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Rac1 Regulation of Microglia Homeostasis and Activation During Neuroinflammation

Tiago de Oliveira Almeida

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Orientador: Doutor Renato Socodato

Co-orientadores: Professor Doutor João Bettencourt Relvas

Professora Doutora Perpétua Pinto do Ó

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Agradecimentos

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Abstract

Abstract

Microglia are the resident macrophage cells of the central nervous system (CNS). They perform several roles in the CNS such as synaptic pruning, neurogenesis, neuronal survival and neuronal cell death. Microglial cells are ramified cells that are constantly extending and retracting their processes to survey the CNS parenchyma. Identifying a signal (pathogens, chemokines, cell debris or even healthy cells), triggers a response involving changes in their morphology that include sending cellular processes towards the detected insult and retracting the others.

Rho GTPases are key players in actin cytoskeleton dynamics and reorganization. Rac1 is a member of the Rho family of GTPases along with RhoA and cdc42, the other two most studied Rho GTPases. Rac1 is classically associated with lamellipodia formation and membrane elongation to be able to perform phagocytosis. It is also involved in cell migration, axon guidance and cytoskeleton organization. It is necessary for the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which leads to reactive oxygen species (ROS) production. Rac1 coordinates the activities of the pro-inflammatory NF-κB and anti-oxidant NRF2 transcription factors.

Morphological changes in microglia require cytoskeleton remodeling and are likely to involve Rac1 function. In this work, we aimed at studying for the first time the roles of Rac1 in microglia in steady state and during neuroinflammation. By crossing a $Cx3cr1^{CreER\text{-}EYFP}$ mouse with a $Rac1^{fl/fl}$ mouse, and inducing Cre recombinase with tamoxifen, we generated mice in which Rac1 depletion was specifically restricted to microglia in the CNS. We observed decreased Ly6C, CD115, CD44, CCR2 and CD62L surface receptor expression and reduced mRNA levels of several homeostasis genes, without changes in the total number of microglia. In response to lipopolysaccharide (LPS) and phosphatidylcholine Rac1 mutant microglia failed to acquire a pro-inflammatory phenotype. There was a significant reduction in the percentage of colocalization of vesicular glutamate transporter 1 (VGLUT1) and post-synaptic density protein 95 (PSD95), indicative of less excitatory synapses in the neocortex and the hippocampus of rac1 mutant mice. This loss of excitatory synapses could explain the diminished sociability behavior observed. Thus, our results suggest that Rac1 specific ablation in microglia is altering their function, possibly by changing their fitness. Future work will

try to identify and characterize the signals and pathways involved in the communication between microglia and neurons responsible for synapse maintenance.

Keywords: Microglia, homeostasis, neuroinflammation, Rho GTPases, Rac1, actin cytoskeleton

Resumo

Resumo

As células da microglia são macrófagos residentes do sistema nervoso central. Esta população é responsável por diversas funções no sistema nervoso central, como por exemplo, pruning sinático, neurogénese, sobrevivência neuronal e morte neuronal. A células da microglia são células ramificadas que estão constantemente a estender e retrair os seus inúmeros processos para patrulhar o parênquima do sistema nervoso central. Quando estas células identificam um sinal (patogénico, quimiocinas, restos celulares ou até mesmo células saudáveis), desencadeia-se uma resposta que envolve mudanças na sua morfologia, incluindo enviar processos celulares na direção da perturbação detetada e retrair os restantes.

As Rho GTPases são peças chave na dinâmica e reorganização do citoesqueleto de actina. A Rac1 é um membro da família Rho de GTPases juntamente com a RhoA e a cdc42, que são as outras duas Rho GTPases mais estudadas. A Rac1 está classicamente associada com a formação de lamellipodia e elongação da membrana para ser capaz de fazer fagocitose. Está também envolvida na migração celular, na condução dos axónios e organização do citoesqueleto. É necessária para a ativação da NADPH oxidase, que leva à produção de espécies reativas de oxigénio. A Rac1 coordena a atividade dos fatores de transcrição pró-inflamatório NFκB e antioxidante NRF2.

As alterações morfológicas na microglia requerem remodelação do citoesqueleto e é provável que envolvam a Rac1. Neste trabalho, o objetivo era estudar, pela primeira vez, os papéis da Rac1 em estado de homeostasia e durante a neuro-inflamação. Cruzando um ratinho $Cx3cr1^{CreER\text{-}EYFP}$ com um ratinho $Rac1^{fl/fl}$, e induzindo a expressão da Cre recombinase com tamoxifen, obtivemos ratinhos nos quais a depleção da Rac1 era especificamente restrita para a microglia, no sistema nervoso central. Observamos uma expressão diminuída dos marcadores de atividade Ly6C, CD115, CD44, CCR2 and CD62L e uma redução nos níveis de mRNA de vários genes homeostáticos, sem alterações no número total de microglia. Em resposta ao LPS e fosfatidilcolina, as microglias sem Rac1 não adquiriram um fenótipo pró-inflamatório. Observou-se uma redução na percentagem de colocalização de VGLUT1 e PSD95, indicativo de menos sinapses excitatórias, no neocórtex e no hipocampo. Esta perda de sinapses excitatórias poderia explicar o comportamento de sociabilidade diminuído. Assim, os nossos

resultados sugerem que a ablação da Rac1 especificamente na microglia está a alterar as suas funções normais, possivelmente alterando as suas aptidões. Trabalhos futuros passarão por tentar identificar os sinais envolvidos na comunicação entre microglia e neurónios e que possam estar a mediar a sustentação das sinapses por parte da microglia.

Palavras Chave: Microglia, homeostasia, neuro-inflamação, Rho GTPases, Rac1, citoesqueleto de actina

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List of Abbreviations

Aβ – Amyloid beta

AD – Alzheimer's Disease

ALS – Amyotrophic Lateral Sclerosis

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

APP – Amyloid Precursor Protein

ATP – Adenosine Triphosphate

BBB – Blood Brain Barrier

BDNF – Brain-Derived Neurotrophic Factor

BSA – Bovine Serum Albumin

cKO – conditional Knock-Out

CMLE – Classic Maximum Likelihood Estimation

CNS – Central Nervous System

CR3 – Complement Receptor 3

CSF1R – Colony Stimulating Factor 1 Receptor

CT – Control

DGAV – Direção Geral de Alimentação e Veterinária

ERK – Extracellular Signal-regulated Kinase

FACS – Fluorescence-Activated Cell Sorting

FBS – Fetal Bovine Serum

FRET – Fluorescence Resonance Energy Transfer

GAPs – GTPase-activating Proteins

GAS6 – Growth Arrest-specific 6

GDIs – Guanine Nucleotide Dissociation Inhibitors

GDNF – Glial cell line-derived neurotrophic factor

GEFs – Guanine Nucleotide Exchange Factors

IGF1 – Insulin-like Growth Factor 1

IL-1β – Interleukin 1β

IRF8 – Interferon Regulatory Factor 8

JNK – c-Jun N-terminal Kinase

KD – Knock-down

LPS – Lipopolysaccharide

LRRK2 – Leucine-rich Repeat Kinase 2

LTD – Long Term Depression

MEFs – Mouse Embryonic Fibroblasts

mRNA – Messenger Ribonucleic Acid

MS – Multiple Sclerosis

NADPH – Nicotinamide Adenine Dinucleotide Phosphate

NF-κB – Nuclear Factor κ B

NGF – Nerve Growth Factor

NMDA – N-methyl-D-aspartate

NO – Nitric Oxide

NRF2 – Nuclear Factor-like 2

P2RY12 - P2Y purinoreceptor 12

PAK – p21 Activating Kinases

PD – Parkinson's Disease

PFA - Paraformaldehyde

PNS – Peripheral Nervous System

PSD95 – Post-synaptic Density Protein 6

PTMs – Post-translational Modifications

qRT-PCR – quantitative Real Time Polymerase Chain Reaction

ROS – Reactive Oxygen Species

RUNX2 – Runt-related Transcription Factor 2

SALL1 – Sall-like protein 1

TAM – TYRO3, AXL and MER

TMEM119 – Transmembrane Protein 119

TNF – Tumor Necrosis Factor

TREM2 – Triggering Receptor Expressed on Myeloid Cells

TLR – Toll Like Receptor

VGLUT1 – Vesicular Glutamate Transporter 1.

Introduction

Introduction

1. Microglia

Rudolf Virchow, in the middle of the 19th century, first identified the glial cells and called them "nerve glue". It was believed that their role was solely to keep neurons together. Later, Santiago Ramón y Cajal and Pío del Río-Hortega were able to distinguish different glial cells in the central nervous system (CNS): astrocytes, oligodendrocytes and a third element, later called microglia. In the peripheral nervous system (PNS), the main glial cell is the Schwann cell.

Microglia are the immune cells of the CNS where they constitute approximately 10% of all the glial cells. They were first described in the beginning of the 20th century by Nissl and Robertson and named by Pio del Rio-Hortega a few years later. Among other roles, they contribute to shape CNS circuitry, to maintain homeostasis of the CNS, and to detect and phagocyte microbes, cell debris, dead cells and protein aggregates.

1.1. Origin and Development

The origin of microglia has been extensively studied. Initially it was thought that microglia arise from the neuroectoderm (just as neurons, astrocytes and oligodendrocytes), but later work from different labs revealed their hematopoietic origin. Recent studies confirmed that in fact microglia derive from erythromyeloid progenitor cells that migrate from the yolk sac, and not from the bone marrow, to colonize the developing nervous tissue before embryonic day 8 (Ginhoux et al., 2010; Kierdorf et al., 2013; Schulz et al., 2012). Ginhoux and colleagues were the first to successfully demonstrate the yolk sac provenience of microglia. They used a mouse expressing an inducible Cre recombinase under the regulation of runt-related transcription factor 1 (Runx1), expressed exclusively in the yolk sac. By crossing this mouse with a *Rosa26* reporter mouse, they were able to trace the labeled cells and observe their presence in the brain. This study also reported that microglia maintain their numbers throughout the

entire life of the organism with no significant hematopoietic contribution (Ginhoux et al., 2010). Schulz et al. also validated the yolk sac origin of these cells and further showed that Myb, a transcription factor necessary for hematopoiesis was not required for microglia generation. Knock-out mice for Myb presented deficits in hematopoietic derived cells (monocytes and macrophages) but not in microglia (Schulz et al., 2012). Kierdorf et al. were able to characterize the population giving rise to microglia as being $CD45^{-}$c-kit^{+} erythromyeloid precursors that later change to $CD45^{+}$c-$kit^{-}CX3CR1^{+}$ microglia (Kierdorf et al., 2013). The process of differentiation depends on two transcription factors, namely interferon regulatory factor 8 (Irf8) and the ETS-domain transcription factor Pu.1. Irf8 deficient mice contains less microglia and Pu.1 ablation led to microglia depletion (Kierdorf et al., 2013). The development and maintenance of this population also depends on the expression of colony stimulating factor 1 receptor (CSF1R) (Elmore et al., 2014). This receptor signals intracellularly, activating protein kinase B (AKT) and ERKs, favoring microglial survival and proliferation. Recently, it was reported that when microglia are ablated, the population is restored by self-renewal of internal pools and independently from bone-marrow-derived macrophages (Bruttger et al., 2015).

Microglia is not the only macrophage-like population in the CNS. There are at least three more groups of CNS macrophages: perivascular, meningeal and choroid plexus macrophages (Goldmann et al., 2016). Among these, only choroid plexus macrophages come from a different origin (Goldmann et al., 2016).

The microglia transcriptome is significantly different from the transcriptomes of other macrophage populations. Microglia transcriptomics allowed the identification and/or validation of specific microglia markers, such as the transmembrane protein 119 (TMEM119) (Bennett et al., 2016), Sal-like protein 1 (SALL1) (Buttgereit et al., 2016), and the P2Y purinoreceptor 12 (P2RY12) (Butovsky et al., 2014). These studies also revealed that in steady state conditions, microglia also have a low expression of CD45 or MHC II, allowing the distinction from other CNS macrophages (**Figure 1**).

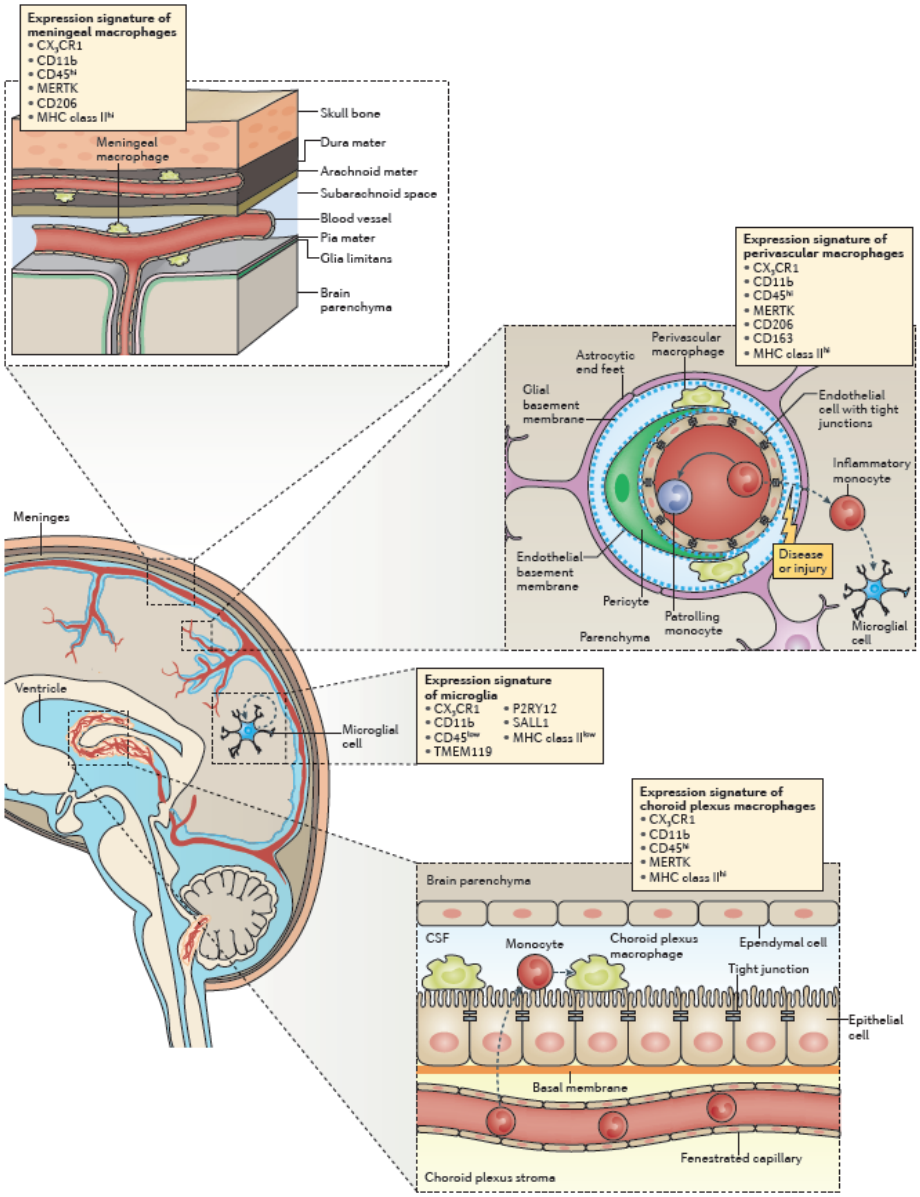

Figure 1 – Different macrophage populations in the CNS and their signature genes (Li & Barres, 2018)

