaptic boutons. A' - Single z-stacks of 0.5 µm of a single bouton. Genotype: *PG-Gal4/+; UAS-CD4-GFP/+*. B – Draper is localized post-synaptically as shown in B' with single Z-planes of 0.5 µm of a single post-synaptic region labelled with an anti-Dlg antibody (4F3). Genotype: *TubGal80^{ts}/+ ; UAS-Ricin/Mdr65-Gal4*. Unstimulated preparations.

We did not observe glial engulfment of neuronal debris in our movies, however, this may be due to the low light emission of debris labelled with genetically encoded reporters. As previous reports demonstrated that glia express Draper and can phagocyte neuronal debris, we stained Draper using a monoclonal antibody (DSHB 5D14) against all isoforms. In all preparations observed, fluorescent puncta in the muscle surrounded all the synaptic boutons (Fig. 12A and co-localized with the post-synaptic marker Dlg (Fig. 12B).

Although PS exposure was found to be important for phagocytosis of neuronal material in sensory neurons, the 'eat me' signal in neuronal debris and ghost boutons not meant to be matured in motor neurons has not yet been identified. Thus, we tested whether PS exposure occurred at the NMJ by staining it with a Cy3-tagged PS-binding protein, Annexin V, and labelling neurons with mCD4-GFP driven by NSyb-Gal4 (neuronal synaptobrevin, pan-neuronal). In two NMJs from different larvae, Annexin V – Cy3 labelled both trachea and the entire axonal terminal (Fig. 13A) but Z-plane analysis (not shown) does not show exclusive binding to the extracellular side of the cell membrane, although the preparation was imaged while alive in a scanning confocal microscope (low image acquisition rate) and thus the movement may account for this mislocalization of Annexin V. In another NMJ trachea were strongly labelled while only a faint signal was detected in the axonal terminal (Fig. 13B). Although the analysis is not conclusive, PS seems to be present at the NMJ.

Altogether, our data on SPG morphology and dynamics supports their role in bouton growth and indicates that they may help determine the site of bouton formation either by local signalling, mechanical constraints or both. However, these are single events and thus more data is required to dissect the players involved in this process.

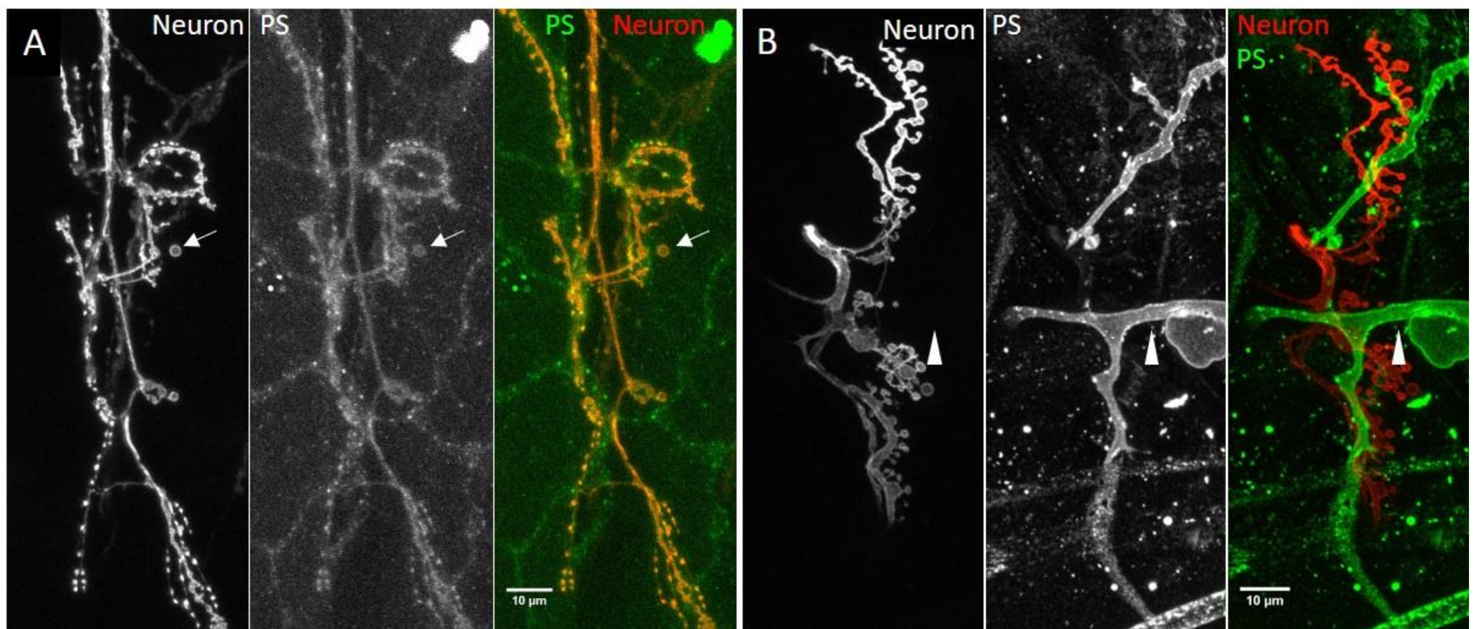


Figure 13 – Phosphatidylserine may be exposed in synaptic boutons. PS staining with Annexin V – Cy3 shows two contrasting scenarios. A – PS labelling is present in most synaptic boutons, including detached boutons (arrow) and lightly in trachea. B – PS staining is absent from the axonal terminal but is highly enriched in tracheal cells. Unstimulated preparation. Genotype: *NSyb-Gal4, UAS-CD4-GFP)*.

5.5. Perineurial glia

Perineurial glia in the PNS usually do not contact neurons, the exceptions being near the dendritic trees of sensory neurons and in their extension into the NMJ. No function has yet been described for PG at the NMJ. To analyse PG morphology and dynamics, we labelled them using either a protein trap, Schnurri-GFP (Shn-GFP) or a Gal4 that labels all perineurial glia[160] (NP6293-Gal4, hereafter referred to as PG-Gal4). In both fixed and live NMJs, PG covered the terminal arborization to different extents (Fig. 14A) and sometimes contacted synaptic boutons via round structures like SPG gliobulbs but usually smaller (not shown, morphology as in figs. 14 A-C arrows). Muscle-bound protrusions were also observed and interestingly, PG often contacted tracheal cells, sometimes at the sites of intersection between neuron and trachea, where PG both intermingled and wrapped the other cells (Fig. 15 live A). This raises the possibility that PG are coupling tracheal vessels with the synaptic terminal to promote adequate oxygen supply to the terminal. Three-dimensional reconstruction of PG and the tracheal lumen highlighted the intimate contacts established between lamellipodium-like PG membranes and trachea (Fig. 14 C), and this coupling was further supported by analysis of the surface area of the contacts (Fig. 14 D).

