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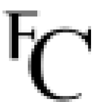
Deciphering the role of RhoA in microglia inflammation

Artur Santos Rodrigues



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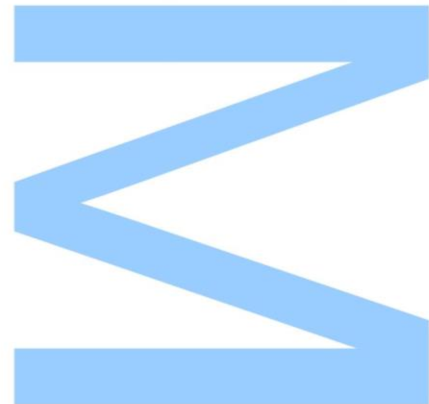

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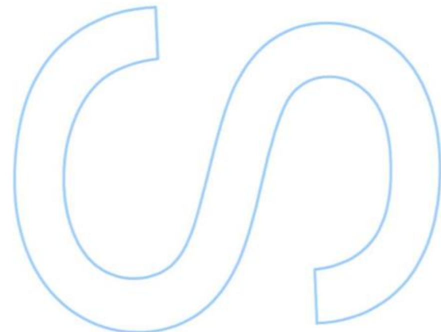

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DECIPHERING THE ROLE OF RHOA IN MICROGLIA INFLAMMATION

ARTUR SANTOS RODRIGUES

2019

Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, ______/______/_________

“The brain is a world consisting of a number of unexplored continents and great stretches of unknown territory”

-Santiago Ramon y Cajal

Agradecimentos

Agradecimentos

Um desafio tão grande como escrever esta dissertação é agradecer em apenas uma página a todas as pessoas que, de forma diferente, fizeram parte desta aventura.

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Um muito obrigado a todos!

Abstract

Abstract

Microglia are resident myeloid cells responsible for the maintenance of central nervous system (CNS) homeostasis. Following CNS damage or infection, microglia become activated, dramatically changing their morphology and transcriptomic signature in order to restore normal brain function. However, exacerbated or prolonged microglial activation may result in neuronal impairment caused by excessive production of proinflammatory mediators, culminating in neuroinflammation, which is a hallmark of several neurological conditions.

Rho GTPases, from which RhoA is one of the most well studied members, are master regulators of cytoskeleton dynamics, playing key roles in cell migration, motility, cell cycle progression, vesicle trafficking and others. Dynamic reorganization of the cytoskeleton is likely to underlie microglial function(s) during activation and microglial activation is associated with profound changes in microglia morphology requiring cytoskeleton reorganization. Therefore, molecules that regulate cytoskeletal organization and dynamics, such as RhoA, are well positioned to critically govern microglia homeostasis.

Here we used in vitro assays with microglial cultures (FRET-based live cell imaging coupled with gain and loss-of-function approaches) and in vivo tissue-specific conditional gene targeting in mice to understand the contribution of RhoA signaling for microglia homeostasis. Our results show that a decrease in microglial RhoA activity is required for microglia metabolic reprograming and proinflammatory polarization to occur. Moreover, in the absence of RhoA, classical inflammatory stimulation and neuroinflammation lead to microglial cell death through the disruption of cytosolic Ca^{2+} and pH homeostasis. Overall, our data suggest that besides being essential for adequate microglia immune activity, tight control of RhoA signaling is also critical for microglial survival during neuroinflammation.

Key words: CNS, FRET, Neuroinflammation, Rho GTPases, Transgenic mice

Resumo

Resumo

Microglia são células mieloides residentes no sistema nervoso central (SNC) responsáveis pela manutenção da homeostasia. Após dano ou infeção no SNC, a microglia transita para um estado ativado, alterando drasticamente a sua morfologia e transcriptoma, de forma a reestabelecer o funcionamento normal do cérebro. No entanto, ativação microglial exacerbada ou prolongada pode resultar em comprometimento neuronal causado pela produção excessiva de mediadores pró-inflamatórios. Tal culmina em neuro-inflamação, um estado caraterístico de diversas condições neurológicas.

As Rho GTPases, das quais a RhoA é um dos membros mais estudados, são importantes reguladores da dinâmica do citoesqueleto, desempenhando papéis relevantes na migração celular, motilidade, progressão do ciclo celular, tráfego vesicular, entre outros. É provável que a reorganização dinâmica do citoesqueleto esteja subjacente à(s) função(ões) da microglia durante a ativação. Além disso, sabe-se que a ativação microglial está associada a mudanças profundas na morfologia da microglia que requerem reorganização do citoesqueleto. Portanto, moléculas que regulam a organização e a dinâmica do citoesqueleto, como a RhoA, estão bem posicionados para desempenhar um papel crítico na regulação da homeostasia da microglia.

Neste estudo recorremos a ensaios *in vitro* com culturas celulares de microglia (tecnologia FRET com captação de imagens de células vivas associada a ensaios de ganho e perda de função), bem como à deleção condicional de genes *in vivo* especificamente nestas células, de forma a perceber a contribuição da RhoA para a homeostasia da microglia. Os nossos resultados mostram que é necessária uma diminuição da atividade da RhoA na microglia para que a reprogramação metabólica e a polarização pro-inflamatória ocorram. Mostram também que na ausência de RhoA, um estímulo inflamatório clássico ou condições de neuro-inflamação resultam em morte celular através da disrupção da homeostasia do Ca^{2+} e pH citosólico. De forma geral, os nossos dados sugerem que, para além de essencial para a função imune normal da microglia, um controlo meticuloso da sinalização e atividade da RhoA é critico para a sobrevivência destas células durante a neuro-inflamação.

Palavras-chave: SNC, FRET, Neuro-inflamação, Rho GTPases, Ratinho transgénico

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List of abreviations

AD – Alzheimer's Disease

AJs – Adherens junctions

AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate

AMPK – 5' adenosine monophosphate-activated protein kinase

AnV – Annexin A5

APP – Amyloid Precursor Protein

ATP – Adenosine Triphosphate

Aβ – Amyloid beta

BAI1 – Brain-specific Angiogenesis Inhibitor 1

BBB – Blood Brain Barrier

BDNF – Brain-Derived Neurotrophic Factor

BSA – Bovine Serum Albumin

C1q – Complement component 1q

C3 – Complement component 3

CCL5 – Chemokine (C-C motif) ligand 5 (RANTES)

CD200 – Cluster of Differentiation 200

CFP – Cyan Fluorescent Protein

cKO – Conditional Knock-Out

CNS – Central Nervous System

CR3 – Complement Receptor 3

CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats

CSF1R – Colony Stimulating Factor 1 Receptor

CT – Control

CUL3 – Cullin 3

DAM – Disease-associated Microglia

DAMP – Damage-associated Molecular Pattern

DAP12 – DNAX Activation Protein of 12kDa

DAPI – Fluorescent stain (4′,6-diamidino-2-phenylindole)

DGAV – Direção Geral de Alimentação e Veterinária

DMEM – Dulbecco's Modified Eagle Medium

DTT – Dithiothreitol

EDTA – Ethylenediamine Tetraacetic Acid

EMPs – Erythromyeloid Percursors

FACS – Fluorescence-Activated Cell Sorting

FBS – Fetal Bovine Serum

FL – Fetal Liver

FRET – Fluorescence Resonance Energy Transfer

GAPs – GTPase-activating Proteins

GDIs – Guanine Nucleotide Dissociation Inhibitors

GDNF – Glial Cell line-derived Neurotrophic Factor

GDP – Guanosine-5'-diphosphate

GEFs – Guanine Nucleotide Exchange Factors

GM-CSF – Granulocyte-macrophage Colony-stimulating Factor

GTP – Guanosine-5'-triphosphate

HD – Huntington's Disease

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid

HRC – Histidine Rich Calcium Binding Protein

IFN-γ - Interferon Gamma

IGF-1 – Insulin-like Growth Factor 1

IKK – Inhibitor of nuclear factor Kappa Kinase

IL-1β – Interleukin 1β

IL-34 – Interleukin 34

IL-6 – Interleukin 6

IRAK – Interleukin-1 Receptor-associated Kinase

IRES – Internal Ribosome Entry Site

IRF8 – Interferon Regulatory Factor 8

KD – Knock-down

LIMK – LIM Kinase

LPS – Lipopolysaccharide

LTP – Long Term Potentiation

MAPK – Mitogen-activated Protein Kinase

MCP-1 – Monocyte Chemoattractant Protein-1

mDIA – Diaphanous-related Formin-1

MERTK – Proto-oncogene Tyrosine-protein Kinase MER

MFG-E8 – Milk Fat Globule-EGF Factor 8 protein (lactadherin)

MFI – Mean Fluorescence Intensity

MLC – Myosin Light Chain

mRNA – Messenger Ribonucleic Acid

mTOR – Mammalian Target of Rapamycin

NADPH – Nicotinamide Adenine Dinucleotide Phosphate

NF-κB – Nuclear Factor κ B

NLR – Nucleotide-binding Oligomerization Domain-like Receptors

NO – Nitric Oxide

NPCs – Neural Percursor Cells

OXPHOS – Oxidative Phosphorylation

P2RY12 - P2Y Purinoreceptor 12

PAMP – Pathogen-associated Molecular Pattern

PBS – Phosphate-buffered Saline

PD – Parkinson’s Disease

PFA – Paraformaldehyde

PKA – Protein Kinase A

PKG – Protein Kinase G

PRR – Pattern Recognition Receptor

PS – Phosphatidylserine

PSF – Point Spread Function

PTMs – Post-translational Modifications

RBS – Rho-binding-site

RIPA Buffer – Radioimmunoprecipitation Assay Buffer

ROCK – Rho-associated Protein Kinase

ROI – Region of Interest

ROS – Reactive Oxygen Species

RUNX1 – Runt-related Transcription Factor 1

SALL1 – Sall-like Protein 1

SCF complex – Skp, Cullin, F-box containing complex

TAM – TYRO3, AXL and MER

TBS-T – Tris-buffered saline + Tween20

TCA Cycle – Tricarboxylic Acid Cycle (Krebs cycle)

TGF-β1 - Transforming Growth Factor β1

TIM4 – T-cell Membrane Protein 4

TLR – Toll Like Receptor

TMEM119 – Transmembrane Protein 119

TNF – Tumor Necrosis Factor

TREM2 – Triggering Receptor Expressed on Myeloid Cells

YFP – Yellow Fluorescent Protein

YS – Yolk Sa

Chapter 1
Introduction

1. Introduction

1.1. Microglia

1.1.1. Discovery and ontogeny of microglial cells

The perception of the CNS as an extremely complex system has always led scientists to question how such intricate relations between all its components are controlled and maintained under homeostatic conditions. Early in history, glial cells were described as the "glue", with the simplistic function of providing structural support to the CNS (Virchow et al., 1858, *vide* Kierdorf and Prinz, 2017). Years later, Pio del Rio-Hortega sheds light to a different view by ontogenically and functionally differentiating a population of non-neural and non-astrocytic cells from the other glia, calling it the "third element". This newly-discovered group included (apart from oligodendrocytes) a cell type from mesodermal origin and with potential to change from an amoeboid to a ramified morphology, baptized by Hortega as microglia (Del Rio-Hortega, 1919, *vide* Cherry et al., 2014).

Nowadays microglia are recognized as the resident myeloid cells of the CNS, representing 5-10% of total brain cells. Together with perivascular macrophages, meningeal macrophages and choroid plexus macrophages, microglia represent the immune guards of the CNS, benefiting from a location that makes them the only population of myeloid resident cells in the CNS parenchyma (Herz et al., 2017).

Until recently, the ontogeny of microglial cells was a controversial subject, with studies referring that they derive directly from blood monocytes. However, new fate-mapping studies show that microglia arise from embryonic yolk sac (YS) precursors, similarly to other tissue macrophages (Alliot et al., 1999, Ginhoux et al., 2010, Gomez Perdiguero et al., 2015). Ontogenically distinct from other CNS cell populations, such as astrocytes and oligodendrocytes, which originate from neuroectodermal progenitors (Rowitch and Kriegstein, 2010), microglia genesis **(Figure 1)** starts with YS erythromyeloid precursors (EMPs) that differentiate into YS macrophages – precursor cells with migratory ability that colonize the embryonic brain (Gomez Perdiguero et al., 2015). In fact, these macrophages evolve from the A1 initial state (start expressing CD45 and decrease expression of the tyrosine kinase receptor KIT) into A2 macrophages (KIT expression are replaced by CX_3C chemokine receptor – CX_3CR1 expression), which populate the early nervous system rudiment starting from embryonic day 9.5 (E9.5) (Kierdorf et al., 2013). Although independent of the transcription factor MYB, which is

expressed on fetal liver (FL) monocytes, microglial development requires PU.1 and RUNX1 in the initial phases, as well as interferon regulatory factor 8 (IRF8) for the maturation of migratory macrophage precursors (Kierdorf et al., 2013). CSF1 receptor (CSF1R) is also critical for microglial development and maintenance in such way that mice lacking either one of its ligands – CSF1 or IL-34, display decreased microglia numbers, whereas in PU.1 deficient mice, microglia are absent (Beers et al., 2006, Ginhoux et al., 2010). Furthermore, environmental cues such as TGF-1β , epigenetic modifications or the expression of SALL1, function as microglia-specifying factors, which ultimately establish microglia identity (Butovsky et al., 2013, Buttgereit et al., 2016, Gosselin et al., 2017).

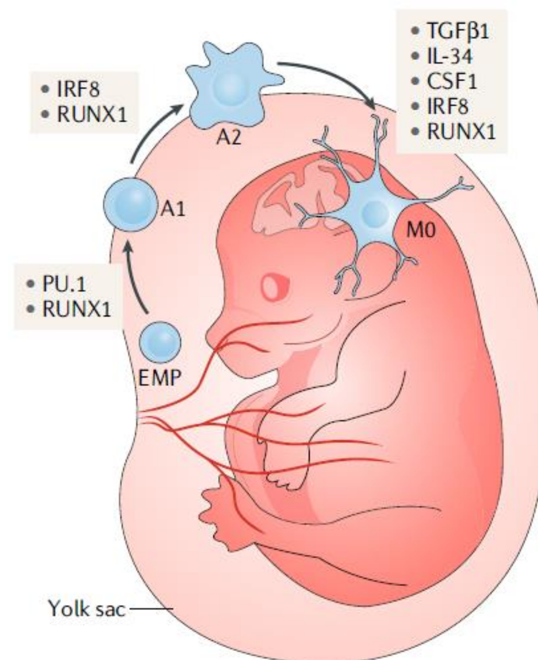

Figure 1- **Mammalian microglia ontogeny and development.** Main genes expressed in the different microglia developmental stages. Adapted from Butovsky and Weiner,2018.

1.1.2. Microglia and brain homeostasis – *Housekeeping function*

As other peripheral tissue-specific macrophages, microglial cells integrate the innate immune system and are classically known for their role in host defense against pathogens during infections. For a long time, the microglia under homeostatic conditions in a healthy brain were referred to be in a “resting state”. However, *in vivo* observations completely abrogated this terminology and today it is known that microglia are immunosurveillant cells, continuously scanning the microenvironment by extending and retracting their fine processes searching for any damage or threat that can compromise the CNS parenchyma (Nimmerjahn et al., 2005). In fact, such accurate sensing ability is a feature of the "microglia sensome" **(Figure 4)**, which is composed of several membrane receptors grouped according to the type of ligands they recognize (Hickman et al., 2013). Most of these receptors are common to microglia from different brain regions, reinforcing their role as key factors for microglia to perform so-called "housekeeping" functions (Hickman et al., 2018). For instance, purinergic receptors such P2ry12 detect ATP and are important to monitor neuronal activity and redirect microglia to neuronal injury sites (Haynes et al., 2006). Moreover, the sensome is also required for microglia defense function and activation in response to self and non-self stimulation. Overall, this system is a prerequisite for the different microglia functional states in the maintenance of CNS homeostasis (Kierdorf and Prinz, 2017).

1.1.2.1. Microglial function in the developing CNS

Besides regulating major aspects of brain immune activity, microglia are responsible for many other brain-specific functions. Those capabilities are possible due to the release of factors in the CNS environment as soon as microglial progenitors enter the early developing brain. This ultimately results in upregulation of several cell surface receptors such as Tmem119, P2ry12 and Sall1, which compose the microglial transcriptional signature and are crucial for microglia identification and differentiation from other brain myeloid cells (Lavin et al., 2014, Mass et al., 2016). Considering the fact that brain colonization by myeloid precursors occurs simultaneously with neurogenesis (but before astrocytes and oligodendrocytes generation), microglia are believed to play an important and prominent role in this early phase of CNS development (Martynoga et al., 2012, Frost and Schafer, 2016). In line with this are studies reporting severe structural defects

on brain architecture in animals where microglial cells were depleted (Parkhurst et al., 2013, Paolicelli and Ferretti, 2017, Erblich et al., 2011).

Accordingly, as proficient phagocytes, microglia play critical roles in the clearance of apoptotic neurons in the CNS (Dekkers et al., 2013, Ashwell, 1990). This process, which is also present in the adult state, is possibly carried out by the so called "eat me" signals such as phosphatidylserine (PS) that is exposed by dying cells and recognized by phagocytic receptors (e.g. MFG-E8, BAI1, TIM4) or TREM2/DAP12 machinery, located in microglia surface (Hanayama et al., 2002, Takahashi et al., 2005, Liu et al., 2013, Mazaheri et al., 2014). Moreover, microglia *per se* are capable of inducing cell death, not only by engulfing neural precursor cells (NPCs) to control both embryonic and adult cell pools, but also through the release of ROS in a process resembling neutrophil oxidative burst (Marin-Teva et al., 2004, Cunningham et al., 2013). Nevertheless, microglia also have an important role in neurogenesis, providing trophic support mainly through the secretion of neurotrophic factors such as insulin-like growth factor 1 (IGF-1) or brain-derived neurotrophic factor (BDNF) to promote neuronal survival during postnatal development (Ueno et al., 2013, Bathina and Das, 2015). Additionally, microglia are important to promote neuronal wiring and circuit assembly with the contribution of CX_3CR1 and DAP12 surface receptors (Squarzoni et al., 2014).

The prenatal stage is also a critical period for the establishment of a proper brain vasculature in order to allow an efficient delivery of nutrients and oxygen to all brain cells. Therefore, the connection between sprouting vessels is carried out by the microglial cells of the neuroectoderm, which not only produce soluble factors that promote branching but also induce anastomose between neighboring vessel tip cells, generating vascular loops (Checchin et al., 2006, Arnold and Betsholtz, 2013). Concomitantly, the reduction of microglial numbers is correlated with a decrease in vascular density and branching (Greenberg and Jin, 2005, Kubota et al., 2009).

The developing brain is also characterized by a constant remodeling of the neuronal circuits, which occurs with the contribution of microglial cells. These brain "architects" shape postnatal neural circuits in an activity-dependent manner using the complement system, a signaling cascade exclusive of microglia in the brain (Veerhuis et al., 2011). Therefore, the close interaction with pre- and post-synaptic elements allows microglia to sense synaptic activity and to tag weak or desynchronized synapses with the complement components C3 and C1q, ultimately resulting in the microglia-mediated synapse engulfment through CR3 **(Figure 2)** (Stephan et al., 2012). This process, known as "synaptic pruning", is an apoptosis-independent process to refine synaptic circuitry and avoid excessive number of synapses. Thus, not only network activity is improved,

but this system is also related with plasticity and learning, which are features required for proper brain development (Paolicelli et al., 2011).