1.2. Microglia in Physiology

Microglia are ramified cells with multiple processes coming from the cell soma. These cellular processes have secondary branches and lamellipodia structures. In the past, microglia under steady state conditions were described as quiescent and resting cells, However, it was showed that microglia are highly dynamic cells that extend and retract their processes to survey and scan the CNS environment (Davalos et al., 2005; Nimmerjahn, Kirchhoff, & Helmchen, 2005), searching for tissue damage, infections and guaranteeing the functionality of synapses. Davalos and colleagues performed a series of experiments in living mice that demonstrated the response of microglia to different injuries in vivo in the brain. Contrary to what was expected, using two-photon microscopy, they were able to show that microglia extended their processes towards the lesion site while their cell bodies remained static. Their work identified ATP as an

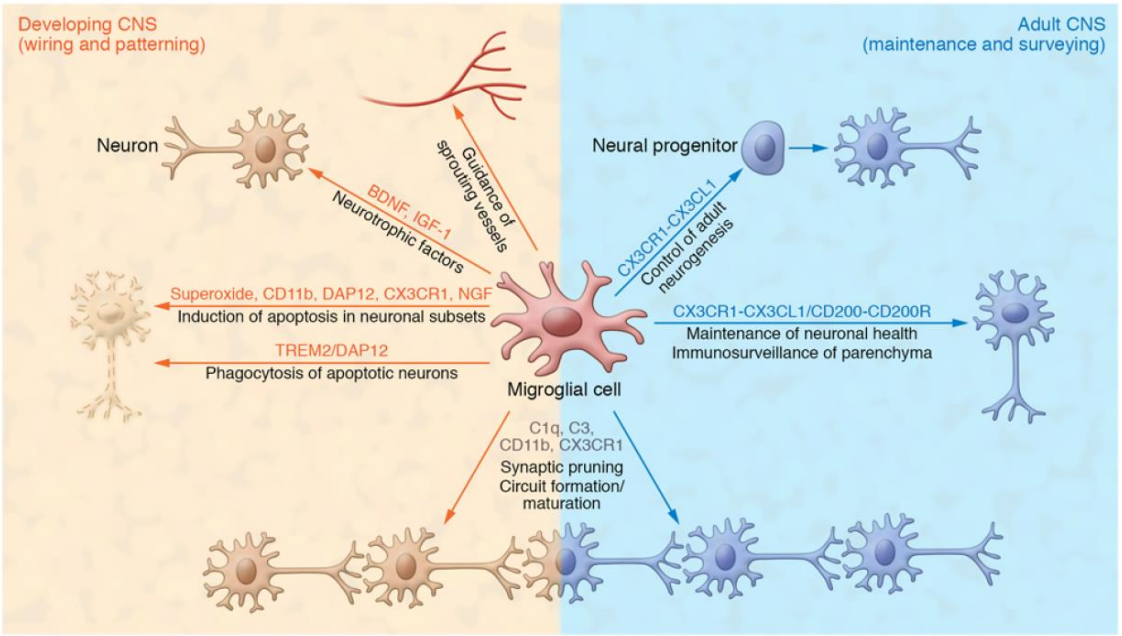

Figure 2 – Microglia functions in the developing and adult CNS (Kierdorf & Prinz, 2017)

important chemotactic signal for microglia to extend their processes towards the lesion site (Davalos et al., 2005). Allying these findings to the early presence of microglia in the developing CNS, it is expected that microglia are relevant, not only during adulthood but also in early stages of CNS development (**Figure 2**).

1.2.1. Role in Neurogenesis and Neuronal Cell Death

Neurogenesis is the process by which new neurons are formed. This happens mainly during embryonic development but also to a lesser extent in the adult brain. One of the most important responses of microglia is the phagocytosis of cell debris, infectious agents or even healthy cells. It is described that during development, Purkinje cells from the cerebellum are engulfed by microglia, which promote this process by producing ROS (Marín-Teva et al., 2004). The same phenomenon was observed in the hippocampus (Wakselman et al., 2008). In neurogenesis, neural precursor cells give rise to neurons. Microglia phagocytose these precursor cells, thus controlling their pool (Cunningham, Martinez-Cerdeno, & Noctor, 2013; Sierra et al., 2010). The engulfment is conducted by protein S, growth-arrest-specific protein 6 (Gas6), Tyro3, Axl and Mer (TAM) receptor tyrosine kinases (Fourgeaud et al., 2016) (**Figure 3**).

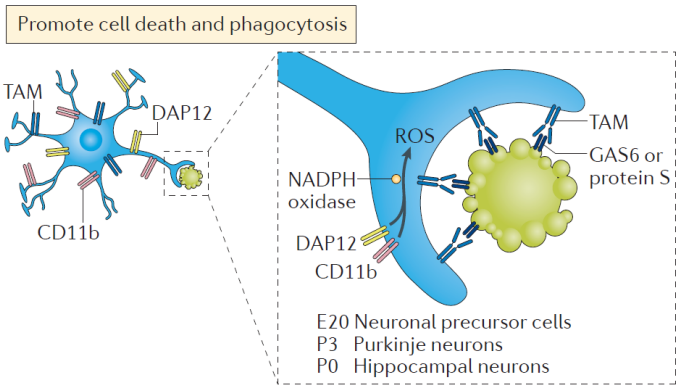

Figure 3 – Microglia drive cell death and engulfment mediated by TAM, GAS6 and protein S (Li & Barres, 2018)

1.2.2. Role in Neuronal Survival

Microglia can secrete a great diversity of molecules, both during development and in adulthood. By releasing neurotrophic factors, microglia are essential for the survival of neurons. During postnatal development, survival of cortical neurons from layer V is supported by the release of insulin-like growth factor 1 (Igf-1) by microglia (**Figure 4**) (Ueno

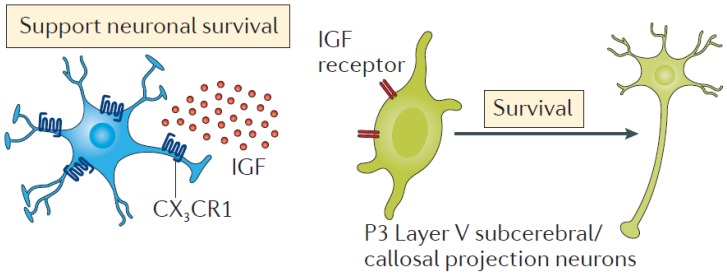

Figure 4 – Microglia promote neuronal survival by releasing IGF (Li & Barres, 2018)

et al., 2013). Moreover, this work also reported that CX3CR1 is required for the survival of layer V neurons. Other trophic factors such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) are also secreted by microglia, conferring these cells crucial roles in neuronal survival and functioning (Nakajima et al., 2001).

1.2.3. Role in Synaptic Pruning

Microglia are the major contributors, in the brain, for the expression of complement components. Microglial cells intervene actively in synaptic pruning during postnatal development of the CNS through phagocytosis (Paolicelli et al., 2011). The first clue that this function could be performed by microglia was the establishment of transient contacts between microglia and synapses (Wake, Moorhouse, Jinno, Kohsaka, & Nabekura, 2009). During this developmental stage, neurons establish more connections than necessary. When these synapses do not receive functional inputs, they are engulfed by microglia (Schafer et al., 2012). This process can be mediated through two pathways: complement and CX3CR1 (**Figure 5**). C1q and C3, two important proteins from the complement cascade, localize within these synapses to be recognized by complement receptor 3 (CR3) (Stevens et al., 2007). CX3CL1 (fractalkine) interaction with CX3CR1 was also demonstrated to be important for synaptic pruning as lack of these interactions using CX3CR1 deficient mice led to decreased synaptic pruning (Paolicelli et al., 2011).

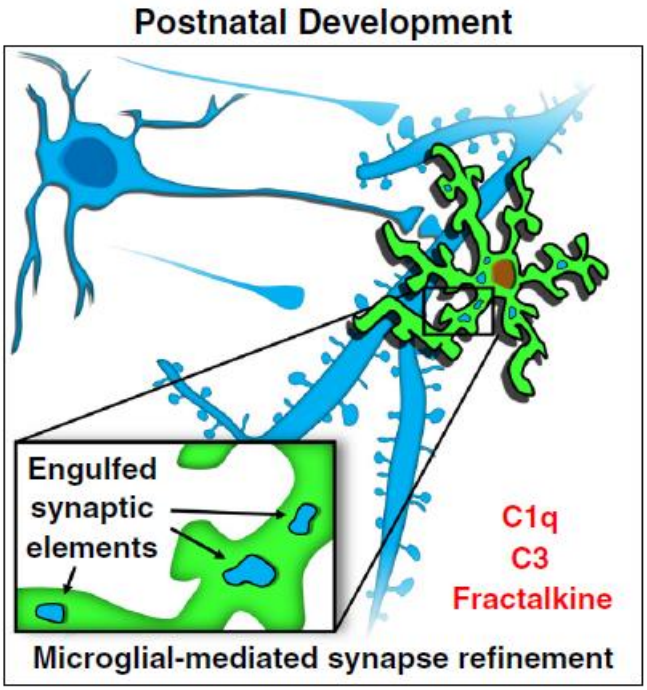

Figure 5 – Synaptic pruning during development through complement (C1q and C3) or CX3CL1 (Fractalkine) (Hong, Dissing-Olesen, & Stevens, 2016)

1.2.4. Role in Synaptic Plasticity

Synaptic strengthening and plasticity are also affected by microglial cells. One of the mechanisms that leads to reduction of synaptic strength is long-term depression (LTD) induced by glutamate signaling. This will lead to the activation of NMDA receptors from microglia, activation of NADPH oxidase and consequent ROS release. This induces a decrease of AMPA receptors in the neuronal membrane, lowering the strength of these synapses (Zhang et al., 2014). Since there is an activity reduction, these synapses can undergo synaptic pruning. BDNF secreted by microglia is also relevant for this plasticity. When BDNF was depleted in $CX3CR1^{+}$ microglial cells, there was a decrease in learning-dependent synaptic plasticity (Parkhurst et al., 2013).

1.3. Microglia in Pathology and Disease

As stated previously, when certain stimuli are detected by microglia, they trigger a response, involving drastic modification of their morphology and gene expression, to reestablish homeostasis. However, if these stimuli are persistent over time, there is an exacerbated or chronic activation of microglia, which can lead to excessive inflammation, synaptic pruning and neuronal damage (Block, Zecca, & Hong, 2007). The response of microglia is characterized by the increased production and release of reactive oxygen species (ROS), nitric oxide (NO), glutamate (which is neurotoxic when exposure is prolonged or in high concentrations) and pro-inflammatory cytokines (interleukin-1β (IL-1β) and tumor necrosis factor (TNF)) (Block et al., 2007).

It is accepted that the inflammatory component might contribute for the progression of most neurodegenerative diseases. One of the regulatory proteins that balances activation and pro-inflammatory responses in myeloid cells is the triggering receptor expressed in myeloid cells 2 (TREM2). TREM2 can recognize LPS (it is also recognized by Toll-like receptor 4) and phospholipids (Daws et al., 2014; Y. Wang et al., 2015). Variants of this protein have been associated with an increased risk for the development of Alzheimer's disease (AD) (Guerreiro et al., 2013; Jonsson et al., 2013). Microglia that are around amyloid beta (Aβ)-containing senile plaques express TREM2 that could

constitute a putative therapeutic target to prevent disease progression. Interestingly, microglia that are around Aβ plaques seem to have a neuroprotective function as they reduce the toxicity of Aβ fibrils by decreasing the interaction of Aβ with neurons (Yuan et al., 2016). Since TREM2 is involved in phagocytosis and inflammatory pathways alongside DAP12, mutations in this gene may lead to defects in microglial functions, such as neuroinflammation, phagocytosis of amyloid or cell survival. However, it is increasingly accepted that microglial response is not linear or binary (Gomez-Nicola & Perry, 2015). It is a highly controlled and regulated process, which is context dependent in relation to the stimulus and the physiological fitness of the cells.

TREM2 is not the only gene identified as a risk gene for AD. CD33 is implicated in neuroinflammation and Aβ clearance. In a mouse model of tauopathy (a hallmark of AD), neurofibrillary tangles only form after microglial activation, suggesting that microglia may be important in the progression of the disease (Maphis et al., 2015; Yoshiyama et al., 2007). There are several complement system components also upregulated in some AD mouse models, which inhibition prevents synapse loss (Soyon Hong et al., 2016). This indicates that microglia might contribute to synaptic loss in the disease context, suggesting also that the mechanisms involved in synaptic pruning are dysregulated in AD and drive synapse loss. Amyotrophic lateral sclerosis (ALS) is a disease in which motor neurons are gradually lost. In ALS, some of the mutated genes include superoxide dismutase 1 (SOD1) and C9orf72. Using a mouse model for the disease, it was observed that when SOD1 was removed from myeloid cells, there was a reduction in disease progression and increased survival (Boillee et al., 2006). C9orf72 has been associated with ALS and frontotemporal dementia and microglia with a mutation in this gene increase expression of neuroinflammatory factors (O'Rourke et al., 2016). Multiple sclerosis (MS) is another neurodegenerative disease characterized by the loss of the myelin sheath around axons, which eventually causes damage to neurons and leads to their death. In MS, microglia are thought to have a protective role by cleaning myelin debris (Yamasaki et al., 2014). It is also described that microglia might favor the remyelination process by inducing oligodendrocyte precursor differentiation (Miron et al., 2013). Parkinson's disease (PD) is a neurodegenerative disease where misfolded α-synuclein forms fibrillary inclusions (Lewy bodies) and dopaminergic neurons are lost from the *substantia nigra*. α-synuclein is neurotoxic and activates microglia through TLR1/2, leading to inflammation (Daniele et al., 2015). There is also increased expression

of the Axl receptor in microglia in a mouse model of PD. Blocking this receptor, along with Mertk, reduces the progression of the disease (Fourgeaud et al., 2016). Therefore, deregulation of microglia activity is associated with neurodegeneration and these cells should be considered a crucial element, performing pivotal roles in the progression of these diseases.

Since they are important regulators of synaptic strength and plasticity, microglia dysfunction might also be involved in psychiatric diseases. In the absence of Hoxb8 in myeloid cells, mice showed excessive grooming, with hair loss and injuries in their skin, a phenotype resembling trichotillomania, which is a compulsive disorder in humans (S. K. Chen et al., 2010). Remarkably, when those mice were lethally irradiated and grafted with bone marrow, they started behaving normally, suggesting that this phenotype is caused by the absence of Hoxb8 in myeloid cells. However, the exact origin of the myeloid cells that are responsible for this recovery is not known because lethal irradiation opens the blood-brain-barrier (BBB). The role of microglia in autism spectrum disorder is more controversial. Rett syndrome, recently classified as a neurodevelopmental disorder, is characterized by a reduction of MeCP2 in neurons. MeCP2 is also important for the normal functioning of microglia (Cronk et al., 2015). However, a recent study suggests that microglial levels of MeCP2 are not relevant for synapse loss in a mouse model of Rett syndrome. It is postulated that microglia contribute to the disease later in these mice, by engulfing weakened synapses where there is decreased expression of MeCP2 (Schafer et al., 2016).

