

Unveiling the roles of small Rho GTPase Rac1 in microglia homeostasis and activation during neuroinflammation

Tiago de Oliveira Almeida

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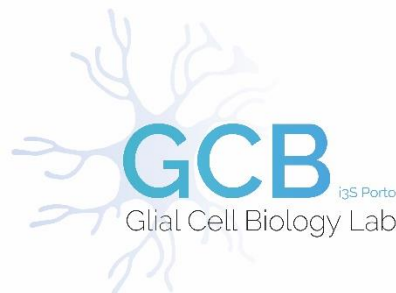
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“I seem to have been only like a boy playing on the seashore and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me.”

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Abstract

Microglia are the main myeloid population in the brain representing 5-10% of total brain cells. They are a long-lived autonomous cell population, distinct from other tissue-resident myeloid cells and circulating monocytes.

Under physiological conditions, they are highly branched and dynamic cells that continually extend and retract their processes to survey the CNS parenchyma, monitoring the functionality of synapses, and searching for tissue damage and infections. Reorganization of the actin cytoskeleton is thought to be critical for the physiological roles of these plastic cells.

Thus, proteins that modulate the actin cytoskeleton dynamics and intracellular processes, such as the proteins of the Rho family of GTPases, emerge as attractive regulators of microglia function. Rac1, a member of this family, was extensively studied in the CNS and other myeloid cells. Its involvement in axonal guidance, NADPH oxidase complexes, regulation of NF- κ B pathway activation, and phagocytosis is well documented. Given the existing gap in the regulatory effect of Rac1 in microglia, the present thesis aims at studying and identifying novel roles for Rac1 in microglia biology, both in homeostatic and neuroinflammatory conditions.

In this study, we unveil essential non-redundant roles of Rac1 in governing microglial functions, significantly extending our understanding of its contributions beyond traditional immune responses. Using conditional gene ablation techniques in mice to achieve Rac1 ablation specifically in microglia, coupled with *in vitro* experiments, we gave the first steps into understanding the mechanistic relevance of Rac1 in microglia. Firstly, we characterized Rac1 mutant mice and showed that microglial engulfment of synapses and redox state do not require Rac1 during homeostasis. However, Rac1 critically regulates the communication between microglia and synapses via microglia-synapse contacts and microglia-secreted growth factors. Loss of microglial Rac1 rearranges the synaptic phosphoproteome landscape and abrogates the cognitive enhancements induced by exposure to novel experiences. Instructed by our multi-omics data, we explored GDNF as a potential missing link between microglia and synapses. Our results suggest that Rac1 regulates GDNF availability via mTORC1 and secretion through Rab27a. Our data also posit that this Rac1-dependent GDNF secretion by microglia modulates excitatory synaptic numbers influencing learning and memory. Lastly, in the context of neuroinflammation induced by systemic LPS administration, microglia dramatically reconfigure their phosphoproteomic signature, and this process is highly dependent on Rac1. Furthermore, upon LPS exposure Rac1 is necessary for reactive oxygen species production, NF- κ B pathway activation, and AMPK

activation. In line with this, neuroinflammation-driven microgliosis and microglial reactivity were abrogated in Rac1 mutant mice.

Overall, we demonstrated that Rac1 is not only pivotal for mediating the dynamic interplay between microglia and synapses, essential for synaptic plasticity and cognitive performance driven by novel experiences, but also crucial in orchestrating the microglial response to the brain neuroinflammatory milieu. This dual role of Rac1 highlights its potential as a therapeutic target as its modulation could offer novel approaches for enhancing cognitive functions and mitigating neuroinflammatory responses. Future works should explore the translational potential of Rac1 modulation to refine therapeutic strategies aimed at optimizing neurological health, particularly in contexts where synaptic function is impaired and inflammatory responses are dysregulated, such as in neurodegenerative diseases and aging.

Keywords: Microglia; Actin Cytoskeleton; Rho GTPases; Rac1; Microglia-synapse crosstalk; Environmental Enrichment; Neuroinflammation.

Resumo

As células da microglia constituem a principal população mieloide do cérebro e representam 5 a 10% do total das células deste tecido. Esta população é autônoma e duradoura, o que a distingue de outras populações células mieloides residentes em tecidos e de monócitos em circulação.

Em condições fisiológicas, as microglias, células bastante ramificadas e dinâmicas, estendem e retraem os seus processos celulares continuamente para patrulhar o parênquima do sistema nervoso central, monitorizar o funcionamento das sinapses e procurar lesões e infeções nos tecidos. A reorganização do citoesqueleto é potencialmente crítica para as funções fisiológicas destas células altamente plásticas.

Desta forma, proteínas capazes de modular a dinâmica do citoesqueleto de actina e outros processos intracelulares, tais como as GTPases da família Rho, surgem como potenciais reguladores das funções microgliais. A Rac1, um dos membros desta família de proteínas, tem sido amplamente estudada no sistema nervoso central e noutras células mieloides. A contribuição desta proteína no direcionamento dos axónios, nos complexos NADPH oxidase, na regulação da ativação da via do NF- κ B e na fagocitose estão já bem descritos na literatura. Dada a lacuna existente no que diz respeito às funções regulatórias da Rac1 na microglia, a presente tese ambiciona estudar e identificar novos papéis da Rac1 na biologia da microglia, tanto em condições homeostáticas como neuroinflamatórias.

Neste estudo, revelamos papéis essenciais e não redundantes da Rac1 na modulação das funções microgliais, expandindo o nosso conhecimento atual das suas contribuições para lá das respostas imunes. Através do uso de técnicas de ablação genética condicionais em murganhos para promover ablação da Rac1 especificamente na microglia, em conjunção com abordagens *in vitro*, demos os primeiros passos para a compreensão da relevância mecanística da Rac1 na microglia. Inicialmente, caracterizamos os murganhos mutantes para Rac1 e demonstramos que esta proteína não é crítica para o englobamento de sinapses nem para o estado oxidativo da microglia em condições homeostáticas. No entanto, a Rac1 regula a comunicação entre microglia e sinapses, através dos contactos diretos microglia-sinapse e da secreção de fatores de crescimento pela microglia. A perda de Rac1 na microglia remodelou o perfil fosfoproteómico das sinapses e anula as melhorias cognitivas induzidas pela exposição a novas experiências. Direcionados pelos nossos dados ómicos, exploramos o GDNF como possível elo em falta entre a microglia e as sinapses. Os nossos resultados sugerem que a Rac1 regula a disponibilidade de GDNF através de mTORC1 e a sua secreção através de Rab27a. Os nossos dados também

indicam um potencial papel modulatório da secreção de GDNF dependente de Rac1 no número de sinapses excitatórias e, conseqüentemente, na aprendizagem e memória. Por último, num contexto de neuroinflamação induzido pela administração sistêmica de LPS, verificamos que a microglia readapta profundamente o seu perfil fosfoproteômico, num processo altamente dependente de Rac1. Para além disso, em resposta à estimulação com LPS, a Rac1 é necessária para a produção de espécies reativas de oxigénio, ativação da via do NF- κ B e ativação de AMPK. Em conformidade com estas observações, a microgliose induzida pela neuroinflamação, assim como o aumento da reatividade da microglia neste contexto, foram abolidas nos animais mutantes para Rac1 na microglia.

Assim, demonstramos que a Rac1 é crítica para a mediação da comunicação dinâmica entre microglia e sinapses, um processo essencial para a plasticidade sináptica e performance cognitiva promovida por novas experiências, e para orquestrar a resposta microglial num ambiente cerebral neuroinflamatório. Este envolvimento duplo da Rac1 realça o seu potencial como alvo terapêutico, uma vez que a modulação da sua atividade poderá oferecer novas abordagens para potenciar funções cognitivas e mitigar as respostas neuroinflamatórias. No futuro, o potencial translacional da modulação de Rac1 poderá ser explorado para melhorar estratégias terapêuticas direcionadas para a otimização da saúde neurológica, em particular em contextos de doenças neurodegenerativas e envelhecimento, caracterizados pela debilidade de funções sinápticas e desregulação das respostas inflamatórias.

Palavras Chave: Microglia; Citoesqueleto de Actina; Rho GTPases; Rac1; Comunicação Microglia-sinapse; Enriquecimento Ambiental; Neuroinflamação.

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Abbreviations

4'OHT	4-hydroxytamoxifen
AAV	Adeno-associated virus
Aβ	Amyloid beta
ABPs	Actin binding proteins
AD	Alzheimer's disease
ADF	Actin depolymerizing factor
AMPK	5' AMP-activated protein kinase
AP	Anteroposterior
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
Ca²⁺	Calcium
cAMP	Cyclic adenosine monophosphate
Cdk1	Cyclin-dependent kinase 1
CE	Control environment
CLRs	C-type lectin receptors
CNS	Central Nervous System
Csf1	Colony stimulating factor 1
Csf1r	Colony stimulating factor 1 receptor
DAMPs	Danger-associated molecular patterns
DAP12	DNAX activating protein of 12kDa
DEGs	Differentially expressed genes

DGAV	Direção Geral da Alimentação e Veterinária
DH	Dbl-homology
DHE	Dihydroethidium
DHR	Dock Homology Domain
DI	Discrimination index
DV	Dorsoventral
E7.5	Embryonic day 7.5
ECM	Extracellular matrix
EE	Enriched environment
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
GAPs	GTPase activating proteins
GDI	Guanine nucleotide dissociation inhibitors
GDNF	Glial-derived neurotrophic factor
GEFs	Guanine exchange factors
GPCRs	G protein-coupled receptors
GSEA	Gene set enrichment analysis
HMC3	Human microglia clone 3
Hmgb1	High mobility group box 1
Hsp	Heat shock proteins
IFN	Interferon
Igf-1	Insulin-like growth factor 1
IL-1β	Interleukin 1-beta
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-34	Interleukin 34

Irf8	Interferon regulatory factor 8
KD	Knockdown
LPS	Lipopolysaccharide
LRRK2	Leucine-rich repeat kinase 2
MCM	Microglia conditioned medium
mDia1	Mammalian diaphanous-related formin 1
ML	Mediolateral
mTORC1	Mammalian target of rapamycin complex 1
MWM	Morris water maze
Myb	Myeloblastosis
Myh9	Myosin heavy chain isoform 9
Myh10	Myosin heavy chain isoform 10
NADPH	Nicotinamide adenine dinucleotide phosphate
NCAM1	Neural cell adhesion molecule 1
NF-κB	Nuclear factor kappa B
NGF	Nerve growth factor
NLRs	Nod-like receptors
NOR	Novel object recognition
NOX	NADPH oxidase complex
Nrf2	Nuclear factor erythroid 2-related factor 2
ORA	Over-representation analysis
PAKs	p21-activating kinases
PAMPs	Pathogen-associated molecular patterns
PH	Pleckstrin
PPI	Protein-protein interaction
PRRs	Pattern recognition receptors

PSD95	Post-synaptic density 95
PSMs	Peptide spectrum matches
PTMs	Post-translational modifications
Rheb	Ras homolog enriched in brain
RNA-seq	RNA sequencing
ROI	Region of interest
ROS	Reactive oxygen species
Runx	Runt-related transcription factor 1
SOD1	Superoxide dismutase 1
SRE	String ranked enrichment
TAM	Tyro3, Axl and Mer
TLRs	Toll-like receptors
TNFα	Tumor necrosis factor alpha
Trem2	Triggering receptor expressed on myeloid cells 2
WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP family verprolin homologous protein
WT	Wild type

General Introduction

Microglia

Glial cells were first described in 1856 by Rudolph Virchow who called them the *Nervenkitt* or the “nerve glue” (Virchow, 1856) since they were thought to be within the central nervous system (CNS) with the sole purpose of structurally supporting neurons. The current name also derives from this notion, since the word “glia” in Ancient Greek “glía”, means “glue”. It was only in 1895 that Mihaly Lenhossék introduced the term astrocytes (von Lenhossék & Mills, 1895). Only a few years later, at the beginning of the 20th century, through the astounding work of Santiago Ramón y Cajal and Pío del Río-Hortega, the remaining components of the “third element” (Ramón y Cajal, 1913) were fully distinguished. By developing a new staining method – the ammoniacal Silver carbonate method – Río-Hortega concluded that the third element was constituted by two distinct cell types: oligodendrocytes and microglia (Hortega et al., 1919; Río-Hortega, 1919a, 1919b, 1919c).

Microglia displaying a smaller cell body compared to those of other glial cells, serve as a first line of defense against infectious agents and injury-related products in the CNS parenchyma (Ousman & Kubes, 2012) and are the focus of the present work.

Origin and Development of Microglia

Since the identification of microglia, Río-Hortega suggested that these cells were capable of phagocytosing dendritic spines and debris and had a mesodermal origin (Hortega et al., 1919; Río-Hortega, 1919a, 1919b, 1919c), possibly arising from blood monocytes, given their similarities with macrophages. In 1939, John Kershman studied the origin of microglia in the human brain and reported the presence of migrating microglia during embryonic development, suggesting an embryonic origin to these cells (Kershman, 1939). However, the exact identity of the microglial progenitors remained elusive until recently. In 2010, using a fate-mapping mouse model, Ginhoux and colleagues discovered that microglia was derived exclusively from yolk sac erythromyeloid progenitors. By crossing a *Rosa26* reporter mice with a mouse expressing a tamoxifen-inducible Cre recombinase under the regulation of the runt-related transcription factor 1 (Runx1) promoter, Ginhoux and colleagues observed that microglia originate exclusively from erythromyeloid progenitors that migrate from the yolk sac (Ginhoux et al., 2010). This was achieved by injecting 4-hydroxytamoxifen (4'OHT) at different embryonic developmental stages. At mouse embryonic day 7.5 (E7.5), the only Runx1⁺ cells were the yolk sac progenitors, whereas at E8.5 or later, 4'OHT administration would favor tagging of other hematopoietic progenitors.

This study reported that 4'OHT injection at E7.25 led to a great number of tagged microglia in the CNS and that this number profoundly decreased when 4'OHT was injected from E8 onwards, reaching almost zero around E8.5. Additionally, this work also showed that the microglial population in the CNS is stable for the entire life of the organism with no hematopoietic contribution (**Figure 1**) (Ginhoux et al., 2010). This yolk sac origin was further confirmed by Schulz et al, who showed that microglia did not require the transcription factor myeloblastosis (Myb), contrarily to monocytes and macrophages (cells derived from definitive hematopoiesis), by knocking out Myb. Furthermore, they also reported that microglia depends on the transcription factor Pu.1 (Schulz et al., 2012). Further studies were able to characterize the microglia yolk sac progenitors as c-kit⁺CD45⁺ cells that later differentiate into c-kit⁺CD45⁺CX₃CR1⁺ microglia *in vivo* and *in vitro* (Kierdorf et al., 2013). The same progenitors were also able to differentiate into F4/80⁺CX₃CR1⁺ and F4/80⁺CX₃CR1⁺ macrophages that colonized the developing brain surface.

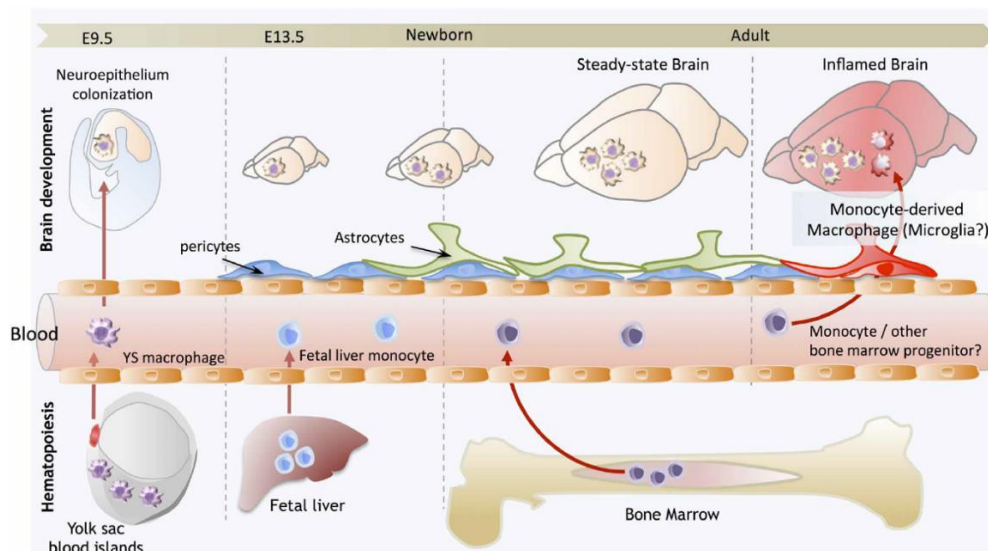


Figure 1 – Origin of microglia. Early colonization of the brain parenchyma by yolk-sac progenitors establishes a long-lasting microglia population. (Reproduced from (Ginhoux et al., 2013)).

While the origin and development of microglia have seen substantial progress in the past 15 years, the critical elements that regulate the microglia differentiation process are only partially known. The differentiation process is dependent on two transcription factors: the ETS-domain transcription factor Pu.1 and interferon (IFN) regulatory factor 8 (Irf8). Pu.1 deficient mice are devoid of several myeloid cells, including brain microglia (Kierdorf et al., 2013). Additionally, Irf8 gene ablation decreased microglial cell density. Pu.1 and Irf8 ablation distinctly affected the microglial progenitors at different developmental stages. Pu.1 is also expressed in adult microglia and it is critical to *in vitro* human adult microglia viability (Smith et al., 2013). Although their specific roles are still undetermined, colony stimulating

factor 1 (Csf1) and its receptor (Csf1r) are essential during the developmental stages of microglia since the absence of any of them results in a profound decrease in microglia numbers (Ginhoux et al., 2010; Yoshida et al., 1990). Later work identified interleukin-34 (IL-34) as another ligand of Csf1r (Lin et al., 2008). IL-34 ablation also reduced microglia numbers, supporting the importance of Csf1r as a survival signal during this process. A recent study showed that there is a dynamic relationship between Csf1 and IL-34 throughout development until adulthood (Easley-Neal et al., 2019). Blockage of IL-34 in pregnant mice (E6.5 and E7.5) with a blocking antibody did not alter microglia density in the newborn brain (P 0.5). Csf1 blocking, however, caused a sustained decrease in microglia density, suggesting that only Csf1 is required during prenatal development. These observations were distinct when the blocking was performed early postnatally, in which both IL-34 and Csf1 blocking resulted in regional microglia depletion. In adult mice, IL-34 was shown to be necessary for microglia maintenance in gray matter, while Csf1 was more preponderant in white matter. This suggests that there is a spatiotemporal regulation of the Csf1/IL-34 – Csf1r signaling axis that is critical for microglia, extending from development to adulthood (Easley-Neal et al., 2019).

Delving further into the proliferation and distribution of microglia in the developing brain niche, it was recently hypothesized that space availability could also modulate this process (Barry-Carroll et al., 2023). This work showed that microglia expansion during pre and post-natal development accompanies the changes in brain growth. Furthermore, increase in microglial cell numbers is achieved through clonal expansion, with heterogeneity in clone size, until the mosaic distribution observed in the adult brain was achieved (Barry-Carroll et al., 2023). These results further support a potential regulation of the the spatial distribution of microglia via contact inhibition mechanism (Zhan et al., 2019) and raise questions on whether this process is recapitulated in the adult brain upon microglia depletion.

In contrast with previous views, it is currently established that microglia in the adult brain are long lived cells that maintain their population by proliferation-apoptosis cycles of self-renewal, at a slower rate than macrophages (~0.69% per day in mice) (Askew et al., 2017), with no significant contribution from peripheral myeloid cells (Ajami et al., 2007; Ginhoux et al., 2010; Hashimoto et al., 2013). Adult brain microglia under homeostatic conditions are characterized by the expression of Csf1r, Cx3cr1, Hexb, P2ry12 and Tmem119 (Vecchiarelli & Tremblay, 2023).

Physiological Roles of Microglia

Microglia are considered to be the main innate immune defenders of the brain, protecting the brain parenchyma from damage and infections. While this holds true, studies performed over the past 20 years revealed involvement of microglia in a plethora of physiological processes in the CNS, from embryonic development until adulthood (**Figure 2**). Early migration of microglia progenitors to the developing brain suggested that microglia could have an active role in CNS development. Microglia regulate vasculogenesis (Fantin et al., 2010) and neural circuit development at several levels, mainly through molecule secretion and engulfment/phagocytosis (Diaz-Aparicio et al., 2020; Sierra et al., 2010). Microglia-induced apoptosis of excessive neurons was demonstrated firstly in the chick retina, in which microglia-derived neuronal apoptosis was induced by the production of neurotrophic growth factor (NGF) by microglia (Frade & Barde, 1998). Microglia release of tumor necrosis factor- α (TNF) was also demonstrated to promote apoptosis in the mouse spinal cord (Frédéric et al., 2004). Superoxide radical production by microglia also promotes cerebellar Purkinje cell (Marín-Teva et al., 2004) and hippocampal neuron programmed cell death (Wakselman et al., 2008). Complementing this process, microglia phagocytose and clear apoptotic neurons, neuronal progenitors and cellular debris from the CNS parenchyma (Cunningham et al., 2013; Marín-Teva et al., 2004). Engulfment of apoptotic cells in the neurogenic niches involves Tyro3, Axl and Merck (TAM) receptor tyrosine kinases and its ligands Gas and protein S6 throughout the entire life of the organism (Fourgeaud et al., 2016). Additionally, DNAX-activating protein of 12 kDa (DAP12) and triggering receptor expressed in myeloid cells 2 (Trem2) are also involved the elimination of apoptotic neurons (Wakselman et al., 2008). Microglia also promote clearance of astrocytes (Puñal et al., 2019), oligodendrocyte progenitors (Nemes-Baran et al., 2020) and myelin sheaths (Hughes & Appel, 2020). In addition, microglia are also capable of clearing the extracellular matrix (ECM) to facilitate synaptic plasticity (Nguyen et al., 2020). Microglia-released neurotrophic factors are also critical for neuronal survival. Cx3cr1-mediated insulin-like growth factor 1 (Igf-1) secretion by microglia supports cortical layer V neurons survival (Ueno et al., 2013). Microglial brain-derived neurotrophic factor (BDNF) signaling has also been implicated in neural precursor proliferation in the adult brain (Harley et al., 2021).

Regulation of neurons by microglia is not restricted to controlling the overall population levels. In fact, microglia plays critical roles in CNS circuitry refinement at the synapse level by engulfing and eliminating excessive synapses, in a process known as synaptic pruning (Paolicelli et al., 2011; Schafer et al., 2012; Stevens et al., 2007). Microglia extend their processes and contact neurons in an activity-dependent manner (Tremblay et al., 2010;

Wake et al., 2009) to monitor their activity and eventually eliminate them. The synaptic pruning process is regulated via “eat me” signals, such as phosphatidylserine (Scott-Hewitt et al., 2020) and the complement protein C1q (Schafer et al., 2012; Stevens et al., 2007), and “don’t eat me” signals, such as CD47 (Lehrman et al., 2018). Other critical elements involved in this process are Cx3cr1 (Paolicelli et al., 2011), Trem2 (Filipello et al., 2018) and P2ry12 (Sipe et al., 2016). Of note, evidence suggests that microglia remodel the synaptic landscape by eliminating specifically presynaptic terminals and promoting postsynaptic structure formation (Weinhard et al., 2018). Adding to this last observation, microglia-secreted BDNF (Parkhurst et al., 2013) and microglia-dendrite contacts are critical for synapse formation during development (Miyamoto et al., 2016). In the adult brain, Cx3cr1 signaling has been demonstrated to be involved in synaptic formation in the neurogenic niches (Reshef et al., 2017).

It is worth reinforcing that neuronal survival, monitoring of neuronal activity, synaptic pruning and synapse regulation by microglia go beyond the CNS pre and postnatal development and are maintained during adulthood (Basilico et al., 2022; Parkhurst et al., 2013; Reshef et al., 2017; Sierra et al., 2010).

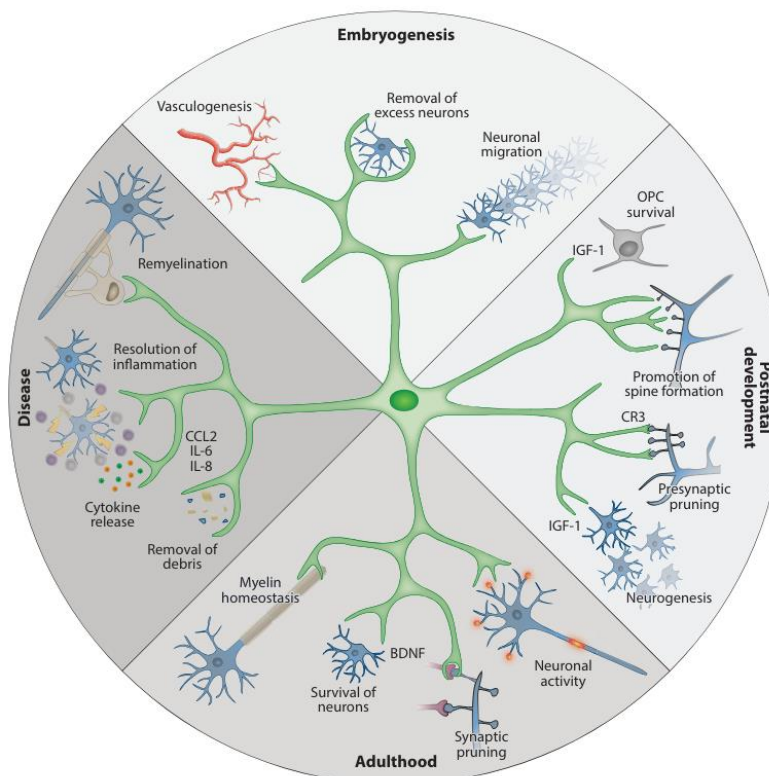


Figure 2 – Microglia in health and disease. The plethora of microglia functions from embryonic development to adulthood. (Reproduced from (Prinz et al., 2021)).

Microglia in Pathology and Disease

As the innate immune defenders of the CNS, microglia are constantly patrolling and sensing their microenvironment (Davalos et al., 2005; Nimmerjahn et al., 2005). Changes in brain homeostasis, that can either result from tissue lesions, pathogens, dying neighbouring cells or accumulation of toxic substances, promote microglia morphological remodeling, characterized by the extension of their cellular processes towards the trigger. Recognition of danger-associated molecular patterns (DAMPs), such as high mobility group box 1 (Hmgb1), heat shock proteins (Hsp) and adenosine triphosphate (ATP), or pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) and viral nucleic acids, is achieved by microglia through pattern recognition receptors (PRRs) (Akira et al., 2006), including toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs) or Trem2 (Ransohoff & Brown, 2012). Characteristic of this adaptive process is the increase in glutamate and reactive oxygen species (ROS) release, as well as in interleukin 1 beta (IL-1 β), interleukin 6 (IL-6) and TNF α , pro-inflammatory cytokines. This is potentiated by nuclear factor kappa B (NF- κ B) pathway activation, in an effort to reestablish tissue homeostasis. While this initial mechanism is considered beneficial, dysregulation of the immunological pathways that prevent overactivation, the microglial immune checkpoints, can become detrimental to the surrounding cells and exacerbate pathology and neurodegeneration (Deczkowska et al., 2018).

The involvement of microglia in various neurodegenerative disorders is well documented. (Gao et al., 2023; Hickman et al., 2018). Example of this is the capacity of microglia to sense and clear amyloid-beta (A β) (Condello et al., 2015) and α -synuclein (Choi et al., 2020), protecting the brain from neurodegeneration. Additionally, R47H mutation in Trem2, which is almost exclusively expressed in microglia in the CNS, is the one with the highest risk association with Alzheimer's disease (AD) (Jay et al., 2017). Recent advances in the field are now reinforcing the potential role of microglia as an initiator of the disease, instead of being just responders to changes in their microenvironment (Bido et al., 2021; Socodato, Portugal, et al., 2020).