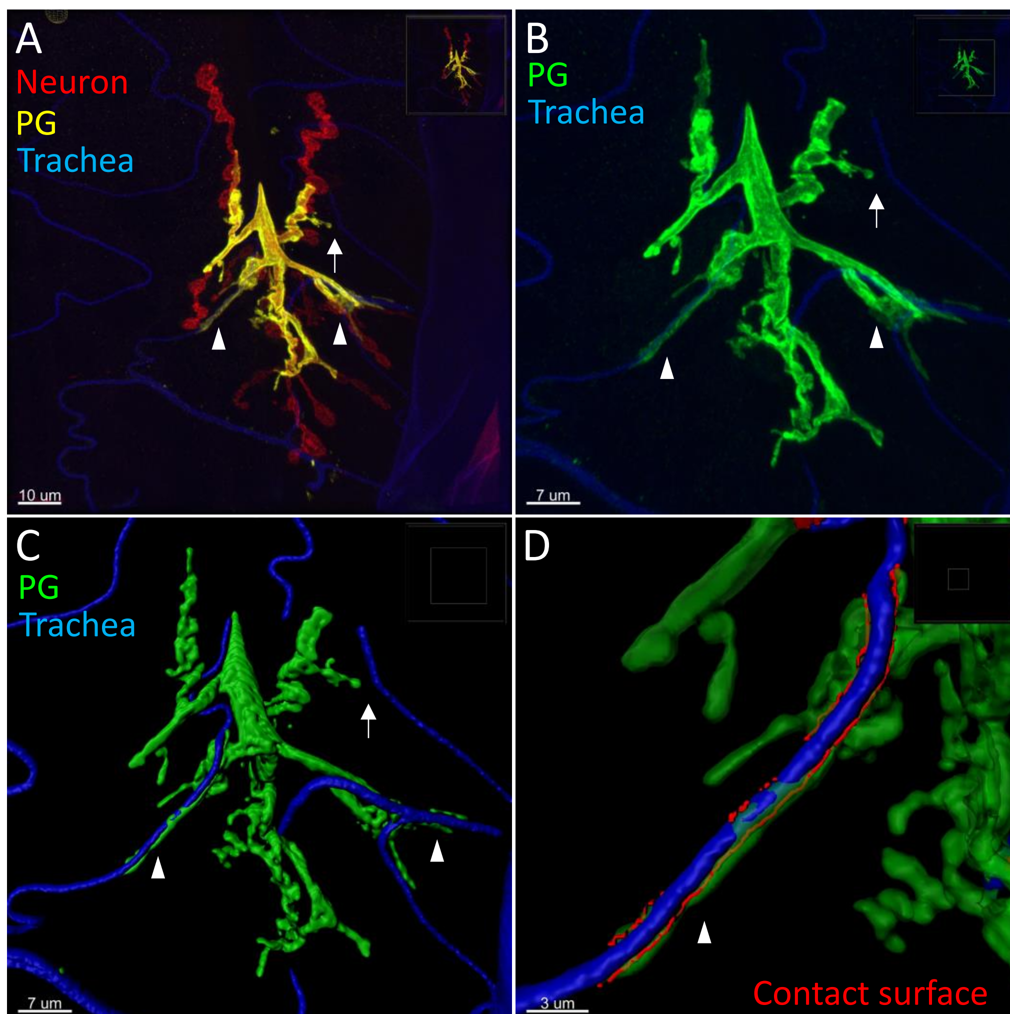


Figure 14 – Perineurial glia interact with neurons and trachea. A and B – Confocal microscopy images showing perineurial membranes cover the main axonal branch and the initial segments of secondary branches, contacting synaptic boutons and extending processes, with both lamellipodial (arrowheads) and bulbous (arrows) morphologies, towards trachea. C – 3D reconstruction of the perineurial cell and the tracheal lumen shows these structures and intimately connected. D – The contact surface shared by PG and the tracheal lumen. Unstimulated preparation. Genotype: *PG-Gal4/+; UAS-CD4-GFP/+*.

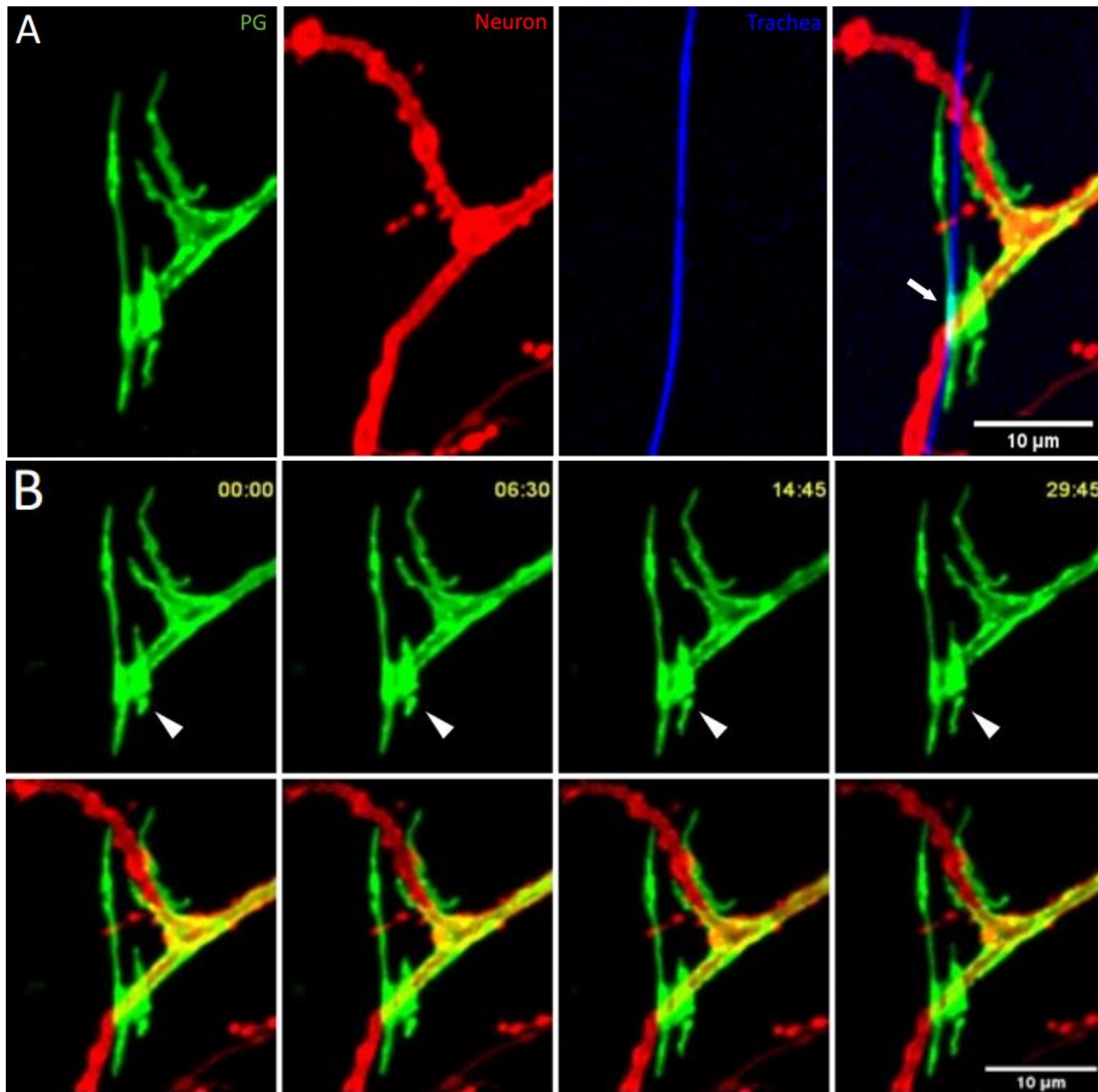


Figure 15 - Perineurial glia contact trachea and are dynamic. A – Perineurial glia, in green, partially covers the arbor, branching out and contacting nearby tracheoles that supply oxygen to the muscle and synaptic terminal. The arrow denotates the site of physical contact between the three cells. B – Perineurial glial processes are dynamic, slowly extending and retracting (arrowheads). Unstimulated preparation. Genotype: *PG-Gal4/+; UAS-mCD4-GFP/DVGlut-LexA, LexAOp-mCherry*. Video available as supplementary material.