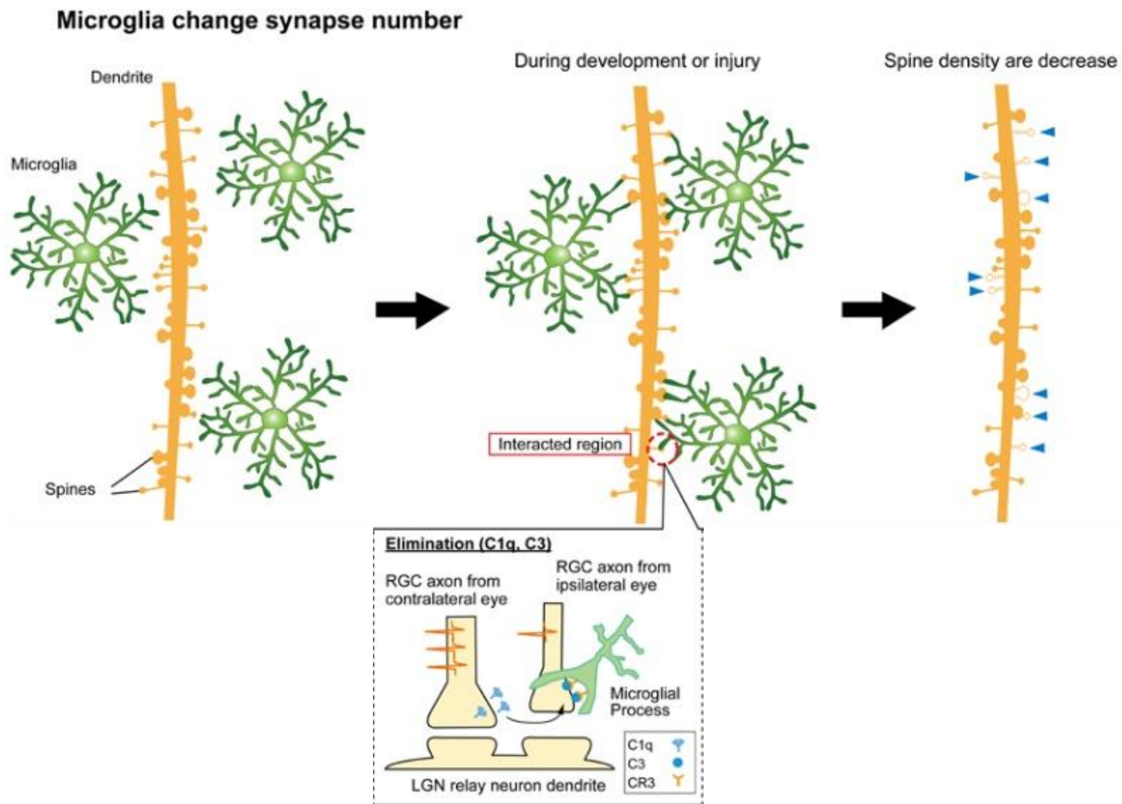

Figure 2- **Microglia-mediated synaptic pruning.** Microglia use complement system components to target and eliminate week synapses, leading to decreased spine density. Adapted from Miyamoto et al., 2013

1.1.2.2. Microglial function in the adult CNS

Although with the main neuronal network already established in the different regions, the adult brain is still highly dynamic, being characterized by constant circuitry remodeling, mostly at the synaptic level (Gu et al., 2013). In fact, processes such as learning-mediated plasticity and homeostasis maintenance are dependent of microglia activity and their close interaction with neurons. Accordingly, microglia-neuron interactions via CX_3CR1/fractalkine (CX_3CL1) or CD200/CD200R are examples of how this synergy takes place (Biber et al., 2007, Kierdorf and Prinz, 2013). Although both pairs are important for the preservation of microglial homeostatic phenotype (avoiding unwanted activation), CX_3CR1/ CX_3CL1 is also a decisive link in adult neurogenesis by supporting hippocampal neurogenic niche (Bachstetter et al., 2011). Moreover, this regulation is supported by the regular elimination of apoptotic neurons and cellular

debris, which is carried out by microglia via receptor tyrosine kinase MerTK (MERTK or TAM) (Sierra et al., 2010).

Various studies also correlate microglial cells with the modulation of both synaptic plasticity and strengthening. Here, microglia appear to be able to decrease synaptic efficacy/strength by promoting AMPA receptors internalization on neurons (Zhang et al., 2014a). Interestingly, in addition to its neurotrophic functions, microglial-derived BDNF is also crucial in learning-dependent structural plasticity by activating Trk receptors on neurons (Parkhurst et al., 2013, Coull et al., 2005).

On balance, such multitasking role of microglia throughout life is implicated on the preservation of several CNS microenvironmental factors under restrict levels, which characterize a fully-functional brain. The slightest change on any of these processes is likely to compromise homeostasis, possibly enabling a pathological state.

1.1.3. Microglia activation – *Defense function*

Besides all housekeeping functions referred previously, microglia are also known for its immune role in CNS protection. This defense behavior has the microglia sensome as a prerequisite **(Figure 4)**, with the expression of pattern-recognition receptors (PRRs) on the cell surface that recognize both pathogen-associated molecular patterns (PAMPs) and tissue damage-associated molecular patterns (DAMPs). These last (e.g. ATP, glutamate, DNA, RNA) are released by injured cells that may also deliver Ca^{2+} waves, ultimately attracting microglia to the injured site (Takeuchi et al., 2002, Boche et al., 2013). Among microglia PRRs are the NOD-like receptors (NLRs) that can operate with Toll-like receptors (TLRs) which typically recognize structurally conserved molecules present in various pathogens (e.g. lipopolysaccharides detection by TLR4) **(Figure 3)** (Olson and Miller, 2004).

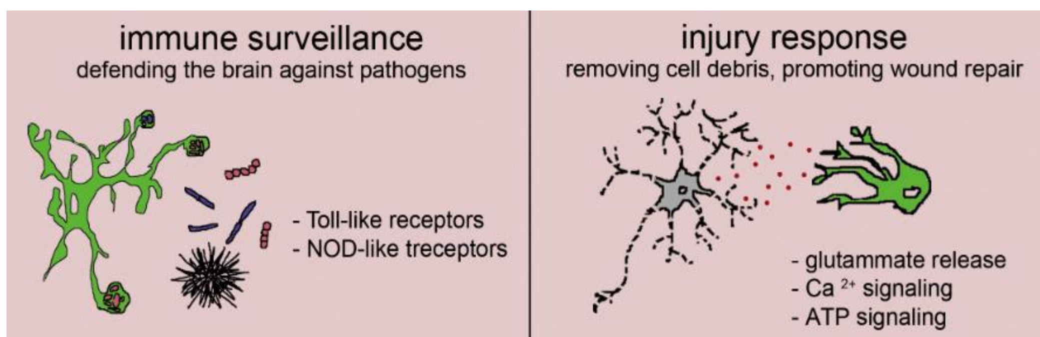

Figure 3- **Microglia immune-related functions.** Through the expression of several membrane receptors, microglia are able to identify and eliminate possible threats (left panel) as well as perceive signals from other cells and repair any damage (right panel) in order to preserve tissue homeostasis. Adapted from Casano and Peri, 2015

In fact, stimulus such bacterial infection or physical trauma, when detected by sensome receptors, can trigger microglia activation. This shift is associated with a change in microglia functional phenotype, with cells shifting from a ramified to a more amoeboid shape (motility improvement) (Stence et al., 2001, Torres-Platas et al., 2014, Uhlemann et al., 2016) and synthesizing a battery of cytokines, chemokines and other molecular mediators that, depending on the causative stimulus, will have a different impact on surrounding cells (Block et al., 2007).

Until recently, microglia activation was seen as a polarized process and macrophage terminology M1/M2 was used to describe a classical or alternative activation, respectively. In this system, M1 phenotype is associated with an inflammatory phenotype, classically induced by triggering of TLR, IFN-γ and NF-kB signaling pathways, where microglia produce pro-inflammatory cytokines (IL-1β, IL-6, TNF-α), glutamate and ROS (Block et al., 2007, Orihuela et al., 2016). On the other hand, M2 activation is associated with neuroprotection and microglial cells express anti-inflammatory mediators (IL-10, IL-12, TGF-β) and BDNF in order to control inflammation and promote damage repair (Varin and Gordon, 2009, Cherry et al., 2014). However, currently this dichotomy is considered an oversimplification and recent genome-wide transcriptomic and epigenetic analysis had demonstrated that microglia activation is a complex process where both M1 and M2 phenotypes may be simultaneously present and vary according to the region and context of study (Ransohoff, 2016b).

Nevertheless, microglia-mediated inflammatory response, when transient, is a necessary step for homeostasis reestablishment by promoting pathogen elimination/injury repair and phagocytosis of remaining dead cells (Aguzzi et al., 2013). However, the failure of anti-inflammatory mechanisms (Cherry et al., 2014), the loss of "resting signals" (e.g. CX_3CR1/CX_3CL1 and CD200/CD200R) (Cardona et al., 2006, Biber et al., 2007) or the long-term exposure to the pathogen or injury (Sochocka et al., 2017) may exacerbate microglia activation, ultimately leading to neuronal damage.

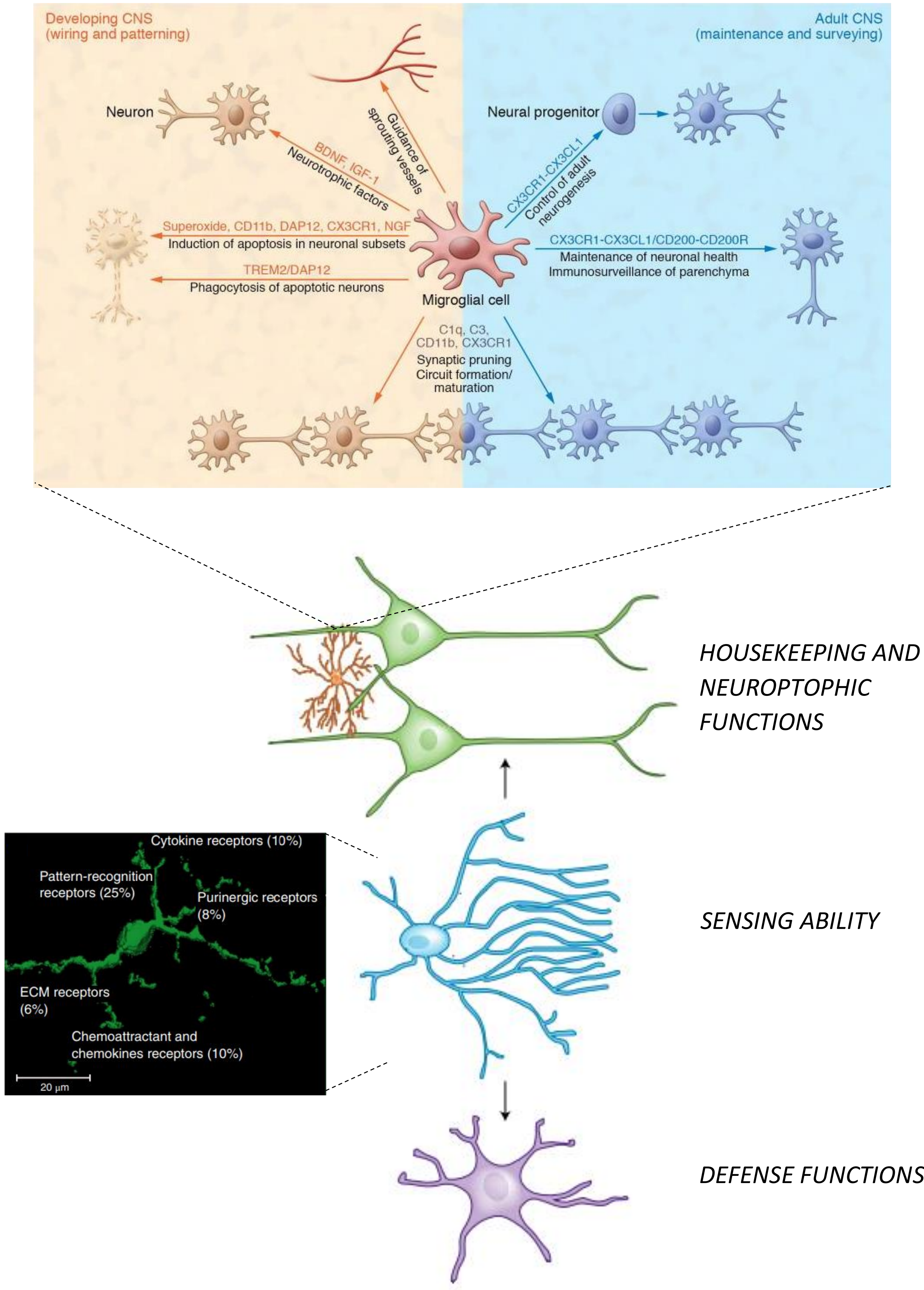

Figure 4- **Overall view of the different functional states of microglia and ontology of the cell sensome.** Microglia sensome (dark panel) is the main prerequisite for all their functions. It includes microglia **homeostatic functions** in the developing and adult CNS, generally by performing housekeeping and neurotrophic tasks (upper part). Sensome is also the basis of microglia **immune functions** as they initiate an inflammatory response and play a defensive role against potential harmful factors (bottom part). Adapted from Kierdorf and Prinz, 2017 and Hickman et al., 2018.

1.1.3.1. Microglia activation and brain inflammation

Microglia overactivation is seen as an important landmark in the transition from a transient inflammatory episode to a chronic inflammatory state, observed in various brain pathologies, including neurodegenerative disorders. In fact, chronic neuroinflammation is characterized by the exacerbated production of pro-inflammatory cytokines, chemokines, ROS and glutamate by CNS glial cells (Block et al., 2007). The accumulation of these mediators in the brain milieu ultimately results in neuronal damage caused by glutamate excitotoxicity (Barger and Basile, 2001) and ROS-mediated oxidative stress (via NADPH oxidase) (Gao et al., 2012). Moreover, pro-inflammatory cytokines such TNF-α, IL-1β and IL-6 as well as the chemokines MCP-1 and CCL5 (RANTES), in the most severe cases, are among those responsible for BBB disruption and peripheral immune cells infiltration (Shigemoto-Mogami et al., 2018), worsening the inflammatory state. Altogether, these events result in a self-perpetuating neurotoxicity cycle, where neuronal damage induces reactive microglia-mediated production of neurotoxic factors that will, in turn, aggravate neuronal damage and result in progressive degeneration **(Figure 5)**.

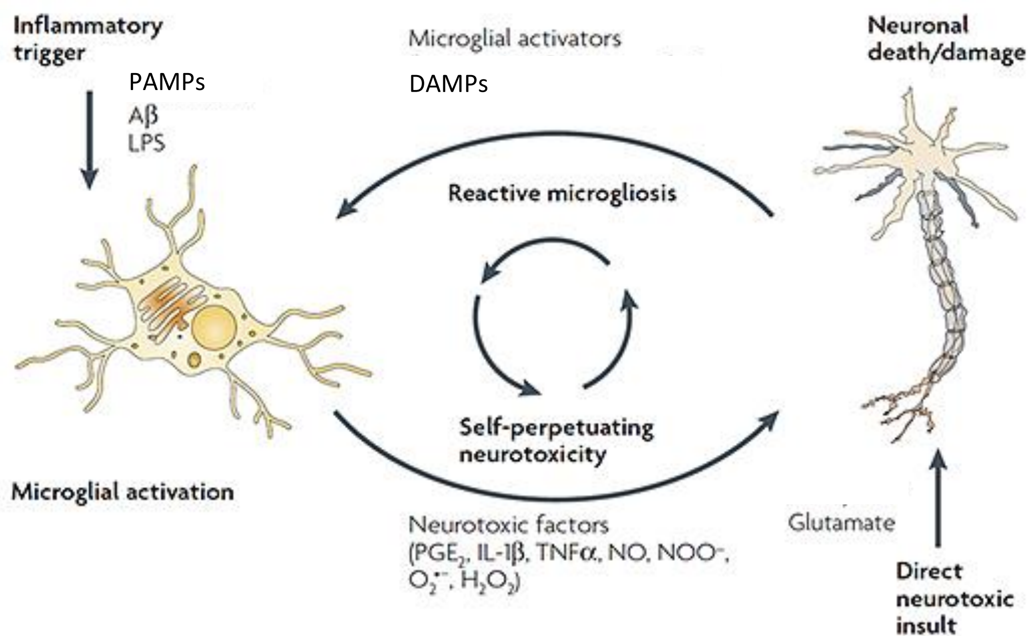

Figure 5- **Self-perpetuating neurotoxicity cycle induced by exacerbated microglia activation.** A progressive cycle of microglia activation and neuronal damage, with the accumulation of several neurotoxic factors is a major feature of chronic neuroinflammation. When sustained, this condition may lead to continuous neurodegeneration. Adapted from Block et al., 2007

Accordingly, this detrimental inflammatory state is a feature of several neurodegenerative disorders such as Alzheimer's disease (AD) (Itagaki et al., 1989), Parkinson's disease (PD) (McGeer et al., 1988) and Huntington's disease (HD) (Sapp et al., 2001). Interestingly, recent studies based on single-cell RNA sequencing analysis address the possible role of microglia-mediated inflammation in pathologies by introducing the terminology "degeneration- or disease-associated microglia" (DAM) (Keren-Shaul et al., 2017, Deczkowska et al., 2018, Song and Colonna, 2018). DAM is characterized by a particular transcriptional and functional signature that incorporates genes associated with various neurodegenerative diseases, most of them typical of both M1 and M2 phenotypes. The transition from homeostatic microglia to DAM was shown to be TREM2-dependent (Wang et al., 2015b) and together with extensive morphological changes was verified the downregulation of "homeostatic" microglia genes such P2ry12, Tmem119, Cx3cr1 and CD33 and the upregulation of genes involved in lysosomal, phagocytic and lipid metabolism, classically associated with disease (Keren-Shaul et al., 2017, Ajami et al., 2018). It is important to note that although the DAM phenotype was firstly observed in AD models where beta amyloid (Aβ) plaques, protein aggregates and cell debris act as an activation trigger, studies have been showing conservation among different mouse models for brain diseases (Song and Colonna, 2018).

Notwithstanding, there is an extensive lack of knowledge regarding the mechanisms involved in the induction of microglia activation and its physiological modulation. The extensive morphological changes that take place in the transition to a more amoeboid shape constitute one of the first steps of the shift to a proinflammatory phenotype (Stence et al., 2001, Uhlemann et al., 2016). In such manner, it is possible that molecules implicated in cytoskeletal remodeling have an important role in this process. Among them, Rho GTPases are more than key regulators of cytoskeleton dynamics, being also important regulators of signaling transduction pathways and gene regulation in multiple cell processes (Zhou and Zheng, 2013). Therefore, are good candidates to be involved not only in the modulation of cell morphology but also in the microglia response to neuroinflammation.

