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Mechanical principles driving microglia activation

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Mechanical Principles Driving Microglia Activation

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Abstract

Microglia are the resident innate immune cells of the central nervous system. Their ramified morphology and dynamics of their membrane protrusions are essential for their functions in development, homeostasis and disease. Microglia rely on a constant extension and retraction of protrusions to survey the surrounding microenvironment and, upon sensing damage, mediate a return to homeostasis through redirection of cellular protrusions and the release of soluble mediators and phagocytosis. To enact the shape changes required for their function, microglia (like other cells) rely on actomyosin cytoskeleton dynamics. Although recent studies have shown that actin dynamics and cytoskeleton regulators are important in the control of microglia shape and function, the underlying regulatory mechanisms remain largely unknown. Notably, and given that morphological changes are classically linked to variations in the type II myosin-mediated distribution of mechanical forces at the cortical actomyosin cytoskeleton, the control and, it is expected that these molecular motors and their regulators play an important role in microglia biology. In this study, we evaluated if regulators and effectors of actin cytoskeleton tension are important for controlling microglia shape and if they also affect the function of these cells.

Using the retina as a model, we developed an experimental system that allows an in-depth study of microglia morphology in situ, together with the implementation of a semi-automated image analysis workflow (*MorphoMacro*). Such a system allowed for the quantification of several morphological parameters from microglia in steady state conditions and in response to chemical perturbation (ATP stimulation, which elicits extension of microglia protrusions). We then applied such a system to study the effect of conditional genetic ablation of the classical type II myosin and actin dynamics regulator RhoA and observed that although this small GTPase is not important for the generation of new protrusions, it is important for their extension. To understand the possible mechanisms that are operating downstream of RhoA, we used an in vitro CRISPR-Cas9 model of RhoA-ablated microglia to analyse the actomyosin cytoskeleton and its regulators in these cells. Such study revealed that the impaired extension of microglia protrusions is likely due to decreased actin polymerization, itself owing to low cofilin activity, uncovering a mechanism for microglia protrusion extension. It also revealed that RhoA ablation specifically decreased the expression of the type II myosin isoform Myh10, while not affecting Myh9, prompting us to evaluate the possible differential contribution of each isoform in microglia. We observed that the in vitro lentiviral mediated silencing of these two proteins resulted in altered microglia morphology only in the case of Myh9 ablation and that the increased number of cellular protrusions is

linked to a decrease in cortical tension (measured by a stress-sensing FRET probe), which allows efficient actin polymerization to drive the formation of the protrusion. Pharmacological inhibition of type II myosin motors in retinal explants also resulted in an increase of microglia morphological complexity in vivo, uncovering a mechanism for the control of protrusion formation in these cells. Furthermore, upon evaluation of the effect of type II myosin isoform ablation in the cell's function, we observed that Myh10 ablated cells presented an inflammatory phenotype with increased reactive oxygen species and pro-inflammatory cytokine production. Attempts to dissect the possible mechanism revealed the involvement of the classical NF- κ B and Src pathways and suggested the possible contribution of the mechanosensory-associated YAP pathway in the inflammatory promoting effect of Myh10 ablation in microglia.

In sum, our results revealed that i) Myh9 controls cortical tension levels and affects microglia protrusion formation, ii) cofilin-mediated maintenance of actin turnover regulates microglia protrusion extension, iii) Myh10 influences microglia inflammatory activation. Overall, we uncover molecular pathways that regulate microglia morphology and identify type-II myosins as important regulators of microglia biology with differential roles in the control of cell shape (Myh9) and functions (Myh10).

Resumo

A microglia é a célula imune inata residente do sistema nervoso central. A sua morfologia ramificada e a dinâmica das suas protrusões membranares são essenciais para as suas funções durante o desenvolvimento, homeostasia e doença. Estas células apresentam uma constante extensão e retracção de protrusões que lhes permitem monitorizar o microambiente circundante e, em caso de detecção de dano, mediar um regresso à homeostasia através de um redireccionamento das protrusões, libertação de mediadores solúveis e fagocitose. Para efectuar as mudanças de forma necessárias para a sua função, a microglia (tal como outras células) depende da dinâmica do seu citoesqueleto de actomiosina. No entanto, e apesar de estudos recentes terem demonstrado que a dinâmica de actina e os reguladores do citoesqueleto são importantes para o controlo da forma e função da microglia, os mecanismos reguladores subjacentes permanecem amplamente desconhecidos. Notavelmente, e dado que as alterações morfológicas celulares estão classicamente associadas a variações na distribuição das forças mecânicas produzidas pela acção de miosinas do tipo II no citoesqueleto cortical, é expectável que estes motores moleculares e os seus reguladores desempenhem um papel importante na biologia da microglia. Neste estudo, avaliamos se os reguladores e agentes da tensão do citoesqueleto de actina são importantes para o controlo da forma da microglia e se afetam também a função destas células.

Utilizando a retina como modelo, desenvolvemos um sistema experimental que permite um estudo aprofundado da morfologia da microglia *in situ*, juntamente com a implementação de um sistema de análise de imagem semi-automatizado (*MorphoMacro*). Este sistema permitiu a quantificação de vários parâmetros morfológicos da microglia em condições basais e em resposta a perturbações químicas (estimulação com ATP, que provoca a extensão de protrusões na microglia). Em seguida, aplicámos este modelo para estudar o efeito da ablação genética condicional de RhoA, um regulador clássico da acção de miosinas do tipo II e também da dinâmica de actina, e observámos que, apesar desta GTPase não ser importante para a formação de novas protrusões, ela é importante para a sua extensão. De modo a entender os possíveis mecanismos que operam a jusante da RhoA, usámos de seguida um modelo *in vitro* de microglia cuja RhoA foi removida por CRISPR-Cas9, de modo a analisar o seu citoesqueleto de actomiosina e reguladores associados. Tal estudo revelou que a extensão incompleta de protrusões na microglia se deve a uma diminuição da polimerização da actina, por sua vez associada a baixos níveis de atividade de cofilina, revelando um mecanismo para a extensão de protrusões nestas

células. O estudo também revelou que a ablação de RhoA diminui especificamente a expressão da isoforma Myh10 de miosina do tipo II, sem afectar Myh9, levando-nos a avaliar a potencial contribuição diferencial de cada uma destas isoformas na microglia. Recorrendo ao silenciamento *in vitro* destas duas proteínas por lentivírus, foi possível detectar uma alteração na morfologia exclusivamente em células sem Myh9 e que o aumento observado do número de protrusões celulares está associado a uma diminuição nos níveis de tensão cortical (medidos por uma sonda FRET sensível a stress), o que por sua vez permite a polimerização eficiente de actina essencial para a formação da protrusão. A inibição farmacológica dos motores de miosina do tipo II em explantes de retina também resultou num aumento da complexidade morfológica da microglia *in vivo*, revelando um mecanismo para o controlo da formação de protrusões nestas células. De seguida, ao avaliar o efeito da ablação das isoformas de miosina do tipo II na função celular, observámos que as células silenciadas para Myh10 apresentaram exclusivamente um fenótipo inflamatório com aumento de produção de espécies reactivas de oxigénio e de citocinas pró-inflamatórias. Tentativas subsequentes de elucidação do possível mecanismo subjacente a esta alteração revelaram o envolvimento das vias clássicas de sinalização NF- κ B e Src, sugerindo também a possível contribuição da via mecanossensitiva YAP no efeito pró-inflamatório da remoção de Myh10 na microglia.

Em suma, os nossos resultados revelaram que i) a isoforma Myh9 controla os níveis de tensão cortical e afeta a formação de protrusões na microglia, ii) a manutenção da renovação de actina mediada pela cofilina regula a extensão de protrusões na microglia, iii) a isoforma Myh10 influencia a activação inflamatória da microglia. Foram assim descobertas vias moleculares que regulam a morfologia da microglia e identificadas as miosinas do tipo II como reguladoras importantes da biologia destas células, com papéis diferenciados no controle da forma celular (Myh9) e das suas funções (Myh10).

Abbreviations

AAV – Adeno-Associated Virus;

ADP – Adenosine di-Phosphate;

ANOVA – Analysis of Variance;

ATP – Adenosine tri-Phosphate;

BDNF – Brain-Derived Neurotrophic Factor;

cAMP – Cyclic Adenosine mono-Phosphate;

CD206 – Cluster of Differentiation 206;

CD47 – Cluster of Differentiation 47;

CNS – Central Nervous System;

CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats;

Csk – C-terminal Src kinase;

DAPI - 4',6-diamidino-2-phenylindole;

DMSO – Dimethylsulfoxide;

Ena/VASP - Enabled/Vasodilator-Stimulated Phosphoprotein;

FRET – Fluorescence resonance energy transfer;

GAPDH – Glyceraldehyde-3-phosphate dehydrogenase;

GAPs - GTPase-activating proteins;

GDI - Guanine dissociation inhibitors;

GEFs - Guanine exchange factors;

GFP – Green fluorescence protein;

gRNA – guide RNA;

GTP – Guanidine tri-phosphate

HDAC – Histone deacetylase;

|Abbreviations

HMC3 – Human microglia clone 3;

IL-1 β – Interleukin 1 β ;

IL-6 – Interleukin 6;

IPL – Inner plexiform layer;

IRES – Internal ribosomal entry site;

LIMK – LIM-motif containing kinase;

LUT – Lookup table;

LV – Lentivirus;

MLC2 – Myosin light chain 2;

Myh10 – Non muscle myosin heavy chain isoform 10;

Myh9 – Non muscle myosin heavy chain isoform 9;

NF- κ B – Nuclear factor κ B;

NLRP3 - Nucleotide-binding oligomerization domain 3;

NMIIA – Non-muscle myosin IIA;

NMIIB – Non-muscle myosin IIB;

NMIIC – Non-muscle myosin IIC;

ONL – Outer nuclear layer;

OPL – Outer plexiform layer;

P2RY12 – Purinergic receptor Y12;

PCR – Polymerase chain reaction;

PKC – Protein kinase C;

PRC2 – Polycomb repressive complex 2;

RNS – Reactive nitrogen species;

ROCK – Rho-associated protein kinase;

ROS – Reactive oxygen species;

SEM – Standard error of the mean;

SNT – Single neurite tracer;

TGF β 1 – Transforming growth factor β 1;

TNF α – Tumour necrosis factor α ;

WASP – Wiskott-Aldrich syndrome protein;

WAVE - WASP family verprolin homologous protein;

WT – Wild type;

YAP – Yes associated protein;

YFP – Yellow fluorescence protein;

General Introduction

Microglia

The existence of Glia has been recognized for nearly two centuries now, specifically, since 1856. It was first described by Rudolph Virchow (Virchow, 1856, as cited in (Somjen, 1988)) as the *Nervenkitt*, a sort of glue that permeates the structure of the central nervous system (CNS) and sustains neuronal cells. Further work identified the cellular components of the *Nervenkitt*, which Santiago Ramón y Cajal separated into two groups: the second element (today's astrocytes) and the third element (Ramón y Cajal, 1913, as cited in (Bereciartu, 2020)). Through the innovative staining techniques of Pío Del-Rio Hortega in 1919, the latter was revealed to be composed of two distinct cells, oligodendrocytes and microglia (Rio-Hortega, 1919, as cited in (Sierra et al., 2016)). The separate identity of today's most widely known glial cells has thus only been recognized for little more than a century now. Since then, much has been discovered about their function and role in the biology of the CNS. Astrocytes provide structural and nutritional support to neurons, play an essential part in the regulation and recycling of neurotransmitters at neuronal synapses and are an important part of the blood brain barrier (Allen & Eroglu, 2017; Lyon & Allen, 2022). On the other hand, oligodendrocytes are notably responsible for insulating neuronal projections in the form of myelin sheaths, allowing for a more efficient and rapid conduction of electrical signals between neurons (Stadelmann, Timmler, Barrantes-Freer, & Simons, 2019). Finally, microglia are the CNS resident innate immune cells. Since they are the glial cells that the present work focuses on, the next sections will summarize what is known about their origins, development in the CNS, functional and cellular characteristics.

The origins of microglia

The phagocytic nature of microglia was noticed by del-Rio Hortega as soon as he identified these cells. Microglia early presence in the neural tissues during embryonic stages suggested that they could be generated from primitive hematopoiesis. On the other hand, their functional similarity to macrophages also led to the hypothesis that they could be derived from circulating monocytes. These views persisted for decades until more recent studies unequivocally demonstrated that microglia derive exclusively from erythromyeloid progenitors that originate from the Yolk Sac at around E7.0 (Bertrand et al., 2005; F. Ginhoux et al., 2010; Kierdorf et al., 2013), and then migrate through the embryonic bloodstream to enter the developing brain at E9.5 and the developing retina at E11.5

(Florent Ginhoux, Lim, Hoeffel, Low, & Huber, 2013; Santos et al., 2007). After a postnatal proliferation stage, microglia settle as a Sal1^+ , Iba1^+ , $\text{CX}_3\text{CR1}^+$, P2yR12^+ , Tmem119^+ , C1qa^+ , Tgfbr1^+ (F. C. Bennett et al., 2018; Butovsky et al., 2014) stable compartment with no contribution from circulating monocytes under homeostatic conditions (Ajami, Bennett, Krieger, Tetzlaff, & Rossi, 2007; F. Ginhoux et al., 2010).

In the developing brain, microglia arrive at different locations at slightly different developmental stages, migrating at first tangentially along the CNS surface and radially into the parenchyma, following primitive neuronal tracts (Navascués, Calvente, Marín-Teva, & Cuadros, 2000; Rigato, Buckinx, Le-Corronc, Rigo, & Legendre, 2011; Swinnen et al., 2012). After entry into the developing regions, they progressively colonize the parenchyma. Similarly, in the retina, microglia first migrate along the vitreal surface and, over time, radially towards the photoreceptor layer, at the back of the retina (Santos et al., 2007). Once development is complete, this region will contain cone and rod cells that sense photons and transduce them into neuronal impulses (Demb & Singer, 2015). These will be transmitted to an intermediate layer of neurons, the bipolar and amacrine cells, which then relay them to retinal ganglion cells (Demb & Singer, 2015), the neurons below the vitreal border whose projections deliver the signals to the visual cortex through the optic nerve. During the initial stages of retinal development, microglia remain limited to the nascent Inner Plexiform Layer (IPL) (Santos et al., 2007). With the continuing development of the tissue and its intermediate neuronal layers, microglia also migrate toward the outer retina and from P9 onwards settle in both the IPL, the layer between the retinal ganglion cells and intermediate neurons, and in the Outer Plexiform Layer (OPL), the layer between the photoreceptors and intermediate neurons, attaining their final distribution in the retinal tissue (F. Li, Jiang, & Samuel, 2019; Santos et al., 2007).

Once established as a stable population, microglia numbers are maintained throughout adulthood by local proliferation and apoptosis (Askew et al., 2017), leading to a relatively rapid turnover of these cells during an organism's lifetime (Réu et al., 2017), which has also been shown to increase in a model of neurodegeneration (Füger et al., 2017).

The functions of microglia within the CNS

For many years, the status of microglia as resident innate immune cells of the CNS was seen as an indication that their main role was to respond to and control tissue infection. Many studies have since confirmed the essential role of microglia in the context of viral,

bacterial, fungal or parasitic infections (Rock et al., 2004). Their inflammatory response not only contributes to the active elimination of microorganisms by phagocytosis and production of reactive oxygen and nitrogen species (ROS/RNS), as in the case of *Escherichia coli*, *Streptococcus pneumoniae* (Ribes et al., 2010; Ribes et al., 2013) and West Nile Virus infection (Quick, Leser, Clarke, & Tyler, 2014), but is also essential in the recruitment and subsequent activation of peripheral T cells by chemokine secretion, for example in the control of *Toxoplasma gondii* (Harris et al., 2012; Norose, Kikumura, Luster, Hunter, & Harris, 2011) or murine coronavirus infection (Mangale et al., 2020).

However, a series of studies performed over the last 15 years showed that the contribution of microglia to CNS biology goes far beyond responding to infection. As mentioned previously, microglia enter the developing CNS tissues early in development (E9.5), with neurogenesis starting at E10 (Chen et al., 2017; Takahashi, Nowakowski, & Caviness, 1996). This early presence makes microglia uniquely poised to actively participate in the development of the CNS. Indeed, microglia were shown to not only control the number of neuronal progenitors by apoptosis and phagocytosis in the subventricular zone (Cunningham, Martínez-Cerdeño, & Noctor, 2013; Shigemoto-Mogami, Hoshikawa, Goldman, Sekino, & Sato, 2014) but also to support survival of neurons by secretion of neurotrophic factors (Ueno et al., 2013). They play an active role in the development of the neurovasculature (Fantin et al., 2010) and influence the positioning of neurons in the neocortex during development (Squarzone et al., 2014). Microglia also have a profound impact in the regulation of neuronal synapses. They contact synapses in an activity-dependent manner, constantly monitoring their activity (Tremblay, Lowery, & Majewska, 2010; Wake, Moorhouse, Jinno, Kohsaka, & Nabekura, 2009), and can selectively eliminate them (Schafer et al., 2012). This process of synaptic pruning is essential for the normal regulation of neuronal activity and development (Paolicelli et al., 2011), and is in part regulated by a balance between complement signalling (Schafer et al., 2012; Stevens et al., 2007), that provides an “eat me” signal, and CD47-mediated “don’t eat me” signals (Lehrman et al., 2018). This remodelling by microglia occurs both by removal of presynaptic elements and induction of post-synaptic structures (Weinhard et al., 2018). Microglia can also promote the formation of new synapses during CNS development through either direct contact with neurons or the secretion of soluble mediators such as BDNF (Miyamoto et al., 2016; Parkhurst et al., 2013; Reshef et al., 2017).

Interestingly, further studies show that the role of microglia in neuronal and synaptic regulation is not restricted to the CNS developmental window, with control of precursor cell numbers (Sierra et al., 2010), synapse turnover (Basilico et al., 2022; Cangalaya, Stoyanov, Fischer, & Dityatev, 2020; Parkhurst et al., 2013; Socodato et al., 2021), fine tuning and

circuit maintenance (Liu et al., 2020; C. Wang et al., 2020) continuing to occur in adulthood. Although much of the work on these neuroglial interactions has focused mostly on the brain, similar roles for microglia in the development of retinal vasculature (Dudiki et al., 2020) and interactions of the resident microglia with retinal neurons have also been described, regulating Retinal Ganglion Cell numbers (S. R. Anderson et al., 2019) and removing apoptotic cells (Blume, Lambert, Lovel, & Mitchell, 2020) during development, promoting the maturation of photoreceptors (Jobling et al., 2018) or affecting neuronal synapses in the ONL (X. Wang et al., 2016).

Microglia also play an important role in the response to tissue damage. They are not only essential to clear debris to allow subsequent regeneration, as demonstrated in optic nerve crush models (Norris et al., 2018), but also to mobilize and regulate immune infiltrates that regularly follow and promote regeneration after injury, as for example in the case of retinal detachment (Okunuki et al., 2018).

Finally, considering the remarkable interaction between microglia and neurons, and given microglia's capacity for triggering or contributing to inflammation, a role for microglia in neurodegenerative and autoimmune pathologies of the CNS has also been more and more recognized (Hickman, Izzy, Sen, Morsett, & Khoury, 2018; Madore, Yin, Leibowitz, & Butovsky, 2020; Song & Colonna, 2018), with microglia assuming a potentially causative role in the pathophysiological process of Alzheimer's, Parkinson's or Amyotrophic Lateral Sclerosis animal models (Bido et al., 2021; Joshi et al., 2019; Socodato et al., 2020). The involvement of microglia in these degenerative pathologies is once again not limited to the brain, since they have also been shown to contribute to retinal neurodegeneration for example in retinitis pigmentosa (Blank et al., 2018; L. Zhao et al., 2015), glaucoma (Ramírez et al., 2020; Tribble, Harder, Williams, & John, 2020), age-related macular degeneration (Ma et al., 2019), diabetic retinopathy (Mills et al., 2021) and in the context of autoimmunity (Okunuki et al., 2019).

Despite the wide range of interactions and tasks in which microglia are involved, their actions rely on a common set of cellular functions: phagocytosis and secretion of mediators (Hanisch & Kettenmann, 2007). The shift from tissue caretakers to active players in pathophysiological processes is currently believed to be dependent on a balance of environmental signals derived from neighbouring cells that act as microglia checkpoints which, in case of deregulation or loss, trigger an unbalanced response from microglia, with excessive production of pro-inflammatory mediators (such as IL-1 β , IL-6, TNF α and ROS), and their subsequent deleterious effects in the surrounding cells and tissue (Deczkowska, Amit, & Schwartz, 2018).

As such, microglia not only play a role in the development of the CNS, but also in the maintenance of its homeostasis, response to injury, tissue regeneration and pathophysiology, assuming a central role in CNS biology that goes far beyond what was initially expected for a simple immune resident cell.

Microglia Morphology and Dynamics

The extended spectrum of functions that microglia play in the CNS mentioned previously require them to properly sense their surrounding environment, effectively probe the tissue and interact with other cells. Microglia achieve this through means of their remarkable morphology. Indeed, their intricate shape remains a hallmark characteristic of these cells and was appreciated from the very beginning by del-Río Hortega, who masterfully captured them in his illustrations (Sierra, Paolicelli, & Kettenmann, 2019). Microglia owe their name to their smaller size, when compared with other glial cells and neurons. However, their smaller size is compensated by their highly ramified shape. The high number of cellular protrusions increases the surface area that is in contact with the surrounding tissue. On the other hand, the long and thin processes ensure that microglia can pinpoint specific regions of interest and establish precise points of contact with other cells. Aside from this highly complex shape, microglia were also described by del-Río Hortega and others to adopt a rounded amoeboid shape during most of the CNS developmental stages and under conditions where CNS damage is present. These observations initially established the notion that these cells could transition between the two morphological extremes. This was later confirmed by studies of microglia in tissue slices, showing that, in response to tissue damage, microglia shift from a ramified shape to amoeboid by sequential retraction of their processes (Stence, Waite, & Dailey, 2001) and subsequent migration towards the site of injury (Heppner, Skutella, Hailer, Haas, & Nitsch, 1998), where they engage in phagocytosis of debris (Brockhaus, Möller, & Kettenmann, 1996). Since these changes in shape occurred in response to an insult, and microglia became engaged in tissue clearance, this originally led to the classification of ramified cells as resting microglia and amoeboid cells as active microglia. However, the advent of intravital microscopy challenged this view, since resting microglia were revealed to be extremely dynamic cells, constantly extending and retracting their processes (Nimmerjahn, Kirchhoff, & Helmchen, 2005). Using this motility capacity of protrusions, microglia maximize their ability to thoroughly scan the CNS parenchyma and maintain a tight surveillance of the surrounding environment. Further studies demonstrated that purinergic signalling, mediated by the release of ATP from injured sites and sensed by microglia through P2RY12 receptors is responsible for eliciting the extension and guidance

of microglia's protrusions towards damaged sites (Davalos et al., 2005). The morphological response of microglia to damage can thus be more local, with reorientation of processes towards injury, or more pronounced, with full retraction of processes and acquisition of an amoeboid shape and motility. Some functions of microglia only require very local changes in process dynamics or orientation, such as the ones necessary for synaptic pruning (Weinhard et al., 2018), while others, such as overt tissue clearing and mediator secretion following damage force them to change their shape more drastically (Carbonell, Murase, Horwitz, & Mandell, 2005; Du, Zhou, Zhang, & Du, 2021). This shows that these cells cycle through a variety of morphologies to appropriately respond to the needs of the tissue surrounding them. In fact, alterations in microglia morphology, specifically their degree of ramification, have long been used to evaluate their reactive state, since changes in their shape are overwhelmingly associated with active or inflammatory responses, and continue to be used today as an indicator of departure from tissue homeostasis (Kettenmann, Hanisch, Noda, & Verkhratsky, 2011).

As such, morphological changes in microglia are a constant in these cells, which start as migratory amoeboid cells that invade the developing tissue, spread throughout it, and acquire a ramified shape (Orłowski, Sołtys, & Janeczko, 2003), which exhibits fine process control and that can be reverted to an amoeboid or less complex state when necessary. Such remarkable morphological plasticity requires underlying cellular machinery and mechanisms that are capable of enacting rapid shape changes. Cellular actin and actomyosin dynamics are responsible for this morphological plasticity. As such, the remainder of this introduction will first provide a general overview of the actomyosin cytoskeleton, followed by a short description of its known roles in microglia morphology and function.

The actomyosin cytoskeleton

Cells have an internal structure of various filaments that are essential for their survival, proliferation and function (Thomas D. Pollard & Goldman, 2018). The three main eukaryotic cytoskeletal networks are composed of actin, microtubules or intermediate filaments. From the three, intermediate filaments are the least studied, but form the most stable filaments, conferring a resilient structure that anchors and positions the nucleus, allows the cell to withstand internal tension and modulate signal transduction (Cheng & Eriksson, 2017; Herrmann & Aebi, 2016). Microtubules form stiff filaments that originate in the perinuclear

region and spread to the edge of the cell, performing a crucial role in cell division by enabling correct chromosomal segregation, are essential components of cilia, and are further used as tracts for intracellular transport by their motor proteins (Barlan & Gelfand, 2017; Goodson & Jonasson, 2018). Finally, actin filaments are located underneath the plasma membrane, creating the cellular cortex, which together with myosin motors is responsible for the force generation necessary for cell locomotion and cytokinesis, and also used as a scaffold to direct short-distance transport of cellular vesicles (T. Svitkina, 2018).

Although the three filament networks operate as mechanical elements in the cell, the actin cytoskeleton is the most dynamic of them, being responsible for orchestrating rapid changes in cell shape. As such, and given that it is a focal point of the work performed in this thesis, its components and regulators will be briefly introduced in the next subsections.

Actin and actin filament basic dynamics

Monomeric actin, also called globular or G-actin is the unit basis of actin filaments. It is a 42 kDa protein with an ATP binding site and intrinsic ATPase activity. Despite its globular shape, G-actin is asymmetric, with a so-called pointed region and a barbed region, located at opposing sides of the monomer (Huxley, 1963). The formation of actin filaments (F-actin) occurs from the stepwise polymerization of monomers that, given the asymmetry at the monomeric level, leads to the formation of polar filaments with similarly two distinct ends: the pointed end and the barbed end. Although monomers can naturally interact with and bind to each other, their spontaneous association rate is very low (Frieden, 1983), meaning that, for example, when two actin monomers have assembled it is far more likely that they will disassemble back to individual subunits than successfully polymerize a third monomer. However, when 3 or more G-actin subunits assemble, then subsequent polymerization into long filaments is enhanced (Sept & McCammon, 2001). The process through which this critical minimum number of monomers is achieved is called nucleation and is the starting step for the formation of actin filaments. Once this proto-filament nucleus has been formed, its stability is highly increased, and it acts as a seed for sustained actin polymerization and filament growth. This elongation happens preferentially at the filament's barbed end site, given the higher association rate there when compared to the pointed end side (T. D. Pollard, 1986). This leads to net polymerization on one side of the filament, with oriented filament growth at barbed ends. Once ATP-G-Actin is incorporated into the filament, its intrinsic ATPase activity hydrolyses the nucleotide to ADP and P_i . The release of phosphate from the filament is very slow, but once it happens, the remaining ADP-actin unit has an increased tendency to dissociate from the filament (Carrier, Pantaloni, & Korn, 1987; Korn,

Carlier, & Pantaloni, 1987). For each filament end there is a threshold value, the critical concentration, that dictates whether net polymerization or depolymerization of actin occurs at that site. As such, under steady state conditions, the net addition or removal of monomers to actin filaments is simply dictated by the concentration of free actin monomers, and whether its value lies above or below the critical value (T. D. Pollard, 1986). As such, we have seen that a seemingly simple system of monomers and their ATP-dependent ability to associate and grow or dissociate and shrink can form dynamic cytoskeletal filaments. However, the random nature of spontaneous nucleation to generate new filaments and the strict dependency on the concentration of free actin to direct the site and timing of filament elongation or depolymerization would make this system wholly inadequate for a cell that requires filament changes in seconds. Cells address this caveat by employing a diverse group of actin-binding proteins, which we will briefly address next.

Actin-binding proteins and actin structures

In order to ensure a timely, localized and highly dynamic generation, growth, maintenance and disassembly of actin filament networks, cells use a variety of proteins that regulate all aspects of actin dynamics by binding actin monomers, promoting nucleation, filament elongation, capping, severing, depolymerization, and crosslinking (Thomas D. Pollard, 2016).

Profilin and Thymosin- β 4 are two actin monomer binding proteins. Profilin binds to ADP-actin and facilitates the removal of the nucleotide, favouring its exchange for ATP, increasing the availability of actin for polymerization (Courtemanche & Pollard, 2013). Furthermore, it delivers ATP-actin to elongation factors (Ferron, Rebowski, Lee, & Dominguez, 2007), and so its overall effect is to promote actin polymerization in the cell. On the other hand, thymosin- β 4 binds ATP-actin but sequesters it in the cell, making it unavailable for polymerization. Both profilin and thymosin- β 4 compete for G-actin, with nearly all of a cell's monomeric actin being bound to one or the other.

The formation of new actin filaments occurs by the action of nucleation factors such as Arp2/3 and Formins. The Arp2/3 complex binds the side of pre-existing actin filaments and promotes the nucleation of a new filament at that site. Once the secondary filament is formed, Arp2/3 remains attached as an anchor point, and so it stimulates the formation of branched actin networks (Rouiller et al., 2008). Formins nucleate actin filaments by stabilizing the formation of actin dimers and trimers. Furthermore, they interact with the barbed ends of actin filaments, where they supply profilin-bound ATP-actin to be

incorporated at that site (Paul & Pollard, 2009; Pring, Evangelista, Boone, Yang, & Zigmond, 2003). After incorporation of a new monomer to the barbed end, formins remain attached processively to the filament end, promoting the incorporation of further actin subunits, effectively acting as an elongation factor, culminating in the formation of long, parallel and non-branched filaments (Otomo et al., 2005). Formins also inhibit the capping of filaments, by blocking the binding of such proteins (e.g. capping protein and tropomodulin) to filament ends. Finally, Ena/VASP proteins promote filament elongation by preventing capping and recruiting profilin-actin (Ferron et al., 2007; Hansen & Mullins, 2010).