Microglia profoundly adapt their transcriptomic states in a context-dependent manner (Paolicelli et al., 2022). This includes alterations in the expression of otherwise considered stable signature microglial genes Tmem119, Csf1r, Cx3cr1 or P2ry12 (Chen & Colonna, 2021). Furthermore, the advent of single-cell transcriptomics has brought to light the heterogeneity of microglial states, not only in homeostasis but specially in pathological and disease contexts such as LPS-induced neuroinflammation, ageing and neurodegenerative

diseases (Hammond et al., 2019; Keren-Shaul et al., 2017; Sousa et al., 2018). While this should be complemented with other methodological approaches that are closer to the biological function (proteomics, phosphoproteomics or metabolomics), the already available information can open new avenues of exploration in the identification of novel biomarkers and potentiate microglia-based therapeutic approaches in neuropathological conditions.

Cytoskeleton

Every cell supports its structure and morphology in a network of several filaments that constitute the cytoskeleton. Besides structural support, the cytoskeleton is critical for a myriad of cellular processes, including signaling, cell motility and intracellular trafficking (Ramaekers & Bosman, 2004). This structure has three main components: actin filaments, microtubules and intermediate filaments. Actin filaments, the most dynamic structures of the three, are mainly located closer to the cell borders and promote cell movement and membrane trafficking (Svitkina, 2018). Microtubules form long filaments that start from the perinuclear region and spread radially towards the cell edge, working as tracks for intracellular trafficking and being also involved in the mitotic spindle (Barlan & Gelfand, 2017). Intermediate filaments are distributed in the perinuclear space, creating a ring-like structure and can also spread towards the cell edge. They are responsible for supporting the nuclear location inside the cell, as well as enduring mechanical stress (Herrmann et al., 2007).

In line with the constant surveillance of the brain parenchyma by microglia, which requires a fast extension and retraction of its protrusions, as well as other morphological modifications to which microglia are subjected, the actin cytoskeleton and its regulators emerge as potential key elements for microglia.

The Actin Cytoskeleton

The actin monomer, G-actin, is a highly conserved, flat molecule composed of two asymmetric subunits that are located in opposite sides of the protein: the pointed end and the barbed end. Actin filaments, F-actin, are formed through the polymerization of G-actin monomers. Given their asymmetry, the actin filaments are polarized and their ends can be distinguished using the same terminology of the G-actin (Huxley, 1963). Actin filament polymerization can be divided in three distinct phases. The first step is nucleation, in which

a nucleus with a minimum number of 3 actin monomers is formed (Sept & McCammon, 2001). The second step is elongation, where G-actin subunits are added to both ends of the structure. This growth, however, is not equal on both ends, occurring favorably on the barbed end region of the filament (five to ten times faster than in the pointed end) (Pollard, 1986). The addition of actin monomers is a reversible process. Dissociation of actin can occur when ATP-bound actin monomers in the actin filament, due to their intrinsic ATPase activity, hydrolyze ATP, promoting the release of monomers from the filament (Korn et al., 1987). The polymerization rate is dependent of the concentration of free monomers (Pollard, 1986). Thus, differences in the critical concentration at both ends of the actin filament result in different polymerization/depolymerization at the barbed and pointed ends, respectively. Once the equilibrium between polymerization and dissociation is achieved, actin polymerization is in its third phase: the steady state.

Actin assembly and disassembly is tightly controlled by actin binding proteins (ABPs). This group of proteins can control the pool of available actin monomers, nucleation or elongation and promote filament severing and crosslinking (Pollard, 2016).

Profilin is one of the ABPs capable of binding to monomeric actin. It is responsible for catalyzing the exchange from ADP-bound to ATP-bound G-actin, thus improving its availability and promoting actin polymerization, mainly at the barbed end of the actin filament (Courtemanche & Pollard, 2013; Vinson et al., 1998). Almost all available monomeric actin is bound to either profilin or thymosin- β 4, another ABP that sequesters G-actin, preventing them from polymerizing.

To facilitate nucleation, cells rely on the Arp2/3 complex to branch already formed actin filaments and on formins to elongate unbranched filaments. Actin dimers are very unstable and will tend to rapidly dissociate (Cooper et al., 1983). Arp2 and Arp3 are the two first subunits of a branched filament. They reorganize and alter their conformation to form a dimeric structure to which actin monomers can bind to give rise to the new filament. Arp2/3 promotes branching by anchoring the pointed end of the newly formed branch to the mother filament. The barbed end is then elongated to produce a branched actin filament (Rouiller et al., 2008). The Arp2/3 complex can be activated by several factors (Rottner et al., 2010) such as the Wiskott-Aldrich syndrome protein (WASP) or the WASP family verprolin homologous protein (WAVE). Interaction of these factors with the Arp2/3 complex is regulated, among others, by proteins of the Rho GTPase family (Schaks et al., 2018). Formins can promote the beginning of nucleation by stabilizing actin dimers (Pring et al., 2003) for the formation of unbranched actin filaments. In addition, formins can also attach to the barbed ends of actin filaments to promote elongation, by facilitating the integration of

profilin-bound ATP-G-actin. At the same time, formins prevent the capping of the barbed end, further promoting the elongation of actin filaments. Ena/VASP proteins can also promote elongation by delivering actin monomers and preventing the capping of the barbed end (Edwards et al., 2014; Ferron et al., 2007; Hansen & Mullins, 2010).

Cofilin, otherwise called actin-depolymerizing factor (ADF), is responsible for actin filament severing. Having a greater affinity to ADP-bound actin, cofilin attaches to the sides of actin filaments and promotes their severing, potentially due to rigidity differences between flexible cofilin-bound segments and stiffer parts of the actin filament (Ngo et al., 2015). Severing generates free ends for depolymerization, favoring the turnover of actin filaments (Andrianantoandro & Pollard, 2006). Cofilin can be activated via dephosphorylation of its Ser3 by slingshot phosphatase and inhibited by phosphorylation by LIM kinases (which are part of the Rho GTPase signaling).

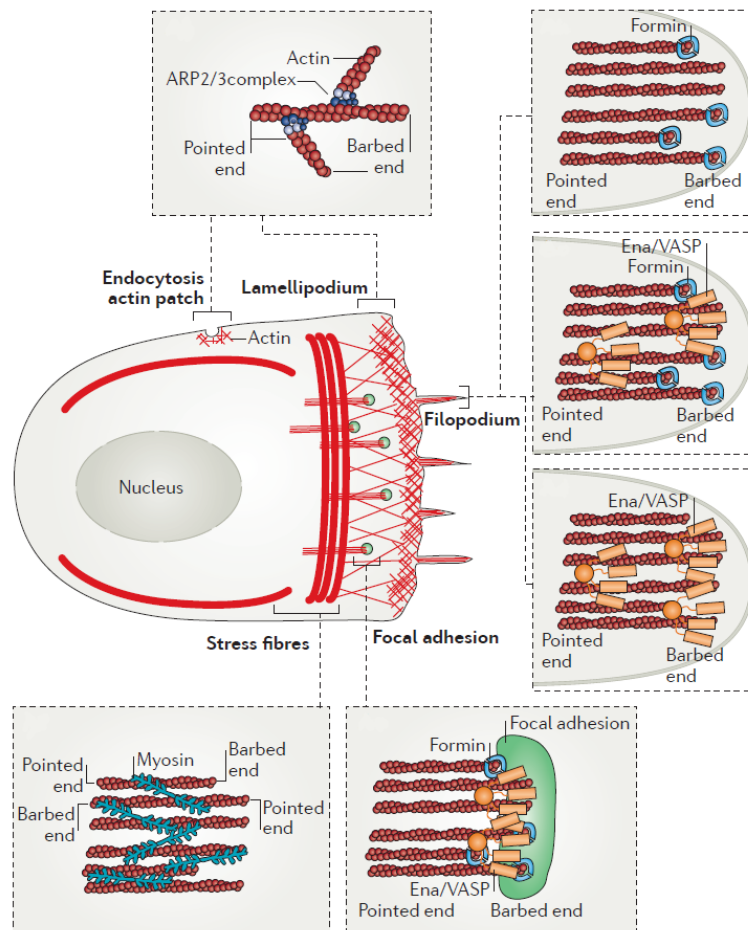


Figure 3 – The actin cytoskeleton. Participation of actin binding proteins in elongation, branching and crosslinking, to form lamellipodia, filopodia, focal adhesions and stress fibers. (Adapted from (Suarez & Kovar, 2016).

Crosslinking ABPs are responsible for stabilizing complex actin structures by connecting actin filaments to each other (Matsudaira, 1994). Actin bundles consisting of several actin filaments arranged in a parallel orientation, are critical for supporting other actin filament based structures. Lamellipodia are a mesh of actin branched filaments located near the cell borders. Filopodia are thin, perpendicular cell protrusions composed of parallel actin bundled filaments, frequently supported by lamellipodia on the cell edge (**Figure 3**). Together with other actin-based structures, these are necessary for oriented cell movement, protrusion dynamics and phagocytosis (restricted to some cell types).

Rho GTPases

Tight regulation of the actin cytoskeleton dynamics is critical for cellular morphological adaptations and cell functions. Cells rely on a family of small proteins that are key regulators of actin cytoskeleton dynamics and reorganization known as Rho GTPases.

The Rho family of small GTPases is part of the Ras superfamily of guanine nucleotide-binding proteins. In humans, there are 20 Rho GTPases identified to date. Proteins of this family are divided in the Rho, Rac, Cdc42, RhoBTB and Rnd subfamilies, according to their homology (**Figure 4**).

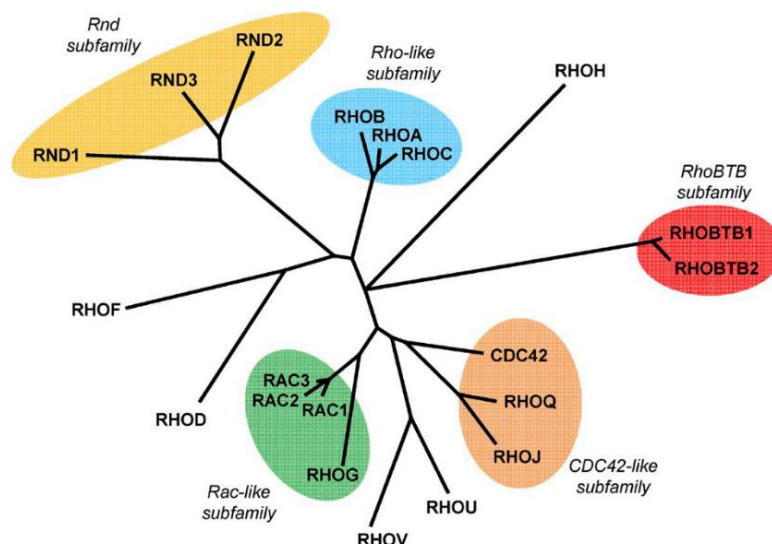


Figure 4 – The family of Rho GTPases. The 20 human Rho GTPases organized by their homology, and respective subfamilies. (Adapted from (Lawson & Ridley, 2017)).

These proteins can also be categorized as typical or atypical GTPases. Typical Rho GTPases, where the most well-known and studied proteins of this family RhoA, Rac1 and Cdc42 are included, can cycle between an active, GTP-bound state, and an inactive, GDP-bound state. This switch mechanism is regulated by guanine exchange factors (GEFs), that promote GTPase activation through the exchange from GDP to GTP, by GTPase activating proteins (GAPs), which inhibit Rho GTPases by facilitating their inherent GTPase capacity of hydrolyzing GTP, and by guanine nucleotide dissociation inhibitors (GDIs), that sequester and prevent GTPase mobilization to the plasma membrane to be activated (**Figure 5**) (Bos et al., 2007; DerMardirossian & Bokoch, 2005). There are at least 85 GEFs, 66 GAPs and 4 GDIs reported (Mosaddeghzadeh & Ahmadian, 2021). RhoGEFs reduce the binding affinity of ADP to Rho GTPases, disrupting their bond and promoting the association with GTP. These proteins typically contain Dbl-homology (DH) and Pleckstrin homology (PH) domains or Dock homology regions (DHR). The activity of these proteins is mainly autoregulated intramolecularly since their structural conformation does not suffer substantial changes. Autoinhibition mediated by N-terminal or C-terminal domains and upregulation promoted by C-terminal domains are two examples of the autoregulatory mechanisms RhoGEFs undergo. RhoGAPs contain a conserved catalytic domain that enhances the GTPase activity of Rho GTPases: These catalytic domains are also responsible for RhoGAPs autoinhibition and membrane interactions. Contrarily to RhoGEFs and RhoGAPs, RhoGDIs rely mainly on phosphorylation to regulate their activity and interaction with Rho GTPases. Additionally, it is suggested that Rho GEFs can interact directly with Rho GTPase effectors to promote specific spatiotemporal activation (Cherfils & Zeghouf, 2013). Thus, the concerted action of RhoGEFs, RhoGAPs and RhoGDIs is critical for typical Rho GTPases signaling dynamics. Atypical GTPases, instead, are constitutively bound to GTP and their regulation relies on changes of the expression level and post-translational modifications (PTMs). Likewise, typical Rho GTPases are also regulated by PTMs, including phosphorylation, prenylation, ubiquitylation and sumoylation (Hodge & Ridley, 2016).

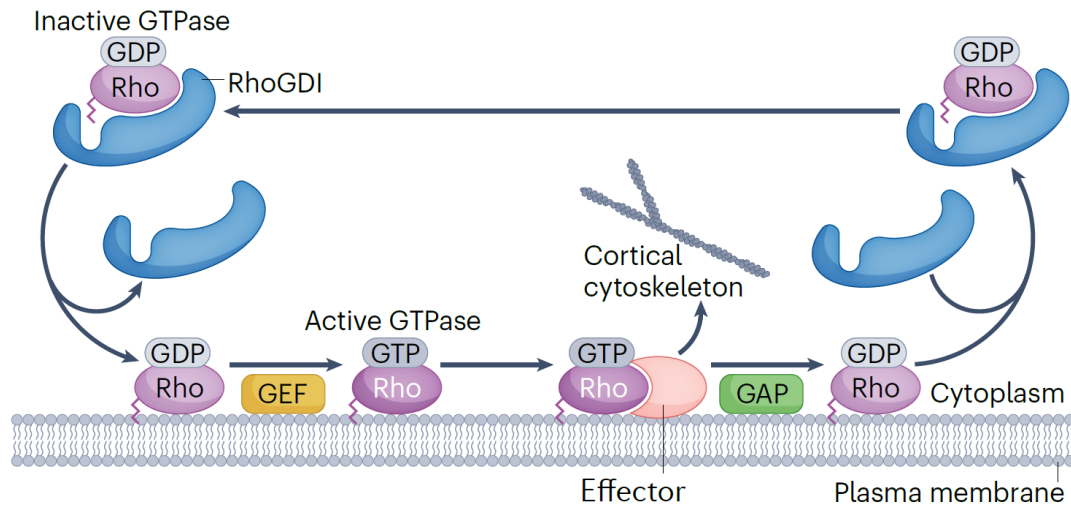


Figure 5 – Rho GTPase regulation by GEFs, GAPs and GDIs. Interchange between RhoGTPase activation states, mediated by GEFs and GAPs. Rho GTPase membrane availability is regulated by GDIs. (Adapted from (Bement et al., 2024)).

There are several studies that pinpoint the specific involvement of each of those main Rho GTPases in the regulation of the actin cytoskeleton. RhoA is associated with stress fibers and focal adhesion formation (Ridley & Hall, 1992), whereas Rac1 is involved in lamellipodia formation (Ridley et al., 1992). Cdc42 is responsible for filopodia formation (Nobes & Hall, 1995). As previously mentioned, Rho GTPases regulate the actin cytoskeleton at the actin binding protein level. RhoA, by activating LIM kinase, can lead to the phosphorylation of cofilin, thus decreasing the severing of actin filaments and impairing its turnover (Arber et al., 1998). RhoA can also bind to the mammalian diaphanous-related formin 1 (mDia1), promoting its actin nucleation capacity (Watanabe et al., 1999). Cdc42 and Rac1 can also regulate other mDia formins, promoting actin nucleation and polymerization. Rac1, with the intermediation of the insulin receptor tyrosine kinase p53, interacts with the WAVE proteins, that in turn will bind to the Arp2/3 complex to promote actin filament branching (Miki et al., 2000). Rac1 can also regulate the actin cytoskeleton via p21-activated kinases (PAKs) that phosphorylate LIM kinase and ultimately lead to cofilin inactivation in the lamellipodia (Delorme et al., 2007).

The roles of Rho GTPases extend far beyond the control of cell morphology and motility. Due to their close proximity with the cell membrane and upstream position in several signaling pathways, Rho GTPases are key signaling integrators that can be activated by integrins, cadherins, G protein-coupled receptors (GPCRs) or cytokine receptors. Activation of these receptors will trigger a response, resulting from the concerted effort from specific Rho GTPases, GEFs and downstream effectors, that often are stimulus, cell-type and context-specific.

Rho GTPases have complex signaling regulatory networks that can lead to the regulation of other Rho GTPases, usually through the action of GEFs and GAPs (Boulter et al., 2012). Therefore, observed phenotypes resulting from the ablation of a single RhoGTPase may not solely reflect the individual functions of that RhoGTPase, but could also be influenced by the crosstalk and compensatory mechanisms among other RhoGTPases.

Since the focus of the present work is Rac1, the following section will further describe this protein and its participation in biological processes.

Rac1

Ras-related C3 botulinum toxin substrate 1 (Rac1) is a 21kDa protein of the Rho GTPase family. Identified for the first time in 1989 (Didsbury et al., 1989), Rac1 is part of the Rac subfamily of Rho GTPases, along with Rac2, Rac3 and RhoG. It is ubiquitously expressed and its knockout is embryonically lethal (Sugihara et al., 1999). Rac1 has been extensively studied in several cell types inside and outside the CNS. Consequently, various functions have already been identified for Rac1 (**Figure 6**). In neurons, Rac1 is involved in axonal growth and guidance (Chen et al., 2007) and in the regulation of amyloid precursor protein transcription (APP) (Wang et al., 2009). Synaptic vesicle endocytosis also requires a balance between Rac1/RhoA signaling via its downstream effectors mDia1/3 respectively, in hippocampal neurons (Oevel et al., 2024). In macrophages and monocytes, Rac1 is critical for the assembly and activation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex (NOX), responsible for ROS production (Abo et al., 1991; Zhao et al., 2003). Rac1 is also involved in immunoglobulin receptor-mediated phagocytosis (Caron & Hall, 1998). Additionally, Rac1 coordinates inflammation through the activation of the NF- κ B and the antioxidant nuclear factor erythroid 2-related factor 2 (Nrf2) (Cuadrado et al., 2014). Engulfment of apoptotic cells requires initial Rac1 activation to form phagocytic cups and inactivation for phagocytic cup closing (Nakaya et al., 2008).

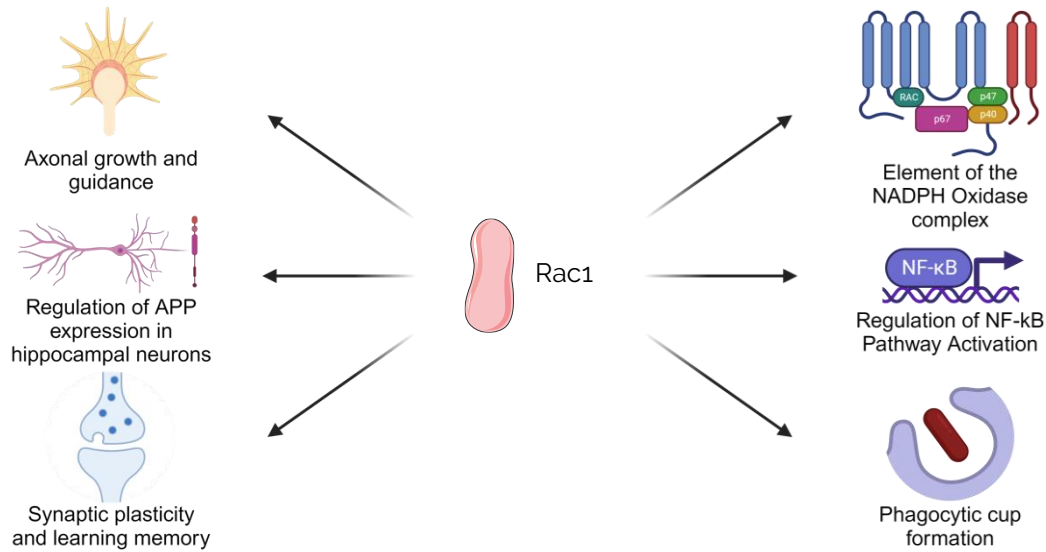


Figure 6 – The spectrum of Rac1 functions. Rac1 contribution in the CNS (left side) and in immune cells (right side). This representation was created with Biorender.

Due to its participation in such a significant number of biological processes, Rac1 signaling is associated with various diseases. This includes infectious diseases (Criss et al., 2001), cancer (Ma et al., 2023) and neurodegenerative disorders (Stankiewicz & Linseman, 2014). In relation to Alzheimer's disease, besides regulating APP expression, Rac1 also is involved in APP cleavage (Boo et al., 2008), suggesting a role in A β production (product of APP cleavage) and A β plaque formation. Rac1 is decreased in brains from AD patients (Borin et al., 2018). This study also reports that, in the 3xTg-AD mouse model, there is a significant activation of Rac1 in 6-week-old mice, that then evolves to an overall reduction in Rac1 expression at 7 months. Administration of an active Rac1 peptide could prevent the spine loss observed in non-treated mice (Borin et al., 2018). A recent study also suggests that inhibition of Bcr, a Rac1 GAP, enhanced aversive learning (Wang et al., 2023). In Parkinson's disease, Rac1 can rescue neurite retraction induced by a mutation in leucine-rich repeat kinase (LRRK2), by modulating its binding with Rac1 (Chan et al., 2011). Moreover, Rac1 signaling modulates alpha-synuclein accumulation and promotes the survival of dopaminergic neurons (Kim et al., 2018). In amyotrophic lateral sclerosis, mutations in superoxide dismutase 1 (SOD1) promote dysregulated Rac1 and, consequently, NADPH oxidase activation, that can ultimately lead to neurotoxicity (Harraz et al., 2008).

Rac1 signaling is therefore highly relevant for cell biology, both in homeostatic and disease contexts, and can be a potential therapeutic target to be further explored.

The Microglia Cytoskeleton

The unique morphology of microglia in the brain, with constant extension and retraction of their several cellular protrusions, coupled with the morphological adaptations that these cells are subjected to when triggered (Davalos et al., 2005; Nimmerjahn et al., 2005), suggest a particular need for a highly dynamic cytoskeleton. For a long time, morphological changes in microglia were viewed as a direct proxy for its function. Currently, it is suggested that the relationship between a morphological change and the resulting functional consequences need to be considered on a case-by-case basis (Paolicelli et al., 2022). In homeostatic microglia, actin is mainly found in their protrusions. In addition to the high-order large protrusions, Bernier et al identified filopodia structures in microglia *in vivo* that extend and retract constantly, in apparently random directions in the homeostatic brain, promoting surveillance at the nanoscale (Bernier et al., 2019). In contrast with the larger protrusions, these filopodia are only actin-dependent. Protrusion extension towards lesion sites is mediated through P2RY12, which responds to ATP released at the injury site as a chemotactic cue (Davalos et al., 2005; Haynes et al., 2006). Control of filopodia formation and extension was regulated by P2RY12-mediated modulation of cyclic adenosine monophosphate (cAMP) (Bernier et al., 2019).

Microtubule remodeling is also necessary for microglial morphological rearrangements. Two recent studies delved into the mechanisms associated with the morphological transition from homeostatic to responsive microglia (Adrian et al., 2023; Rosito et al., 2023). Homeostatic microglia nucleate microtubules at Golgi outposts, satellite organelles where acentrosomal microtubule nucleation can occur. Upon stimulation with LPS-interferon- γ or interleukin-4 (IL-4), microtubules acquired a radial distribution, centered near the nucleus in a pericentrosomal region. Furthermore, γ -tubulin distribution was also altered and critical for cytokine release (Rosito et al., 2023). Adrian and colleagues further identified cyclin-dependent kinase 1 (Cdk1) as a critical regulator of this microtubule rearrangement and cytokine trafficking (Adrian et al., 2023).

Rho GTPases, due to their critical role in coordinating actin and myosin dynamics, are on the interface between the regulation of cytoskeleton and microglial functions. In fact, recent work from our lab revealed that RhoA ablation specifically in microglia resulted in an AD-like phenotype, with A β deposition, synapse loss and cognitive impairments (Socodato, Portugal, et al., 2020). In a neuroinflammatory context, a decrease in RhoA activity was necessary for microglia metabolic reprogramming (Socodato, Rodrigues-Santos, et al., 2023). Microglia morphology and inflammatory response are also dependent on the RhoA

downstream effectors non muscle myosin heavy chain isoform 9 (Myh9) and 10 (Myh10), as well as cofilin (Melo et al., 2021).

Currently, information on Rac1 regulation of microglial functions is scarce. Given the already described functions for Rac1 signaling in other cells and considering the immune nature of microglia, it is tempting to suggest that Rac1 will occupy a central spot in microglia biology. In this work I tested this hypothesis.

Objectives

Objectives

The main goal of the present thesis is to understand with molecular detail Rac1 control of microglia function and to determine whether and to what extent it impacts microglia-mediated neuroinflammation and/or CNS homeostasis. To achieve this, the experimental work targeted the following specific goals/tasks:

1. Study the impact of adult microglia-specific Rac1 ablation in the steady state and characterize the consequent changes in microglia homeostasis, from morphology to function.
2. Determine whether and how does Rac1 ablation in microglia impact neurons and synapses and the resulting behavioral outcomes.
3. Investigate the effect of LPS by identifying how it modulates microglia signaling networks.
4. Explore the requirement of Rac1, *in vivo* and *in vitro*, for the microglial response to LPS-induced neuroinflammation.