1.2. Rho GTPases

1.2.1. Regulation and function

Ras-homologous (Rho) protein family is a distinct member of the Ras superfamily constituted by low molecular weight (~21 kDa) guanine nucleotide binding proteins (also known as G proteins) (Madaule and Axel, 1985, *vide* Ridley, 2012). In humans, Rho GTPases family comprises 20 members classified into 8 subfamilies, including the three extensively studied members RhoA, Rac1 and Cdc42 (Heasman and Ridley, 2008) **(Figure 6)**. These evolutionarily conserved proteins were early established as key regulators of cytoskeletal dynamics in eukaryotic cells (Nobes and Hall, 1995, *vide* DeGeer and Lamarche-Vane, 2013). Classically, they associate cell-surface receptors (for instance integrins, cadherins, Tyr kinase, cytokine and G proteins coupled receptors) with the assembly and organization of the actin cytoskeleton as well as with the control of microtubule dynamics, cell polarity, membrane trafficking and gene transcription in different cell types (Jaffe and Hall, 2005).

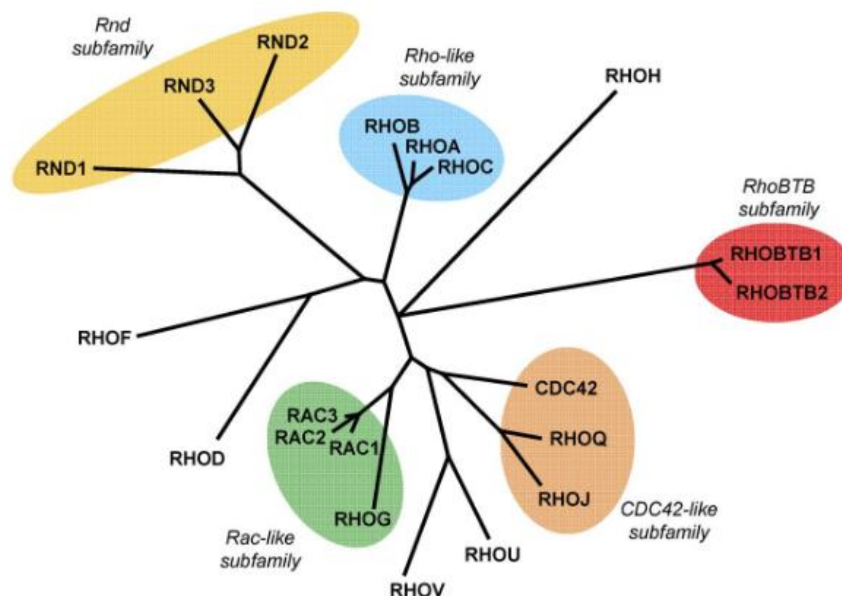

Figure 6- **Rho GTPase family.** Representation of the 20 human Rho GTPase family members grouped in subfamilies. Adapted from Lawson and Ridley, 2018

RhoA is mainly associated with the formation of focal adhesions and stress fibers (Ridley and Hall, 1992), whereas Rac1 mediates lamellipodia formation (Ridley et al., 1992) and Cdc42 induces filopodia formation (Kozma et al., 1995) **(Figure 7A)**. This

modulation of cellular responses to exogenous stimuli (e.g. growth factors) occurs because of the ability of typical Rho GTPases to cycle between an inactive (GDP-bound) form and an active (GTP-bound) form, molecularly operating as binary switches. This process is further modulated by the intervention of three regulatory proteins: guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs). Whereas GEFs catalyze the exchange of GDP to GTP culminating in Rho GTPase activation (Rossman et al., 2005), GAPs have an antagonistic function, enhancing intrinsic GTPase activity that leads to GTP hydrolysis and consequent GTPase inactivation (Tcherkezian and Lamarche-Vane, 2007). GDIs are responsible for confining the GDP-bound form of the Rho GTPase into the cytosol, precluding translocation to the plasma membrane and, consequently, preventing GEFs-mediated activation (DerMardirossian and Bokoch, 2005) **(Figure 7B)**.

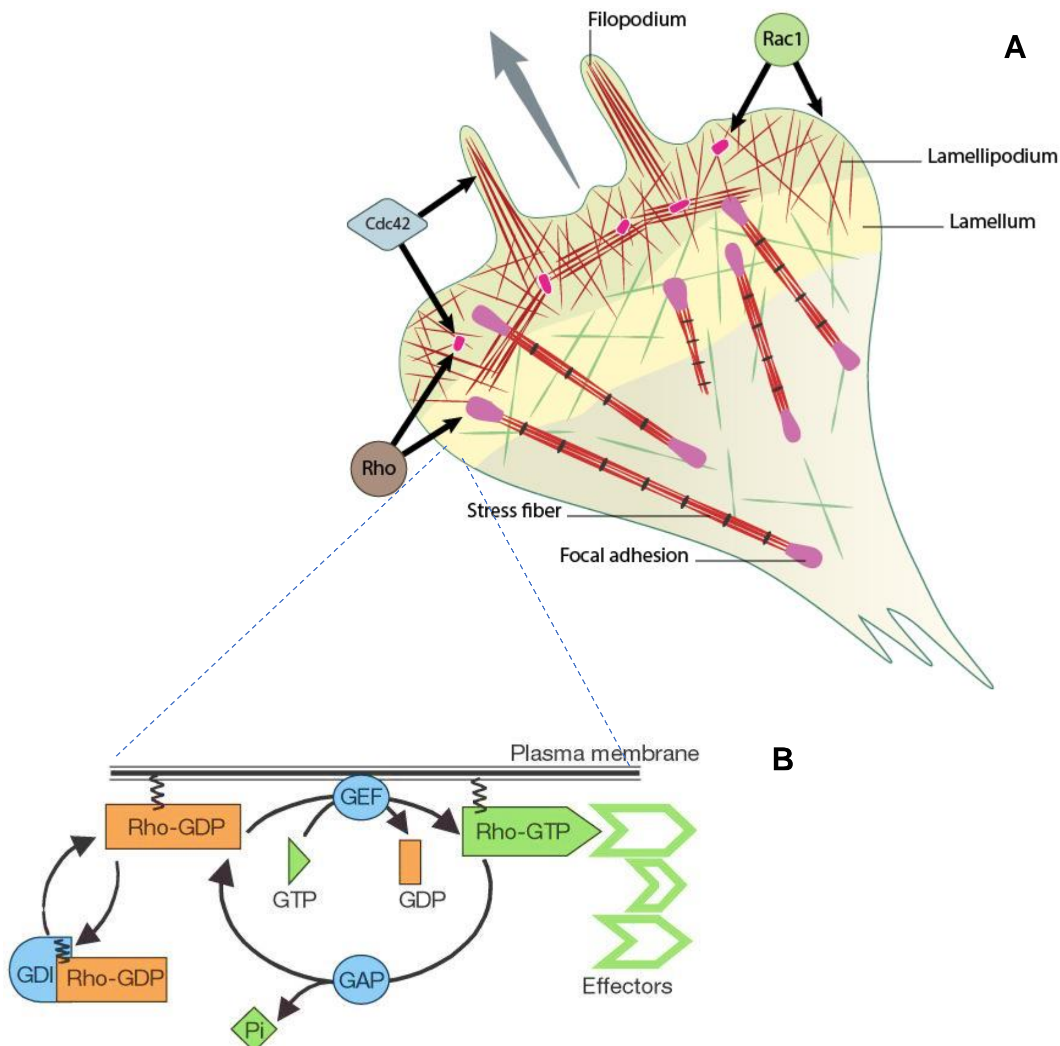

Figure 7- **Rho GTPases function and typical activation mechanism.** RhoA is mainly associated with the formation of focal adhesions and stress fibers, whereas Rac1 mediates lamellipodia formation and Cdc42 induces filopodia formation **(A)**. GEFs and GAPs mediate Rho GTPases transition from inactive to an active form in which they modulate downstream effectors **(B)**. Adapted from Etienne-Manneville and Hall, 2002 and mechanobio.info

Based on regulatory mechanisms, Rho GTPases can be classified as “typical”, if regulated mainly through the previous described GDP-GTP cycling, or “atypical”, for constitutively GTP-bound forms. In the latter, signaling modulation relies on differential gene expression levels (Croft and Olson, 2011) and post-translational modifications (PTMs) (Aspenstrom et al., 2007). In fact, many studies described the importance of these PTMs in regulating both classical Rho GTPases (RhoA, Rac1 and Cdc42) and their regulatory proteins (GEFs, GAPs and GDIs) (Hodge and Ridley, 2016). Whereas lipid modifications as prenylation and palmitoylation control the subcellular localization of Rho GTPases (Linder and Deschenes, 2007, Berg et al., 2010, Navarro-Lerida et al., 2012), phosphorylation and sumoylation are key factors in regulating Rho GTPase activity and interaction with downstream effectors (Lang et al., 1996, Castillo-Lluva et al., 2010). Rho GTPase protein amounts can also be controlled by ubiquitylation, which may target them to proteasome degradation, further controlling GTPase function and turnover (Wang et al., 2003). RhoA inactivation processes can be used as an example since the phosphorylation of RhoA illustrates the importance of PTMs for regulation of Rho GTPase activity. Accordingly, RhoA phosphorylation by protein kinase A or G (PKA/PKG) promotes its interaction with GDIs, culminating in RhoA translocation to the cytosol and preventing its activation (Lang et al., 1996, Tkachenko et al., 2011). Also, three different E3 ligase complexes (SCF, CUL3 and SMURF1) are responsible for RhoA ubiquitylation with consequent proteasome degradation and reduction of cellular protein amounts (Wang et al., 2003, Chen et al., 2009, Wei et al., 2013).

Indeed, the complex interplay between classical regulatory mechanisms and PTMs allows proper response to environmental stimuli that can be cell-specific and different at the subcellular level (Hodge and Ridley, 2016). Therefore, the dynamic balance between activated/inactivated states of Rho GTPases and their regulatory proteins control processes such as vesicle trafficking (Ellis and Mellor, 2000), cell cycle progression (Olson et al., 1995) and cell migration (Ridley, 2001), which are critical functions for a cell to interpret and react to its surrounding microenvironment.

1.2.2. RhoA

RhoA is typically associated with the formation of actin stress fibers and focal adhesion complexes, which were firstly identified in fibroblasts exposed to growth factors (Ridley and Hall, 1992). This occurs mainly due to RhoA ability to induce actin polymerization and actomyosin contractility, ultimately allowing extensive cytoskeleton remodeling. These phenomena are associated with the interaction of activated RhoA with two classic effectors – Rho-associated protein kinase (ROCK) and mDia (Narumiya et al., 2009) **(Figure 8)**. ROCK activation can promote both contraction of actomyosin via the MLC complex and avoid actin polymerization by inhibition of cofilin through LIMK activation (Amano et al., 2000) . Although these classical mechanisms work collectively as an engine that allows cell migration (with RhoA being continuously activated in the cell rear and transiently activated in the front) (Ridley, 2015, Nguyen et al., 2018), several other effectors have been characterized, and consequently, novel RhoA functions have been described. Among them are the roles of RhoA in gene transcription (including MAPK and NF-kB) (Jaffe and Hall, 2005), cell cycle progression (Olson et al., 1995), cell polarization (Fukata et al., 2003), Golgi function (Mardakheh et al., 2016) and chemotaxis (Wang et al., 2013). Therefore, such broad involvement in vital cellular mechanisms makes RhoA signaling dysregulation a likely candidate to be involved in various pathologies like tumor formation and neurological disorders.

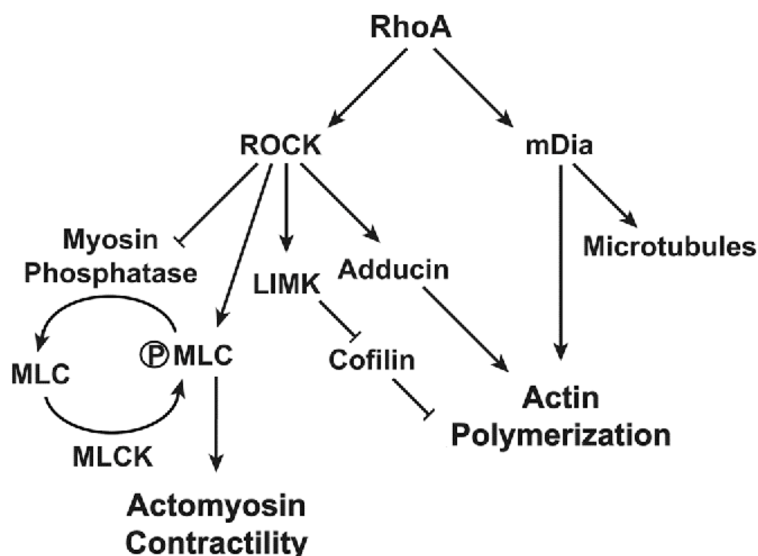

Figure 8- **Classical RhoA associated pathways.** RhoA facilitates actin polymerization by positively regulating multiple effectors and kinases (arrows) as well as through the negative regulation of cofilin by the ROCK-LIMK pathway (blunted lines). Adapted from O'Connor and Chen, 2013

1.2.2.1. RhoA in the nervous system

The complex architecture of the nervous system requires strict control over the processes involved in its development and operation. From neuroepithelial formation to neuronal differentiation and migration, cytoskeleton remodeling is a transversal requirement for the establishment of a functional intercommunicative network. Because Rho GTPases are critical players in cytoskeletal dynamics, a plethora of studies focused on their roles during CNS development and adulthood (referred below).

RhoA was recognized as key player in the first stages of nervous system development, being essential to normal neural tube closure together with maintenance of adherens junctions (AJs) in midbrain/forebrain and spinal cord, ultimately avoiding cases of dysplasia or malformations. Moreover, RhoA is important in cell cycle regulation, modulating the cellular population of neuronal progenitors in the neocortex and spinal cord (Katayama et al., 2011, Herzog et al., 2011, Wallingford et al., 2013). Afterwards, the progression of brain development reaches a point where newly formed neuronal cells leave the Ventricular Zone (VZ), migrating towards their definitive position (e.g. cortical or olfactive neurons) and start establishing synaptic connections (Rakic, 2009). In this case, RhoA activation inhibits neuronal migration and is required as a "stop indicator" when neurons reach their final location, avoiding overgrowth and misleading positioning/ disposition (Iguchi et al., 2008, Ota et al., 2014).

Not only during development but also throughout life, axon branching and extension, neurite outgrowth and dendritic spine formation are required for synapse establishment. In these processes, once again, RhoA appears to have an inhibitory role by promoting growth cone collapse in response to repellent environmental cues and consequently causing axon retraction (Luo, 2000, Wu et al., 2005, Gallo, 2006). In opposition to Rac1 and Cdc42, studies refer RhoA/ROCK as an antagonistic pathway of neurite outgrowth and remodeling (Da Silva et al., 2003), likely through an increase in actomyosin contractility and dephosphorylation of intermediary filaments (Govek et al., 2005). Accordingly, RhoA-induced neurite retraction is reflected on Alzheimer's Disease (AD) brains where degenerating neurites exhibit high levels of activated RhoA (Huesa et al., 2010). Moreover, RhoA/ROCK activation has been associated with gamma secretase-dependent cleavage of APP and consequent production of Aβ fragments (Zhou et al., 2003).

RhoA can also promote axon branching or neurite outgrowth, possibly via Diaphanous-related formin-1 (mDia1) activation (also responsible for AJs maintenance) and actin polymerization (Sit and Manser, 2011). Moreover, diverse factors such as

neurotrophins may act as modulators of Rho GTPases' function by interacting with membrane receptors (Spillane and Gallo, 2014, Rex et al., 2007). For instance, mTOR–mediated *de novo* synthesis of RhoA was described as a crucial event for long-term potentiation (LTP) in hippocampal neurons (Briz et al., 2015).

Besides neurons and neuroepithelial cells, RhoA has been implicated in Schwan cell migration, proliferation, and myelin formation. This was observed after RhoA downregulation, which resulted in migratory and proliferative impairment along with a phenotype of hypomyelination in sciatic nerves (Wen et al., 2017). Congruently, inhibition of RhoA activity in astrocytes gives rise to dramatic changes in morphology, inducing a highly branched and stellate phenotype (Zeug et al., 2018). This is typically observed in activated astrocytes during glial scar formation, where IL-1β induces this phenotype via RhoA inhibition (John et al., 2004).

Regarding microglia, very few studies are available and most of them are focused on the role of ROCK as a direct RhoA effector, rather than in RhoA itself. For instance, Scheiblich and colleagues reported the inhibition of RhoA/ROCK via Ibuprofen and Y-27632, respectively, as a method to decrease both nitric oxide (NO) release and neuronal fragments engulfment during brain inflammation (Scheiblich and Bicker, 2017). The same inhibitory method was also described as neuroprotective in a study reporting RhoA/ROCK as mediators of the microglial inflammatory response after Aβ-mediated microglia activation in the an animal model of AD (Zhang et al., 2019). A very recent work also proposes microglial RhoA/ROCK signaling as a key intervenient in neuropathic pain. This occurs after ATP-mediated P2Y12 activation and induces morphological changes and cytokines release, leading to neuronal hyperactivity and consequent increase in pain sensitivity (Yu et al., 2019).

In fact, although none of these studies addresses specifically RhoA function in microglia, they shed light into a probable involvement of this Rho GTPase in inflammatory processes triggered by brain-resident immune cells. Despite several works have implicated RhoA in a multitude of processes regarding neuronal network establishment and modulation, whether RhoA plays any specific role in the physiological and morphological changes that occurs in microglia during brain inflammation remains elusive. Therefore, concerning the prevalence of neuroinflammation as a common feature in the development and progression of most brain disorders, the understanding of those mechanisms would open a door to more accurate therapeutic approaches, aiming at reducing the detrimental consequences of brain inflammation.

Chapter 2

Aims

2. Aims

As the resident immune cells of the CNS, microglial response to harmful stimuli is a prerequisite for the preservation of brain homeostasis. In fact, the disruption of this response either by its exacerbation or its prevention is detrimental to brain homeostasis. Is also widely accepted that microglia response to stimuli, classically known as 'microglia activation', is associated with extreme morphological changes requiring extensive cytoskeletal rearrangements. Therefore, Rho GTPases, as master regulators of cytoskeleton dynamics and other important cell functions, are well-positioned to modulate microglia homeostasis.

Previous studies from our laboratory revealed that the small GTPase RhoA is critical for microglia homeostasis under physiological conditions. With this in mind, it is reasonable to hypothesize that RhoA is also important for microglia response during neuroinflammation.

Thus, the main goal of this work was to test the hypothesis and study the role of RhoA in classical microglia activation induced by LPS. In order to so, I addressed 3 main questions using complementary *in vitro* and *in vivo* approaches:

1. Does LPS-mediated microglia activation modulate RhoA activity?
2. Does sustain RhoA activity have any impact on microglia inflammatory response?
3. What is the impact of RhoA deficiency for microglia activation during neuroinflammation?

Chapter 3

Methodology

3. Methodology

3.1. Animals

All mice experiments were approved by Direção Geral de Alimentação e Veterinária (DGAV) and by the animal ethics committee of IBMC-i3S, Porto. Animal facilities and the people directly involved in animal experimentation were also certified by DGAV. All animal experiments considered the Russell and Burch 3R's principle and followed the European guidelines for animal welfare (2010/63/EU Directive), ensuring minimal animal suffering. Animals were maintained in standard laboratory conditions with a 12h/12h light/dark cycle and were allowed free access to food and water.