Perineurial glia were also dynamic, extending and retracting small filopodial-like structures towards the muscle (arrowheads in Fig. 15B). While it is unclear what is the function carried out by these processes, one possible explanation is the probing of extracellular space for cues, be it for finding tracheal or neuronal processes to interact with or to deliver cues to the muscle or another unknown reason.

Overall, PG seem to behave differently than SPG at the NMJ, contacting the arbor, including boutons, as SPG do, but not actively participating in boutonogenesis. Instead, they appear as stabilizers of trachea-neuron interactions which are important for proper oxygenation at the NMJ.

Their dynamicity suggests they are adaptable to the environment, but whether PG can sense synaptic activity or oxygenation remains unknown. However, given the reduced number of observations, these conclusions require further analysis.

5.6. Tracheal cells

Our first observations of tracheal cells in moody-Gal4; UAS-mCD8-GFP larvae prompted us to study trachea interactions at the NMJ in more detail. We labelled tracheal cells by expressing Actin-GFP in tracheal cells only under control of Btl-Gal4 and performed confocal imaging in both fixed and live preparations. Several tracheal processes were found to lack a lumen and to extend laterally from tracheoles towards the muscle and at the tip of tracheoles where intimate trachea-bouton contacts were established (Fig. 16). This branching and extracellular probing is characteristic of the oxygen-demand dependent phase of tracheal development. Lumen-containing vessels also extended along the axon terminal branches in a non-overlapping fashion so that most boutons have a tracheal vessel in the vicinity (Fig. 16).

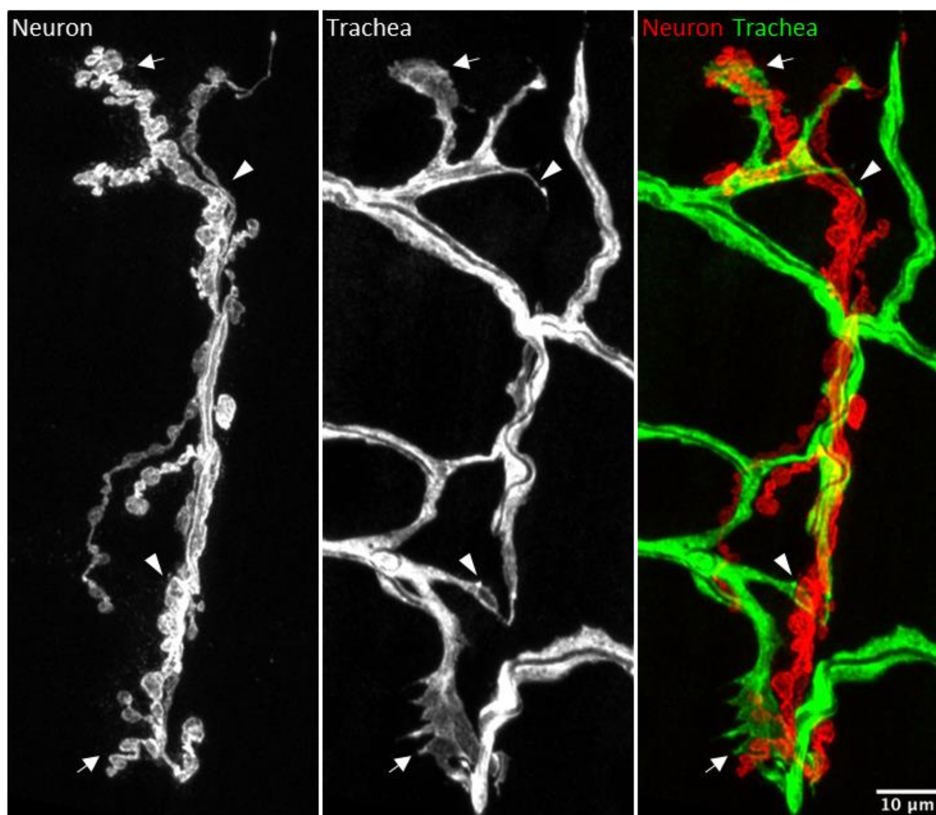


Figure 16 – Trachea physically interact with neurons. Tracheal cells extend processes towards the arbor, contacting individual boutons with lamellipodial (arrows) and thinner, filopodial-like morphologies (arrowheads). Unstimulated preparation. Genotype: *Btl-Gal4, UAS-Actin-GFP*.

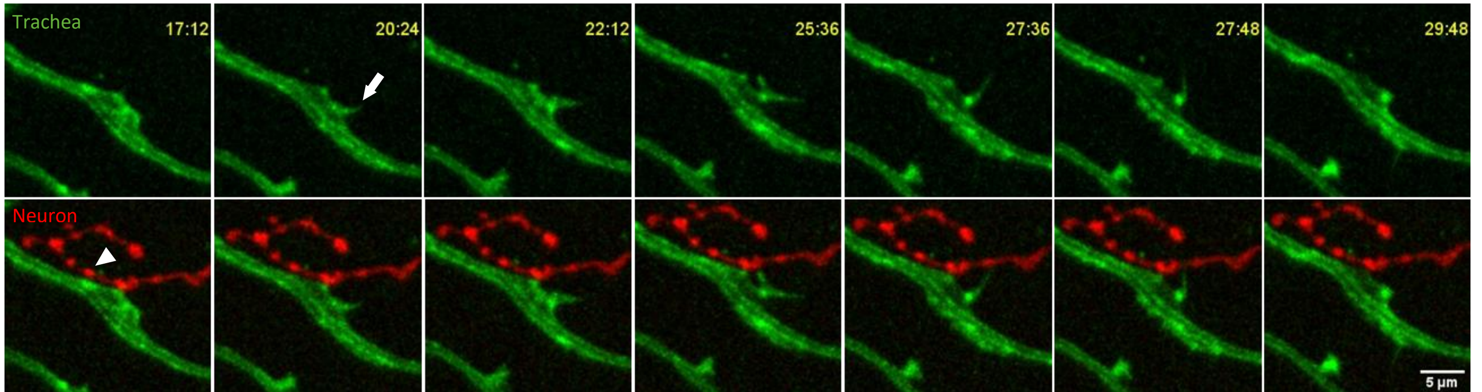


Figure 17 - Trachea probe the extracellular space and closely associate with neurons. A lumen-containing tracheole and a neuronal branch in close proximity (arrowhead). Dynamic protrusions (arrows) constantly probe the extracellular space in unstimulated conditions. Genotype: *btl-Gal4, UAS-ActinGFP/+; DVGlut-LexA, LexAOp-mCherry*. Video available as supplementary material.