Materials & Methods

Materials

Antibodies

All antibodies used in experiments for this thesis are listed in the following table:

Antibody	Company	Catalog No	Dilution
Rac1 antibody [0.T.127]	Abcam	ab33186,	1:250 (FACS) 1:50 (IF-cells)
Iba1	Wako	019-19741	1:500
CD11b clone M1/70.15	BD Biosciences	MA1-045	1:100
PSD95 clone 6G6- 1C9	Thermo Fisher Scientific	MA1-045	1:600 (IF-tissue) 1:1000 (WB)
vGlut1	Synaptic Systems	135 303	1:1000
Anti-PSD95	Synaptic Systems	124 308	1:500 (IF-tissue)
CD68 clone FA-11	Bio-Rad	MCA1957T	1:400
Fc Receptor Blocking Solution	BioLegend	156603	1:50
NeuN	Millipore	MAB377	1:400
GDNF	Abcam	ab18956	1:100 (IF-cells) 1:200 (IF-tissue)
Rab27a clone 1G7	Abnova	H00005873-M02	1:200 (IF-cells)
Rab27a	Merck	HPA001333	1:200 (IF-cells)
APC anti- mouse/human CD11b antibody	BioLegend	101212	0.25 µg to 10 ⁶ cells
Alexa Fluor® 647 anti-mouse/human CD11b antibody	BioLegend	101218	0.25 µg to 10 ⁶ cells
PE anti-mouse CD45 antibody	BioLegend	103106	0.25 µg to 10 ⁶ cells
PerCP/Cy5.5 anti- mouse Ly6C antibody	BioLegend	128012	0.25 µg to 10 ⁶ cells
BV421 anti-mouse MHC-II antibody	BioLegend	107631	0.25 µg to 10 ⁶ cells
PE/Cy7 anti-mouse Annexin-V antibody	BioLegend	640950	0.25 µg to 10 ⁶ cells
PE/Cy7 anti-mouse Ki67 antibody	BioLegend	652425	0.25 µg to 10 ⁶ cells
CaMKII (pan) (D11A10) Rabbit mAb	Cell Signaling Technology	4436	1:1000
Phospho-CaMKII (Thr286) (D21E4) Rabbit mAb	Cell Signaling Technology	12716	1:1000
Mouse Anti- Glyceraldehyde-3- Phosphate Dehydrogenase (GAPDH) Antibody, Unconjugated	HyTest	5G4-9B3	1:100000

Peroxidase-AffiniPure Donkey Anti-Rabbit IgG (H+L)	Jackson ImmunoResearch Labs	711-035-152	1:10000
Peroxidase-AffiniPure Goat Anti-Mouse IgG (H+L)	Jackson ImmunoResearch Labs	115-035-146	1:15000
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488	Thermo Fisher Scientific	A-11001	1:400 – 1:500 (IF-cells) 1:800 – 1:1000 (IF-tissue)
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 568	Thermo Fisher Scientific	A-11004	1:1000 (IF-tissue)
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 568	Thermo Fisher Scientific	A-11011	1:1000 (IF-tissue)
Goat anti-Rat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 568	Thermo Fisher Scientific	A-11077	1:1000 (IF-tissue)
Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ Plus 647	Thermo Fisher Scientific	A32728	1:1000 (IF-tissue)
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 647	Thermo Fisher Scientific	A-21244	1:400 – 1:500 (IF-cells)
Goat Anti-Guinea Pig IgG (H+L) Highly Cross-adsorbed Antibody, Alexa Fluor 647 Conjugated	Thermo Fisher Scientific	A-21450	1:1000 (IF-tissue)

Primers

All primers used in experiments for this thesis are listed in the following table:

Gene	Forward	Reverse
Arg1	CTCCAAGCCAAAGTCCTTAGAG	AGGAGCTGTCATTAGGGACATC
C1qB	AGACACAGTGGGGTGAGGTC	GGTCCCCTTTCTCTCCAAAC
CD14	GCTCAACTTTTCCTGCGAAC	CCCGCAGTGAATTGTGACTA
CD163	TGGTGTGCAGGGAATTACAA	AGCTCCACTCTTCCCTCACA
Csfr1	CCCTAGGACAAAGCAAGCAG	GATGTCCCTAGCCAGTCCAA
Gclc	CACAGACCCAACCCAGAG	TGGCACATTGATGACAACCT
GNDF	TGGGTCTCCTGGATGGGATT	ATTGGAGTCACTGGTCAGCG
Gr	CACGACCATGATTCCAGATG	CAGCATAGACGCCTTTGACA
Hmox1	GAAGGGTCAGGTGTCCAGAGAA GG	CGCTCTATCTCCTCTTCCAGGG C
Icam1	CGAAGGTGGTTCTTCTGAGC	GTCTGCTGAGACCCCTCTTG
IL-18	CAGACAACCTTGGCCGACTT	GGGTTCAGTGGCACTTTGAT
IL-1b	GCCCATCCTCTGTGACTCAT	AGGCCACAGGTATTTTGTCTG
IL-6	CACAAGTCCGGAGAGGAGAC	CAGAATTGCCATTGCACAAC
Irak3	TGGAGGTTCTAACGGGCTG	CCATCAGTTCCATGAGGAGGTC
Mertk	GCCCACAATGACAAAGGACT	GGGAGTAGCCATCAAAACCA
Mhc-II	ATGACCCAGGACCATGTGAT	ATCTTCCAGTTCACGCCATC
Nrlp3	ATGCTGCTTCGACATCTCCT	AACCAATGCGAGATCCTGAC
Pros1	GATTCTCGCTCTGGAACGTC	GGTGTGGCACTGAAGGAAAT
Pu.1	CAGTTCTCGTCCAAGCACAA	TTTCTTCACCTCGCCTGTCT
Rac1	GAAGCTTCCCATCACCTACCC	GGGGACAGAGAACCGCTCGGA TAG
Slc23a2	CACTGATAGAAGTGGTCAT	AACACTAGGAAAATCGTCAG
Tlr2	TTGCTCCTGCGAACTCCTAT	GCTTTCTTGGGCTTCCTCTT
Tlr7	TGGAAATTTTGGACCTCAGC	TTGCAAAGAAAGCGATTGTG
Tnf	GCCACCACGCTCTTCCTGTCT	TGAGGGTCTGGGCCATAGAAC
Tnxd1	CAGGGTGACTGCTCAATCCACA AAC	CTCTTCCTACCGCCAGCAACAC TG
Trem2	AACTTCAGATCCTCACTGGACC	CCTGGCTGGACTTAAGCTGT
Tspo	TGGGAGGTTTCACAGAGGAC	GCCAGGTAAGGGTACAGCAA
Ywhaz	GATGAAGCCATTGCTGAACTTG	GTCTCCTTGGGTATCCGATGTC

Plasmids and Biosensors

All plasmids and biosensors used in experiments for this thesis are listed in the following table:

Plasmid	Source	Catalog No.
Daglas-pm1	(Sato et al., 2006)	N/A
pcDNA-D1ER	(Palmer et al., 2004)	Addgene #36325
CMV-R-GECO1	(Zhao et al., 2011)	Addgene #32444
CKAR	(Violin et al., 2003)	Addgene #14860
GFP.Cre	Gift from Tyler Jacks	Addgene #20781
p47-roGFP	(Furusato et al., 1990)	N/A
pLKO-empty	(Karabiyik et al., 2018)	N/A
pLKO-Rac1 shRNA	(Karabiyik et al., 2018)	N/A
scrambled-mCherry shRNA control	(Karabiyik et al., 2018)	N/A
mCherry-Rac1 shRNA	(Karabiyik et al., 2018)	N/A
pRK5-myc-Rac1-wt	Gift from Gary Bokoch	Addgene #12985
pRK5-myc-Rac1-T17N	Gift from Gary Bokoch	Addgene #12984
pGP-CMV-GCaMP6f	(Chen et al)	Addgene #40755
mCherry-Lifeact-7	Gift from Michael Davidson	Addgene #54491
Cyto-ABKAR	(Miyamoto et al., 2015)	Addgene #61510
pcDNA3-PM-TORCAR	(Zhou et al., 2015)	Addgene #64930
Raichu Rac1		N/A
pFRET-HSP33 cys	(Guzy et al., 2005)	Addgene #16076
plkB α -miRFP703	(Shcherbakova et al., 2016)	Addgene #80005
YFP-FKBP (YF)	(Inoue et al., 2005)	Addgene #20175
Lyn11-targeted FRB (LDR)	(Inoue et al., 2005)	Addgene #20147
YFRac1(CA)	(Inoue et al., 2005)	Addgene #20150
pLKO-Rab27a shRNA	N/A	Sigma TRCN0000279985

Methods

Animals

All mice experiments were reviewed and approved by the i3s animal ethical committee (CEA) and by Direção-Geral da Alimentação e Veterinária (DGAV). Mice were housed under standard housing conditions, in an inverted 12h/12h light-dark cycle and with access to food and water *ad libitum*. All experiments were planned and performed in agreement with the 3Rs principles and the European Directive 2010/63/EU.

Cx3cr1^{CreER-EYFP} mice were purchased from Jackson Laboratories and used as before (Socodato, Portugal, et al., 2020). In these mice, the *Cx3cr1* promoter drives high expression of the CreER cassette in microglia (Parkhurst et al., 2013). Mice homozygous for the *Rac1* floxed allele (Chrostek et al., 2006) were backcrossed for at least ten generations and were kept at the I3S animal facility. PCR determined all genotypes on genomic DNA. *Rac1* floxed mice were crossed with Cx3cr1^{CreER-EYFP} mice. Progeny of interest were: Controls (*Rac1*^{fl/fl} or Cx3cr1^{CreER+}) and mutants (Cx3cr1^{CreER+/-}:*Rac1*^{fl/fl}). Mice (4/5-weeks-old) were given tamoxifen (5 mg *per animal* by oral gavage; 1 mg daily for five days or 20 mg *per animal* by oral gavage; 4 mg daily for five days) and then analyzed post-tamoxifen. Experiments were performed in mice kept on a C57BL/6 background.

Environmental enrichment protocol

Mice with 4-5 weeks of age were weighted and placed in Type III cages for three days with two different housing conditions: control environment (CE) and enriched environment (EE). Littermates were randomly assigned to one of the housing conditions, with *Rac1*^{fl/fl} and Cx3cr1^{CreER+/-}:*Rac1*^{fl/fl} mice in every cage. CE only contained bedding material (corn cob, grade 12) and tissue paper. EE had bedding material, tissue paper, a small cardboard tube, four small aspen bricks, a wood ladder, a vertical plastic running wheel with a steep metal grid, a dome home, a plastic Lego-like block, and sunflower seeds. Mice were weighted immediately before tamoxifen administration and then weighted once per week during home-cage change for the first three weeks after tamoxifen administration. Mice were kept in the same housing conditions until the end of the protocol (18 weeks) with access to food and water *ad libitum*. Home cages in CE and EE conditions were changed weekly. Enrichment material was checked every week: new tissue paper and sunflower seeds were

added weekly, while cardboard tubes, dome homes, and aspen bricks were replaced when destroyed or highly degraded. The remaining enrichment material was constantly transferred to the new cage during weekly home-cage changes. The spatial organization of the enrichment material was maintained throughout the experiment. Manipulation of mice during the behavioral test period was reduced to the minimum necessary.

In vivo LPS administration

Rac1^{fl/fl} and Cx3cr1^{CreER+/+}:Rac1^{fl/fl} mice (23 weeks) were administered with 4mg/kg *Escherichia coli* 0111:B4 LPS (Merck) intraperitoneally or with a saline solution (0.9% NaCl) (B. Braun). Animals were sacrificed 24 hours after administration and brains were collected for posterior experiments.

Behavioral tests

Procedures were conducted in the dark phase of the light/dark cycle and performed blind to genotypes. The order and interval between tests were used as acclimation for the next test and performed in the following order: (1) three-chamber sociability; (2) novel object recognition; (3) Y-maze; (4) step-down passive avoidance. The experimenter recording the behavioral parameters during test sections was blind to genotypes.

Three-chamber sociability: Mice were first habituated to the empty apparatus, a three-chamber box consisting of three interconnected lined compartments, for 10 minutes. After the habituation phase, mice were tested in the sociability task. In this phase, subject mice socialize with a conspecific mouse in a cage or explore an empty cage in opposite external compartments. All phases during 10 minutes and the placement of stranger mice on the left or right side of the chamber are systematically altered between trials. The time spent in each compartment (four paws have entered the chamber) and the exploration time (direct contact or stretching in 3 – 5 cm around the mouse or empty cage) were measured. All parameters were evaluated by Boris software (Friard & Gamba, 2016). Because female control mice did not significantly enhance sociability parameters, only male mice (both genotypes) were used in this test. Statistical analyses were conducted using randomized-block two-way ANOVA (mice were considered the experimental units; statistical significance was defined as $p < 0.05$) with sphericity assumption and the Turkey correction for multiple comparisons.

Novel object recognition (NOR): The NOR test used experimental procedures similar to the three-chamber test. The test consists of three phases. During the habituation phase, mice can explore the apparatus for 10 minutes (used to perform habituation in three-chamber test, 24 hours before). The next day, during the object familiarization/acquisition phase, two identical objects were placed at the center of each outer chamber. Mice were allowed to explore the objects for 10 minutes freely. Then mice were returned to their home cage, and after 4 hours (inter-trial interval, ITI), the retention phase was performed. In this phase, one of the familiar objects is changed by a novel object, and animals are allowed to explore these objects for 3 minutes. Exploration was defined as follows: the mouse touched the object with its nose, or the mouse's nose was directed toward the object at a distance shorter than 2 cm. The discrimination index (DI) was calculated as before (Socodato, Portugal, et al., 2020) and used as an index of memory function, $DI = (\text{time exploring the novel object}) / (\text{total time spent exploring both objects})$. All parameters were evaluated by Boris software (Friard & Gamba, 2016). Statistical analyses were conducted using randomized-block two-way ANOVA (mice were considered the experimental units; statistical significance was defined as $p < 0.05$) with sphericity assumption and the Turkey correction for multiple comparisons.

Y-maze: Wrong arm returns (# errors), a measure of spatial working memory, were assessed by allowing mice to explore all three arms of the maze, motivated by an innate interest of rodents to explore previously unvisited areas. The mice are positioned in the center of the apparatus and allowed to explore freely for 8 minutes. During the test, visual clues were placed on the walls. Entries in the same arm during the spontaneous alternation phase were considered wrong arm returns and used as the number of working memory errors. Statistical analyses were conducted using randomized-block two-way ANOVA (mice were considered the experimental units; statistical significance was defined as $p < 0.05$) with sphericity assumption and the Turkey correction for multiple comparisons.

Step-down passive avoidance: The step-down test was used to assess the long-term memory and consisted of two phases. First, in the training phase, each mouse was placed in the center of an elevated platform (15 mm above the grid floor), and the step-down from the platform with four paws was immediately followed by a foot shock (0.5 mA) for 2 seconds. The latency to step-down was measured (maximum of 120s; minimum 10 seconds). Afterward, the animals were presented to the retention phase 24 hours after training. This phase was conducted in the absence of shock, and the step-down latency from the platform was recorded (up to 120 seconds) and evaluated as an indicator of memory retention. Statistical analyses were conducted using randomized-block two-way ANOVA with sphericity assumption. The Turkey correction for multiple comparisons was

conducted in both analyses. Moreover, mice were considered the experimental units, and statistical significance was defined as $p < 0.05$.

Morris water maze (MWM): MWM was used to evaluate learning and spatial memory. The apparatus consisted of an opaque grey circular pool (110 cm diameter) filled with water ($21 \pm 1^\circ\text{C}$). Visual cues were placed equidistant in the pool walls. Cued learning was performed during the first two days. A non-visible escape platform (7 x 8 cm) was submerged 1 cm below the water surface in the quadrant center. In this phase, mice were trained to find the hidden platform which had a visual cue. Animals were subjected to four consecutive swimming trials, which had a different start and goal position. In the acquisition phase (4 trials/day for five days), mice were given up to 60 seconds per trial to find the hidden platform and were required to remain seated on the platform for 10 seconds. The platform location was the same during the acquisition phase (center of target quadrant). Escape latency (time to locate the platform) was measured during the cued learning and acquisition phases. During the probe test (day 8), the platform was removed from the pool and each mouse was given up to 30 seconds to search the platform's position. All parameters were evaluated automatically by SMART v3.0 software (Panlab, Spain). Statistical analyses to assess learning were performed using repeated measures two-way ANOVA (mice were considered the experimental units; statistical significance was defined as $p < 0.05$) with the Greenhouse-Geisser correction (to combat sphericity violation). In the probe test, statistical analyses were conducted using unpaired t-test (mice were considered the experimental units; statistical significance was defined as $p < 0.05$).

Tissue preparation and immunofluorescence

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 a sucrose gradient in a row (15 and 30%). After 24 hours, brains were mounted in an OCT embedding medium, frozen, and cryosectioned in the CM3050S Cryostat (Leica Biosystems). Coronal sections from brains (30 μm thickness) were collected non-sequentially on Superfrost ultra plus slides. Tissue sections from controls and experimental mice encompassing identical stereological regions were collected on the same glass slide and stored at -20°C . Frozen sections were defrosted for 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. Alternatively, free-floating tissue sections were collected to 10mL PBS with 0.1%

sodium azide and kept at 4°C. Sections were blocked (10% FBS, 0.2% Triton X-100) for 1 hour. Primary antibodies were incubated in a blocking solution in a humidified chamber overnight at 4°C. Secondary antibodies were incubated for 2 hours (frozen sections) or overnight (free-floating sections) in a blocking solution. After the secondary antibody, sections were washed three times for 10 minutes with PBS. Slides were coverslipped using Fluoroshield (Merck) as mounting medium.

Confocal imaging and morphometric analysis

Images from tissue sections of the neocortex and the dorsal hippocampus were acquired in 8-bit sequential mode using standard TCS mode at 400 Hz. The pinhole was kept at one airy in the Leica TCS SP8 confocal microscope (Leica Microsystems). Images were illuminated with different laser combinations and resolved at 512 x 512 or 1024 x 1024 pixels format using HyD detectors, and entire Z-series were acquired from tissue sections. Equivalent stereological regions were obtained for all tissue sections within a given slide.

To quantify microglia (Iba1⁺ cells) and neurons (NeuN⁺ cells), the number of cells was manually scored, as before (Socodato, Henriques, et al., 2020; Socodato, Portugal, et al., 2020), in stereological identical regions of the neocortex of stained sections (3 images per section; 3 sections per mouse for each experimental group). Statistical analyses comparing genotypes were conducted the Mann-Whitney test (mice were considered the experimental units; statistical significance was $p < 0.05$). Statistical analyses comparing the effect of LPS between genotypes were conducted using two-way ANOVA (tissue sections were considered the experimental units; statistical significance was defined as $p < 0.05$) with the Šidák correction for multiple comparisons.

Sholl analysis was performed in ImageJ by drawing concentric circles around the microglial cell body at defined radius increments. Statistical analyses were conducted using randomized-block two-way ANOVA (single microglia were considered the experimental units; statistical significance was defined as $p < 0.05$) with sphericity assumption and the Šidák correction for multiple comparisons.

To quantify synapses, images from stereological identical neocortical regions from each experimental group (3 images per section; 3 sections per animal for each experimental group) were acquired using a Leica HC PL APO CS 40x /1.10 CORR water objective at 1024 x 1024 resolution with 8-bit bidirectional non-sequential scanner mode at 400 Hz and pinhole at one airy in the Leica TCS SP5 II confocal microscope (Leica Microsystems). Z-

stacks were converted to maximum projection images using the LAS AF routine. Z-projections were background-subtracted using the rolling ball background subtraction built-in algorithm in FIJI, and then images were upsampled using a bicubic interpolation routine. The number of double-positive PSD-95/vGlut1 puncta per μm^2 was manually scored for each image. Statistical analyses comparing two genotypes were conducted using the Mann-Whitney test (tissue sections (3 per mice) were considered the experimental units; statistical significance was defined as $p < 0.05$).

Colocalization analyses were carried out in FIJI using the Coloc2 plugin (https://imagej.net/Coloc_2). Statistical analyses were conducted using randomized-block two-way ANOVA (single microglia were considered the experimental units; statistical significance was defined as $p < 0.05$) with sphericity assumption and the Šidák correction for multiple comparisons.

IMARIS Analysis

All smoothing values and rolling ball radii were empirically determined and then applied to every image. All threshold values were determined specifically for each image.

Microglia morphological analysis: Morphological analysis of microglia was conducted using the filaments function of the IMARIS software (version 10.1.0) (Oxford Instruments). Initially, all images were uploaded and pre-processed using a background subtraction filter with a rolling ball radius of 10 μm . An exaggerated surface object was subsequently created from the Iba1 channel, ensuring the inclusion of every process, using the default smoothing of 0.283 μm . This surface was used to mask the Iba1 channel, which facilitated the creation of the filaments object. During the creation of the filaments object, the soma diameter was adjusted to each cell and reconstructed when possible. The seed point threshold was determined for every image, and the seed points were manually classified. The segment classification option was deactivated and no filter was applied to the segments at the end. Finally, after the filament object creation, every segment was individually verified and any missing or incorrect segments were manually corrected and redrawn using either the auto-depth or auto-path drawing mode.

CD68 and PSD-95 analysis: For each cell, CD68 was masked using the same microglia surface as before and vesicles were reconstructed using the Surface object with a surface smoothing of 0.120 μm . The surfaces were filtered by volume to exclude any that were under 0.01 μm^3 . After reconstruction, the vesicles were checked to ensure they were

all inside the microglia being reconstructed. These surfaces were then used to mask the PSD-95 channel to calculate the volume of PSD-95 inside phagolysosomal vesicles. The same process was used to create the PSD-95 surfaces, but no filtering was performed this time.

PSD-95 spots analysis: To calculate the PSD-95 spot density, the PSD-95 channel was initially pre-processed by subtracting the background using a 1 μm rolling ball radius. A Spots object was then created using a 0.450 μm diameter with the Point Spread Function (PSF) modeling enabled and 0.900 μm elongation. The Different Spot Sizes option was also turned on using local contrast. After performing this for every image, the density was obtained using a constant Region of Interest (ROI) and dividing the number of spots in that ROI by its volume. To calculate PSD-95-microglia contacts, the MATLAB routine 'Spots Close to Filaments' included in the software was used with the Absolute Distance option and three different distances (0.1, 0.2 and 0.3 μm).

Flow cytometry and cell sorting

Microglia were collected from the brains and other tissues of ice-cold PBS-perfused control and mutant mice using density gradient separation as before (Socodato, Henriques, et al., 2020; Socodato, Portugal, et al., 2020). Briefly, tissues were dissected and mechanically homogenized on ice-cold RPMI. Cell suspensions passed through a 100 μm cell strainer and centrifuged in a 70%/30% Percoll gradient. Cells separated at the interface were collected, pelleted, washed and then counted using Countess™ cell counting chamber slides (ThermoFisher Scientific). Single-cell suspensions (5×10^5 cells) were incubated with different mixes of FACS antibodies for 30 minutes at 4°C in the dark. Compensation settings were determined using the spleen from both the control and mutants. Cell suspensions were evaluated on a FACS Canto II analyzer (BD Biosciences). Cell suspensions were seeded in a U bottom 96-well plate. For GDNF staining, cells were treated with Brefeldin A (10 $\mu\text{g/mL}$) for 3 hours at 37°C in RPMI supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco) and 1% Penicillin/Streptomycin (Pen/Strep) (Gibco). Cells were incubated with CD45, CD11b, Ly6C, MHC-II, Annexin-V, or Ki67. After antibody washing, for intracellular staining, cells were fixed in 2% PFA for 30 minutes, washed in PBS, and permeabilized with permeabilization buffer (Life Technologies). Intracellular antibody staining mix was prepared in permeabilization buffer and incubated with the cells for 1 hour at 4°C in the dark. After washing in permeabilization buffer, cells were incubated with Alexa Fluor 488 secondary antibody for 30 minutes at 25°C in the dark. After that, cells were

washed twice in permeabilization buffer, resuspended in FACS staining buffer (2% BSA, 0.1% sodium azide), and analyzed in FACS Canto II (BD Biosciences). Antibody controls for Rac1 and GDNF staining (FMO) were prepared in mixes with CD45-PE and CD11b-Alexa Fluor 647 plus secondary antibodies alone. Cell sorting was performed on a FACS ARIA cell sorter. Data were analyzed by FlowJo X10 software (BD Biosciences).

Statistical analyses comparing three different genotypes were performed using ANOVA (mice were considered the experimental units; statistical significance was defined as $p < 0.05$) with the two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli correction for multiple comparisons. Statistical analyses comparing the effect of EE or CE between genotypes were conducted using two-way ANOVA (mice were considered the experimental units; statistical significance was defined as $p < 0.05$) with the Šidák correction for multiple comparisons.

MACS isolation of microglia

Mice (males) were perfused with ice-cold PBS, and their brains were removed. The right hemisphere was mechanically dissociated in ice-cold Dounce buffer (15mM HEPES; 0,5% Glucose; and DNase) by six strokes in a tissue potter. Homogenate was pelleted by centrifugation, resuspended in MACS buffer (0.5% BSA; 2 mM EDTA in PBS), followed by incubation with 80 μ L myelin removal microbeads. Homogenate was negatively selected using LS columns, pelleted, washed twice, resuspended in MACS buffer, and incubated 10 μ L CD11b microbeads (Miltenyi Biotec) or MojoSort™ Mouse P2RY12 Selection Kit (BioLegend). CD11b⁺ or P2RY12⁺ fraction was selected using LS columns according to the manufacturer's instructions. Enriched fractions were centrifuged (9300 g; 1 min; 4°C) and reserved for RNA isolation or preparation of protein extracts.

RNA sequencing

Library preparation, sequencing and bioinformatics: RNA was isolated using the RNeasy Plus Micro Kit (Qiagen). RNA integrity was analyzed using the Bioanalyzer 2100 RNA Pico chips (Agilent Technologies). Ion Torrent sequencing libraries were prepared according to the AmpliSeq Library prep kit protocol as we did before. Briefly, 1 ng of highly intact total RNA was reverse transcribed. The resulting cDNA was amplified for 16 cycles by adding PCR Master Mix and the AmpliSeq mouse transcriptome gene expression primer

pool. Amplicons were digested with the proprietary FuPa enzyme, and then barcoded adapters were ligated onto the target amplicons. The library amplicons were bound to magnetic beads, and residual reaction components were washed off. Libraries were amplified, re-purified, and individually quantified using Agilent TapeStation High Sensitivity tape. Individual libraries were diluted to a 50 pM concentration and pooled equally. Emulsion PCR, templating, and 550 chip loading were performed with an Ion Chef Instrument (ThermoFisher Scientific). Sequencing was performed on an Ion S5XL™ sequencer (ThermoFisher Scientific) as we did before (Canedo et al., 2021).

Data from the S5 XL run was processed using the Ion Torrent platform-specific pipeline software Torrent Suite v5.12 to generate sequence reads, trim adapter sequences, filter and remove poor signal reads and split the reads according to the barcode. FASTQ and BAM files were generated using the Torrent Suite plugin FileExporter v5.12. Automated data analysis was done with Torrent Suite™ Software using the Ion AmpliSeq™ RNA plugin v.5.12 and target region AmpliSeq_Mouse_Transcriptome_V1_Designed as we did before (Canedo et al., 2021).

Raw data was loaded into Transcriptome Analysis Console 4.0 (ThermoFisher Scientific) and first filtered based on ANOVA eBayes using the Limma package and displayed as fold change. Significant changes had a p-value < 0.05 and FDR < 0.2. Functional enrichment analyses were performed using Gene Set Enrichment Analysis (GSEA) with WEB-based Gene Set Analysis Toolkit (WebGestalt) (Liao et al., 2019), and STRING ranked enrichment (SRE) (Szklarczyk et al., 2019). The whole transcriptome gene list was ranked (linear fold change*-log10 FDR) and submitted to WebGestalt (<http://www.webgestalt.org>) or STRING (https://string-db.org/cgi/input?sessionId=bFzQ6nknJDLN&input_page_show_search=on). Pathway enrichment was performed using the REACTOME database with default settings. Enrichment scores for gene sets were calculated using an FDR cutoff of 0.05.

Enriched pathways were manually recategorized to core transcriptomic modules and are displayed as a network (constructed using Cytoscape). Contingency analyses covering different microglial phenotypes were performed using Fisher's exact test and the Baptista-Pike method to calculate the odds ratio.

RNA-seq data was deposited to the Gene Expression Omnibus repository with the dataset identifier GSE244773.

Gene expression by qRT-PCR

RNA was extracted from flow-cytometry sorted microglia using the Direct-zol™ RNA MiniPrep kit according to the manufacturer's instructions. cDNA synthesis was performed using 300 ng of total RNA (DNase I treated) with SuperScript® III First-Strand Synthesis SuperMix (ThermoFisher Scientific). qRT-PCR was carried out using iQ™ SYBR® Green Supermix on an iQ™5 multicolor real-time PCR detection system (Bio-Rad). The expression of PCR transcripts was calculated using the $2^{-\Delta\Delta Ct}$ with *Yhwaz* serving as the internal control gene. Statistical analyses were performed on raw $2^{-\Delta\Delta Ct}$ values to detect differentially expressed transcripts between sampled groups.