3.1.1. Conditional RhoA ablation in microglia

Conditional RhoA-deficient mice were generated using two different mice: $Cx3cr1^{CreER\text{-}EYFP}$ mice (purchased from Jackson Laboratories) in which the Cx3cr1 promoter drives high expression of the CreER cassette in microglia (Parkhurst et al., 2013) and mice homozygous for the RhoA floxed allele (Herzog et al., 2011, Jackson et al., 2011). RhoA floxed mice were backcrossed at least for 10 generations and kept at the I3S animal facility. Genotype determination was done by PCR on genomic DNA. Primers used for RhoA floxed alleles were: AGC CAG CCT CTT GAC CGA TTT A (forward); TGT GGG ATA CCG TTT GAG CAT (reverse). Primers for CreER insertion were: AAG ACT CAC GTG GAC CTG CT (WT forward); AGG ATG TTG ACT TCC GAG TG (WT reverse); CGG TTA TTC AAC TTG CAC CA (mutant reverse).

RhoA floxed mice were crossed with $Cx3cr1^{CreER\text{-}EYFP}$ mice from which resulted the progeny of interest: $RhoA^{fl/fl}$ (control mice) and $RhoA^{fl/fl}$:$Cx3cr1^{CreER+}$ (RhoA cKO mice). In order to conditionally ablate RhoA in microglia, control and RhoA cKO mice were administered with tamoxifen (10 mg *per* animal by oral gavage) at P26 and P28 and then analyzed between P100 and P110. Mice was kept on a C57Bl/6 background in all experiments.

3.1.2. LPS administration

Control and RhoA mutant mice (100-110 day-old) were intraperitoneally injected with LPS (4mg/kg) from Escherichia coli 0111:B4 (Sigma Aldrich). Both genotypes were also

similarly administered with a saline solution (NaCl). Twenty four hours post-administration, animals were sacrificed by CO_2 inhalation and brains were extracted.

3.2. Flow cytometry

To identify microglia population, the markers used were: CD45-PE (103106 BioLegend) and CD11b-APC (101212 BioLegend, USA). The assessment of necrotic/apoptotic microglia was done using both Annexin V (640906 BioLegend, USA) and Zombie Violet Dye (77477 BioLegend, USA) cell death markers.

Microglia cells were collected from brain, blood, spleen and liver of both control and RhoA cKO mice. Mice were anesthetized with sodium pentobarbital (0.2ml *per* 30g of mice body weight) and then perfused (transcardial perfusion) with ice-cold PBS. To obtain single cell suspensions, the hole brain was quickly removed from the head, placed on ice-cold Gibco® RPMI 1640 (Thermo Fisher, USA) and mechanically homogenized. The obtained cell suspension was passed through a 100μm cell strainer and centrifuged over a discontinuous 70%/30% Percoll (Sigma-Aldrich, USA) gradient for 30 minutes. Cells located on the interface were collected, pelleted, resuspended in FACS buffer (2% BSA; 0.1% Sodium Azide in PBS) and then counted on Countess™ automated cell counter (Thermo Fisher) using trypan blue exclusion to estimate the number of live cells. A single cell suspension (5×10^5 cells) was incubated with the different FACS antibodies for 30 minutes at 4°C in the dark. Compensation settings were determined using spleen from both control and RhoA cKO mice. A FACS Canto II analyzer (BD Immunocytometry Systems, USA) was used to evaluate cell suspensions. All data were analyzed by FlowJo X10 software® (TreeStar, USA) using a sequential gating strategy.

3.3. MACS® isolation of adult microglia

Mice were sacrificed by CO_2 inhalation and the brains were isolated. The right hemisphere was mechanically dissociated in ice-cold Dounce buffer (15mM HEPES; 0,5% Glucose; and DNAse) by 6 strokes in a tissue potter. Then, the homogenate was passed through a 70 μm cell strainer, centrifuged and cells were counted in a TC10 Automated Cell Counter (Bio-Rad, USA) using Trypan Blue to exclude dead cells. 4×10^7 cells were pelleted by centrifugation (9300 g; 1 min; 4ºC) and resuspended in 360 μL of MACS® Buffer (0.5% BSA; 2 mM EDTA in PBS) followed by incubation with 40 μL CD11b Microbeads (130-093-634 Miltenyi Biotec, Germany). $CD11b^+$ fraction was selected using LS columns (130-042-401 Miltenyi Biotec) in a MACS separator (Miltenyi

Biotec) according to the manufacturer's instructions. Eluted CD11b enriched fraction was centrifuged (9300 g; 1 min; 4ºC), the pellet was lysed in RIPA-DTT buffer and used for protein isolation.

3.4. Brain tissue preparation and immunohistochemistry

After animal perfusion with ice-cold PBS (15 ml), brains were fixed by immersion in 4% PFA in PBS, pH 7.2 overnight. After that, brains were washed with PBS and then cryoprotected using gradually increased sucrose concentrations in a row (15 and 30% w/v). After 24 hours, brains were embedded in OCT medium, frozen (-80ºC) and cryo-sectioned in the CM3050S Cryostat (Leica Biosystems, Germany). Coronal sections from brains (30µm thickness) were collected non-sequentially on Superfrost Ultra Plus® slides. Brain sections from control and RhoA cKO mice (both administered with LPS or NaCl) encompassing identical stereological regions were placed side by side, on the same glass slide. Slides were stored at -20°C until processed for immunohistochemistry. Frozen sections were thawed by at least 1 hour and hydrated with PBS for 15 minutes. Sections were permeabilized with 0.25% Triton X-100 for 15 minutes, washed with PBS for 10 minutes and blocked (5% BSA, 5% FBS, 0.1% Triton X-100) for 1 hour. Primary antibody Anti-Iba1 (1:500; Wako, Japan) was incubated in blocking solution in a humidified chamber overnight at 4°C. The secondary antibody Anti-rabbit Alexa 647 (1:500) was incubated for 2 hours in blocking solution. After the secondary antibody, sections were washed three times for 10 minutes with PBS, incubated for 30 minutes with DAPI (1µg/ml) and rinsed twice in PBS. Slides were cover slipped using Fluoroshield™ (Sigma Aldrich) and visualized under a Leica TCS SP5 II confocal microscope.

3.4.1. Image reconstruction

Images from tissue sections (cortex region) were acquired using a Leica HC PL APO Lbl. Blue 20x /0.70 IMM/CORR water objective in 8-bit sequential mode using standard TCS mode at 400 Hz and the pinhole was kept at 1 airy in the Leica TCS SP5 II confocal microscope. Images were resolved at 1024 x 1024 pixels format illuminated with 2-5% DPSS561 561 nm wave laser using a HyD detector in the BrightR mode and entire Z-series were 41 acquired from mouse brain sections. Equivalent stereological regions were acquired for all tissue sections within a given slide. Image series were deconvolved

using the Hyugens Professional using the Classic Maximum Likelihood Estimation (CMLE) algorithm together with a determined theoretical PSF established using a routine-based implementation for the Hyugens software. Images from different sections within a given slide were acquired on the same day, always by the same operator and with identical microscope parameters (e.g., same laser-line potency; same power for the confocal Argon laser; same objective; same fluorescence exposure times and offset for a given fluorophore; same pinhole aperture; same camera binning, zoom and ROI magnification; same pixel size; same TCS scanner mode and speed; same z-stack step size and optical sectioning and same line averaging).

3.5. Cortical microglia cultures

Primary cortical microglial cell cultures were performed as previously described (Portugal et al., 2017, Socodato et al., 2015a). In brief, mice pups (2-day-old) were sacrificed, their cerebral cortices were dissected in HBSS, pH 7.2, and digested with 0.07% trypsin plus 50 µL (w/v) DNAse for 15 minutes. Next, cells were gently dissociated using a glass pipette in DMEM F12 GlutaMAX™-I (Thermo Fisher) supplemented with 10% FBS, 0.1% gentamicin. Cells were plated in polyD-lysine-coated T-flasks (75 cm^2) at $1.5x10^6$ cells per cm^2. Cultures were kept at 37°C and 95% air/5% CO_2 in a humidified incubator. Culture media was changed every 3 to 4 days up to 20 days. Culture flasks were subjected to orbital shaking at 200 rpm for 2 hours to obtain purified microglial cell cultures. Next, culture supernatant was collected and centrifuged at 453 g for 5 minutes at room temperature. The supernatant was discarded and the pellet, containing microglia, was re-suspended in culture medium. Ultimately, cells were seeded in poly-D-lysine-coated 6 or 12-well culture plates at $2.5x10^5$ cells/cm^2 with Dulbecco's Modified Eagle Medium (DMEM) F12 + GlutaMAX™-I (Thermo Fisher) supplemented with 10% FBS, 0.1% gentamicin and 1 ng/ml M-CSF or 1 ng/ml GM-CSF. Purified microglia were cultured for 5-8 days. Only microglia cultures in which CD11b immunolabeling showed a purity of 95-99% were used in this work.

3.6. Immunocytochemistry

Rat cortical primary microglia were fixed with 4% PFA, washed three times for 5 minutes in PBS, permeabilized with 0.1% Triton X-100 for 10 minutes, washed again and incubated for 1 hour in blocking solution (5% BSA). The primary antibody for GTP-RhoA

(1:100; NewEast Biosciences, USA) was added in blocking solution and coverslips were maintained in a humidified chamber for 1 hour. Coverslips were washed three times for 10 minutes with PBS and incubated with the secondary antibody for 1 hour in blocking solution. After three PBS washes of 10 minutes, the coverslips were incubated for 1 minute with 1 mg/ml DAPI and rinsed twice in PBS. Coverslips were mounted with using Fluoroshield™ and visualized in a Leica DMI6000B inverted epifluorescence microscope using PlanApo 63X/1.3NA glycerol immersion objective. Images were acquired with 4 x 4 binning using a digital CMOS camera (ORCA-Flash4.0 V2, Hamamatsu Photonics). For quantification, images were exported as raw 16-bit tiff using the LAS AF software. Background was subtracted in FIJI using the roller-ball ramp in between 35-50% pixel radius. Images were segmented in FIJI using the local Otsu threshold. Thresholded images were converted to binary mask using the dark background function. Binary mask images were multiplied for their respective original channel images using the image calculator plug-in to generate a masked 32-bit float images relative to each channel. Original coordinate vectors were retrieved from the ROI manager and FIJI returned the mean fluorescent intensity in gray values contained within any single microglia using the multi-measure function. Mean fluorescent intensity for each single microglia were exported and statistically evaluated using GraphPad Prism® software.

3.7. Microglia cell line cultures

Human microglia clone 3 (HMC3) cell line was obtained through SV40-dependent immortalization of human embryonic microglial cells and authenticated by the American Type Culture Collection (ATCC® CRL-3304™) (Janabi et al., 1995). These cells were cultivated with DMEM + GlutaMAX™-I (supplemented with 10% FBS and 1% Penicillin/Streptomycin) and maintained at 37°C, 95% air and 5% CO2 in a humidified incubator. RhoA KO HMC3 microglia cell line, obtained using CRISPR-Cas9 technology was kindly supplied by Pedro Melo from Glial Cell Biology Lab in I3s, Porto.

3.8. Live cell imaging and FRET assay

HMC3 cells were plated at a density of 25000 cells/dish on plastic-bottom culture dishes (μ-Dish 35 mm, iBidi) with Dulbecco's Modified Eagle Medium (DMEM) + Glutamax® (supplemented with 5% FBS and 1% Penicillin/Streptomycin). Cells were transfected with the different biosensors **(table 1)** using the JetPrime DNA transfection

reagent (Polyplus Transfection SA., USA) in a proportion of 2 µL of reagent per 1 µg of DNA. Total medium was changed 4 hours after transfection.

Förster resonance energy transfer (FRET) relies on probes with two kinds of fluorophores: the donor and the acceptor. When excited with the adequate light, donor emits energy that excites the acceptor fluorophore which then emits detectable fluorescence. This only occurs when donor and acceptor fluorophores are in close proximity. For instance, to monitor RhoA activity in living microglia, cultures were transfected with Raichu RhoA FRET biosensor (Nakamura et al., 2006). This probe belongs to a family (Raichu probes) designed to change configuration according to Rho GTPases activation state: The GTP-bound form (activated RhoA) leads to conformational change which causes Donor (CFP) to Acceptor (YFP) energy transfer with ultimate emission of 530nm radiation (YFP emission wavelength). Otherwise, when GTP is hydrolyzed to GDP (inactivated RhoA) there is no Rho – Rho-binding-site (RBS) ligation. In this case, CFP and YFP are too distant for transmission and the emitted radiation has only 475nm (CFP emission wavelength) **(Figure 9)**.

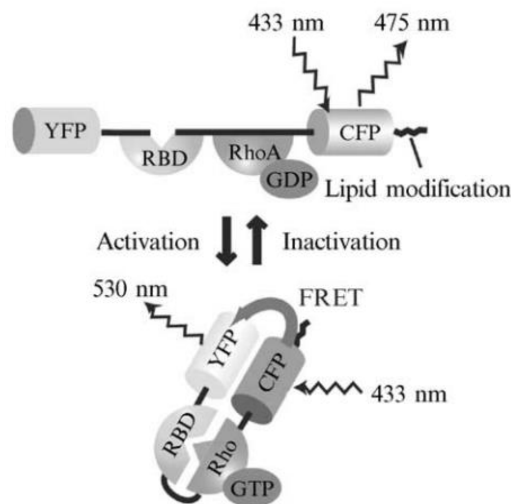

Figure 9- **Raichu RhoA FRET probe conformation and operating mode.** Adapted from Nakamura et al., 2006.

Imaging was performed 24 hours post transfection using a Leica DMI6000B inverted microscope. During the assay, cells were kept under 37ºC in HBSS with $CaCl_2$ and $MgCl_2$ (Thermo Fisher) buffered with HEPES 15mM (Thermo Fisher) with the exception of cells transfected with Calcium and pH biosensors where the imaging was performed using DMEM D-Glucose, L-Glutamine, HEPES without phenol red (Thermo Fisher). Cells were recorded before (5 minutes; baseline) and after that stimulation with LPS (1ug/ml).

Table 1-List of biosensors used throughout the work

Biosensor	Amount of DNA	Addgene catalog #	Reference
pTriEx-RhoA FLARE.sc Biosensor WT- Herein defined as **RhoA FLARE WT**	0.5 µg	plasmid 12150	(Pertz et al., 2006)
pTriEx-RhoA FLARE.sc Biosensor Q63L- Herein defined as **RhoA Q63L**	0.5 µg	plasmid 12151	
pTriEx-RhoA FLARE.sc Biosensor T19N- Herein defined as **RhoA T19N**	0.5 µg	plasmid 12152	
*pRK5-myc-**RhoA WT***	0.5 µg	plasmid 12962	Gift from Gary Bokoch (unpublished)
*pRK5-myc-**RhoA Q63L***	0.5 µg	plasmid 12964	
*pRK5-myc-**RhoA T19N***	0.5 µg	plasmid 12963	
GW1-pHRed Herein defined as **pH biosensor**	0.8 µg	plasmid 31473	(Tantama et al., 2011)
GW1CMV-Perceval Herein defined as **ATP biosensor**	0.5 µg	plasmid 21737	(Berg et al., 2009)
Laconic/pcDNA3.1(-) Herein defined as **Lactate biosensor**	0.5 µg	plasmid 44238	(San Martin et al., 2013)
Pyronic /pcDNA3.1(-) Herein defined as **Pyruvate biosensor**	0.5 µg	plasmid 51308	(San Martin et al., 2014)
pcDNA3.1 FLII12Pglu-700uDelta6 Herein defined as **Glucose biosensor**	0.5 µg	plasmid 17866	(Takanaga et al., 2008)
Cyto-ABKAR Herein defined as **AMPK biosensor**	0.5 µg	plasmid 61510	(Miyamoto et al., 2015)
pFRET-HSP33cys Herein defined as **ROS biosensor**	0.5 µg	plasmid 16076	(Guzy et al., 2005)
pGP-CMV-GCaMP6F Herein defined as **Ca^{2+} biosensor**	0.5 µg	plasmid 40755	(Chen et al., 2013)
mCherry-Lifeact-7 Herein defined as **Lifeact**	0.2 µg	plasmid 54491	Gift from Michael Davidson (unpublished)
Raichu-RhoA	0.5 µg		Kindly supplied by Dr. M. Matsuda (Kyoto Uni, Japan)

The excitation light source was a mercury metal halide bulb integrated with an EL6000 light attenuator. High-speed low vibration external filter wheels (equipped with CFP/YFP excitation and emission filters) were mounted on the microscope (Fast Filter Wheels, Leica Microsystems). A 440-520nm dichroic mirror (CG1, Leica Microsystems) and a PlanApo 63X 1.3NA glycerol immersion objective were used for CFP and FRET images. Images were acquired with 2x2 binning using a digital CMOS camera (ORCA-Flash4.0 V2, Hamamatsu Photonics). At each time-point, CFP and FRET images were sequentially acquired using different filter combination (CFP excitation plus CFP emission (CFP channel), and CFP excitation plus YFP emission (FRET channel), respectively). Quantification of biosensors **(table 1)** was performed using FIJI software. Briefly, images were exported as 16-bit tiff files to the software and the background was dynamically removed from all frames from both channels. Ratiometric images were generated using the PFRET plugin for ImageJ. Finally, a whole cell/subcellular domain analysis was performed and the mean values for each timepoint were extracted.

3.9. Cell lysates

Cell cultures (HMC3) and MACS isolated microglia were lysed using RIPA-DTT buffer (150 mM NaCl, 50 mM Tris, 5 mM EGTA, 1% Triton X-100, 0.5% DOC, 0.1% SDS) supplemented with complete-mini protease inhibitor mix, 1 mM DTT and phosphatase inhibitor cocktail. Samples were sonicated (7 pulses of 1 sec at 60Hz) and centrifuged at 16,000 g, 4°C for 10 minutes. After supernatants collection, protein concentration was determined by the BCA method. All samples were denatured with sample buffer (0.5 M Tris-HCl pH 6.8, 30% glycerol, 10% SDS, 0.6 M DTT, 0.02% bromophenol blue) at 95°C for 5 minutes and stored at −20°C until use.

3.10. Western Blotting

Samples were separated by SDS-PAGE electrophoresis in a 12% SDS-page gel with a voltage of 120V (adjustments overtime were made). Precision Plus Protein™ Dual Color Standards (1610374; Bio-Rad) was used as a molecular weight marker. Proteins were transferred from gel to the Immun-Blot® PVDF membrane (Bio Rad) using a Trans-Blot® Turbo™ Transfer System (Bio-Rad). The transference was performed for 10 minutes in diluted Trans-Blot® Turbo™ 5X Transfer Buffer (20% buffer, 20% ethanol and

60% ultrapure H_2O). Membranes were blocked for 60 minutes in blocking solution composed of 5% skimmed milk diluted in tris based saline with 0.1% Tween (TBS-T) pH 7.6 and incubated with the primary antibodies Anti-RhoA (1:1000; Cell Signaling), Anti-GAPDH (1:20000; HyTest Ldt.) and Anti-Src $pTyr^{416}$ family (1:1000; Cell Signaling) diluted in blocking solution overnight at 4ºC. Membranes were then washed 3 times, for 10 minutes each, with TBS-T and incubated with peroxidase-conjugated secondary antibodies: HRC conjugated anti-rabbit (1:10000; Promega) and HRC conjugated anti-mouse (1:15000; Promega). Membranes were developed using a Pierce™ ECL Fast Western Kit (Thermo Fisher) and revealed using ChemiDoc™ XRS System (Bio-Rad). Images were finally quantified and processed by FIJI Software®.