By studying how microglia behaves in different conditions or when presented with a variety of stimuli, one can start unraveling the molecular mechanisms that fine-tune the response of these cells. This can be achieved by analyzing parameters of microglia that are associated with their function. As stated before, microglia are highly dynamic cells that are constantly moving their processes scanning the CNS parenchyma. They have a crucial phagocytic role in the nervous tissue and change their morphology when activated. All these processes require cytoskeletal dynamics and impairment of cytoskeleton-associated activity might be associated with the deregulation of microglia. Thus, looking for molecules that control different aspects of cytoskeleton dynamics might be one of the ways of fine-tuning microglial activity in the healthy or diseased CNS.

2. Rho GTPases

The Rho GTPase family of proteins consists in a group of low molecular weight proteins, from the Ras superfamily of guanidine nucleotide binding proteins. This family is divided into several subfamilies, such as the Rho, Rac, Cdc42 and RhoD subfamilies (**Figure 6**). The most studied Rho GTPases are RhoA, Rac1 and Cdc42. These proteins (typical GTPases) alternate between an active GTP-bound state and an inactive GDP-bound state. Activation of Rho GTPases is mediated by guanine exchange factors (GEFs) and inactivation is promoted by GTPase activating proteins (GAPs) (Bos, Rehmann, & Wittinghofer, 2007). GEFs allow the exchange of GDP for GTP and GAPs promote the inherent ability of Rho GTPases to hydrolyze GTP to GDP. There are also guanine nucleotide dissociation inhibitors (GDIs) that prevent some GTPases from going to the membrane or being activated by GEFs (DerMardirossian & Bokoch, 2005) (**Figure 7**). Atypical Rho GTPases do not alternate between an active and an inactive state because they are constitutively bound to GTP. Therefore, regulation of these rely on expression levels, post-transcriptional or post-translational modifications (PTMs) other than by GEFs and GAPs. Typical Rho GTPases are also regulated by PTMs. Rac1 is phosphorylated by ERK, which leads to its translocation to the nucleus (Tong, Li, Ballermann, & Wang, 2013), and by AKT, which decreases Rac1 activity by inhibiting GTP binding (conformational alteration in the GTP binding site) (Kwon, Kwon, Chun, Kim, & Kang, 2000). Src also phosphorylates Rac1 and acts as a negative regulator of its activity (Chang, Lemmon, Lietha, Eck, & Romer, 2011). These PTMs can also be mediated through lipid modifications, such as prenylation or palmitoylation. In fact, prenylation of Rac1 followed by palmitoylation is important for Rac1 stability and targeting to membrane regions linked with modulating the actin cytoskeleton (Navarro-Lerida et al., 2012). When this palmitoylation does not occur, cells show deficient migration.

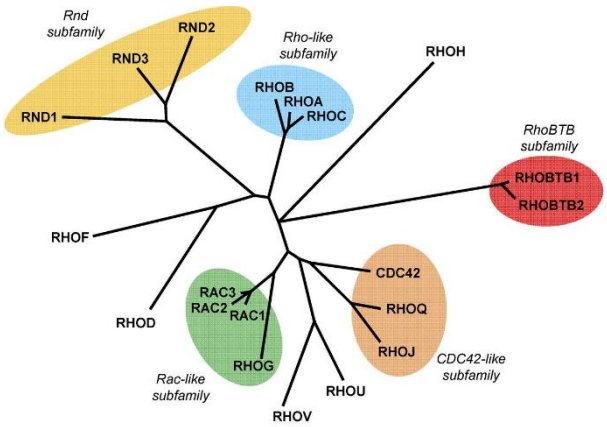

Figure 6 – Phylogenetic tree representing the 20 human Rho GTPases and their organization in subfamilies (Lawson & Ridley, 2017).

Proteins from the Rho GTPase family can be activated by several receptors in the cell surface, such as integrins, G protein-coupled receptors, cadherins, Tyr kinase receptors or cytokine receptors (Rossman, Der, & Sondek, 2005). Once activated, they communicate with various effectors inside the cell, initiating a plethora of downstream signaling pathways. These effectors can be actin regulators or kinases. Activation of a Rho GTPase can trigger a variety of responses, depending on the extracellular signal.

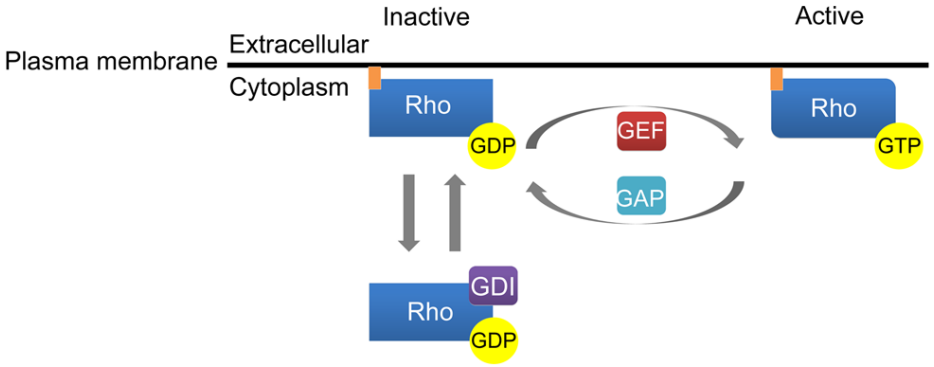

Figure 7 – Typical Rho GTPases regulation by GEFs and GAPs (adapted from (Stankiewicz & Linseman, 2014))

Rho GTPases are master regulators of the actin cytoskeleton dynamics and organization. This translates into important roles such as microtubule dynamics, cell motility and polarity, axon guidance, gene expression (Hill, Wynne, & Treisman, 1995) or even cell cycle progression (Olson, Ashworth, & Hall, 1995). Classically, RhoA activation is associated with the formation of focal adhesions and stress fibers (Ridley & Hall, 1992), Rac1 leads to lamellipodia formation (Ridley, Paterson, Johnston, Diekmann, & Hall, 1992) and Cdc42 to the formation of filopodia (Nobes & Hall, 1995).

2.1. Rac1

Rac1 was first identified in 1989 (Didsbury, Weber, Bokoch, Evans, & Snyderman, 1989). Along with Rac2, Rac3 and RhoG, they constitute a sub-family of Rho GTPases. These proteins are associated with lamellipodia formation and membrane elongation to perform phagocytosis (Heasman & Ridley, 2008). Rac1 has also been associated with cell migration, axon guidance (L. Chen et al., 2007) and production of superoxide radicals (Zhao, Carnevale, & Cathcart, 2003). Rac1 is ubiquitously expressed and it establishes interactions with various effectors. The first effectors described for Rac1 were the family

of p21 activating kinases (PAK). Stimulation of their activity when they bind to Rac1 promotes cytoskeleton reorganization (Brown et al., 1996). PAK activate c-Jun N-terminal kinase (JNK) and the JNK signaling pathway (Brown et al., 1996).

2.1.1. Rac1 in Physiology

Rac1 has been extensively studied outside de CNS and in other myeloid and macrophage-like populations. Rac1 knockout in mice proved to be lethal embryonically (Sugihara et al., 1999). Therefore, the strategy to keep studying this protein has been the generation of knockout mice lacking the protein in specific tissues. Regarding actin dynamics, it is already well established that Rac1 activation results in the formation of lamellipodia. Primary mouse embryonic fibroblasts (MEFs) deficient in Rac1 lost focal adhesions and actin stress fibers, leading to cell death (Guo, Debidda, Yang, Williams, & Zheng, 2006). In endothelial cells, mast cells or lymphocytes, Rac1 also drives actin polymerization (Hall, 1998). Rac1 has also been implicated in endocytosis and vesicle trafficking, as these processes are highly dependent on the actin cytoskeleton (Bosco, Mulloy, & Zheng, 2008). Some of the already described roles are: induction of membrane protrusions enriched in actin during pinocytosis; phagocytosis by localized actin polymerization; necessary for the activation of NADPH oxidase in macrophages and monocytes (Abo et al., 1991; Zhao et al., 2003), which leads to ROS production; in macrophages, for phagocytosis mediated by immunoglobulin receptors, considering also the activation of JNK and MAPK signaling pathways (Caron & Hall, 1998). Rac1 also coordinates the activities of the pro-inflammatory NF-κB and anti-oxidant NRF2 transcription factors (Cuadrado, Martin-Moldes, Ye, & Lastres-Becker, 2014).

2.1.2. Rac1 in Pathology and Disease

Rac1 signaling is involved in various human diseases such as cancer (Sahai & Marshall, 2002), infectious diseases (Criss, Ahlgren, Jou, McCormick, & Casanova, 2001), pathological inflammation or neurodegenerative disorders (Stankiewicz & Linseman, 2014). This Rho GTPase has a protective role by reducing neurodegeneration mediated by apoptosis. In ALS, constitutively active Rac1 expression reduces cell death in SOD1 mutants (Pesaresi et al., 2011). Alsin is a Rac1 GEF whose mutations have been associated with ALS (Hadano, Kunita, Otomo, Suzuki-Utsunomiya, & Ikeda, 2007). When this protein is knocked down, there is a reduction of neurite outgrowth and cell death induction (Jacquier et al., 2006). In PD, Rac1 expression recovers neuronal death observed in leucine-rich repeat kinase (LRRK2) mutants (Chan, Citro, Cordy, Shen, & Wolozin, 2011). Thus, downregulation of Rac1 expression in neurons seems to be required for the occurrence of neurodegenerative diseases. However, when SOD1 is mutated in microglia, there is an increased activity of Rac1, which promotes NADPH oxidase activation and ROS production (Harraz et al., 2008), leading to degeneration (Wu, Ré, Nagai, Ischiropoulos, & Przedborski, 2006). In AD, reports demonstrated that Rac1 is implicated in the regulation of expression and transcription of amyloid precursor protein (APP), crucial for Aβ cleavage (P. L. Wang, Niidome, Akaike, Kihara, & Sugimoto, 2009). This might suggest that Rac1 function is important for Aβ plaques formation in AD.

Rac1 behavior varies according to the cell type in question. In microglia, a macrophage-like cell population with myeloid origin, little has been studied regarding the role of Rac1, either during homeostasis or during inflammation. Undoubtedly, due to the high requirements of a very dynamic cytoskeleton for microglial function, Rac1 might be directly involved in modulating the physiology of these cells.

Objectives

Objectives

As previously referred, Rac1 roles in microglia are still largely unknown. However, Rac1 has been extensively studied in myeloid cell populations outside the CNS. Since microglia and macrophages are similar, it is tempting to say that some of the functions described in macrophages will also be observed in microglia. Microglia are constantly changing their morphology, not only in steady state to survey the CNS parenchyma, but also in response to activating stimuli. Therefore, actin cytoskeleton dynamics and reorganization are crucial for microglial cells functions. Rac1, as other proteins from the Rho GTPase family, are key players in actin cytoskeleton dynamics. This raises the possibility for Rac1 being an important molecule for microglia in both homeostasis and neuroinflammation.

Thus, the main research goal of this work was to study for the first time the *bona fide* roles of Rac1 in microglia homeostasis and during neuroinflammation. To do so, we followed these specific goals:

1. Investigate the effects of Rac1 depletion specifically in microglia in steady state.

2. Study the response of microglia to classical activation stimuli such LPS and phosphatidylcholine *in vitro* and *in vivo*.

3. Explore the effects of microglia-specific Rac1 depletion on the neuronal compartment by analyzing synapse and neuronal number in the brain.

Materials and Methods

Materials and Methods

1. Animals

To generate mice with Rac1-deficient microglia, two different mice were used: $Cx3cr1^{CreER\text{-}EYFP}$ mice (Jackson Laboratories), in which the Cx3cr1 promotor drives CreER expression specifically in microglia in the CNS (Parkhurst et al., 2013), and homozygous floxed mice for the exon 3 of Rac1 gene. These mice were crossed to generate $Rac1^{fl/fl}$ $Cx3cr1^{CreER\text{-}EYFP+}$ (Rac1 cKO) and $Rac1^{fl/fl}$ (control) mice. Genotypes were determined by PCR from genomic DNA. For conditional ablation of Rac1, $Rac1^{fl/fl}$ $Cx3cr1^{CreER\text{-}EYFP+}$ (Rac1 cKO) and $Rac1^{fl/fl}$ (control) mice received tamoxifen (Sigma-Aldrich) (10 mg/kg by oral gavage per animal) at P26 and P28 and analyzed at different time points. All mice were kept on a C57Bl/6 background with no sex difference, except in behavioral tests where only male mice from both CT and Rac1 cKO groups were used. Administration of LPS intraperitoneally (4 mg/kg) from Escherichia coli 0111: B4 (Sigma Aldrich) was performed at P150 CT and Rac1 cKO mice and 24 hours later the animals were sacrificed. All experiments performed were approved by i3s Animal Ethics Committee and Direção Geral de Alimentação e Veterinária (DGAV).

2. Flow cytometry and cell sorting

To characterize microglia and macrophages, the markers used were: CD45-PE (BioLegend 103106), CD11b-APC (BioLegend 101212), CD11b-Alexa Fluor 647 (BioLegend 101218), CD44-Brilhant Violet 510 (BioLegend 103044), CCR2-PE/Cy7 (BioLegend 150612), CD115-APC/Cy7 (BioLegend 135531) and Ly6C-PerCP/Cy5.5 (BioLegend 128012). Microglia and macrophages were collected from brain, blood and spleen of both control and Rac1 cKO mice. Mice were anesthetized with 0.2 ml of sodium pentobarbital per 30 g of mouse body weight and then perfused with ice-cold PBS. Tissues were dissected, placed on ice-cold Gibco® RPMI 1640 (Thermo Fisher), mechanically homogenized and passed through a 100 µm cell strainer. Cells were centrifuged in a 70%/30% Percoll (Sigma-Aldrich) gradient. Cells from the interface were

collected, centrifuged, washed and resuspended in FACS buffer. Cells were incubated with the antibodies for 1 h at 4°C in the dark. Compensation settings were determined using spleen from both CT and Rac1 cKO mice. Cell suspensions were evaluated on a FACS Canto II analyzer (BD Immunocytometry Systems). Cell sorting was performed on a FACS ARIA cell sorter and data were analyzed with FlowJo X10 software (TreeStar).

3. Social interaction experiment

This behavioral test was performed in the dark phase of the light/dark cycle. Before each session, mice were transported from their home cages to the testing rooms (illuminated with 100 lux and attenuated noise). Crawley's sociability and preference for social novelty test is performed in a three-chambered box as described (Moy et al., 2004). Briefly, the compartments were isolated and subject mice were placed in the middle chamber for habituation. One empty wired cup was placed in each lateral compartment. A naïve male mouse from the same background of the experimental mice was placed in one of the wired cups and the entire apparatus was opened to be freely explored by CT or Rac1 cKO mice. Recording was immediately initiated, and the following parameters were evaluated: duration of contacts between the experimental mouse and the empty wired cup or the naïve mouse in the other wired cup and time spent in each compartment.

4. Tissue Collection and Sectioning

Mice were anesthetized with 0.2 ml of sodium pentobarbital per 30 g of mouse body weight and then perfused with ice-cold 4% PFA. Brains were collected and placed in 4% paraformaldehyde (PFA) overnight, washed with PBS 1x and changed to a sucrose solution twice (15% and 30%). Brains were mounted in OCT freezing medium (Leica Biosystems) and stored at -80 °C. Coronal sections were obtained using the CM3050S Cryostat (Leica Biosystems) with a thickness of 30 µm and collected on Superfrost™ Ultra Plus adhesion slides (ThermoFisher Scientific). Slides were stored at -20ºC.