Once filaments have been nucleated and elongated, the cell must have a way through which they can be depolymerized or reduced in size. Severing factors are used by cells to achieve these functions. The two main families of severing proteins are ADF/cofilin and gelsolin. Cofilin cooperatively binds actin filaments and induces their severing, creating free ends where depolymerization can occur, ensuring the disassembly of actin filaments in the cell (Andrianantoandro & Pollard, 2006). Gelsolin both severs and caps actin filaments, contributing also to their disassembly (Nag, Larsson, Robinson, & Burtneck, 2013). The action of these proteins replenishes the monomeric actin pool, establishing an essential recycling system that allows for continuous rounds of polymerization and depolymerization of actin filaments.

Finally, after building and establishing a turnover of actin filaments within a cell, they can be reinforced by the employment of actin-crosslinking proteins. These molecules bridge two actin filaments, stabilising them and allowing the construction of robust higher-order actin structures, such as actin bundles (promoted by α -actinin or fascin crosslinking) or actin networks (promoted by spectrin or filamin) (Matsudaira, 1994). The reinforcing action of different types and numbers of crosslinkers ensures that the actin structures throughout the cell can mechanically withstand internal or externally generated forces without compromising the integrity of the cytoskeleton structure.

Using the above-mentioned actin binding proteins, the cell is then capable of generating, maintaining and removing a variety of cellular actin structures, which the cell employs in many of its functions.

The cell cortex is a complex actin network of branched actin and crosslinked bundles that underlies the plasma membrane, providing it structural support and defining the overall shape of the cell (Chugh & Paluch, 2018). It is a continuous structure that envelops the entire cell, acting as a shell that, at specific locations gives rise to different actin structures. At points where there is contact with the extracellular matrix, the cell can establish connection points via integrin molecules (L. R. Anderson, Owens, & Naylor, 2014) that act

as a bridge between matrix components and the cell's actin cytoskeleton. These adhesion points (focal points or focal adhesions) are composed of long, crosslinked actin bundles that anchor the actin cytoskeleton, acting as mechanosensors of the extracellular environment and as traction points through which the cell can drive the generation of force (Bachmann, Kukkurainen, Hytönen, & Wehrle-Haller, 2019). The cellular cortex also gives rise to two essential actin structures that are responsible for advancing the plasma membrane forward, allowing the cell to explore new territory and ultimately gain motility, if necessary. For the plasma membrane to advance, it must be actively pushed forwards by a force that can overcome its natural elastic resistance. That force comes from the polymerization of the actin cytoskeleton against the plasma membrane. At lamellipodia, branched actin filaments, generated mainly by the action of the Arp2/3 complex, grow against the membrane forming a thin actin sheet that spreads outwards, creating a forwards-advancing broad edge in the cell (Vinzencz et al., 2012). Conversely, at filopodia, bundles of parallel actin filaments grow perpendicular to and against the plasma membrane, built mainly by the action of formins and Ena/VASP and stabilized by crosslinkers, leading to the formation of very thin, tendril-like protrusions that are seen either at the edge of lamellipodia or on their own (T. M. Svitkina et al., 2003; Yang et al., 2007). Both structures allow the cell to probe the environment or initiate movement, while sensing the newly explored areas, and help determining if and in which direction the cell should move (Anne J. Ridley, 2011). As such, using an array of actin binding proteins, the cell can generate robust structures that can be used for complex cellular functions.

Type-II Myosins

We have seen above how the creation, maintenance, stabilization and disassembly of actin networks is successively achieved in a cell by the action of several actin binding proteins. Their action is further augmented by a family of actin motor proteins, Myosins, which, together with the actin network, constitute the actomyosin cytoskeleton.

The myosin superfamily is vast, with around 40 different elements in humans alone, and is currently divided into 15 classes based on phylogenetic analysis (Sellers, 2000). A more accessible classification scheme separates them into conventional and non-conventional myosins. Conventional myosins form filaments in muscle and non-muscle cells, and together constitute the Type II class. They are the ones that, through their association to actin filaments mainly generate contractile forces in muscle and non-muscle cells. All other myosin classes belong to the non-conventional type (Sellers, 2000), and their interaction with actin is generally associated with cargo transport (e.g., myosins Types V and VI) and

membrane remodelling (e.g., myosin Type I). Although the structure of myosins shows slight variation between different classes, they all share a common motor domain that binds actin and ATP and possesses intrinsic ATPase activity that moves them along actin filaments in the direction of the barbed end (the sole exception being myosin VI, which moves toward the pointed end of filaments). Given the essential role of Type II myosins in the dynamics of the actin cytoskeleton and that they will be key molecules in the present thesis, we will focus more on their description here.

Type II myosins are the most abundant myosin type and are present in every cell (Sellers & Heissler, 2019). They are hexameric proteins composed of 2 pairs of heavy chains, 2 pairs of essential light chains and 2 pairs of regulatory light chains. The heavy chains are long, approximately 230 kDa proteins, with a globular head domain that contains the motor region, a neck region and rod region (Krendel & Mooseker, 2005). The essential light chains bind to the neck region of the heavy chains and are critical stabilizers of the entire complex (Heissler & Sellers, 2014). The pair of regulatory chains is responsible for regulating the conformational state of the complex and the activation of its motor activity, notably by phosphorylation of the Serine 19 residue (Tan, Ravid, & Spudich, 1992). The rod region of one heavy chain can form a coiled coil interaction with the homologous region of another heavy chain. The subsequent association of the essential and regulatory light chains creates the functioning myosin complex monomer which, via antiparallel association with other monomers, generates bipolar myosin filaments. To move actin filaments, myosin-heads bind to an actin filament, via their actin-binding domain, in the absence of ATP. Hydrolysis of the nucleotide into ADP and P_i releases the actin filament and changes the motor into a straight conformation that allows it to contact the actin filament once again. Release of P_i induces a drastic conformational change that rotates the neck region, translocating the actin filament. After ADP is released, myosin remains bound to actin, with the bond being released only when ATP is replenished in the motor domain, restarting the mechanochemical cycle (Sweeney & Holzbaur, 2018). The movement of the myosin filaments that transmits the translocation force to the actin filaments is called the myosin powerstroke. When the two ends of myosin II filaments bind different actin filaments, successive powerstrokes lead to the sliding of the two actin filaments past each other. This sliding action effectively creates tension within actin filaments, leading to contraction of the actin network at places where myosin II is activated (Bendix et al., 2008). Also, since myosin filaments can bind actin filaments regardless of their motors being active or inactive, they also act as important actin crosslinkers within the cell. This complementary function has been proven to be essential for constriction of actomyosin rings during cytokinesis (Mendes Pinto, Rubinstein, Kucharavy, Unruh, & Li, 2012).

The force-generating activity of type II myosins and their ability to crosslink actin filaments are the basis of their functions within a cell. For example, localized contraction of the actin network at lamellipodia leads to retrograde flow of the network. Such an action is used by the cell to efficiently retract existing protrusions without having to rely on actin disassembly alone (Lin, Espreafico, Mooseker, & Forscher, 1996; Wylie & Chantler, 2003). It also allows the cell to condense the actin network in a specific region, where subsequent disassembly of the bundled actin filaments can occur, promoting the recycling necessary for the maintenance of a stable pool of actin monomers (T. W. Anderson, Vaughan, & Cramer, 2008; Sonal et al., 2019). Myosin II-driven localized constriction also underlies cell division (cytokinesis). The tension generated by type II myosins along the actin bundles of focal points and focal adhesions is essential for their maturation and maintenance (Pasapera, Schneider, Rericha, Schlaepfer, & Waterman, 2010; Zaidel-Bar, Cohen, Addadi, & Geiger, 2004). Type II myosin mediated tension is also crucial for the morphogenesis of epithelial tissues (Heisenberg & Bellaïche, 2013; Lecuit & Lenne, 2007). Overall, the stresses and tension that type II myosins impart onto the actin network are responsible for changes in cellular shape (Salbreux, Charras, & Paluch, 2012) as well as the behaviour and integrity of tissues (Gross, Kumar, & Grill, 2017).

Although the mechanochemical cycle is common to all myosins, the specific activity and role of myosin ii is defined by the type of heavy chain present in the complex. Mammalian cells have three distinct type ii myosin heavy chain isoforms, coded by the Myh9, Myh10 and Myh14 genes. The functioning myosin complexes that they create together with the light chains are called NMIIA, NMIIB and NMIIIC, respectively. Myh9 and Myh10 are the isoforms that are most responsible for the generation of contractile forces in cells, since their specific kinetic properties endow them with higher ATPase activity, a greater number of myosin monomers per filament and longer engagements over time with actin filaments (Billington, Wang, Mao, Adelstein, & Sellers, 2013). These differences in mechanochemical properties are likely responsible for the differential functions of type II myosin isoforms. During cell motility, Myh9 and Myh10 tend to segregate into different regions of the cell, with myh10 being responsible for the definition of the cell rear and the cell's forward translocation (Vicente-Manzanares, Koach, Whitmore, Lamers, & Horwitz, 2008). Myh10 is also essential for nuclear placement and integrity during cell motility (Thomas et al., 2015), for example, while Myh9 is crucial for the stability of cell-cell junctions (Ivanov et al., 2007; Shewan et al., 2005).

Whether through global or isoform-specific contributions to actomyosin cytoskeleton dynamics, the importance of the contribution of type II myosins to cellular function is further strengthened by the impact of type-ii myosin isoform deletion experiments. Ablation of Myh9

in mice results in defects in cell-cell adhesion leading to failure of the formation of polarized visceral endoderm and resulting in embryonic lethality at E6.5 (Conti, Even-Ram, Liu, Yamada, & Adelstein, 2004), while Myh10 deletion in mice cause impaired cell adhesion and migration that leads to cardiac and brain defects that become lethal at E14.5 (Tullio et al., 1997; Tullio et al., 2001).

As such, through the concerted action of actin, actin binding proteins and myosin motors, the actomyosin cytoskeleton allows cells to move, extend and retract protrusions, divide, move, deform and adapt to changing conditions in their surrounding environment, ensuring their diverse functions.

RhoGTPases and the regulation of actin and myosin dynamics

The appropriate spatial and timely action of actin binding proteins, myosins and the subsequent effects in cell shape and function require tight regulation. Cells achieve this through a group of small molecule regulators: Rho GTPases.

The Rho GTPase family consists of 20 members which are divided into classical and atypical GTPases. The activation state of classical members, as implied by their name, is dependent of the binding of a GTP nucleotide, which upon hydrolysis returns the GTPase to its inactive state (Hodge & Ridley, 2016). Their activity is regulated by guanine exchange factors (GEFs), that activate the GTPase by promoting the exchange of GDP for GTP, GTPase-activating proteins (GAPs), that promote GTP hydrolysis and thus inactivate the GTPase, and guanine dissociation inhibitors (GDIs), that sequester the GTPase away from the plasma membrane and prevent the action of GAPs (Bos, Rehmann, & Wittinghofer, 2007; Garcia-Mata, Boulter, & Burridge, 2011; Jaffe & Hall, 2005). As such, Rho GTPases constantly cycle through active, GTP-bound states and inactive, GDP-bound states, with GAPs, GEFs and GDIs performing the essential regulatory functions of these proteins. On the other hand, the activity of atypical members is regulated by mechanisms that are independent of GTP-binding (Heasman & Ridley, 2008). There are currently at least 80 GEFs and 70 GAPs reported in the literature (Hall, 2012), which indicates high redundancy and complexity in the regulation of Rho GTPases (Bagci et al., 2019).

The most widely investigated Rho GTPases are the classical RhoA, Rac1 and Cdc42. Studies have identified specific contributions of each of these proteins to the regulation of actomyosin dynamics. For instance, expression of constitutively active Cdc42 in a cell leads to filopodia formation at the cell edge, while constitutively active Rac1 stimulates lamellipodia formation, and constitutively active RhoA induces cellular contraction, with

formation of stress fibers (Nobes & Hall, 1995; Anne J. Ridley & Hall, 1992; Anne J. Ridley, Paterson, Johnston, Diekmann, & Hall, 1992). Further studies revealed the signalling networks that link the activation of these regulators with the activation of specific actin binding proteins or myosin regulators (Schwartz, 2004). Among other targets, Cdc42 activates mDia formins, allowing for the nucleation and polymerization of actin bundles required for filopodia formation. Rac1 notably stimulates the Arp2/3 complex by activation of WASP, essential for the formation of the branched actin network that constitutes the lamellipodia. Finally, RhoA activates ROCK, which inactivates MLCP (myosin light chain phosphatase) and activates MLCK (myosin light chain kinase), which culminates in increased phosphorylation of myosin regulatory light chains and consequent activation of type II myosins (Totsukawa et al., 2000). It also classically activates LIMK, which is responsible for decreasing the turnover of actin filaments to enhance the force-generating action of type II myosin activation (Arber et al., 1998), making RhoA the classical regulator of actomyosin contractility in the cell.

Together, through their ability to specific and spatiotemporally control the action of actin binding proteins and myosin motors, these three Rho GTPases are master regulators of actomyosin cytoskeleton dynamics, with subsequent contributions to a variety of cell functions, such as division, motility, signalling and behaviour (Heasman & Ridley, 2008; Luo, 2000; Phuyal & Farhan, 2019; Sadok & Marshall, 2014).

Microglia and the actomyosin cytoskeleton

The complex ramified morphology displayed by microglia and the changes in shape that they undergo suggest an important role for the actomyosin cytoskeleton in these cells. Indeed, recent studies have demonstrated the importance of actin dynamics and of actin cytoskeleton regulators in microglia. When microglia sense ATP within the environment, the in-situ extension of protrusions towards the site of release is driven by actin polymerization (Hines, Hines, Mulligan, & Macvicar, 2009) in a process that involves intracellular cAMP levels downstream of P2Y₁₂R (Bernier et al., 2019). The extension of microglia processes towards damage sites in situ also involves kindlin-3 signalling (Meller et al., 2017), a molecule that is important for adhesion-induced actin rearrangement. Podosomes, actin-rich structures that actively help degrade extracellular matrix components and that are known to facilitate three dimensional migration, have also been observed in microglia (Siddiqui, Lively, Vincent, & Schlichter, 2012). The importance of actomyosin cytoskeletal

forces in the assembly of tunnelling-nanotubes that are used by microglia to share phagocytosed material has additionally been described in these cells (Scheiblich et al., 2021). Furthermore, new studies have shown that absence of RhoA in microglia affects their morphology and notably alters their state from surveillant to inflammatory, disrupting CNS homeostasis (Socodato et al., 2020), while Rac1 deletion changes the shape of microglia and further renders them unresponsive, affecting synapse homeostasis (Socodato et al., 2021). A different type of study focused on the potential impact of pharmacological disruption of the actin network in in vitro microglia and found that it reduced the secretion of inflammatory mediators in response to LPS (lipopolysaccharide) (Uhlemann et al., 2016). Ablation of cofilin also resulted in impaired response to LPS (Alhadidi & Shah, 2017).

All of these studies clearly demonstrate that actin dynamics is important in the definition of microglia shape, and that changes in upstream actin regulators can severely influence the function of these cells. However, they focused mostly on the impact in microglia function, while shedding very little light on specific mechanisms for control of actin dynamics, protrusion formation and microglia shape. Together, such studies and observations open new research opportunities that will be explored in the present thesis.

Hypothesis

The morphology of microglia is commonly used as an indicator of deviation from homeostasis since it is usually associated with inflammatory activity and consequent negative effects on the CNS tissue. The normal functions of microglia also require tight regulation of cellular protrusions, to sense their surrounding microenvironment. Given that changes in shape and the extension of protrusions in cells are tightly linked with mechanical forces acting at the level of the cell's cortex we hypothesize that **actomyosin cytoskeletal tension generation and its control are important to control microglia shape.**

Additionally, since actomyosin force generation is classically associated with the activity of type II non-muscle myosins, we also hypothesize that these **motor proteins will be of importance to the control of microglia morphology.**

Information gathered by several years of research and to which our group has contributed has shown that the regulation of the actin cytoskeleton in microglia, as well as some of its regulators has an impact on the normal function of these cells. As such, and given the expected effect of the myosin motors on the shape of microglia, we further hypothesize that **interfering with these key cytoskeleton components will also influence the function of microglia.**

Objectives

The main goal of this thesis is to determine the validity of the hypothesis described in the previous section, to contribute to a better understanding of the basic mechanisms that control actin dynamics in microglia, and to know how these can in turn regulate protrusion dynamics. To achieve this, the work focused on 4 sequential objectives/tasks:

- Develop an experimental model that allows the adequate study of microglia morphology in situ.
- Perform an in-depth study of the role of the classical actomyosin force regulator RhoA in the shape of microglia.
- Ascertain the impact of RhoA ablation on its classical downstream targets (actin binding proteins and type II myosin motors), the actin cytoskeleton itself, and how such changes affect the morphology of microglia.
- Determine if changes in type II myosin motors change the function of microglia and, if so, through which molecular mechanisms.

The experiments performed used the retina as an ex vivo experimental model together with in vitro culture of a microglia cell line and primary cells.

Materials and Methods

Materials

All the antibodies used during work for this present thesis are listed in the following table, for convenience:

Antibody	Clone	Species	Company	Dilution
Iba1	Polyclonal	Rabbit	Wako	1:500
CD16/32 (Fc-Block)	93	Rat	BioLegend	1:500
CD11b	M1/70	Rat	BioLegend	1:200
CD45	30-F11	Rat	BioLegend	1:1000
Myh9	3672	Rabbit	Cell Signalling Technology	1:1000 (WB) 1:100 (IHC)
Myh10	Poly19099	Rabbit	BioLegend	1:1000 (WB) 1:250 (IHC)
pCofilin(S3)	77G2	Rabbit	Cell Signalling Technology	1:1000
Cofilin	D59	Rabbit	Cell Signalling Technology	1:1000
pProfilin1(S137)	-	Rabbit	-	1:1000
Profilin1	Polyclonal	Rabbit	Abcam	1:1000
YAP	Polyclonal	Rabbit	Abcam	1:1000
pYAP(S127)	D9W2I	Rabbit	Cell Signalling Technology	1:1000
pYAP(Y357)	Polyclonal	Rabbit	Abcam	1:1000
Csk	C74C1	Rabbit	Cell Signalling Technology	1:1000
MLC2	Polyclonal	Rabbit	Cell Signalling Technology	1:1000
pMLC2(S19)	Polyclonal	Rabbit	Cell Signalling Technology	1:1000
GAPDH	6C5	Mouse	HyTest	1:100000
Rabbit IgG (AlexaFluor 594)	Polyclonal	Goat	ThermoFisher	1:1000
Mouse IgG (HRP)	Polyclonal	Goat	SouthernBiotech	1:15000
Rabbit (IgG (HRP)	Polyclonal	Donkey	SouthernBiotech	1:10000

Methods

Animals and Ethics

Mice (*Mus musculus*) were maintained under standard conditions at the I3S animal facility with a normal 12 hour light/12 hour dark cycle with ad libitum access to food and water. All experiments were reviewed by the I3S Animal Ethics Committee and approved by Direcção Geral de Alimentação e Veterinária (DGAV). All experiments were performed in adult mice (2-6 months of age) in adherence to the 3Rs principles and the European Union Animal Welfare laws and guidelines. Maintenance and husbandry of mice homozygous for RhoA floxed allele (Herzog et al., 2011; Jackson et al., 2011) and mice carrying CX₃CR1CreER-IRES-YFP (Parkhurst et al., 2013), both kept on a C57BL/6 background, was performed as previously described (Socodato, Portugal, et al., 2020). Crossing of these lines generated the control (RhoA^{fl/fl} : CX₃CR1^{+/+}) and mutant (RhoA^{fl/fl} : CX₃CR1^{CreER-IRES-YFP/+}) mice used in the experiments. Genotypes were confirmed by gDNA PCR. To induce Cre-mediated recombination, mice received 10mg tamoxifen by oral gavage at P26 and P28, and analysed at least one month later (no earlier than P60) to allow efficient elimination of RhoA from microglia and sufficient time for replenishment of other CX₃CR1⁺ cells from unrecombined CX₃CR1⁻ bone marrow precursors (Socodato, Portugal, et al., 2020).

Retina flatmount preparation

Mice were sacrificed and their eyes immediately enucleated into 4% PFA in PBS for direct preparation of flatmounted retinas. The cornea was removed to improve PFA access and the tissue was fixed overnight at 4°C in the dark. Retinas were then dissected from the ocular tissue and gently transferred with a plastic Pasteur pipette in PBS to 96-well plates (one retina per well) for immunohistochemistry.

Ex vivo culture of retinal explants

Mice were sacrificed and their eyes immediately enucleated into Ringer solution (125mM NaCl, 5mM KCl, 1.5mM CaCl₂, 0.75mM MgCl₂, 1.25mM NaH₂PO₄, 10mM glucose, 20mM HEPES, pH 7.35). Retinas were then gently dissected from the ocular tissue in fresh Ringer solution and radially cut with a scalpel into 4 explants. These were then carefully transferred onto 0.45µm Millipore filters (Merck) that were pre-wet in fresh Ringer solution. Explants on filters were cultured on wells of 24-well plates (Corning) containing either 500µL fresh

Ringer solution (unstimulated) or 500μL fresh Ringer solution containing 1mM ATP (Sigma) for control and RhoA mutant retinas. After 15 minutes of incubation at 32°C the filters with the explants were transferred with thin forceps to new wells of a 24-well plate containing 4% PFA in PBS. For the experiments assessing the impact of type-II myosin inhibition in retinal microglia morphology, explants from control RhoA^{fl/fl} mice were similarly cultured in Millipore filters on 24-well plates containing fresh Ringer solution (unstimulated) or fresh Ringer solution containing DMSO (control) or 50μM blebbistatin (Sigma). Transfer of explants into 4% PFA in PBS was performed as described above after 30 minutes of incubation at 32°C. All explants were fixed for 1 hour, washed once in PBS and transferred after cutting off excess filter into wells of a 96-well plate (one explant per well) with fresh PBS for immunohistochemistry.

Immunohistochemistry

All steps were performed on wells of a 96-well plate. Whole retinas or retinal explants were permeabilized and blocked in 5% BSA, 5% Triton X-100 in PBS for 1 hour at room temperature. Staining with rabbit anti-Iba1 (Wako 1:500) in PBS 0.5% Triton-X100 was performed for 72 hours under low orbital agitation (to promote antibody access by gentle tissue movement) at 4°C in the dark. After washing 3x5 minutes with PBS the explants were incubated with secondary antibody (anti rabbit-AlexaFluor594, ThermoFisher 1:1000) in PBS for 2 hours at room temperature, in the dark, under low agitation. After washing 3x5 minutes with PBS explants and whole retinas were gently transferred to glass slides with forceps or a plastic Pasteur pipette. Using small spring scissors four radial cuts were made in whole retinas to facilitate flattening of the tissue. Explants and wholemounts were finally mounted with coverslips using FluoroShield (Sigma) and stored in the dark at 4°C until image acquisition by confocal microscopy.

Confocal microscopy of retina wholemounts and explants

Images from whole-retina flatmounts or retina explants were acquired using an HC PL APO 20x /0.75 IMM/CORR CS2 glycerol objective at 8000Hz (resonant mode, bidirectional), zoom 1.25x, with pinhole set to 1 airy unit in a Leica TCS SP8 confocal microscope (Leica Microsystems, Germany). Excitation was achieved with a HeNe 594nm laser (2-5%) and images were generated with a 1024x1024 pixel format, 8-bit depth using a HyD detector. Using the LAS-X Navigator suite to spirally acquire spatially sequential images we first generated a global image that covered the entire retina tissue. This allowed us to then select

areas of the retina that had no signs of tissue damage (tears or wholes), in order to exclude the possible effect of such artefacts on microglia morphology. We then defined two areas per retinal explant, per animal or eight areas per whole retina (2 areas per quadrant) per animal for imaging. For each area we acquired z-stacks starting at the ganglion cell layer through to the outer nuclear layer of the retina, in order to successfully capture both the inner and outer plexiform layers, the two retinal compartments where microglia reside (Silverman & Wong, 2018). Stacks had a depth of 40-100µm and were always acquired with a step size of 0.42µm.

Quantitative image analysis of retinal microglia

Confocal z-stacks of Iba1-stained microglia were analysed using the image analysis software ImageJ/Fiji Fiji. For each of the acquired retina fields of view we generated two substacks (defined by the user after inspection): one for slices containing inner plexiform layer (IPL) microglia and another for slices containing outer plexiform layer (OPL) microglia. All the slices from both substacks were background subtracted using the mean pixel value of a region containing no cells defined by the user. IPL and OPL microglia were subsequently analysed separately, to exclude possible differences arising from their particular retinal compartments. All morphological data on control and RhoA mutant retinal cells (direct in vivo as well as ex vivo ATP stimulated) are from IPL microglia. Data on the effect of blebbistatin (type-II myosin inhibitor) on morphology of cells are from OPL microglia, since at the longer time point (30 minutes of exposure to drug) control unstimulated IPL microglia had a more amoeboid shape, with shortening and thickening of protrusions and enlarged cell bodies, usually indicative of cell activation, while no dramatic changes in control unstimulated OPL microglia were seen. For each field of view individual cell regions were manually drawn, excluding all cells whose protrusions extended beyond the edges of the image as well as cells that significantly overlapped with others in both the xy as well as z directions. Then, for each cell a maximum projection was generated, the image was median filtered to enhance cell edges and then thresholded using the Li algorithm, with values manually corrected by the user. Since simple thresholding was rarely enough to accurately differentiate the entire cell from the background the mask was then manually refined in order to remove segments that did not belong to the cell or to join unlinked segments, successfully creating a mask of the cell. From it, we generated a skeleton representation of the cell, using the skeletonize function, that preserves the cell's shape characteristics and complexity (Fig. S1A). Using the Analyze Skeleton plugin we extracted from the skeleton the cell's number of junction points (Fig. S1B). For the cell

territory we used the cell mask to compute the area of the cell's convex hull (Fig. S1C). To determine the average length of the cell's branches, we used the MorphoLibJ plugin to generate the geodesic distance map of the skeleton starting from the cell soma (manually identified by the user) and using the quasi-euclidean method for distance calculation. From it, we extracted the distance from each of the skeleton's end points (obtained through the Analyze skeleton plugin) to the cell soma and then averaged the lengths of all protrusions per cell (Fig. S1D). We developed a group of ImageJ macros to semi-automatically implement this image analysis workflow.

Culture of HMC3 microglia

Human microglia clone 3 (HMC3) cells were obtained from ATCC (CRL-3304) and cultured in high glucose DMEM GlutaMAX (Gibco) supplemented with 10% heat-inactivated FBS (Gibco) and Penicillin-Streptomycin (Gibco), in a humidified incubator at 37°C and an atmosphere of 95% air and 5% CO₂. Cells were passaged for a maximum of 4 times and then discarded to prevent effects of prolonged-culturing, starting a new culture from frozen stocks.

Primary microglia culture

Microglia were purified from mice (between 30 and 100 days old) using the protocol described in (Bohlen, Bennett, & Bennett, 2019). Briefly, dissected brains from which the cerebellum and brainstem were removed were gently dissociated in a Dounce homogenizer and the resulting cell suspension passed through a 70µm cell strainer. Myelin was removed by density gradient centrifugation using a single layer of 24% Percoll (Sigma), after which the remaining cells were submitted to magnetic separation using CD11b-directed beads (Miltenyi Biotech 130-093-634) and MS columns (Miltenyi Biotech 130-042-201), according to the manufacturer's instructions. Purified microglia were cultured in DMEM/F12 (Gibco) supplemented with 10% FBS (Gibco), Penicillin/Streptomycin (Gibco) and 1ng/mL GM-CSF (PeproTech). A portion of the cells was stained for CD45 (Biolegend clone 30-F11) and CD11b (Biolegend clone M1/70), after anti-mouse CD16/CD32 blocking (Biolegend Clone 93) and cells analysed in a BD FACS Canto II to assess microglia purity.

CRISPR-Cas9 knockout on HMC3 microglia

For deletion of RhoA and Myh10 on HMC3 microglia we followed the method in (Bauer, Canver, & Orkin, 2014). Guide-RNA design and selection was performed with the webtool ChopChop (Labun, Montague, Gagnon, Thyme, & Valen, 2016). For RhoA we selected a pair of gRNAs (gRNA1: AATCACCAGTTTCTTCCGGA; gRNA2: ATCGACAGCCCTGATAGTTT) to achieve deletion of exons 2 and 3 (start codon at exon2) by non-homologous end joining (NHEJ). For Myh10 another pair (gRNA1: TTGGATCGTTCCATTTACAA; gRNA2: CTGAAGGATCGCTACTATTC) targeted exon 3 for deletion (start codon at exon 3) by NHEJ. Selected gRNAs had either no predicted off-targets or a maximum of 2, all containing at least 3 mismatches to the predicted off-target sequences, which were all located in intronic regions of genes that are not expressed by microglia. Individual gRNAs were cloned into the pSpCas9(BB)-2A-GFP plasmid (PX458, Addgene #48138) (Ran et al., 2013) after BbsI vector restriction and ligation with pre-annealed gRNAs (sense and anti-sense) containing BbsI-compatible overhangs. Insertion of the gRNA sequence into the plasmid was assessed by resistance to BbsI digestion of plasmid DNA extracted from transformed DH5 α E. coli colonies, and confirmed by Sanger sequencing of the insert from both sides using the primers CAAGGCTGTTAGAGAGATAATTGGA (fwd) and GCCATTTACCGTAAGTTATG (rev). HMC3 cells were then transiently transfected with a mixture of the 2 plasmids carrying the 2 gRNAs for RhoA or Myh10 (1 μ g of each plasmid) using JetPrime® transfection reagent (Polyplus Transfection) according to the manufacturer's instructions. Cas9-mediated targeting of the genes of interest and expression of GFP, together with subsequent repair by NHEJ were allowed for 48 hours, after which GFP+ HMC3 microglia were single-cell sorted into wells of flat-bottom 96-well plates by a BD FACS Aria II to establish clonally pure populations. After 14 days of incubation growing clones were picked up and expanded into wells of 24-well plates, in duplicate, to allow further expansion. Finally, clones were evaluated for successful deletion of the gene of interest by western blot, after which selected clones were further expanded and frozen for stock preparation. RhoA and Myh10 knockout clones were again passaged for a maximum of 4 times and then discarded to prevent effects of prolonged-culturing, starting a new culture from frozen stocks.