3.11. Statistical analysis

A 95% confidence interval was used and $p<0.05$ was defined as statistically significant difference in all groups. To compare 2 experimental groups, Mann-Whitney test with equal variance assumption for data with normal distribution was used. To compare 4 experimental groups, one-way ANOVA with Bonferroni multiple comparation test was used. In live cell imaging experiments, two-way ANOVA with Sidak's multiple comparisons test was used. All quantifications were performed using Graph Pad Prism 6.0 software (GraphPad® software).

Chapter 4

Results

4. Results

4.1. Subcellular distribution of RhoA activity in microglia

Although RNA Seq studies revealed that RhoA is expressed in microglia (Zhang et al., 2014b), the establishment of its activity pattern at the subcellular level remains to be determined. Diverse factors modulate the cycling of RhoA between an active and an inactive form, which is critical to regulate cell-specific functions that ultimately result in tight control of cellular homeostasis (Hodge and Ridley, 2016). In order to study the spatial distribution of RhoA activity we transfected primary cortical microglia with the Raichu-RhoA biosensor. This FRET-based probe allows precise monitoring of RhoA activation/inactivation cycle by changing configuration depending on the binding of either GDP or GTP, which leads to changes in Donor (CFP) to Acceptor (YFP) energy transfer. Those differences are represented in the FRET map (FRET/CFP) **(Figure 10A)** where the red and yellow colors (high RhoA activity) are mainly located in the cell processes and near the cell membrane, whereas the blueish and greenish colors (low RhoA activity) located in the cell body.

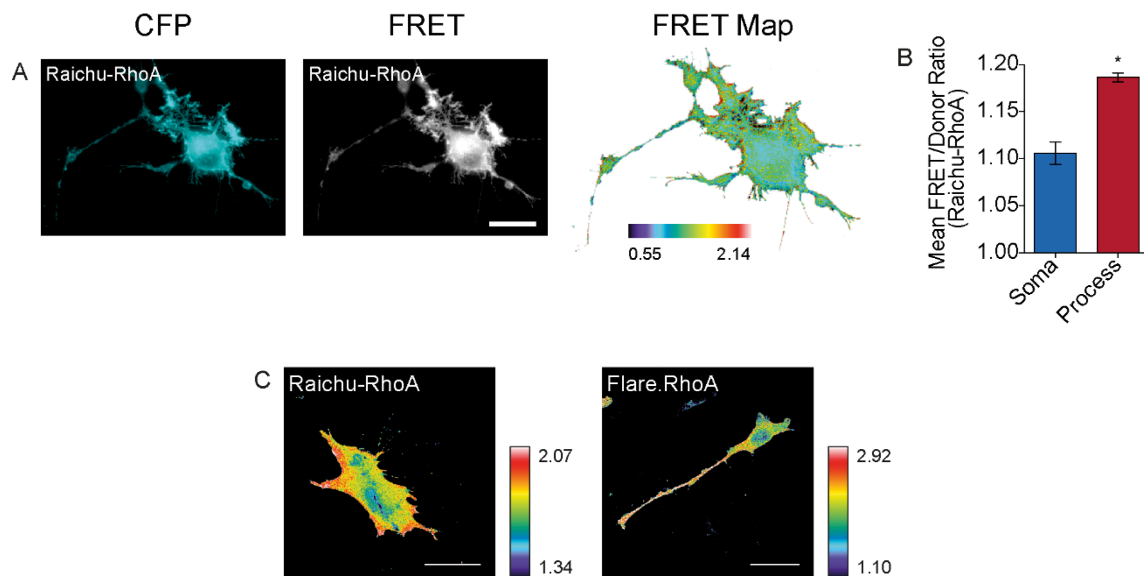

Figure 10-**Spatial distribution of RhoA activity in both rat cortical primary microglia and HMC3 microglia cell line.**

Heat map (FRET/CFP) representing the distribution of RhoA activity in primary rat cortical microglia expressing Raichu-RhoA FRET biosensor. The red color is associated with high RhoA activity and green to blue colors represent low RhoA activity **(A)**. Graphic representation (mean and SEM) of a comparative analysis between RhoA activity in microglia soma and processes (n=8-10 cells from 2 independent experiments) **(B)**. Heat map of HMC3 microglia cell line expressing Raichu-RhoA biosensor (left panel) and RhoA FLARE WT biosensor (right panel) **(C)**. *$p<0.05$ (Mann-Whitney test) vs. CT. Scale bars:20μm.

Indeed, subcellular analysis of RhoA activity demonstrated that RhoA activity was significantly higher in the dendrites than in the cell body of cortical microglia **(Figure 10B)**.

Additionally, the HMC3 microglia cell line was transfected with either Raichu-RhoA or RhoA FLARE biosensors **(Table 1)**, two structurally and functionally similar probes. Similarly to primary microglia, results in the microglia cell line showed higher RhoA activity in the processes than in the cell body **(Figure 10C)**, thereby indicating that the pattern of RhoA activity is similar between cortical microglia and HMC3 microglia.

4.2. Modulation of microglial RhoA activity by LPS

We next examined whether lipopolysaccharide (LPS), a classical stimulus that induces a microglia inflammatory response (Pulido-Salgado et al., 2018), modulates RhoA activity. We first performed immunocytochemistry using an antibody against GTP-RhoA (RhoA active form) on cortical microglia following exposure to LPS. Results showed a significant decrease in GTP-RhoA in microglia treated with LPS when compared with controls **(Figure 11A)**.

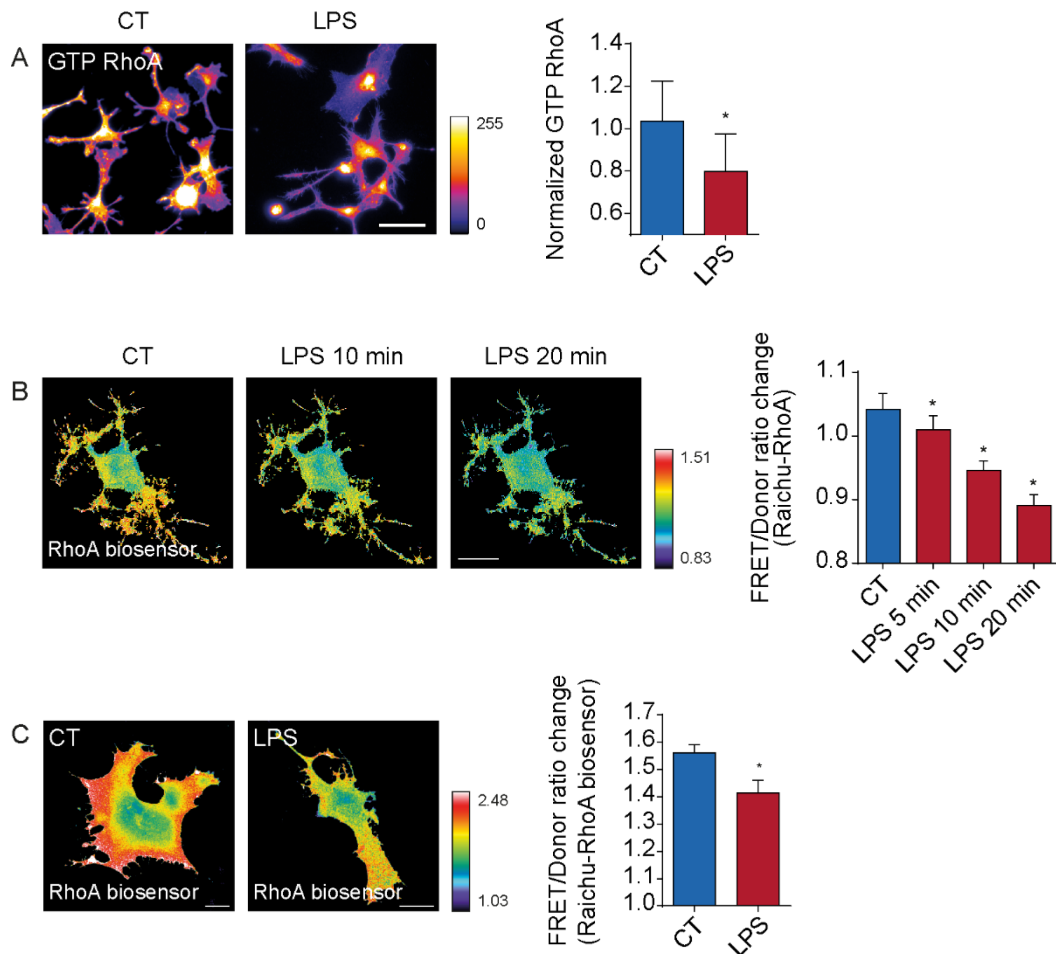

Figure 11- **LPS-mediated modulation of RhoA activity in microglia**

Rat cortical primary microglia treated and non-treated (CT) with LPS (1ug/ml) for 1 hour (n=30 cells from 2 different experiments) and immunostained for GTP-RhoA (yellow to white) **(A)**. Primary rat cortical microglia expressing the Raichu-RhoA FRET probe exposed to 1ug/ml LPS (n=8 cells pooled across 3 different cultures). Panels represent time-lapse FRET/CFP images coded according to the pseudocolor ramp **(B)**. HMC3 microglia expressing Raichu-RhoA biosensor treated and non-treated (CT) with 1 µg/ml LPS for 1 hour (n=65-70 cells per group from 2 different experiments). Pseudocolor ramps represent min/max FRET/CFP ratios **(C)**. Graphs (mean and SEM) display normalized GTP-RhoA content per cell **(A)** or FRET/CFP ratio changes **(C)** normalized at 0 minutes **(B)**. *$p<0.05$ (Mann-Whitney test) vs. CT. Scale bars: 10µm **(A and B)** 20µm **(C)**.

To reinforce this result we measured RhoA activity by FRET using Raichu-RhoA biosensor in both cortical microglia and HMC3 microglia cell line exposed to LPS. Living cortical microglia exhibited a very fast and robust reduction of RhoA activity following LPS treatment **(Figure 11B)**. Consistently, HMC3 microglia treated with LPS also showed a significant decrease in RhoA activity in comparison with non-treated control cells **(Figure 11C)**. Collectively, these data suggest that LPS exposure leads to a decrease in RhoA activity in microglia.

4.3. Regulation of microglial inflammation by RhoA

After showing that microglia inflammation induced by LPS is associated with a decrease in RhoA activity we decided to evaluate if preventing such decrease, by artificially sustaining RhoA activity, would impact the LPS-induced microglia inflammation. In order to do so, we addressed two different aspects of the microglial activation process: (1) microglia metabolic reprograming and (2) microglia proinflammatory polarization.

4.3.1. Regulation of microglial metabolic reprograming by RhoA

It is now accepted that proinflammatory stimuli, such as LPS, induces microglia metabolic reprograming by promoting a shift from oxidative phosphorylation to glycolysis (Borst et al., 2018, Baik et al., 2019). Therefore, since microglia status is reflected on cellular metabolism, we questioned whether sustaining RhoA activity would compromise the metabolic reprograming, and so, microglia inflammation induced by LPS. To answer that, we focused our study on the four major indicators of microglia metabolic reprograming: ATP/ADP balance, glucose consumption, pyruvate levels and lactate levels.

ATP/ADP balance represents the ratio between ATP generation and expense (by conversion into ADP) and is decreased in metabolic processes that are less energetically efficient such as glycolysis (Wilson, 2017). Considering that, we co-transfected HMC3 microglia with a constitutively active RhoA mutant (RhoA Q63L) and an ATP/ADP ratiometric biosensor **(Table 1)** to monitor de variation in ATP levels after challenging microglia with LPS. Live cell imaging results showed that in cells transfected with wild-type RhoA (RhoA WT), LPS exposure induced a decrease in ATP levels or either an increase in ATP consumption **(Figure 12A)**. Such decrease in ATP was prevented by sustaining RhoA activity in microglia expressing the RhoA Q63L mutant **(Figure 12A)**.

Glucose fuels most of microglia metabolic pathways and is also important for ROS production via the electron transport chain. However, during glycolysis, glucose consumption increases rapidly in an attempt to provide sufficient ATP and NADH for normal cell functioning (Bar-Even et al., 2012). In order to evaluate glucose consumption (disregarding its uptake), we performed live cell imaging assays in HMC3 microglia co-expressing the mutant RhoA Q63L and a FRET-based glucose biosensor (Takanaga et

al., 2008) in a glucose-free medium. Similar to what we observed with the ATP/ADP balance, in cells expressing RhoA WT, LPS treatment caused a decrease of glucose levels or an increase in glucose consumption, an effect prevented in microglia expressing the constitutively active RhoA mutant (RhoA Q63L) **(Figure 12B)**.

Pyruvate and lactate are two major by-products of glycolysis. Pyruvate directly results from the glycolytic breakdown of glucose, generating energy (Bar-Even et al., 2012). Afterwards, lactate dehydrogenase reduces pyruvate into lactate, which accumulates in the cytosol and serves as direct output of the glycolytic metabolism (Rogatzki et al., 2015). To evaluate if sustaining RhoA activity impacts either pyruvate or lactate levels after LPS stimulus, HMC3 microglia were co-transfected with RhoA Q63L and either pyruvate or lactate FRET biosensors (San Martin et al., 2013, San Martin et al., 2014). Live cell imaging results showed that LPS induced an increase in the amounts of both by-products in the cytosol of RhoA WT microglia **(Figure 12C and D)**. However, such decrease was blocked in cells expressing mutant RhoA Q63L **(Figure 12C and D)**. Collectively, these results suggest that a decrease of RhoA activity is required for metabolic reprogramming during microglia inflammation.

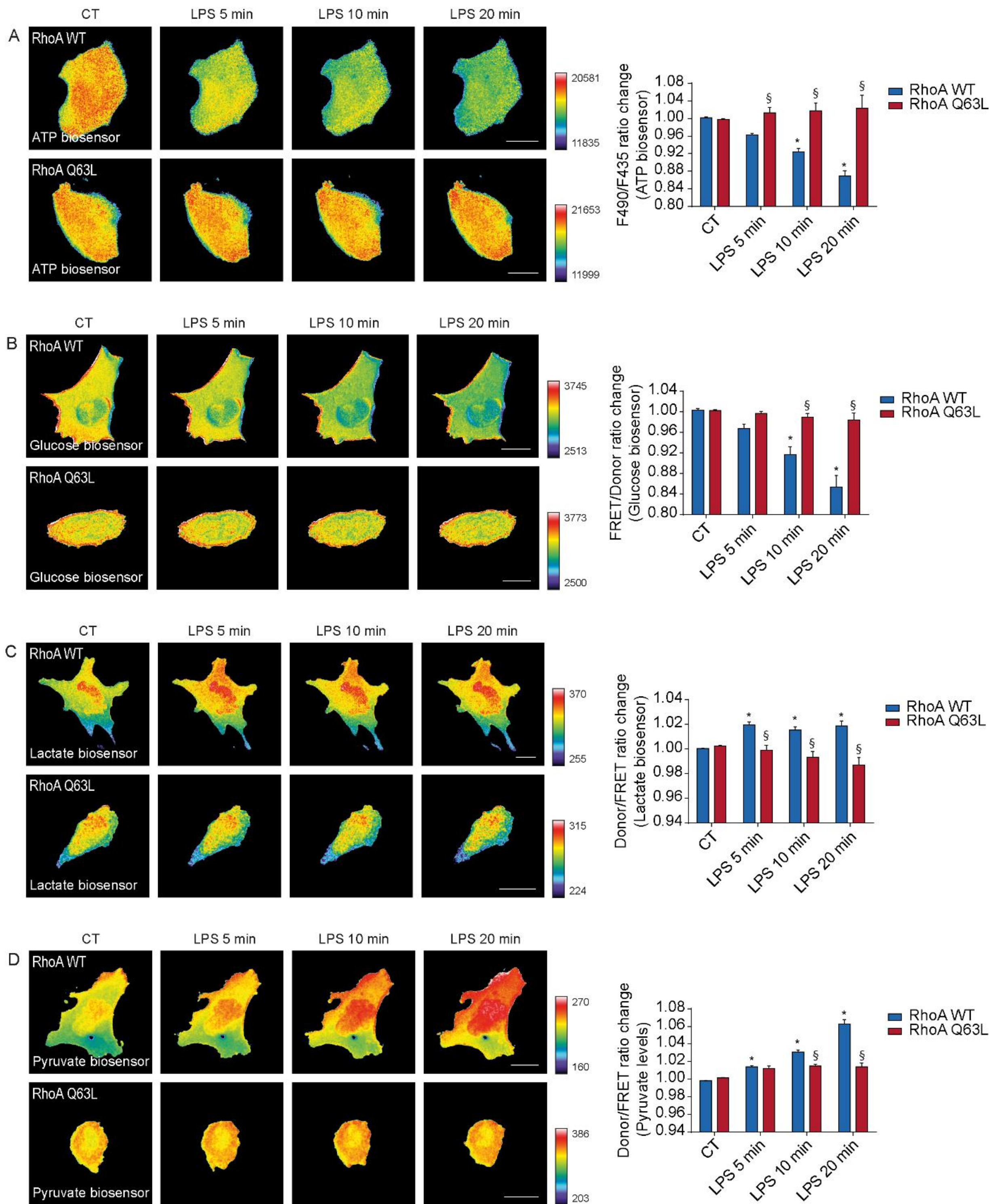

Figure 12- **RhoA regulation of the metabolic reprograming process in HMC3 microglia.**

HMC3 microglia expressing the ATP biosensor **(A)**, Glucose biosensor **(B)**, Lactate biosensor **(C)** or Pyruvate biosensor **(D)** transfected with the RhoA Q63L construct (red bars) or with RhoA WT (blue bars) and exposed to LPS (1 µg/ml) (n=15-30 cells per group from 2 different experiments using each biosensor). Panels show time-lapse ratio images coded according to the pseudocolor ramp. Graphs (means and SEM) that display F490/F435 (A), FRET/Donor **(B)** and Donor/FRET **(C and D)** ratio changes, were normalized at 0 minutes and plotted. *$p<0.05$ (Two-way ANOVA comparing each LPS timepoint with CT), §$p<0.05$ (Two-way ANOVA comparing RhoA Q63L vs. RhoA WT in each LPS timepoint). Scale bars: 20µm

4.3.2. Regulation of microglial classic inflammatory polarization by RhoA

Microglia inflammatory polarization is classically associated with the response to proinflammatory stimulus like LPS (Lund et al., 2006). Besides morphological alterations, this response is characterized by several physiological changes that culminate in the production of different factors that will, somehow, modulate the progress of inflammation (Block et al., 2007). Typically, LPS-activated microglia produce high levels of reactive oxygen species (ROS) as a result of the activation of specific signaling pathways involved in microglia defense functions (Haslund-Vinding et al., 2017). Participating in the regulation of those pathways are tyrosine kinases, including Src kinase (Socodato et al., 2015b), AMP-activated protein kinase (AMPK) and other proteins (Chen et al., 2014), already established as important regulators of the microglia inflammatory response. With this in mind, we questioned whether, by preventing the decrease in RhoA activity induced by LPS, the classic hallmarks of microglia proinflammatory activation would be compromised.