5. Immunohistochemistry

Sections were defrosted for at least 1 hour and hydrated with PBS 1x. Sections were permeabilized with 0.25% Triton X-100 for 10 minutes, washed with PBS 1x and blocked for 1 hour in 5% bovine serum albumin (BSA), 5% fetal bovine serum (FBS) and 0.1% Triton X-100. Primary antibodies (vesicular glutamate transporter 1 (VGLUT-1) (1:1000, Synaptic Systems); post-synaptic density protein 95 (PSD95) (1:600, Cell Signaling); NeuN (1:100, Millipore)) were incubated overnight in blocking solution at 4ºC. Secondary antibodies (1:500 anti-rabbit Alexa647; 1:1200 anti-mouse Alexa568; 1:700 anti-rabbit Alexa568) were incubated for 90 min in blocking solution at room temperature. Slides were cover slipped using Flouroshield (Sigma-Aldrich) and visualized under a Leica TCS SP5 II confocal microscope.

Images from tissue sections were acquired using a Leica HC PL APO Lbl. Blue 20x /0.70 IMM/CORR or a Leica HC PL APO CS 40x /1.10 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 acquired from mouse brain sections. Equivalent stereological regions were acquired for all tissue sections within a given slide. Image series were deconvolved using the Huygens Professional using the Classic Maximum Likelihood Estimation (CMLE) algorithm together with a determined theoretical PSF established using a routine-based implementation for the Huygens software. Reconstruction and generation of 3D volumes of deconvolved images were performed using the ImageJ 3D viewer plugin and cell counts were performed blinded on integral 3D volume-rendered images. All immunostaining in sets of slides were performed together, using the same batch of primary and secondary antibodies, and blocking and washing solutions. Furthermore, images from different sections within a given slide were acquired on the same day, always by same operator and with identical microscope parameters.

$NeuN^+$ neurons were scored manually in maximum projection z-stack converted images of the CA1 region of the hippocampus or the neocortex of NeuN stained sections (4 images per hippocampal/cortex section; 3 hippocampal/cortex sections per genotype).

Images from identical regions from each experimental group (4 images per cortex; 4 cortex sections per experimental group) were acquired using a Leica HC PL APO CS 40x /1.10 CORR water objective at 1024 x 1024 pixels resolution with 8-bit bidirectional non-sequential scanner mode at 400 Hz and pinhole at 1 airy in the Leica TCS SP5 II confocal microscope. Z-stacks were converted to maximum projection images using LAS AF routine and the LAS AF colocalization plugin processed each projection using subtracted background (25–36% offset for both channels) and thresholded foreground (35-45% offset for vGlut-1 channel; 30-40% offset for PSD-95 channel). Values corresponding to the positive area of vGlut-1/PSD-95 colocalization puncta (synapses) and values for the overall image area for each image was extracted using the LAS AF colocalization plugin and statistically evaluated in GraphPad Prism.

6. RNA Extraction

RNA from the hippocampus was extracted using the Trizol extraction coupled with PureLink™ Mini Kit (Invitrogen). Briefly, samples were collected and fast frozen with cold isopentane and stored at -80 °C. After incubation for 5 minutes at room temperature 1 mL of TRIzol® to completely dissociate nucleoprotein complexes, 200 µL of chloroform were added and the samples were vigorously shaken. After centrifugation, the supernatant was retrieved and ethanol 70% (V/V) was added to a final concentration of 35%. Samples were transferred to a spin cartridge and then centrifuged several times with two different wash buffers. The spin cartridge was then introduced in a new collecting tube and RNase free water was added in the center of the spin cartridge. After centrifugation, RNA was collected and its concentration and quality were assessed using a Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific). RNA was stored at -80 °C.

7. cDNA Synthesis

cDNA synthesis was performed using NZY First-Strand cDNA Synthesis Kit (NZYTech). Briefly, a mix was prepared with 10 µL of NZYRT 2x Master Mix, 2 µL of NZYRT Enzyme Mix, 1 µg of RNA (variable volume, dependent on the concentration of

the sample) and DEPC-treated water to a final volume of 20 µL for each sample. Each reaction was incubated firstly at 25 °C for 10 minutes and then at 50 °C for 30 minutes. Reaction was inactivated by heating at 85 °C and after 5 minutes was put on ice. 1 µL of NZY RNase H (*E. coli*) was added and reactions were incubated at 37 °C for 20 minutes. Samples were stored at -20 °C.

8. Quantitative RT-PCR

qRT-PCR was performed using iQ™ iTaq™ SYBR® Green Supermix on an iQ™5 multicolor real-time PCR detection system (Bio-Rad). Efficiency was analyzed using a log-based standard curve. Expression of PCR transcripts between genotypes was calculated using the $2^{-\Delta Ct}$ or the Livak method with Yhwaz serving as the internal control gene. The primers used were:

Sequences (5' – 3')		
	Forward	Reverse
Rac1	GAAGCTTCCCATCACCTACCC	GGGGACAGAGAACCGCTCGGATAG
Csfr1	CCCTAGGACAAAGCAAGCAG	GATGTCCCTAGCCAGTCCAA
P2ry12	CACCTCAGCCAATACCACCT	CACCTCAGCCAATACCACCT
Pu.1	CAGTTCTCGTCCAAGCACAA	TTTCTTCACCTCGCCTGTCT
C1qA	GTGTGCTGACCATGACCCTA	ATTCCCCTGGGTCTCCTTTA
C1qB	AGACACAGTGGGGTGAGGTC	GGTCCCCTTTCTCTCCAAAC
C1qC	GAGGACCCAAGGGTCAGAAG	TGTATCGGCCCTCCACAC
Gpr34	GGTTGCTCTTGCTGGATTTC	CCGGGCTGTTGTAGCATATT
Pros1	GATTCTCGCTCTGGAACGTC	GGTGTGGCACTGAAGGAAAT
Mertk	GCCCACAATGACAAAGGACT	GGGAGTAGCCATCAAAACCA
Trem2	AACTTCAGATCCTCACTGGACC	CCTGGCTGGACTTAAGCTGT
Tlr2	TTGCTCCTGCGAACTCCTAT	GCTTTCTTGGGCTTCCTCTT
Tlr4	GCTTTCACCTCTGCCTTCAC	GCGATACAATTCCACCTGCT
Tlr7	TGGAAATTTTGGACCTCAGC	TTGCAAAGAAAGCGATTGTG

9. FRET-based live cell imaging and biosensor quantification

HMC3 (ATCC® CRL-3304™) cell line was used for all *in vitro* assays. 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 Scrambled mCherry, shRac1 mCherry, pLKO or shRac1 plasmids and HSP33 ROS, DAG LAS PM, D1ER, Lyn AKT and pIκBα-miRFP703 FRET probes (total of 0.8 µg of DNA/dish). Transfection reaction was prepared with 20 µL of JetPrime® Buffer and JetPrime® Reagent (1 µg of DNA: 2 µL of reagent). The medium was changed 4 hours after transfection. Two days after transfection, cells were imaged using a Leica DMI6000B inverted microscope. 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 440-520 nm dichroic mirror (CG1, Leica Microsystems) and a PlanApo 63X 1.3 NA glycerol immersion objective were used for CFP and FRET images. Images were acquired with 2x2 binning using a digital CMOS camera (ORCA-Flash 4.0 V2, Hamamatsu Photonics). Shading illumination was online-corrected for CFP and FRET channels using a shading correction routine implemented for the LAS AF software. At each time-point, CFP and FRET images were sequentially acquired using different filter combinations (CFP excitation plus CFP emission and CFP excitation plus YFP emission, respectively).

Quantification of FRET biosensors was performed as previously described (Socodato et al., 2015). Briefly, images were processed in FIJI software. Background was dynamically subtracted from all frames from both channels and images were filtered using a Kalman stack filter. Segmentation was achieved on a pixel-by-pixel basis using a modification of the Phansalkar algorithm. After background subtraction and thresholding, binary masks were generated for the CFP and FRET images. Original CFP and FRET images were masked, registered and bleach-corrected. Ratiometric images (CFP-CFP (donor to CFP-YFP(FRET) for HSP33 ROS probe and FRET to donor DAG LAS PM probe, D1ER probe and AKT probe) and F-F0 images for IκBα reporter (NF-κB pathway probe) were generated as 32-bit float-point tiff images. Values corresponding to the mean

gray values were generated using the multi calculation function in FIJI and exported as mentioned above.

10. 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 or paired Student t 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).

Results

Results

1. Conditional ablation of Rac1 in adult microglia

To conditionally ablate Rac1 in adult microglia, we used mice bearing a tamoxifen-inducible Cre recombinase and EYFP cassette under the endogenous regulation of the Cx3cr1 promoter (Parkhurst et al., 2013). In $Rac1^{fl/fl}$ $Cx3cr1^{CreER\text{-}EYFP+}$ (Rac1 cKO), tamoxifen administration induces Cre recombinase to translocate to the nucleus leading to recombination of the Rac1 floxed alleles (**Figure 8A**). This system allowed us to synchronize the genetic inactivation of Rac1 to our experimental requirements and to study the bona fide role of Rac1 specifically in adult microglia. Likewise, tamoxifen was administered to CT ($Rac1^{fl/fl}$) and Rac1 cKO ($Rac1^{fl/fl}$ $Cx3cr1^{CreER\text{-}EYFP+}$) mice at P28 and P30 and analyses were performed at P65-75 (young adult) and P180-190 (mature adult) (**Figure 8B**).

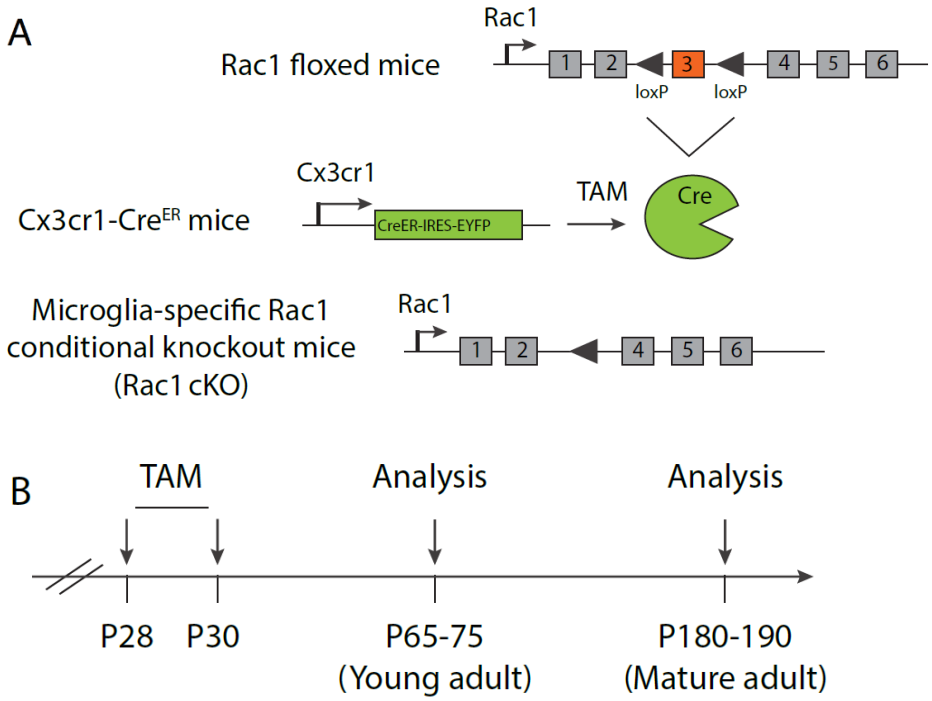

Figure 8 – Animal model breeding scheme and timepoints outline. Rac1 floxed mice (exon 3) were crossed with Cx3cr1-CreER-IRES-EYFP mice to originate mice without Rac1 in microglia after tamoxifen administration (**A**). Tamoxifen administration at P28 and P30 and analysis at P65-75 and P180-190 (**B**).

To validate the ablation of Rac1 in microglia, mRNA levels of Rac1 were analyzed both in flow cytometry-sorted microglia ($CD11b^{+}CD45^{mid}$ cells) and in flow cytometry-sorted blood monocytes ($CD11b^{+}CD45^{high}$ cells). In Rac1 cKO mice, Cx3cr1 promoter drives the expression of Cre recombinase. In the CNS, Cx3cr1 expression is restricted almost exclusively to microglia, but in peripheral organs, other myeloid resident cell populations also express Cx3cr1 (Parkhurst et al., 2013). Therefore, recombination of Rac1 floxed alleles is expected to occur not only in CNS microglia but, to a smaller content, in other peripheral myeloid cells expressing Cx3cr1. However, myeloid cells such as blood monocytes have a high turnover rate (van Furth & Cohn, 1968) whereas microglia have a very low turnover rate and their population is maintained during adulthood with little contribution from hematopoietic progenitors. Thus, recombination in the microglial compartment, but not on peripheral myeloid cells, will be maintained throughout the life of the mouse (Parkhurst et al., 2013). qRT-PCR analyses showed, as

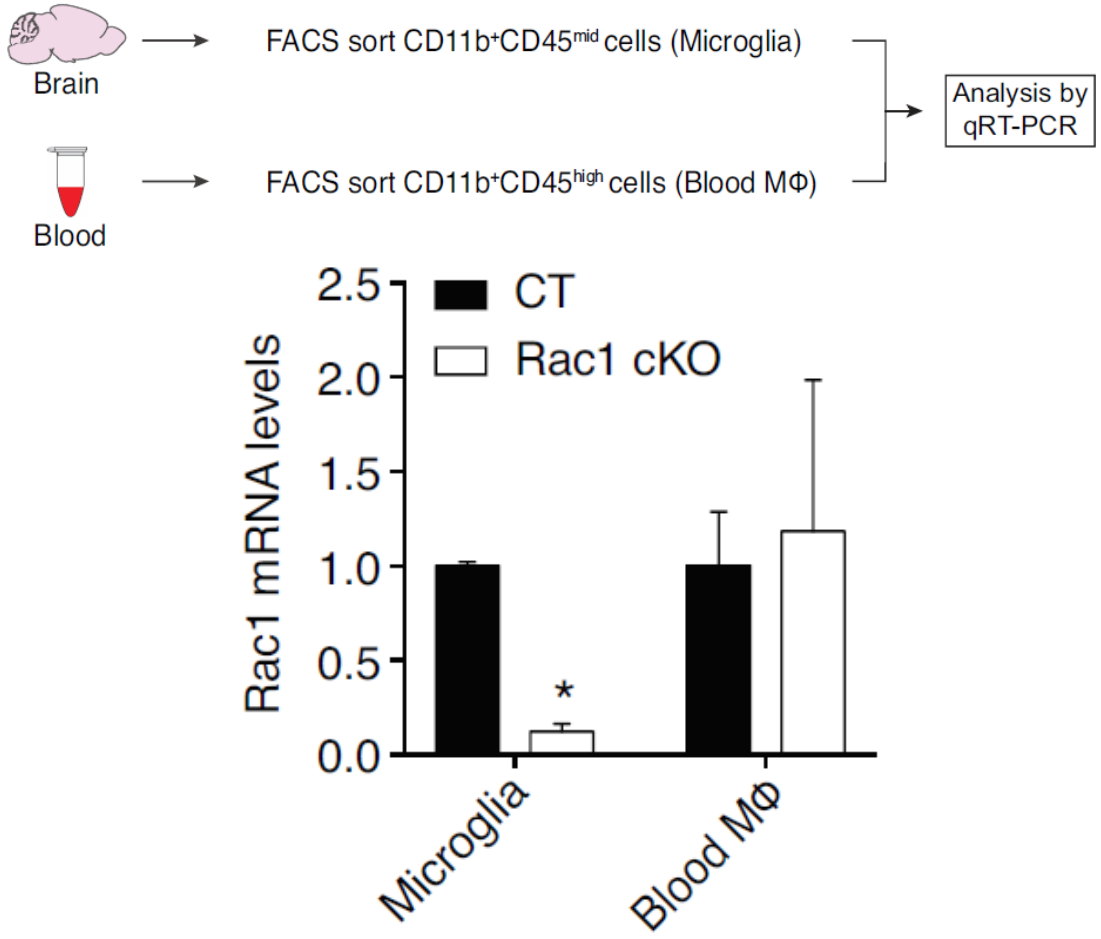

Figure 9 – Animal model characterization by qRT-PCR. mRNA was collected from sorted brain microglia and blood macrophages from CT and Rac1 cKO mice at P65-75 (**top**). Levels of Rac1 mRNA were determined by qRT-PCR to each population (n=3-5 animals) (**bottom**). Graph represent means and SEM (Mann-Whitney test, *p<0.05).

expected, that Rac1 mRNA levels were significantly lower in flow cytometry-sorted

microglia ($CD11b^{+}CD45^{mid}$ cells) from Rac1 cKO brains compared with those from the brains of CT littermates (**Figure 9**). Rac1 mRNA levels were not significantly altered in blood monocytes ($CD11b^{+}CD45^{high}$ cells) sorted from Rac1 cKO and CT littermates (**Figure 9**), confirming that after tamoxifen administration, ablation of Rac1 was restricted to microglia.