Lentiviral-mediated silencing of Myh9, Myh10 and RhoA in HMC3 microglia

MISSION shRNA plasmids containing Myh9-specific (TRCN0000285480), or Myh10-targeting (TRCN0000299094) shRNAs, or RhoA-targeting (TRCN0000068199), as well as non-mammalian targeting shRNA negative control plasmid (termed in the text as scrambled) in the pLKO.1-puro vector were obtained from Sigma. For production of lentiviral particles we followed the protocol of (Tiscornia, Singer, & Verma, 2006) with some modifications. Low passage HEK293T cells were plated in 10cm culture dishes in DMEM GlutaMAX (Gibco) supplemented with 10% FBS (Gibco) and Penicillin/Streptomycin (Gibco), and allowed to reach 80% confluency. They were then co-transfected with 6µg shRNA plasmid together with 3µg pPAX2 and 3µg pVSVG plasmids using JetPrime transfection reagent (PolyPlus Transfection) following manufacturer's instructions. After 4 hours the medium was exchanged for fresh medium and cells incubated for 48 hours. After this, medium with lentiviral particles was collected into falcon tubes, centrifuged at 600g for 15 minutes and the supernatant aliquoted and frozen at -80°C. For HMC3 microglia transduction, cells were seeded onto wells of a 24-well plate and infected with viral particles in their normal culture medium for 24 hours. After removal of the lentiviral particles cells were incubated for a further 24 hours in culture medium before starting selection with 6µg/mL puromycin (Calbiochem). Selection was completed when control, non-infected HMC3 microglia cells died under the same selection conditions. Stable shRNA-expressing HMC3 cells were then expanded (while keeping the same level of selective pressure by puromycin) and the efficiency of silencing was determined by western blot. Stable lines were also passaged no more than 4 times before being discarded to avoid effects of long-culturing, and fresh HMC3 were used to generate new stably-silenced microglia. To evaluate the effect of Myh9 and Myh10 silencing on HMC3 microglia morphology, 4% PFA fixed cells that were seeded onto glass coverslips were imaged by Phase-contrast optical microscopy through an HCX PL FLUOTAR L 20x/0.40 CORR Ph1 objective in a Leica DMI6000 microscope (Leica Microsystems, Germany). Images were analysed in ImageJ/Fiji using the Cell counter plugin by manually assigning cells into one of four morphological categories: 1) round cells without protrusions or with a single protrusion, 2) bipolar cells with two protrusions, 3) cells with three or four protrusions, and 4) cells displaying more than 4 protrusions. For primary microglia transduction, cells were seeded onto wells of a 12-well plate and infected with lentiviral particles for 24 hours, after which the medium was completely exchanged with fresh primary culture medium. Cells were then incubated for 4 days to allow for target protein silencing (confirmed by western blot), and were then processed for immunocytochemistry.

Microglia cell lysates and Western blotting

HMC3 microglia were seeded into wells of a 6-well plate were lysed with RIPA buffer (50mM Tris-HCl, 150mM NaCl, 0.1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate) supplemented with protease inhibitor cocktail (Roche), phosphatase inhibitor cocktail (Roche) and 1mM DTT. Samples were collected on ice and centrifuged at 16.000g for 10 minutes at 4°C. Supernatants were stored at -80°C until use. Protein concentration was ascertained using the Lowry method (BCA Protein Assay Kit – Pierce). Extracts were resolved in 12% (for RhoA, pCofilin, Cofilin, pProfilin and Profilin) or 7% (for Myh9 and Myh10) SDS-PAGE gels loaded with 20µg protein per lane. Proteins were then blotted onto PVDF membranes by wet-transfer using a Mini Trans-Blot Cell (Biorad). Membranes were blocked for 1 hour at room temperature under mild orbital agitation in blocking buffer (5% low-fat milk, 0.1% Tween-20 in TBS (20mM Tris, 200mM NaCl, pH 7.4). Appropriate areas of the membrane were then incubated overnight with primary antibodies (Cell Signalling Technology #3403 rabbit anti-Myh9 1:1000; Biolegend Poly19099 rabbit anti-Myh10 1:1000; Cell Signalling Technology #3313 rabbit anti-pCofilin(S3) 1:1000; Cell Signalling Technology #3318 rabbit anti-Cofilin 1:1000; rabbit anti-pProfilin1(S137) 1:1000 kindly provided by Dr Marc Diamond, UCSF, San Francisco, USA; Abcam 50667 rabbit anti-Profilin1 1:1000; Abcam 39361 rabbit anti-YAP, Cell Signalling Technology #13008 rabbit anti-pYAP(S127); Abcam 62751 rabbit anti-pYAP(Y357); Cell Signalling Technology #4980 rabbit anti-Csk 1:1000; Cell Signalling Technology #3672 rabbit anti MLC2 1:1000; Cell Signalling Technology #3671 rabbit anti-pMLC2(S19) 1:1000; HyTest 6C5 mouse anti-GAPDH 1:100000) diluted in blocking buffer at 4°C under mild agitation. After washing with 20mM Tris, 200mM NaCl, 0.1% Tween-20, membranes were incubated with suitable HRP-conjugated secondary antibodies (SouthernBiotech goat anti-mouse IgG 1:15000 and SouthernBiotech donkey anti-rabbit IgG 1:10000) for 1 hour at room temperature under mild agitation and, after further washing, imaged in a ChemiDoc XRS System (Biorad) using the SuperSignal™ West Pico PLUS Chemiluminescent Substrate (ThermoFisher). Images were quantified using the ImageLab v5.2.1 software (Biorad).

Immunocytochemistry and Fluorescence imaging

HMC3 and primary mouse microglia cultured on glass coverslips were fixed in 4% PFA in MP buffer (60mM PIPES, 27.5mM HEPES, 10mM EGTA, 1.5mM MgSO₄, pH 7.0) and washed 3x5 minutes in PBS. They were then permeabilized for 15 minutes with 0.1% Triton X-100 in PBS, washed once in PBS and then blocked for 1 hour at room temperature with 10% normal goat serum and 2% BSA in PBS. Incubation with primary antibodies (Cell

Signalling Technology #3403 rabbit anti-Myh9 1:100; Biolegend Poly19099 rabbit anti-Myh10 1:250) was then performed overnight at 4°C in a humidified chamber in PBS 2% BSA. After 3x5 minute washes with PBS, the coverslips were incubated for 1 hour at room temperature in the dark with secondary antibody (goat anti-rabbit-Alexa488 1:1000) and Phalloidin-DyLight594 (ThermoFisher 1:100). Finally, the coverslips were incubated for 5 minutes with DAPI in PBS (1µg/mL) and washed twice with PBS before being mounted onto glass slides with FluoroShield (Sigma). Images of the cells were obtained with a Leica DMI6000 epifluorescence microscope (Leica Microsystems, Germany) through an HCX PL APO CS 63x/1.30 glycerol objective, captured by an ORCA-Flash4.0 V2 camera (Hamamatsu, Japan) with 2x2 binning at a 16-bit depth. Quantification of Myh9, Myh10 and F-actin localization relative to the cell edge was performed in ImageJ/Fiji. First, all channels were background subtracted using the mean pixel value of a region defined by the user that contains no cells. Then, a mask of each cell was created by thresholding of the phalloidin channel after median filtering. Using this mask, the background of all channels was eliminated, clearly defining the edge of the cell (first non-zero-valued pixel). Afterwards, using the line tool, lines that are perpendicular to the cell edge, starting from outside the cell and ending near the cell centre with a thickness of 5 pixels were manually drawn, and the average fluorescence intensity value along the line was extracted using the plot profile tool. The pixels with zero value at the start of the profile (corresponding to background) were discarded and individual profiles from different cells were normalized to the maximum intensity value of the profile in Excel and then combined to form an averaged global line profile plot in GraphPad Prism.

FRET analysis of actin cytoskeleton tension

The protocol used was based on the one described in (Socodato, Melo, Ferraz-Nogueira, Portugal, & Relvas, 2020). The plasmid containing the expression cassette for the alpha-actinin tension sensor probe PEG-Actinin-M-sstFRET (Meng & Sachs, 2011) was obtained from Addgene (#61100). The one containing the expression cassette for the Src activation sensor probe Kras-Src FRET was also obtained from Addgene (#78302). HMC3 microglia were seeded onto glass coverslips. On the next day they were transfected with 800ng of the probe's plasmid DNA using JetPrime transfection reagent (PolyPlus Transfection) following the manufacturer's instructions. Cells were incubated for 24 hours to allow expression and targeting of the probe, and then fixed for 10 minutes in 4%PFA in MP buffer (60mM PIPES, 27.5mM HEPES, 10mM EGTA, 1.5mM MgSO₄, pH 7.0) before being mounted onto glass slides with FluoroShield (Sigma). Cells were imaged through an HCX

PL FL L 40x/0.60 CORR Ph2 objective, captured by an ORCA-Flash4.0 V2 camera (Hamamatsu, Japan) with 2x2 binning at a 16-bit depth, using a Leica DMI6000 epifluorescence microscope (Leica Microsystems, Germany). Samples were excited by a mercury metal halide lamp with an integrated light attenuator (EL6000, Leica Microsystems) and through a BP 427/10 nm filter for donor excitation, or through a BP 504/12 nm filter for acceptor excitation. Donor-emitted light was captured through a BP 472/30 nm filter, direct acceptor-emitted light through a BP 542/27 nm filter and fret acceptor-emitted light through a 440/520 nm dichroic mirror and a BP 542/27 nm filter. Images were analysed in ImageJ/Fiji using the 2DFRET-ratiometrics macro, created by Mafalda Sousa at the Advanced Light Microscopy facility of I3S (Sousa, Seixas, & Sampaio, 2020), with automatic thresholding. All cell masks were confirmed and manually refined by the user when inadequate. The ratio of donor/acceptor was selected in order to more easily relate fret levels with tension estimates or Src activation estimates on the ratiometric images (higher ratio indicates higher tension or Src activation). Global cell tension levels or Src activation levels were computed as the average value of the fret ratio of the entire cell (obtained from cell masks that were generated by thresholding). Quantification of tension levels along protrusions was performed similarly as described before for actin and myosin quantification. The background was eliminated using the cell masks mentioned above and lines perpendicular to the cell edge, starting from outside the cell and running parallel and along microglia protrusions were manually drawn with a thickness of 5 pixels. The average fluorescence intensity value along the line was extracted using the plot profile tool. The pixels with zero value at the start of the profile (corresponding to background) were discarded and individual profiles from different cells were combined to form an averaged global line profile plot in GraphPad Prism.

Phagocytosis assay

HMC3 microglia were incubated with 0.5µm fluorescent red latex beads (Sigma) in wells of a 12 well plate at a cell:bead MOI of 1:100 and, 3 hours later cells were washed 3x with PBS to remove remaining beads, and then fixed for 10 minutes in 2% PFA in PBS. After 3 more washes with FACS buffer (2% FBS, 1mM EDTA in PBS) cells were acquired in a BD FACS Canto II. Data was analysed in FlowJo (TreeStar). To generate representative images of phagocytic cells HMC3 microglia were incubated with beads as above but on glass coverslips, washed, fixed and then stained for Phalloidin-Alexa488 (1:100) in PBS 0.05% Triton X-100 and DAPI (1µg/mL) and mounted onto glass slides with FluoroShield (Sigma). Images were acquired through an HC PL APO 20x /0.75 IMM/CORR CS2 glycerol

objective with 1400Hz scanning speed, a zoom factor of 4.5x and pinhole set to 1 airy unit in a Leica TCS SP8 confocal microscope (Leica Microsystems, Germany). DAPI excitation was achieved with a Diode 405nm laser and emission captured by a PMT detector; Phalloidin-Alexa488 excitation was performed with an Argon 488nm laser with emission captured by another PMT detector; Fluorescent beads were excited by a HeNe 594nm laser and emission captured using a HyD detector. Confocal z-stacks with a depth of 5µm and a step size of 0.3µm for all channels were generated with a 1024x1024 pixel format, 8-bit depth. Orthogonal views of the stacks were created alongside the images in ImageJ/Fiji.

ROS production assay

HMC3 microglia seeded in wells of a 12 well plate were incubated for 30 minutes with CellROX® Deep Red reagent (ThermoFisher - 1µM per well) in the dark in their regular culture incubator. Afterwards cells were washed once with PBS, trypsinized, washed once with FACS buffer, transferred to FACS tubes, and immediately analysed in a BD FACS Canto II. In the case of Blebbistatin stimulation, cells were first incubated with 50µM Blebbistatin for 24 hours and then processed as above. They were then Analysis of the data was performed in FlowJo (TreeStar).

RNA extraction and qPCR

Total RNA from HMC3 microglia seeded onto wells of a 6-well plate was extracted using the Quick-RNA MiniPrep kit (Zymo Research, USA) according to the manufacturer's instructions and performing DNase I digestion. RNA concentration and quality were assessed in a Nanodrop ND1000. cDNA was synthesised from 500ng of total RNA using the SuperScript™ IV First-Strand Synthesis kit (Invitrogen). qPCR was performed on a CFX384 Real-time PCR detection system (Biorad) using the iTaq Universal SYBR Green Supermix (Biorad) and gene-specific primers (IL-1β fwd ATGATGGCTTATTACAGTGGCAA and rev GTCGGAGATTCGTAGCTGGA; IL-6 fwd ACTCACCTCTTCAGAACGAATTG and rev CCATCTTTGGAAGGTTTCAGGTTG; TNFα fwd GCTGCACTTTGGAGTGATCG and rev GCTTGAGGGTTTGCTACAACA; GAPDH fwd GCTCTCTGCTCCTCCTGTTC and rev ACCAAATCCGTTGACTCCGA). Relative quantification of the gene's expression was performed using the 2-ΔCt method, using GAPDH as the reference gene.

Statistics

Statistical analysis was performed using GraphPad Prism 8. All data distributions were tested for normality by the Shapiro-Wilk test. Normal distributions were analysed by unpaired two-tailed Student's t test (when comparing 2 groups), one-way ANOVA with Sidak multiple comparisons correction (when comparing 3 groups), two-way ANOVA with Tukey's multiple comparisons correction (when comparing 2 groups with 2 different variables each) or two-way ANOVA with Holm-Sidak multiple comparisons correction (when comparing 3 groups with 2 different variables each). Non-normal distributions were analysed by the Mann-Whitney non-parametric test (when comparing 2 groups) or the Kruskal-Wallis non-parametric test with Dunn's multiple comparisons correction (when comparing 3 groups). Significance levels and corresponding tests for experiments are all indicated in the figure legends.

Results

**Chapter 1 - The retina and *MorphoMacro*
as tools for *in vivo* and *ex vivo*
morphometric studies of microglia**

Introduction

One of the distinctive hallmarks of microglia that has been appreciated and studied ever since these cells were first described by Rio-Hortega more than a century ago is their intricate morphology (Sierra et al., 2016). Under homeostatic conditions, processes originating from a microglia cell body quickly develop into several sub-branches, culminating in a dense and complex arborization that maximizes the cell's capacity to survey the tissue microenvironment (Nimmerjahn et al., 2005). Conversely, under inflammatory or damaging conditions, microglia transition into less complex shapes, which can range from decreased ramification to a full amoeboid shape, where there are no more ramifications left (Karperien, Ahammer, & Jelinek, 2013; Stence et al., 2001). These changes in cell shape have been used as a classic, simple and direct indicator of microglia reactivity that can be easily assessed by imaging (Dubbelaar, Kracht, Eggen, & Boddeke, 2018; Karperien et al., 2013; Kettenmann et al., 2011). Since these shape changes are not always striking, an attempt to study and characterize the effects of candidate molecular regulators on cell morphology and function must be sensitive enough to detect small morphological differences. However, the experimental assessment of microglia shape is inherently complex, requiring the calculation of advanced parameters such as the number of branches, their associated arborization, length or thickness. Although the application of quantitative analysis to extract this type of information about microglia complexity has been a growing area of investigation, the existing options are either not available for free (Heindl et al., 2018; York, LeDue, Bernier, & MacVicar, 2018), are not openly shared (Davis, Salinas-Navarro, Cordeiro, Moons, & Groef, 2017; Fernandez-Arjona, Grondona, Granados-Duran, Fernandez-Llebrez, & Lopez-Avalos, 2017), or are very complex and time consuming (Arshadi, Günther, Eddison, Harrington, & Ferreira, 2021). Here, we propose the combined use of the retina as an experimental platform to image microglia shape, together with an image analysis tool we developed for the free and open access software ImageJ/Fiji as a simple and accessible tool to facilitate the study of changes in microglia morphology *in situ*.

Results

Use of the retina as a platform for studying *in situ* microglia morphology

Studying the detailed morphology of microglia is complicated by the fact that they lose most of their exuberant ramifications when cultured *in vitro* (Witting & Möller, 2011). As such, the best possible system for their study would not only retain or biomimic natural interactions with the surrounding CNS cells, but would also allow easy access to imaging and perturbations to be introduced. Although the work on microglia morphology has mostly focused on the brain, the retina is an equal or even better system. As a part of the CNS, it presents a laminar organization, neuronal complexity, and neurotransmitter diversity similar to other CNS regions (Masland, 2012; Pourcho, 2009), and also contains highly branched microglia cells, located mostly in the inner (IPL) and outer (OPL) plexiform layers (F. Li et al., 2019). Their processes have a dynamic behaviour that is in every way similar to what is displayed by brain microglia, constantly scanning their surrounding environment (Lee, Liang, Fariss, & Wong, 2008). Its anatomy also allows for easier *in vivo* and *ex vivo* manipulation, presenting advantages over the brain as an experimental platform (**Figure 1A**). First, a direct visualization of retina microglia morphology requires only simple dissection and fixation, followed by wholemount Iba1 staining, as opposed to direct visualization of brain microglia, which requires tissue embedding and sectioning after fixation and before cell staining (**Figure 1A top panel**). Secondly, although both tissues allow *ex vivo* stimulation through tissue culture, the dissection of retina into explants is easier to achieve and causes less tissue damage when compared to the use of brain slices, which also call for further specialized equipment to obtain (**Figure 1A bottom panel**). Finally, given the layered structure of the retina and the fact that microglia are mainly circumscribed to either the IPL or OPL layers, their shape is more naturally flattened when compared to brain microglia, increasing their complexity more along the x-y plane when imaged from above and decreasing their complexity along the z-axis (**Figure 1B**). This not only decreases the volume of tissue that needs to be sampled during image acquisition, but also facilitates the subsequent analysis of microglia morphology from the set of acquired images. As such, the retina is a model system that integrates the previously mentioned ideal requirements for the study of microglia morphology.

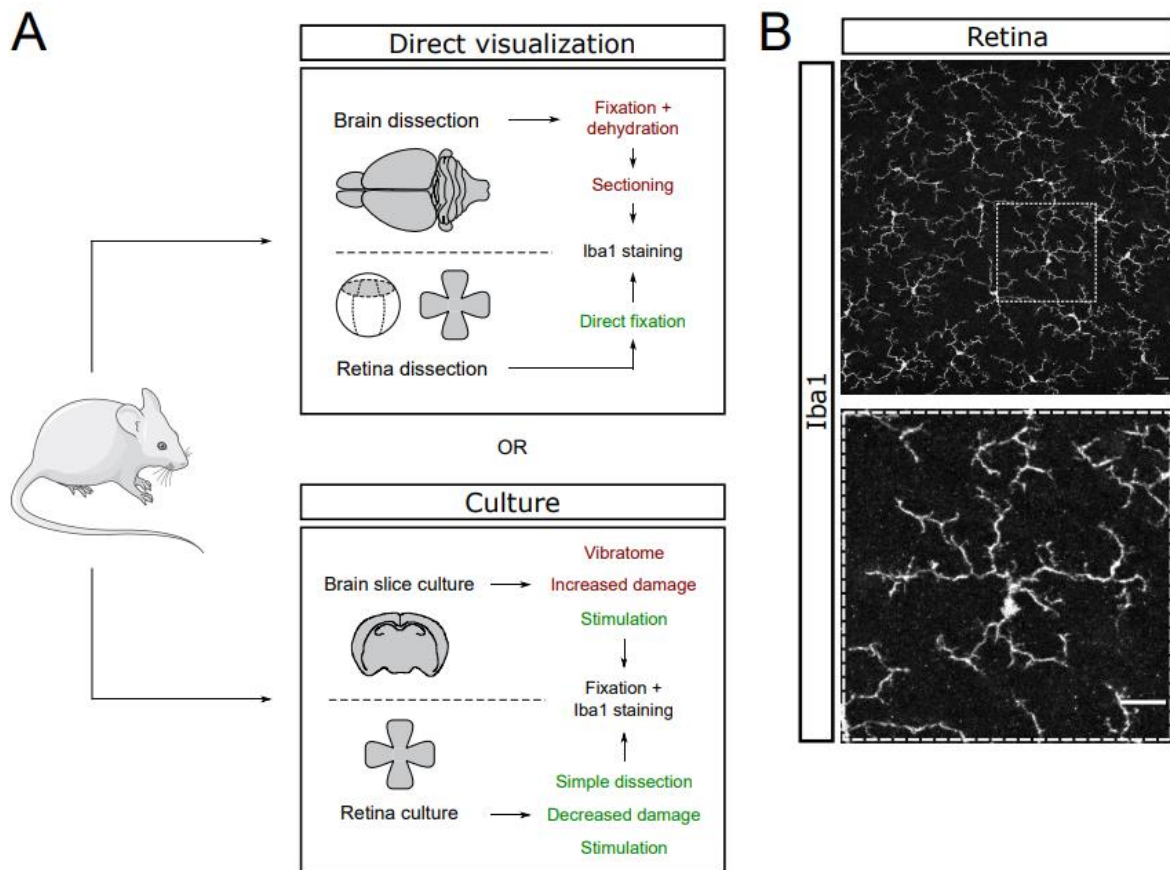


Figure 1 – The retina as a platform for the experimental study of microglia morphology. (A) *In situ* visualization and study of microglia morphology under homeostatic conditions can be achieved by Iba1 staining of murine brain and retina tissues (top panel), but direct fixation and staining of the retina constitutes a faster and simpler experimental setup, with no need for embedding or sectioning, when compared with the use of the brain. Short-term culture for studying the morphological response of microglia to exogenous stimulation can be performed using both CNS tissues (bottom panel), but the retina is more beneficial, since simple dissection reduces damage to the tissue without the need for special equipment. Advantages are highlighted in green, while disadvantages are marked in red. Mouse image was taken from the Servier Medical Art open access image collection.. (B) Representative image of microglia distribution (top panel) and morphology (bottom panel – inset) in mouse retina shows that they are easily visible cells and that their complexity is greater along the x-y axis and lower along the z-axis, facilitating the study of their morphological complexity. Scalebar corresponds to 20µm.

Development of a novel and open access tool for semi-automatic analysis of microglia morphology (*MorphoMacro*)

The complex and sometimes exuberant shape of microglia makes the study of their morphology an inherently difficult task. An acceptable investigation of their shape must look beyond common shape descriptors and complement these with complex parameters like

number and size of processes, their varying degrees of ramification, territorial areas, and their occupancy by the cell. Although Sholl analysis produces a good characterization of microglia complexity, its classical implementation does not provide accurate measurements of cell area or territory, and most importantly, provides no information about the length and number of global or individual processes (Ferreira et al., 2014). Although the methodologies for obtaining some of these parameters have been described, their implementation is difficult since either their code is not shared or is based on the use of software which is behind a pay-wall (e.g. MATLAB, NeuroLucida, Imaris, LMtrace). To overcome this difficulty, we developed a series of macros for the free and open-access image analysis software ImageJ/FIJI that semi-automatically analyse and extract these parameters from microglia confocal images using skeleton analysis. *Morphomacro* is a toolset that consists of eight separate macros that are executed in successive fashion, with each macro taking as input either the raw image data files or the output of previous macros from the toolset (Figure 2).

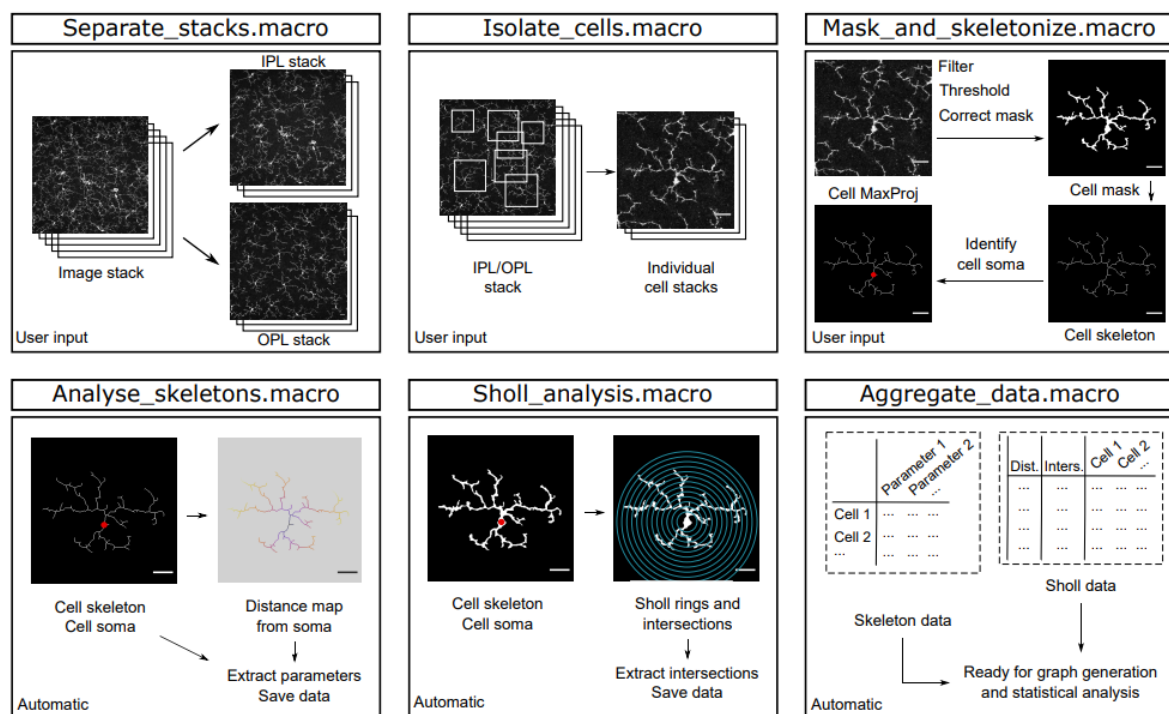


Figure 2 – The *MorphoMacro* toolset for semi-automatic analysis of microglia morphology. The *Separate_stacks* macro takes the raw image data confocal stack of Iba1-stained microglia and splits it into two sub-stacks: one containing IPL microglia and the other OPL microglia. The user must visually evaluate and manually identify the slices at which each of these substacks begins and ends. The macro *Isolate_cells* takes the previously generated substack and uses regions of interest manually defined by the user corresponding to individual cells to save the corresponding cell stack for subsequent analysis. The *Mask_and_skeletonize* macro automatically applies a series of image processing steps and suggests a mask for each individual cell, which the user manually refines, if necessary. The final mask is skeletonized and the user is asked to identify the location of the cell soma. This simpler representation readily allows the extraction of quantitative morphological

(continuation of Figure 2 legend) parameters. *Analyse_skeletons* macro creates a distance map from the cell soma to the cell edges along the skeleton and extracts a series of morphological parameters for the cell (see Figure 3 for more details). The *Sholl_analysis* macro implements Sholl analysis using the previously defined cell soma and cell mask. Finally, the *Aggregate_data* macro compiles the morphological parameters individually extracted for each cell into a single table file. It also creates a single table file containing all the individual cell Sholl curves. Both files facilitate subsequent graphing and statistical analysis.

The *Separate stacks* macro asks the user to inspect the desired stacks of confocal images of stained-retinal microglia and identify the slices that correspond to the IPL and OPL layers. It subsequently slices the original stack into two new sub stacks (IPL and OPL), which are then fed to the *Isolate cells* macro. This tool isolates user-identified cells and saves an individual image stack for each cell. Subsequently the *Mask and skeletonize* macro automatically creates a maximum projection of the stack, and then filters and thresholds it to generate a candidate mask. The user is then instructed to inspect this mask and, if necessary, to manually correct it by adding missing portions or removing incorrect sections. The final mask is then saved and used to generate a skeletonized version of the cell, where the user also identifies the position of the cell soma, capturing the cell's complexity and process information in a more readily analysable representation. The *Analyse skeletons* macro takes these cell skeletons and automatically generates a distance map from the cell soma to all process edges, allowing measurements of process length, and also extracts and saves a series of morphological parameters that are described below. These complement the output of the *Sholl analysis* macro which extracts the classical number of intersections radially out from the cell soma. Finally, the *Aggregate_data* macro collects the parameter values extracted individually per cell and compiles them into a single output table file, facilitating subsequent statistical analysis.