Time-lapse live imaging revealed that tracheal cells at the NMJ are highly dynamic, as lumen-lacking processes constantly explored their surroundings with a variety of morphologies similar to glia, ranging from filopodia (arrowheads in Fig. 16, Fig. 17) to lamellipodia-like (arrows Fig. 16) and bulbous, bleb-like protrusions (Fig. 18). In one event after a massed stimulation protocol, a trachea-derived bulbous structure contacted a neuronal branch from which a new bouton formed. The bouton grew inside the tracheal protrusion and acquired its shape, as confirmed by single Z-planes and 3D reconstruction (Fig. 18). While it is possible that the tracheal bulb promoted and/or guided bouton formation, this was a single observation, and more are required to understand the physiological relevance of this sort of trachea-neuron interactions.

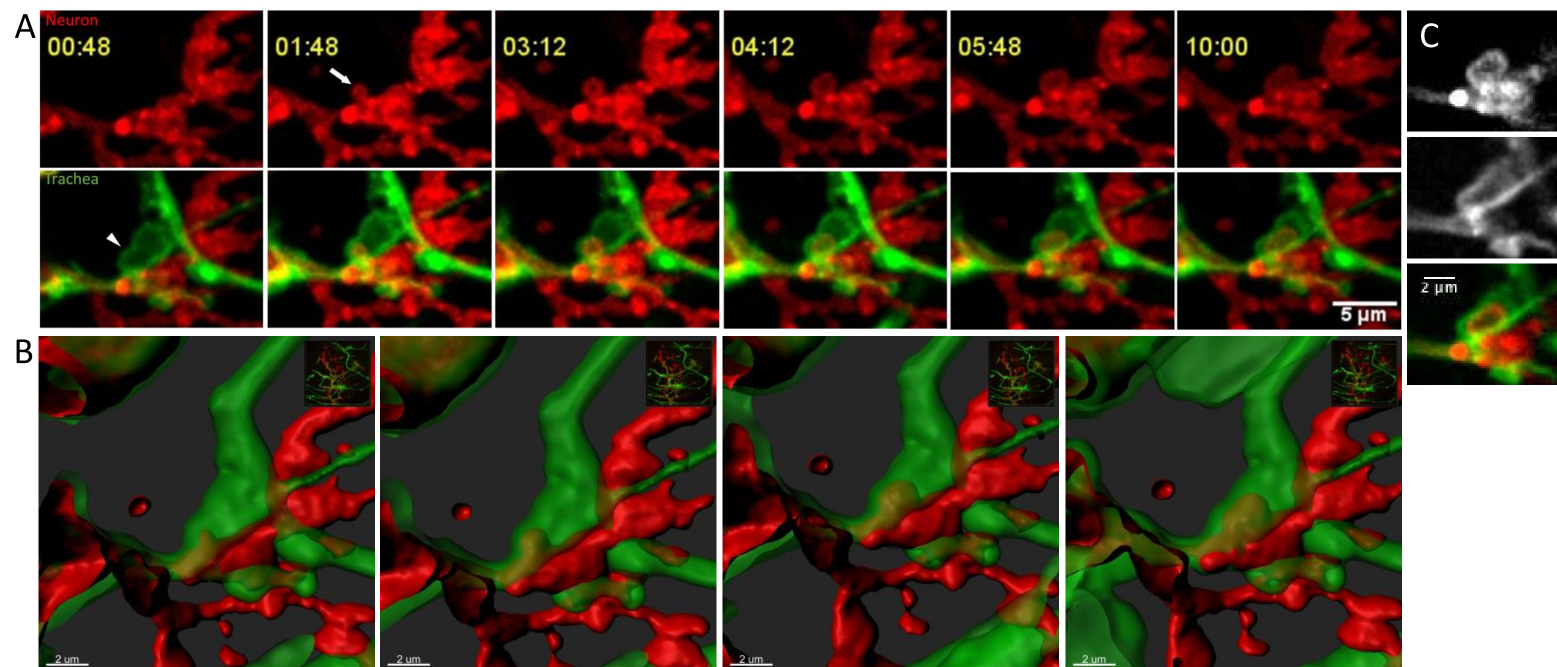


Figure 18 - Synaptic bouton forming in close association with a tracheal membrane extension. A - A tracheal bulbous membrane protrusion (arrowhead) appears to guide/restrict the growth of a new synaptic bouton (arrow) after massed stimulation. B – 3D reconstruction of the event. C – single Z-plane of the last frame of A. Genotype: *moody-Gal4/+; DVGlut-LexA, LexAOp-mCherry/UAS-CD8-GFP*. Video available as supplementary material.

We analysed actin dynamics in tracheal processes to understand their nature and functions by expressing LifeActRuby, a red fluorescent reporter of filamentous actin (F-actin) and mCD8-GFP under the control of moody-Gal4. We observed thin and bulbous-ended filopodial-like processes where actin retrograde flow occurred from the tip to the base of the protrusion throughout the imaging period (Fig. 19). Retrograde actin flow is the result of the addition of actin monomers at the cell membrane facing plus end of actin filaments, being important to push the membrane forward and in generating mechanical forces on focal adhesions together with Myosin II to drive cell migration[161,162].