In microglia, ROS are mainly produced in mitochondria and the cytoplasmic membrane (via NADPH oxidase) (Bordt and Polster, 2014). They are one of the main factors produced by inflammatory microglia not only as secondary messengers, with the ability to modulate inflammatory gene expression (Rojo et al., 2014), but also as inflammatory molecules (Shabab et al., 2017). In this last case, ROS are delivered to the extracellular medium and participate in the defense process directly contributing to inflammation. To evaluate if consistently high RhoA activity would impact microglial ROS production after LPS stimulation, we used the RhoA mutant Q63L in HMC3 microglia, also expressing the ROS FRET biosensor (HSP biosensor) **(Table 1).** As expected, RhoA WT cells exhibited a robust increase in ROS production after LPS treatment **(Figure 13A).** However, in microglia expressing mutant RhoA Q63L, ROS production was completely prevented **(Figure 13A).**

AMPK, more than a key metabolic regulator, is also important in microglia inflammatory response (Chen et al., 2014). In fact, its activation is associated with the suppression of LPS-induced secretion of proinflammatory mediators (Giri et al., 2004, Saito et al., 2019). Considering this, we asked whether RhoA activation would modulate AMPK activity. To assess that we co-transfected HMC3 microglia with RhoA Q63L and, in this case, an AMPK activity FRET biosensor **(Table 1).** Results showed that in RhoA WT cells, LPS induced a decrease in AMPK activation, an effect significantly attenuated in cells expressing the active mutant RhoA Q63L **(Figure 13B).**

The Src family kinases (SFKs) are a family of non-receptor protein tyrosine kinases from which the proto-oncogene Src is the archetype member (Martin, 2001). Activity of SFKs, including Src, regulates several aspects of innate immunity (Lowell, 2011) and inflammation (Page et al., 2009). Src itself, when activated, controls microglial response via increased production and secretion of proinflammatory cytokines and ROS (Socodato et al., 2015a) (Socodato et al., 2015b). Therefore, to evaluate if constitutively active RhoA modulates Src activation after LPS exposure, we performed Western blot analysis with lysates obtained from HMC3 cells expressing RhoA Q63L. Results showed that whereas in RhoA WT cells LPS exposure induces an increase in the amounts of activated Src (Src $pTyr^{416}$), in cells expressing the RhoA Q63L mutant, that increase was significantly reduced **(Figure 13C)**.

Collectively, the results of this comprehensive approach based on two different aspects of microglia inflammation, strongly suggest that a decrease of RhoA activity is an important requirement for microglia inflammatory response induced by LPS.

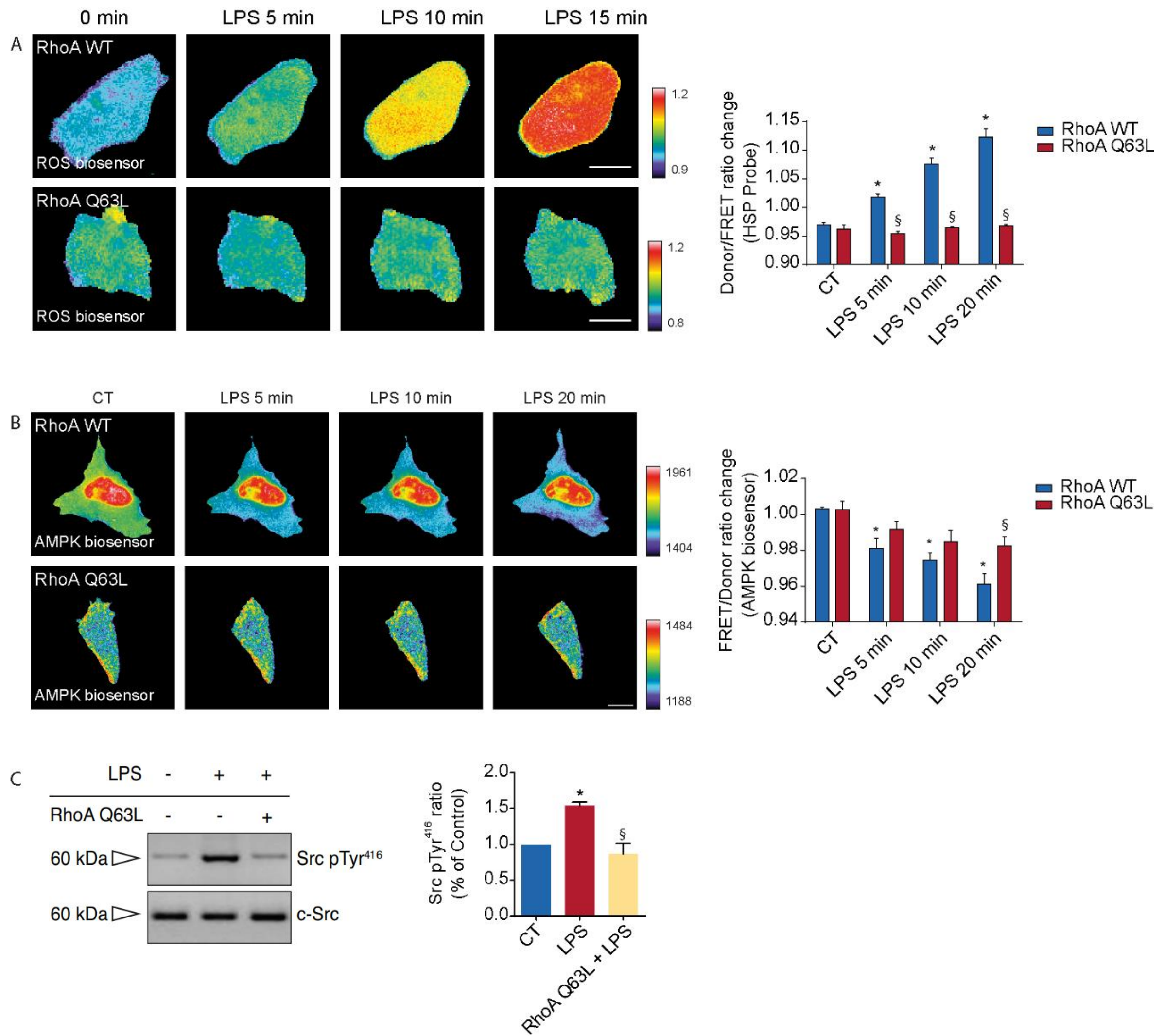

Figure 13- **RhoA regulation of classic inflammatory polarization in HMC3 microglia.**

HMC3 microglia expressing the ROS **(A)** and AMPK biosensors **(B)** were transfected with the RhoA Q63L construct (red bars) or with RhoA WT (blue bars) and then exposed to LPS (1 µg/ml) (n=18-26 cells per group from 2 different experiments using each biosensor). Panels show time-lapse ratio images coded according to the pseudocolor ramp. Graphs (means and SEM) that display CFP/FRET **(A)**, FRET/CFP **(B)** ratio changes, were normalized at 0 minutes and plotted. $^{*}p<0.05$ (Two-way ANOVA comparing each LPS timepoint with CT), $^{\S}p<0.05$ (Two-way ANOVA comparing RhoA Q63L vs. RhoA WT in each LPS timepoint). Scale bars: 20 µm **(A and B)**. Western blot for Src $pTyr^{416}$ on lysates from HMC3 cells expressing RhoA Q63L mutant and treated with LPS (1 µg/ml) for 24 hours (n=3 different cultures) **(C)**. The Graph (mean and SEM) display Src $pTyr^{416}$/Src ratio normalized to the CT values. $^{*}p<0.05$ (One-way ANOVA) vs. CT and $^{\S}p<0.05$ (One-way ANOVA) vs. LPS.

4.4. Conditional ablation of RhoA in adult microglia

Having shown in microglia cultures that a decrease in RhoA activity is critical for microglia to initiate an inflammatory response to LPS, we then questioned whether the absence of RhoA in vivo impacts this response. To do that, we took an approach based on the *in vivo* conditional ablation of the RhoA gene in microglia. For this purpose, we used an experimental set up where RhoA floxed mice ($RhoA^{fl/fl}$) are crossed with mice expressing both EYFP and tamoxifen-inducible Cre recombinase (Cre^{ER}) under the endogenous regulation of Cx3cr1 promoter ($Cx3cr1^{CreER\text{-}IRES\text{-}EYFP}$) (Goldmann et al., 2013, Parkhurst et al., 2013). Following tamoxifen administration Cre translocates to the nucleus inducing RhoA gene inactivation in microglia from $RhoA^{fl/fl}$:$Cx3cr1^{CreER+}$ mice (RhoA cKO mice) **(Figure 14A)**. Overall, this inducible model allows us to adjust the timing of microglial RhoA gene inactivation to our specific experimental requirements.

First, we evaluated if the $Cx3cr1^{CreER\text{-}IRES\text{-}EYFP}$ transgene was expressed in these cells under steady state conditions. We observed that in $Cx3cr1^{CreER\text{-}IRES\text{-}EYFP}$ mice, the population of EYFP-positive cells corresponded exclusively to Iba1-positive cells, indicating that the CreER-IRES-EYFP transgene is transcriptionally active in brain myeloid cells **(Figure 14B)**. Furthermore, this was further validated by flow cytometry where the YFP^+CD11b^+ cell population was almost entirely identified as microglia ($CD45^{mid}CD11b^+$) **(Figure 14C)**.

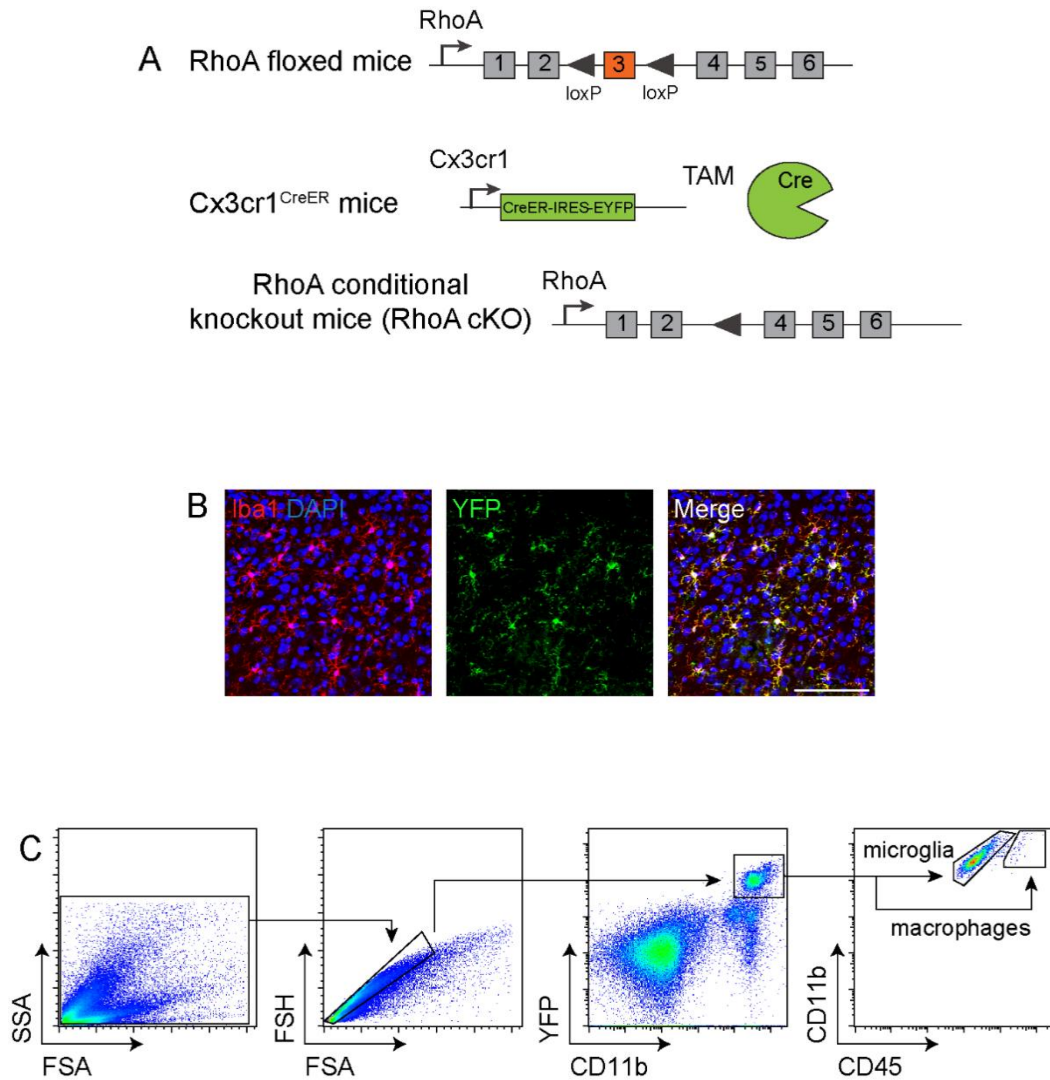

Figure 14- **Ablation of RhoA in RhoA$^{fl/fl}$:Cx3cr1^{CreER+} mice**

Breeding scheme for tamoxifen-inducible microglia-specific RhoA gene inactivation in mice. Crossing mice bearing CreER-IRES-EYFP transgene within the Cx3cr1 locus (middle) with mice in which the exon 3 of the RhoA gene is flanked by loxP sites (top) gives rise to mice in which RhoA deletion in microglia/macrophage is obtained by tamoxifen administration (bottom) **(A)**. Confocal imaging of brain sections from Cx3cr1-EYFP-CreER+ heterozygous mice immunolabeled using anti-Iba1 antibody (red) and anti-YFP antibody (green). Virtually all YFP+ cells were also Iba1+ (images are representative of 4 different mice). Scale bar: 100 µm **(B)**. Gating strategy for sorting microglia from the brains of Cx3cr1-EYFP-CreER+ mice. More than 95% of the EYFP^{high} gated population in the brain corresponded to microglia (CD45midCD11b+), the remaining cells were composed of non-parenchymal brain macrophages (CD45highCD11b+) **(C)**.

4.4.1. Validation of RhoA gene conditional knock-out in microglia.

We then administered TAM by oral gavage to control $RhoA^{fl/fl}$ and to $RhoA^{fl/fl}$:$Cx3cr1^{CreER+}$ mice (RhoA cKO) at P26 and P28. At P100-P110 (adult mice) control and RhoA cKO brains were analysed using flow cytometry and Western blotting to confirm that RhoA was successfully depleted from microglia **(Figure 15A)**. As represented in the **figure 15B**, contrarily to what occurs in the control $RhoA^{fl/fl}$ mice, microglia from $RhoA^{fl/fl}$:$Cx3cr1^{CreER+}$ mice (RhoA cKO) are YFP^{+}, indicating that the transgene CreER-IRES-EYFP is being expressed in the cell. Therefore, following TAM administration, we expect Cre to migrate into the nucleus and recombine the RhoA floxed allele, ultimately inactivating the gene and consequently abolishing RhoA protein expression in RhoA cKO microglia. This was confirmed by the substantial reduction in the percentage of $RhoA^{+}$ microglia as well as RhoA expression levels (MFI) in the microglia population of RhoA cKO mice when compared with controls **(Figure 15B)**. RhoA protein levels were also evaluated by western blot using protein lysates from MACS-isolated microglia from control and RhoA cKO brains. Results showed a substantial decrease of RhoA protein in $RhoA^{fl/fl}$:$Cx3cr1^{CreER+}$ mice compared with $RhoA^{fl/fl}$ mice **(Figure 15C)**, confirming that RhoA was efficiently depleted in RhoA cKO microglia.

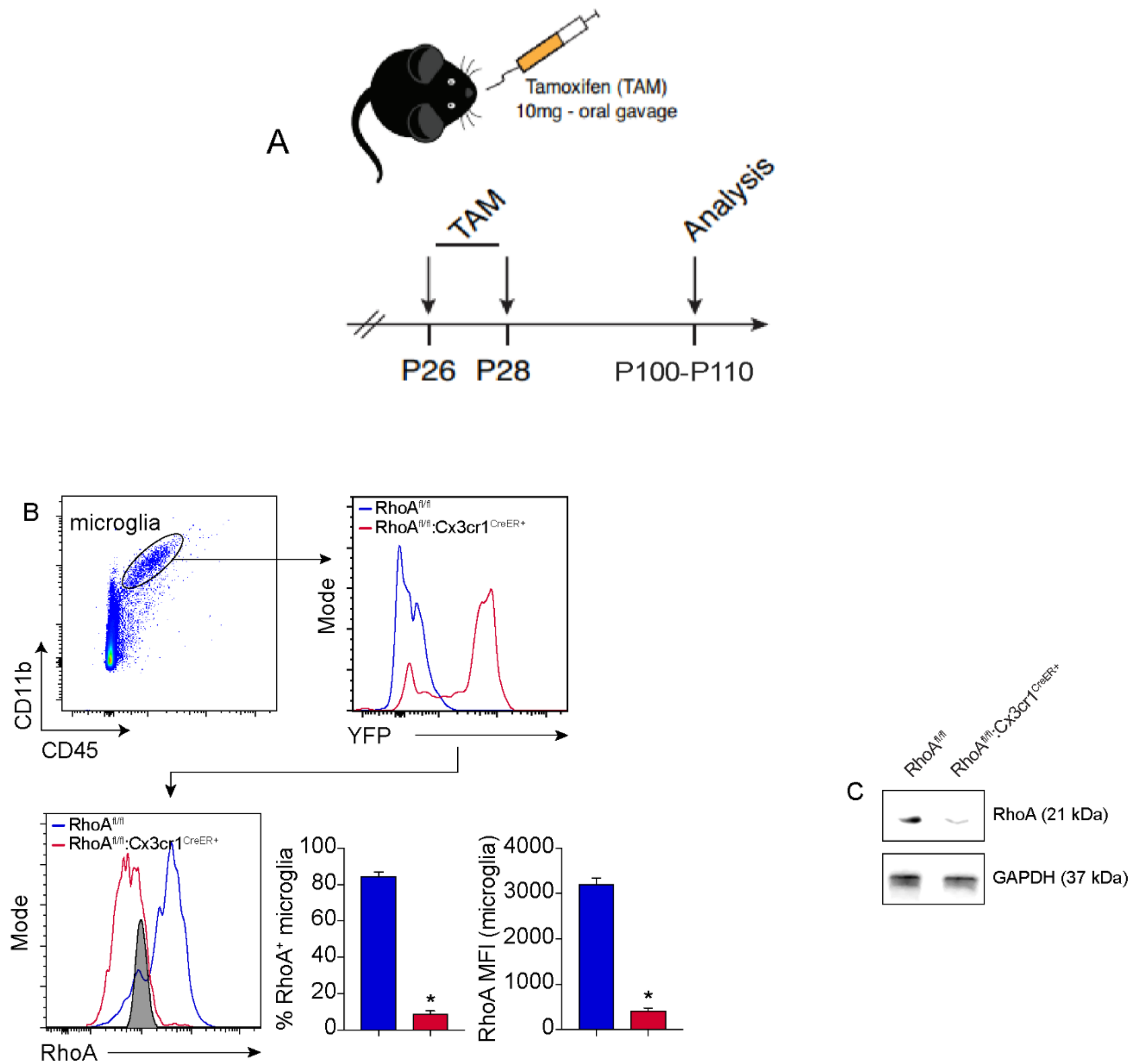

Figure 15- **Validation of RhoA ablation in microglia**

Timeline of procedures for inducing tamoxifen-mediated microglia-specific deletion of RhoA in RhoA$^{fl/fl}$:Cx3cr1^{CreER+} mice. Tamoxifen was administrated via oral gavage (10mg/animal) at P26 and P28, tissue analysis was performed between P100 and P110 **(A)**. Expression of RhoA in microglia from the brains of RhoA$^{fl/fl}$ and RhoA$^{fl/fl}$:Cx3cr1^{CreER+} mice 65-75 days after TAM administration (n=4 animals per genotype). Graphs (mean and SEM) depict RhoA^{+} microglia and microglial RhoA MFI. Cell debris was excluded by size. *P<0.05 (Mann-Whitney test comparing both groups) **(B)**. Western blot for RhoA on lysates from MACS-separated microglia collected from the brains of RhoA$^{fl/fl}$ and RhoA$^{fl/fl}$:Cx3cr1^{CreER+} mice 80 days after TAM administration. GAPDH was the loading control (images are representative of 3 mice per genotype) **(C)**.