Statistical analyses were conducted using Mann-Whitney (mice were considered the experimental units; statistical significance was defined as $p < 0.05$). The primers used in this study were purchased from Thermo Fisher Scientific. Primer sequences are provided in the Materials section.

Synaptosomal isolation

Hippocampal synaptosomes were freshly isolated using the Syn-PER Synaptic Protein Extraction Reagent (ThermoFisher Scientific), following the manufacturer's recommendations. *Rac1^{fl/fl}* and *Cx3cr1^{CreER+/+}; Rac1^{fl/fl}* housed under CE or EE were perfused with ice-cold PBS. The hippocampus was collected and homogenized using a Dounce tissue grinder with approximately 10 strokes. The homogenate was centrifuged at 1200g for 10 minutes and the pellet was discarded. The supernatant was centrifuged at 15000g for 20 minutes. The resulting synaptosomal pellet was resuspended in Syn-PER Synaptic Protein Extraction Reagent supplemented with protease inhibitor cocktail 2 (1:100) (Merck, Germany and phosphatase inhibitor cocktail 2 (1:1000) (Merck). Protein concentration was determined using the BCA Protein Assay Kit – Pierce (ThermoFisher Scientific). Samples were stored at -80°C until use.

Phospho-proteomics sample preparations

Proteins (100-150 micrograms) from MACS-isolated microglia or hippocampal synaptosomes were processed for phospho-proteomic analysis following the solid-phase-enhanced sample-preparation (SP3) protocol. Enzymatic digestion was performed with

trypsin/LysC (2 micrograms) overnight at 37°C at 1000 rpm. The resulting peptide concentration was measured by fluorescence. Enrichment for phosphorylated peptides was performed using Titanium dioxide beads (TiO₂; ThermoFisher Scientific, USA) as described in the manufacturer's protocol.

High-throughput proteomics data acquisition and quantification

Protein identification and quantitation were performed by nanoLC-MS/MS using an Ultimate 3000 liquid chromatography system coupled to a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (ThermoFisher Scientific). Peptides of each sample were loaded onto a trapping cartridge (Acclaim PepMap C18 100 Å, 5 mm × 300 µm i.d., 160454, ThermoFisher Scientific) in a mobile phase of 2% ACN, 0.1% FA at 10 µL/min. After 3 min loading, the trap column was switched in-line to a 50 cm × 75 µm inner diameter EASY-Spray column (ES803, PepMap RSLC, C18, 2 µm, ThermoFisher Scientific) at 300 nL/min. Separation was achieved by mixing A: 0.1% FA and B: 80% ACN, 0.1% FA with the following gradient: 5 min (2.5% B to 10% B), 120 min (10% B to 30% B), 20 min (30% B to 50% B), 5 min (50% B to 99% B), and 10 min (hold 99% B). Subsequently, the column was equilibrated with 2.5% B for 17 min. Data acquisition was controlled by Xcalibur 4.0 and Tune 2.9 software (ThermoFisher Scientific).

The mass spectrometer was operated in the data-dependent (dd) positive acquisition mode alternating between a full scan (m/z 380-1580) and subsequent HCD MS/MS of the 10 most intense peaks from a full scan (normalized collision energy of 27%). The ESI spray voltage was 1.9 kV. The global settings were as follows: use lock masses best (m/z 445.12003), lock mass injection Full MS and chrom. peak width (FWHM) of 15 s. The full scan settings were as follows: 70 k resolution (m/z 200), AGC target 3×10^6 , maximum injection time 120 ms; dd settings: minimum AGC target 8×10^3 , intensity threshold 7.3×10^4 , charge exclusion: unassigned, 1, 8, >8, peptide match preferred, exclude isotopes on, and dynamic exclusion 45 s. The MS2 settings were as follows: microscans 1, resolution 35 k (m/z 200), AGC target 2×10^5 , maximum injection time 110 ms, isolation window 2.0 m/z , isolation offset 0.0 m/z , dynamic first mass, and spectrum data type profile.

The raw data were processed using the Proteome Discoverer 2.5.0.4 software (ThermoFisher Scientific). Protein identification analysis was performed with the data available in the UniProt protein sequence database for the *Mus Musculus* Proteome (2020_02 version, 55,398 entries) and a common contaminant database from MaxQuant (version 1.6.2.6, Max Planck Institute of Biochemistry). Sequest HT tandem mass

spectrometry peptide database search program was used as the protein search algorithm. The search node considered an ion mass tolerance of 10 ppm for precursor ions and 0.02 Da for fragment ions. The maximum allowed missing cleavage sites was set as 2. For the phosphoproteomics, the IMP-ptmRS node, with the PhosphoRS mode (set to false), was used to localize phosphorylation sites. The Inferys rescoring node was considered, and the processing node Percolator was enabled with the following settings: maximum delta Cn 0.05; decoy database search target False Discovery Rate—FDR 1%; validation based on q-value. Protein-label-free quantitation was performed with the Minora feature detector node at the processing step. Precursor ion quantification used the processing step with the following parameters: Peptides: unique plus razor; precursor abundance was based on intensity; normalization mode was based on the total peptide amount; the pairwise protein ratio calculation and hypothesis test were based on a *t*-test (background based). The Feature Mapper node from the Proteome Discoverer software was used to create features from unique peptide-specific peaks within a short retention time and mass range. This was achieved by applying a chromatographic retention time alignment with a maximum shift of 10 min and 10 ppm of mass tolerance allowed for mapping features from different sample files. For feature linking and mapping, the minimum signal to noise (S/N) threshold was set at 5.

Further downstream analyses were performed using Proteome Discoverer 3.0. Peptide groups were filtered by modifications, excluding those that had no phosphorylation and contained oxidation or deamidation. Peptides with carbamidomethylation were maintained. The table file with the final phosphoprotein list was extracted to Microsoft Excel and peptides were further filtered for each comparison using a custom-made Python script using the following parameters: (1) only master proteins detected with high/medium confidence FDR; (2) a protein/phosphoprotein must be detected in more than 50% of samples in each experimental group (except for proteins that were depleted entirely in one of the experimental groups); (3) the P-value adjusted using Benjamini–Hochberg correction for the FDR was set to < 0.05; (4) at least 50% of samples with protein-related peptides sequenced by MS/MS; (5) the peptide spectrum matches (PSMs) was set to ≥ 3 .

The mass spectrometry proteomic data was deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD045719.

Phosphoproteome bioinformatic analyses

DE phosphoproteins of the datasets were inspected and cross-checked using the comprehensive web portal PhosphositePlus (<https://www.phosphosite.org/homeAction.action>) (Hornbeck et al., 2015). Putative protein kinases controlling the phosphorylation of the filtered phosphopeptides were identified using NetworkKIN 3.0 (<http://networkin.info/index.shtml>) (Horn et al., 2014). DE phosphoproteins were then screened for synaptic interactors/partners using Stitch and Mechismo (Betts et al., 2015). Pathway enrichment analyses (FDR cutoff < 0.05 with the Benjamini-Hochberg multiple test adjustments) were carried out in WebGestalt (Liao et al., 2019) and gProfiler with REACTOME and GO as functional databases. Only annotated proteins were included in the statistical background for pathway enrichment. Network construction and clustering were carried out using k-means clustering in STRING (Szklarczyk et al., 2019), or Enrichment Map (Merico et al., 2010) and Omics Visualizer (Legeay et al., 2020) in Cytoscape.

For phosphoproteomics analyses of microglia in the steady-state, *Rac1^{fl/fl}* and *Cx3cr1^{CreER+}* mice datasets were merged and filtered out using Python to assemble a unique phosphoprotein list compensating the modifications in the microglial phosphoproteome caused by *Cx3cr1* haploinsufficiency.

For network integration of synaptic topographic maps, the set of DE phosphoproteins were first inputted in SynGO. The DE proteins/phosphoproteins were flagged to presynaptic or postsynaptic compartments using SynGO cellular components' anthology (dataset release version 20210225). DE proteins of datasets positively mapped to SynGO were subsequently screened for synaptic interactors/partners using protein-binding interfaces via inter-protein cross-links in the Stitch database (<https://xlink.cncr.nl>) (Gonzalez-Lozano et al.).

Western blotting

Synaptosomes were denatured for 10 minutes at 95°C, separated in 10% SDS-PAGE loaded with 2.5 µg protein per lane. Proteins were transferred to nitrocellulose membranes using a Trans Blot Turbo Transfer System (Bio-Rad). Membranes were blocked in 5% nonfat dry milk in 0.1% Tween20 in TBS (TBS-T) for 1 hour with mild shaking and incubated overnight with primary antibodies at 4°C. Membranes were washed for 30 minutes with TBS-T buffer, incubated with suitable peroxidase-conjugated secondary antibodies for at

least 1 hour at room temperature and incubated for 5 minutes with SuperSignal™ West Pico PLUS Chemiluminescent Substrate (ThermoFisher Scientific). Images were acquired in a ChemoDoc System (Bio-Rad) and quantified using FIJI software.

Adeno-associated virus (AAV) injections and quantification of dendritic spines

Mice were anesthetized by isoflurane inhalation and positioned on the stereotaxic frame. Mice eyes were lubricated to prevent cornea dryness. The animal was maintained with a Stoeling™ Rodent Warmer device with a rectal probe to keep its temperature at 37°C. Deep anesthesia was confirmed by the absence of a response to a toe pinch. The following coordinates were used to target the hippocampus: anteroposterior (AP) -2.3, mediolateral (ML) $\pm$ 1.3 and dorsoventral (DV) -2.2 mm, using the Bregma as reference. Only the right hippocampus of each animal was injected with pAAV-hSyn-mCherry (Addgene viral preparation # 114472-AAV5). The viral titer was 7×10^{12} vg/mL. The red fluorescent protein (mCherry) was driven by the synapsin-1 promoter.

The surgical procedure started with a skin incision in the mouse's head to expose the bregma. After the correct x:y localization, a drill was used to puncture the skull. The needle was slowly introduced into the animal's brain until it reached the right z coordinate. We waited 2 minutes for tissue accommodation before AAV administration. To optimize neuronal labeling to subjacent cortical areas, we injected 0.5 μ L of undiluted viral preparation into the right hippocampus at a rate of 0.1 μ L/min. The needle was removed 2 minutes after administration. The skin was sutured and isoflurane flow was stopped. After waking up, mice were put in a new cage for the first 24 hours, with wet food in the cage bedding, paracetamol in the water bottle and dietary supplements (anima strath and Duphalyte®) to avoid weight loss and malnutrition.

Dendritic spines were reconstructed using IMARIS. Briefly, dendrites were traced with the Filaments tool using the AutoPath algorithm. The dendrite diameter was rebuilt using the shortest distance from the distance map algorithm with the local contrast threshold. Spines were detected with seed points set to 0.3 μ m and the maximum spine length at 3 μ m. The spine diameter was rebuilt using the shortest distance from the distance map algorithm with the local contrast threshold. Statistical analyses were conducted using nested t-test (to combat pseudoreplication) with spine density per dendrite stacked within each mouse (statistical significance was defined as $p < 0.05$).

HMC3 Microglial cell line culture

The human microglia clone 3 (HMC3) cell line was obtained from ATCC (ATCC® CRL-3304™) and cultured in DMEM GlutaMAX (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco) and Pen/Strep (Gibco) in an incubator at 37°C and 5% CO₂. Cells were used up until passage 40 and then replaced with new cultures from low-passage frozen stocks.

Biosensor live cell imaging and Förster Resonance Energy Transfer (FRET)

HMC3 microglia were plated on plastic-bottom culture dishes (μ -Dish 35 mm, iBidi) at a density of 25000 cells/dish and transfected with up to 1 μ g total plasmid DNA using the JetPrime transfection reagent (PolyPlus, France), following the manufacturer's recommendations, with different biosensors. Transfection mix was removed 4 hours after incubation and cells were allowed to express the probes for 48 hours before imaging.

Imaging was performed 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/farRed 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 2 x 2 binning using a digital CMOS camera (ORCA-Flash4.0 V2, Hamamatsu Photonics). Quantifications were performed in FIJI (Schindelin et al., 2012), as described in (Socodato, Melo, et al., 2020), using the precision FRET (PFRET) data processing software package for ImageJ (<https://lvg.virginia.edu/digital-downloads/pfret-data-processing-software>). The mean gray intensity values from ratio images were normalized using the $\Delta F/F_0$ for statistics. Representative images are shown in the intensity-modulated display.

Statistical analyses were conducted using repeated measures two-way ANOVA (individual microglial cells were considered the experimental units; statistical significance was defined as $p < 0.05$) with the Greenhouse-Geisser correction (to combat sphericity violation) and the Šidák correction for multiple comparisons.

Immunofluorescence in cultured microglia

HMC3 microglia were cultured in glass coverslips coated with poly-D-lysine and transfected, as described in the previous subsection and in (Socodato, Melo, et al., 2020). Cells were fixed with 4% PFA and washed with PBS. Then, cells were permeabilized with 0.1% TritonX-100 in PBS for 10 minutes, washed with PBS and blocked for 1 hour at room temperature with 3% bovine serum albumin (BSA) and 0.1% TritonX-100 in PBS. Cells were incubated with primary antibodies overnight at 4°C in a humidified chamber in blocking solution. Coverslips were washed 3 times with PBS and incubated for at least 1 hour at room temperature with the corresponding secondary antibodies. Finally, cells were incubated with 1 µg/mL DAPI in PBS and washed once with PBS and then mounted in glass slides with Fluoroshield (Merck). Coverslips were imaged in a Leica DMI6000B inverted epifluorescence microscope using a PlanApo 63X/1.3NA glycerol immersion objective. Images were acquired using a digital CMOS camera (ORCA-Flash 4.0 V2, Hamamatsu Photonics).

Quantification of Rac1 and Rab27a fluorescence was performed as before (Socodato, Henriques, et al., 2020; Socodato, Portugal, et al., 2020). GDNF quantifications were performed using the PFRET data processing software package for ImageJ. The native 32-bit values from ratio images (GDNF over mCherry or YFP) were used for statistics. Ratio images are shown in intensity-modulated display. Statistical analyses were conducted using unpaired t-test (single cells were considered the experimental units; statistical significance was defined as $p < 0.05$).

High-Content Screening (HCS) imaging in cultured microglia

HMC3 microglia were cultured in 96-well plates (PhenoPlate96) (Perkin Elmer) at a density of 3000 cells/well. Cells were transfected 24 hours after seeding with up to 0.1 µg/well of total plasmid DNA following the manufacturer's recommendations. Cells were stimulated with LPS (100 ng/mL), IL-1 β (1 ng/mL) (Peprotech), IL-6 (50 pg/mL) (Invitrogen) or TNF α (50 pg/mL) (Invitrogen) at given timepoints. Cells were fixed 48 hours after transfection with 4% PFA, washed 3 times with PBS and stored at 4°C until imaging. Cells were imaged using an Opera Phenix Plus high content screening system (PerkinElmer). The plate was pre-scanned to identify YFP⁺ or GFP⁺ cells, according to the biosensors, using a 10x 0.3 air objective. Then, cells YFP⁺ or GFP⁺ cells were re-scanned using the 63x 1.15 water

objective, using excitation at CFP and detecting emission at CFP and YFP (for FRET biosensors) or using excitation of GFP and detection of GFP (for the pGP-CMV-GCaMP6f biosensor). Cell acquisition was limited to 15 fields and z-stack (7 planes, 3 μm stack) images were acquired for each cell. Max projection images were used for analysis. Analysis workflows were adjusted for each experiment, given the distinct spatial distribution of each probe expression. Overall, YFP⁺ or GFP⁺ cell regions were identified. For interleukin stimulation experiments, cells with roundness > 0.4 and touching the field borders were excluded. For cytosolic AMPK activity measurement, cells regions with an area < 600 μm^2 , threshold < 200 and touching the field borders were excluded. For mTORC1 activity, cell regions with an area < 700 μm^2 and touching the field borders were excluded. YFP/CFP ratio or GFP fluorescence was calculated for each individual cells that passed the filters. Image analyses were all performed using the HarmonyTM 5.1 software (PerkinElmer).

Statistical analyses comparing the effect of stimuli between genotypes were conducted using two-way ANOVA (cells were considered the experimental units; statistical significance was defined as $p < 0.05$) with the Šidák correction for multiple comparisons. Analyses comparing only genotypes were performed using the Mann-Whitney test (cells were considered the experimental units; statistical significance was $p < 0.05$). Outliers were removed using the ROUT method ($Q = 1\%$).

Protrusion velocity measurements in cultured microglia

Live cell imaging was conducted to assess the protrusion velocity of HCM3 microglia. A Leica FFW inverted microscope equipped with a temperature-controlled chamber and a CO₂ incubator was utilized to maintain optimal cell conditions (Leica Microsystems). The cells were co-transfected with plasmids encoding Rac1-DN or wild-type Rac1 (WT Rac1) and mCherry-lifeact to visualize actin dynamics. To facilitate cell adhesion, plastic bottom dishes coated with poly-D-lysine were used. Imaging was performed at 10-minute intervals over a total duration of 5 hours. During imaging, appropriate filter sets were employed to visualize the mCherry fluorescence emitted by the cells. Time series were acquired using a 40x objective, ensuring comprehensive coverage of the entire cells and enabling clear visualization of microglial protrusions. Low-intensity illumination and short exposure times were employed to minimize photobleaching and phototoxicity, thereby preserving the integrity of the cells during the imaging process.

Kymographs were generated from the acquired time-lapse images using Fiji/ImageJ software to quantify the protrusion velocity. The mCherry-lifeact channel was used to identify

the leading edge of microglial protrusions. Manual tracking was performed by tracing the outline of individual protrusions over time to extract positional information. The "Segmented Line" tool in ImageJ was utilized to measure the distance traveled by the leading edge between consecutive time points. The time intervals between frames were used to calculate the velocity of protrusion extension. The protrusion velocity assessments were performed by an experimenter blinded to the experimental conditions. Statistical analysis was conducted using a two-way ANOVA.

Primary microglial cultures

Primary cultures of cortical microglia were prepared as before (Canedo et al., 2021; Portugal et al., 2017). Cerebral cortices from Wistar pups (P0 and P2) were dissected and digested with trypsin-EDTA (Gibco) in the presence of DNase (Merck) for 15 minutes at 37°C. Cells were seeded in DMEM Glutamax-I medium supplemented with 10% FBS and 1% Pen/Strep. Cultures were maintained in a humidified incubator at 37°C and 95% air/5% CO₂. Flasks were shaken orbitally (200rpm for 2 hours) to detach microglia and obtain purified microglial cultures.

Collection of microglia conditioned media (MCM)

Cultures were infected with lentiviruses carrying pLKO-empty, pLKO-Rac1 shRNA, or Cre recombinase. After viral removal and medium refeed, microglia were cultured for 5-7 days. Then, the culture medium (MCM) was collected, centrifuged for debris removal (1200 rpm, 5 min), and frozen at -80 °C until used.

Primary cultures of cortical neurons

Brain cortices were dissected from embryonic day 18 Wistar rat embryos and dissociated using trypsin (0.25%, v/v). Neurons were plated at a final density of 1×10^4 to 5×10^4 cells per dish and cultured in the presence of a glial feeder layer. Cells were cultured in phenol red-free Neurobasal medium supplemented with B27 supplement (1:50, v/v), 25 mM glutamate, 0.5 mM glutamine, and gentamicin (0.12 mg/ml). To prevent glial overgrowth, neuronal cultures were treated with 5 mM cytosine arabinoside after 3 days in vitro and maintained

in a humidified incubator with 95% air/5% CO₂ at 37°C for up to 2 weeks, feeding the cells once per week by replacing one-third of the medium.

Single-spine calcium imaging

Cortical neurons were plated on plastic-bottom culture dishes (μ-Dish 35 mm, iBidi) and co-transfected using the calcium phosphate method, with mCherry-Lifeact-7 and pGP-CMV-GCaMP6f. Briefly, a mix of equal amounts of both plasmids (up to a total of 10μg DNA), to which was sequentially added Tris-EDTA, CaCl₂ and HEPES buffered saline, with vortex cycles in between, was prepared and allowed to develop precipitates for 30 minutes in the dark. To reduce toxicity, kynurenic acid was added to the neuronal medium before adding the transfection mix. The mix was added dropwise to the culture dishes and cells were incubated for 3 hours. Experiments were carried out by recording neuronal cultures with microglia-conditioned media (MCM) for 10 minutes (baseline) and then in the presence of MCM + KCl 20 mM (neuronal stimulation) for additional 10 minutes.

Imaging was performed using a Leica DMI6000B inverted microscope. The excitation light source was a mercury metal halide bulb integrated with an EL6000 light attenuator. The L5 (Ex: 460-500; BS: 505; Em: 512-542) and TX2 (Ex: 540-580; BS: 595; Em: 607-683) filter set and a PlanApo 63X 1.3NA glycerol immersion objective were used. Images were acquired with 2 x 2 binning using a digital CMOS camera (ORCA-Flash4.0 V2, Hamamatsu Photonics). The mean gray intensity values from spine GCaMP6f were normalized using the $\Delta F/F$.

Data for activity-dependent spine calcium dynamics were fit using a beta growth and decline model ($Y=Y_m*(1+(T_e-X)/(T_e-T_m))*(X/T_e)^{(T_e/(T_e-T_m))}$) during spine expansion or a plateau followed by one-phase exponential decay ($Y=IF(X<X_0, Y_0, Plateau+(Y_0-Plateau)*exp(-K*(X-X_0)))$) during spine retraction. Spines that collapsed or were eliminated during the evoked neuronal activity were excluded from the analysis. The additional parameters were used for data exclusion: spine expansion < 2.5% spine area; spine retraction < 2% spine area; calcium signal < 25% amplitude increase. Statistical analyses were conducted with single spines measurements as the experimental units (statistical significance was defined as $p < 0.05$).

Statistics

Experimenters were blinded to genotypes and testing conditions whenever possible. Statistical tests and their respective significance thresholds are detailed in each subsection of this Methods section. Descriptive statistics for each dataset are defined in Figure Legends. Statistical analysis and graph construction were performed in GraphPad Prism version 9.0.2 (Dotmatics). Figures were assembled in Adobe Illustrator 2020 (version 24.3). Illustrations were designed in BioRender.

Results

Chapter 1 – Microglial Rac1 is essential for
experience-dependent brain plasticity and
cognitive performance

Introduction

Microglia, the largest myeloid cell population in the central nervous system (CNS), are best known for their protective roles in the brain (Croese et al., 2021). Although long-held as quiescent cells under steady-state conditions, microglia continuously sense and decipher molecular information derived from their local environment (Bernier et al., 2019; Davalos et al., 2005; Nimmerjahn et al., 2005). Such capability strongly relies on actin cytoskeleton-dependent filopodia-like membrane protrusions and an underlying intracellular signaling machinery built on plasma membrane receptors and second messenger systems (Bernier et al., 2019). Microglia dramatically change their morphology and adapt their intracellular signaling program in response to environmental signals released by dying neighbor cells or regions undergoing intense synaptic activity.

Targeted gene ablation experiments indicate that microglia-secreted factors are putative modulators of synaptic activity and plasticity (Ndoja et al., 2020; Nguyen et al., 2020; Parkhurst et al., 2013; Socodato, Portugal, et al., 2020). Even though the microglial secretome conceivably regulates many aspects of the microglia-synapse crosstalk, little is known about the magnitude and specificity of the alterations in synaptic signaling and remodeling driven specifically by microglia (Cserép et al., 2021). Remodeling of the synaptic structure and signaling occurs continuously throughout life, and it is crucial for high-order cognitive functions needed for an organism to adapt to different environmental inputs (Mayford et al., 2012).

Control of actin cytoskeleton reorganization and cell shape, intracellular trafficking involved in the integration of extracellular information, and cell-cell communication by the GTPases of the Rho family, including the most well-characterized members Rac1, RhoA, and Cdc42, make these molecules good candidates to govern microglial sensing capacity and homeostatic mechanisms. In line with this hypothesis, RhoA is a critical negative regulator of microglia immune activation, and its ablation in adult microglia leads to an Alzheimer's disease-like neurodegenerative process (Socodato, Portugal, et al., 2020). Although gene-targeting approaches revealed essential modulatory roles for Rac1 in the CNS, including axonal growth and stability (Hall & Lalli, 2010), oligodendrocyte myelination (Thurnherr et al., 2006), and astrocyte morphology (Zeug et al., 2018), no information is currently available on Rac1 regulation of microglial functions.

Here, using conditional gene targeting in mice together with a set of complementary approaches, we demonstrate that the loss of Rac1 in adult microglia severely disrupts the microglial ability to sense and interpret their local environment, affecting the microglia-

synapse crosstalk required for experience-dependent plasticity and cognitive performance. Furthermore, using multi-omics profiling of microglia, we found that experience-dependent plasticity involves extensive regulation of microglial RhoGTPase signaling (predominantly Rac1), and it is likely to require tight regulation of RhoGTPase-dependent pathways. Our data suggest that modulating RhoGTPase signaling in adult microglia might be useful to devise strategies for boosting neuroplasticity in health and disease.

Results

Conditional ablation of Rac1 in adult microglia

Studies have indicated that Rac1 signaling is affected by aging and brain diseases. In fact, aged mice have shown a significant decrease in Rac1 expression in their brain (Afshordel et al., 2014), which can severely affect microglial function and immune response. Moreover, a correlation has been established between lower Rac1 signaling and Alzheimer's disease (Kikuchi et al., 2020; Rong et al., 2020), which can impact microglial-mediated processes in this context. These findings highlight the critical importance of Rac1 as a molecular target, warranting further investigation in the regulation of microglial functional states.

We aimed to study the effects of targeting Rac1 on adult microglial function, similar to our previous study on RhoA (Socodato, Portugal, et al., 2020). We crossed Cx3cr1^{CreER-IRES-EYFP} mice (Goldmann et al., 2013; Parkhurst et al., 2013; Yona et al., 2013) with mice carrying Rac1 floxed alleles (Chrostek et al., 2006) to investigate this (**Fig. 7A**). In Cx3cr1^{CreER+} mice, Cre recombinase is induced by tamoxifen administration, and the CreER-IRES-EYFP transgene is active in Iba1⁺ brain cells, including microglia (Goldmann et al., 2013; Parkhurst et al., 2013; Yona et al., 2013). We administered tamoxifen to controls and conditional microglia Rac1 mice and analyzed their brains eight weeks later (**Fig. 7A**). Our analysis confirmed a significant reduction of Rac1 protein levels (**Fig. 7B**) and mRNA (**Fig. 7C**) in Cx3cr1^{CreER+/-}:Rac1^{fl/fl} microglia compared to the controls. Notably, Cx3cr1 haploinsufficiency had no impact on Rac1 mRNA or protein levels in Cx3cr1^{CreER+} microglia (**Fig. 7C and Fig. 7D**).

The adverse effects of the CreER-IRES-EYFP transgene in Cx3cr1^{CreER+} mice are mostly limited to the neonatal stage (Sahasrabuddhe & Ghosh, 2022), and we found no differences in the frequency of Rac1⁺ microglia between the genotypes when adult mice did not receive TAM (**Fig. 7D**).