The implementation of Skeleton analysis by *MorphoMacro* allows it to complement Sholl Analysis by computing a total of eight morphological parameters for each cell. The skeleton representation of the cell allows it to efficiently analyse and extract three complexity parameters (**Figure 3A**). *End-points* are the regions at which the cell's processes terminate, while *junction-points* are all the points along the skeleton where it branches into two or more sections. These two parameters can also be used to compute a cell *ramification ratio*, corresponding to the ratio of end-points to junction points. Since an increasingly complex cell will have more and more junction points that are not connected to an end-point, its value decreases with increasing cell complexity. One of the advantages of Skeleton analysis is that it generates two precise area parameters (**Figure 3B**): *cell area* and *cell territory*, which is the tissue area that is bound by the cell and provides a measure of its surveillance capacity. The other great advantage is that it allows the analysis of a cell's processes, both

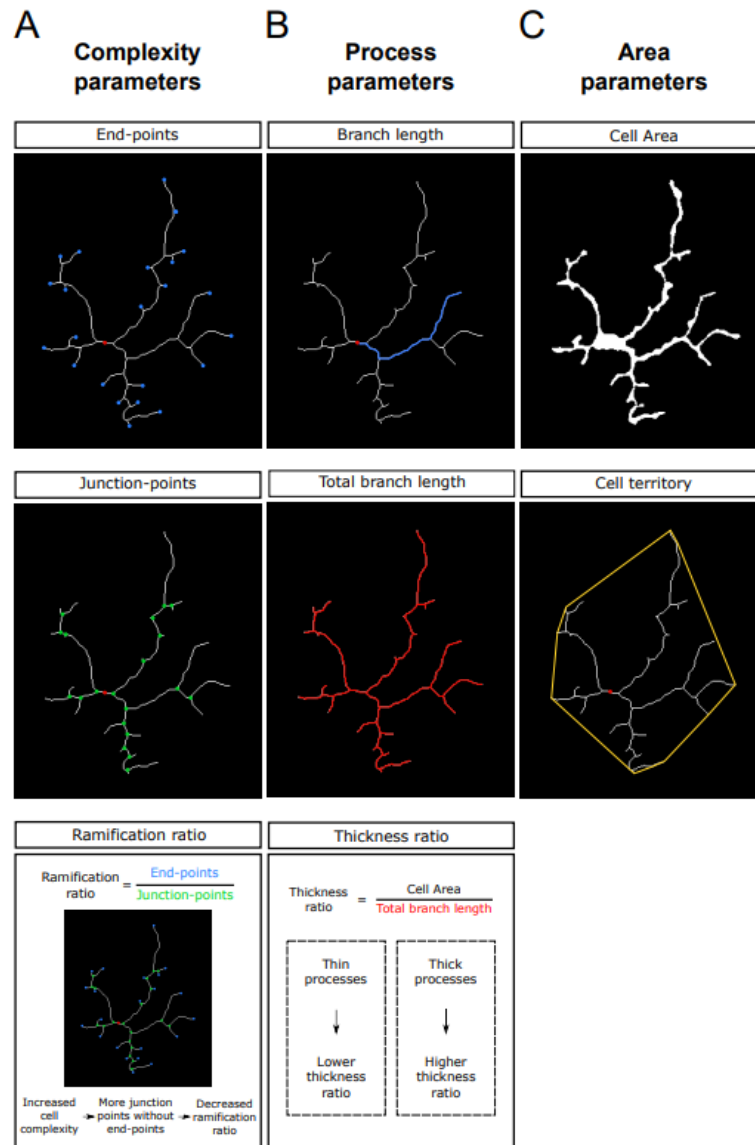


Figure 3 – Morphological parameters computed by MorphoMacro's skeleton analysis. (A) The macro computes three cell Complexity parameters: Cell junction points are defined as any points where the skeleton branches off into two or more segments (marked by green dots in the upper panel). Cell end points are any of the points where the skeleton ends (marked by blue dots in the middle panel). The ramification ratio is the ratio between end and junction points (bottom panel). Since complex cells have more junction points without any end-points linked to them, this ratio decreases as the cell's complexity increases. (B) Three Process parameters are also generated by the macro: Branch length is the distance along the skeleton from the cell soma (marked by red dot) to each of the skeleton's end-points (depicted in blue in the upper panel). Total branch length is the sum of the length of all skeleton segments (marked in red in the middle panel). The thickness ratio is the ratio between cell area and total branch length (bottom panel). Cells with low area and higher branch lengths are cells with thinner processes and a lower thickness ratio. Conversely high area and low branch length correspond to cells with thicker processes and a higher thickness ratio. (C) The area parameters computed by the macro are two: Cell area is the area occupied by the cell itself, computed as the area of the cell mask (upper panel). Cell territory is the tissue area that is bound by the cell (corresponds to the cell's convex hull – depicted in yellow on the bottom panel).

individually and globally, outputting three process parameters (**Figure 3C**). Branch length is the distance along the skeleton from the cell soma to each of the skeleton's end-points. Its values can be averaged to produce a cell mean branch length that provides an idea of how much the processes extend away from the cell soma. Then, in order to have an idea of how the length of each individual skeleton segment is added up to compute the total branch length, which serves as a global indicator of the cell's overall length. Finally, by taking the ratio between the cell's area and its total branch length, we can compute the cell's thickness ratio, which increases if the cell's processes are thick and decreases when they are thinner.

All in all, MorphoMacro's Sholl and skeleton analysis produce 9 morphological parameters that, when combined, have the potential to provide an in-depth characterization of microglia morphology.

***MorphoMacro* captures subtle morphological differences between microglia populations and quantifies their response to exogenous stimulation**

To test whether the outputs of MorphoMacro can be used to successfully study the complexity of retinal microglia cells, we first analysed and compared IPL microglia with OPL microglia from confocal stacks of retinas that were fixed and stained for Iba1 immediately after dissection (**Figure 4A**). Applying Sholl analysis alone to study these two cell populations does not readily detect major differences between them in terms of complexity (**Figure 4B**). The Sholl graph displays a tendency for decreased complexity of OPL cells at the radial distance of 35-45µm, but it does not reach statistical significance. Likewise, the complexity parameters generated by skeleton analysis are aligned with that assessment, since both the number of end points, junction points and ramification ratio do not differ between IPL and OPL cells (**Figure 4C-E**). However, skeleton analysis detects that both the area of the cells themselves (**Figure 4F**) as well as the territory that they occupy (**Figure 4G**) are smaller in OPL microglia. Furthermore, it also reveals that OPL microglia processes do not extend as much as the ones from IPL cells, given the smaller mean branch length (**Figure 4H**) and total branch length (**Figure 4I**), and likely explaining the observed values in cell territory. Finally, no differences in process thickness were observed between the two cell populations, as determined by their thickness ratios (**Figure 4J**). As such, the outputs of MorphoMacro's skeleton analysis detected differences between IPL and OPL microglia that Sholl analysis did not. Even if it did, differences in complexity on a Sholl graph are hard

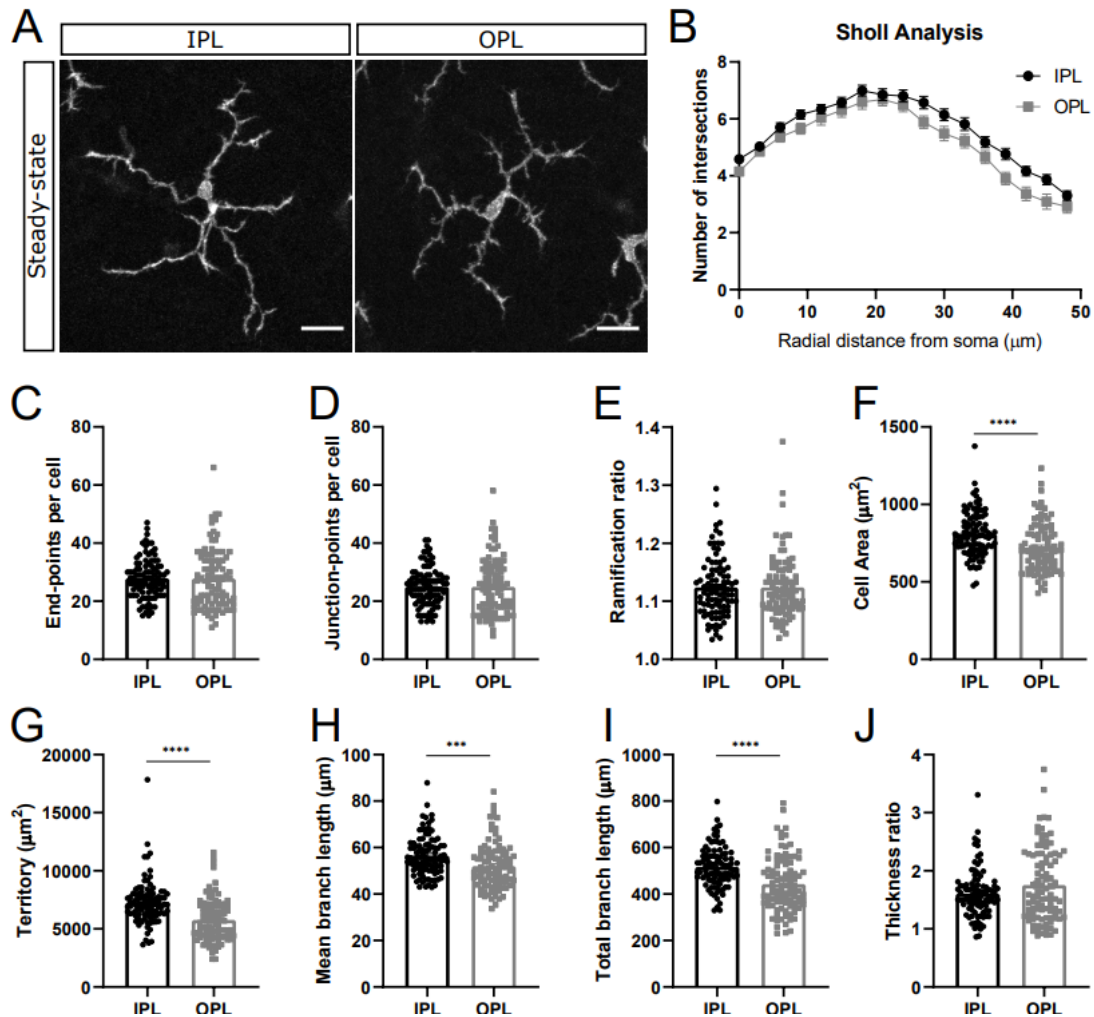


Figure 4 – MorphoMacro’s skeleton analysis detects slight differences between retinal microglia populations that are not detected by Sholl analysis. (A) Representative confocal microscopy images of Iba1-stained IPL (left panel) or OPL (right panel) microglia from the wholemount retinas of WT mice. Scale bars correspond to 20μm. (B) Sholl analysis of IPL and OPL microglia does not distinguish between the two cell populations (n=96 cells coming from 6 animals from 2 independent experiments, Two-way ANOVA with Holm-Sidak’s correction for multiple comparisons). (C-J) Complementary quantification of morphological parameters by skeleton analysis [number of cell end-points (C), number of cell junction points (D), ramification ratio (E), cell area (F), cell territory (G), mean branch length (H), total branch length (I) and thickness ratio (J)] shows that OPL microglia are slightly smaller and cover less tissue due to decreased process length (n=96 cells coming from 6 animals per group, from 2 independent experiments). *** P<0.001, **** P<0.0001 (Mann-Whitney U test). All Plots show mean +/- SEM.

to translate into a specific effect on cell shape, and how other cell parameters might be influenced by them. In this respect, the outputs of skeleton analysis augment the output of Sholl analysis, offering a more complete and detailed view of the differences in shape between cells. Most importantly, these results show that such an analysis method can detect subtle differences between microglia populations.

Given that microglia change shape in response to changes in their environment, we wanted to determine if *MorphoMacro* is also capable of quantifying such morphological differences. To test this, we used the retina as an *ex vivo* model system and easily cultured retinal explants immediately after dissection. This allowed us to expose them for a short time to ATP, a classical stimulus known to elicit microglia protrusion extension *in vivo* (Haynes et al., 2006). We then used *MorphoMacro* to evaluate confocal images of Iba1-stained IPL microglia (**Figure 5A**), since the cells of this layer showed a more robust and consistent response to this stimulant.

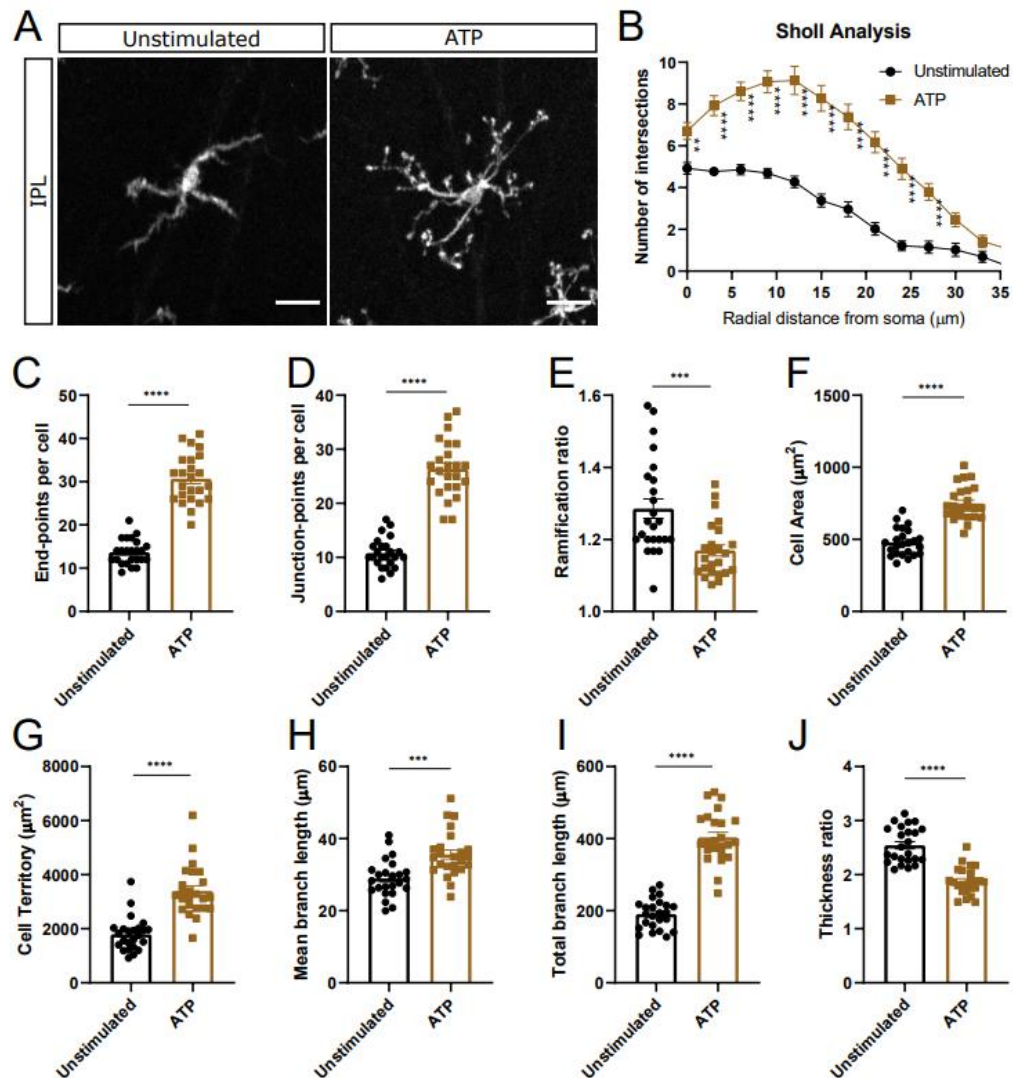


Figure 5 – The increase in microglia morphological complexity in response to ATP is captured by Sholl Analysis and further dissected by MorphoMacro’s Skeleton analysis. (A) Representative confocal microscopy images of Iba1-stained IPL microglia from wholemount retinas of WT mice that were either unstimulated (left panel) or stimulated with 1mM ATP (right panel) for 15 minutes before fixation. Scale bars correspond to 20µm. **(B)** Sholl analysis of unstimulated and ATP-stimulated microglia (n=24 cells coming from 4 animals from 2 independent experiments). **P<0.01, ****P<0.0001 (Two-way ANOVA with Holm-Sidak’s correction for multiple comparisons). **(C-J)** Skeleton analysis-derived morphological parameters [number of cell

end-points **(C)**, number of cell junction points **(D)**, ramification ratio **(E)**, cell area **(F)**, cell territory **(G)**, mean branch length **(H)**, total branch length **(I)** and thickness ratio **(J)** of unstimulated and ATP stimulated microglia (n=24 cells coming from 4 animals per group, from 2 independent experiments). ** P<0.01, *** P<0.001, **** P<0.0001 (Mann-Whitney U test). All Plots show mean +/- SEM.

Importantly, we observed an effect of culturing on the morphology of microglia, with cells displaying a much less complex arborization than seen when fixed and analysed directly after dissection (Fig.5A VS Fig.4A). Regardless, stimulation with ATP resulted in the expected extension of microglia processes, with a visible increase in their arborization. Sholl analysis detects this global increase in complexity, with consistently higher numbers of radial intersections from the cell soma up to a distance of 30 μm **(Figure 5B)**. Skeletonization analysis also confirms these results, given that it outputs an increase in both cell end points **(Figure 5C)** and junction points **(Figure 5D)** as well as a decrease in the arborization ratio **(Figure 5E)**. Additionally, it reveals an increase in both cell area **(Figure 5F)** and cell territory **(Figure 5G)**, supported by the extension of processes, as seen by the increase in both mean branch length **(Figure 5H)** and total branch length **(Figure 5I)**. The thickness of microglia processes also decreases in response to ATP and is clearly reflected by the computed thickness ratio **(Figure 5J)**. This shows that our analysis method is capable of not only detecting but also quantifying the differences in microglia cell shape elicited by *ex vivo* ATP stimulation. Also, they demonstrate how the parameters derived from skeleton analysis complement Sholl analysis, further confirming the validity of *MorphoMacro* as a tool for quantitative studies of microglia morphology.

Discussion

The study of the morphology of microglia is classically used as a first indicator of their activation status, and as such, of their particular function, since their shape changes in response to different events and environments (Dubbelaar et al., 2018; Karperien et al., 2013; Kettenmann et al., 2011). Given the number and complexity of their protrusions, simple qualitative inspections are only adequate when differences in morphology are evident, and are not enough to describe smaller, more subtle differences that can nonetheless occur. Thus, an ideal quantitative assessment of microglia shape calls for an experimental system that allow a complete study of microglia morphology *in situ*, both under steady state conditions but also in response to exogenous stimulation, capturing both more

and less obvious changes of shape. Our first step towards the development of such a system was the use of the retina as an experimental model. Its ease of access and manipulation allowed us to study the morphology of *in situ* microglia right after tissue dissection as well as after chemical stimulation after short term *ex vivo* culture in a much more straightforward way, and with no specialized equipment. Although in the case of explant culture there is an obvious effect on microglia, similar effects have been seen in organotypic brain slice cultures (Ogaki, Araki, Ishikawa, Ikegaya, & Koyama, 2020; Richter et al., 2020). Since the retina is easily accessible and naturally very thin, it can be cultured in explants using a very low number of simple cuts, with damage mostly contained to the area near the cut. In contrast, brain organotypic cultures suffer from more widespread damage due to the necessary slicing along their entire surfaces. The retina is also seen as a window into the brain (London, Benhar, & Schwartz, 2012), with several neurodegenerative conditions from the brain showing manifestations in the eye, such as Alzheimer's disease, Parkinson's disease and Multiple Sclerosis (Archibald, Clarke, Mosimann, & Burn, 2009; Chiquita et al., 2019; Petzold et al., 2010). Also, microglia have been described as critical elements in the pathophysiological process of retinal neuropathies such as diabetic retinopathy and glaucoma (Calkins, 2012; Massengill, Ahmed, Lewin, & Ildefonso, 2018). As such, the use of the retina as an experimental system further offers the possibility of studying in depth the dynamic response of microglia morphology and function in the context of multiple CNS disease models. All in all, we believe that the preserved CNS environment, easy accessibility, capacity for external manipulation and availability of CNS disease models make the retina a simple and valid alternative to the brain as a system for the study of microglia morphology in particular, but also for the study of CNS cell and molecular biology in general, a view that has also been gaining more traction in the field (Alarautalahti et al., 2019; Johnson & Martin, 2008; Murali, Ramlogan-Steel, Andrzejewski, Steel, & Layton, 2019; Schaeffer, Delpech, Albert, Belin, & Nawabi, 2020).

The second step we took towards the conception of a complete system for the quantitative study of microglia morphology was the development of *MorphoMacro*. Our tool sequentially implements both Sholl and Skeleton Analysis on individual microglia cells with as little user input as possible, taking advantage of available ImageJ/Fiji's built in functions and other plugins (Arganda-Carreras, Fernández-González, Muñoz-Barrutia, & Ortiz-De-Solorzano, 2010; Ferreira et al., 2014; Legland, Arganda-Carreras, & Andrey, 2016). This allowed us to extract a series of complex parameters in a semi-automatic way and accelerate the image analysis steps by reducing the amount of time traversing through multiple menus and manually saving data. Although the analysis of microglia in 2 dimensions leads to the loss

of some morphological information, the use of the retina helps reduce this caveat, since retinal microglia are naturally extended along the plexiform layer surfaces. This flat-like nature (especially at the OPL) decreases the likelihood of a three-dimensional analysis providing significantly more information than the simpler two-dimensional one. Also, 3D morphological analysis is inherently much more complex, creating other difficulties during analysis: ImageJ/Fiji is ill equipped to analyse 3D data and we would be introducing another level of uncertainty during whole cell reconstruction (along the z-axis) that would need to be checked and corrected by the user, increasing analysis time. When conducting our 2-dimensional analysis, on the rare cases where any given cell had a greater complexity along the z-axis that did not allow for its accurate reconstruction in 2D, we excluded them from analysis. Also, cells were analysed individually because, for any given acquired confocal stack containing a group of cells, they tended to occupy slightly different positions along the z-axis and also present little variations in Iba1-intensity between each other. Choosing a single sub-stack as well as common thresholding level for all cells would subsequently lead to poorly created masks, that would then need a lot of manual refinement. Although these intermediate steps improve the quality of the automatically generated cell mask candidate, the natural variability in microglia morphology and Iba1 staining did not allow us to find a combination of filtering and thresholding that is capable of fully automating cell segmentation in a satisfactory way, making manual user refinement a necessary and indispensable step. This step is the most time-consuming one among all of MorphoMacro's sub-steps, but is also present in other ImageJ/Fiji morphological analysis frameworks such as SNT (Arshadi et al., 2021) (whose cell tracing process is more complex and much more time consuming than MorphoMacro's) or on proprietary image analysis software like Imaris, NeuroLucida or Matlab. Regardless of the program or tool used, the execution of an accurate and trustworthy morphometrical analysis of cells as complex as microglia always requires a high level of care from the user to ensure that the final cell outline is as accurate as possible. This is especially important when subsequent skeleton analysis is to be applied, since only a complete cell mask allows the generation of a fully connected skeleton, without any breaks along its structure. The analysis of a fully connected skeleton opened the possibility of evaluating the cell's process length and overall branch length, which helps us infer if process formation and extension have occurred. It also made an assessment of process thickness possible by combining that information with the cell's area, a concept adapted from (Tribble et al., 2021). In order to facilitate the processing and comparison of the generated set of morphological parameters between different experimental groups, MorphoMacro automatically compiles all the generated data into a single file, further accelerating data analysis. Finally, we wanted to implement this analysis method on a free and open-access image analysis platform, since the existing alternative methods that could

achieve a similar analysis of cell morphology are either behind pay-walls (NeuroLucida, Imaris, Matlab, LMTrace), or are more complex and time consuming to perform (SNT). By developing this tool in ImageJ/Fiji, we also make it easier for other users to introduce their changes as desired, given the accessibility of the code, once it is published. We are also improving the code so that, in the future, it decomposes the cell skeletons into their primary, secondary and n-ary branches and determines their number and average/total length, further dissecting the cell's morphological complexity. Also, the current implementation of *MorphoMacro* relies on successive calls to different macros. We plan to develop a UI (user interface) that integrates them together in a single tool, making it even easier and more intuitive to use.

The application of *MorphoMacro* to Iba1-stained retinal microglia in both *in vivo* steady state conditions as well as *ex vivo* ATP stimulated ones showed that this analysis method detects both small and large morphological differences in microglia. This sensitivity is important, since it can help us understand not only the possible contribution of any microglia cell regulator in cell shape, but also quantify their specific morphological response to local stimulation or disease process. As such, the combined use of the retina as an experimental system and the application of *MorphoMacro* as an analysis instrument constitutes a valid and useful tool for future in depth studies of microglia morphology *in situ*.

**Chapter 2 - Morphofunctional
programming of microglia requires
distinct roles of type II myosins**

Introduction

Microglia are the resident innate immune cells of the central nervous system (CNS). Originating from erythromyeloid progenitors (Florent Ginhoux & Guilliams, 2016), they migrate early into the CNS, where they actively contribute to its development (Silverman & Wong, 2018; Thion, Ginhoux, & Garel, 2018). Afterwards they play an active role in the maintenance of CNS homeostasis, and also in the context of diseases (Silverman & Wong, 2018; Wolf, Boddeke, & Kettenmann, 2016). Known for their complex ramified morphology, microglia membrane protrusions, composed of dynamic actin filaments (Bernier et al., 2019), are constantly extending and retracting, ensuring proper surveillance of their microenvironment (Nimmerjahn et al., 2005). When they sense an insult, microglia re-orient their protrusions towards it (Davalos et al., 2005) and, through the combined activation of phagocytosis and release of soluble mediators, attempt to ensure a proper return of the tissue to homeostasis (Hanisch & Kettenmann, 2007). In this regard, their capacity for tissue surveillance and actuation are dependent on their ability to form and control membrane protrusions. As in other cell systems, the remarkable ability of microglia morphological change most likely emerges from an interplay between local actin polymerization (Thomas D. Pollard & Cooper, 2009) and variations in cell cortex tension (Chugh & Paluch, 2018; Clark, Wartlick, Salbreux, & Paluch, 2014; Salbreux et al., 2012). Cortical tension can be generated by actin and type-II myosin motors, the so called actomyosin cytoskeleton. In mammals type-II myosin motors are composed of essential and regulatory light-chains and one of three heavy chain isoforms (Myh9, Myh10 and Myh14) (Vicente-Manzanares, Ma, Adelstein, & Horwitz, 2009). The activity of these motors and affinity towards actin is defined by the heavy chain isoform that is present in the complex (Billington et al., 2013). Despite recent evidences reporting how changes in the turnover of actin filaments and/or action of myosin II motors impact tension generation (Chugh et al., 2017) and how these could dynamically control cell morphology, the molecular mechanisms that act at the level of the actomyosin cytoskeleton to allow the formation, maintenance and extension of protrusions in microglia still remain elusive.

In this study, we combine conditional genetic ablation in mice with quantitative image analysis of retinal microglia, *ex vivo* pharmacological studies and *in vitro* genetic manipulation to study the morphology and effector functions of microglia. Our results reveal important mechanisms in the control of microglia morphology and identify different contributions of type-II myosin heavy chain isoforms Myh9 and Myh10 in the regulation of microglia processes/morphology and inflammatory activation.

Results

RhoA plays an active role in the complexity of microglia morphology

Following our previous studies highlighting the importance of the small GTPase RhoA in microglia function (Socodato et al., 2020) and based on the regulatory dependence of actin cytoskeleton and type-II myosins on RhoA (Schwartz, 2004), we first studied how RhoA impacts the morphology of microglia. We used the retina as a model system, given the advantages established previously in this manuscript (Chapter 1 of Results section). We generated RhoA-deficient microglia (**Fig.1A**) by crossing RhoA-floxed mice (Herzog et al., 2011; Jackson et al., 2011) with mice expressing Cre-recombinase under the control of the microglia promoter Cx3Cr1 (Cx3Cr1^{CreER-IRES-YFP} mice) (Goldmann et al., 2013; Parkhurst et al., 2013).

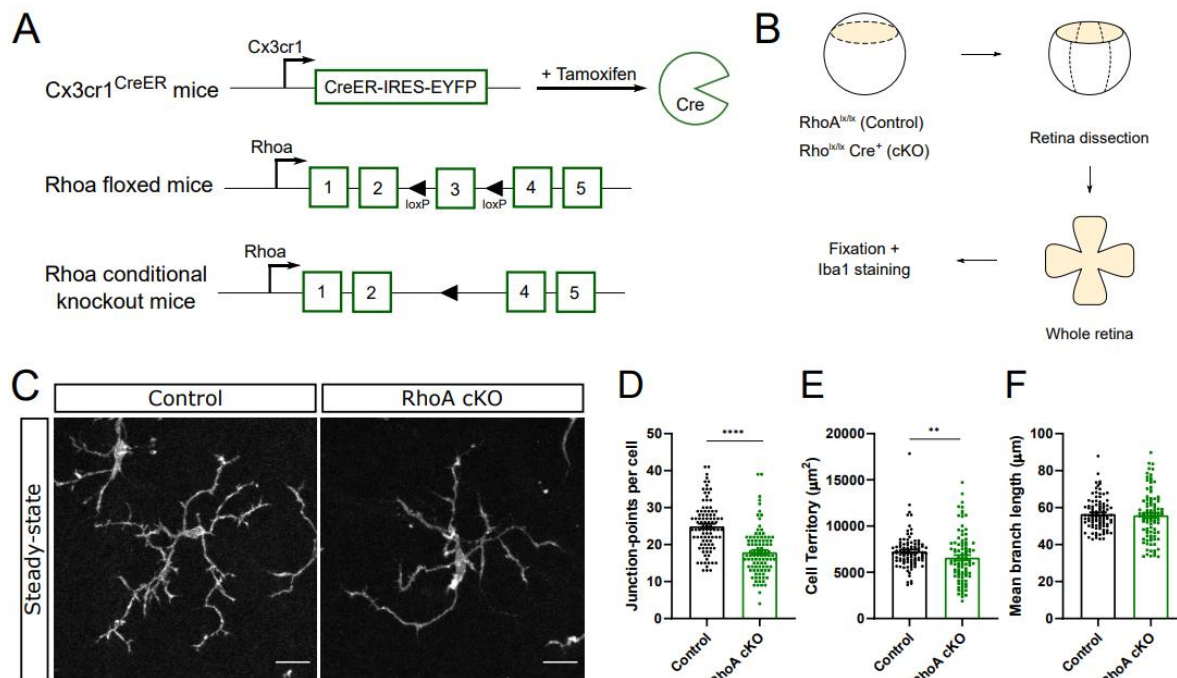


Figure 1: RhoA ablation decreases the complexity of microglia morphology. (A) Animal breeding strategy to ablate RhoA in microglia. Mice harbouring CreER-IRES-YFP in the Cx3cr1 locus (top), crossed with mice whose exon 3 of RhoA is flanked by loxP sites (middle), generate mice in which RhoA ablation in microglia and macrophages is achieved upon tamoxifen administration (bottom). (B) Experimental layout for evaluating retinal microglia morphology. Whole retinæ are dissected from the eyes of control mice (carrying floxed RhoA exon 3) or conditional knockout (cKO) mice (crossed as in panel A) and are immediately fixed and processed for staining of microglia marker Iba1. (C) Representative confocal microscopy images of Iba1-stained microglia from the wholemount retinas of control and RhoA cKO mice. (D-F) Quantification of the impact of RhoA ablation in

microglia morphology (n = 96 cells coming from 6 animals per group, from 2 independent experiments), using 3 parameters: number of cell junction-points (D), cell territory (E) and mean branch length (F). ** P<0.01, **** P<0.0001 (Mann-Whitney U test).