4.5. The impact of RhoA deficiency in microglia during neuroinflammation.

Neuroinflammation is an inflammatory response occurring in the CNS as a defence mechanism against any factor (pathogen, physical injury etc.) that potentially compromises tissue homeostasis (Ransohoff, 2016a). Because microglia are the resident innate immune cells of the CNS, they play a major role in the initiation and regulation of the inflammatory process by increasing the production of several proinflammatory cytokines, chemokines and ROS (Block et al., 2007). Generally, when transient or acute, neuroinflammation exerts a beneficial function for the host organism and the mediators released by microglia contribute for tissue-repairing and neuroprotection (Russo and McGavern, 2015, DiSabato et al., 2016). However, a different scenario occurs when the inflammatory response is sustained and those molecules become neurotoxic, ultimately causing brain damage (Colonna and Butovsky, 2017). *In vivo* LPS administration is a well-studied and extensively used model to induce neuroinflammation (Catorce and Gevorkian, 2016). In fact, when peripherally administered, LPS induces a systemic inflammatory response that results in microglia activation via TLR4 (Beutler, 2000, Zhao et al., 2019). Many studies, including transcriptomic analysis, revealed that LPS recognition via TLR4 is associated with the activation of several inflammation-related signalling cascades, including NF-kB and MAPK/AP-1 pathways that drive the production of proinflammatory cytokines (IL-1β, IL-6 and TNFα) and Interferon regulatory factors (ITFN), that regulate cell proliferation and chemotaxis (Lu et al., 2008, Pulido-Salgado et al., 2018). Therefore, all these features establish LPS administration as a suitable model to study neuroinflammation.

We were interested in evaluating if the absence of RhoA impacts microglia response during neuroinflammation. This was carried out in both control and RhoA cKO mice that were systemically administered with a single LPS (4mg/kg) or saline solution (as control) injection at P100-P110. Mice brains were extracted for analysis 24 hours later **(Figure 16A)**.

To test this hypothesis, we first evaluated whether LPS-induced neuroinflammation would compromise microglia viability in RhoA cKO mice. To do that, we performed flow cytometry with cell death markers to assess the percentage of necrotic ($Zombie^{+}AnV^{-}$) and apoptotic ($Zombie^{-}AnV^{+}$) cells present in the microglia population from control and RhoA cKO animals. We found that although in control mice LPS-mediated neuroinflammation do not cause cell death neither by necrosis or apoptosis, in RhoA

cKO animals, neuroinflammation was highly cytotoxic, inducing both microglia necrosis and apoptosis **(Figure 16B)**.

To reinforce these results, we performed a qualitative evaluation of microglia morphology by immunostaining brain sections from the four experimental mice groups with the myeloid marker Iba1. As expected, in $RhoA^{fl/fl}$ mice, LPS-induced neuroinflammation resulted in a decreased number of microglia processes, as well as the acquisition of a more amoeboid cell morphology when compared with the saline control with no overt signs of cell death **(Figure 16C)**. Although $RhoA^{fl/fl}$:$Cx3cr1^{CreER+}$ mice treated with saline already exhibited impaired processes, in $RhoA^{fl/fl}$:$Cx3cr1^{CreER+}$ mice treated with LPS, microglia displayed an even more aberrant morphology, characterized by severe loss of cell processes and extensive cell body swelling compared with $RhoA^{fl/fl}$ mice treated with LPS **(Figure 16C)**. Therefore, these results, associating flow cytometry data with cell morphology observations, collectively suggest that in the absence of RhoA, neuroinflammation leads to microglial cell death.

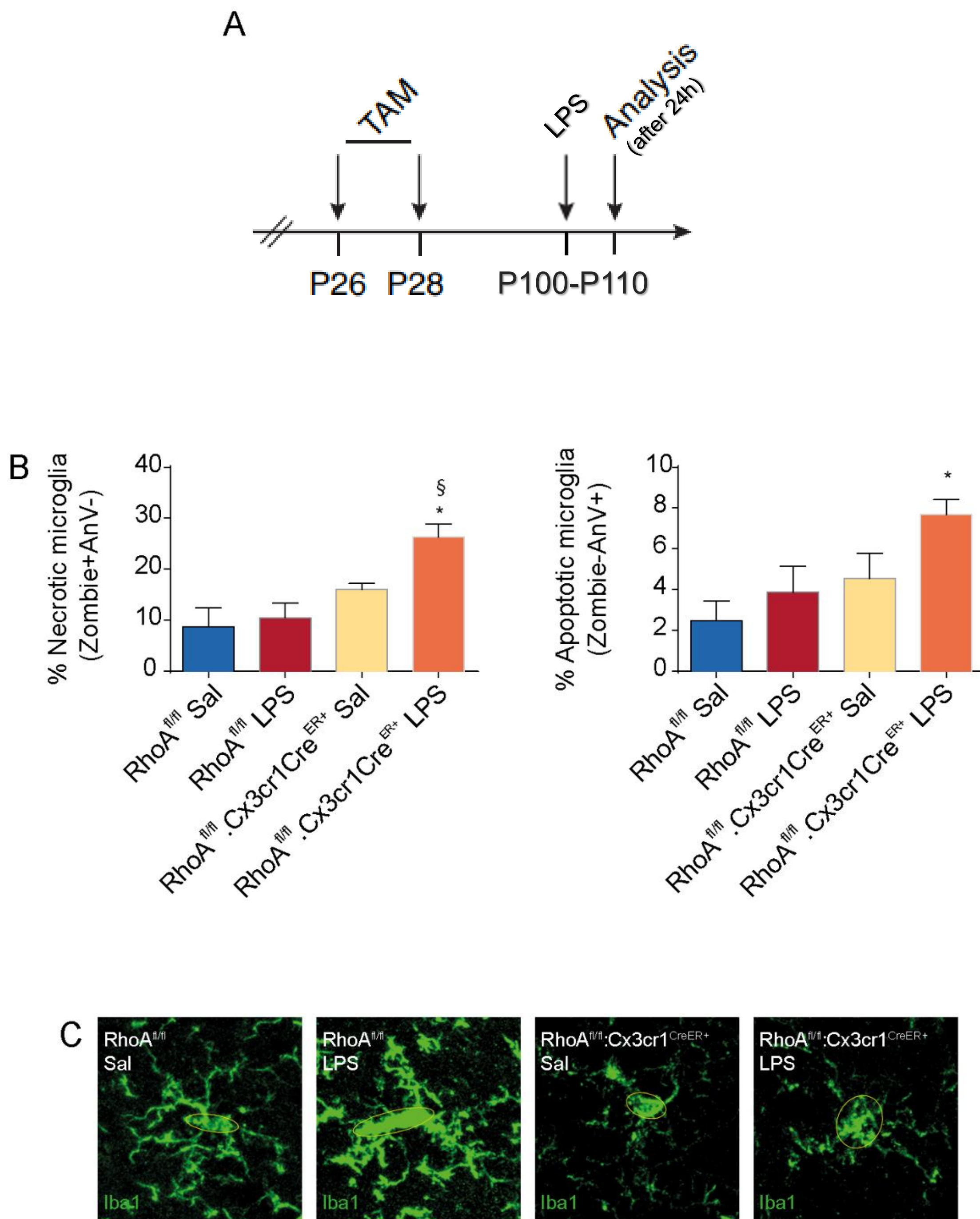

Figure 16- ***In vivo* study of the impact of RhoA deficiency in microglia during neuroinflammation.**

Timeline of procedures to induce neuroinflammation in $RhoA^{fl/fl}$ and $RhoA^{fl/fl}$:$Cx3cr1^{CreER+}$ mice. Control and RhoA KO were administrated with LPS (1 intraperitoneal injection in a 4mg/kg dose, 24 hours prior to perfusion) **(A)**. Flow cytometry analysis of cell death markers in microglia from $RhoA^{fl/fl}$ and $RhoA^{fl/fl}$:$Cx3cr1^{CreER+}$ mice 72-82 days after TAM administration (n=6 animals per group). Graphs show necrotic and apoptotic microglia (mean and SEM). Cell debris were excluded by size. *$p<0.05$ (One-way ANOVA) vs. $RhoA^{fl/fl}$ LPS and $^{§}p<0.05$ (One-way ANOVA) vs. $RhoA^{fl/fl}$:$Cx3cr1^{CreER+}$ Sal **(B)**. Histological confocal qualitative analysis for Iba1 on tissue sections from the neocortex of $RhoA^{fl/fl}$ and $RhoA^{fl/fl}$:$Cx3cr1^{CreER+}$ mice treated with LPS or saline, 72-82 days after TAM administration. Cell body fitting ellipse obtained using ImageJ is represented in yellow **(C)**.

4.6. Potential pathways involved in LPS-mediated cytotoxicity.

Having shown that in the absence of RhoA neuroinflammation causes microglial cell death, we next questioned which cellular mechanisms/pathways could be impaired, potentially compromising microglial viability. Because the basis of this study is the lack of RhoA in microglia, we took two different approaches in order to promote RhoA inhibition *in vitro*. In the first one we used HMC3 microglia overexpressing a dominant-negative RhoA mutant (RhoA T19N), whereas the second relies on the use of a RhoA knockout HMC3 cell line (RhoA KO), generated using the CRISPR/Cas9 system (Pedro Melo, unpublished). In cells expressing RhoA T19N, RhoA inhibition was confirmed by a decrease in RhoA activity reported by the Flare.RhoA FRET biosensor **(Figure 17A)**. Moreover, RhoA T19N overexpression clearly induced a disruption of the actin cytoskeleton organization, reflected in the decrease of actin stress fibers observed in cells expressing the RhoA mutant form comparatively with RhoA WT transfected microglia **(Figure 17B)**. To evaluate protein amounts, we performed Western blot analysis on cell lysates from RhoA KO and microglia overexpressing RhoA WT and RhoA T19N constructs. Results confirm the complete absence of RhoA in the KO microglia cell line **(Figure 17C)**.

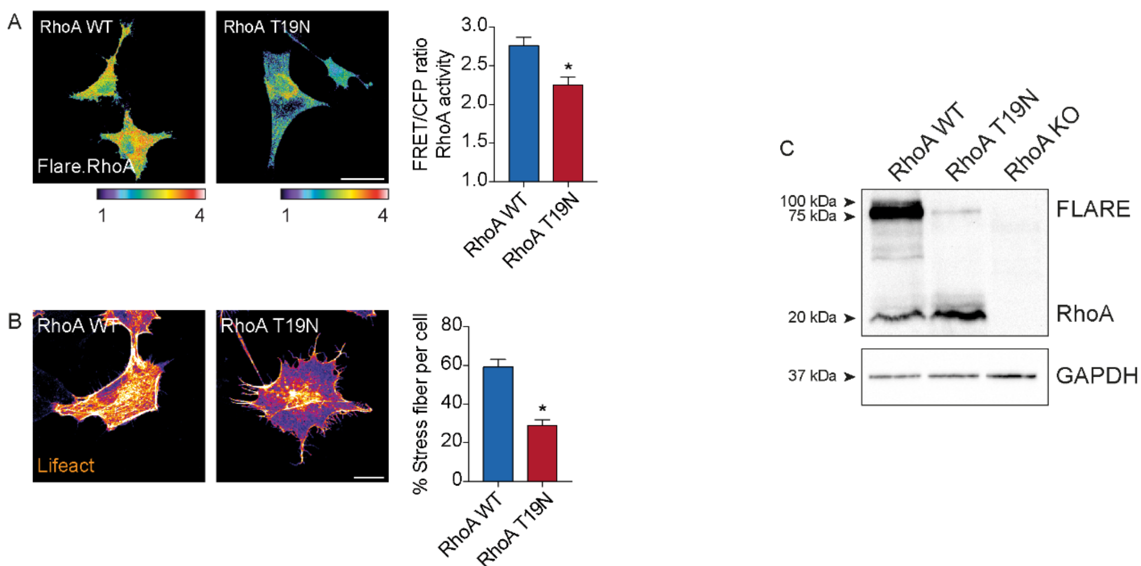

Figure 17- **In vitro inhibition of RhoA activity**

Representative images of HMC3 microglial cultures expressing a RhoA FRET biosensor (Flare.RhoA) **(A)**, or Lifeact **(B)** transfected with wild-type (WT) RhoA or dominant-negative (T19N) RhoA mutant (n=25 cells per group from 2 different experiments in A, n=60 cells per group from 2 different experiments in B). Graphs (means and SEM) display FRET/CFP ratio images **(A)** and stress fibers content (B). Pseudocolor ramps represent min/max FRET/CFP ratios **(A)** and lifeact intensity **(B)**. *P<0.05 (Mann-Whitney test) vs. RhoA WT **(A and B)**. Scale bars: 20 µm in **A**, 10 µm in **B**. Western blot for RhoA on lysates from HMC3 microglia transfected with wild-type (WT) RhoA, dominant-negative (T19N) RhoA mutant or CRISPR cas9-obtained RhoA KO cells. GAPDH was the loading control **(C)**.

Aiming at studying the cytotoxicity observed in RhoA KO microglia during neuroinflammation, we evaluated Ca^{2+} signaling and pH equilibrium as two of the parameters with major roles in the regulation of cell homeostasis. Because, when dysregulated, both compromise vital functions (Lagadic-Gossmann et al., 2004, Zhivotovsky and Orrenius, 2011), they are considered important hallmarks of cell death and therefore potential candidates to be related with our phenotype.

The Ca^{2+} ion is one of the fundamental signaling elements in eukaryotic cells where it plays a primary role in signal transduction pathways as a secondary messenger (Clapham, 2007). Such function requires tight control over Ca^{2+} levels in order to potentiate an effective response when cell receptors interact with an external stimulus. In addition, because Ca^{2+} cannot be chemically altered, it has to be properly compartmentalized (mainly in mitochondria and endoplasmic reticulum) in order to keep low cytoplasmic levels and preserve cell homeostasis (Rizzuto and Pozzan, 2006). Considering that the slightest disturbance of this equilibrium entails severe consequences for cell function, Ca^{2+} signaling has long been known to be critically involved in both initiation and progression of cell death (Orrenius et al., 2003). In fact, necrotic cell death is classically associated with intracellular Ca^{2+} overload, a phenomenon that frequently cooperate with the activation of several proteases (e.g. caspases and calpains) and endonucleases, involved in the apoptotic process (Zhivotovsky and Orrenius, 2011). With this premise, we carried out live cell imaging experiments to evaluate whether dysregulation of Ca^{2+} homeostasis is implicated in the LPS-mediated toxicity observed in microglia. For that, the three cell groups – RhoA WT, RhoA T19N and RhoA KO – were co-transfected with a Ca^{2+} biosensor (Chen et al., 2013). As expected, after LPS treatment, RhoA WT microglia exhibited a slight increase in the Ca^{2+} levels that is typical of a homeostatic cellular response. However, both RhoA T19N and RhoA KO cells, showed a massive increase of intracellular Ca^{2+} following the inflammatory stimulus **(Figure 18A)**, indicating that when RhoA is inhibited or depleted, LPS stimulation causes Ca^{2+} overload in microglia.

Intracellular pH is another parameter that is tightly regulated on mammalian cells. Such stringent control relies on the great pH variability across different organelles, which is a requirement for the correct fulfillment of their individual functions (Casey et al., 2010). Generally, cytosolic pH is neutral (7-7.4), resulting from the balance between H^+ and HCO_3^- ion concentrations mediated by pH-regulatory transporters and cation/anion channels embedded in the cytoplasmic membrane (Grinstein et al., 1986). This continuous ion exchange across the membrane is controlled by highly responsive sensors that not only adjust intracellular pH according to environmental conditions but

also trigger response-related pH fluctuations. In fact, both apoptotic and necrotic cell death have long been associated with cytoplasmic acidification (Lagadic-Gossmann et al., 2004). This occurs mainly after caspase activation, leading to mitochondria and/or lysosome dysfunction and impaired ion transporters (Liu et al., 2000, Wang et al., 2018). These critical events culminate in aberrant H^+ accumulation and cell death.

Accordingly, we examined whether pH dysregulation could also be involved in the toxicity observed in RhoA-deficient microglia following LPS treatment. Like the previous described Ca^{2+} data, we addressed this question using live cell imaging in microglia with compromised RhoA activity (i.e. RhoA T19N and RhoA KO microglia) and exposed them to LPS. We monitored pH homeostasis by transfecting those cells with a genetically encoded pH sensor obtained from mutagenesis of the red pH sensitive protein – mKeima (Tantama et al., 2011). We observed that RhoA WT microglia undergo a subtle acidification of the cytoplasm following LPS exposure. However, LPS induced extensive cytosolic acidosis in microglia in which RhoA was absent (RhoA KO) or had compromised RhoA activity (RhoA T19N) **(Figure 18B)**.

Overall, our data suggest that in the absence of RhoA, LPS stimulation causes disruption of Ca^{2+} and pH homeostasis, which is ultimately positively correlated with the increased cell death observed in microglia lacking RhoA.