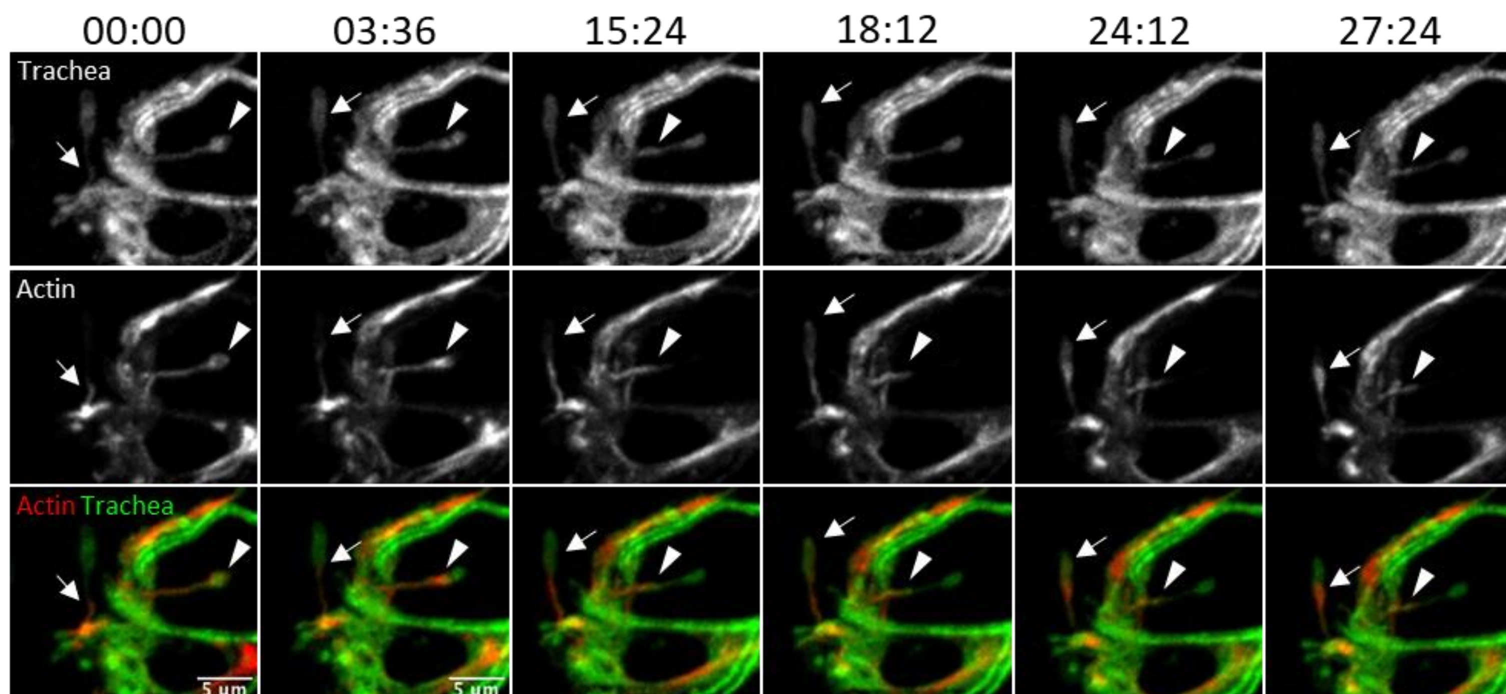


Figure 19 – Filamentous actin dynamics in tracheal cells. Two tracheal processes exhibit retrograde F-actin flow and partially retract. Arrows and arrowheads point towards the visible actin at its closest to the tip of the protrusions. Massed stimulation. Genotype: *moody-Gal4/+; UAS-CD4-GFP/UAS-LifeActRuby*. Video available as supplementary material.

5.7. Genetic ablation of glial subtypes

To dissect the functions of each glial subtype in the development and plasticity at the NMJ, we selectively killed each subtype by expression of the Ricin A chain driven by specific Gal4 drivers. To eliminate any leaky expression of the UAS-Ricin construct and to maintain the stock healthy, a tubulin-Gal80^{ts} construct was also present, also allowing temporal control of Ricin expression by shifting flies, embryos or larvae to 29ºC, which leads to Gal80^{ts} degradation. We shifted embryos from 18ºC to 29ºC soon after egg-laying in order to kill developing glial cells and prevent their growth and extension to the NMJ. We then analysed NMJ morphology and quantified ghost bouton formation and debris shedding in unstimulated and stimulated 3rd instar larvae (Fig. 20). The overall morphology of the NMJ was grossly normal in all genotypes, although we observed satellite boutons only in SPG-ablated NMJs.

Whereas in control $w^{1118};\frac{TubGal80^{ts}}{+};\frac{UAS{-}Ricin}{+}$ larvae and when WG were ablated (Nrv2-Gal4>UAS-Ricin) the spaced stimulation protocol (Short stimulation, SStim) successfully elicited bouton formation, with a mean of ghost boutons per NMJ of 4.63 ± 4.81 (SD) in control and of 5.89 ± 4.96 in WG-lacking larvae, in larvae were SPG were ablated (Mdr65-Gal4>UAS-Ricin) the stimulation was not able to induce the same level of boutonogenesis, averaging 2.79 ± 2.78 new boutons per NMJ (Fig. 21). This result supported both previous and our observation that SPG promote or facilitate bouton formation.

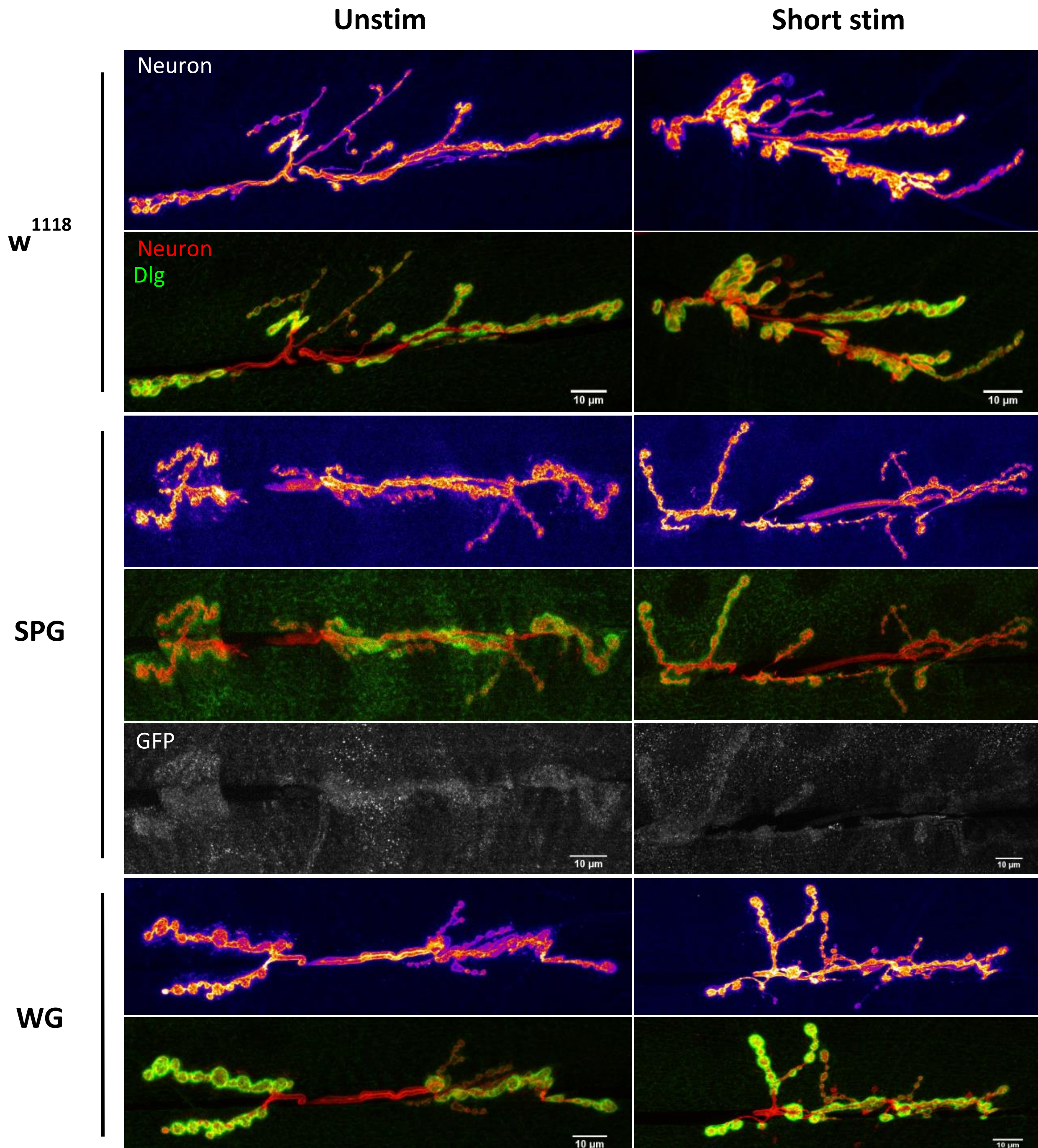


Figure 20 – NMJ morphology upon ablation of WG or SPG. Neurons stained with anti-HRP. Dlg stained with anti-Dlg (4F3). Anti-GFP staining confirmed the absence of SPG at the NMJ. Genotypes: w1118 - *w1118; TubGal80ts/+; UAS-Ricin/+*. SPG – *w; TubGal80ts/UAS-CD4-GFP; UAS-Ricin/Mdr65-Gal4*. WG – *w; TubGal80ts/Nrv2-Gal4; UAS-Ricin/+*.