Tamoxifen administration leads to RhoA ablation in all Cx3cr1⁺ cells but, after one month, only ablated microglia remain, since other Cx3cr1⁺ macrophage/monocyte populations with fast turnover have in that time been naturally replaced from non-recombined bone marrow precursors (Socodato et al., 2020). Characterization of retinal microglia morphology was performed on dissected whole retinas that were fixed immediately after dissection, stained against the microglia marker Iba1 (Sasaki, Ohsawa, Kanazawa, Kohsaka, & Imai, 2001) and imaged by confocal microscopy (**Fig.1B**). In order to quantitatively analyse the morphology of microglia we used *MorphoMacro*, as discussed and validated previously in this thesis manuscript (Chapter 1 of Results section). Since some of the parameters that it generates are redundant (end-points, junction points, arborization ratio and the Sholl plot, for example), we focused on a set of 3 parameters that still capture both the complexity of the cells, their surveillance capacity and the properties of their processes: junction points, territory and mean branch length. Using this novel approach, we compared the morphology of control microglia (RhoA-floxed mice) with that of RhoA-deficient microglia (RhoA cKO) (**Fig.1C**). We observed that under steady state conditions microglia lacking RhoA are less complex, displaying a smaller number of protrusions, as evidenced by fewer junction points per cell (**Fig.1D**). Concomitantly we also observed a decrease in the territory that they occupy (**Fig.1E**). Since the average branch length was similar in both conditions (**Fig.1F**), the loss in territory is due to the decrease in number of protrusions, and not due to them being shorter.

RhoA controls protrusions length and stability

Microglia membrane protrusions are highly dynamic structures, constantly extending, probing and retracting (Nimmerjahn et al., 2005). Given the observed decrease in complexity in RhoA deficient microglia we wondered if this could be due to impaired formation of protrusions or to increased instability of existing ones. In order to experimentally determine this we cultured retinal explants immediately after dissection and exposed them for a short time to adenosine tri-phosphate (ATP) (**Fig.2A**), a classical stimulus known to elicit microglia protrusion extension *in vivo* (Haynes et al., 2006), and once again evaluated cell morphology after confocal imaging of Iba1-stained microglia. Importantly, we observed an effect of culturing on the morphology of microglia, with cells

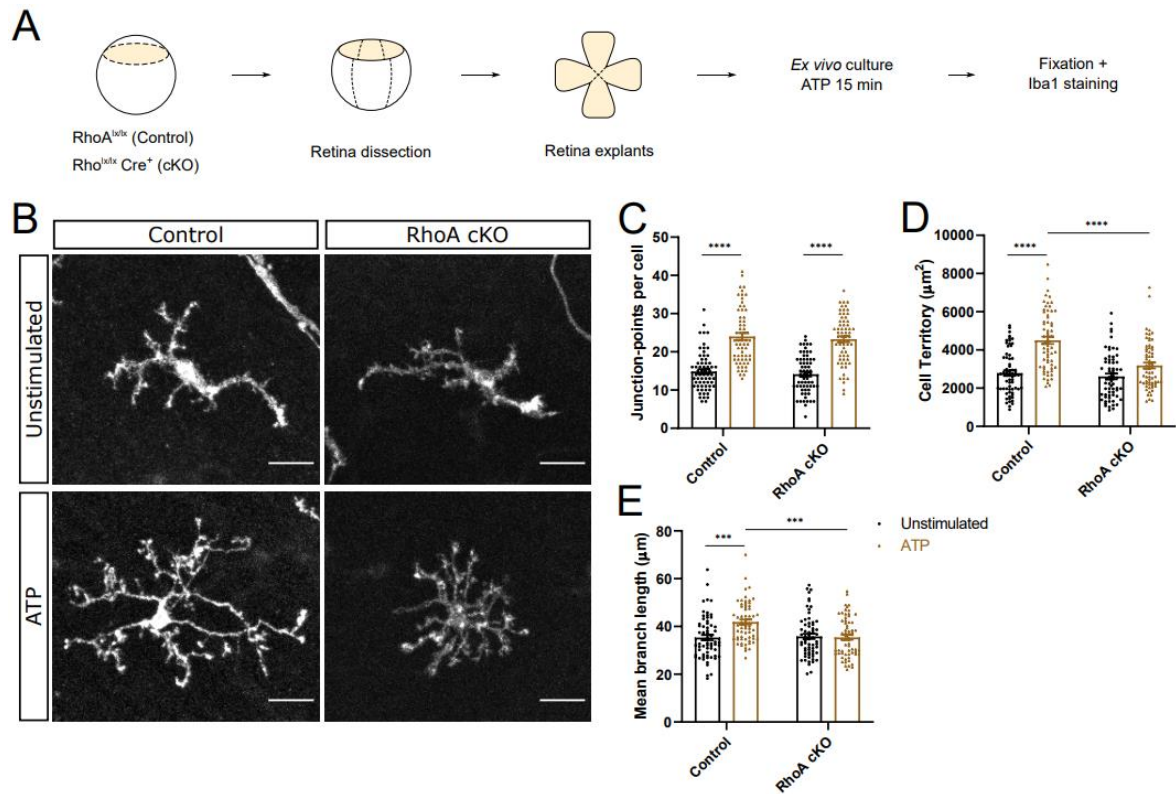


Figure 2: RhoA is not necessary for the extension of new microglia protrusions, but influences their maintenance and length. (A) Experimental layout for evaluating the morphological response of retinal microglia to ATP, a stimulus that induces extension of new protrusions. Whole retinæ are dissected from the eyes of wild-type mice, cut into 4 explants, and cultured *ex-vivo* in Ringer solution with or without 1mM ATP for 15 minutes, before fixation and staining with microglia marker Iba1. (B) Representative confocal microscopy images of Iba1-stained microglia in the presence or absence of 1mM ATP on retinas from control and RhoA cKO mice. (C-E) Quantification of the impact of RhoA ablation in microglia's morphological response to ATP, by assessing the number of cell junction-points (C), cell territory (D) and mean branch length (E). (n = 65 cells coming from 4 animals per group, from 2 independent experiments). *** P<0.001, **** P<0.0001 (Two-way ANOVA with Tukey's correction for multiple comparisons). All scale bars correspond to 20μm. All Plots show mean +/- SEM.

displaying a much less complex arborization than seen when fixed and analysed directly after dissection (**Fig.2A** VS **Fig.1C**). Under these conditions we no longer observed a difference in the morphology of control and RhoA knockout microglia in the absence of stimulation, likely due to these cells being basally activated by the culturing process. However, addition of ATP allowed us to observe that RhoA-deficient microglia reacted differently to this stimulus than control cells (**Fig.2B**). While both cells responded to ATP by increasing their complexity, as evidenced by a similar increase in the number of junction points per cell (**Fig.2C**), only control microglia showed an increase in the amount of territory surveyed (**Fig.2D**). This was due to an inability of RhoA-deficient microglia to extend the newly-formed protrusions to the same level as control cells (**Fig.2E**). These results show that RhoA is dispensable to the formation of new protrusions in microglia, instead acting as

an important regulator of their length once they are formed. As such, since the formation of new protrusions is not impaired by RhoA ablation, this regulator is also likely to play a role in the stability of microglia protrusions, explaining the results seen in direct *ex vivo* retinal microglia (results from Fig.1).

RhoA ablation impairs Cofilin activity and actin depolymerisation

Although it is known that ATP elicits microglia protrusion extension (Haynes et al., 2006), it remains poorly understood the downstream mechanisms that occur at the level of the actomyosin cytoskeleton. Given that RhoA is a classical regulator of actin polymerization and depolymerization via profilin and cofilin (A. J. Ridley, 2001), and that we uncovered a role for RhoA in microglia protrusions, we characterized by western blot the status of these actin-binding proteins, in an attempt to identify some of these mechanisms. Since an accurate measurement of these proteins in microglia is not feasible in whole retinal extracts and that isolation of retinal microglia would dramatically disrupt their steady state, we used a CRISPR-Cas9 strategy (**Fig.3A**) to generate *in vitro* a RhoA knockout HMC3 microglia cell model (**Fig.3B**). Phosphorylation of profilin at S137 is associated with a decrease in actin polymerization (Sathish et al., 2004; Shao, Welch, DiProspero, & Diamond, 2008), while S3-phosphorylation of cofilin inhibits its depolymerizing/severing capacity (Moriyama, Iida, & Yahara, 1996). Using protein extracts from HMC3 microglia we assessed the levels of these markers to understand if RhoA ablation in microglia affects actin polymerization or depolymerization. We detected a significant increase in p-cofilin(S3) (**Fig.3C**), indicating that actin severing/depolymerization is compromised in microglia that lack RhoA. Regarding profilin we observed no significant differences in the levels of p-profilin(S137) (**Fig.3D**) indicating its ability to enhance polymerization is not compromised in the absence of RhoA in microglia. However, although profilin facilitates actin polymerization by enhancing actin's exchange of ADP for ATP (Witke, 2004), its activity *per se* cannot ensure sustained actin polymerization in the absence of actin monomers. Replenishment of actin monomers is achieved via severing and depolymerization of existing filaments, predominantly achieved through cofilin activity (Kiuchi, Ohashi, Kurita, & Mizuno, 2007). Furthermore, cofilin activity also generates free barbed-ends from which actin polymerization can occur at the cell edge (Ghosh et al., 2004). As such, we wondered if the observed low activity of cofilin could be negatively affecting actin polymerization in our cells. To verify this hypothesis we assessed the levels of filamentous actin in control and RhoA-deficient cells by staining them with fluorescent phalloidin. Interestingly we observed a decreased level of F-actin in cells ablated

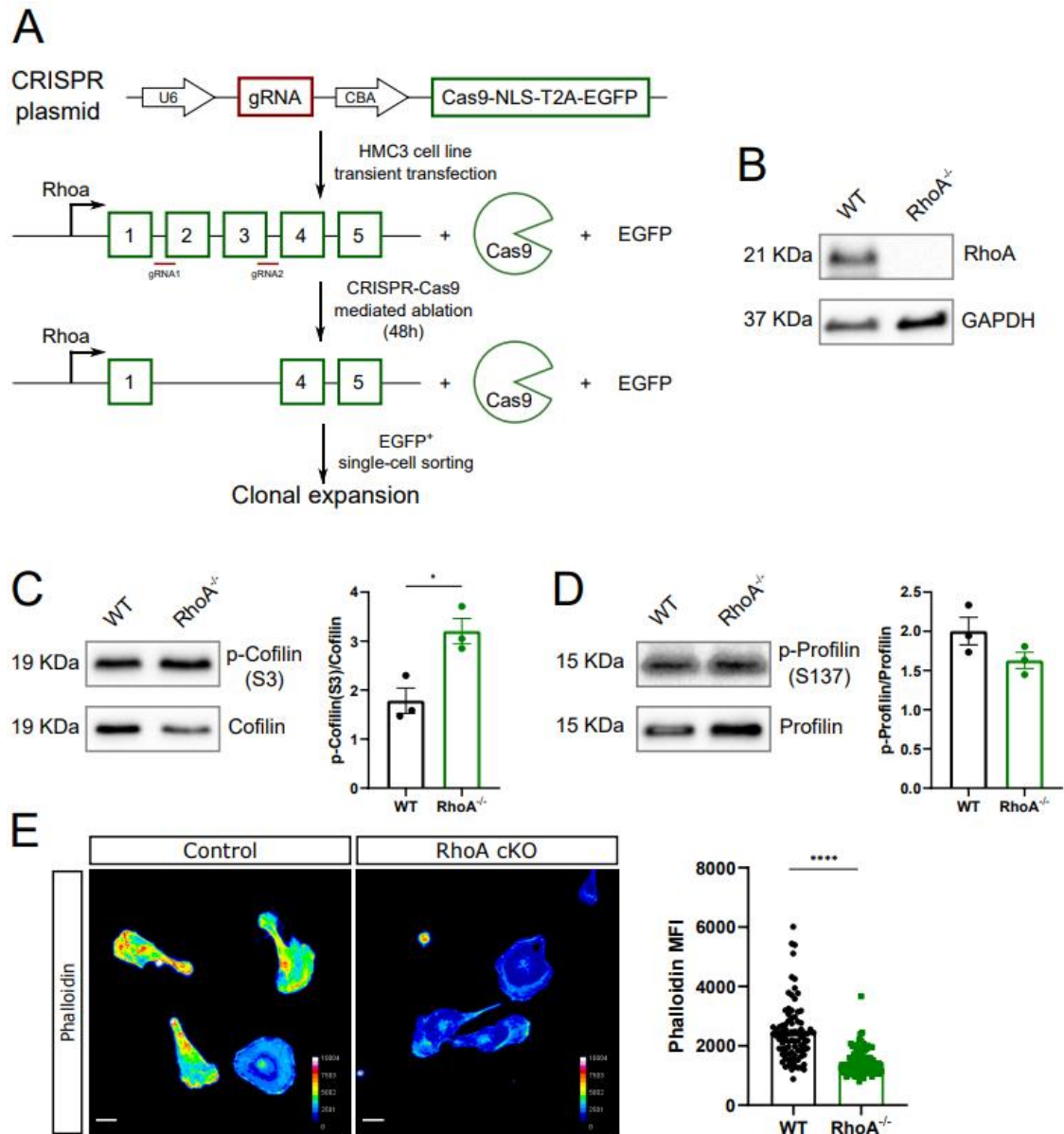


Figure 3: Decreased actin polymerization via inhibition of severing occurs in the absence of RhoA. (A) Strategy for RhoA ablation in HMC3 microglia *in vitro* via CRISPR-Cas9. A single plasmid encoding Cas9, GFP and guide RNA (gRNA) is transiently transfected into cells (top). A combination of two gRNAs directs Cas9 to the RhoA gene downstream of exon 2 and upstream of exon 3 (middle), resulting in RhoA deletion and expression of GFP, which is used to isolate single-cell clones by sorting (bottom). **(B)** Western blot depicting absence of RhoA protein from extracts of WT and RhoA^{-/-} HMC3 microglia cells generated according to the strategy in A. GAPDH was used as loading control. **(C)** Representative western blot (left) for pCofilin (S3) and cofilin. Quantification (right) shows an increase in inactivated cofilin expression in RhoA^{-/-} HMC3 microglia cells (n=3 independent cultures). * P<0.05 (unpaired t-test). **(D)** Representative western blot (left) for pProfilein (S137) and profilin. No differences in profilin activation are observed in HMC3 microglia RhoA^{-/-} cells (right, n=3 independent cultures, unpaired t-test). **(E)** Representative images of Phalloidin-stained WT and RhoA^{-/-} HMC3 microglia (left) show a decrease in actin filaments in the absence of RhoA (right). Graph depicts mean +/- s.e.m, n= at least 88 cells per group from 3 independent experiments. **** P<0.0001 (Mann-Whitney U test). Scale bar corresponds to 20µm. Ramp represents min/max Phalloidin intensity using the 16 colors LUT. All Plots show mean +/- SEM.

for RhoA (**Fig.3E**), as well as in primary microglia cells silenced with RhoA shRNA (**Fig.4**), indicating that actin polymerization is indeed impaired in these cells. Since actin polymerization is essential for cells to extend at leading edges (T. Svitkina, 2018), these results explain the observed impairment in protrusions extension of RhoA-deficient cells in response to ATP. They also identify cofilin-mediated actin turnover as a mechanism used by microglia to achieve adequate extension of protrusions, by ensuring availability of monomeric actin.

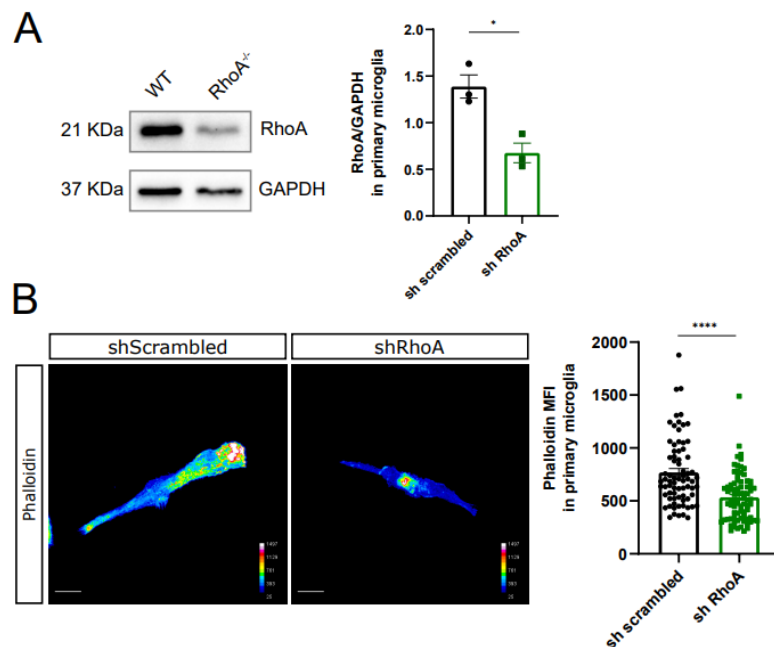


Figure 4: shRNA silencing of RhoA in mouse primary microglia decreases actin polymerization. (A) Western blot showing silencing of RhoA on primary microglia transduced with lentiviral particles carrying control (scrambled) or RhoA-shRNA. GAPDH was used as loading control. **(B)** Representative images of Phalloidin-stained control and shRhoA-silenced primary microglia show a decrease in actin filaments in the absence of RhoA. Graph depicts mean $\pm$ s.e.m, n= at least 74 cells per group from 3 independent experiments. **** $P < 0.0001$ (Mann-Whitney U test). Scale bar corresponds to 20 μ m. Ramp represents min/max Phalloidin intensity using the 16 colors LUT. All Plots show mean $\pm$ SEM.

Myosins type-II differentially impact the morphology of microglia *in vitro*

Aside from changes in polymerization and depolymerization, the dynamics of the actin cytoskeleton are further enhanced by the action of myosin motors, generating changes in tension responsible for alterations in cell shape (Salbreux et al., 2012). Since cytoskeletal tension in cells can be mediated by the action of type-II myosin motors (Vicente-

Manzanares et al., 2009), and that RhoA is a classical regulator of these molecules we next assessed the impact of RhoA on their activation state by evaluating the levels of S19-phosphorylated MLC2 (myosin regulatory chain) in protein extracts from HMC3 microglia by Western blot (**Figure 5A**), which showed an expected decrease in the amount of pMLC2(S19) in the absence of RhoA.

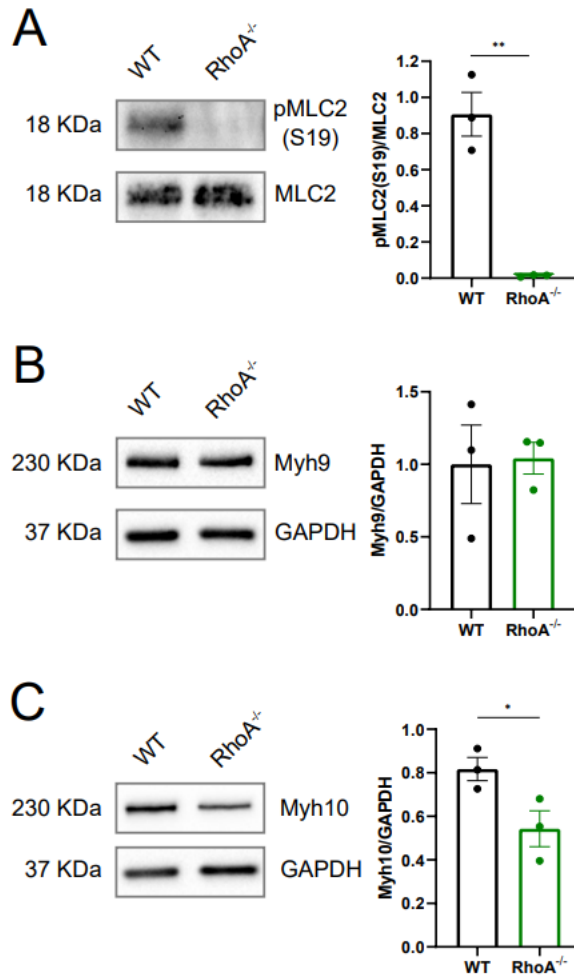


Figure 5: Absence of RhoA impairs Type-II myosin activation and differentially affects heavy chain expression. (A) Representative Western blot (left) for pMLC2 (S19) and MLC2 from extracts of WT and RhoA^{-/-} HMC3 microglia. Quantification (right) shows a decrease in activated MLC2 in the absence of RhoA. (n=3 independent cultures). * P <0.01 (unpaired t-test). **(B)** Representative western blot (left) for Myh9 from extracts of WT and RhoA^{-/-} HMC3 microglia. GAPDH was used as loading control. No differences in Myh9 levels are detected in HMC3 microglia RhoA^{-/-} cells (right, n=3 independent cultures, unpaired t-test). **(C)** Representative western blot (left) for Myh10. GAPDH was used as loading control. Quantification (right) reveals a decrease in Myh10 expression in HMC3 microglia RhoA^{-/-} cells (n=3 independent cultures). * P <0.05 (unpaired t-test).

Interestingly, when we evaluated the expression of Myh9 and Myh10, the myosin-ii heavy-chain isoforms expressed by microglia (M. L. Bennett et al., 2016), our analysis also revealed that RhoA ablation did not change the expression of Myh9 (**Fig.5B**), but specifically decreased the expression of Myh10 (**Fig.5C**). Given this differential impact of RhoA on myosin-ii expression, and knowing that these isoforms play different roles in mouse development (Conti et al., 2004; Takeda, Kishi, Ma, Yu, & Adelstein, 2003; Tullio et al., 1997), in cell junctions (Smutny et al., 2010), intracellular membrane dynamics (Milberg et al., 2017) and leading edge protrusion and cell migration (Betapudi, Licate, & Egelhoff, 2006), we wondered if these isoforms could have separate contributions to microglia

morphology. To test this, we used lentiviral delivery of shRNA (**Fig.6A**) to silence Myh9 and Myh10 on HMC3 microglia (**Fig.6B and C**), since CRISPR-mediated attempts to knock these proteins out only worked for Myh10 and failed for Myh9, likely given its role in cytokinesis.

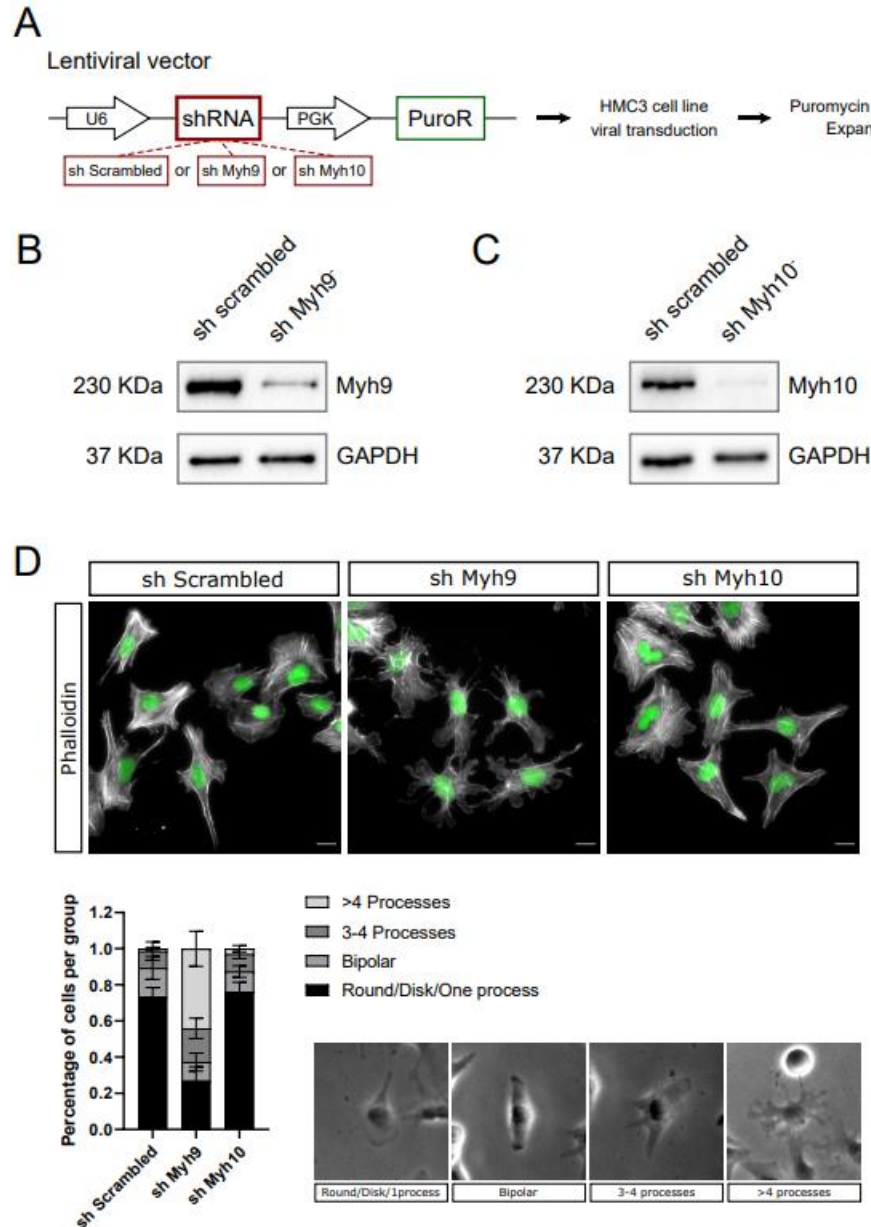


Figure 6: Myh9-silencing impacts the morphology of microglia *in vitro*, increasing the cell's complexity.

(A) Strategy for myosin ablation in HMC3 microglia *in vitro* using shRNA. Lentiviral delivery of control (scrambled), Myh9 or Myh10-targeting shRNA, together with acquisition of puromycin resistance leads generates stable cell lines where specific myosin-ii isoforms are silenced. (B) Western blot showing silencing of Myh9 on HMC3 microglia after puromycin selection of cells transduced with lentiviral particles carrying control (scrambled) or Myh9-shRNA. GAPDH was used as loading control. (C) Western blot showing silencing of Myh10 on HMC3 microglia after puromycin selection of cells transduced with lentiviral particles carrying control (scrambled) or Myh10-shRNA. GAPDH was used as loading control. (D) Representative wide-field microscopy images of control (top left panel) and myosin isoform-silenced HMC3 microglia (top middle and right panels),

| Chapter 2 - Morphofunctional programming of Microglia requires distinct roles of type II myosins

stained with DAPI (nuclear) and Phalloidin (F-actin). The graph quantifies cells according to their number of protrusions, showing that Myh9-silencing increases the complexity of *in vitro* HMC3 microglia, while Myh10-silencing does not affect the cell's morphology (n= at least 50 cells per field of view, from 3 independent experiments with 3 fields of view per experiment). Small figures are phase contrast example images of each of the four categories quantified. All scale bars correspond to 20µm. All Plots show mean $\pm$ SEM.

Phalloidin staining of control (scrambled) or myosin-ii shRNA showed that Myh9-silencing impacted the morphology of HMC3 microglia, with cells becoming more ramified, as evidenced by the increase in the number of cells with 3 or more protrusions (**Fig.6D**), while no changes were seen in cells silenced for Myh10. To exclude the possibility that these results were due to compensation by increased expression of the non-silenced isoform, we analysed the levels of Myh10 in Myh9-silenced cells (**Fig.7A**) and of Myh9 in Myh10-silenced cells (**Fig.7B**) but detected no changes in their expression when compared to control cells.

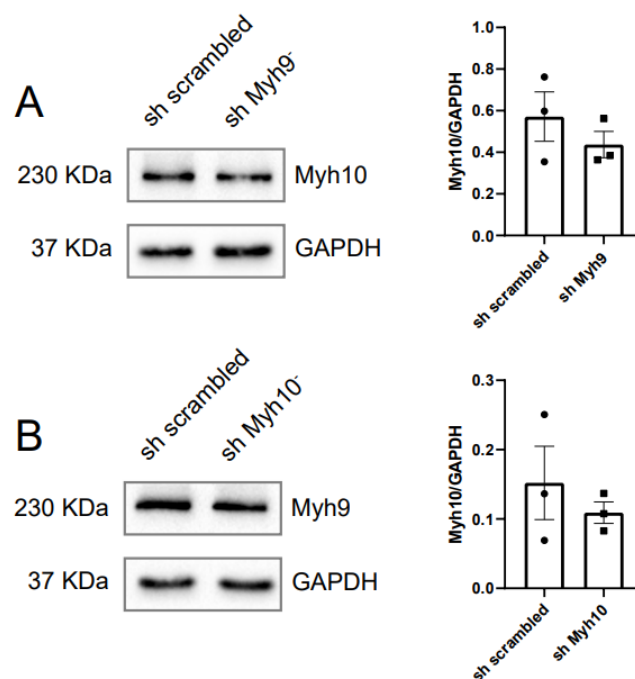


Figure 7: shRNA myosin-ii knockdown does not affect the expression of the other myosin isoform. (A) Western blot images (left) of Myh10 in Myh9-silenced cells and its quantification (right). GAPDH was used as loading control. **(B)** Western blot images (left) of Myh9 in Myh10-silenced cells and its quantification (right). GAPDH was used as loading control. All Plots show mean $\pm$ SEM. n=3 independent experiments. No statistically significant difference was detected (unpaired student's t test).

As such, our results show that Myh9, but not Myh10, regulates the shape of microglia and that its absence dictates an increase in morphological complexity.

Myh9 controls cortical stress and microglia morphological complexity

To understand why microglia morphology is specifically linked to changes in Myh9 we assessed the subcellular distribution of the two myosin isoforms together with actin filaments. For this, we fluorescently stained control HMC3 microglia and primary mouse microglia cells for Myh9 or Myh10, and F-actin and imaged the cells by microscopy (**Fig.8A and Fig.9A**).