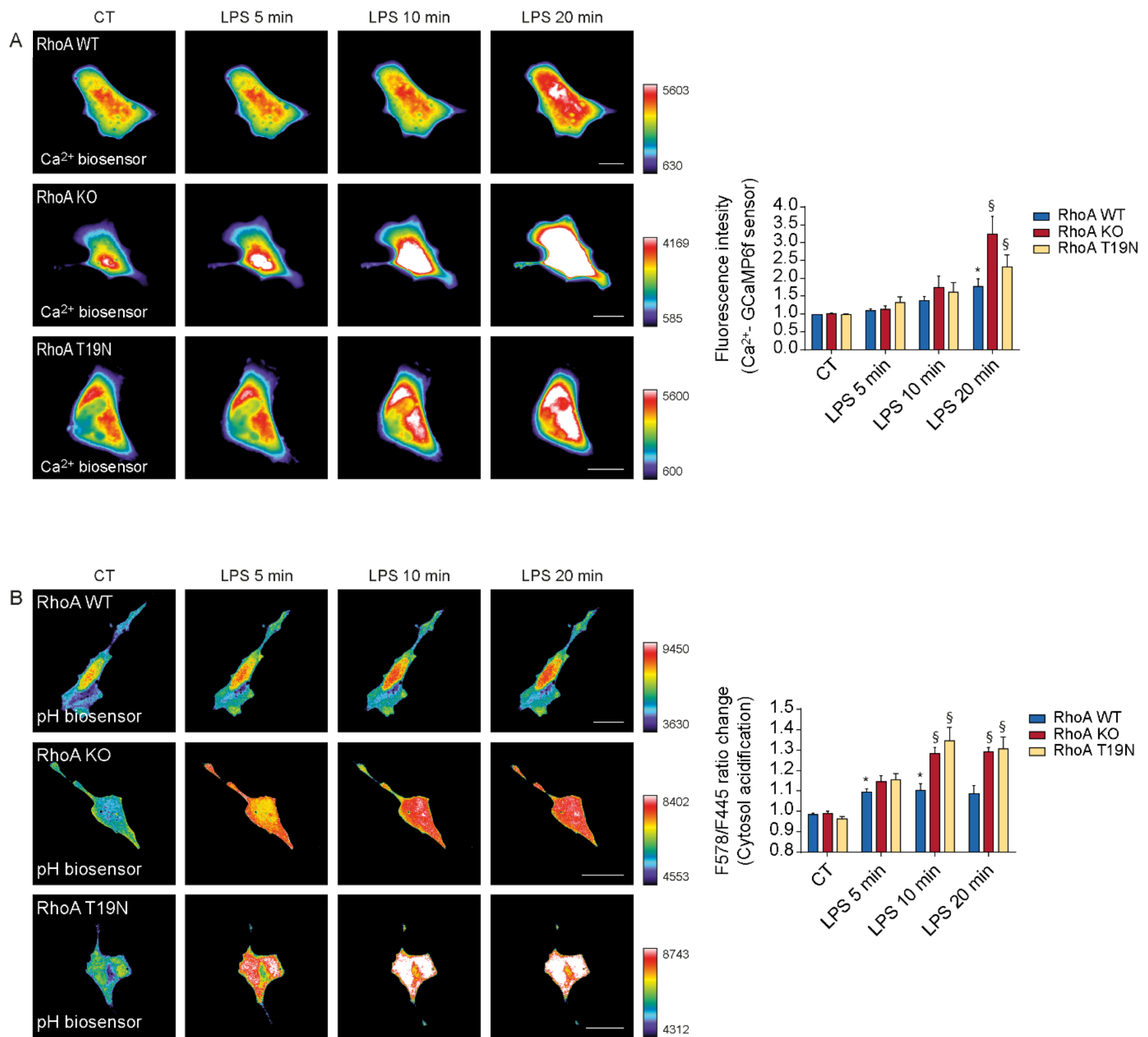

Figure 18- **Ca^{2+} and pH as potential pathways involved in LPS-mediated cytotoxicity in RhoA absent microglia.**

HMC3 microglia cultures expressing the Ca^{2+} biosensor **(A)** or pH biosensor **(B)** transfected with RhoA WT (blue bars), dominant-negative (T19N) RhoA mutant (yellow bars) or knockout for RhoA gene (red bars) exposed to LPS (1 µg/ml) (n=15-30 cells per group from 2 different experiments using each biosensor). Panels show time-lapse ratio images coded according to the pseudocolor ramp. Graphs (means and SEM) that display Fluorescent intensity **(A)** and F578/F445 ratio change **(B)** were normalized at 0 minutes and plotted. *p<0.05 (Two-way ANOVA comparing each LPS timepoint with CT), §p<0.05 (Two-way ANOVA comparing RhoA T19N/RhoA KO vs. RhoA WT in each LPS timepoint). Scale bars: 20 µm **(A and B)**.

Chapter 5

Discussion

5. Discussion

By allying high-end live cell imaging with *in vivo* cell-specific conditional gene ablation in mice, my work addressed the role of the small GTPase RhoA in microglial cells during neuroinflammation. Microglia are the resident immune cells of the CNS and besides serving several *housekeeping* functions, they exert a pivotal role in brain defence by initiating an inflammatory response against modified-self and non-self injurious agents (Carson et al., 2006). This response is typically associated with morphological and transcriptomic changes that induce microglia migration to the affected site and production of several pro- and anti- inflammatory factors (abd-el-Basset and Fedoroff, 1995, Block et al., 2007). As master regulators of cytoskeletal dynamics, Rho GTPases, and more specifically RhoA, are likely involved in this microglial inflammatory response (Stankiewicz and Linseman, 2014).

Although previous transcriptomic studies already confirmed that RhoA is expressed in both human and mouse microglia (Zhang et al., 2014b, Zhang et al., 2016), here we demonstrated that RhoA activity changes according to the subcellular region. In fact, by means of a FRET analysis with a RhoA biosensor, we showed that RhoA activity was higher in microglia processes and regions close to the plasma membrane than in the cell body. This pattern of distribution is in line with the classical view of Rho GTPases regulatory mechanisms, where RhoGTPases closely associate with plasma membrane receptors in order to activate downstream effectors (Hodge and Ridley, 2016). This association is also potentiated by the existence of polybasic residues connected to the Rho GTPase, as well as PTMs like prenylation, that facilitate the attachment to the cell membrane (Michaelson et al., 2001). Additionally, RhoA classical association with the formation of focal adhesion and stress fibers also supports its higher activity in microglia periphery, where it can actively contribute to cell migration and process dynamics (Lawson and Ridley, 2018).

Interestingly, after exposing microglia to LPS, we observed a complete transformation of the previously described activity pattern, with an overall decrease in RhoA activity. Such alteration not only indicates that the microglial response to an inflammatory stimulus is associated with the modulation of RhoA activity but also suggests that RhoA itself may play a role in this process. Accordingly, various studies that were carried out in other cell types such as neutrophils (Fessler et al., 2007), monocytes (Chen et al., 2002) and macrophages (Moon et al., 2010, Wang et al., 2015a) already report this association. However, in all of those cases, LPS exposure induces an increase of RhoA activity, contrasting with the decrease we observed in microglia. This

suggests that modulation of RhoA activity is cell specific and may have distinct functional effects among immune cells. In fact, conditional-gene targeting studies demonstrate that many of the specific pathways under the regulation of a given Rho GTPase are cell type and stimulus specific, in such manner that knowing the role of RhoA in a given cell type cannot necessarily predict its function and signaling mechanisms in a different one (Pedersen and Brakebusch, 2012). Our observations also suggest a relation between TLR4 (LPS receptor in microglia) and RhoA, which is a reasonable premise given the classical association that membrane receptors have with Rho GTPases, and the multitude of pathways in which RhoA appears to be involved. In agreement with this is a study carried out in human monocytes where LPS modulates RhoA activity in a process dependent of interleukin-1 receptor-associated kinase (IRAK) (Chen et al., 2002). Interestingly, this factor is classically involved in the MyD88-mediated TLR4 signaling, that subsequently activates Nf-kB and AP-1 (via IKKs and MAPKs) and promotes the release of proinflammatory cytokines (Lu et al., 2008). Moreover, RhoA was shown to participate in macrophage inflammatory response to LPS by regulating TLR4 signaling and internalization via p120-catenin (Yang et al., 2014). However, given the previously mentioned cell specificity of the functions, the role of RhoA in the microglial response to LPS remains to be elucidated.

Looking forward to that, we asked whether preventing the decrease of RhoA activity in microglia would impact the inflammatory response triggered by LPS. In result, the use of cells expressing a constitutively active RhoA mutant (RhoA Q63L) entirely compromised the two major indicators of microglia inflammation: metabolic reprograming and classic inflammatory polarization. In immune cells, including microglia, metabolic reprograming takes place during inflammatory activation and is characterized by a shift from oxidative phosphorylation (OXPHOS) to aerobic glycolysis in a phenomenon known as Warburg effect (Freemerman et al., 2014, Galván-Peña and O'Neill, 2014, Baik et al., 2019). Because the inflammatory response is associated with an increase in cell proliferation, phagocytosis and secretion of several factors, some studies suggest that the metabolic reprograming occurs in order to fulfill the new energy requirements (Lunt and Vander Heiden, 2011, Cheng et al., 2014). In fact, although glycolysis is considered to be less efficient than OXPHOS when it comes to ATP production, the rate of glucose metabolism is much faster, fulfilling energy-intensive processes and enabling an immediate cellular response (Marelli-Berg et al., 2012). In complete agreement with our results, more than the obvious increase in glucose consumption, the change to a glycolytic metabolism implies a decrease in the ATP:ADP ratio, which results from the negative balance between the low amounts of ATP produced and the energetically

expensive cellular processes (Rambold and Pearce, 2018). Moreover, such high rate of glucose breakout (and possibly the impairment of the TCA cycle reported in inflammatory macrophages) (Jha et al., 2015), result in the accumulation of the by-product pyruvate that at some point is converted into lactate, and is continuously secreted to the extracellular medium (Borst et al., 2018). In accordance with the literature (Voloboueva et al., 2013, Gimeno-Bayon et al., 2014), we showed that microglia exposed to LPS became glycolytic, however, this metabolic shift was arrested when RhoA activity was sustained in microglia. This indicates that the previously described decrease in RhoA activity upon LPS stimulation might be a critical step for proinflammatory microglia polarization, as without such decrease, microglia cannot initiate an inflammatory response.

This hypothesis is further reinforced by biochemical results, showing that Src activation, a typical requirement for LPS-induced microglia inflammation (Socodato et al., 2015b), did not occur when RhoA activity was sustained. Because active Src is able to activate the NF-kB pathway and promote the secretion of proinflammatory cytokines, the lack of active Src will also compromise the production of those factors, which are a hallmark of LPS-challenged microglia.

The production of ROS and the decreased activation of AMPK are other two classical features of LPS-mediated microglia inflammation, that were significantly inhibited by sustaining RhoA activity. In accordance, RhoA/ROCK pathway is associated with NADPH oxidase, a major ROS productor in microglia (Rodriguez-Perez et al., 2015, Moon et al., 2013). Regarding AMPK, its activation is sufficient to reduce LPS-induced inflammation in microglia (Chen et al., 2014). Furthermore, neuroprotective properties of various compounds have been tested by their capacity to induce AMPK activation in LPS stimulated microglia, in order to regulate brain inflammation (Park et al., 2016, Lee et al., 2018). Overall, the robust outcome we obtained by addressing such different aspects of microglia inflammation, strongly validates our hypothesis. Accordingly, preventing the decrease in RhoA activity that occurs after LPS stimulation also arrested microglia inflammatory polarization. This turns out to be even more curious in light of the results suggesting that RhoA activity participate in this process at several levels, once its decrease is not only key for triggering microglia inflammation-related pathways but also for the cell to reprogram its metabolism according to its energetic requirements.

The opposite scenario, where no RhoA is present, was studied *in vivo* as a strategy to further characterize the *bona fide* role of RhoA in microglia during neuroinflammation. This was addressed using a microglia-specific Cre line, based on the insertion of the Cre^{ER} cassette into the Cx3cr1 locus (Parkhurst et al., 2013). In this case, by crossing

Cx3cr1$^{CreER-eYFP}$ (Parkhurst et al., 2013) mice with RhoA floxed mice (Herzog et al., 2011), we were able to conditionally ablate RhoA in microglial cells. Importantly, the use of this method overcome the lack of specificity of previous approaches such the use of C3 exoenzyme, that besides RhoA also inhibits RhoB and RhoC (Hoffmann et al., 2008) or the use of mice overexpressing dominant mutants, where unwanted trans-dominant effects can occur (Moon et al., 2013).

With a single LPS administration in mice with RhoA-deficient microglia we induced an acute neuroinflammatory condition that was sufficient to cause microglia cell death by necrosis and, to a lower extent, apoptosis. This cytotoxic effect was further validated using a histological qualitative analysis of the microglia morphology in which we found clear hallmarks of cell death, including process loss/beading and dysmorphic shape accompanied by extensive cell body swelling (Jurkowitz-Alexander et al., 1992, Grooten et al., 1993, Festjens et al., 2006). Such drastic outcome is not completely unexpected if we consider that previous studies form our laboratory report that the conditional ablation of RhoA in microglia under physiological conditions, compromise its fitness and induce an activated phenotype. It is plausible that without RhoA, instead of initiating inflammation-related pathways that would lead to an activated phenotype, microglia respond to neuroinflammation by triggering necrotic and apoptotic pathways which culminate in cell death.

Furthermore, our study also clarifies some of the possible signaling underlying this cytotoxicity, showing that after LPS exposure there is a disruption of calcium and intracellular pH homeostasis in both RhoA KO and RhoA T19N microglia. As previously referred in the *Results* section, the observed calcium overload and cytosolic acidosis may have a multitude of causes, most of them associated with impaired functioning of membrane transporters and consequent ionic dysregulation. Therefore, it is possible that in the absence of RhoA, the inflammatory stimulus dysregulated those transporters (e.g. ATPases or Na^{2+}/Ca^{2+} exchangers) leading to the increase of Ca^{2+} and H^+ in the cytoplasm. Following that, such excessive Ca^{2+} may lead to the activation of proteases, endonucleases and phospholipases that are classically involved in cleavage of organellar membranes (Zhivotovsky and Orrenius, 2011). For instance, increased lysosome permeability is sufficient for its content (including additional proteases) to be released into the cytoplasm, contributing to the observed acidification and consequent cell death (Wang et al., 2018).

Overall, we demonstrated for the first time the importance of RhoA in microglia during neuroinflammation. Whereas activation of RhoA prevented microglial inflammation to occur, inflammatory stimulation in microglia with impaired RhoA signaling led to cell

death **(Figure 19)**. This duality leads us to conclude that tight control of RhoA activity is critical for microglia physiological response to an inflammatory stimulus. This is highly relevant considering that microglia immune function is required for the sustainment of neuronal activity/plasticity and CNS homeostasis.

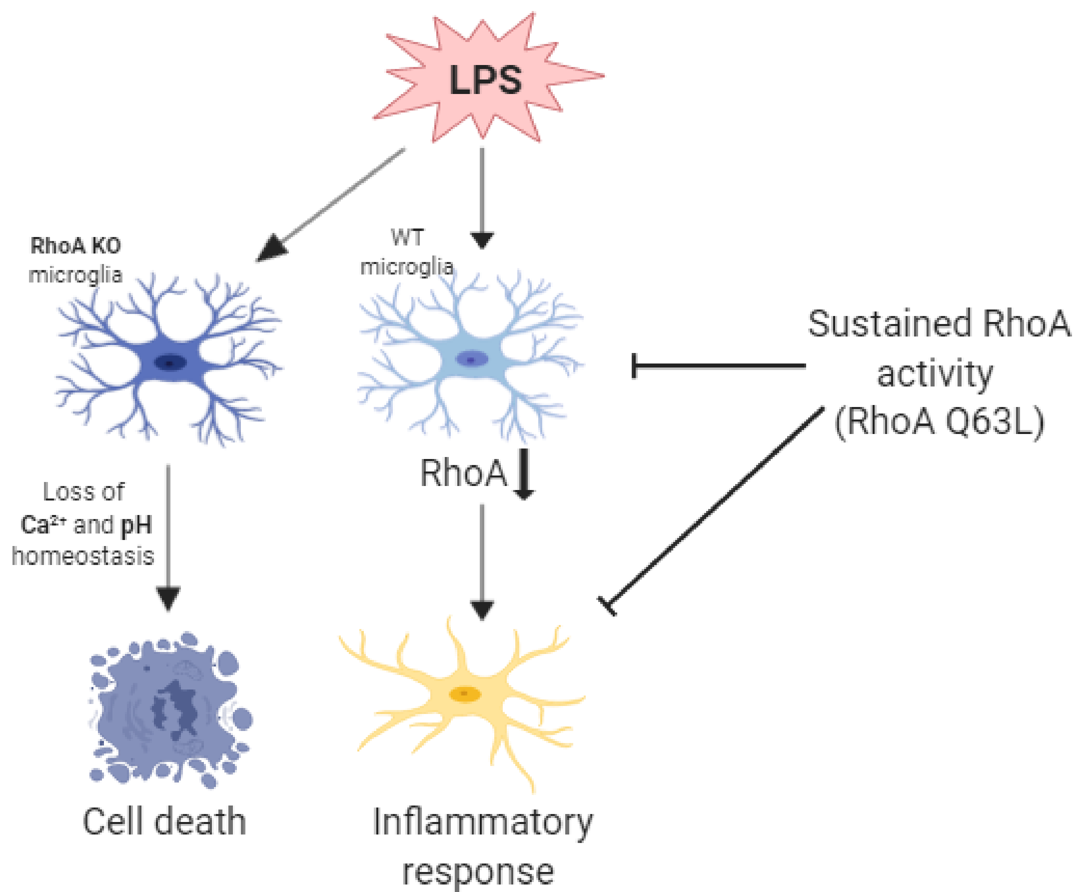

Figure 19- **Overview of the role of RhoA in LPS-induced microglial inflammatory response.**

Microglia immune function requires a tight and continuous regulation of RhoA activity. Accordingly, LPS-induced microglia inflammatory response requires a decrease in RhoA activity in such way that sustaining it (via RhoA Q63L) prevents overall microglia inflammation. On the other hand, in RhoA KO microglia, LPS-induced neuroinflammation caused cell death, likely through dysregulation of Ca^{2+} and pH homeostasis.

Chapter 6

Concluding Remarks

6. Concluding remarks

As suggested by the main title, this work aimed at shedding light into the role of the small GTPase RhoA in microglial inflammatory response. The association of live cell imaging with FRET technology allowed a real time characterization of the mechanisms by which RhoA was implicated in microglia inflammation. Additionally, the application of a well stablished *in vivo* model of neuroinflammation, combined with *in vivo* microglia-specific conditional RhoA ablation contributed to a more accurate analysis based on authentic features of a neuroinflammatory state. Therefore, in the course of this work, different hypothesis were progressively tested, with our results retrieving the following main conclusions:

1. RhoA activity is higher in microglia processes than in the cell body;
2. LPS-mediated inflammatory microglia response decreases RhoA activity;
3. Decrease of RhoA activity is required for microglia inflammatory response induced by LPS. Accordingly, sustaining this activity prevents both metabolic reprograming and initiation of classic inflammatory activation;
4. In the absence of RhoA, *in vivo* acute neuroinflammation is cytotoxic, leading to microglia cell death;
5. In the absence of RhoA, LPS stimulation causes disruption of Ca^{2+} and pH homeostasis which, potentially, is ultimately correlated with the increased microglial cell death.

Overall conclusion: A **tight control of RhoA activity** is critical for microglia physiological response to an inflammatory stimulus.

To the best of our knowledge, this is the first characterization of microglia inflammation in the perspective of Rho GTPases function. Interestingly, it emerged as a very promising aspect, considering that our data point out to a dependency of tight RhoA activity modulation for microglia response to occur and, therefore, for the overall microglia immune function. We started this work by referring that Rho GTPases are much more than cytoskeletal regulators, performing a large spectrum of cell functions and consequently, regulating and being regulated by a multitude of pathways. Corroborating that, our study present clear and robust data showing some of the main processes that RhoA is managing during microglia inflammatory response. However, a more in-depth analysis of the pathway(s) by which RhoA regulates it remain to be determined.

In the future we plan to address that by performing RNA sequencing, in order to find out which genes are being differently expressed among WT and RhoA cKO mice brain under neuroinflammation. It will not only increase the knowledge about RhoA operation in the cell, but also represents a major step to decode the biology of microglia inflammatory activation. In fact, modulation of that response is indicated as a promising therapeutic approach to control neuroinflammation, a condition that is common to several neurodegenerative disorders and has been making many neuroscientists reconsider the typical "*neuron-centric*" perspective and pay more attention to microglia.

Some experiments I performed for this work were included in a scientific paper that is currently under review (minor revisions phase) for Cell Reports Journal.

Title: *RhoA ablation in adult microglia triggers spontaneous activation leading to changes in neuronal function*

1st author: Renato Socodato

Manuscript number: CELL-REPORTS-D-18-03816R1

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