Regarding debris accumulation, surprisingly, ablating WG, but not SPG, led to an increase in HRP⁺ debris in the muscle area surrounding the arbor when compared to control larvae in both unstimulated and stimulated conditions. Also unexpectedly, stimulation did not lead to the accumulation of debris in none of the genotypes tested (Figs. 21 and 22, WG: mean unstim 2.2 ± 0.75 mean Sstim 2.2 ± 0.74; SPG: mean unstim 1.6 ± 0.56, mean Sstim 1.5 ± 0.59; $w^{1118}; \frac{TubGal80^{ts}}{+}; \frac{UAS-Ricin}{+}$: mean unstim 1.46 ± 0.95, mean Sstim 1.59 ± 0.8).

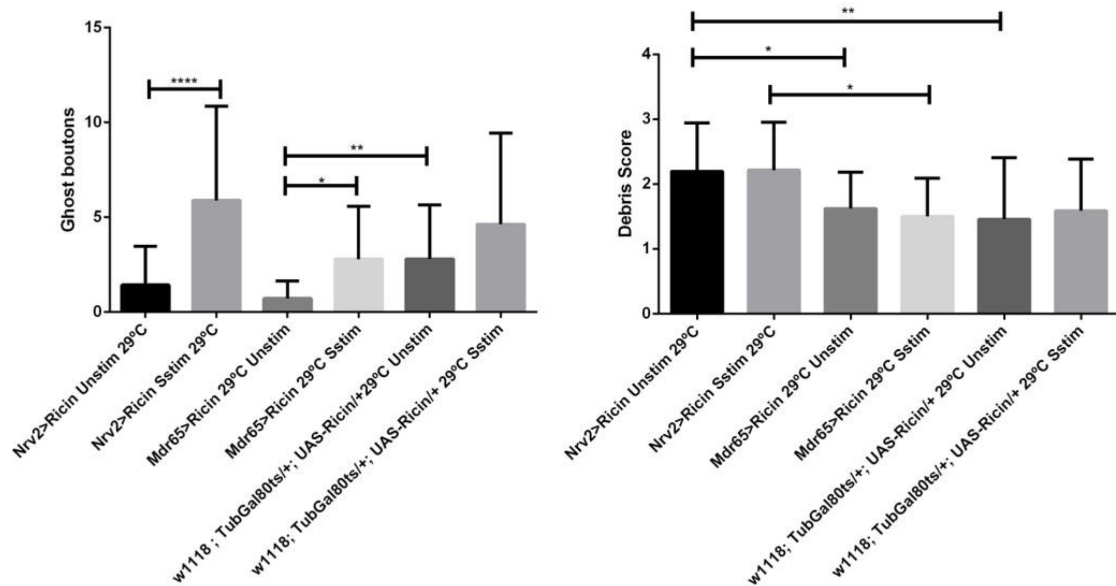


Figure 21 – Quantification of neuronal debris levels and ghost bouton numbers in glia-ablated NMJs. Only A2 to A4 segments analysed. The statistical tests used can be found in the Materials and Methods section. * - P value ≤ 0.05; ** - P ≤ 0.01; **** - P ≤ 0.0001. Full genotypes are detailed in Fig. 22. Debris n = 41, 28, 30, 24, 35, 26 NMJs for each genotype, respectively. Ghosts n = 39, 28, 29, 24, 35, 24 NMJs, respectively.

We also attempted to ablate PG and tracheal cells, but the ablation was incomplete, and thus plasticity and debris accumulation were not quantified, as both cell types were present at the NMJ despite showing some morphological defects (Figs. 22 and 23).

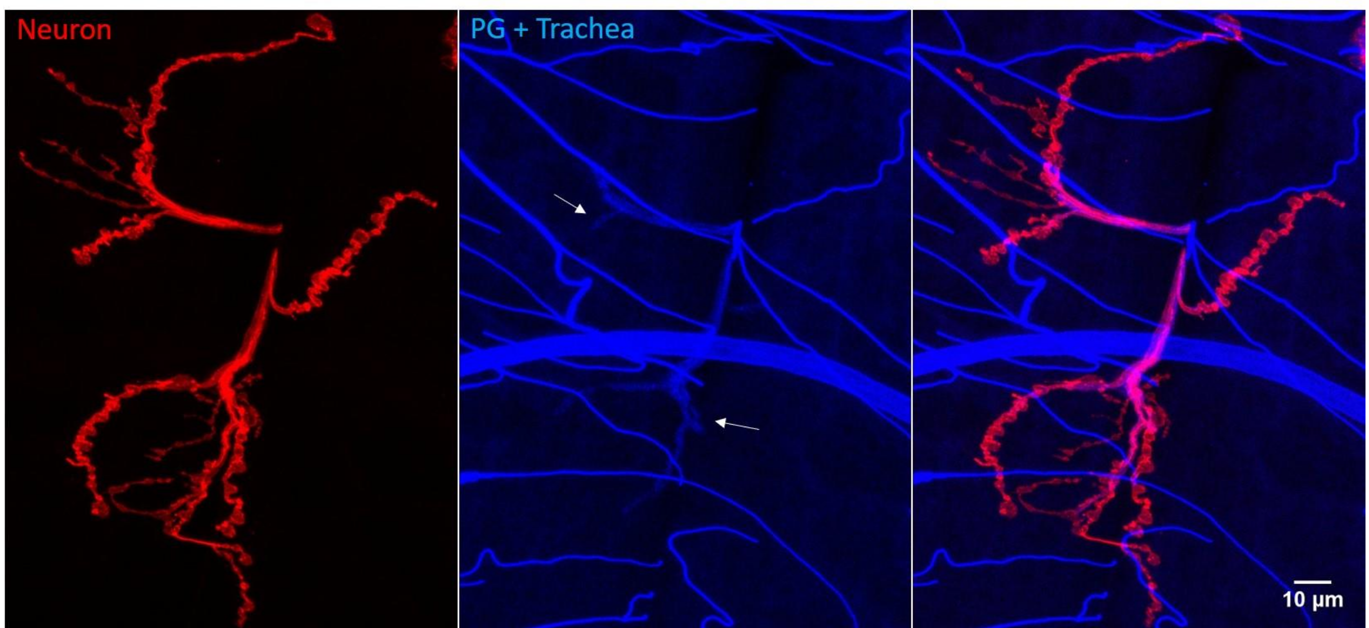


Figure 22 – Perineurial glia ablation failed. Expression of Ricin under control of PG-Gal4 did not ablate PG, as noticeable by the presence of BFP⁺ glial processes at the NMJ, indicating that PG did not fail to develop. Genotype: *TubGal80^ts/PG-Gal4; UAS-Ricin/UAS-tagBFP*.