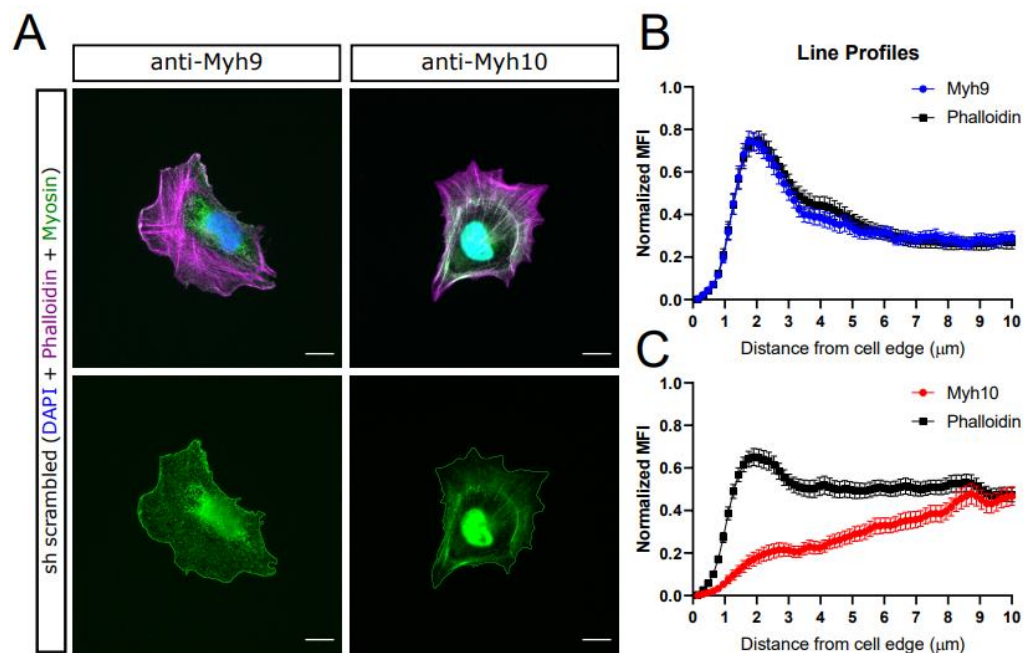


Figure 8: Myh9 and Myh10 are differently distributed throughout microglia cells. (A) Representative wide-field microscopy images of scrambled (control) HMC3 microglia stained with DAPI (blue), phalloidin (magenta) and anti-Myh9 (green, left panels) or Myh10 antibodies (green, right panels). Lower panels display only the myosin signal, with the cell's edge drawn as a line for reference. (B) Line profile of the fluorescence intensity of phalloidin (black) and Myh9 (blue) along a line drawn perpendicular to the cell edge and into the cell, normalized to the maximum value along that line, showing that Myh9 is locally concentrated near the edge and follows the local distribution of phalloidin. (C) Line profile of the fluorescence intensity of phalloidin (black) and Myh10 (red) along a line drawn perpendicular to the cell edge and into the cell, normalized to the maximum value along that line, demonstrating that Myh10 is concentrated further away from the border. n = at least 12 cells per group from 3 independent experiments.

Quantification of the signal intensities from the edge to the centre of the cell showed a peak for Myh9 distribution near the edge (approximately 2μm from the border) coinciding with the local distribution of actin, seen by phalloidin staining. (**Fig.8B and Fig.9B**). As for Myh10,

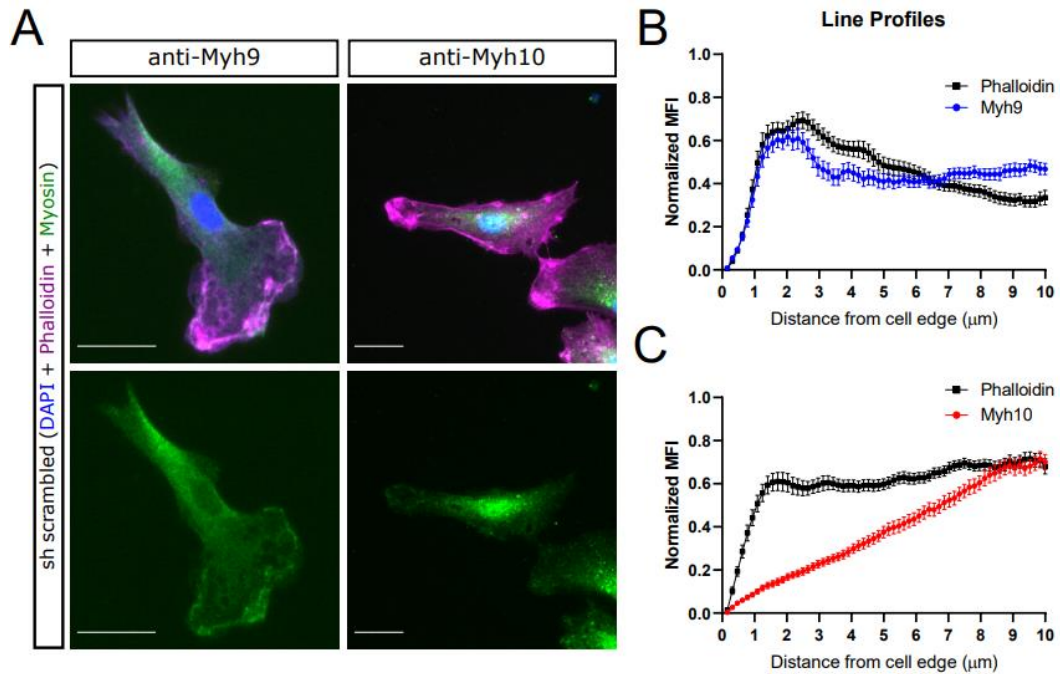


Figure 9: Myosins in primary microglia show the same distribution of HMC3 microglia cells. (A) Representative wide-field microscopy images of primary microglia stained with DAPI (blue), phalloidin (magenta) and anti-Myh9 (green, left panels) or Myh10 antibodies (green, right panels). Lower panels display only the myosin signal. **(B)** Line profile of the fluorescence intensity of phalloidin (black) and Myh9 (blue) along a line drawn perpendicular to the cell edge and into the cell, normalized to the maximum value along that line, showing that Myh9 is locally concentrated near the edge and follows the local distribution of phalloidin. **(C)** Line profile of the fluorescence intensity of phalloidin (black) and Myh10 (red) along a line drawn perpendicular to the cell edge and into the cell, normalized to the maximum value along that line, demonstrating that Myh10 is concentrated further away from the border. $n = 12$ cells per group from 3 independent experiments. All scale bars correspond to $20\mu\text{m}$. All Plots show mean $\pm$ SEM.

its distribution displays a peak further behind, closer to the centre of the cell (approximately $10\mu\text{m}$ from the border), where it likewise coincides with the local distribution of actin (**Fig.8C and Fig.9C**). These results show that in microglia Myh9 is more peripherally distributed, near the edge of the cell, while Myh10 is more centrally located. Since both can engage local actin filaments to generate tension, their differential distribution suggests that Myh9 contributes more to peripheral tension generation. Tension in the cortex hinders the extension of protrusions by countering local actin polymerization (Burnette et al., 2011; T. Svitkina, 2018). Since protrusion number and length were increased in the absence of Myh9, we hypothesized that Myh9-mediated cortical tension hinders protrusion formation and therefore a release in cortex tension is necessary for microglia protrusion generation and extension to occur. To determine if this was indeed happening, we expressed a molecular tension-sensing probe, Actinin-sst FRET (Meng & Sachs, 2011), in control and type-II myosin-silenced HMC3 microglia, in order to map the spatial distribution of stress

throughout the cortex of the cells using FRET microscopy (**Fig.10A**). Interestingly, we did not observe any effect of Myh9 or Myh10 ablation on global cortical stress levels (**Fig.10B**), indicating that even in the absence of a particular myosin-ii isoform, microglia are able to compensate and maintain whole-cell cortical tension levels constant. However, when we focused on the cell's border (**Fig.10A insets**) we observed a local decrease in cortical stress in the region adjacent to the edge (1-3 μ m) in cells silenced for Myh9, but not for Myh10 (**Fig.10C**).

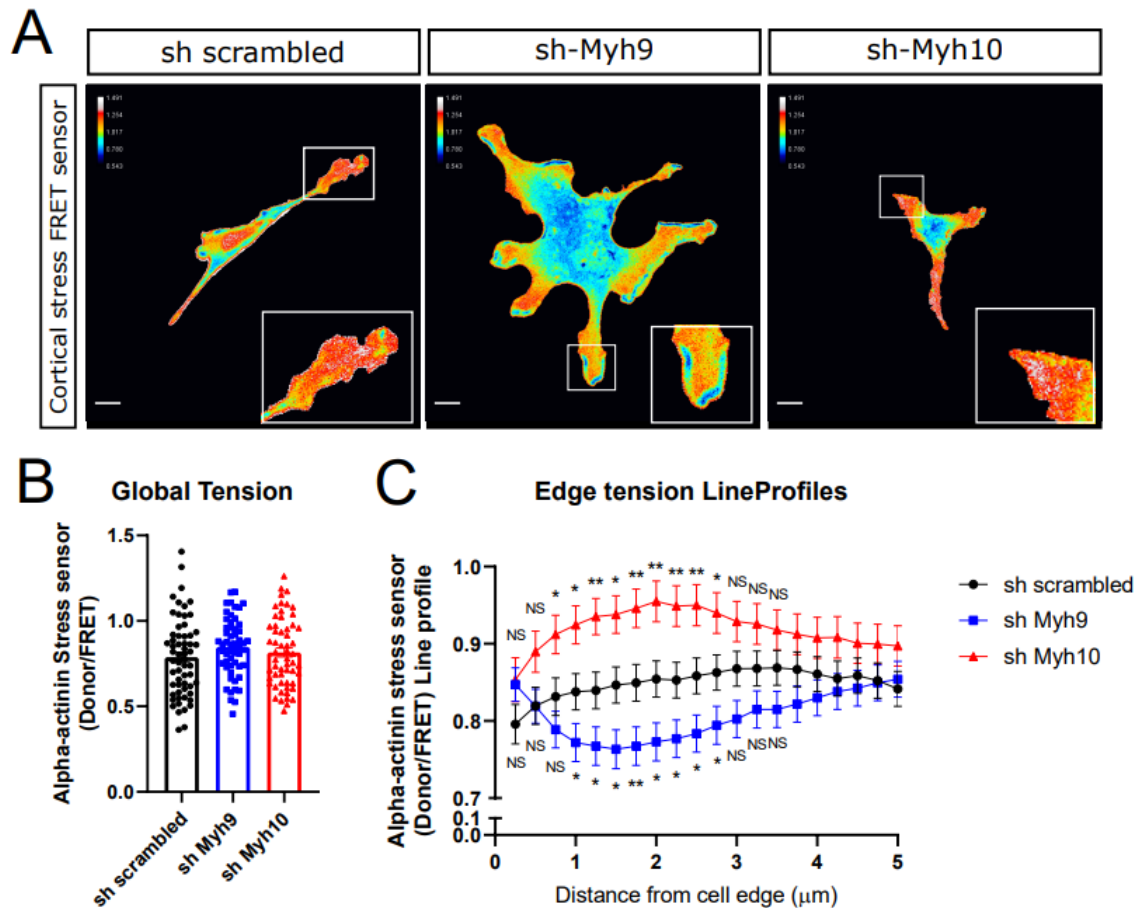


Figure 10: Myh9 alters the shape of microglia *in vitro* by localizing near the cell edge and changing cortical stress levels near the edge of protrusions. (A) Representative images of control or myosin-ii-silenced HMC3 microglia expressing a FRET cortical stress-sensor (Actinin-sstFRET). Ramps represent min/max FRET ratio values using the Royal LUT. Insets are magnifications of the regions identified by white rectangles and show the edge of protrusions in greater detail. **(B)** Quantification of the average whole-cell FRET ratio shows no difference in global cortical stress levels between control and myosin-silenced cells. $n =$ at least 54 cells per group from 3 independent experiments (One-way ANOVA with Sidak's correction for multiple comparisons). **(C)** Line profile of the FRET ratio starting at the edge of the cell and going along the protrusion reveals a decrease in cortical stress in the region underlying the border of Myh9-silenced cells, and an increase in Myh10-silenced cells. $n =$ at least 80 protrusions coming from at least 54 cells per group from 3 independent experiments. * $P < 0.05$, ** $P < 0.01$ (Two-way ANOVA with Holm-Sidak's correction for multiple comparisons). All scale bars correspond to 20 μ m. All Plots show mean $\pm$ SEM.

These results show that local cortex tension relaxation occurs only in Myh9-silenced cells, allowing them to generate and extend more membrane protrusions, and confirming our hypothesis. Furthermore, they reveal Myh9-dependent control of cortical tension as a mechanism that microglia use to elicit the formation and extension of new protrusions.

Type-II myosin inhibition impacts morphology of *ex vivo* retinal microglia

To confirm that this mechanism of cortical tension release for role of type II myosin in the control of cell morphology was not an artefact of culturing HMC3 microglia *in vitro*, we exposed retinal explants to the specific type-II myosin inhibitor blebbistatin (Allingham, Smith, & Rayment, 2005; Limouze, Straight, Mitchison, & Sellers, 2004), which blocks the myosin ATPase motor domain and impacts their ability to slide actin filaments past each other. Using previously established protocols for concentration and incubation time of this inhibitor that minimize toxicity while preserving the inhibitory effect (Koppen, Fernandez, Carvalho, Jacinto, & Heisenberg, 2006; Rauscher, Gyimesi, Kovács, & Málnási-Csizmadia, 2018), we evaluated its impact in *ex vivo* microglia stained for Iba1 and imaged by confocal microscopy. (**Fig.11A**). We observed an increase in the complexity and extension of protrusions of retinal microglia treated with the inhibitor when compared to control DMSO stimulated explants (**Fig.11B**), similar to what was seen in Myh9 silenced cells *in vitro*, as evidenced by an increase in microglia junction points (**Fig.11C**). This change in complexity was also accompanied by an increase in protrusion length (**Fig.11D**), expanding the territory covered by each cell (**Fig.11E**). As such, and even though blebbistatin simultaneously inhibits both Myh9 and Myh10 isoforms, these results parallel what was seen in the *in vitro* model. Since we previously did not observe any impact on the shape of Myh10-silenced microglia, the effect of blebbistatin on retinal microglia morphology is likely due to its inhibitory effect on Myh9. These results further support the view that cortical tension release leads to microglia protrusion generation and extension, with Myh9 assuming the role of a nanomechanical regulator of microglia morphology.

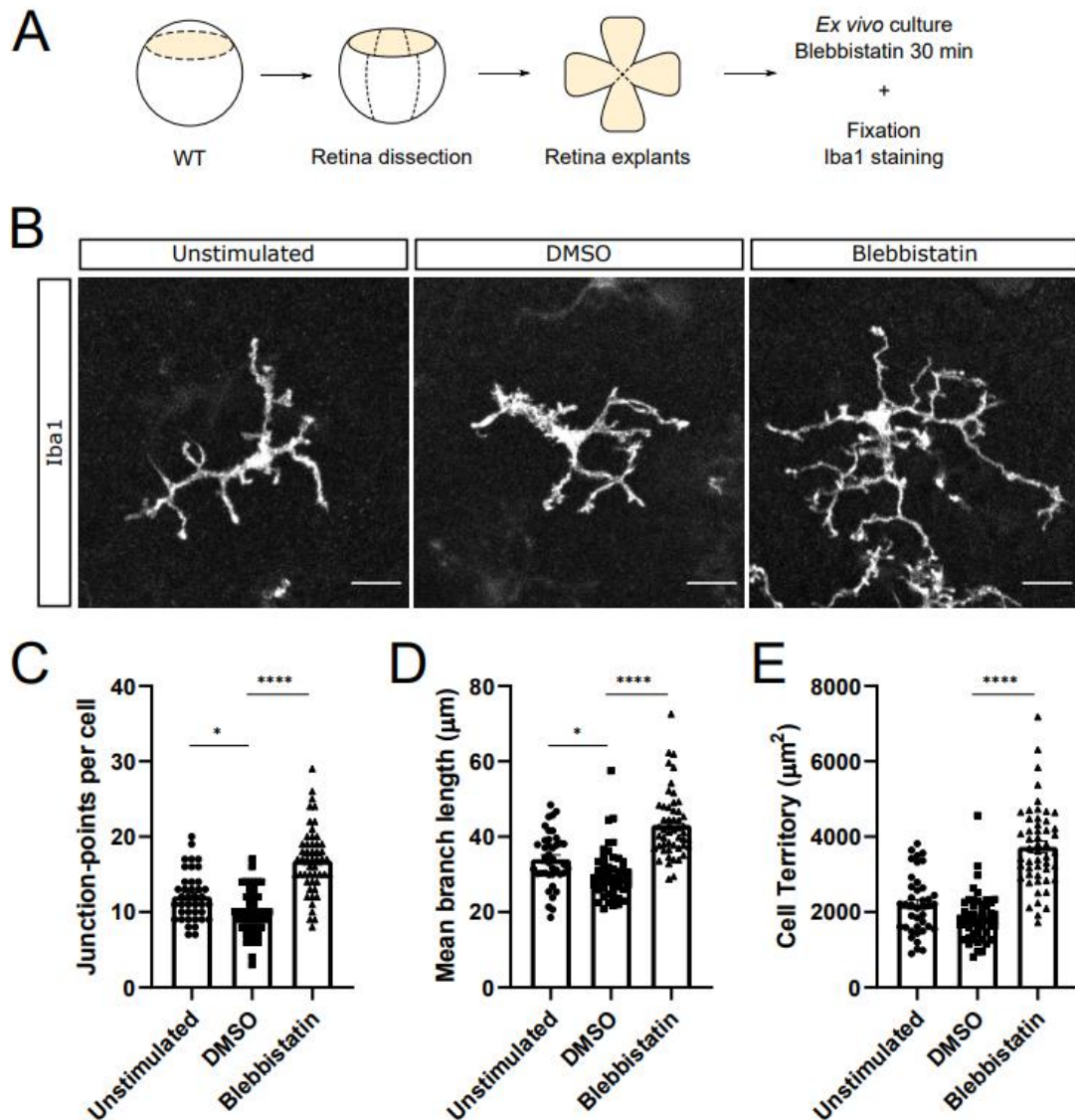


Figure 11: Ex vivo pharmacological inhibition of myosin-ii activity alters retinal microglia morphology, increasing their complexity. (A) Experimental layout for evaluating retinal microglia morphological response to blebbistatin, a specific inhibitor of myosin-ii motor activity. Whole retinae were dissected from the eyes of wild-type mice, cut into 4 explants, and cultured ex-vivo in Ringer solution alone or with 50 μM blebbistatin or DMSO (control) for 30 minutes, before fixation and staining with microglia marker Iba1. (B) Representative confocal microscopy images of Iba1-stained microglia in retinal explants of wild type mice that were untreated or treated with DMSO or blebbistatin. (C-E) Quantification of the impact of myosin pharmacological inhibition on retina microglia morphology, using 3 parameters: number of cell junction-points (C), mean branch length (D) and territory (E). (n = at least 40 cells coming from 7 mice from 3 independent experiments). * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ (Kruskal-Wallis test with Dunn's multiple comparisons correction). All scale bars correspond to 20 μm . All Plots show mean $\pm$ SEM.

Type-II myosins differentially affect phagocytosis, ROS production and cytokine expression

Given the role of protrusion extension for microglia surveillance and effector response, it is possible that changes in an element or regulator of the actomyosin cytoskeleton (such as Myh9) can also impact the function of these cells. Indeed, we have previously demonstrated that the ablation of the small GTPase RhoA, a central regulator of both actin dynamics and type II myosin motors (Schwartz, 2004), induces an inflammatory phenotype in cortical microglia, that ultimately leads to disruption of tissue homeostasis (Socodato et al., 2020). As such, we wondered if type-II myosins could also impact the function of these cells. To study this we evaluated if Myh9 or Myh10 silencing in HMC3 microglia influenced phagocytosis or secretion of inflammatory factors, two crucial cellular processes that characterize the activity of these cells (Hanisch & Kettenmann, 2007). To assess the phagocytic potential of microglia, we exposed these cells to fluorescently labelled beads (**Fig.12A**) and examined the uptake levels by flow cytometry (**Fig.12B**). We observed that both isoforms contribute to phagocytosis, since silencing of either Myh9 or Myh10 decreased the fraction of phagocytic cells (**Fig.12C**), as well as the amount of beads that each cell engulfed (**Fig.12D**). These results demonstrate that type-II myosins are positive regulators of microglia phagocytosis and uncover a role for these molecular motors in one of most important effector functions of microglia. Once activated, microglia produce reactive oxygen species (ROS) and pro-inflammatory cytokines (Q. Li & Barres, 2017). To evaluate the effect of myosin-ii silencing on the production of ROS we incubated microglia with a fluorescent ROS reporter, and once again inspected the cells by flow cytometry (**Fig.12E**). Surprisingly, this revealed that Myh9-silenced cells were no different than scrambled-control cells, but that Myh10-silenced microglia increased ROS production (**Figures 12F and G**). Likewise, when we quantified the expression levels of the classical pro-inflammatory cytokines IL-1 β , IL-6 and TNF α by qPCR we observed that Myh10-silenced cells increased the expression of these mediators, as opposed to Myh9-silenced microglia, which showed no difference in expression when compared to controls (**Fig.12H**). To rule out the effect of some possible artefact of Myh10 silencing, we confirmed these results in the CRISPR-Cas9 Myh10-knockout cell line that we successfully generated (**Fig. 13A and B**), once again detecting impaired phagocytic potential (**Fig. 13C**), increased ROS production (**Fig. 13D**) and increased pro-inflammatory cytokine expression (**Fig. 13E**) in Myh10 ablated microglia.

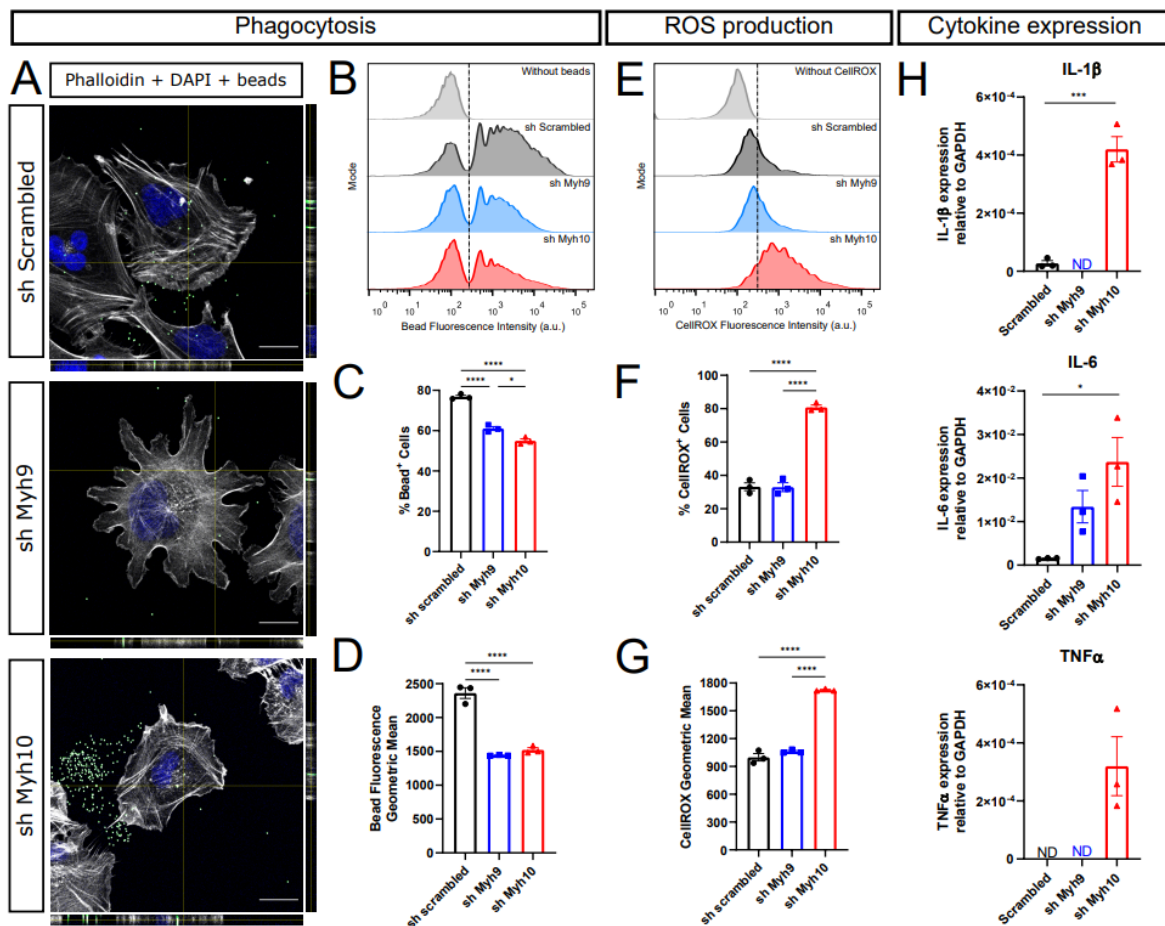


Figure 12: Myh10-silencing affects microglia function, leading to pro-inflammatory activation and a decrease in phagocytic potential. (A) Control and myosin-silenced HMC3 microglia cells were incubated with fluorescent latex beads to assess their phagocytic capacity. Figure panels are representative maximum projection and orthogonal views of confocal microscopy images of cells (stained with phalloidin - in white) with bead uptake (beads in green). Scale bar = 20 μ m. (B-D) Flow cytometry analysis (B -fluorescence histograms) shows that Myh9 and Myh10-silenced cells have a smaller phagocytic population (C) and impaired phagocytic uptake (D) compared with control cells. n = 5000 cells per experiment from 3 independent experiments. **P<0.01, ***P<0.001, ****P<0.0001 (One-way ANOVA with Sidak multiple comparison correction). (E-G) Control and myosin-silenced HMC3 microglia cells were incubated with CellROX, a fluorescent indicator of reactive oxygen species (ROS) production. Flow cytometry analysis (E – fluorescence histograms) reveals no impact of Myh9-silencing, but an increase in ROS production in Myh10-silenced cells (F and G). n = 5000 cells per experiment from 3 independent experiments. ****P<0.0001 (One-way ANOVA with Sidak multiple comparison correction). (H) Quantitative PCR analysis of inflammatory cytokines from the RNA of control and myosin-silenced HMC3 microglia cells shows a specific impact of Myh10-silencing on IL-1 β , IL-6 and TNF α expression. *P<0.05, ***P<0.001 (One-way ANOVA with Sidak multiple comparison correction). All Plots show mean $\pm$ SEM.

As such, absence of Myh10 results in an activated, inflammatory phenotype for microglia.

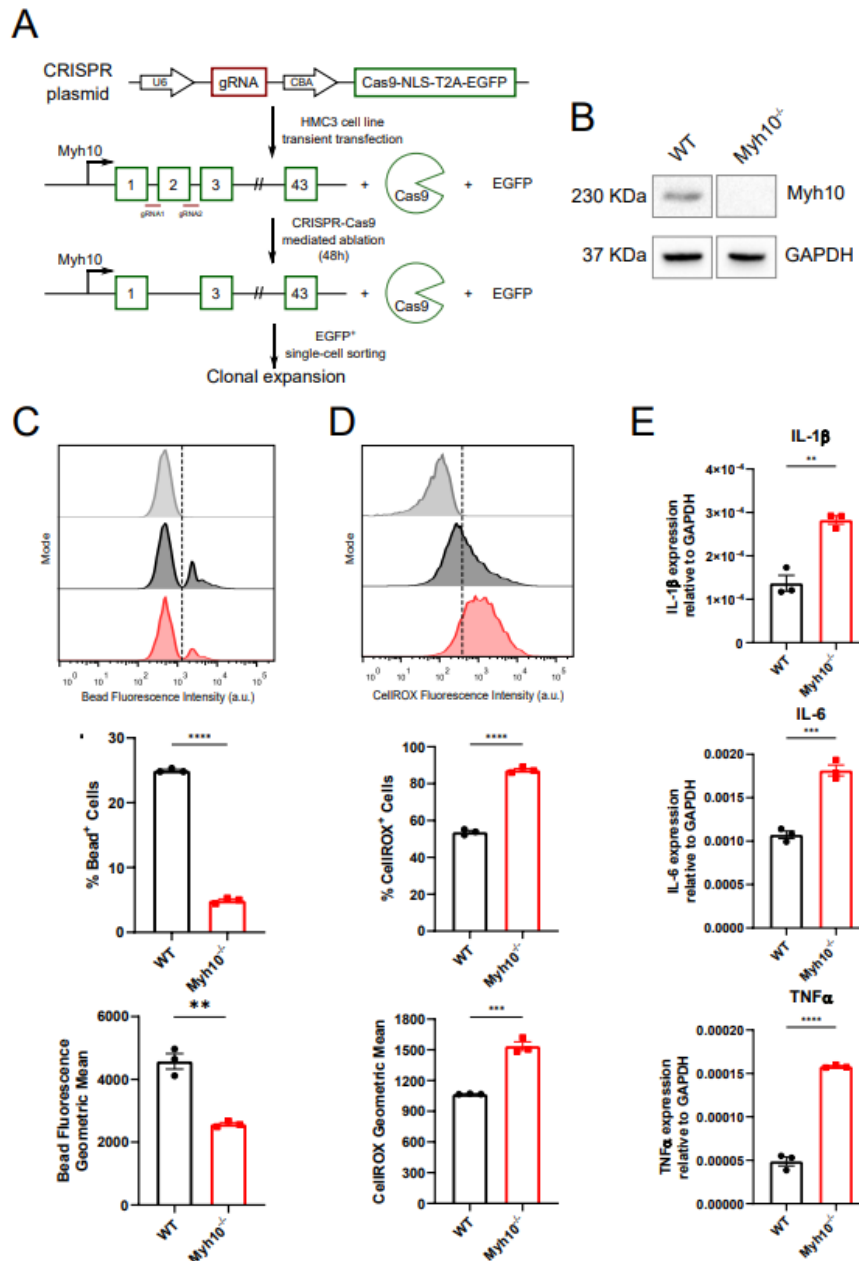


Figure 13: CRISPR knockout of Myh10 in HMC3 microglia confirms the impact in cell function seen in Myh10-silenced cells: decreased phagocytic potential with increased ROS and pro-inflammatory cytokine expression. (A) Strategy for Myh10 ablation in HMC3 microglia *in vitro* via CRISPR-Cas9. A single plasmid encoding Cas9, GFP and guide RNA (gRNA) is transiently transfected into cells (top). A combination of two gRNAs directs Cas9 to the Myh10 gene downstream and upstream of exon 2 (middle), resulting in Myh10 deletion and expression of GFP, which is used to isolate single-cell clones by sorting (bottom). (B) Western blot depicting absence of Myh10 protein from extracts of WT and Myh10^{-/-} HMC3 microglia cells generated according to the strategy in A. GAPDH was used as loading control. (C) WT and Myh10^{-/-} HMC3 microglia cells were incubated with fluorescent latex beads to assess their phagocytic capacity. Flow cytometry analysis (histograms) shows that Myh10-ablated cells have a smaller phagocytic population (top graph) and impaired phagocytic uptake (bottom graph) compared with control cells. n = 5000 cells per experiment from 3 independent experiments. **P<0.01, ****P<0.0001 (student's t test). (D) Control and Myh10^{-/-} HMC3 microglia cells were incubated with CellROX, a fluorescent indicator of ROS production. Flow cytometry analysis (histograms) shows

an increase in ROS production in myosin null cells (top and bottom graphs). $n = 5000$ cells per experiment from 3 independent experiments. $***P < 0.001$, $****P < 0.0001$ (student's t test). **(E)** Quantitative PCR analysis of inflammatory cytokines from the RNA of control and Myh10^{-/-} HMC3 microglia cells shows a specific impact of Myh10 deletion on IL-1 β (top), IL-6 (middle) and TNF α (bottom) expression. $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ (student's t test). All Plots show mean $\pm$ SEM.