2. Ablation of Rac1 alters microglia homeostasis

Next, we evaluated if loss of Rac1 could compromise microglial homeostasis at the steady state. First, we assessed total microglia numbers in the brains of CT and Rac1 cKO mice by flow cytometry in two time points (35 days (P65 - young adult) and 150 days (P180 - mature adult)) after tamoxifen administration. We observed that the total number of microglia in Rac1 cKO brains was not significantly different from the numbers found in the brains of CT littermates in both time points analyzed (**Figure 10**).

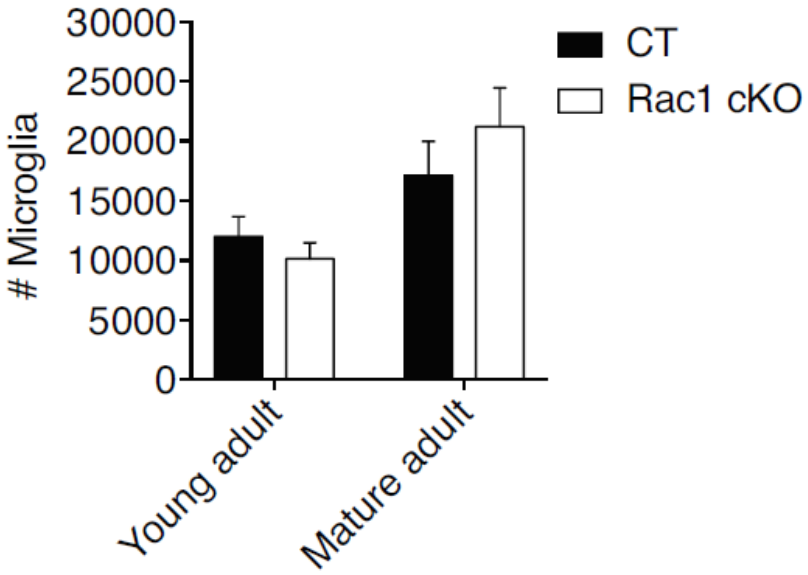

Figure 10 – Total number of microglia by FACS. Microglial cell number for both CT and Rac1 cKO brains was determined by FACS at P65-75 (n=9-14 animals) and P180-190 (n=15-16 animals). Graph displays mean and SEM for microglia cell counts (Mann-Whitney test).

Then, using flow cytometry analysis, we studied the expression of microglial surface receptors thought to be important for the modulation of their immune functions. We found that the loss of Rac1 changed the frequency of microglia expressing several of these cell surface receptors (**Figure 11**). Specifically, there was significantly lower frequencies of $Ly6C^+$, $CD115^+$, $CD44^+$, $CCR2^+$ and $CD62L^+$ microglia in Rac1 cKO mice compared with CT littermates (**Figure 11**, left graphs for each marker). We also detected that the expression levels (using the median fluorescence intensity – MFI) of Ly6C, CD115 and

CD44, but not of CCR2 and CD62L, were also significantly lower in microglia from Rac1 cKO brains compared to those from CT brains (**Figure 11**, right graphs for each marker).

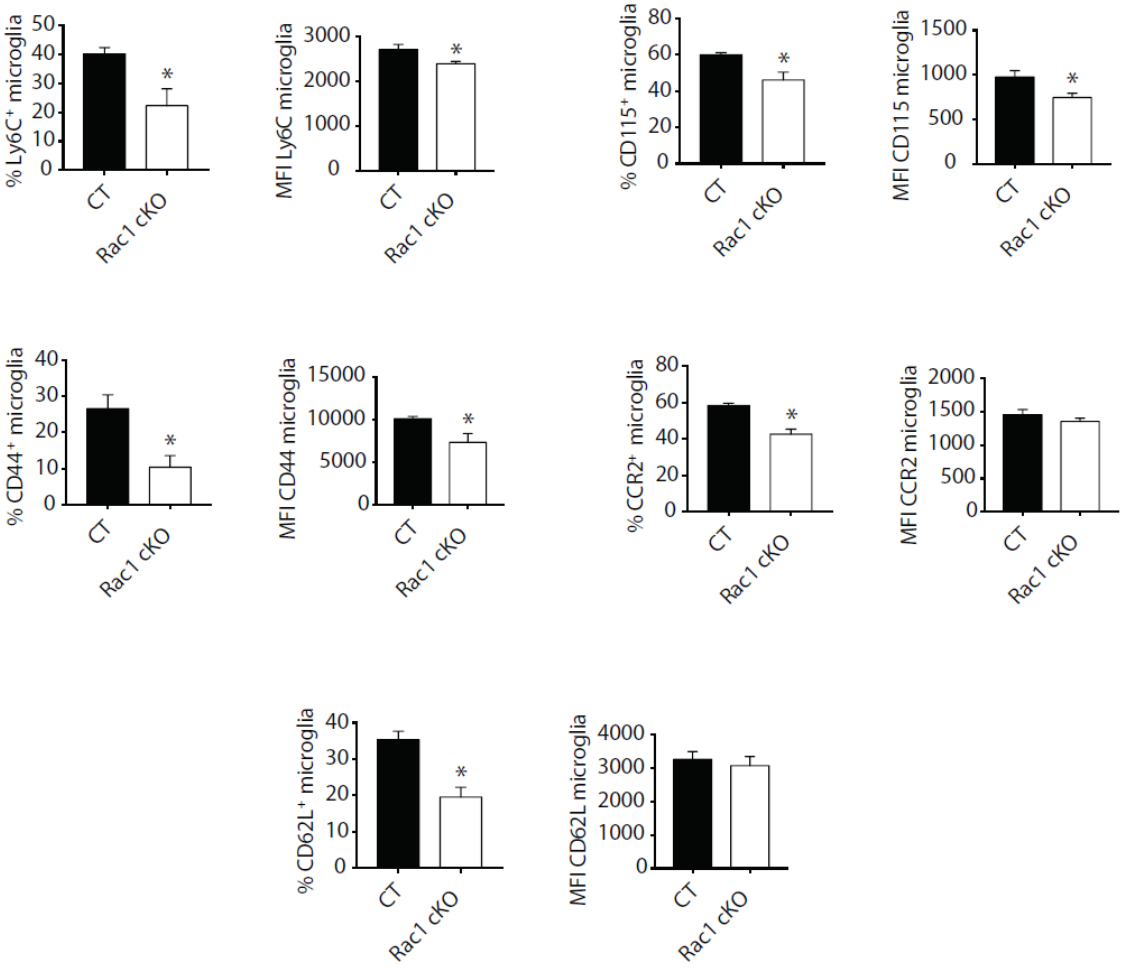

Figure 11 – Expression of activity markers in microglia. Ly6C, CD115, CD44, CCR2 and CD62L expression in microglia represented as frequency of microglia positive for each marker (**left graphs for each marker**). Median fluorescence intensity (MFI) of Ly6C, CD115, CD44, CCR2 and CD62L gated on the microglia population (**right graphs for each marker**) (n=4-6 animals). Graphs show mean and SEM (Mann-Whitney test, *p<0.05).

To further analyze the possible effects of Rac1 ablation in microglia physiology, we evaluated by qRT-PCR the mRNA expression of several genes important for microglia homeostasis and function. These genes included: Csfr1 that is important for microglia proliferation (Olmos-Alonso et al., 2016); P2ry12 that is necessary for microglia to migrate and extend their processes in the direction of a lesion site (Haynes et al., 2006); Pu.1 that maintains the viability of microglia (Smith et al., 2013); C1qA, C1qB and C1qC that are part of the classical complement pathway and control the synaptic function of microglia (Schafer et al., 2012; Stevens et al., 2007); Gpr34 that is relevant for microglial

phagocytosis (Preissler et al., 2015); Pros1 that is the gene that encodes protein S, involved in phagocytosis, as well as Mertk (Fourgeaud et al., 2016); Trem2 that is responsible for lipid sensing and microglia metabolic fitness during neurodegeneration (Y. Wang et al., 2015); TLR2, TLR4 and TLR7 that are important for the recognition of pathogens. We found that almost all those genes presented a significant reduction of their mRNA expression levels in the brains of both young and mature adult Rac1 cKO mice compared with those in the brains of age-matched CT littermates (**Figure 12**). Specifically, the heat map below shows the values obtained for each individual mouse used of each genotype (**Figure 12A**) while the bar graph shows the mean mRNA expression levels normalized to CT for each gene on the indicated time periods (**Figure 12B**). Overall, these results suggest that Rac1 deficiency alters microglia homeostasis and that this might impact on microglial fitness and/or immune function.

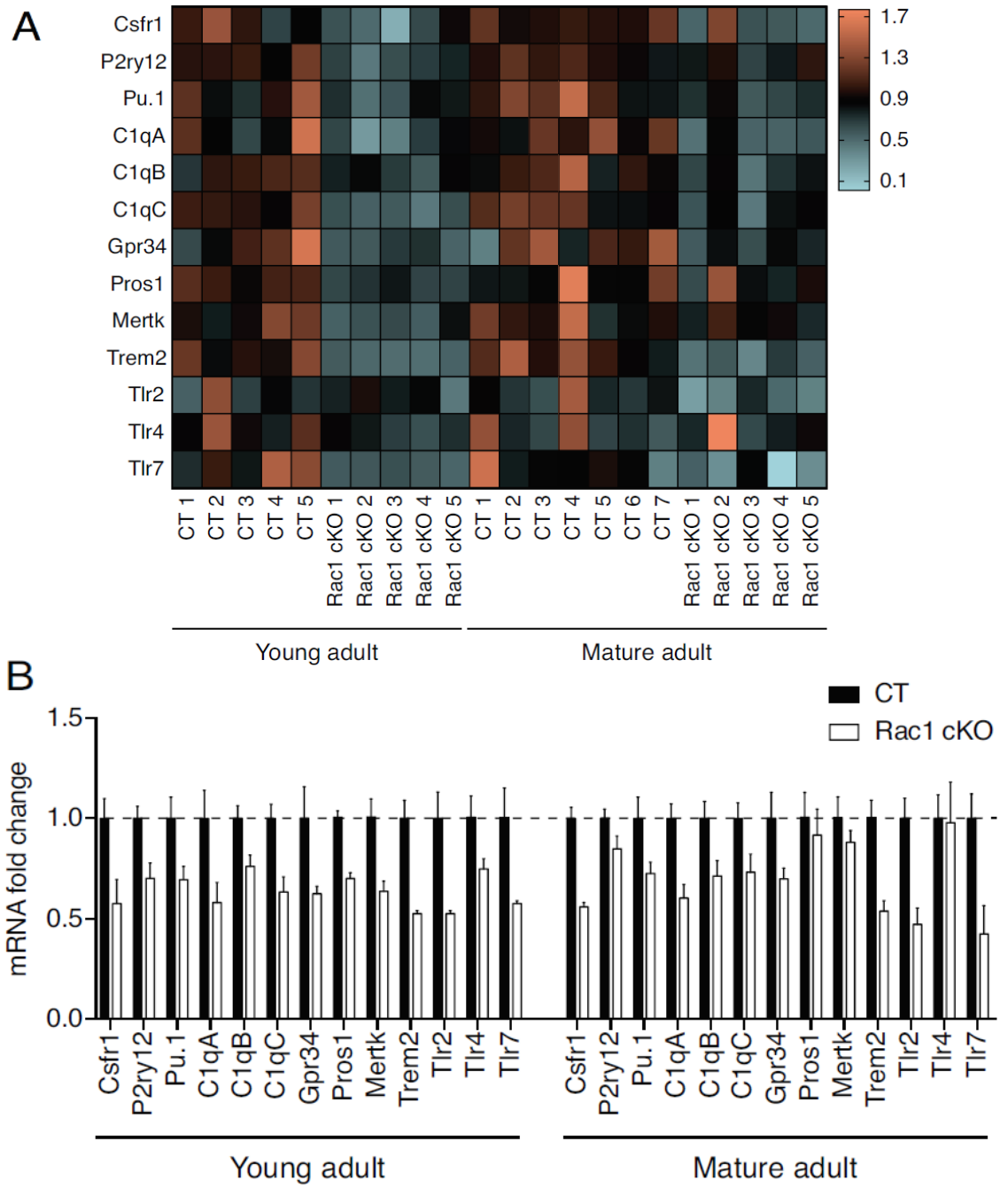

Figure 12 – qRT-PCR analysis of microglial homeostatic genes. mRNA levels of the indicated transcripts from the hippocampus of CT and Rac1 cKO mice at P65-75 (young adult) and P180-190 (mature adult) (n=5-7 animals for each time point), represented in a heat map (**A**) and in a graph (**B**). Graph represents mean and SEM (Mann-Whitney test, *p<0.05).

3. Microglial activation by external ligands

Since there was a general downregulation of several important molecules and genes pivotal for microglia homeostatic functions, we decided to study how microglia would respond to classical stimuli that normally result in their pro-inflammatory polarisation.

3.1. LPS stimulation

The first stimulus used was LPS. This component of the Gram (-) bacteria cell wall is a classical activator of microglia *in vivo*. It is mainly recognized by TLR4 and ultimately leads to NF-κB activation and ROS production (Sanlioglu et al., 2001). Using biosensors for the NF-κB pathway (Shcherbakova et al., 2016) and ROS production (Waypa et al., 2006), we were able to observe the response to LPS of HMC3 microglia expressing normal amounts of Rac1 or microglia knocked down for Rac1. Specifically, microglial cells were co-transfected either with an empty vector (plus biosensor) (CT) or with a vector coding Rac1 shRNA (plus biosensor) (Rac1 KD). We found, as expected, that CT cells increased the production of ROS when challenged with LPS (**Figure 13A, black circles**). However, the production of ROS induced by LPS in living microglia depleted of Rac1 (Rac1 KD cells) was significantly reduced compared with CT microglia (**Figure 13A, white circles**).