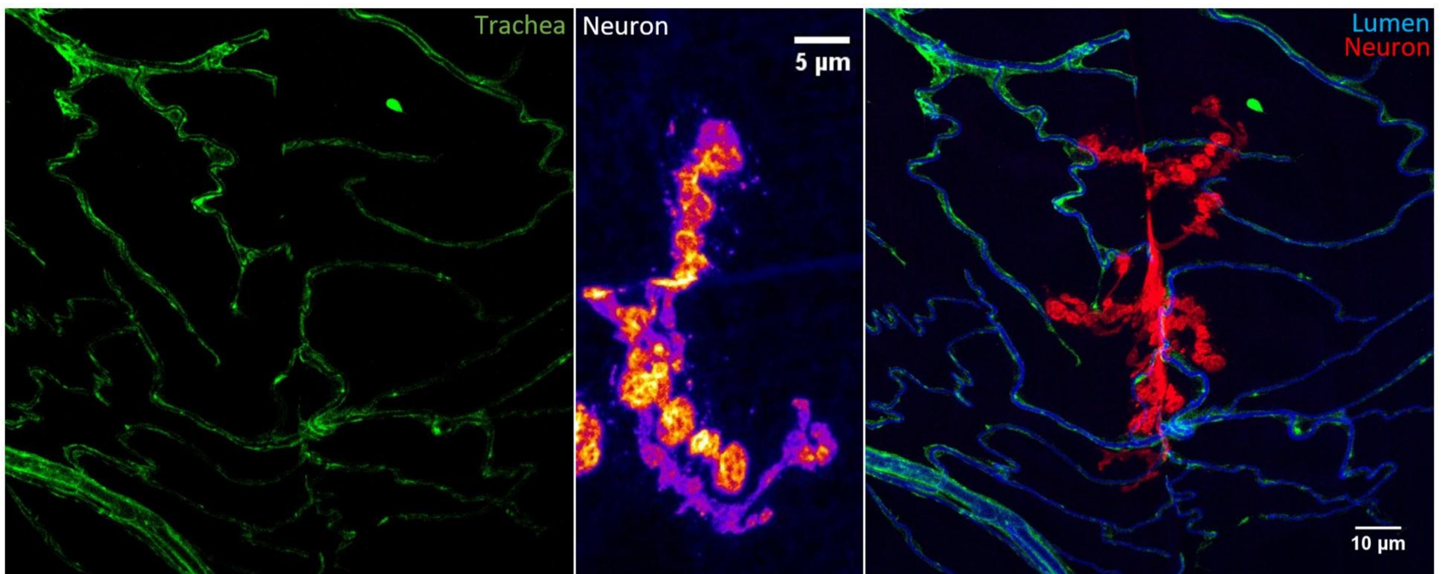


Figure 23 – Tracheal cell ablation failed. Expression of Ricin under control of Btl-Gal4 did not ablate trachea, as noticeable by the presence of thin GFP+ tracheal membranes surrounding the lumen, indicating that trachea did not completely fail to develop. Note the presence of neuronal debris even in the absence of tracheal ablation. Scale bar in rightmost panel also applies to the leftmost one. Genotype: *TubGal80^{ts}/Btl-Gal4, UAS-Actin-GFP; UAS-Ricin/+*.

6. Discussion and conclusions

6.1. Glial morphology at the NMJ

Our analysis of glial morphology revealed a wide range of cell-cell physical interactions at the *Drosophila* NMJ. As previously reported, the two outermost layers of peripheral glia, subperineurial and perineurial glia, extend along axons as their terminal arborizations become embedded in the muscle, whereas wrapping glia usually stop extending at this point. Flat glial processes covered the initial segment of the arbor and some of the primary branches, and from these other thinner and more diverse membrane protrusions emerge, some resembling filopodia and others having a round, bulbous ending with varying size. We found that, besides contacting the main axonal branches and synaptic boutons, glia at the NMJ also contact the muscle cell and the tracheoles that oxygenate the tissue.

6.2. Subperineurial glia

We examined the contribution of each glial subtype to these interactions, which revealed different 'preferences' in the contacts that SPG and PG make. Subperineurial processes often had rounded endings that were apposed to both mature and immature 'ghost' synaptic boutons. Time-lapse live imaging revealed that SPG processes contact mature boutons during bouton formation events in two different ways – either the SPG membrane contacted the mature bouton and did not change in shape during the event, or the SPG protrusion wrapped around the bouton and 'squeezed' it, increasing confinement and promoting bouton budding in a slow, sustained way. As there were no visible physical rearrangements of glial membranes in the first event, we propose that local signalling may be promoting bouton formation.

Secreted factors such as the TGF-β Maverick and the Wnt-1 Wingless are good candidates to perform this function. Mav and Wg are secreted from SPG at the NMJ, where Mav promotes Gbb-dependent muscle-neuron signalling, facilitating bouton growth through Trio activation of Rac-1, which leads to actin cytoskeleton remodelling. Wg, on the other hand, acts directly and locally on boutons, also promoting boutonogenesis and stabilization via microtubule rearrangements[144]. Additionally, local Mav signalling was found to mediate dendrite growth and space-filling in *Drosophila* sensory neurons by acting on neuronal Ret receptors and modulating adhesion to the ECM[163]. Given that *in vivo* bouton formation was characterized as using membrane blebbing to remodel (unpublished results from our laboratory), and that this process relies on both cytoskeleton remodelling and low-adhesion environments, it is plausible that local secretion of Mav and/or Wg by subperineurial membrane processes is directing bouton formation. Time-lapse live imaging of endogenous Mav and Wg by using GFP protein traps, available for both, will certainly uncover whether this is the case.

The other observed mode of glial participation in bouton budding is through contraction, increasing the confinement of the bouton and 'forcing' the budding of a new bouton. Besides the cytoskeleton and adhesion, high hydrostatic intracellular pressure and confinement are the two other main factors favouring bleb formation supporting our hypothesis that SPG contraction can drive bouton formation. Actomyosin contraction is required for blebbing, as it increases the internal pressure. This can also be achieved by pressing cells with an agar overlay, which causes blebbing indicating that external forces play a role in bleb formation[164]. Imaging of non-muscle myosin II dynamics at the NMJ revealed that contractility of non-neuronal sources of myosin also correlates temporally with neuronal blebbing events. We reasoned glia are likely the source of this NMII due to our previous observations of glial contraction, even though the NMII staining allows clear visualization of nearby tracheal cells whose involvement in the event is unclear and

that could be the source of the NMII-rich, lumen-lacking processes observed. This further demonstrates that contraction of cells other than the muscle can promote bouton formation at the NMJ, possibly providing a mechanical role for glia in boutonogenesis at the NMJ.