These results show that, although it was Myh9 that affected microglia morphology, it is Myh10 that influences these cell's effector functions, revealing a specific contribution of Myh10 for the activation of microglia. They also demonstrate that the role of type-II myosins goes beyond regulating tension and cell morphology, but also influences microglia function, identifying novel and differential contributions of these molecular motors for microglia physiology.

Discussion

Cofilin-mediated actin turnover in the regulation of microglia protrusion extension

Despite the knowledge of extracellular cues that alter microglia morphology, like ATP (Haynes et al., 2006), and some of the accompanying intracellular events, such as a decrease in cAMP levels (Bernier et al., 2019) and potassium (Madry et al., 2017), or an increase in calcium (Pozner et al., 2015), the mechanisms and relevant players that act at the level of the actomyosin cytoskeleton for the associated creation, maintenance and extension of protrusions in microglia have remained unexplored. Using the retina as a platform we were able to study the morphology of microglia within their natural environment. The application of our image-analysis routine to the study of RhoA knockout microglia, together with follow-up *in vitro* and *ex-vivo* studies allowed us to identify for the first time several mechanisms and molecular players in the control of microglia morphology (**Fig.14**). The observed decrease in complexity of RhoA knockout retinal microglia is in accordance to what our group identified in cortical microglia (Socodato et al., 2020). Moreover, previous evidence (Parkhurst et al., 2013; Socodato et al., 2020) as well as control experiments that we performed with wild type and Cx3cr1^{CreER-IRES-YFP/+} mice (data not shown) reinforces that the results are not a consequence of Cx3cr1 haploinsufficiency. Studying the role of RhoA in protrusion extension by ATP stimulation and using a complementary *in vitro* biochemical

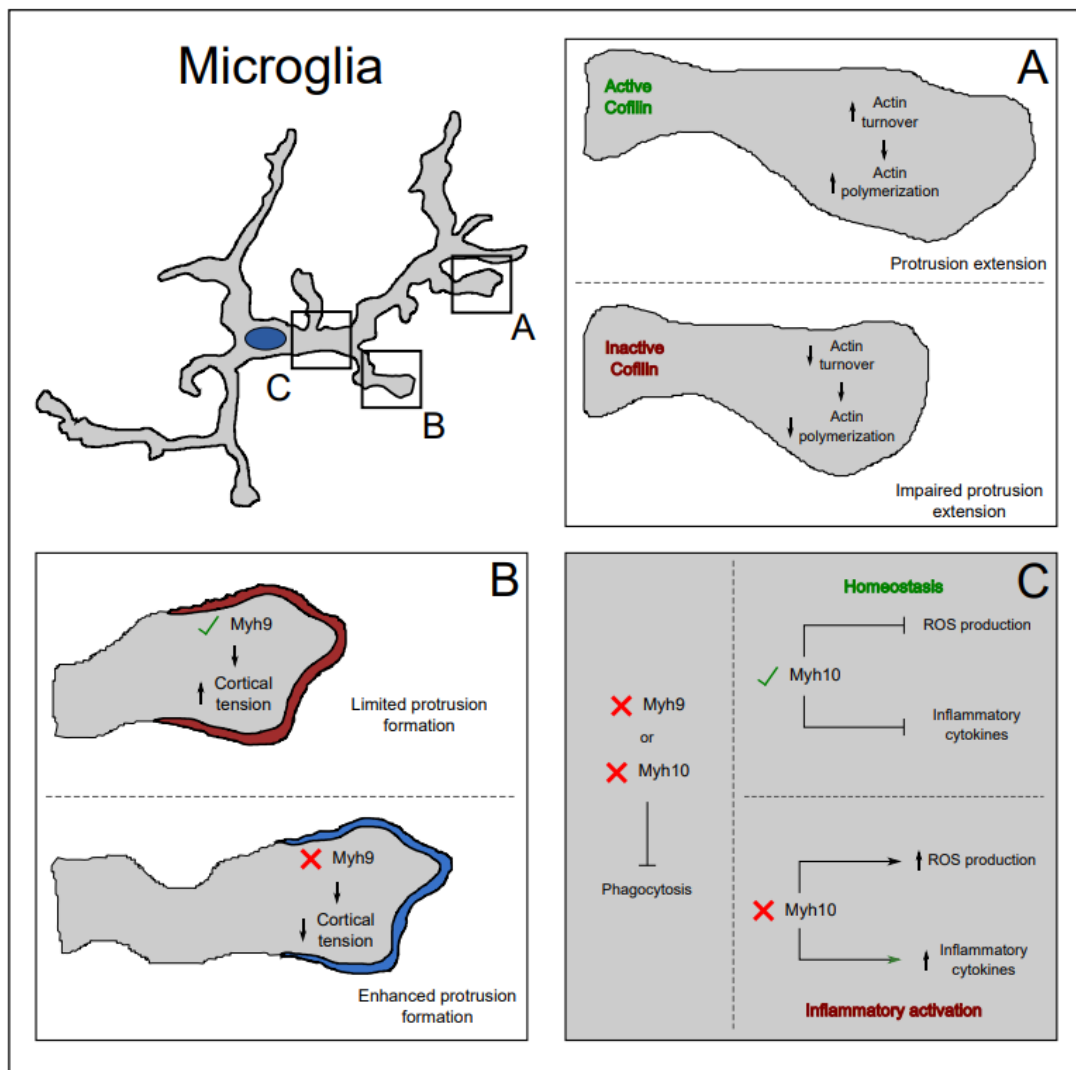


Figure 14: Type-II myosins differentially affect microglia morphology and function. (A) The extension of protrusions relies on actin polymerization, which in turn needs a steady supply of monomeric actin to keep actin polymerizing at the cell's edge. Cofilin activity, which is known to sever and depolymerize actin filaments, contributes to replenish the pool of monomeric actin available for polymerization (top). A decrease in this activity, like the one seen in *RhoA*^{-/-} microglia, compromises this mechanism, decreasing actin polymerization and leading to the development of shorter protrusions, with possible effects on the ability of microglia to reach for their targets (bottom). **(B)** In order for actin polymerization to generate new protrusions and extend them, it must overcome the existing cortical tension in the network. Myh9, which is enriched at the edge of the cell, contributes to an even development of cortical tension via its motor activity, which contracts the actin network (top) and resists the extension of the protrusion. In the absence of Myh9, however, there is a local decrease in cortical tension at the edge of the cell, facilitating both the formation and extension of microglia protrusions (bottom). **(C)** The action of type-II myosins on microglia also go beyond their effect on morphology, contributing to the effector functions of these cells. Both isoforms contribute to the phagocytic capacity of microglia, while Myh10 influences microglia activation state, since in its absence, and through a yet unknown pathway, there is increased ROS production and expression of pro-inflammatory cytokines, affecting the homeostasis of these cells.

analysis of actin regulators from a RhoA knockout microglia cell line and primary mouse microglia allowed us to propose a mechanism by which microglia extend their membrane protrusions: Cofilin activity increases the turnover of actin, replenishing the pool of monomeric actin that is necessary to sustain continuous actin polymerization at the front of the protrusion (**Fig.14A**). In neurons, cofilin activity has also been shown to enhance axonal extension by promoting actin turnover (Tedeschi et al., 2019). A similar mechanism has also been proposed for protrusion extension of astrocytes (Baorto, Mellado, & Shelanski, 1992), while the extension of the broad protrusions of myelinating oligodendrocytes during neuronal wrapping also critically depends on cofilin-mediated actin turnover (Nawaz et al., 2015; Zuchero et al., 2015). Classically, cofilin activity is regulated by RhoA via LIMK (Arber et al., 1998; Maekawa et al., 1999), which inactivates cofilin by phosphorylation. Within such a framework, the expectation was that absence of RhoA in microglia would decrease cofilin phosphorylation. Interestingly, under such conditions we observed instead an increase in pCofilin. This is a clear instance of a cell-specific effect of RhoGTPases (Zhou & Zheng, 2013), which highlights the importance of studying their role and downstream signalling in different cell types. Possible regulators that are maintaining pCofilin levels in the absence of RhoA are RhoC, which has been shown to inactivate cofilin in epithelial cells via the classical LIMK axis (Jose J. Bravo-Cordero et al., 2011; J. J. Bravo-Cordero et al., 2013), or TES kinases, which can directly phosphorylate cofilin (Toshima et al., 2001). Interestingly, although this mechanism of actin-turnover control seems to be shared by the main CNS cell types for protrusion extension, recent data has shown that in the absence of RhoA neurons do not present changes in cofilin activation (Dupraz et al., 2019), which indicates that the control of this mechanism is not shared between them (in neurons RhoA does not operate as a controller, while in microglia it can act as such), making this another example of a RhoGTPase cell-specific effect (L. Wang & Zheng, 2007). Aside from decreased cofilin activation we also observed a specific impact of RhoA ablation on Myh10 expression. It is possible that this further contributes to the observed deficit in polymerization, since the action of type-II myosin motors has also been shown to enhance the turnover of actin (Sonal et al., 2019). As such, RhoA regulates the levels of actin turnover that is essential for the control of protrusion extension by microglia.

Myh9-mediated cortical tension release in the control of microglia protrusion formation and extension

The study of the impact of RhoA ablation on several actin binding proteins pointed to a possible differential effect of type-II myosin isoforms in the control of microglia morphology.

Our approach of *in vitro* shRNA delivery and use of a tension-sensing FRET probe, complemented by pharmacological manipulation of retinal microglia pointed to a possible mechanism for the generation of membrane protrusions in microglia cells: edge cortical tension, under the control of Myh9, but not of Myh10, opposes the protrusive effort of actin polymerization in the formation and extension of a protrusion. Absence of Myh9 leads to a reduction in cortical tension, facilitating the initiation and elongation of new protrusions (**Fig.14B**). Our use of an *in vitro* microglia cell line, whose morphology does not differ significantly from that of primary cells, constituted an advantage since it allowed for a more efficient silencing of both myosin isoforms (greater turnover in culture) and also made it possible to probe cortical stress levels via successful transfection with the FRET construct. We observed a local decrease in cortical tension specifically on cells silenced for Myh9, while for Myh10-targeted cells local tension levels near the edge of the cell actually increased (**Fig.10C**). The two isoforms have similar structures, but display slightly different mechanochemical properties: the higher ATPase activity of Myh9 (Kovács, Wang, Hu, Zhang, & Sellers, 2003) increases the speed with which it slides actin filaments past each other. As such, it is possible that in the absence of Myh10 this increase in filament sliding due to Myh9 produces more tension in actin crosslinkers, which are picked up by the FRET probe (itself a crosslinker), resulting in the measured increase in cortical tension.

A previous study had identified that cortical tension in spherical actin networks hindered polymerization, and that breakage of the network caused a release in tension that allowed local actin polymerization to occur (Carvalho et al., 2013). Furthermore, it hypothesized that an analogous tension release in cells could initiate local actin protrusions. Our proposed mechanism of microglia protrusion formation and extension is the first confirmation of this hypothesis in a cellular model, given our observed local decrease in cortical tension. It is possible that this mechanism also occurs in other CNS cells, since *in vivo* simultaneous knockout of Myh9 and Myh10 has been shown to enhance retinal ganglion cell axonal extension after optic nerve crush (X.-W. Wang et al., 2020) and that a similar mechanism had also been proposed for the formation and extension of protrusions during oligodendrocyte differentiation (Domingues et al., 2020). Of note, in this last case, Myh10 is the isoform that regulates protrusion extension (H. Wang et al., 2012), demonstrating once again cell-specific differences in the control of a shared mechanism.

Type-II myosins are known to create tension in actin networks independently from their power stroke, a phenomenon that has been observed in yeast (Mendes Pinto et al., 2012) and mammalian cytokinesis (X. Ma et al., 2012) and that depends solely on their ability to crosslink actin filaments (Sun, Walcott, & Wolgemuth, 2010). Despite the possibility of such a contribution in microglia, our results on the pharmacological inhibition of type-II myosin

activity in retinal explants (**Fig.6**) point to a predominately motor-dependent function of Myh9 in its control of cortical tension. We propose that a local control of Myh9 activity is responsible for the local decrease in cortical tension, allowing the initiation and extension formation of microglia protrusions. Once the protrusion is formed, it is also possible that the decreased activity of myosin further supports process extension by decreasing actin retrograde flow (Lim, Sabouri-Ghomi, Machacek, Waterman, & Danuser, 2010; Marchenko et al., 2017). How this such local regulation happens, we do not know, but it will be interesting to see if links between purinoceptors (responsible for the signal transduction of ATP, which induces protrusion formation and extension) and type-II myosin regulation in microglia are discovered. Also, given that Myh9 inhibits microglia protrusion generation and lengthening, and that studies on axonal retraction have shown the importance of Myh9 for this process (Gallo, 2006; Wylie & Chantler, 2003), it is tempting to speculate that Myh9 (or Myh10, given the cell-specific roles observed for these isoforms) may play an important role in controlling microglia protrusion retraction, a phenomenon that is associated with microglia activation (Stence et al., 2001).

Regarding our results on the effect of blebbistatin on retinal microglia shape, despite the specificity of this molecule towards members of the type-II myosin family (Limouze et al., 2004), we cannot exclude the possibility that some effect of blebbistatin on other retinal cells is indirectly conditioning the morphology of microglia. Absolute confirmation of the Myh9-specific effect on microglia shape in an *in vivo* context as well as a live observation of its effect on process extension and retraction would require the use of conditional gene ablation and *in vivo* or *ex vivo* microscopy imaging, something we believe would be interesting to explore in the future.

The specific influence of Myh10 on microglia activation

Our *in vitro* model of type II myosin silencing also allowed us to address the specific contributions of each isoform in microglia effector functions. We were able to determine that both Myh9 and Myh10 contribute to the phagocytic capacity of microglia, demonstrating the importance of type II myosins in one of these cell's central effector functions (**Fig.14C**). Indeed, phagocytosis is used by microglia in synapse pruning (Bialas & Stevens, 2013; Norris et al., 2018; Schafer et al., 2012; Weinhard et al., 2018), removal of debris (Olah et al., 2012), or elimination of cells (S. R. Anderson et al., 2019; Sierra et al., 2010; L. Zhao et al., 2015). As such, our results suggest that modulation of microglia Myh9 or Myh10 can have far-reaching consequences in CNS biology. Phagocytosis also serves as an important sensing tool. Some intracellular receptors are only able to recognize their ligand after it has

been internalized and degraded, a process to which phagocytosis is important (Underhill & Goodridge, 2012). The relevance of such a process in microglia is shown for example in the context of viral infection, where the ability of microglia to ingest infected material from the surrounding environment is essential to generate a protective antiviral response (Moseman, Blanchard, Nayak, & McGavern, 2020), or in the context of neurodegeneration, where the response of microglia to Abeta depends on the ability of sensing it by the intracellular inflammasome component NLRP3 (Halle et al., 2008). This shows that both Myh9 and Myh10, via phagocytosis impairment, also have the potential to affect microglia's proper sensing of the environment and its ensuing effector response.

Aside from their impact in microglia phagocytosis we also uncovered a specific role for Myh10 in the activation state of microglia. In the absence of Myh10, but not of Myh9, microglia acquire a pro-inflammatory profile with increased expression of pro-inflammatory cytokines and production of ROS (**Fig.14C**). This showed that type-II myosins can also influence the function of microglia, this time with a specific contribution from Myh10. Although we do not know the exact mechanism through which this myosin is changing microglia activation, evidences from other cell types allow us to propose two hypotheses. Since Myh9-mediated sensing of mechanical strain can change the epigenetic landscape of human epithelial progenitor cells (Le et al., 2016) and that Myh9 or Myh10 silencing alters the gene expression profile of primary tumour cells via the mechanosensitive transcriptional regulator YAP/TAZ (Halder et al., 2019), Myh10 can be a regulator of microglia mechanosensitive pathways, influencing gene expression either directly by controlling the action of transcription factors, indirectly via changes in chromatin accessibility, or through a combination of both. Although our FRET data shows that Myh10 ablation does not affect global levels of tension in microglia, regional changes may be sufficient to modulate these mechanosensitive cascades. It is also possible that Myh10 mediates microglia activation through a tension-independent direct change in signalling, by acting as a scaffold for other signalling proteins. In fact, studies in *S. cerevisiae* show that myo1 (the myosin II equivalent in yeast) acts as a scaffold for signalling proteins during cytokinesis (Wloka et al., 2013). In mammalian cells Myh10 has also been detected in complexes together with PAK and PKC (Even-Faitelson & Ravid, 2006). As such, it is possible that Myh10 is acting as a hub for different signalling molecules in microglia, and that its absence perturbs signalling cascades that culminate in the observed changes in ROS production and increased cytokine expression. It is also important to note that in any of the hypothesis raised, the effect of Myh10 on microglia activation may or may not depend on its motor activity. Regardless of the mechanism that is operating, our results uncover a role for type-II myosins in controlling the activation of microglia, expanding the number of immune cell types (beyond B and T-

cells) where this motor is known to influence their function (Hoogeboom et al., 2018; Ilani, Vasiliver-Shamis, Vardhana, Bretscher, & Dustin, 2009).

Finally, when evaluating the possible effects of type-II myosins in the activation of microglia, we were surprised to find that the isoform that regulates the morphology of these cells (Myh9) was not the isoform associated with changes in their function (Myh10), and vice versa. We believe that an *in vivo* validation of these results, once again using conditional gene ablation, would be important to perform in the future, because if confirmed, it would raise the puzzling possibility that, under certain circumstances, the shape of microglia cannot be used as an indicator of its function, something that is routinely done in the field. Furthermore, it would identify the molecular players that are responsible for such a decoupling.

Overall, our results demonstrate that the actomyosin cytoskeleton is intimately linked with the morphology of microglia, and that changes in its components *per se* are sufficient to influence the activation state of these cells. Furthermore, they show that the homeostatic state of microglia depends on differential roles played by type-II myosins: Myh9 as a regulator of cell shape and Myh10 as a regulator of their activation state.

Chapter 3 - Exploring the mechanisms of Myh10-mediated regulation of microglia inflammatory activation

Introduction

The activation of microglia is recognized as a key component of neuroinflammatory and neurodegenerative disorders (Gilhus & Deuschl, 2019; Song & Colonna, 2018). Continued activation of microglia can result in further damage to the CNS as well as hinder its recovery after the challenge has been resolved (Deczkowska et al., 2018). A better sense of the mechanisms that are behind microglia activation could contribute to a better understanding of disease pathogenesis but also uncover potential therapeutic targets. NF- κ B signalling is a classical pathway that controls the expression of inflammation-associated genes, such as inflammatory cytokines and chemokines (Zhang, Lenardo, & Baltimore, 2017). c-Src signalling is another essential component in the regulation of microglia activation (Socodato et al., 2015). Recently, Hippo signalling, a key developmental pathway that controls nuclear entry of the transcriptional co-activator complex YAP/TAZ (Totaro, Panciera, & Piccolo, 2018; Varelas, 2014) and that is known to mediate cellular mechanotransduction (Dupont et al., 2011), has also been associated with inflammation in innate immune cells (S. Wang et al., 2020). Results previously obtained by our group and described in the previous chapter of this thesis showed that Myh10 specifically influences the inflammatory activation status of microglia (Melo, Silveira, Pinto, & Relvas, 2021). However, we still do not understand how the absence of Myh10 causes this. Here, we explore some of the possible mechanisms that may be involved in such a change.

Results

Myh10's effect on microglia function is independent of its motor activity

Given the role of Myh10 as an actin molecular motor, we started by understanding if its effect was dependent on its motor activity. To test if this was true, we inhibited it using the type-ii myosin-specific inhibitor blebbistatin (Allingham et al., 2005), and assessed the cell's production of ROS by flow cytometry using the fluorescent reporter CellROX. Interestingly,

we saw no differences between inhibitor-treated HMC3 microglia when compared to vehicle-only control cells (**Figure 1A**). We also evaluated the expression levels of the

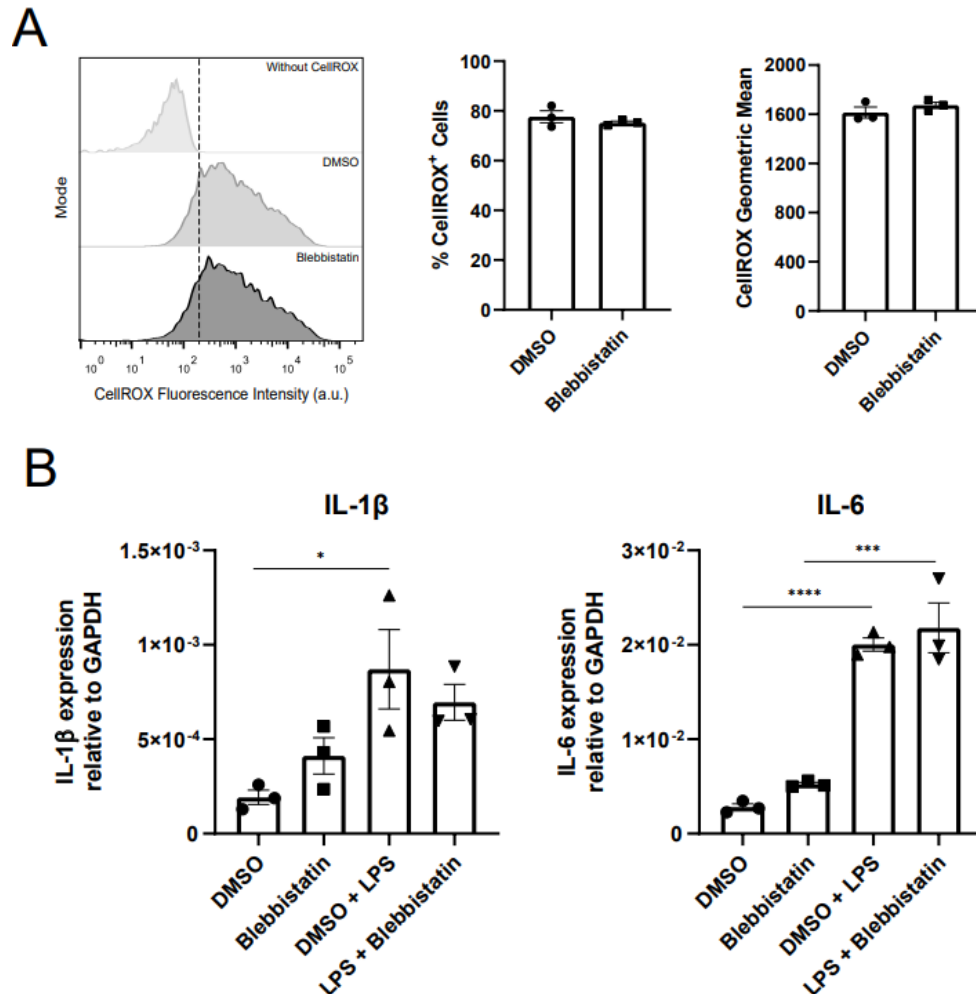


Figure 1: Blebbistatin inhibition does not recapitulate the inflammatory activation seen in the absence of Myh10. (A) HMC3 microglia cells were treated with the type-ii myosin inhibitor Blebbistatin for 24h and then incubated with CellROX, a fluorescent indicator of ROS production. Flow cytometry analysis (histograms) shows no difference in ROS production in type-ii myosin-inhibited cells (graphs, student's t test). n = 10000 cells per experiment from 3 independent experiments. (B) Quantitative PCR analysis of inflammatory cytokines from the RNA of HMC3 microglia cells incubated with DMSO (vehicle), 25μg/mL Blebbistatin, DMSO plus LPS 1μg/mL (positive control) or Blebbistatin together with LPS for 24h shows no impact of type-ii myosin inhibition on IL-1β (left) or IL-6 (right) expression. * P<0.05, *** P<0.001, **** P<0.0001 (One-way ANOVA with Sidak multiple comparison correction). All Plots show mean +/- SEM.

previously studied inflammatory cytokines IL-1β, IL-6 and TNFα in HMC3 microglia under the influence of blebbistatin for 24h. Again, we detected no effect of the inhibitor on IL-1β or on IL-6 expression (**Figure 1B**). Stimulating the cells with LPS for the same amount of

time showed that they are capable of responding such a challenge (**Figure 1B**). Finally, to determine if type-ii myosin motor inhibition could influence the cell's response to an exogenous chemical stimulus, we cultured HMC3 microglia in the presence of both LPS and blebbistatin. Once more, the inhibitor did not affect the levels of IL-1 β or IL-6 elicited by the stimulus (**Figure 1B**). No TNF α transcripts were detected at this time point on any of the conditions tested. Since blebbistatin-treated cells showed no changes in ROS production or inflammatory cytokine expression, our results demonstrate that the effect of Myh10 on the activation state of microglia does not seem to depend on its motor activity.

Microglia activation in the absence of Myh10 follows classical inflammatory pathways

The transcription factor NF- κ B is a classical regulator of a cell's inflammatory activation (Lawrence, 2009), and is known to be associated with the expression of pro-inflammatory cytokines in microglia (Socodato et al., 2015). To test if the pro-inflammatory effect of Myh10 ablation in microglia follows this classical pathway we fluorescently stained WT and Myh10^{-/-} HMC3 microglia for DAPI and the p65 subunit of NF- κ B (**Figure 2A**) and evaluated the levels of transcription factor present in the nucleus. Quantification showed increased nuclear presence of p65 (**Figure 2B**), indicating enhanced NF- κ B signalling in the absence of Myh10. Another important regulator of microglia activity is the non-receptor tyrosine kinase c-Src, the activity of which our group has previously shown to be essential for the activation of microglia (Socodato et al., 2015). In order to determine if removal of Myh10 also results in increased c-Src activity we expressed a molecular c-Src probe (Ouyang, Sun, Chien, & Wang, 2008) in WT and Myh10^{-/-} HMC3 microglia and inspected them using FRET microscopy (**Figure 2C**), and observed a global increase in c-Src activity in Myh10-ablated cells (**Figure 2D**). The activation of c-Src is itself controlled by C-terminal Src kinase (Csk) (Okada, 2012), so we evaluated the levels of this protein were affected in Myh10 ablated cells. Western blot analysis revealed that the amount of Csk is indeed decreased when compared to WT control cells (**Figure 2E**), indicating that Myh10's effect on c-Src activity is possibly achieved via Csk. Together, these results show that the effect of cell activation seen in Myh10^{-/-} microglia follows canonical inflammatory signalling pathways.

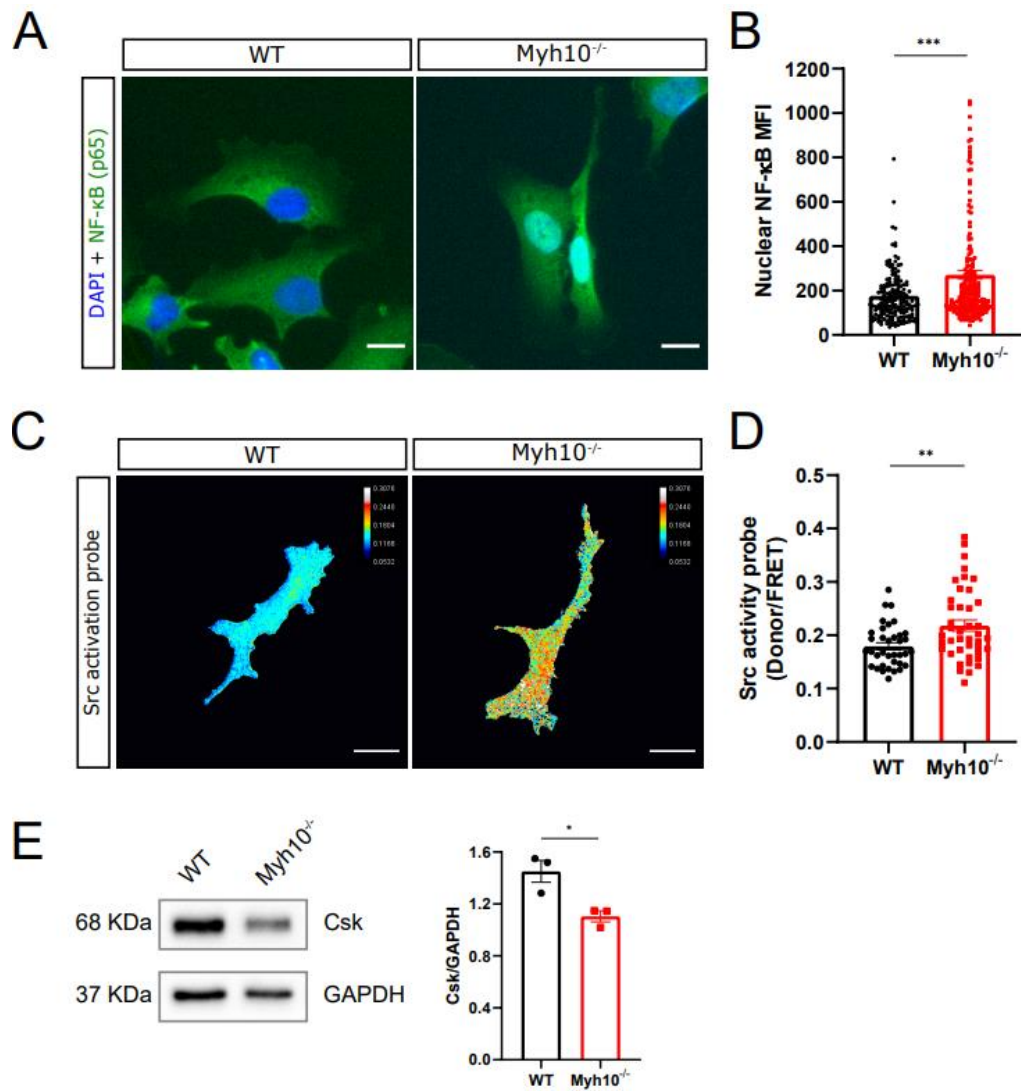


Figure 2: NF-κB and c-Src are activated in Myh10-ablated microglia. (A) Representative images of WT and Myh10^{-/-} HMC3 microglia (left) stained with DAPI (in blue) and NF-κB (p65, in green) show an increase in nuclear NF-κB in the absence of Myh10 (right). Graph depicts mean $\pm$ s.e.m, n = at least 150 cells per group from 2 independent experiments. *** $P < 0.001$ (Mann-Whitney U test). **(B)** Representative images of WT or Myh10^{-/-} HMC3 microglia (left) expressing a c-Src-activity FRET sensor (KRAS-Src). Ramps represent min/max FRET ratio values using the Royal LUT. Quantification of the average whole-cell FRET ratio (right) shows an increase in c-Src activity between control and Myh10-ablated cells. n = at least 36 cells per group from 2 independent experiments. ** $P < 0.01$ (Mann-Whitney U test). All scale bars correspond to 20 μ m. **(C)** Representative western blot (left) for Csk from extracts of WT and Myh10^{-/-} HMC3 microglia. GAPDH was used as loading control. Quantification (right) reveals a decrease in Csk expression in the absence of Myh10 ($n=3$ independent cultures). * $P < 0.05$ (unpaired t-test). All Plots show mean $\pm$ SEM.