NF-κB pathway biosensor signal corresponds to an inhibitor of NF-κB (Shcherbakova et al., 2016). It is expected that when the pathway is activated, the inhibitor is destroyed (reduction in the signal of the biosensor), representing an increased pathway activation (Shcherbakova et al., 2016). In concordance with what was observed with ROS levels, exposure of CT living microglia to LPS led to a notorious activation of the NF-κB pathway (**Figure 13B, black circles**) and this effect was significantly attenuated in microglia knocked down for Rac1 (**Figure 13B, white circles**).

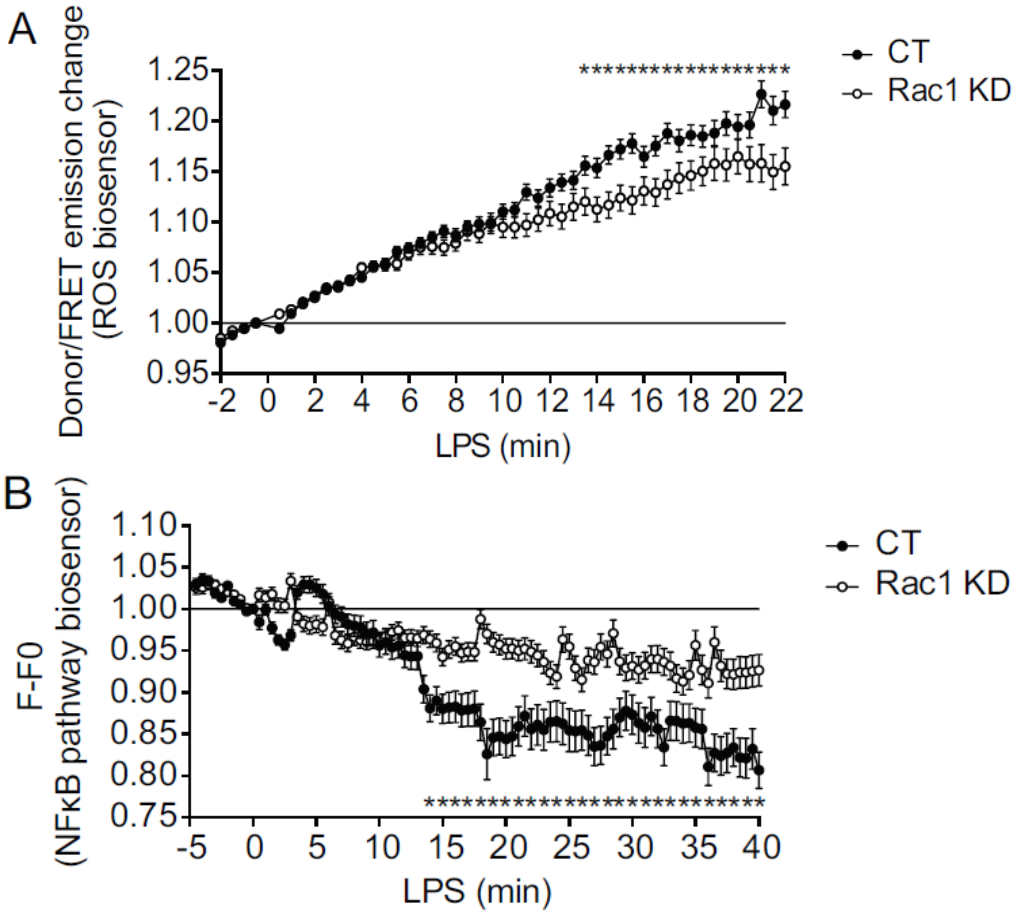

Figure 13 – Effect of LPS in ROS production and NF-κB activation in HMC3 cells. HMC3 cells were transfected with a ROS FRET biosensor (HPS33) (n=61-64 cells from 3 independent experiments) (A) or a NF-κB pathway FRET biosensor (pIκBα-miRFP703) (n=38-43 cells from 3 independent experiments) (B) and pLKO (CT) or Rac1 shRNA (Rac1 KD). Graphs show mean and SEM of time-lapse donor/FRET ratio images recorded with saline solution before (t=0 min) and after treatment with LPS (500 ng/mL) (Two-way ANOVA with Sidak's multiple comparisons test, *p<0.05).

3.2. Phosphatidylcholine stimulation

Microglia are crucial to remove cell debris, like dead neurons, during neurodegeneration or injury (Sokolowski & Mandell, 2011). To do so, microglia recognizes phospholipids present on the plasma membrane of neurons. Phosphatidylcholine is one of the most abundant phospholipids present in cell membranes and it is also one of the most lipid components recognized by microglia. Therefore, we used phosphatidylcholine to activate the lipid sensing signaling cascade in microglia and evaluated the impact of knocking down Rac1 in this pathway. Three biosensors were used to address different steps of the lipid sensing cascade in microglia (X. Gao et al., 2011;

Palmer, Jin, Reed, & Tsien, 2004; Sato, Ueda, & Umezawa, 2006): (1) a biosensor indirectly measuring the activity of phospholipase C at the plasma membrane, (2) a biosensor measuring the mobilization of calcium from the endoplasmic reticulum and (3) a biosensor measuring the activity of AKT, a downstream effector of the lipid sensing cascade. Increase in free DAG in the plasma membrane, as a result of phospholipase C activation, represents one of the first steps in the activation of the lipid sensing pathway (Berridge, 1993). We first used a DAG biosensor and observed an increase of the free DAG content at the plasma membrane of living microglia exposed to phosphatidylcholine (**Figure 14A, black circles**). However, we observed that DAG levels did not increase in Rac1 KD cells upon exposure to phosphatidylcholine (**Figure 14A, white circles**). Activating the phospholipase C pathway leads to the mobilization of Ca^{2+} from the endoplasmic reticulum into the cytosol because of the production of DAG and IP3 We used a biosensor detecting the specific efflux of Ca^{2+} from the endoplasmic reticulum into the cytosol and observed an increase of cytosolic Ca^{2+} mobilization in living microglia exposed to phosphatidylcholine (**Figure 14B, black circles**). In Rac1 KD microglia, the efflux of Ca^{2+} triggered by phosphatidylcholine was abrogated (**Figure 14B, white circles**). It is also described that this lipid sensing pathway leads to the activation of several downstream protein kinases and AKT is one of them (Kortholt, King, Keizer-Gunnink, Harwood, & Van Haastert, 2007). Using a biosensor measuring the activity of AKT (X. Gao et al., 2011), we observed that exposing CT microglia to phosphatidylcholine indeed led to AKT activation (**Figure 14C, black circles**) and that this effect was completely blocked in Rac1 KD microglia (**Figure 14C, white circles**).

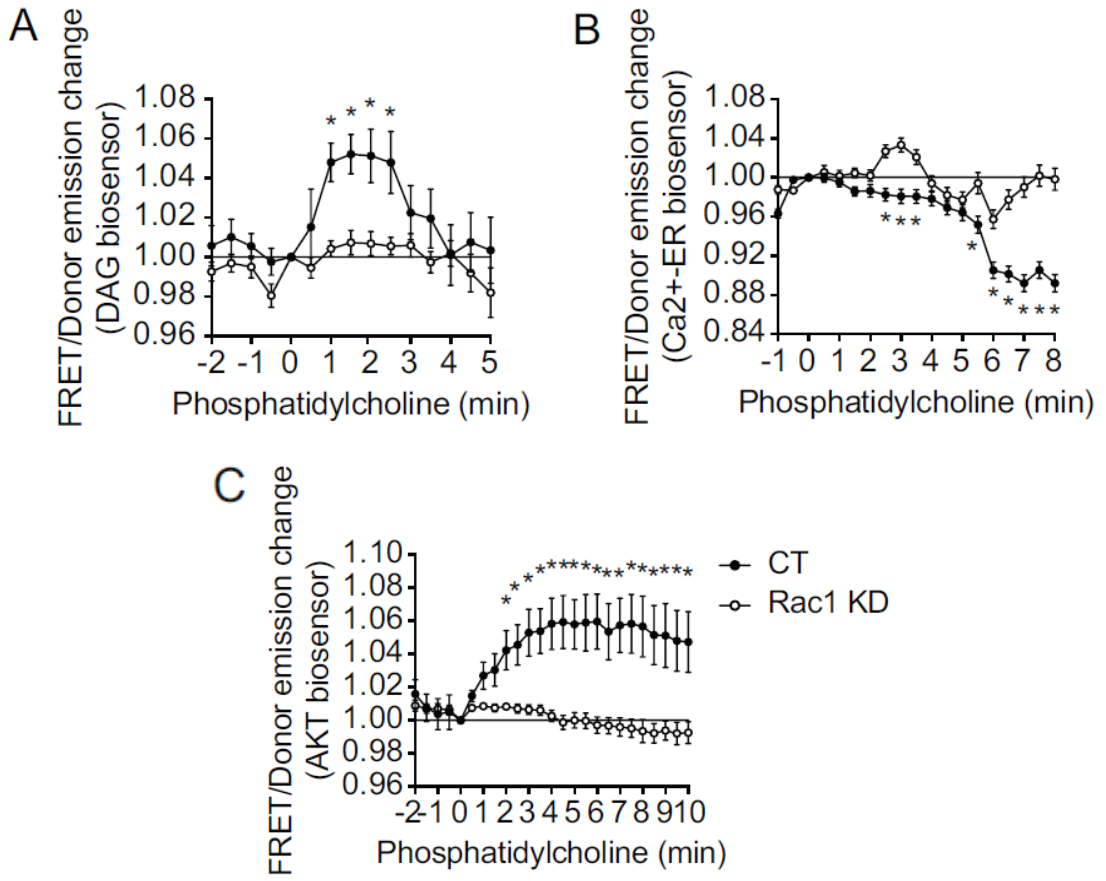

Figure 14 – Effect of phosphatidylcholine in the lipid sensing pathway in HMC3 cells. HMC3 cells were transfected with a DAG FRET biosensor (DAG LAS PM) (n=20-34 cells from 3 independent experiments) (A), a Ca^{2+}-ER FRET biosensor (D1ER) (n=91-109 cells from 3 independent experiments) (B) or AKT FRET biosensor (Lyn AKT) (n=16-24 cells from 3 independent experiments) (C) and Scrambled mCherry (CT) or shRac1 mCherry (Rac1 KD). Graphs show mean and SEM of time-lapse donor/FRET ratio images recorded with saline solution before (t=0 min) and after treatment with phosphatidylcholine (10 ng/mL) (Two-way ANOVA with Sidak's multiple comparisons test, *p<0.05).

Overall these results demonstrate that loss of Rac1 in microglia renders these cells completely irresponsive to classical activating stimuli such as LPS (a PAMP) and phosphatidylcholine (a DAMP), which reflects that Rac1 deficiency might cause problems on microglial fitness.

3.3. Rac1 deficiency affects microglia activation during neuroinflammation

During neuroinflammation caused by tissue damage or neurodegeneration microglia become activated and proliferate, a phenomenon known as microgliosis. Microgliosis, triggered by different set of stimuli, is a stereotypical physiological response to danger signals believed to contribute for reestablishing tissue homeostasis after insults to the nervous tissue (Aguzzi, Barres, & Bennett, 2013). Because microglia without Rac1 were non-responsive to LPS *in vitro,* we decided to study the how microglia deficient for Rac1 would respond to neuroinflammation. We induced neuroinflammation by injecting LPS systemically. Saline solution or LPS was intraperitoneally injected into P150 Rac1 cKO mice and CT littermates and their brains were analyzed 24 h later (**Figure 15A**). Flow cytometry analysis demonstrated that, as expected, in CT mice there was an increase in the number of microglia when LPS was injected (**Figure 15B**). It was also observed that the number of $Csfr1^{high}$ microglia or microglia positive for the activity markers Ly6C, CD44, CCR2 and CD62L was also increased in CT mice injected with LPS compared with CT mice injected with saline (**Figure 15B**). Thus, neuroinflammation induced by systemic LPS administration led to microgliosis in the brain of CT mice. However, when we evaluated the number of microglia in Rac1 cKO brains after LPS administration, we did not observe the same effect. Interestingly and paralleling the in vitro phenotype of microglia knocked down for Rac1, the loss of Rac1 in microglia in vivo rendered these cells irresponsive to neuroinflammation. Indeed, the total number of microglia was significantly lower in Rac1 cKO mice injected with LPS than in CT mice injected with LPS. Accordingly, the levels of the microglia homeostatic marker Csfr1 and the microglia reactivity markers Ly6C, CD44, CCR2 and CD62L were also significantly lower in Rac1 cKO injected with LPS compared with those in LPS injected controls (**Figure 15B**). The total number of microglia and the number of reactive microglia ($Csfr1^{high}$, $Ly6C^{+}$, $CD44^{+}$, $CCR2^{+}$ and $CD62L^{+}$) was comparable between CT mice injected with saline and Rac1 cKO littermates injected with LPS (**Figure 15B**). These data further support the hypothesis that loss of Rac1 causes deficits on microglial fitness and activation.

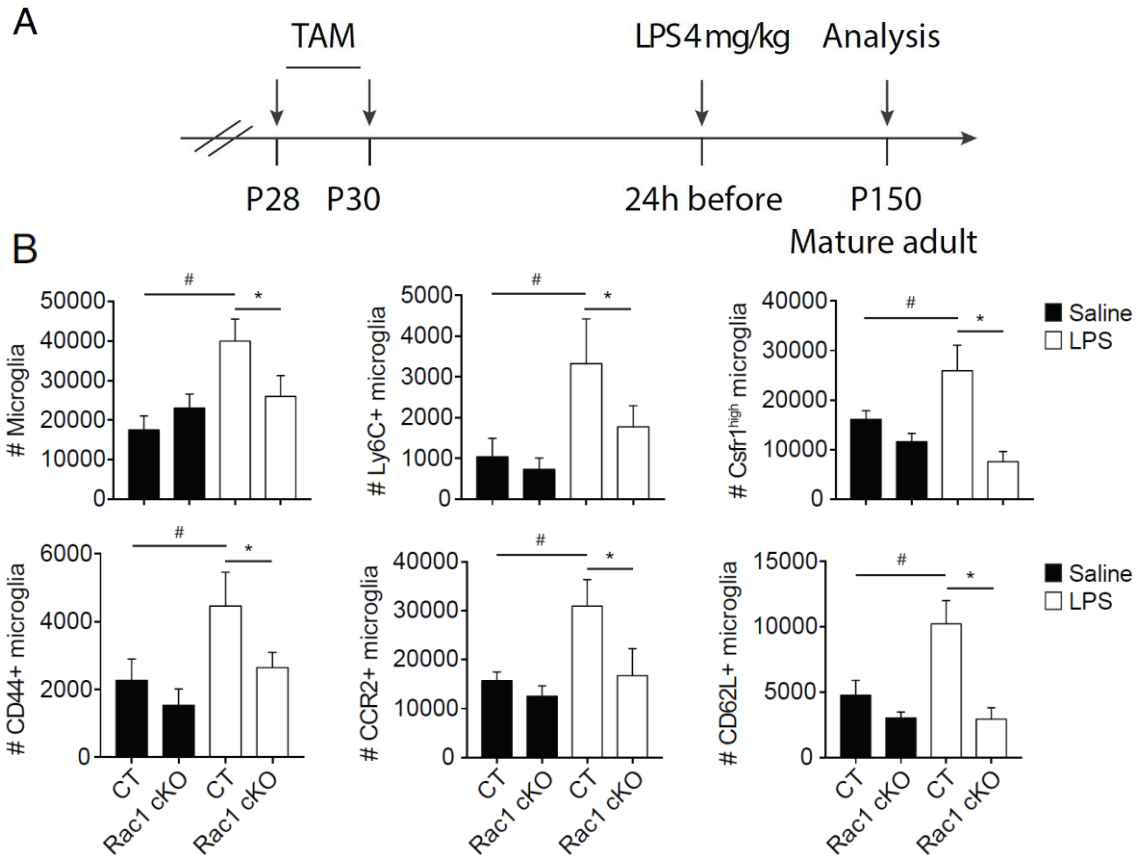

Figure 15 – Effect of LPS in microglia activation *in vivo*. Scheme of LPS administration (1 intraperitoneal injection 24 hours prior to perfusion) to CT and Rac1 cKO mice at P150 (**A**). Number of total microglia and microglia positive for the represented markers from CT and Rac1 cKO mice injected with saline or LPS (4 mg/kg per animal) was analyzed by FACS (n=4-6 animals) (**B**). Graphs display mean and SEM (One-way ANOVA with Bonferroni multiple comparation test, *p<0.05, #p<0.05).