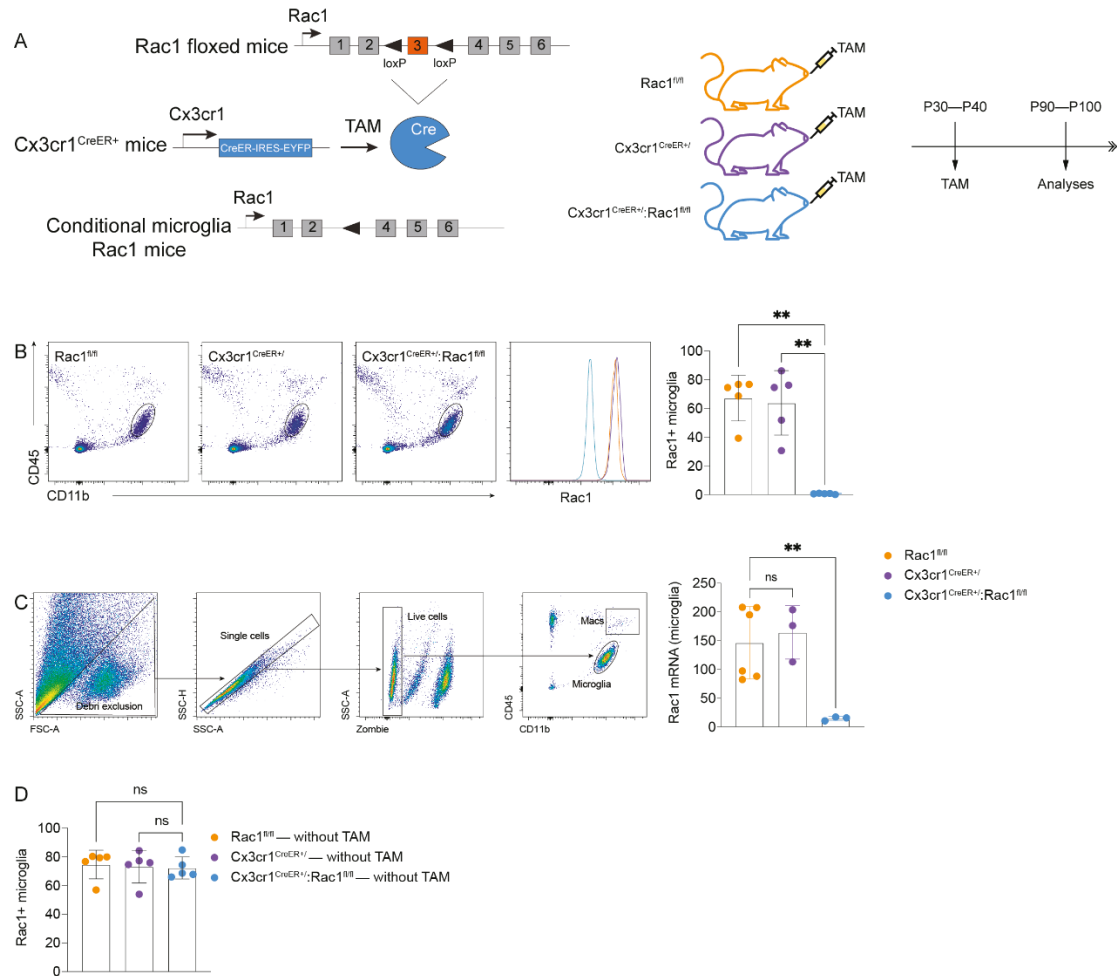


Figure 7 – Rac1 ablation validation in adult microglia. (A) Schematic representation of the strategy used for conditionally ablating Rac1 in adult brain microglia. (B) Flow cytometry analysis showing Rac1 expression levels in microglia from Rac1^{fl/fl}, Cx3cr1^{CreER+} and Cx3cr1^{CreER+}; Rac1^{fl/fl} mice (n = 5 animals per genotype). The histogram represents the mean with standard deviation (SD) of Rac1⁺ microglia. **p < 0.01 (One-way ANOVA). (C) Rac1 mRNA amounts evaluated by qRT-PCR in microglia sorted from Rac1^{fl/fl}, Cx3cr1^{CreER+} and Cx3cr1^{CreER+}; Rac1^{fl/fl} mice (n = 3-6 mice per genotype). **p < 0.01 (One-way ANOVA). (D) Flow cytometry analysis showing Rac1 expression levels in microglia from Rac1^{fl/fl}, Cx3cr1^{CreER+} and Cx3cr1^{CreER+}; Rac1^{fl/fl} mice without tamoxifen (n = 5 mice per genotype) (One-way ANOVA).

In Cx3cr1^{CreER+} mice, the CreER-IRES-EYFP transgene is active, to a lesser extent, in border-associated macrophages in the brain (Goldmann et al., 2016; Socodato, Portugal, et al., 2020). However, we found no significant alterations in Rac1 mRNA in brain resident macrophages (CD11b⁺Cd45^{hi} population) or the frequency of brain macrophages in Cx3cr1^{CreER+}; Rac1^{fl/fl} mice (Fig. 8A). Similar to brain macrophages and contrasting to microglia, we found no significant changes in Rac1 mRNA or the frequency of macrophage populations in the blood or spleen between the genotypes (Fig. 8B and Fig. 8C).

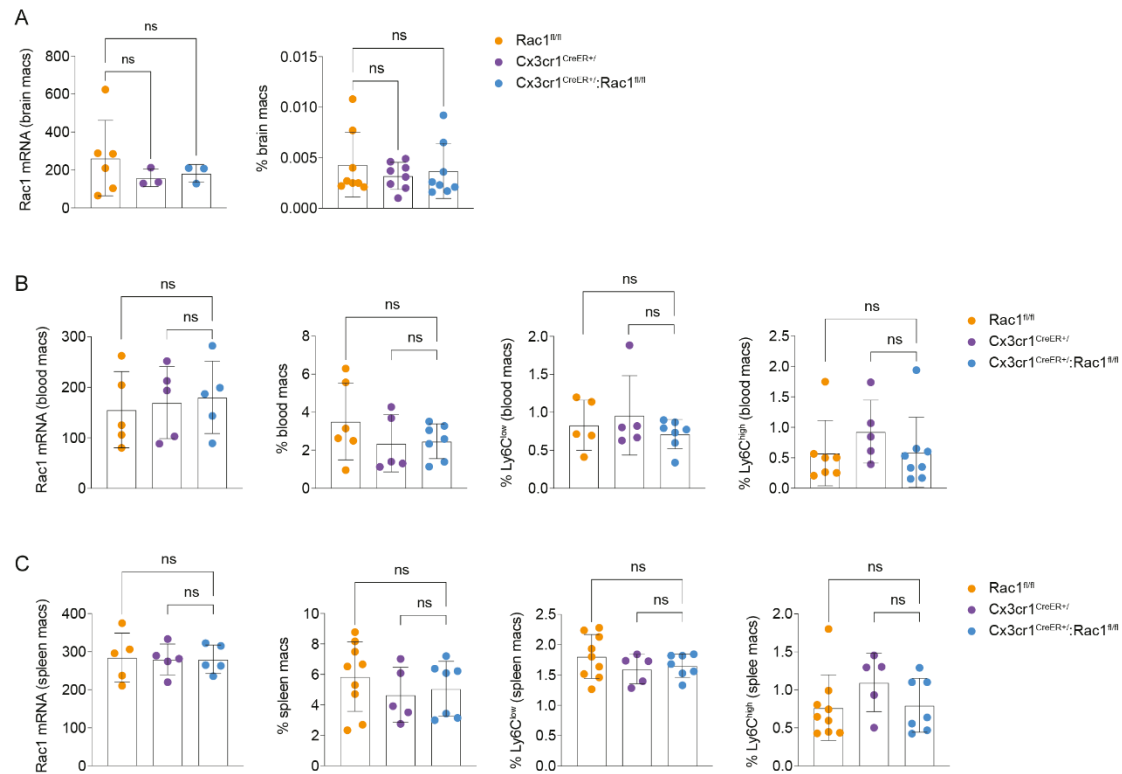


Figure 8 – *Rac1* expression in different macrophage populations. (A, B, C) *Rac1* mRNA amounts evaluated by qRT-PCR in different macrophage populations from $Rac1^{fl/fl}$, $Cx3cr1^{CreER+/+}$ and $Cx3cr1^{CreER+/+}; Rac1^{fl/fl}$ mice ($n=3-9$ mice per genotype) and the frequency of different macrophage populations in the blood and spleen. Histograms display means with SD (One-way ANOVA).

We then evaluated the requirement of *Rac1* for microglia homeostasis in the adult brain. In agreement with a classical role for *Rac1* as a cell shape modifier, we found that adult microglia from $Cx3cr1^{CreER+/+}; Rac1^{fl/fl}$ mice were hyper-ramified (**Fig. 9A**). The impact of *Rac1* on microglial morphology strongly suggests its potential modulation of microglial protrusion dynamics. To investigate this effect, we conducted a comparative analysis of protrusion velocities in HCM3 microglia. Specifically, we overexpressed a dominant negative form of *Rac1* (*Rac1*-DN) and compared it to wild-type (WT) *Rac1*. Our results show a significant decrease in protrusion velocity in microglia overexpressing *Rac1*-DN when compared to cells overexpressing WT *Rac1* (**Fig. 9B**). These findings underscore a potential regulatory role of *Rac1* in microglial protrusion dynamics and cell motility.

These observed morphological changes in microglia were independent of any *Rac1*-related effects on microglial cell numbers. Flow cytometry analysis (**Fig. 9C**) and *Iba1* immunofluorescence on brain tissue sections (**Fig. 9D**) revealed no difference in microglial cell count between $Cx3cr1^{CreER+/+}; Rac1^{fl/fl}$ and control groups. Additionally, no significant differences were found in microglial cell death (annexin V⁺ cells), proliferation (Ki67⁺ cells), surface expression of CD11b and CD45, or the number of major histocompatibility complex II⁺ microglia between the genotypes (**Fig. 9E-I**). These findings suggest that *Rac1*

modulates microglial morphology but is not necessary for regulating adult microglial numbers or reactivity under steady-state conditions.

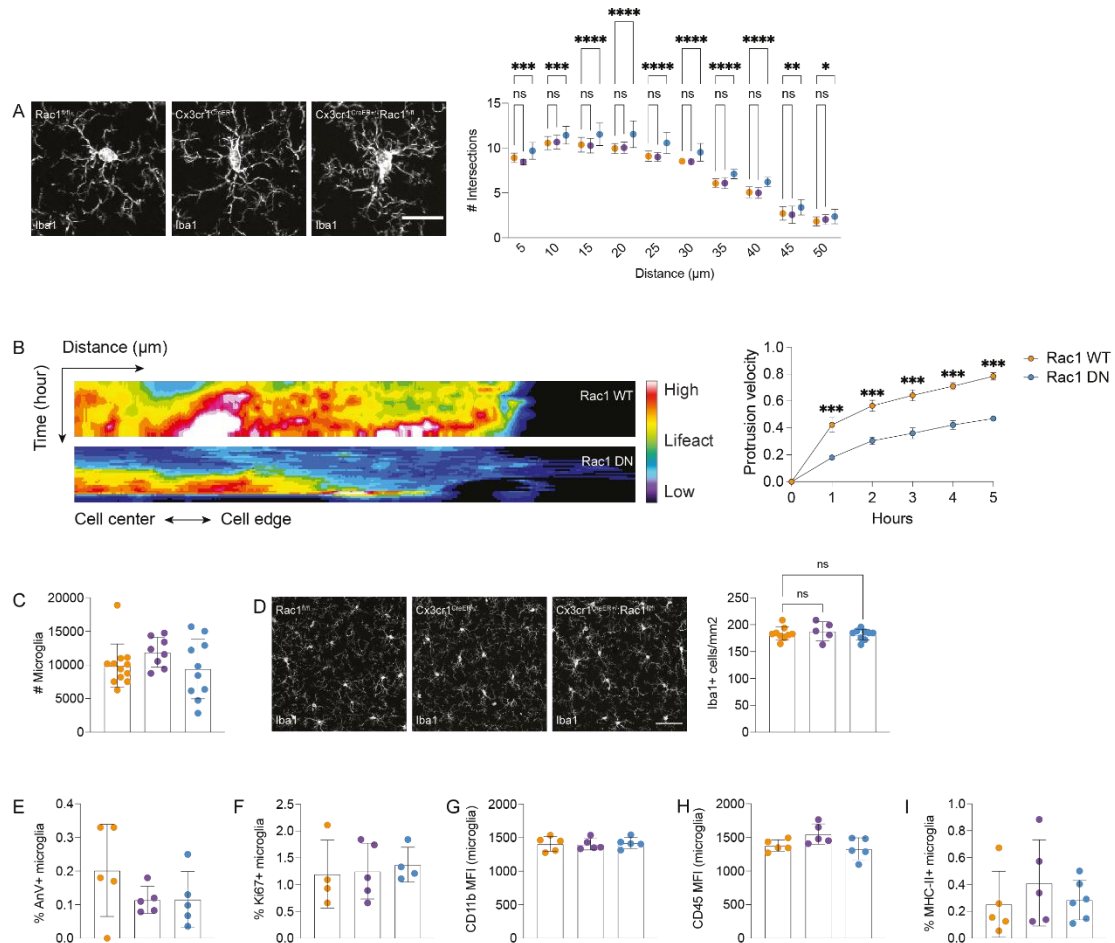


Figure 9 – Rac1 ablation in adult microglia. (A) Sholl analysis of Iba1 immunostaining on tissue sections obtained from neocortices of *Rac1^{fl/fl}*, *Cx3cr1^{CreER+}* and *Cx3cr1^{CreER+}; Rac1^{fl/fl}* mice (n = 25 cells from 3 mice per genotype). The histogram displays the mean with SD. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 (Two-way ANOVA comparing each distance between genotypes). Scale bar: 10 μ m. (B) Kymographs illustrating the comparison of protrusion velocities between HMC3 microglia overexpressing Rac1-DN and WT Rac1 (*Rac1* WT) (n = 9 cells from 3 independent experiments). In both conditions, microglia were transfected with mCherry-Lifeact. Protrusion velocities were measured in μ m/min using kymograph analysis. The histogram shows the mean with SD. ***p < 0.001 (Two-way ANOVA comparing each time point between WT and DN). (C, D) Microglial cell count by flow cytometry (C) or immunofluorescence (D) in the brains of *Rac1^{fl/fl}*, *Cx3cr1^{CreER+}* and *Cx3cr1^{CreER+}; Rac1^{fl/fl}* mice (n = 5-12 mice). Histograms display mean with SD. Scale bar: 50 μ m. (E-H) Microglia cell death (E), proliferation (F), expression of CD11b (G) or CD45 (H), and MHC-II frequency (I) estimated by flow cytometry. Histograms display means with SD.

Rac1 is dispensable for microglia phagocytosis or ROS production in the steady-state

The regulation of active phagocytosis of external ligands (mainly through complement or Fc receptors (Caron & Hall, 1998; Castellano et al., 2000; Cox et al., 1997; Massol et al., 1998)) and the production of reactive oxygen species (ROS) via assembly of NADPH oxidases

(NOXs) (Hordijk, 2006; Werner, 2004) are the best-known roles for Rac1 in immune cells. Thus, we evaluated the requirement of Rac1 for phagocytic function and ROS production in steady-state microglia.

To conduct these analyses, we used RNA sequencing (RNA-seq) on microglia isolated through CD11b selection. We chose this method because Rac1 ablation did not affect other myeloid populations during steady state, which reduces any confounding factors in the study of microglial transcriptomics. Furthermore, this approach is widely used in the microglia field and allows for meaningful comparisons with existing transcriptomics datasets.

Following RNA-seq analysis in MACS-separated microglia (flow cytometry confirmed high microglial enrichment by MACS) (**Fig. 10A**), we investigated the requirement of Rac1 for microglia phagocytosis. As such, we performed contingency analysis between the differentially expressed genes (DEGs) regulated by Rac1 in microglia (**Fig. 10B**) and a microglial engulfment module (Ayata et al., 2018) or a module of prospective phagocytic macrophages (A-Gonzalez et al., 2017). Interestingly and unexpectedly, analyses indicated no significant association between the Rac1-regulated DEGs and the microglia engulfment module (odds ratio = 2.12, P-value = 0.38) or the phagocytic macrophage module (odds ratio = 2.31, P-value > 0.99) (**Fig. 10C**). Then, using double-labeling immunofluorescence on brain tissue sections, we found no significant alterations in the expression of CD68, a glycoprotein mainly associated with the lysosomal compartment and a marker of microglia activity, between $Cx3cr1^{CreER+/-};Rac1^{fl/fl}$ and $Rac1^{fl/fl}$ microglia (**Fig. 10D**). To further explore the role of microglia in synaptic engulfment, we examined the phagocytosis of post-synaptic elements, which serves as a direct measure of complement-dependent microglial synaptic engulfment (Stephan et al., 2012). We found that the amount of postsynaptic material (PSD95⁺) co-localizing with microglial phagocytic structures (CD68⁺) was similar in both $Cx3cr1^{CreER+/-};Rac1^{fl/fl}$ and $Rac1^{fl/fl}$ littermates (**Fig. 10E**). In line with the RNA-seq data, we confirmed by PCR in flow-cytometry sorted microglia from $Cx3cr1^{CreER+/-};Rac1^{fl/fl}$, $Rac1^{fl/fl}$, and $Cx3cr1^{CreER+}$ mice the lack of alteration in the amounts of selected mRNA transcripts from the microglia engulfment module (**Fig. 10F**). These findings suggest that the steady-state engulfment activity of adult microglia does not rely on the presence of Rac1. Moreover, this lack of effect on engulfment activity did not impact neuronal numbers, as the density of NeuN⁺ cells was comparable between $Cx3cr1^{CreER+/-};Rac1^{fl/fl}$ and $Rac1^{fl/fl}$ littermates (**Fig. 10G**).

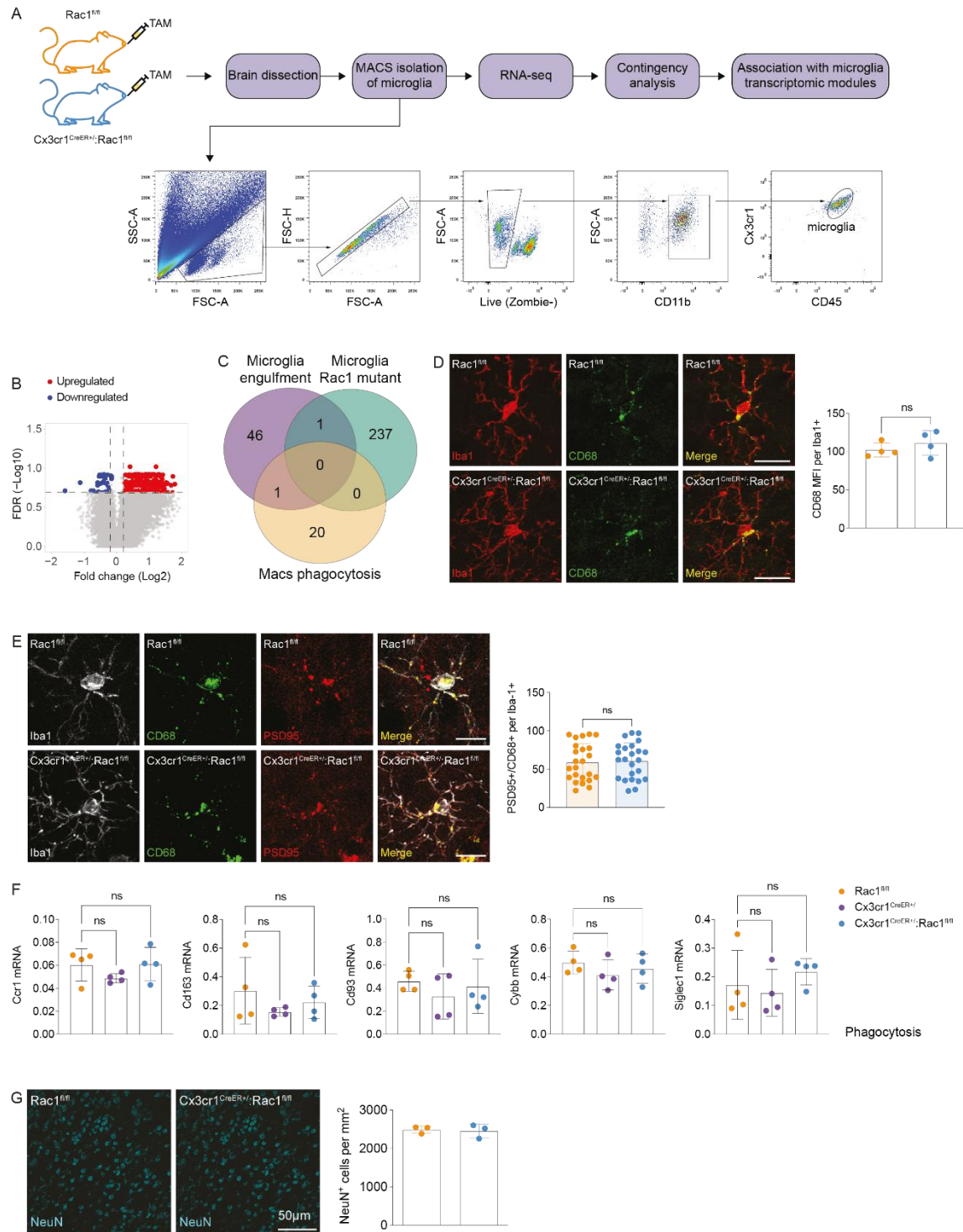


Figure 10 – *Rac1* is dispensable for microglial engulfment of synaptic elements in homeostatic conditions. (A) Workflow of microglial isolation, RNA-seq, and downstream bioinformatics analysis. (B) Volcano plot showing differentially expressed (DE) transcripts retrieved from RNA-seq. After applying FDR correction, the value of p was less than 0.05. (C) Venn diagram with contingency analyses between the DE transcripts (RNA-seq) in $Cx3cr1^{CreER+/+}; Rac1^{fl/fl}$ vs. $Rac1^{fl/fl}$ and transcriptomic programs for microglial engulfment or macrophage phagocytosis. (D, E) Confocal analysis of Iba1, CD68, and PSD-95 on tissue sections from neocortices of $Rac1^{fl/fl}$ and $Cx3cr1^{CreER+/+}; Rac1^{fl/fl}$ mice ($n = 4$ mice per genotype (D) or $n = 24$ cells from 4 different mice per genotype (E)). Histograms show the mean with SD. Scale bars: 20 μm (D) or 10 μm (E). (F) mRNA amounts evaluated by qRT-PCR in microglia sorted from the brains of $Rac1^{fl/fl}$, $Cx3cr1^{CreER+/+}$, and $Cx3cr1^{CreER+/+}; Rac1^{fl/fl}$ mice ($n = 4$ mice per genotype). Histograms display means with SD. (G) Confocal analysis of NeuN on tissue sections from neocortices of $Rac1^{fl/fl}$ and $Cx3cr1^{CreER+/+}; Rac1^{fl/fl}$ mice ($n = 3$ mice per genotype). Scale bar: 50 μm .

To investigate the requirement of Rac1 for steady-state microglial ROS generation, we performed a contingency analysis between the microglial RNA-seq-derived DEGs and a transcriptomic module of microglial oxidative stress (Mendiola et al., 2020). These analyses indicated no significant association between the microglia oxidative stress module (odds ratio = 0.62, P-value = 0.53) and the Rac1-regulated DEGs (**Fig. 11A**). Furthermore, we used flow cytometry to study whether the absence of Rac1 in microglia affects their production of ROS. To do this, we injected control and Rac1 mutant mice with the redox-sensitive probe dihydroethidium (DHE) and measured oxidative stress levels in their microglia. The results revealed no significant difference in the frequency of DHE-positive microglia between the genotypes (**Fig. 11B**). This indicates that removing Rac1 from microglia does not have a significant impact on their oxidative stress levels.

Lastly, using a NOX biosensor (Pal et al., 2013), we measured baseline ROS generation derived specifically from NOX in primary microglia lacking Rac1. Primary microglial cultures prepared from Rac1^{fl/fl} brain cortices were first infected with control (empty) lentiviral particles or virus-carrying Cre recombinase (validation of Rac1 ablation is shown in **Fig. 11C**) and then transfected with the NOX biosensor — composed of redox-sensitive GFP fused to the NOX organizer protein p47 (Pal et al., 2013). As shown in **Fig. 11D**, the amounts of NOX-derived ROS were comparable between control and Cre-infected cultures. In line with the RNA-seq data, we confirmed by PCR in flow-cytometry sorted microglia from Cx3cr1^{CreER+/+}:Rac1^{fl/fl}, Rac1^{fl/fl}, and Cx3cr1^{CreER+} mice the lack of alterations in the amounts of selected mRNA transcripts from the microglia oxidative stress module (**Fig. 11E**). We concluded that Rac1 is not a primary redox driver in adult microglia under steady-state conditions.

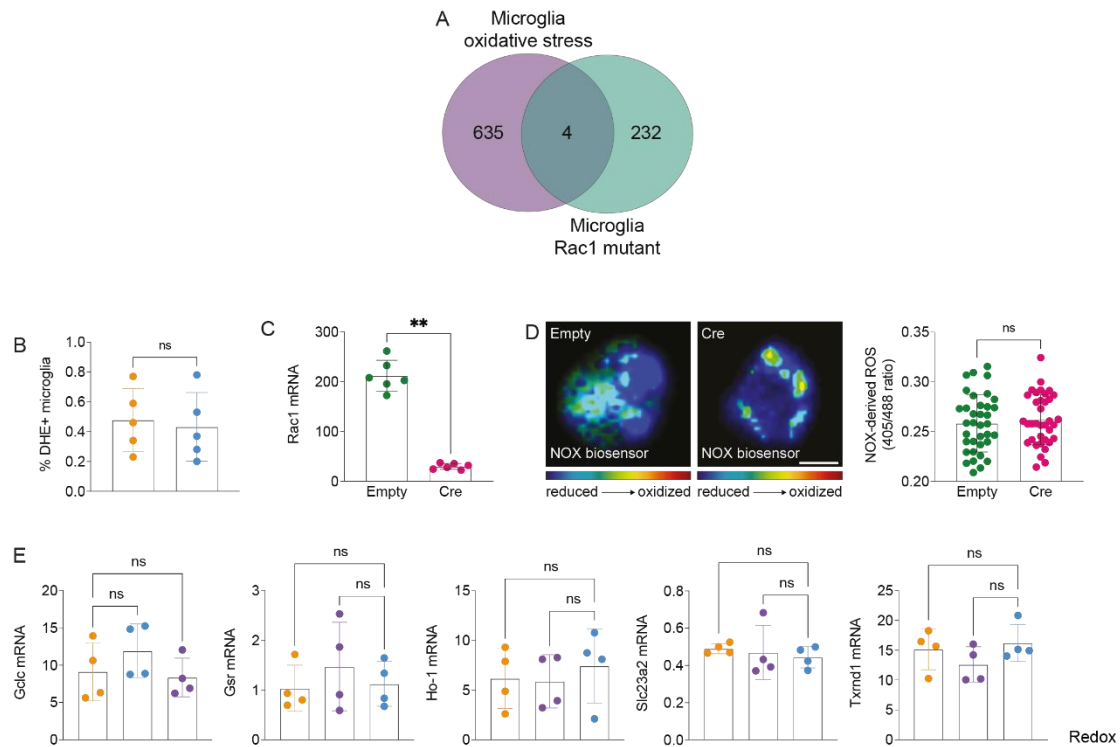


Figure 11 – *Rac1* is dispensable for microglial ROS production in the steady-state. (A) Venn diagram with contingency analyses between the DE transcripts (RNA-seq) in $Cx3cr1^{CreER+/+}; Rac1^{fl/fl}$ vs $Rac1^{fl/fl}$ and a transcriptomic program of microglial oxidative stress. (B) Flow cytometry analysis of DHE frequency in microglia from $Rac1^{fl/fl}$ and $Cx3cr1^{CreER+/+}; Rac1^{fl/fl}$ mice ($n = 5$ mice per genotype). Histogram shows the mean with SD. (C) *Rac1* mRNA amounts evaluated by qRT-PCR in primary cortical microglia from $Rac1^{fl/fl}$ mice. Cultures were transduced with lentiviruses carrying control or Cre recombinase ($n = 6$ independent cultures). Histogram displays mean with SD. $**p < 0.01$ (Mann-Whitney test). (D) Control or Cre-transduced primary cortical microglia from $Rac1^{fl/fl}$ mice expressing a NOX biosensor were compared. Histogram (mean with SD) shows ratio image changes ($n = 36$ cells for each condition pooled across 6 different experiments). Pseudocolor ramps display the levels of reduced-to-oxidized ratio values of the biosensor. Scale bar: 10 μm . (E) mRNA amounts of redox-associated transcripts evaluated by qRT-PCR in microglia sorted from brains of $Rac1^{fl/fl}$, $Cx3cr1^{CreER+/+}$, and $Cx3cr1^{CreER+/+}; Rac1^{fl/fl}$ mice ($n = 4$ mice per genotype).