Absence of Myh10 primes microglia for future challenge

Having established the involvement of NF- κ B and c-Src signalling in Myh10-depleted cells, we wondered if their increased levels could affect the response of Myh10-ablated microglia to classical inflammatory stimulation. To determine if this was the case, we stimulated both WT and Myh10^{-/-} HMC3 microglia with LPS for 4h and evaluated the expression levels of IL-1 β , IL-6 and TNF α by qPCR (**Figure 3**). As expected, both cell types responded to the endotoxin by increasing the transcription of the three inflammatory cytokines. However, this increase was much greater in the case of LPS-treated Myh10-deficient cells, when compared to the WT LPS cells. As such, Myh10-ablation *per se* not only results in basal pro-inflammatory activation, but also primes microglia to respond more vigorously when stimulated.

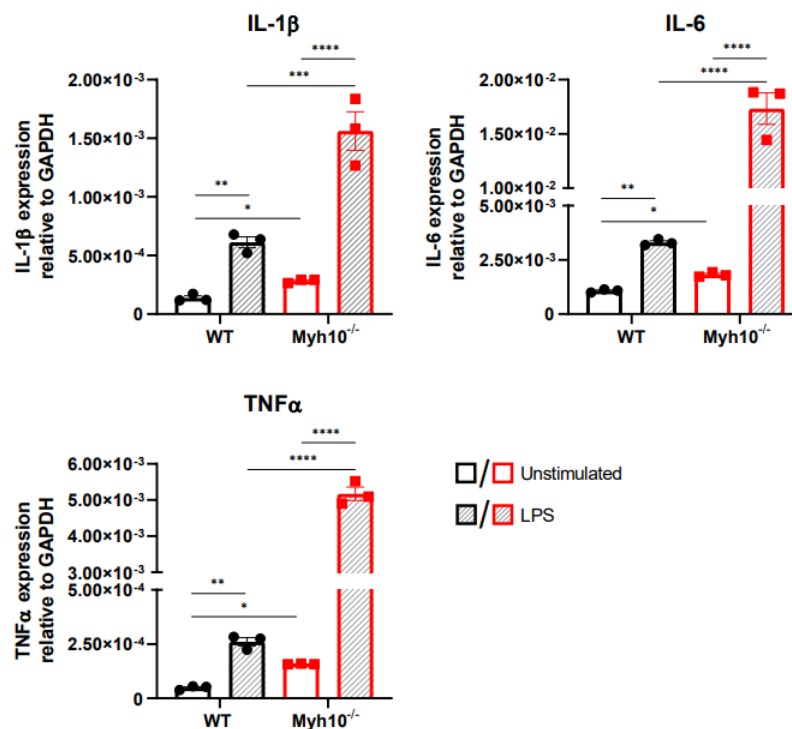


Figure 3: Myh10-deletion enhances microglia expression of pro-inflammatory cytokines in response to LPS. Quantitative PCR analysis of inflammatory cytokines from the RNA of control and Myh10^{-/-} HMC3 microglia that were either unstimulated or incubated with 1 μ g/mL LPS for 4h. Cells show an even greater response to LPS in the case of Myh10 deletion on IL-1 β (top left), IL-6 (top-right) and TNF α (bottom) expression. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001 (2-Way ANOVA with Tukey's multiple comparison test). All Plots show mean $\pm$ SEM.

YAP signalling plays a potential role in Myh10-mediated microglia activation

The priming effect and the involvement of NF- κ B signalling seen above in Myh10-ablated microglia suggests the possible involvement of transcriptional co-activators, since these can influence and further enhance gene expression (Spiegelman & Heinrich, 2004). Since YAP is one of these regulators, and has previously been shown to enhance the activity of NF- κ B (Ye et al., 2018), we wondered if YAP signalling could be associated with the activation of microglia in the absence of Myh10. For this, we determined the levels of nuclear YAP in WT and Myh10^{-/-} HMC3 microglia fluorescently stained for DAPI and YAP (**Figure 4A**).

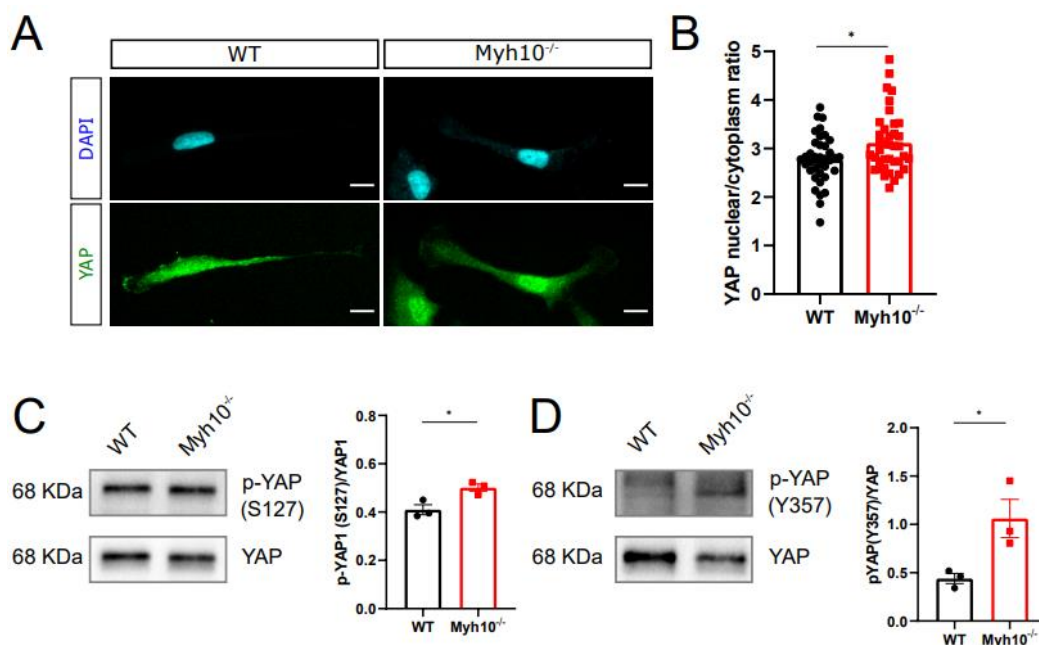


Figure 4: YAP signalling is increased in Myh10-knockout microglia. (A) Representative images of WT and Myh10^{-/-} HMC3 microglia (left) stained with DAPI (in blue) and YAP1 (in green) show an increase in nuclear YAP in the absence of Myh10 (right). Graph depicts the ratio of nuclear to cytoplasmic intensity of YAP +/- s.e.m, n = at least 38 cells per group from 2 independent experiments. *** P<0.001 (Mann-Whitney U test). Scale bar corresponds to 20 μ m. (B) Representative western blot (left) for pYAP (S127) and YAP. Quantification (right) shows an increase in phosphorylated S127-YAP in Myh10^{-/-} HMC3 microglia cells (n=3 independent cultures). * P<0.05 (unpaired t-test). (C) Representative western blot (left) for pYAP (S127) and YAP. Absence of Myh10 increases the levels of phosphorylated Y127-YAP (right, n=3 independent cultures). (D) Representative western blot (left) for pYAP (Y357) and YAP. Absence of Myh10 also increases the levels of phosphorylated Y357-YAP (right, n=3 independent cultures). * P<0.05 (unpaired t-test). All Plots show mean +/- SEM.

Quantification of the nuclear to cytoplasmic ratio revealed that there is indeed an increase in YAP nuclear levels in the absence of Myh10 when compared to control cells (**Figure 4B**). The subcellular distribution of YAP is regulated by phosphorylation, most commonly at

position S127, dictating an exit from the nucleus and increased residence at the cytoplasm (B. Zhao, Li, Tumaneng, Wang, & Guan, 2010). Using protein extracts from WT and Myh10^{-/-} HMC3 microglia we determined the levels of this marker by western blot, but surprisingly detected a slight increase in pYAP(S127) in myosin-ablated cells (**Figure 4C**). Although uncommon, there have been instances where YAP phosphorylation at S127 alone it is not sufficient to exclude it from the nucleus (Wada, Itoga, Okano, Yonemura, & Sasaki, 2011), in which case other residues assume the control of the protein's subcellular distribution. One such residue is Y357, which has been shown to stabilize YAP in the nucleus when phosphorylated (Chakravarti et al., 2020; Sugihara et al., 2018; Vlahov et al., 2015). As such, we evaluated the levels of this marker by western blot and found that there is indeed an increase in pYAP(Y357) in Myh10^{-/-} cells (**Figure 4D**), possibly explaining the higher levels of YAP in the nucleus of these cells. Together, these results identify YAP as a possible mediator of the effect of Myh10 in microglia and suggest a potential mechanism for the activation and priming seen in these cells.

Discussion

We have recently shown that the absence of Myh10 in microglia leads to the inflammatory activation of these cells (Melo et al., 2021). Since Myh10 is the heavy chain of the type-ii myosin complex and contains the motor domain that defines its motor activity (Billington et al., 2013), the effect seen after its ablation could be the result of either the lack of its presence or the absence of its molecular motor activity. Our experiments with the type-ii myosin inhibitor blebbistatin favour the latter hypothesis since no effect was seen on ROS production or inflammatory cytokine expression. Although blebbistatin inhibits both Myh9 and Myh10 motor activity, we believe that this simultaneous effect does not affect the results seen, given that we previously determined that myh9 ablation has no influence in microglia activation (Melo et al., 2021). We did not study the effects of blebbistatin on phagocytosis because its positive contribution to this cellular process has been previously identified (Barger, Gauthier, & Krendel, 2019; Olazabal et al., 2002). Nonetheless, it was interesting to see that the effect of Myh10 ablation on microglia activation does not require its motor activity, since the overwhelming majority of the known functions of this protein depend on it (Vicente-Manzanares et al., 2009). However, this result does not allow us to exclude a

mechanical effect of Myh10 on the inflammatory activation of microglia, since blebbistatin inhibition blocks the function, but not the connection of the myosin motor to actin (Takács et al., 2010). As such, inhibited motor molecules can remain bound to actin filaments and affect the mechanical properties of the actin cytoskeleton through this cross-linking effect, something that no longer occurs in the absence of the Myh10 molecule. We must further mention that we cannot definitively exclude the possibility that the effect of the absence of Myh10 on microglia function is mediated by a “gain of function” of Myh9.

In an attempt to further understand the mechanism that is operating, we were able to identify two key molecules that are activated by the absence of the motor protein: NF- κ B and c-Src, indicating that these two canonical signalling pathways of microglia activation are associated with Myh10 deletion. We also identified Csk as the likely upstream regulator of c-Src that is responsible for the increased activity of this kinase in the absence of myosin. The regulation of this kinase lies mostly in the control of its migration to the plasma membrane, increasing the proximity to its targets (Okada, 2012). Although we do not know if Myh10 is also influencing the spatial distribution of Csk, a previous study has detected myosin heavy chains in direct contact with Csk in muscle cells (Goel & Dey, 2002), while another has identified the heavy chain myh9 as a target of c-Src (Almeida et al., 2015). It would be interesting to understand if analogous Csk-Myh10 and c-Src-Myh10 interactions occur in microglia, since such a complex would suggest that Myh10 can act as a scaffolding protein with the ability to regulate signalling pathways, as we previously hypothesized in Chapter 2 of this thesis.

Aside from the enhanced NF- κ B and c-Src signalling, we also detected increased nuclear presence of YAP, suggesting a role for this transcriptional co-activator in the response of microglia to the absence of Myh10. YAP has been linked to enhanced NF- κ B activity in macrophages (Boro, Singh, & Balaji, 2016; Tang et al., 2021) and sarcoma cells (Rivera-Reyes et al., 2018; Ye et al., 2018), influencing their production of cytokines and chemokines, likely by altering chromatin accessibility (Halder et al., 2019; Monroe et al., 2019). Interestingly, an upstream signalling partner of YAP (Mst1) has been suggested to increase NF- κ B signalling in microglia (S. Zhao et al., 2016) by inducing the degradation of I κ B α , the main inhibitor of NF- κ B, pointing to a direct cross-talk between the two signalling pathways. Furthermore, c-Src signalling can also be influencing YAP activity, since both have been linked in the context of epithelial cell signalling (Lamar et al., 2019; Si et al., 2017). Finally, YAP is also known to play an essential role in mechanotransduction (Dupont et al., 2011). Although the regulation of such a process is complex and still the subject of active research, the presence of YAP in the nucleus has been linked to F-actin in fibroblasts and epithelial cells (Das, Fischer, Pan, & Waterman, 2016; Wada et al., 2011). Given our

previously hypothesized effect of Myh10 on the mechanical properties of the actin-cytoskeleton, YAP may also behave as a mechanotransducer in the absence of Myh10, and in this way further contribute to the regulation of microglia inflammatory activation. Given all these possible roles for YAP in mediating the effect of Myh10 ablation in microglia, we believe it would be important to knockdown YAP in Myh10^{-/-} cells and evaluate the levels of nuclear NF-κB and inflammatory cytokine expression.

Our results on YAP also offer a putative explanation to the priming results seen in response to LPS, since the possible enhancement of NF-κB activity and increased chromatin accessibility could pre-condition cells to respond to inflammatory stimuli. This tendency to demonstrate an increased response to a challenge is an example of trained immunity, a concept that has seen increased attention, given the potential that it has to influence global immune responses to either infectious organisms, vaccine challenges, autoimmune disorders or even neurodegenerative diseases (Divangahi et al., 2021; Netea et al., 2020). Although trained immunity has already been recognised in microglia (Perry & Holmes, 2014; Wendeln et al., 2018), our results after LPS challenge identify Myh10 as a novel factor mediating priming in these cells.

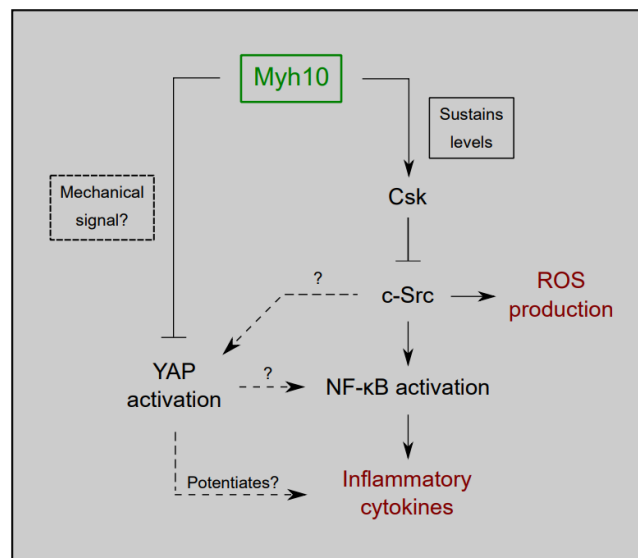


Figure 5: Putative mechanisms of Myh10 regulation of microglia activation. Myh10 sustains the expression of Csk, which in turn inhibits c-Src activity, which in turn reduces the production of ROS and NF-κB activation, decreasing the expression of inflammatory cytokines. YAP activation is also decreased by Myh10, possibly via a mechanochemical effect or through a c-Src mediated direct or indirect effect. This decrease in YAP signalling can further contribute to downregulate the expression of inflammatory cytokines by impacting NF-κB signalling or reducing chromatin accessibility. Solid lines indicate known effects, while dashed lines indicate possible interactions or associations. See the discussion for more details.

Overall, our results identify key regulatory pathways that can mediate the inflammatory activation of microglia in response to Myh10 ablation (**Figure 5**), and although they do not pinpoint the entire mechanism, they raise the possibility of these regulatory pathways acting together, resulting in a cascade of biochemical and mechanochemical signalling that culminates in priming and inflammatory activation of microglia.

Global Discussion

Global Discussion

Microglia are remarkably plastic cells regarding both their functions and their shape. When we started our work on studying morphological changes in microglia, we realized that a straightforward, accessible and easy to use experimental model for such a task was largely missing. The work and results shown in Chapter 1 tried to address this unmet need by proposing and using the retina as a simple, reliable and powerful experimental system that is very well suited for such a purpose. Although we used it for a static study of microglia morphology either directly after tissue dissection or after short-term culture in response to exogenous stimulations, we believe this model system can also be used in more dynamic studies of microglia shape change using live cell imaging. Confocal ocular microscopy has been successfully used to non-invasively study retinal microglia in living animals (Alt, Runnels, Mortensen, Zaher, & Lin, 2014; Bosco et al., 2015), without the need for surgical implantations of imaging windows like the ones necessary for the live imaging of brain microglia. Alternatively, retina's amenability to *ex vivo* culturing also allows for live imaging of microglia using simple laser scanning confocal microscopes (Lee et al., 2008) although we believe that multi-photon confocal microscopes are better suited to such an approach, given the inherent light-sensitivity of the tissue and the ability to use less damage-inducing laser wavelengths during imaging. Aside from the experimental model itself, the development of MorphoMacro further allowed us to quantitatively analyse retinal microglia morphology. The combination of these tools addressed the 1st objective of this thesis and was instrumental for the subsequent work performed. Indeed, without the tools developed in Chapter 1, we would not have been able to probe the contribution of RhoA in microglia shape and process extension like we did in Chapter 2.

The final element of our experimental model toolkit was the use of the HMC3 microglia cell line. Although using a cell line always raises the question of the effect of immortalization on a cell's behaviour, in our specific case we would not have been able to use primary cells to study the impact of RhoA, Myh9 or Myh10 ablation on microglia, since the lack of cell turnover in culture did not allow us to achieve efficient silencing (in the case of myosin heavy chains) or did not allow microglia to recover from it (in the case of RhoA – data not shown). Such situations are precisely where cell lines can be used as an alternative model, to inform on the effect of molecules or proteins that would otherwise remain experimentally inaccessible in *in vitro* microglia. It was only through the combination of our *in vitro* analysis of actin cytoskeleton regulators in RhoA-ablated HMC3 microglia, together with the morphological data provided by the retinal model and MorphoMacro that we were able to propose the mechanisms of protrusion generation and extension, successfully completing

our second objective. The results from Chapter 2 also confirmed our hypothesis that actin cytoskeleton tension is important in the control of microglia shape. Given our proposed role of cortical stress release in the generation of new protrusions and the importance of actin turnover to sustain protrusion extension, it is entirely possible that any regulator of these two processes, aside from Cofilin and Type-II myosins, can influence the dynamics of microglia protrusions, and as such their morphology. In neurons, for example, profilin regulates the morphology of dendritic spines (Ackermann & Matus, 2003) and affects global cell shape (Michaelson et al., 2010), with a similar role being played in astrocytes (Schweinhuber, Meßerschmidt, Hänsch, Korte, & Rothkegel, 2015). As such, it will be interesting to see if in the future this or other actomyosin regulators also have an impact on microglia shape.

Aside from contributing to the morphology of microglia via the control of protrusion generation and extension, our results further revealed a role for type-ii myosins in the control of microglia function. Specifically, the absence of Myh10 and not Myh9 promotes a pro-inflammatory activation state, with an increase in ROS production and inflammatory cytokine expression (Melo et al., 2021). These results, together with our group's previous work showing a similar inflammatory activation in the absence of RhoA, allow us to confirm our proposed hypothesis that changes in cytoskeletal components themselves, as well as cytoskeleton regulators, indeed impact the global function of microglia. Also, the fact that ablation of My10 resulted in inflammatory activation with no concomitant alteration of the cell shape, while Myh9 ablation changed the cell's shape with no apparent effect on the cell's activation state casts an interesting light on the classical form and function relationship in microglia. Although in the vast majority of reported cases the shape of microglia does correlate with changes in their functional state (enough to warrant such an association in the first place), there have been rare cases where no such correlation occurs (Esques, Juillerat-Jeanneret, Leuba, Honegger, & Monnet-Tschudi, 2003; Ohgidani et al., 2016). This shows that it is possible that these cells do not strictly obey this rule. Still, it does not necessarily imply that the form-function relationship is wrong or misleading, it only shows that we must take care in defining exactly what is meant by function: If their function when reactive is simply to secrete factors or locally phagocytose a small target, then it is understandable that microglia can maintain their shape or not vary it significantly and still achieve it. Conversely, if their function when reactive is instead to migrate across the tissue, engulf large particles or address widespread damage, then yes, in that case it is expected that such functions would be compromised if the cell's form does not change substantially.

Having observed the effect of the lack of Myh10 on the activation state of microglia, the last objective of our work was addressed in Chapter 3 by studying possible mechanisms that

explain why the absence of this cytoskeleton member impacts the function of these cells. Given the possibility of mechanotransduction playing a role in the inflammatory activation of microglia in the absence of Myh10, one can wonder if differences in the mechanical properties of the surrounding tissue can affect these cell's activation state or condition their response to a stimulus. We know that microglia sense and react to different substrate rigidities, displaying durotaxis, a preference towards stiffer structures (Bollmann et al., 2015). Furthermore, substrate rigidity influences the expression of genes such as CD206 and TGF β 1 both in *in vitro* (Blaschke et al., 2020) and *in vivo* microglia (Dudiki et al., 2020). As such, it would be relevant to understand if type-ii myosins, particularly Myh10, contribute to microglia mechanosensing of different substrate regimes, mediating this biophysical effect on the cell's function. On this note, it is also known that the nucleus itself is sensitive to mechanical alterations via mechanotransduction through the cytoskeleton (Tajik et al., 2016) in a process that changes gene expression by the action of histone deacetylase (HDAC) enzymes (Killaars, Walker, & Anseth, 2020; Y. Li et al., 2011). Epigenetic control of microglia function has been observed in studies where ablation of HDAC in a model of neurodegeneration showed increased phagocytosis of amyloid plaques and improved disease outcome (Datta et al., 2018). Similarly other studies show that PRC2 (Polycomb repressive complex 2) restricts the microglia program associated with neuronal clearance, with loss of this epigenetic regulator triggering an activated phenotype with aberrant elimination of neurons (Ayata et al., 2018). As such, it might also be relevant to understand if changes in nuclear shape and mechanics, as well as changes in HDAC activity or other epigenetic regulators are occurring in microglia in the absence of Myh10.

When looking at the results from Chapters 2 and 3 it is interesting to note that the effect of the lack of Myh9 on cell morphology seems to be dependent on its motor function (given the *ex vivo* data on the retina with blebbistatin), while the lack of Myh10 and its effect on microglia function instead does not rely on its role as an actin motor. This again reinforces the notion that type-ii myosin heavy chains have differential functions. Given our proposed role for Myh9 in regulating the levels of tension/stress at the cell's cortex, it is easier to understand that such an action would depend on its motor function. Still, and similarly to what we see for Myh10, there have been instances where motor independent functions for type ii myosins have been described, although so far only in the context of cell motility (Laevsky & Knecht, 2003; Xuefei Ma, Bao, & Adelstein, 2007; Xu et al., 2001). Our results, together with these observations, warrant a different look into the roles of this class of molecular motor, one that tries to go beyond its classical function and examines these proteins as possible direct or indirect signalling hubs with the capacity to influence cell behaviour in a much bigger scale than what would be initially expected. In this context, it

will be interesting to see if other motor-independent functions of type-ii myosins will be described in the future, in which cell types, in which contexts and with what outcomes.

Although more is needed to fully understand how microglia control the extension of their processes, fine tune their shape and control their activation state, the work we performed and that is presented in this thesis proposes some of its principles: Myh9 controls cortical tension and affects microglia protrusion formation, cofilin-mediated maintenance of actin turnover regulates microglia protrusion extension and Myh10 influences microglia inflammatory activation via the classical regulators NF- κ B and c-Src by mechanisms that we do not fully understand yet, but that likely involve direct changes in signalling pathways, perturbations of mechanotransduction or a combination of both. Overall, our results identify novel regulators and reveal the importance of contractility and mechanochemical effects mediated by type-ii myosins on the morphology and function of microglia, offering new perspectives and opening future research avenues that can contribute to a more complete understanding of these important and iconic CNS cells.

General Conclusions

General Conclusions

The work performed during this PhD and presented in the current thesis allow us to conclude the following:

- RhoA is an important regulator of microglia morphology under homeostatic and in response to endogenous stimuli (ATP);
- Cofilin (a target of RhoA) is responsible for maintaining actin turnover, necessary for sustaining microglia protrusion extension;
- Downstream of RhoA there is dichotomous signalling at the level of type-II myosins;
- Myh9 influences cortical tension and impacts microglia protrusion formation;
- Myh10 influences microglia inflammatory activation through the involvement of canonical pathways (NF- κ B and cSrc) and likely by mediating changes in cellular signalling and mechanotransduction;
- Mechanochemical effects can influence the biology of microglia;
- Type-II myosins are novel differential regulators of microglia protrusions, shape and function.

Since this work also opened new research avenues, the present thesis will be closed by a brief exploration of these possibilities in the next section.

Future Work

Future Work

Having already identified throughout the previous sections some relevant areas and specific experiments that could be of interest to perform, this final section will emphasize the experimental work that we believe is the most important to focus on in the near future.

The starting point, and as previously mentioned in Chapter 2, would be the study of the effect of Myh9 and Myh10 deletion in mice using the same conditional genetic ablation technique as the one described and used to knockout RhoA. Such work would be essential to validate the *in vitro* results obtained in this thesis and would further give us the opportunity to isolate and culture primary microglia already recombined from these animals, expanding the possibility of *in vitro* experiments that could be performed. One such experiment would be to confirm if YAP signalling is indeed responsible for mediating the inflammatory effects of Myh10 ablation, using shRNA-mediated silencing or, in case of insufficient ablation, using the pharmacological inhibitor of YAP verteporfin (Bierbaumer et al., 2021; Liu-Chittenden et al., 2012). Since it is also possible that Myh10 absence changes the epigenetic landscape of microglia, we could also employ ATAC-seq to confirm if this is happening and to identify the sites where chromatin is opened or closed. In case YAP is correlated with the effects of Myh10 absence, we could also complement this set of data with that of YAP-targeted ChIP-seq, building a global picture of both chromatin accessibility and sites under the influence of YAP's transactivation effect.

Regarding the possibility of Myh10 acting as a signalling hub, and that its absence changes the cell's signalling pathways we could again use the *in vitro* primary microglia derived from conditional Myh10-ablated mice to first unbiasedly evaluate the global signalling landscape of these cells by employing phosphoproteomic analysis. This would not only confirm that cell signalling is perturbed by the absence of Myh10 but also identify the pathways that change the most. Furthermore, to understand how Myh10 might be behaving as a molecular hub for signalling, we could perform interactomic studies, using either Myh10 co-immunoprecipitation or proximity labelling (BioID), followed by mass spectrometry sequencing. Novel interacting partners of Myh10 could thus be identified and used to understand how Myh10 affects the signalling pathways identified in the phosphoproteomics approach. Together, these *in vitro* experiments would help us further clarify the molecular mechanisms responsible for the effects of Myh10 on microglia function.

Also, given the expected inflammatory behaviour of Myh10-ablated retinal microglia, it would be interesting to determine if there is any functional impact of microglia Myh10 ablation on the retina itself. On a similar note, it would also be relevant to determine if RhoA-

ablated microglia affect the normal functioning of the retina, since our group saw an effect of RhoA-knockout microglia on cortical homeostasis (Socodato et al., 2020).

Finally, we believe it would be important to have an experimental system that would allow genetic manipulation and exogenous protein expression in *in situ* retinal microglia. Recent attempts to generate such a system have relied on AAV-mediated delivery (Maes, Wögenstein, Colombo, Casado-Polanco, & Siegert, 2021), with limited success. Since Lentiviral-mediated delivery shows increased *in vivo* microglia transduction (Åkerblom et al., 2013), this would constitute a better delivery system to try and implement. Although the development of such an experimental model would undoubtedly take time and effort to put in place, we believe it would be very much worth it, since its combination with the *ex vivo* retinal culture system used in this Thesis, either alone or together with multi-photon live imaging microscopy would allow *in situ* visualization of retinal microglia subcellular protein distribution, use FRET probes and target (by shRNA) genes for which no conditional (floxed) line exists.

The development and implementation of the techniques and experiments described here would not only validate the observations and predictions of this thesis *in vivo*, they would complement our mechanistic understanding on the control of *in situ* microglia process formation and extension, expand our knowledge of the mechanisms that control microglia activation and would constitute an invaluable tool that could be used by us and the scientific community to study the role of many more genes and proteins in the biology of retinal microglia.

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