Interestingly, the observed blebbing event during SPG contraction was slow and sustained, happened in the absence of muscle contraction and without stimulation. On the other hand, the event where NMII is labelled is faster and accompanied by muscle contraction that likely results from the previous stimulation, suggesting that coordinated contraction of glia and muscle cells may favour the occurrence of 'explosive' events. Speculatively, glial contraction may be a mechanism to maintain basal levels of boutonogenesis during development even in periods of diminished activity, for example in more posterior abdominal segments during feeding, which involves reduced locomotion when plenty of food is available.

Additionally, one can imagine that this type of squeezing mechanism where glia contraction around axons can help structural rearrangements could potentially be useful for CNS bouton addition, of which very little is known. We cannot exclude that local signalling is also occurring during 'squeezing' events, which would further facilitate bouton formation, proving the bouton all the factors necessary for blebbing. The idea of glia contractility being capable to participate in the process of axonal bouton formation in the CNS is certainly worthy of testing.

Ablation of SPG led to reduced bouton formation after stimulation and to an increase in satellite bouton number, which is consistent with live imaging observations of SPG functions. The appearance of satellite boutons has been described as a compensatory mechanism to inefficient synaptic transmission, but these structures are also often observed in suboptimal conditions such as hypoxia[153]. This can indicate that SPG do not act upon all boutons, since type Ib boutons are the usually contacted by SPG, and thus the formation of small boutons would maintain proper muscle activation when the number of type Ib boutons is reduced.

Our observation of wild-type levels of debris in the absence of SPG seemingly contradict previous reports of SPG clearance of debris via Draper. Unexpectedly also, our analysis did not reveal any change in debris accumulation after stimulation protocols. It should be noted, however, that the debris quantification method used here and in most studies is based on a subjective scale and not on intensity or number of particles quantification. Since the fluorescence intensity of debris labelled with αHRP-Cy3 or mCherry is low, it is also possible that our settings did not allow for appropriate detection. Thus, more precise ways to measure neuronal debris will be important to understand how debris is cleared at the NMJ.

In our preparations, the engulfment receptor Draper was post-synaptically localized and surrounded all synaptic boutons in all NMJs observed. No accumulation of Draper puncta was visible in other regions of the NMJ in a way that reminisced glial processes, which was expected due to its role in SPG-mediated debris clearance. While it is possible that Draper only localizes to synapse-contacting regions of glial processes, co-labelling of SPG and Draper is required to properly assess this.

Analysis of phosphatidylserine exposure revealed that PS is present at the NMJ, although when the labelling was coincident with neuronal membranes it was so in all boutons, precluding the determination of whether PS is the 'eat me' signal displayed by neuronal membranes primed for elimination. Identification of such factor would allow the study of how maturation *versus* elimination of newly formed boutons is determined and how elimination of existing boutons occurs.

6.3. Perineurial glia

Perineurial glia also cover the initial segments of the terminal axonal arbor and some of the primary branches, then extending membranes with morphologies such as flat, lamellipodia-like, and thin, filopodia-like, sometimes with small rounded endings. Filopodial protrusions reached into boutons and non-innervated muscle areas and are dynamic, extending and retracting slowly. The lamellipodia often contacted the tracheoles that crossed the innervated area of the muscle, wrapping around tracheal cells. At sites of close neuron-trachea proximity, PG simultaneously interacted with both cells, invading the space in between and sometimes wrapping them together. Coupling between trachea and neurons was already suggested as a role of perisynaptic glia in adult NMJs[165,166], and here we report it as a novel interaction of PG, possibly revealing an unreported function. However, not all neuron-trachea interactions were mediated by glia, indicating that factors other than glial coupling, such as neuron-trachea adhesion molecules and muscle-derived cues, contribute to the coordination between neurons and tracheal cells.

Glia-trachea/endothelium interactions mediate gaseous exchanges at the neuron-vascular level and the identification of PG as mediators of trachea-neuron interactions opens the way to investigate how PG and trachea achieve this coordination and whether they play a role in each other's development as it happens with endothelial, astrocyte and oligodendrocyte intercellular communication during mammalian brain development.

6.4. Tracheal cells

The NMJ is oxygenated by tracheal vessels that grow in an evenly-spaced pattern near neuronal branches to assure all boutons are supplied with oxygen. Although the signalling pathways involved in tracheal development are well-known – tracheal cells expressing Btl and all target tissues expressing Bnl, including muscle cells[167,168] – it is not clear whether neurons or glia at the NMJ express Bnl and whether NMJ vascularization is solely dependent on muscle-derived Bnl. We observed that tracheal cells closely contact neurons, including synaptic boutons at the larval NMJ with both filopodial and rounded protrusions. These membrane extensions were highly dynamic and constantly probed the extracellular space, which we interpreted as a search for oxygen demand-dependent guidance cues. It will certainly be of interest to explore whether neuronal activity, which leads to increased oxygen consumption, can act on trachea, either directly or indirectly via PG or the muscle, to regulate branching towards hypoxic areas.

On the other hand, we found evidence that trachea participate in bouton formation. We observed the sprouting of a new bouton in a trachea-covered neuronal branch, and the bouton grew enclosed by the tracheal membrane, acquiring its shape. It makes sense that boutons grow towards trachea due to oxygen availability, and while it is reported that tracheal ablation and hypoxia affect bouton number and morphology[153], how bouton formation is regulated by oxygenation status and tracheal cells has not been explored yet. The observed bouton formation event occurred after stimulation and in the presence of muscle contraction, whereas no change in the tracheal process shape was visible. This could mean that local signalling is taking place, and it is tempting to speculate that neuronal activity induces local hypoxia, which leads to recruitment of tracheal membranes that create a favourable environment for bouton formation. However, it cannot be excluded that the boutons formed in the observed events did not mature and become functional and are a form of pruning of neuronal membranes.

6.5. Genetic ablation of glial subtypes

Genetic cell ablation is useful when dissecting the role of different cell types in the same environment. Both SPG and WG ablation led to surprising results despite the limitations of the

approach used to quantify debris. Whereas SPG ablation did not increase debris accumulation, WG ablation did so regardless of the stimulation status. This effect of WG ablation can be interpreted in many ways, such as defective axonal insulation that would alter the pattern of neuronal activity and thus neuronal membrane dynamics at the NMJ, or alternatively, a disruption of other glial subtypes development would leave only the muscle to clear all the synaptic debris. Besides expressing toxic or cell death proteins, mutants for genes essential for cell fate determination, such as *trachealess*, can prevent the development of a certain cell type, in this case trachea. We aim to, in the future, dissect the role of each glial subtype for NMJ development, maintenance and plasticity by ablating each cell type at different points in development and labelling the other cell types at the NMJ to understand how these cells coordinate their growth.

6.6. Conclusions

Overall, we identified a variety of intercellular interactions at the *Drosophila* neuromuscular junction, with glia as a central player in mediating this interplay. We distinguished between the active role of subperineurial glia in pre-synaptic bouton formation and the structural support and gas-exchange function of perineurial glia as anchors for trachea at the NMJ. Our findings on tracheal dynamicity and interactions with growing boutons suggest new tracheal functions other than oxygen delivery.

Finally, this work highlights the importance of non-neuronal cell types in neuronal development and function, which are emerging as potential targets for therapies to treat nervous system disorders.

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