In addition, contingency analyses (Fig. 12A) using known microglial signature modules (including inflammation (Sousa et al., 2018) — odds ratio = 0.69, P-value = 0.003; disease-associated (Keren-Shaul et al., 2017) — odds ratio = 1.37, P-value = 0.52; injury-related (Hammond et al., 2019) — odds ratio = 0.25, P-value = 0.27; and aging (Hammond et al., 2019) — odds ratio = 0.61, P-value = 0.99) showed no relevant association with the *Rac1*-regulated DEGs in steady-state conditions, which was corroborated by PCR (using selected genes of each cluster) in flow-cytometry sorted microglia from $Cx3cr1^{CreER+/+}; Rac1^{fl/fl}$, $Rac1^{fl/fl}$, and $Cx3cr1^{CreER+/+}$ mice (Fig. 12B-D).

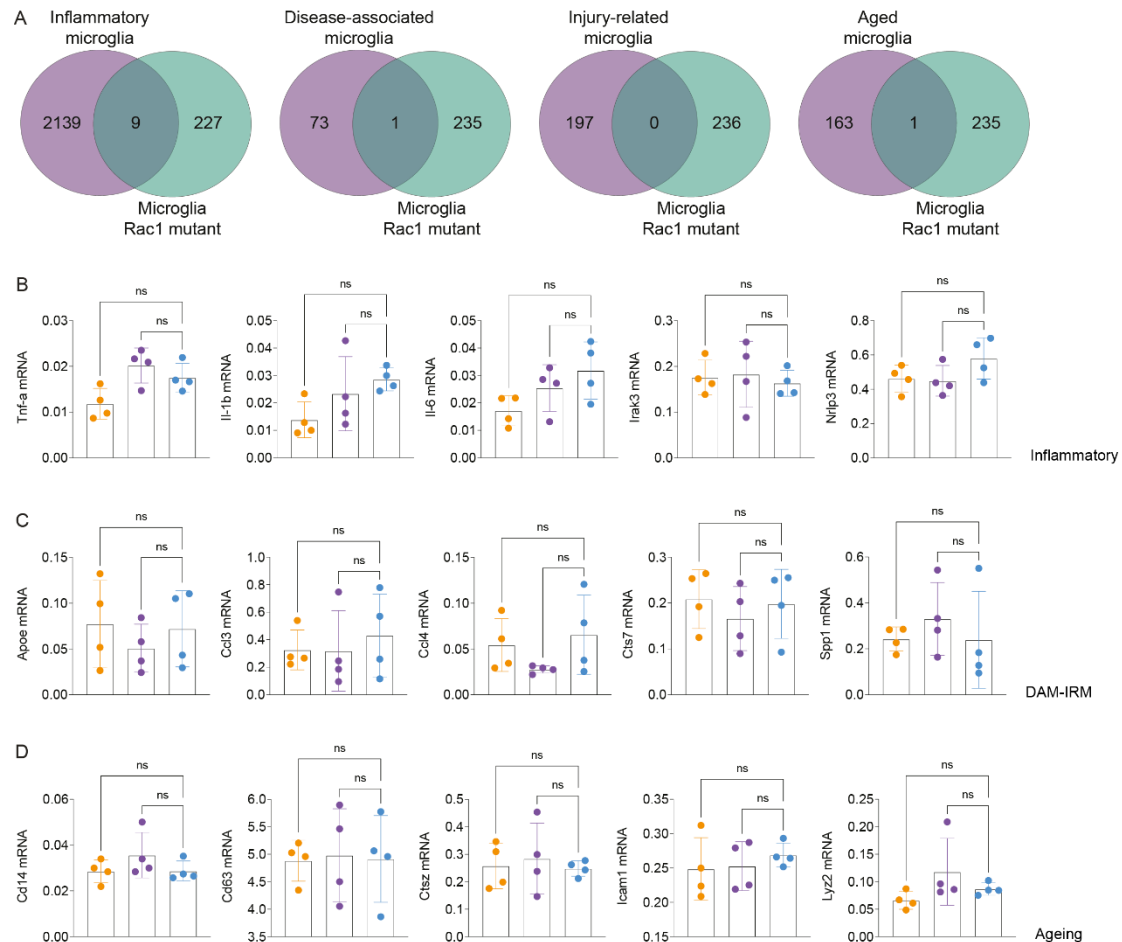


Figure 12 – *Rac1* cKO microglia do not shift towards inflammatory, disease/injury or aged phenotypes. (A) Venn diagrams with contingency analyses between the DE transcripts (RNA-seq) in *Cx3cr1^{CreER+/+};Rac1^{fl/fl}* vs *Rac1^{fl/fl}* and other microglial transcriptomic programs. (B-D) mRNA amounts of inflammatory (B), DAM-IRM (C), and ageing (D) associated transcripts evaluated by qRT-PCR in microglia sorted from the brains of *Rac1^{fl/fl}*, *Cx3cr1^{CreER+/+}*, and *Cx3cr1^{CreER+/+};Rac1^{fl/fl}* mice (n = 4 mice per genotype).

Rac1 coordinates a signaling program required for microglia-synapse crosstalk

To pinpoint the most relevant biological pathways altered in the microglial transcriptome after *Rac1* ablation, we performed pre-ranked gene set enriched analysis (GSEA) and String Ranked Enrichment (SRE). GSEA and SRE revealed significant upregulation of 57 and downregulation of 198 biological pathways derived from the microglia transcriptome following *Rac1* ablation. Manual trimming and analogy recategorizing relevant pathways to build logical signaling modalities led to the classification of main microglial transcriptomic programs regulated by *Rac1*: microglial environmental sensing and microglia-synapse crosstalk (Fig. 13A).

The microglia-synapse crosstalk program included two modules: intracellular protein trafficking, sorting, and export/secretion, and extracellular matrix (ECM)/growth factor

signaling (**Fig. 13A**). The protein trafficking/export/secretion module included pathways related to Rab, Ras, and Rho GTPases, Rab-dependent protein trafficking, and cargo flux through microglial endomembranes. The ECM/growth factor signaling module included adhesion/ECM, glial-derived neurotrophic factor/neural cell adhesion molecule (NCAM)-dependent pathway, EPH-ephrin signaling, and synaptic function/transmission (**Fig. 13A**).

To understand better the signaling network affected by Rac1 in microglia we used quantitative label-free phosphoproteomics in MACS-isolated microglia obtained from Cx3cr1^{CreER+/+}:Rac1^{fl/fl} and control (Rac1^{fl/fl} and Cx3cr1^{CreER+}) mice. After unbiased high-confidence mapping and meticulous phosphosite annotation, we found that the microglial phosphoproteome modulated by Rac1 featured modifications in the abundance of 273 phosphopeptides (corresponding to 206 phosphoproteins), 90 upregulated and 183 downregulated (**Fig. 13B**). To extract consensual information related to microglial signaling transduction, we combined pathway analyses and gene ontology using the 206 matched phosphoproteins and mapped the phosphoproteome-based microglial signaling controlled by Rac1 (**Fig. 13C**).

Supporting the Rac1-dependent microglia-synapse crosstalk program (retrieved from RNA-seq data), the Rac1-induced changes in the microglial phosphoproteome were mainly associated with six signaling modules: RhoGTPase and MAPK signaling, intracellular trafficking/vesicular transport, Ca²⁺/CaM/PLC pathway, protein-protein interaction at synapses, and synaptic transmission (**Fig. 13C**).

The RhoGTPase signaling module displayed alterations mainly in the Rho activity cycle, Rho-dependent activation of PAKs and ROCKs, and RhoGTPases effectors (including Ppp1r12a, Dync1i1, Prkcb, and non-muscle myosins (Myh9 and Myh10)). The MAPK signaling module displayed alterations in BRAF, RAF1, and MAPK1 and MAPK3 pathways. The intracellular trafficking and vesicular transport module displayed alterations in membrane and vesicle trafficking, clathrin-mediated endocytosis, and COPI-mediated anterograde transport. The module of Ca²⁺/CaM/PLC pathway displayed alterations specifically in calmodulin-induced events, and DAG and IP3 signaling leading to phospholipase C-regulated events. The module of protein-protein interaction at synapses displayed alterations in neuroligins and neuroligins, interactions between semaphorins, L1CAM, and L1-ankyrins, and EPH-ephrin signaling. The synaptic transmission module displayed alterations in the release of several neurotransmitter systems along with post-synaptic activity and plasticity, including pathways related to glutamatergic transmission.

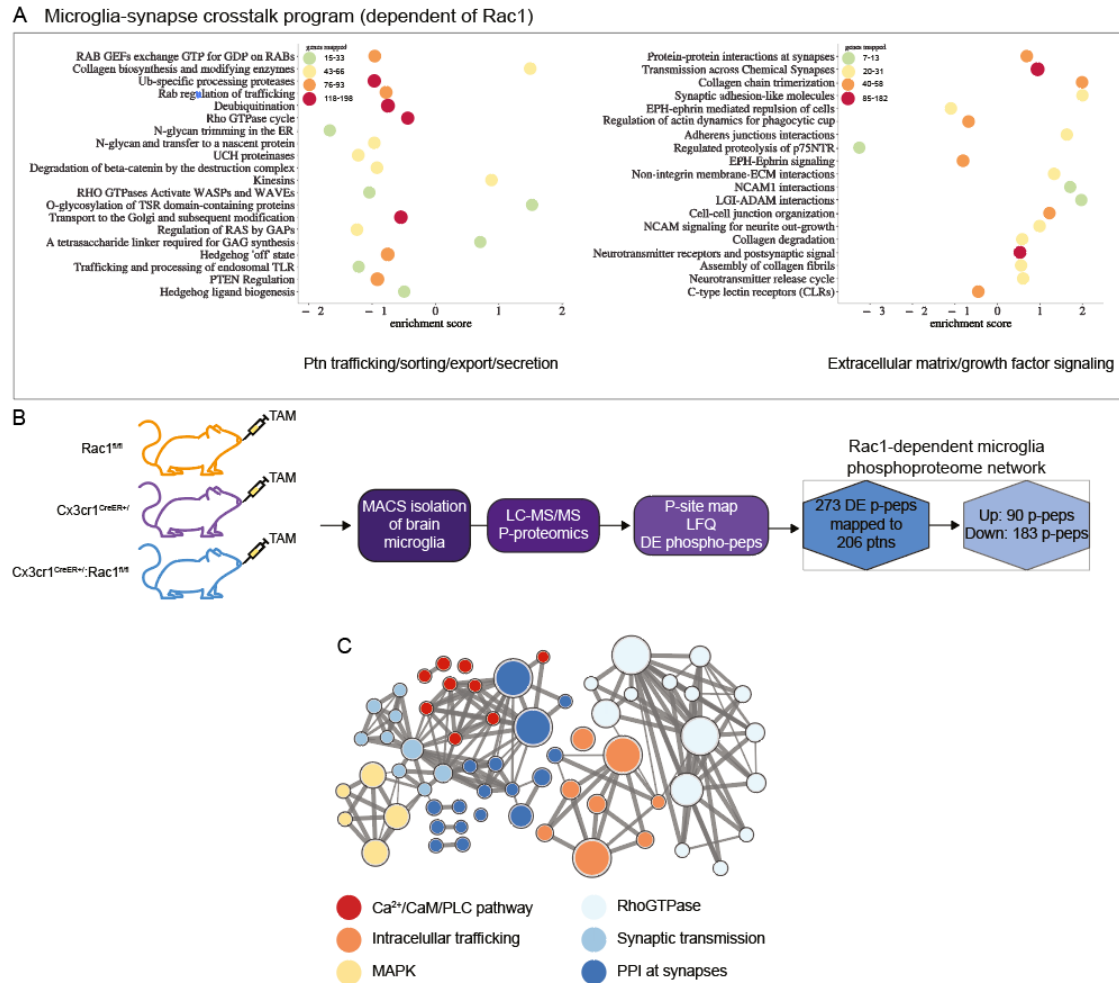


Figure 13 – The microglial *Rac1* signature. (A) *Rac1*-dependent microglia transcriptomic signature revealed by analogy concatenation of GSEA and SRA ($n = 3$ mice per genotype). Interaction networks display the top enriched pathways. (B) Workflow of microglial isolation, high-throughput phosphoproteomics, p-site mapping, and downstream bioinformatics analysis for generating *Rac1*-dependent phosphoproteomic networks. After applying FDR correction, the value of p was less than 0.05 for each phosphosite. (C) *Rac1*-dependent phosphoproteomic networks ($n = 0$ 4-5 mice per genotype). Networks display the top enriched pathways within each module. Node (pathway) is scaled according to the number of phosphoproteins within it. Edge thickness is scaled according to the degree of pathway crosstalk.

To functionally validate the microglial phosphoproteome data, we studied microglial signaling integration via calcium-related pathways using fluorescent biosensors and live-cell imaging in microglial cultures (**Fig. 14A**), as we reported previously (Canedo et al., 2021; Socodato, Henriques, et al., 2020; Socodato, Portugal, et al., 2020; Socodato et al., 2018). As key external cues driving calcium-related pathways, we used phosphatidylcholine (PC; a major modulator of microglial lipid cascade) or ATP (a classical danger-associated molecular pattern for microglia). To analyze the lipid sensing cascade, we exposed microglia to PC and measured, in real-time, the local production of Dag (a second messenger generated by phospholipase C activation) and the dynamics of Ca^{2+} between the endoplasmic reticulum (ER) and the cytosol. Using the DagLas biosensor (Sato et al., 2006), we observed that PC caused a significant increase in Dag production in the

protrusions of control microglia, an effect abrogated in Rac1 knocked down (KD) microglia (**Fig. 14B**). Using the D1ER biosensor (Palmer et al., 2004), we also found that Rac1 KD significantly blocked the PC-induced release of Ca^{2+} from the ER into the cytosol of microglia (**Fig. 14C**). Then, we studied ATP signaling in Rac1-deficient microglia. We monitored global cytosolic Ca^{2+} mobilization and PKC activity in living microglia to study ATP signaling. Using the Ca^{2+} sensor RGECO (Wu et al., 2013) and the FRET-based PKC activity reporter CKAR (Violin et al., 2003), we observed that ATP increased Ca^{2+} mobilization and PKC activation in microglia overexpressing WT Rac1 (**Fig. 14D and Fig. 14E**); however, overexpressing a dominant-negative Rac1 mutant (Rac1^{T17N}) attenuated the ATP effect significantly (**Fig. 14D and Fig. 14E**).

Overall, our combined RNA-seq, phosphoproteome, and live-cell imaging data raise the hypothesis that microglia modulation of synaptic plasticity through Ca^{2+} -dependent secretion of chemokines, cytokines, and growth factors requires Rac1 signaling.

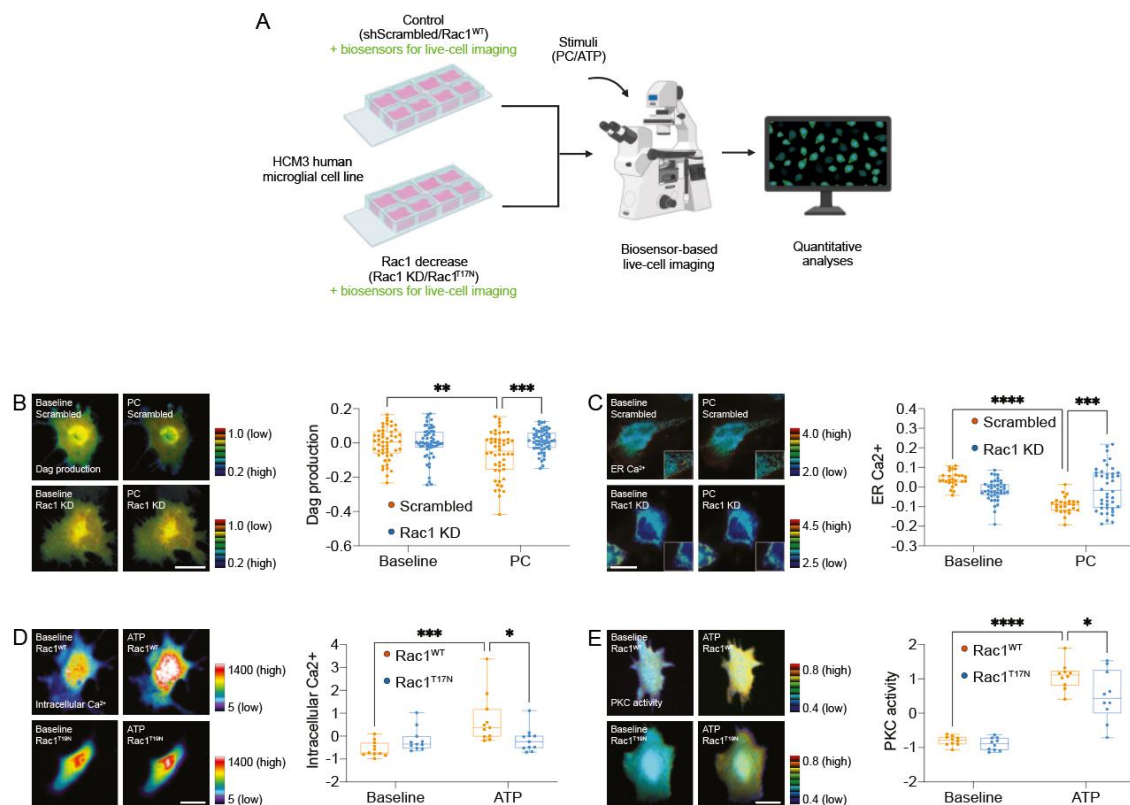


Figure 14 – Rac1-dependent microglia signaling integration via Ca^{2+} -related pathways. (A) Workflow of biosensor-based live-cell imaging in microglia. (B) Control (scrambled) or Rac1 KD HMC3 microglia expressing a Dag FRET biosensor were recorded in saline before (t = 0 min; baseline) and after treatment with 10 ng/mL PC. Whisker plots (median and min/max) show time-lapse FRET/donor ratio changes (n = 50-58 cells pooled across 3 different experiments). Pseudocolor ramps display representative ratio values. **p < 0.01; ***p < 0.001 (Two-way ANOVA). Scale bar: 5 μm . (C) Control (scrambled) or Rac1 KD HMC3 microglia expressing an ER Ca^{2+} FRET biosensor were recorded in saline before (t = 0 min; baseline) and after treatment with 10 ng/mL PC. Whisker plots (median and min/max) show time-lapse FRET/donor ratio changes (n = 26-42 cells pooled across 3 different experiments). Pseudocolor ramps display representative ratio values. ***p < 0.001; ****p < 0.0001 (Two-way ANOVA). Scale bar: 5 μm . (D) Control (Rac1^{WT}) or Rac1 dominant-negative (Rac1^{T17N}) HMC3 microglia expressing a cytosolic Ca^{2+} biosensor were recorded in saline before (t = 0 min; baseline) and after treatment with 100 μM ATP. Whisker plots (median and min/max) show

time-lapse fluorescence changes ($n = 11$ cells per group pooled across 3 different experiments). Pseudocolor ramps display representative fluorescence values. $*p < 0.05$; $***p < 0.001$ (Two-way ANOVA). Scale bar: 20 μm . **(E)** Control (Rac1^{WT}) or Rac1 dominant-negative ($\text{Rac1}^{\text{T17N}}$) HMC3 microglia expressing a PKC FRET biosensor were recorded in saline before ($t = 0$ min; baseline) and after treatment with 100 μM ATP. Whisker plots (median and min/max) show FRET/donor ratio changes ($n = 10$ cells per group pooled across 3 different experiments). Pseudocolor ramps display representative ratio values. $*p < 0.05$; $****p < 0.0001$ (Two-way ANOVA). Scale bar: 20 μm .

Experience remodels the microglial signaling network in a Rac1-dependent manner

Experience-dependent plasticity is the capacity of brain cells and circuits to change and adapt to environmental inputs and learning (Fu & Zuo, 2011) and occurs in brain synapses but also in glial cells. Thus, we ought to demonstrate the magnitude and signaling specificities driven by experience in adult microglia in the presence or absence of Rac1. For that, we used an environmental enrichment (EE) paradigm in which increasing sensory, motor, and cognitive stimulation over standard mouse housing conditions, is used to promote experience-dependent brain plasticity. Then, we used quantitative label-free phosphoproteomics in MACS-isolated microglia obtained from $\text{Rac1}^{\text{fl/fl}}$ and $\text{Cx3cr1}^{\text{CreER+/-}};\text{Rac1}^{\text{fl/fl}}$ mice housed under EE or control environment (CE) (**Fig. 15A**) to pinpoint experience-dependent changes in the microglia.

To carry out our analyses, we MACS-isolated microglia through P2RY12 selection. This selection method helps to minimize contamination from non-microglial cells, as P2RY12 is exclusively expressed in microglia. The reason for this selection strategy was due to uncertainties regarding the effects of EE on non-microglial myeloid populations. Unlike CD11b, which is expressed by other brain myeloid cells, P2RY12 selection circumvents potential bias in evaluating the impact of EE on microglia in both control and Rac1 mutants.

Analyses of the microglial phosphoproteome driven by experience identified a total of 13,018 raw peptides. Following the removal of low peptide-spectrum matches and non-related post-translational modifications, the number of detected phosphosites was refined to 4,521. Subsequently, applying an FDR cutoff of <0.05 revealed experience-dependent phosphoproteome modifications in control ($\text{Rac1}^{\text{fl/fl}}$) mice, comprising alterations in 370 phosphopeptides, corresponding to 273 distinct phosphoproteins. Among these modifications, 61 phosphopeptides were upregulated, while 309 phosphopeptides were downregulated (**Fig. 15B**). To extract consensual information related to microglial signaling transduction, we combined pathway analyses and gene ontology using the 273 matched phosphoproteins and found that experience profoundly remodeled the microglial phosphoproteome in control ($\text{Rac1}^{\text{fl/fl}}$) mice (**Fig. 15C**).

Trimming and analogy recategorization of the phosphoproteome dataset allowed the identification of five microglia signaling modules regulated by experience. Specifically, RhoGTPase signaling was the most affected module, followed by synapse crosstalk, MAPK signaling, cell death-related pathways, and RNA metabolism (**Fig. 15C**). Importantly, unbiased pathway clustering showed a hierarchical organization of classical RhoGTPase signaling in microglia during experience-dependent plasticity, with Rac1 activity cycle being modulated more prominently by experience than Cdc42 or RhoA activity cycle (**Fig. 15C**). Protein-protein interaction (PPI) network integration between Rac1/Cdc42/RhoA modules revealed, as expected, a large redundancy of cytoskeleton-related phospho-guanine nucleotide exchange factors (GEFs) and phospho-GTPase-activating proteins (GAPs) (10/18), with RASGRF2 (S924), ARHGAP45 (S23, S577), and GMIP (S436, S440) being common to the three RhoGTPases modules (**Fig. 15D**). In contrast, SOS1 (S1147), TIAM1 (S358), and NCF2 (T233) were unique to the Rac1 module, FGD1 (T710), and ITSN1 (S971) were specific to the Cdc42 module, and DIAPH1 (S22), ARHGAP6 (S348), and TJP2 (S239) were specific to RhoA module (**Fig. 15D**). These data suggest that RhoGTPase signaling (Rac1 predominantly) might play a pivotal role in the microglia phosphoproteome remodeling during experience-dependent plasticity.

To understand the requirement of Rac1 for experience-dependent phosphoproteome remodeling of microglia, we used integrated analyses with Cx3cr1^{CreER/+}:Rac1^{fl/fl} mice housed under EE or CE. Then, we combined set theory, gene ontology, and bioinformatics to map the microglia phosphoproteomic modifications related to Rac1 during experience-dependent plasticity. Out of 437 phosphoproteins affected by experience in this context, 343 (77%) were modulated by Rac1 (**Fig. 15E**). Indeed, the modules associated with microglia RhoGTPase signaling and microglia-synapse crosstalk demonstrated significant dependence on Rac1, whereas the impact of Rac1 on cell death-related pathways and RNA metabolism was relatively minor (**Fig. 15F**). Importantly, Rac1 did not exhibit any effect on the MAPK signaling module in this specific context (**Fig. 15F**).

Furthermore, we observed that EE specifically modulated the phosphorylation levels of various proteins involved in the regulation of Rac1/Cdc42/RhoA signaling modules in control microglia (**Fig. 15G**). However, the ablation of Rac1 hindered the EE-induced changes in the phosphorylation patterns of multiple GAPs and GEFs associated with these signaling modules (**Fig. 15G**). This finding suggests that Rac1 plays a critical role in the hierarchical organization of the microglial RhoGTPase network during experience-dependent plasticity.