Neuroinflammation triggered by LPS administration opens the Blood-Brain-Barrier (BBB) allowing the infiltration of blood-circulating cells into the brain parenchyma (Banks et al., 2015). Therefore, we decided to study whether non-resident myeloid cells infiltrating the brain would be affected by the loss of Rac1 in microglia during neuroinflammation. Infiltrating monocytes ($CCR2^+$ MΦ), pro-inflammatory monocytes ($CCR2^+Ly6C^+$ MΦ), patrolling monocytes ($Ly6C^-$ MΦ), phagocytic peripheral monocytes ($CD44^+$ MΦ) and blood-born monocytes ($CD62L^+$ MΦ) were analyzed. We observed that CT mice injected with LPS presented significantly higher number of cells for all those cell populations compared with CT littermates injected with saline (**Figure 16**), thereby indicating that neuroinflammation led to the infiltration of non-resident myeloid cells into the brain (**Figure 16**). Importantly, ablation of Rac1 in microglia directly impacted the number of those myeloid non-resident cells within the brain during neuroinflammation. Indeed, we observed that Rac1 cKO mice injected with LPS had a

significantly lower number of $CCR2^+$ MΦ, $CCR2^+Ly6C^+$ MΦ, $Ly6C^-$ MΦ, $CD44^+$ MΦ and $CD62L^+$ MΦ compared with CT littermates injected with LPS (**Figure 16**). These results suggest that Rac1 signaling in microglia is essential for these cells to govern the dynamics of different myeloid populations infiltrating the brain during neuroinflammation.

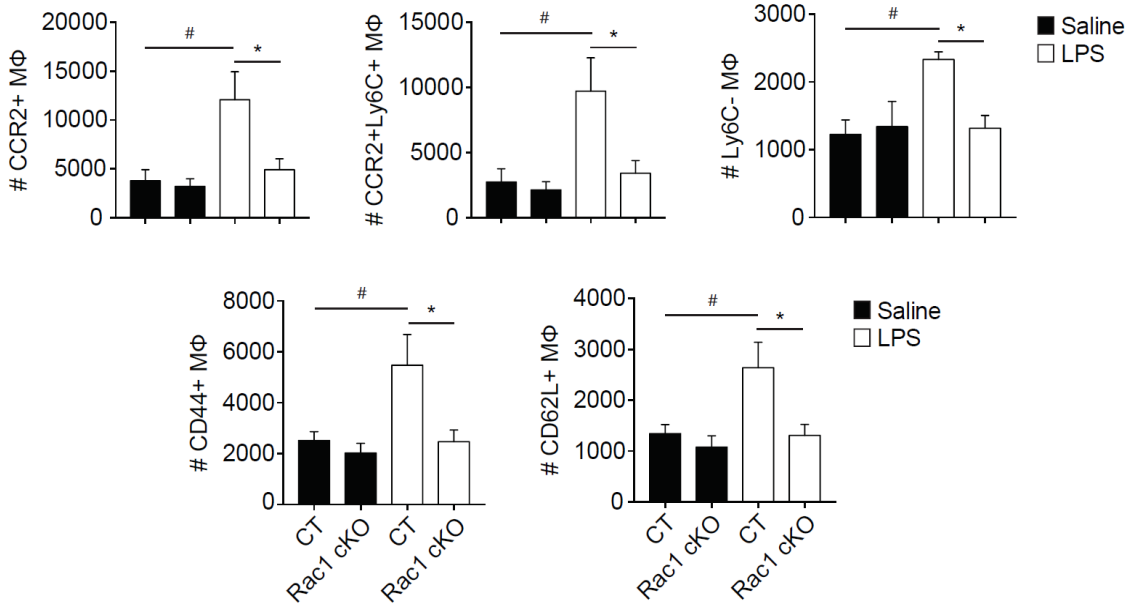

Figure 16 – Effect of LPS in non-resident myeloid populations *in vivo*. Number of total cells of each non-resident myeloid cell population from CT and Rac1 cKO mice injected with saline or LPS (4 mg/kg per animal) was analyzed by FACS (n=4-6 animals). Graphs display mean and SEM (One-way ANOVA with Bonferroni multiple comparation test, *p<0.05, #p<0.05).

4. Impact of microglial Rac1 in the neuronal compartment

Given the importance of microglia to the CNS parenchyma and the effects observed in non-resident myeloid populations, we found relevant to evaluate if the absence of Rac1 in microglia affected brain neurons. We first evaluated synapses using double-labeling immunohistochemistry with the synaptic markers VGLUT1 and PSD95. VGLUT1 is a protein responsible for the uptake and storage of glutamate into synaptic vesicles and is considered a pre-synaptic marker of glutamatergic synapses. PSD95 is a protein responsible for the assembly of the post-synaptic density in several neuronal cell populations, including excitatory glutamatergic neurons. Using VGLUT1 and PSD95 colocalization, we observed a significant reduction in the percentage of

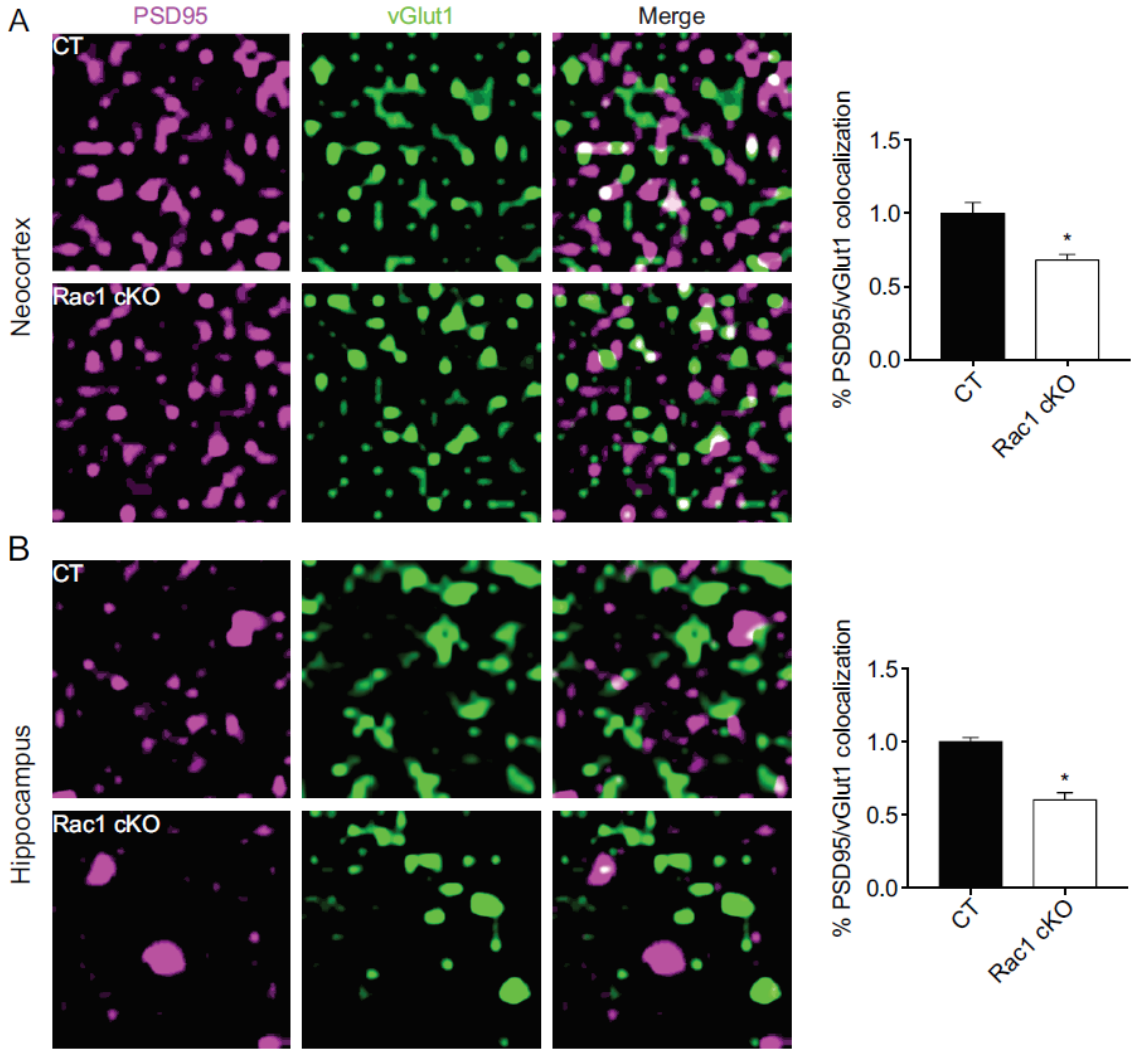

Figure 17 – Synapse number evaluation by double labelling immunohistochemistry. Confocal analysis of CT (n=4 animals) and Rac1 cKO mice (n=4 animals) at P65-75. VGLUT-1 (green) and PSD95 (purple) immunolabeling on 30 µm tissue sections from the neocortex (**A**) and the hippocampus are shown (**B**). Graphs show mean and SEM of PSD95/VGLUT-1 colocalization puncta percentage, normalized to the CT values (Mann-Whitney test, *p<0.05).

$VGLUT1^{+}PSD95^{+}$ double-positive puncta, clearly indicating a decrease in the number of

excitatory synapses, both in the neocortex (**Figure 17A**) and in the hippocampus (**Figure 17B**) of Rac1 cKO mice compared with age-matched CT littermates.

The number of neurons is also an important aspect to take into account, not only as a parameter to verify changes induced by microglia but also to substantiate if the observed

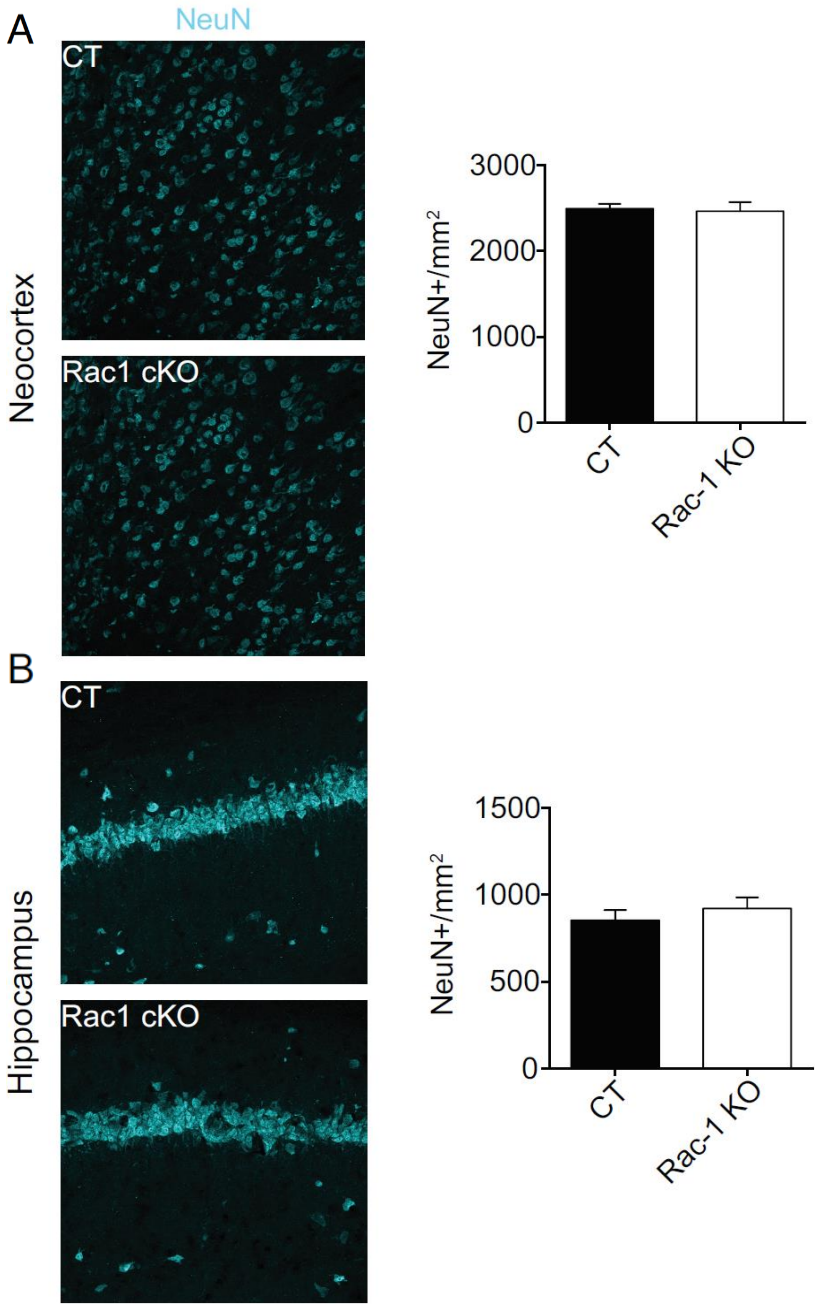

Figure 18 – Neuron number evaluation by immunohistochemistry. Confocal analysis of CT (n=3 animals) and Rac1 cKO mice (n=3 animals) at P65-75. NeuN (blue) immunolabeling on 30 µm tissue sections from the neocortex (**A**) and the hippocampus are shown (**B**). Graphs show mean and SEM of the number of $NeuN^+$ cells per mm^2 (Mann-Whitney test).

decrease of synapses in Rac1 cKO mice was directly correlated with neuronal cell loss. Thus, neuronal number was assessed by immunohistochemistry with NeuN, a neuron-

specific protein that binds to DNA. We found no significant differences in the number of $NeuN^+$ cells in the neocortex (**Figure 18A**) or in the hippocampus (**Figure 18B**) of Rac1 cKO mice and CT littermates.