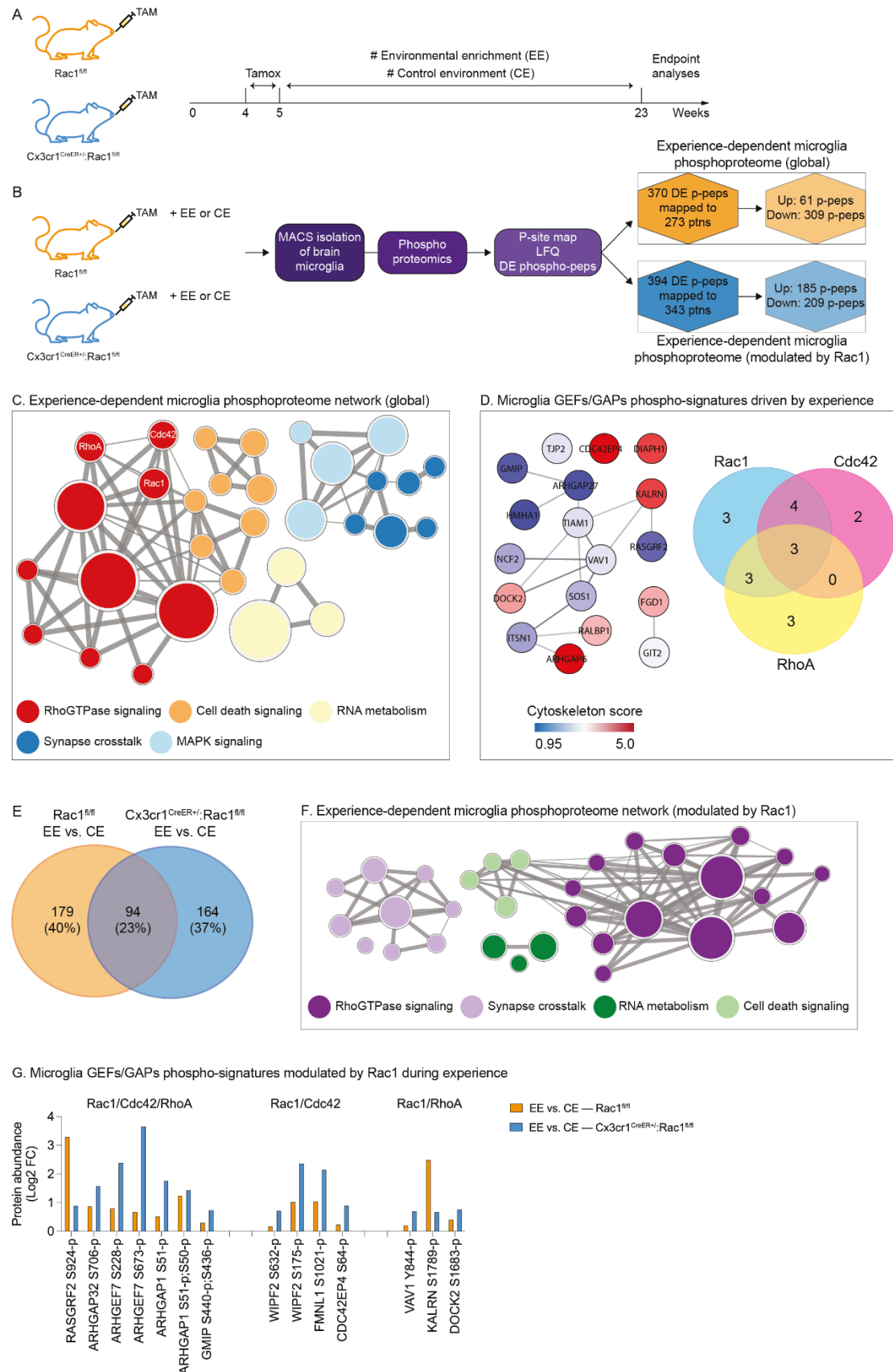


Figure 15 – Experience remodels the microglial phosphoproteome via Rac1. (A) Housing conditions to elicit experience-dependent brain plasticity. (B) Workflow of phosphoproteomic profiling in microglia after experience-dependent plasticity. After applying FDR correction, the value of p was less than 0.05 for each phosphosite. (C) Microglial pathways modulated by experience (EE vs CE) revealed by phosphoproteomic analyses ($n = 4$ $Rac1^{fl/fl}$ – CE mice and 5 $Rac1^{fl/fl}$ – EE mice). Networks display the top enriched pathways

within each module. Node (pathway) is scaled according to the number of phosphoproteins within it. Edge thickness is scaled according to the degree of pathway crosstalk. **(D)** Phospho-PPI hubs of Rac1, Cdc42 and RhoA GEFs and GAPs relative to EE vs CE in $Rac1^{fl/fl}$ mice (retrieved from RhoGTPase signaling module in (C)). Nodes are scaled according to a cytoskeleton score based on the String annotation. The Venn diagram shows the segregation of phosphor-GEFs and GAPs across Rac1, Cdc42 and RhoA signaling hubs. **(E)** Venn diagram allocating the differentially expressed phosphoproteins across housing conditions and genotypes. **(F)** Segmentation of microglial pathways modulated concomitantly by experience and Rac1 ($n = 4$ $Rac1^{fl/fl}$ – CE mice; 5 $Rac1^{fl/fl}$ – EE mice; 4 $Cx3cr1^{CreER+/-}:Rac1^{fl/fl}$ – CE mice; 5 $Cx3cr1^{CreER+/-}:Rac1^{fl/fl}$ – EE mice). **(G)** Abundance of selected Rac1, Cdc42 and RhoA phospho-GEFs and GAPs retrieved from the RhoGTPase signaling module in F. Histograms show mean with SD ($n = 4$ -5 mice per group).

Because Rac1 is a key regulator of microglial phosphoproteomic modifications related to experience-dependent plasticity, we investigated whether these molecular changes influenced microglial morphology. To address this, we examined the morphological parameters of microglia in response to EE in both control and Rac1-deficient microglia. First, we quantified the morphological features of microglia, including the number of segments, segment length, segment volume, and the number of segment terminal points, in $Cx3cr1^{CreER+/-}:Rac1^{fl/fl}$ and control $Rac1^{fl/fl}$ mice housed under either EE or CE. In control ($Rac1^{fl/fl}$) microglia under EE conditions, we observed significant changes in all the measured parameters compared to $Rac1^{fl/fl}$ microglia in the CE group. Specifically, the microglia exhibited increased complexity, as reflected by a significantly higher number of segments, larger segment volumes, and an increased number of segment terminal points (**Fig. 16A and Fig. 16B**). However, when Rac1 was ablated in $Cx3cr1^{CreER+/-}:Rac1^{fl/fl}$ mice, we found that the EE-driven changes in microglial morphology were significantly prevented (**Fig. 16A and Fig. 16B**). These results demonstrate that Rac1 plays a critical role in shaping the morphological response of microglia during experience-dependent plasticity.

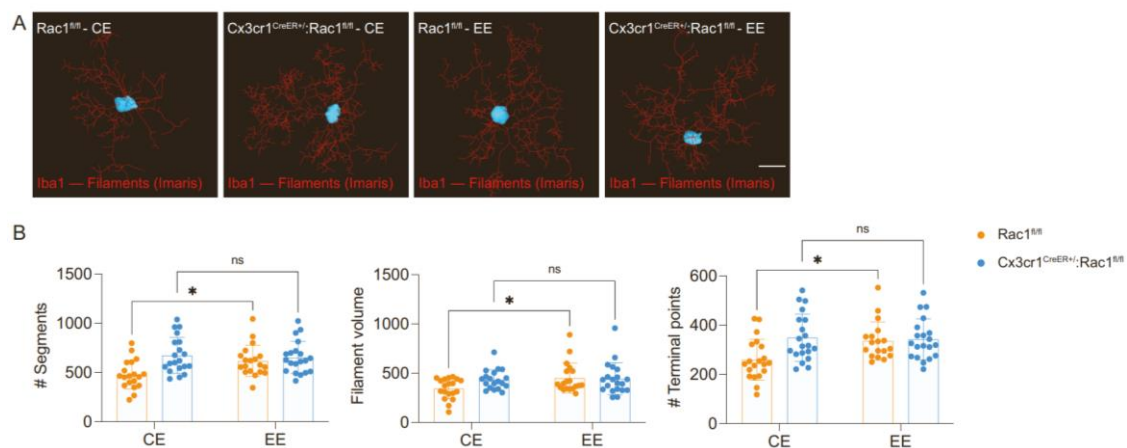


Figure 16 – Rac1-dependent microglia morphology alterations driven by experience. **(A)** Imaris segment rendering of Iba1 on tissue sections from hippocampi of $Rac1^{fl/fl}$ and $Cx3cr1^{CreER+/-}:Rac1^{fl/fl}$ mice ($n = 18$ -20 cells pooled from 4 animals per group). **(B)** Histograms display mean with SD. * $p < 0.05$ (Two-way ANOVA). Scale bar: 10 μm

Experience-dependent synaptic phosphoproteome plasticity requires Rac1 signaling in microglia

Could microglial Rac1 signaling affect microglia-synapse crosstalk to impact experience-dependent synaptic remodeling? Experience-dependent synaptic remodeling relies on multistep phosphorylation of synaptic proteins (Woolfrey & Dell'Acqua, 2015). Thus, we used quantitative phosphoproteomics in hippocampal synaptosomal preparations obtained from *Rac1^{fl/fl}* and *Cx3cr1^{CreER+/+}:Rac1^{fl/fl}* mice housed under EE or CE (**Fig. 17A**) to elucidate changes in the remodeling of the synaptic signaling modulated by microglial Rac1.

We identified 4136 raw peptides in the synaptic phosphoproteome influenced by experience. Applying a false discovery rate cutoff of <0.05 and unbiased high-confidence mapping, we observed modifications in the abundance of 622 phosphopeptides (of 3602), representing 389 phosphoproteins in control mice exposed to EE (**Fig. 17A**). To extract consensual information related explicitly to synaptic pathways, we used over-representation analysis (ORA) with the 389 matched phosphoproteins and found that EE significantly modified over 600 biological processes, 152 molecular functions, and 206 cellular components in the synapses (**Fig. 17B**). Pre- and post-synaptic organization, SNARE complex assembly, secretion of neurotransmitters, GABA and glutamate-dependent synaptic signaling, and post-synaptic cytosolic calcium levels were among the top signaling pathways modulated by EE in *Rac1^{fl/fl}* hippocampus (**Fig. 17B**).

Next, employing set theory, we partitioned the pool of synaptic phosphoproteins to isolate those exclusively regulated by microglial Rac1 during EE (**Fig. 17C**). By integrating ORA, Gene Ontology, and unbiased PPI clustering, we successfully delineated the synaptic signaling modules that were uniquely associated with microglial Rac1 in response to EE (**Fig. 17D**). Specifically, we found that global neurotransmitter release/recycle mechanisms, spine maintenance, post-synaptic architecture, excitatory neurotransmission, and regulation of actin nucleation were the most representative pathways modulated by EE through microglial Rac1 (**Fig. 17D**).

To provide further mechanistic insight into how microglial Rac1-dependent regulation of dynamic phosphorylation events was connected to synaptic function (for instance, neurotransmitter release and excitatory post-synaptic activity) during experience-dependent plasticity, we focused on the prominent Ca^{2+} -dependent synaptic integrator Camk2. After sequence analysis and literature curation, we identified a context- and microglial Rac1-dependent Camk2 circuitry operating simultaneously at pre and post-synaptic compartments (**Fig. 17E**).

Phosphoproteomics profiling detected synaptic supra-phosphorylation of canonical T286 in Camk2a (validated by western blot analyses; **Fig. 17F**) and T287 in Camk2b, both known to cause kinase overactivation in a microglial Rac1-regulated manner (**Fig. 17E**). Increased phosphorylation of S330, known to control binding to Camk2b, Camk2d, and Camk2g, was also modulated by microglial Rac1 during experience-dependent plasticity (**Fig. 17E**).

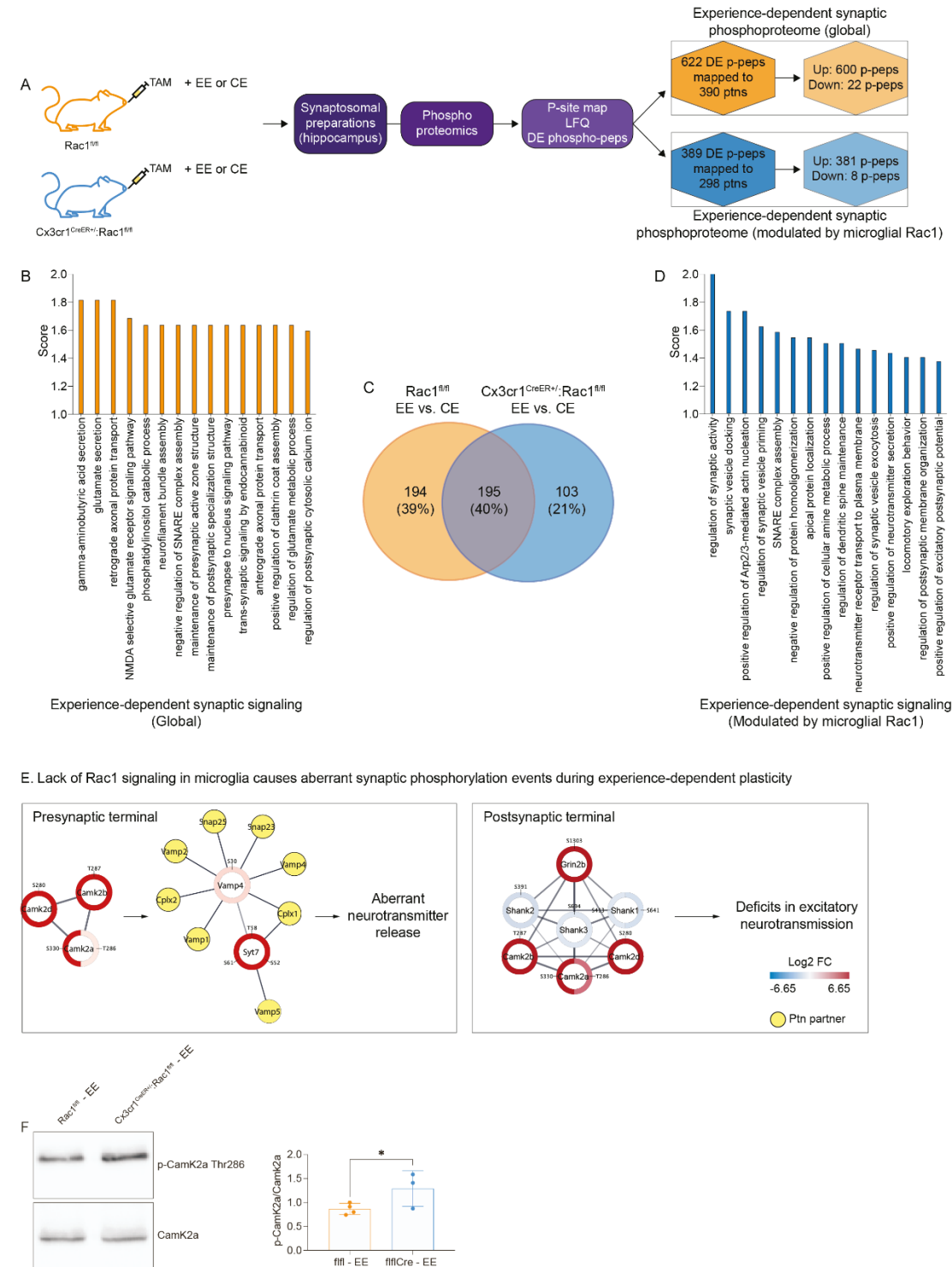


Figure 17 – Microglial Rac1 modulates the phosphoproteome landscape of synapses. (A) Workflow of phosphoproteome profiling in hippocampal synaptosomes after experience-dependent plasticity. After

applying FDR correction, the value of p was less than 0.05 for each phosphosite. **(B)** Synaptic pathways modulated by experience (EE vs CE) revealed by phosphoproteomic analysis ($n = 3$ $Rac1^{fl/fl}$ – CE mice; 5 $Rac1^{fl/fl}$ – EE mice). The histogram displays the top enriched pathways. **(C)** Venn diagram allocating the differentially expressed phosphoproteins across housing conditions and genotypes. **(D, E)** Segregation of synaptic pathways **(D)** and Camk2-associated phosphoprotein hubs **(E)** modulated concomitantly by experience and microglial $Rac1$ ($n = 3$ $Rac1^{fl/fl}$ – CE mice; 5 $Rac1^{fl/fl}$ – EE mice; 5 $Cx3cr1^{CreER+/-}; Rac1^{fl/fl}$ – CE mice; 5 $Cx3cr1^{CreER+/-}; Rac1^{fl/fl}$ – EE mice). Histograms display the top enriched pathways **(D)**. Hubs with Camk2-associated phosphoproteins are shown in **(E)**. Nodes are scaled as $\log_2$ fold-change of phosphoprotein abundance. **(F)** Western blots for phospho-CamK2a (Thr-286) and total CamK2a derived from hippocampal synaptosome fractions. * $p < 0.05$ (Two-way ANOVA).

At the pre-synapse, microglial $Rac1$ -mediated overactivation of Camk2a most likely disarrayed the fusion machinery for neurotransmitter release (composed of increased phosphorylated forms of Syt7 and Vamp4 and their synaptic partners), leading to an aberrant increase of neurotransmitter release **(Fig. 17E)**. At the postsynapse, microglial $Rac1$ -mediated overactivation of Camk2a, elicited by hyperactivity of GluN2B-containing NMDA receptors (hyperphosphorylated at S1303) and derangements of PSD scaffolds (illustrated by decreased phosphorylation of Shank1, 2, and 3), likely compromised the integration of post-synaptic potentials leading to deficits in excitatory neurotransmission **(Fig. 17E)**.

These results raise the hypothesis that microglial $Rac1$ controls neuronal Ca^{2+} dynamics during synaptic activity. To test this hypothesis, we incubated primary cortical neurons with conditioned media obtained from primary microglia expressing control (scrambled MCM) or $Rac1$ short hairpin RNA ($Rac1$ KD MCM) and evaluated single-spine Ca^{2+} dynamics during synaptic activity **(Fig. 18A)**. Single-spine Ca^{2+} imaging (using the Ca^{2+} sensor GCaMP6f (Chen et al., 2013)) revealed largely different kinetics of evoked Ca^{2+} responses between scrambled MCM spines ($1/\text{slope} = 595 \text{ au.s}^{-1}$) and $Rac1$ KD MCM spines ($1/\text{slope} = 2099 \text{ au.s}^{-1}$). In agreement with the synaptic phosphoproteome data, such different time-dependent Ca^{2+} kinetics correlated with higher **(Fig. 18B)** and faster **(Fig. 18C)** activity-dependent Ca^{2+} responses in $Rac1$ KD MCM spines, indicating that loss of $Rac1$ signaling in microglia leads to aberrant Ca^{2+} dynamics in neuronal spines during synaptic activity.

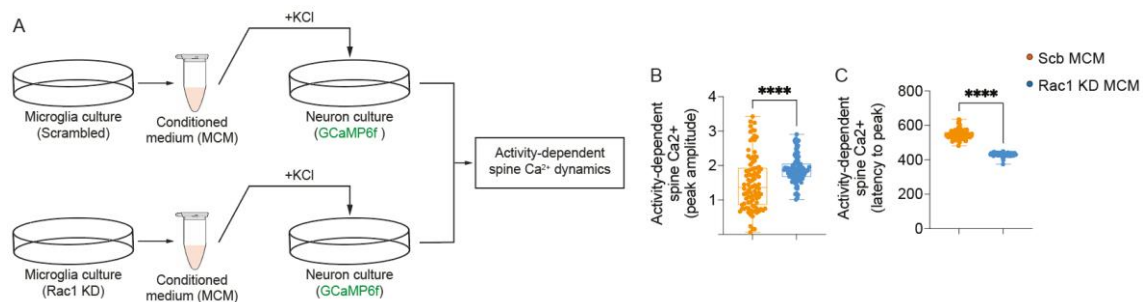


Figure 18 – Microglial $Rac1$ controls neuronal Ca^{2+} dynamics. **(A-C)** Primary cortical neuronal cultures co-expressing GCaMP6f were recorded in saline for 10 min and then recorded for 10 min upon stimulation with conditioned media from primary microglial cultures (MCM) carrying scrambled sequence or $Rac1$ short

hairpin RNA. In all conditions, neurons were co-stimulated with KCl. Whisker plots (median and min/max) show single-spine time-lapse fluorescence changes (n = 90-95 spines pooled across 6 independent neuron cultures). ****p < 0.0001 (Mann-Whitney test).

Overall, these data suggest that Rac1 signaling in microglia is essential for experience-dependent synaptic remodeling.

Rac1 modulates microglia-synapse contacts during experience-dependent plasticity

The interaction between microglia and synapses is crucial in regulating neural circuits. While our data indicate that the microglial secretome may affect the synapse in a Rac1-dependent way, recent evidence shows that microglia-synapse contacts also play a vital role in regulating synapses (Whitelaw et al., 2023). To delve into this further, we observed the frequency of microglia-synapse contacts and the resulting synaptic plasticity after exposing control and Rac1 mutant animals to EE.

In control mice, we observed a significant increase in microglia-PSD contacts following EE (as shown in **Fig. 19A**). Moreover, we found that EE promoted structural synaptic plasticity, such as enhanced engulfment of post-synaptic elements (**Fig. 19B**), a higher density of post-synaptic density (PSD) (**Fig. 19C**), and increased synaptic PSD-95 levels (**Fig. 19D**). These findings suggest that EE not only increases microglia-synapse contacts but also promotes structural plasticity through the action of microglia. In contrast, in microglial Rac1 mutants, the expected increase in microglia-synapse contacts following EE was absent (**Fig. 19A**). Furthermore, the anticipated increase in post-synaptic element engulfment, PSD-95 density and expression levels did not occur (**Fig. 19B-D**). These results support the dependence of microglia-synapse crosstalk and synaptic plasticity on microglial Rac1.

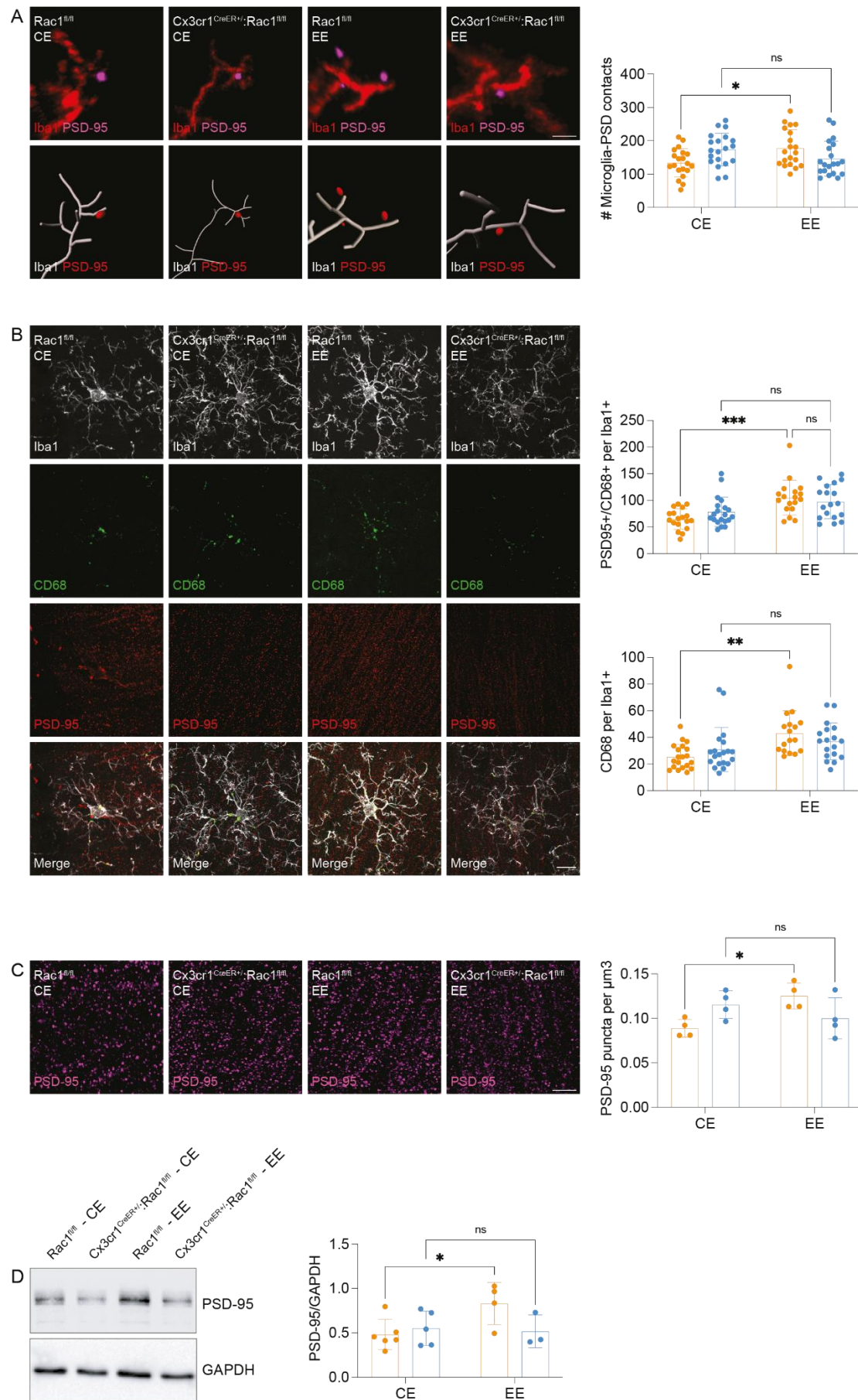


Figure 19 – Rac1 modulates microglia-synapse contacts during experience-dependent plasticity. (A) Contacts between microglia and synapses visualized using immunofluorescence and Imaris software. The microglia are labeled with Iba1, and the synapses were labeled with PSD-95. Scale bar: 2 μ m. (B) Confocal image showing the colocalization of Iba1, CD68, and PSD-95, indicating the interaction/engulfment of synaptic elements by microglia. Scale bar: 10 μ m. (C) Confocal image depicting the density of PSD-95. Scale bar: 10 μ m. (D) Western blot for PSD-95 derived from hippocampal synaptosome fractions. GAPDH was the loading control. The graphs represent the mean with SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Two-way ANOVA). Genotypes include $Rac1^{fl/fl}$ and $Cx3cr1^{CreER+/-};Rac1^{fl/fl}$ mice. Sample sizes in (A) and (B) were 18-20 cells pooled across 4 mice per group; in (C), images were averaged from 4 mice per group; for (D), $n = 3-6$ mice.

Behavioral testing indicates that microglial Rac1 instructs experience-dependent cognitive performance

Dynamic remodeling of protein phosphorylation networks is critical for cognitive function (James et al., 2010). So, by remodeling the synaptic phosphoproteome triggered by EE, microglial Rac1 should also impact cognitive performance following EE. In such a way, we conducted different behavioral tests to evaluate the cognitive performance in $Rac1^{fl/fl}$ and $Cx3cr1^{CreER+/-};Rac1^{fl/fl}$ mice housed under EE or CE (**Fig. 20A**).

First, we assessed contextual memory in the step-down passive avoidance test (Ögren & Stiedl, 2010). Multiple comparisons test revealed an intergroup variation in memory recall (test session latency; **Fig. 20B**), suggesting that EE and CE might have affected memory retention differently across genotypes. Indeed, comparing the latencies to step-down revealed that EE significantly increased memory retention in $Rac1^{fl/fl}$ mice (**Fig. 20B**), whereas in $Cx3cr1^{CreER+/-};Rac1^{fl/fl}$ mice the EE-induced improvement in memory retention did not occur (**Fig. 20B**).

Second, we used the novel object recognition (NOR) test to evaluate recognition memory (Lueptow, 2017) in $Rac1^{fl/fl}$ and $Cx3cr1^{CreER+/-};Rac1^{fl/fl}$ mice housed under EE or CE. We found that EE increased the discrimination degree of the novel object over the familiar object (**Fig. 20C**) in $Rac1^{fl/fl}$ mice (**Fig. 20C**), indicating that EE improved recognition memory. In contrast, the EE-induced improvement in the NOR performance was blocked entirely in $Cx3cr1^{CreER+/-};Rac1^{fl/fl}$ mice (**Fig. 20C**).

Third, we used the Y-maze test to evaluate working memory (Kraeuter et al., 2019) in $Rac1^{fl/fl}$ and $Cx3cr1^{CreER+/-};Rac1^{fl/fl}$ mice housed under EE or CE. We found that EE significantly decreased the number of arm entry errors, or incorrect arm returns, during the spontaneous maze alternation in $Rac1^{fl/fl}$ mice (**Fig. 20D**), indicating that EE improves spatial working memory. However, the number of wrong arm returns was comparable between EE and CE in $Cx3cr1^{CreER+/-};Rac1^{fl/fl}$ mice (**Fig. 20D**). Here, we concluded that the EE effect in enhancing spatial working memory requires microglial Rac1.

Lastly, we used the three-chamber social affiliation test (Kaidanovich-Beilin et al., 2011) to evaluate social behavior in $Rac1^{fl/fl}$ and $Cx3cr1^{CreER/+};Rac1^{fl/fl}$ mice housed under EE or CE. Whereas EE significantly increased $Rac1^{fl/fl}$ mice's preference for exploring the compartment with a stranger animal, EE failed to improve social proximity choices in $Cx3cr1^{CreER/+};Rac1^{fl/fl}$ mice (**Fig. 20E**). Here, we concluded that microglial $Rac1$ signaling modulates experience-dependent social interaction.

Overall, these data suggest that microglial $Rac1$ instructs experience-dependent learning, memory, and sociability by rewiring the synaptic phosphoproteome.