Loss of cortical and hippocampal excitatory synapses can lead to changes in the neuronal circuitry/activity associated with deficits in social behaviour (R. Gao & Penzes, 2015). Because Rac1 cKO mice contained less excitatory synapses in the neocortex and hippocampus we next decided to subject these animals to the Crawley's sociability test, a behavioral paradigm that assesses social interaction in mice (Moy et al., 2004). This test allows the identification of sociability deficits by analyzing and comparing the time spent by the experimental mouse (CT or Rac1 cKO, in this case) contacting an inanimate object (empty chamber - object) or a living mouse (chamber with a naïve mouse - intruder) (**Figure 19A**). Because mice are social animals, it is expected that they will spend more time contacting the chamber with the live mouse than the empty chamber. Indeed, this stereotyped behaviour was observed in our CT animals, as they significantly spent more time contacting the intruder than the empty object (**Figure 19B**). However, the amount of time that Rac1 cKO mice spent contacting the intruder was not significantly different than the time they spent contacting the empty object (**Figure 19B**). The overall time that CT and Rac1 cKO mice spent exploring each compartment of the apparatus was also analyzed and we detected no significant differences between the genotypes (**Figure 19C**), thereby indicating that the sociability deficits observed on Rac1 cKO animals was not due to a lack of exploratory behaviour or locomotor impairment. Taken togheter, these results indicate that deficiency of Rac1 in adult microglia leads to loss of excitatory synapses and deficits in social behaviour.

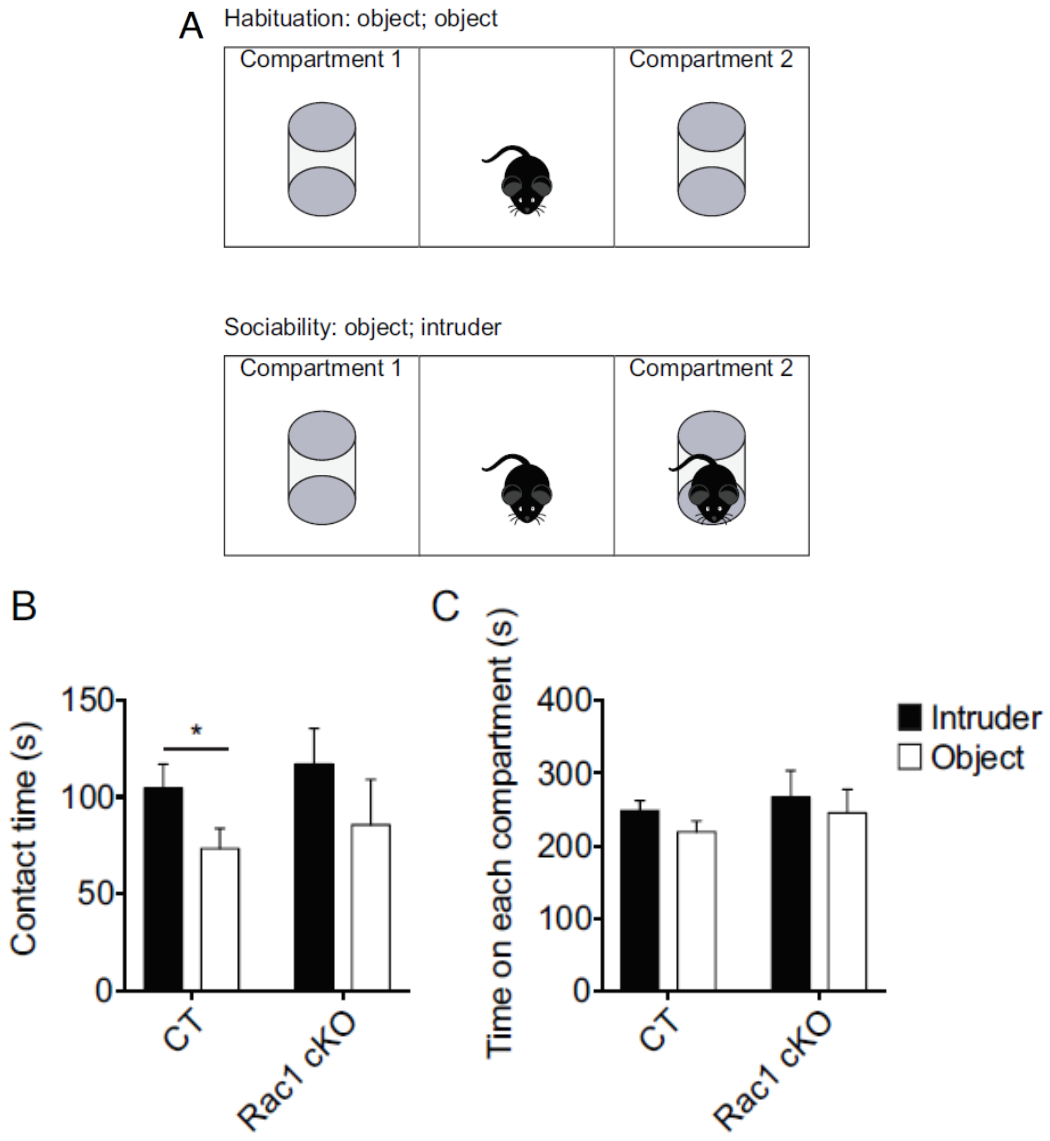

Figure 19 – Evaluation of social interaction with Crowley's sociability and preference for social novelty test. Representative scheme of the apparatus used in the behavioral test (**A**). CT and Rac1 cKO mice at P180-190 were evaluated in this social interaction experiment (n=7 animals). Graphs display mean and SEM for the contact time between the animal and the object or the intruder (**B**) and the time spent by the animals in each compartment of the apparatus (**C**) (Paired t test, *p<0.05)

Discussion

Discussion

By inducing a microglia-specific ablation of Rac1 in adult mice, the present work aimed to study the *bona fide* roles of Rac1 in microglia homeostasis both in steady state conditions and during neuroinflammation. Depletion of Rac1 in microglia did not change total microglial number but resulted in decrease of microglia expressing several activation/homeostatic markers. Loss of Rac1 also led to lower mRNA levels of several homeostatic/signature genes in microglia. When microglia were challenged *in vitro* with LPS, there was a reduced production of ROS and a reduced activation of the NF-κB pathway in Rac1 KD microglia compared with CT ones. With phosphatidylcholine, a reduction in several steps of the lipid sensing signalling cascade was also observed. Free DAG levels and Ca^{2+} efflux from the endoplasmic reticulum were reduced in Rac1 KD microglia, just as AKT levels. *In vivo*, neuroinflammation, mediated via systemic LPS challenge, induced microgliosis (population expansion and activation) in CT mice but not in mice lacking Rac1 specifically in microglia (Rac1 cKO). In Rac1 cKO mice, there was also a lack of effect in non-resident myeloid populations during neuroinflammation induced by systemic LPS administration. Reduction in the number of synapses and maintenance of neuron number in the neocortex and the hippocampus was also observed as a consequence of Rac1 ablation in microglia. In Crowley's sociability test, Rac1 cKO mice lacked social interaction behavior compared with CT mice. These results suggest that Rac1 in microglia is necessary for their homeostasis in steady state and also assumes an important role during activation of these cells.

In the absence of Rac1 in microglia, there was a reduction in the number of microglia positive for certain activity markers important for microglia homeostatic functions. There were less cells positive for Ly6C, CD115, CD44, CCR2 and CD62L. Within these cells, they were expressing less Ly6C, CD115 and CD44. For example, CCR2 is related with chemotaxis and it was already showed that its deficit in microglia was related with Alzheimer's diesease progression (El Khoury et al., 2007). CD115 (CSF1R) is related with the survival of the microglial population (Elmore et al., 2014). mRNA of several genes relevant for microglia function, such as TLR4, TREM2, C1qA or Pu.1 were also downregulated in Rac1 cKO mice. Some of these markers comprise surface receptors for LPS or are constituints of the complement cascade. Thefore, these results suggest that

ablation of Rac1 in microglia leads to an impairment in microglial fitness and or immune function.

Microglia challenging was performed to verify if activation would occur normally in the absence of Rac1. Two classical pathways were analyzed: one induced by LPS and the other by phosphatidylcholine. LPS is mainly recognized by TLR4, which triggers the pathway, leading to NF-κB translocation to the nucleus and ROS production. Due to the roles already described for Rac1 in the NADPH oxidase complex in macrophages and monocytes (Abo et al., 1991; Zhao et al., 2003) and as an important regulator of NF-κB (Cuadrado et al., 2014), it was expected to observe an effect of Rac1 KD in microglia. Therefore, the results obtained in the present work are concordant with the observations already described for other myeloid cell populations outside the CNS. Phosphatidylcholine is one of the best recognized phospholipids present in situations of neuroinflammation (Y. Wang et al., 2015). When phospholipase C is activated, IP_3 goes to activate Ca^{2+} sensitive channels and DAG becomes free in the surface of the cell. A Ca^{2+} efflux from the endoplasmic reticulum is induced, leading to protein kinase C activation and activation of other downstream effectors such as AKT.

It is already described that LPS challenge *in vivo* leads to an expansion and activation of microglial cells (microgliosis). In our experiments, we observed this process when we compared CT mice injected with a saline solution or with LPS. In Rac1 cKO mice injected with LPS, there were no changes in the number of total microglia and in the number of microglia positive for the activity markers compared to the ones injected with a saline solution. TLR4 and TREM2 are two of the receptors responsible for the recognition of LPS. These were two of the genes identified in the present work with lower mRNA levels in Rac1 cKO mice compared with CT mice. This might explain the absence of response from microglia to LPS. Interestingly, when we analyzed non-resident myeloid populations present in the brains of these mice, whe did not observe an in increase in the number of infiltrating monocytes ($CCR2^+$ MΦ), pro-inflammatory monocytes ($CCR2^+Ly6C^+$ MΦ), patrolling macrophages ($Ly6C^-$ MΦ), phagocytic peripheral monocytes ($CD44^+$ MΦ) and blood-borne monocytes ($CD62L^+$ MΦ). One of the effects of LPS administration is the disruption of the BBB (Banks et al., 2015). In situations of neuroinflammation, there is a chronic activation of microglial cells. When this occurs, in this case promoted by LPS, microglia produce chemokines and cytokines that attract myeloid cells from the periphery (Häusler et al., 2002). Thus, these non-resident myeloid

populations can cross the barrier and enter the CNS. In our experimental model, this effect was not observed at the same extent as in CT mice, as microglia did not respond to LPS administration. It is also possible that the dynamics of non-resident myeloid populations are altered but further studies are required to assess this possibility.

Microglia are pivotal for the maintenance of CNS homeostasis. Therefore, addressing the effects of microglia-specific ablation of Rac1 in the CNS parenchyma was another of the goals of this work. When we analyzed the excitatory synapses by immunohistochemistry, we detected a reduction in the percentage of colocalization of VGLUT1 and PSD95 in both the neorcortex and hippocampus of Rac1 cKO mice compared with CT mice, indicative of a reduction in the number of excitatory synapses in those regions. A decrease in the number of neurons could be an explanation for this result. Strikingly, when we studied the number of neurons per mm^2, we did not observe significant differences between CT mice and Rac1 cKO mice.

Microglia are known for their phagocytic capacity and for being responsible for synaptic pruning. Rac1 role in phagocytosis is well documented in the literature (Mao & Finnemann, 2015). As Rac1 is required for the phagocytic cup formation (Cox et al., 1997), we would predict that in Rac1 cKO mice, the phagocytic capacity of microglia would be impaired. In fact, preliminary experiments (not shown) performed in our lab regarding phagocytosis *in vitro*, demonstrated a tendency to a decrease in engulment of beads by Rac1 KD HMC3 cells. Thus, it is not expected that microglia are conducting excitatory synapse loss in the neocortex and the hippocampus by means of synaptic "eating". It is possible that, instead of actively reducing the amount of synapses, microglia are providing trophic support crucial for synapse formation and/or maintenance. Rac1 in microglia might be, somehow, necessary for this process, as its absence is triggering this phenotype. Microglia release neurotrophic factors such as BDNF (Dougherty, Dreyfus, & Black, 2000). BDNF produced by microglia is described as being important in the regulation of synapse formation induced by learning (Parkhurst et al., 2013).

Modifications in synapses are highly associated with cognitive and sociability problems. Mice are often described as being social animals. Therefore, their normal behavior when they contact with a new mouse is to spend more time contacting with the other animal than with an inanimate object. In Rac1 cKO mice, this normal behavior was not observed. The time Rac1 cKO mice spent contacting with the other mice and empty

cup was not statistically different and the animals spent aproximmately the same time exploring both compartments. The decrease found on neocortical and hippocampal excitatoty synapses could be sufficient to explain this deficit in social behaviour, but further studies should be performed to explore this result.

Overall, our results suggest that Rac1 in microglia is necessary for the homeostasis of these cells and Rac1 depletion impaired microglial response to classical pro-inflmmatory stimuli both *in vitro* and *in vivo*. Moreover, Rac1 signaling in microglia is also critical impacting the neuronal parenchyma, in this case, deficiency of Rac1 signaling in microglia leads to loss of excitatory synapses and consenquently to defects in social behaviour on these mice.

Conclusions and Future Perspectives

Conclusions and Future Perspectives

With this work, we were aiming to understand more about Rac1 and its importance for microglial functions. Ablation of Rac1 specifically in microglia was the strategy used to pursue this goal. By studying the effects caused by its absence, we were able to infer about its importance and the dependence of microglial cells on Rac1 to perform their normal roles, not only in steady state but also when it is challenged. The main conclusions that can be retrieved from the results already obtained are:

1. Rac1 conditional knock-out in microglia does not impact on microglia number but alters the expression of some activity markers and homeostatic genes important for their functions in steady state.
2. Rac1 deficiency leads to a disruption of microglia's physiological response to LPS and phosphatidylcholine *in vitro*.
3. Lack of Rac1 in microglia *in vivo* alters the dynamics of non-resident myeloid populations during neuroinflammation.
4. Depletion of Rac1 in microglia did not alter the number of neurons per mm^2 in the neocortex and the hippocampus but lead to a reduction of excitatory synapses. Mice without microglia deficient in Rac1 presented sociability deficits, a possible consequence of less synapses.

However, it was not possible to determine exactly in which point of the signaling cascades is Rac1 missing or the mechanisms underlying the alterations caused to microglia. Further studies will be performed to complement the present work. Some of the possibilities include:

1. Approaching other microglia functions such as phagocytosis. In this case, continue with the preliminary experiments already performed to further understand if phagocytosis is effectively impaired.
2. Explore how microglia are impacting non-resident myeloid populations by observing modifications in cytokine and chemokine released by microglia lacking Rac1. Also study the impact on neurons to reduce synapse number, by looking to the mRNA levels of BDNF and glial cell line-derived neurotrophic factor (GDNF) in sorted microglia.

3. Clarify if synapse reduction is causing other kinds of problems besides sociability deficits by performing other behavioral tests indicative of cognitive problems.
4. Analyze the immune checkpoints of microglia to try to identify, for example, an intracellular transcription factor that might be involved in the signaling cascade. Also evaluate interactions with other cells or soluble factors possibly released by other CNS cells that might be restraining the response of microglia (Deczkowska, Amit, & Schwartz, 2018).
5. Recover the phenotype by searching and testing agonists for some of the downregulated genes, to counteract the effects observed in our experimental model and to try to make it resemble the CT ones.

Microglia are being more and more studied as time goes by. The perspective that glial cells are more than just mere spectators in the nervous system that are there only to support neurons is changing. The importance given to these cells led to an increase of studies in which they were the focus. Nowadays, glial cells are extensively studied. They are starting to be considered as crucial as neurons, even in what disease beginning concerns. Until some years ago, the idea that glial cells could be triggering neuroinflammation or CNS disorders was not even considered. Works as the one presented are small steps towards the demonstration that problems in microglia can also trigger numerous effects, not only in their own population but also in other populations that depend on their normal functions.

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