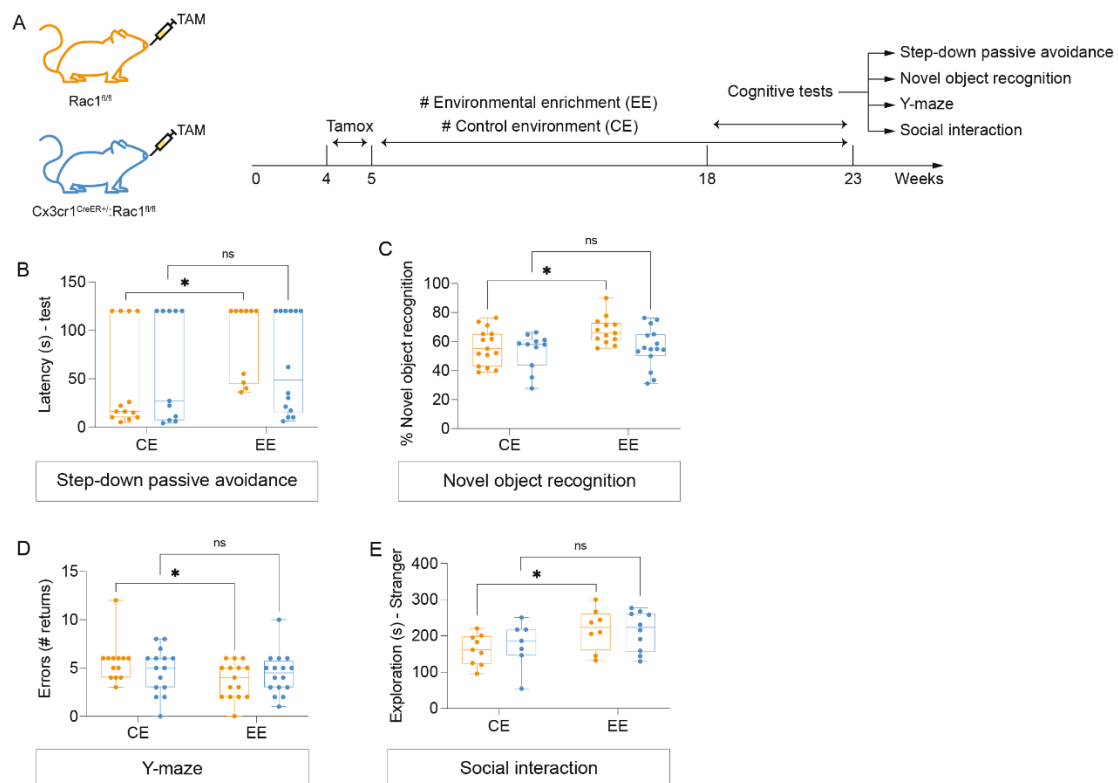


Figure 20 – Experience-dependent cognitive performance requires microglial $Rac1$ signaling. (A) Schematics for assessing cognitive performance driven by experience. (B-E) Evaluation of $Rac1^{fl/fl}$ and $Cx3cr1^{CreER/+};Rac1^{fl/fl}$ mice in the step-down passive avoidance (B), NOR (C), Y-maze (D), and three-chamber social interaction tests (E). Whisker plots show the median, quartiles, and min/max. Each dot represents a mouse. * $p < 0.05$ (Two-way ANOVA).

Discussion

Using inducible microglia-specific gene targeting in mice, RNA-seq, high-throughput phosphoproteomics, and other methodologies, we found that the RhoGTPase Rac1 was required for microglia to sense and interpret their local microenvironment and for microglia-neuron exchanges needed for experience-dependent neuroplasticity.

RhoGTPases work as binary relay switches integrating information from the outside or inside of the cell and disseminating it to various effectors controlling essential cellular functions. The specificity of the information is dictated by the nature of the stimuli, the expression and spatiotemporal activation of GEFs and GAPs, which control RhoGTPase activity (GTP-to-GDP exchange), and effectors downstream of each Rho family member. It is, therefore, no surprise that *in vivo* conditional-gene targeting experiments have revealed that many specific pathways and functions regulated by a given RhoGTPase are cell type, stimulus specific, and context dependent (Zhou & Zheng, 2013). In line with this, and despite functions classically attributed to Rac1, including the formation of the phagocytic cup (Pick, 2014), and assembly of the NOX complex (Zhao et al., 2003), we found no significant association between the Rac1-dependent transcriptomic or phosphoproteomic program and phagocytosis/engulfment or NOX-related ROS signaling in microglia in steady-state conditions. Moreover, no significant changes in the physiological engulfment of post-synaptic elements or ROS production were observed between the brains of control and microglial Rac1 mutants.

Our results also show remarkable specificity for RhoGTPase function in microglia. Comparing the phenotypic outcomes of ablating RhoA (Socodato, Portugal, et al., 2020) or Rac1 in microglia illustrates such specificity. While the lack of RhoA causes spontaneous microglia activation, resulting in neuroinflammation, neuronal loss, and amyloidosis (Socodato, Portugal, et al., 2020), the absence of Rac1 caused no spontaneous microglia activation, and the synaptic alterations found in microglial Rac1 mutants did not relate to neuroinflammation, oxidative stress, or neuronal loss.

Based on the RNA-seq and phosphoproteome profiling of Rac1-deficient microglia, we hypothesized that microglial activation triggered by extracellular ligands required Rac1 signaling. We combined FRET-based biosensors and live-cell imaging approaches to validate these data functionally. We found that Rac1 controlled many signaling pathways critical for ligand-induced microglial function, including Ca²⁺ mobilization, PKC activity, and lipid sensing.

To investigate Rac1-dependent changes in microglia in a controlled and accessible manner, we used HMC3 cells for live-cell imaging and easier experimental manipulation. Our findings in HMC3 cells demonstrated a strong correlation with *in vivo* data from phosphoproteomics analyses of microglia from Rac1 conditional mice, which suggests that HMC3 cells retain crucial functional properties representative of microglial behavior in the context of our Rac1 study. Collectively, these data suggested that Rac1 signaling regulated pathways necessary for sensing and integrating information from the microglia's local microenvironment.

To further investigate this hypothesis, we used an EE paradigm, exposing mice to increased sensory, motor, and cognitive stimulation beyond standard housing conditions. This approach is known to promote brain plasticity in glial cells and synaptic connections. We then conducted a comprehensive examination of both the microglial signaling network and morphology changes induced by EE, utilizing unbiased phosphoproteomics and confocal-based Imaris 3D rendering. The phosphoproteome analysis revealed that microglial RhoGTPase signaling was the pathway most significantly affected by EE. Within this context, we identified a network of phosphorylated GEFs and GAPs, in which Rac1 emerged as the principal hierarchical regulatory node, followed by Cdc42 and RhoA. Notably, the absence of Rac1 disrupted the architecture of this microglial phospho-GEF/phospho-GAP network. Our findings also demonstrate a critical role for Rac1 in the morphological response of microglia to EE. While some degree of morphological remodeling was still present in Rac1-deficient microglia under EE conditions, it became evident that Rac1 was necessary for the full extent of EE-driven changes in microglial morphology. This strongly suggests that Rac1 plays an important role in the hierarchical organization of the microglial RhoGTPase network, which in turn governs microglial responses to experience-dependent plasticity, encompassing both signaling modifications and morphological adaptations.

Since microglia are critical regulators of synaptic function and plasticity, we investigated synaptic plasticity in the context of EE following the ablation of microglial Rac1. Microglia-synapse crosstalk may occur through direct physical contact between microglia and synaptic elements (Hong & Stevens, 2016), microglia-mediated ECM remodeling around synapses (Nguyen et al., 2020) or activation of synaptic receptors by microglia-secreted molecules (secretome) (Parkhurst et al., 2013; Socodato, Portugal, et al., 2020). Remodeling of neuronal circuitries allows the brain to continuously change and adapt to new experiences and relies mainly on multistep modifications of synaptic proteins, including the modulation of their expression (Holt et al., 2019) and phosphorylation (Woolfrey & Dell'Acqua, 2015).

We employed a comprehensive, multi-disciplinary strategy that involved RNA sequencing, phosphoproteomics, tissue immunolabeling, and live-cell imaging to deepen our understanding of the potential influence of microglial Rac1 on synaptic plasticity. Our results revealed a significant role of Rac1 signaling in the microglia-triggered release of various substances, including chemokines, cytokines, and growth factors, all of which may have a profound impact on synaptic plasticity. Moreover, our data underscored an increased frequency of physical interactions between microglia and synapses during experience-dependent plasticity. Such contacts could drive multiple synaptic processes, such as pruning, remodeling, and plasticity, all of which are also paramount for synaptic function. Thus, our findings suggest that the microglia-mediated modulation of synaptic plasticity is orchestrated not only by the secretory products released into the synapses but also through direct physical interactions with synaptic elements. This illuminates the importance of microglial Rac1 signaling in shaping synaptic plasticity driven by EE.

Microglia-specific ablation of Rac1 combined with high-throughput phosphoproteomics allowed us to map and specify a microglia-dependent synaptic phosphoproteome signature driven by experience. As such, our phosphoproteome data further support the emergent notion of proteome plasticity, in which the proteome signature of an organism adapts to long-term changes in the external environment (Domnauer et al., 2021).

Our data suggest that the microglia-dependent synaptic phosphoproteome arranged itself in an intricate signaling network that probably crosstalks continuously with the local synaptic transcriptome (Cajigas et al., 2012) during activity-dependent plasticity. The substantial overlap of synaptic phosphoproteins (our datasets) and many mRNA species present at synaptic sites (e.g., CamK2a, PKC, PKA, NMDA receptor subunits, and actin cytoskeleton-related) (Cajigas et al., 2012) not only illustrates a bidirectional modulation between the synaptic transcriptome and phosphoproteome (Nguyen et al., 2020) but also supports a role for microglia in modulating experience-dependent plasticity via Rac1 signaling.

ECM remodeling can induce NCAM-dependent spine formation and post-synaptic assembly (Irala et al., 2016), whereas loss of NCAM decreases synapse numbers (Dityatev et al., 2000) and disrupts learning and memory (Arami et al., 1996). A post-synaptic adhesion complex formed by NCAM, β I-spectrin, and NMDA receptors promotes post-synaptic Ca^{2+} dynamics, recruiting and activating CamK2a to modulate synapse formation (Sytnyk et al., 2006). Our functional live-cell imaging assays demonstrated aberrant activity-dependent Ca^{2+} dynamics and spine remodeling in neurons exposed to the conditioned media of Rac1-deficient microglia. In line with this, the synaptic phosphoproteome profiling

showed alterations in NCAM, spectrins, NMDA receptors, and CamK2a in the synaptosomes of microglial Rac1 mutants during experience-dependent plasticity.

The fact that microglial Rac1 shaped the phosphoproteome signature of the synapses logically suggested that it also impacted cognition. Accordingly, previous studies using conditional gene ablation have reported that microglia are involved in cognitive functions (Nguyen et al., 2020; Parkhurst et al., 2013; Peng et al., 2019; Socodato, Portugal, et al., 2020; Voet et al., 2018). Our behavioral testing indicated that microglial Rac1 also controlled experience-dependent cognitive enhancement by remodeling the synaptic phosphoproteome. Moreover, based on the microglia's phosphoproteome, we could predict the alterations in the synaptic signaling that we later obtained by directly profiling the phosphoproteome in the synaptosomes.

Synaptic remodeling ultimately governs the encoding and storage of new experiences in the brain, consequently dictating behavioral adaptations to different environmental scenarios. We triggered experience-dependent synaptic remodeling using an EE paradigm. It is well accepted that the molecular and cellular changes that EE produces in the brain correlate with enhanced cognitive function (Gubert & Hannan, 2019). Indeed, we found that control mice housed under EE displayed notorious improvements in learning, memory, and social plasticity, confirming that EE enhances cognitive performance. However, EE produced no cognitive enhancement in microglial Rac1 mutants. The vast differences in the phosphoproteome signature in the hippocampal synapses of microglial Rac1 mutants somewhat explain why EE failed to function as a cognitive enhancer in these mice. Hence, synaptic phosphoproteome profiling and behavioral testing indicated that microglia were required to trigger synaptic plasticity-related pathways connected to high-order cognitive functions.

Overall, we described essential and specific roles of Rac1 for microglial homeostatic functions, microglia-synapse crosstalk, and adult brain plasticity. Combining microglial Rac1 ablation with synaptosome proteomic profiling allowed us to characterize a microglia-dependent synaptic phosphoproteome signature driven by experience. Furthermore, integrating behavioral analyses in mice lacking Rac1 in adult microglia revealed microglia as a modulator of experience-driven cognitive enhancement (**Fig. 21**). Ultimately, mapping and specifying the synaptic changes mediated by microglia might have broad implications for devising strategies to enhance cognitive performance or compensate for the cognitive decline during aging or brain disease.

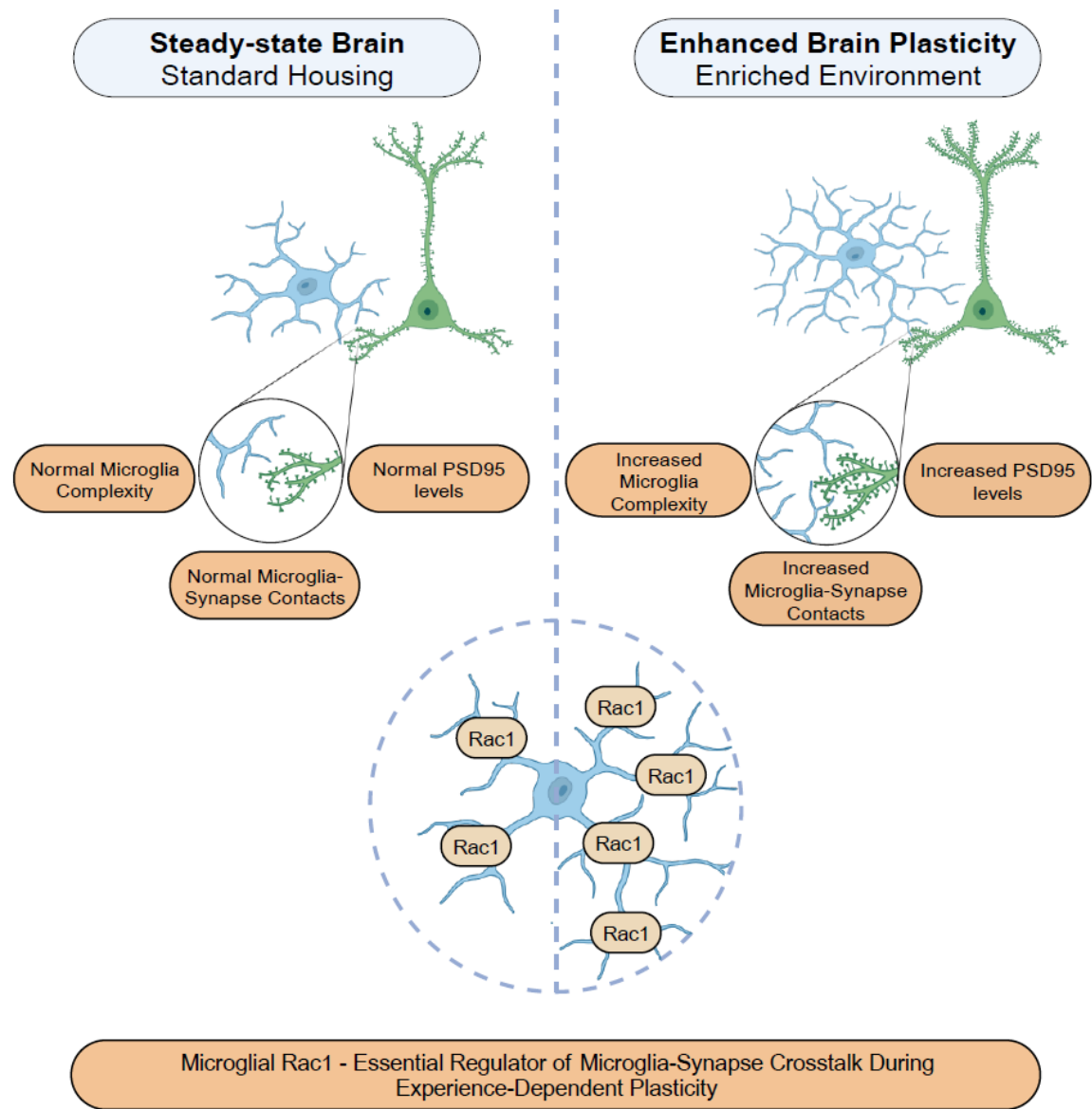


Figure 21 – Microglial Rac1 is an essential regulator of microglia-synapse crosstalk during experience-dependent plasticity.

Chapter 2 – Rac1 promotes microglia-synapse crosstalk via a Rab27-to-GDNF signaling axis

Introduction

Microglia-synapse crosstalk, characterized by a bidirectional communication between microglia and synapses, is a fundamental process in CNS homeostasis. Microglia interaction towards synapses can be attained either by direct contact between microglia and synapses (synaptic pruning and phagocytosis of synaptic elements) (Wake et al., 2009) or through the secretion of signaling molecules such as cytokines, neurotransmitters, or growth factors (Lin et al., 2019; Saw et al., 2020). While several players in this communication are already well documented, some of the specific molecules and the mechanisms that control their transport and secretion remain elusive. Growth factors, from which brain-derived neurotrophic factor (BDNF) is the most well-characterized in this context, are known to modulate synaptic plasticity and exert critical roles in neuronal survival.

Among their plethora of functions within the cell, Rho GTPases, including Rac1, Cdc42, and RhoA, regulate the intracellular trafficking mechanisms of endo and exocytosis (Olayioye et al., 2019). Rac1 modulates vesicle transport in other cells by interacting with IQGAP1. This protein, in turn, interacts with proteins present in endo and exocytic vesicles (such as Rab GTPases), creating a complex with these three elements. Rab GTPases are small GTPases that, similar to Rho GTPases, can cycle between an active GTP-bound state and an inactive GDP-bound state. Regulation of their activation is then critical for the intracellular trafficking process.

Glial-derived neurotrophic factor (GDNF) is a growth factor that interacts with the neuronal extracellular matrix (ECM) via neural cell adhesion molecule 1 (NCAM1) and ephrins to modulate synapse formation and plasticity and is sorted in and secreted from vesicles enriched in Rab GTPases (Liina et al., 2010). Besides, our previous data revealed that Rac1 ablation in microglia led to transcriptomic changes in protein trafficking/sorting/export/secretion and in the extracellular matrix/growth factor signaling. Of note, pathways associated with NCAM1 interactions and ephrin signaling were robustly altered in this context (Socodato, Almeida, et al., 2023). Thus, we raised the hypothesis that GDNF release by microglia could be modulated by Rac1, ultimately impacting synapse plasticity and learning.

Here, combining microglia-specific gene ablation with immunofluorescence, flow cytometry, and *in vitro* experiments, we give further insight into how Rac1 modulates microglial-synapse crosstalk, by identifying a Rac1/Rab27a/GDNF signaling axis that might contribute to this communication.

Results

Rac1 regulates GDNF availability via Rab27a in microglia

Instructed by our previous RNA-seq results (Socodato, Almeida, et al., 2023), we reasoned that Rac1 could modulate intracellular trafficking through the microglial endomembrane system. Indeed, reassessing the microglial phosphoproteomic dataset of Cx3cr1^{CreER/+}:Rac1^{fl/fl} and control (Cx3cr1^{CreER/+} and Rac1^{fl/fl}) microglia (Socodato, Almeida, et al., 2023) using GO annotations retrieved from the String database confirmed that Rac1 ablation significantly altered several components of the microglial endomembrane system, more specifically, the secretory vesicle system (GO:0099503) with a potential connection between proteins of the cytoskeleton-dependent secretion system (including Apc, Bin1, Ctnna1, Map6, and Stx1a and b) and proteins of the dense core granules (including Kif1a, Vps13a, and Syt2) in microglia (**Fig. 22**).

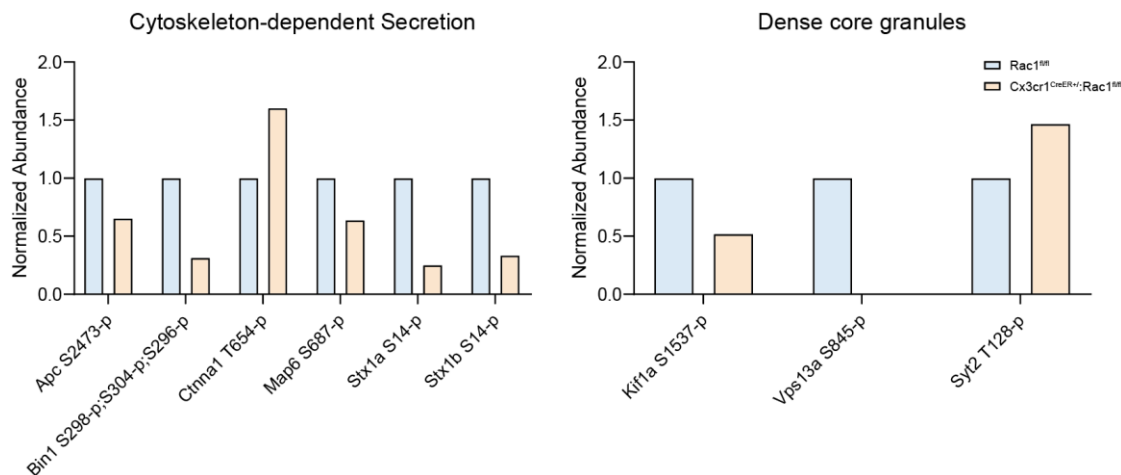


Figure 22 – Rac1 mediates the phosphoproteomic profile of the microglial endomembrane system. Abundance of endomembrane-associated phosphopeptides significantly (FDR < 0.05) modulated by microglial Rac1. Graphs show relative abundance normalized to the Rac1^{fl/fl} levels for each phosphopeptides (n = 5 mice per group).

Given that the cytoskeleton-dependent secretion system is intimately involved with Rac1 activity, and dense core granules are key in the storage and release of neurotrophic factors, investigating how Rac1 influences the availability of growth factor associated with synaptic function (like Glial cell line-derived neurotrophic factor (GDNF)) could uncover critical insights into the molecular dynamics that support microglia-dependent synaptic plasticity.

In this context, GDNF emerged as a prime candidate for two main reasons. Firstly, GDNF is known for its potent neuroprotective effects, which are crucial in supporting neuronal survival under stress conditions and during neurodegeneration (Ge et al., 2024; Yoshihiro

et al., 2014). Secondly, any modulation in the trafficking and secretion pathways of GDNF could have profound implications for neuronal health and disease. Finally, to the best of our knowledge, no direct connection between Rac1 and GDNF had been previously established in microglia, presenting an unexplored area ripe for investigation.

Therefore, we decided to study the association of Rac1 and GDNF content in microglia. Accordingly, Rac1 and GDNF colocalized in brain microglia (**Fig. 23A**) and microglia in culture (**Fig. 23B**). Using flow cytometry, we observed decreased protein amounts of GDNF in microglia from Cx3cr1^{CreER+/+}:Rac1^{fl/fl} mice compared to microglia from Rac1^{fl/fl} mice (**Fig. 23C**). Moreover, whereas the knockdown of Rac1 decreased the GDNF content (corroborating the flow cytometry data in Cx3cr1^{CreER+/+}:Rac1^{fl/fl} brains) (**Fig. 23D**), switching on Rac1 activation (using chemogenetically-controlled Rac1 activity) increased the GDNF content in microglia in culture (**Fig. 23E**). Activation of the mammalian target of rapamycin complex 1 (mTORC1) in neurons by Ras homolog enriched in brain (Rheb), has been shown to induce GDNF production (Yun et al., 2020). Additionally, our previous phosphoproteomics data from Cx3cr1^{CreER+/+}:Rac1^{fl/fl} vs Rac1^{fl/fl} microglia revealed that S1389 phosphorylation of Tsc2, an inhibitor of Rheb, was significantly decreased in Rac1-ablated microglia (Socodato, Almeida, et al., 2023). Although the specific regulatory relevance of S1389 phosphorylation of Tsc2 has not yet been described, it suggests downstream modulation of mTORC1 activity. Thus, we evaluated mTORC1 activation, using the pcDNA3-PM-TORCAR FRET biosensor (Zhou et al., 2015), in HMC3 microglia overexpressing WT Rac1 (Rac1 WT) and microglia overexpressing a dominant-negative form of Rac1 (Rac1 T17N). We observed that there was a significant decrease in mTORC1 activity (**Fig. 23F**) in Rac1 T17N microglia, suggesting that Rac1 might modulate GDNF expression in microglia via mTORC1 signaling.

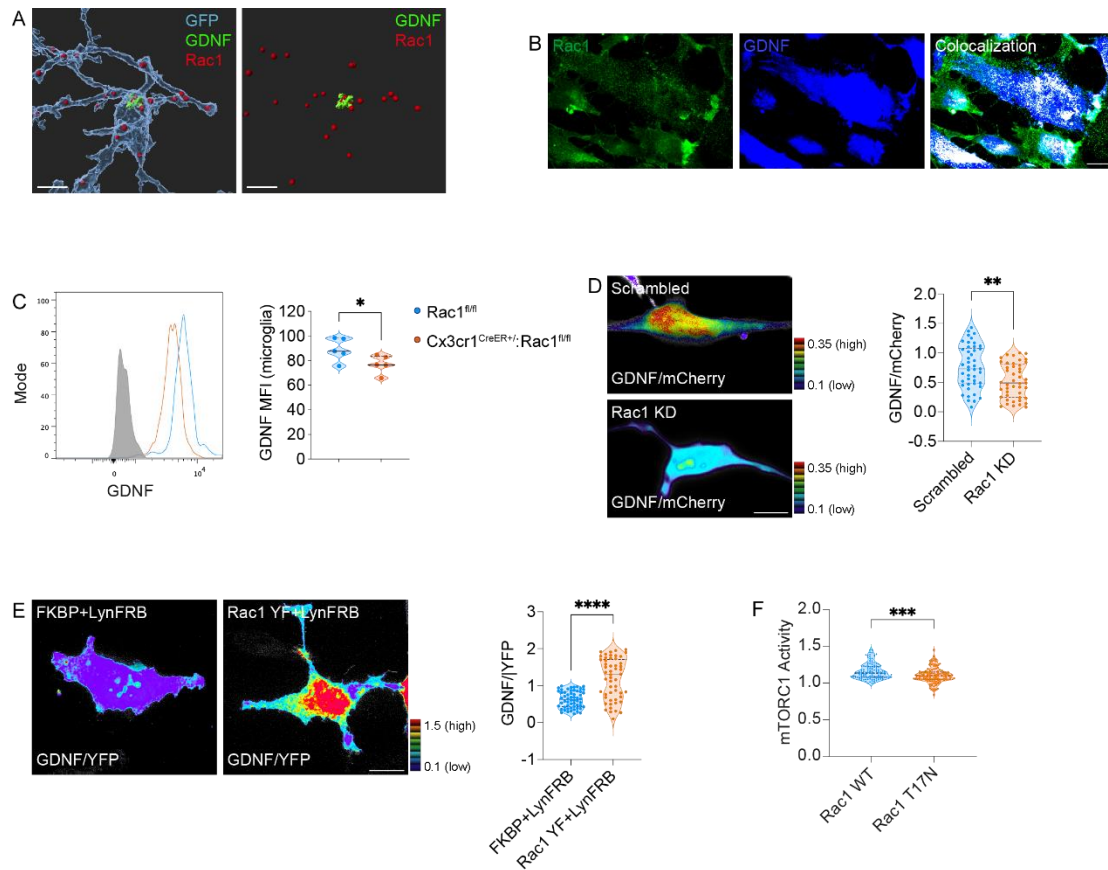


Figure 23 – Rac1 modulates GDNF expression in microglia. **(A)** Imaris rendering of confocal images from neocortical tissue sections of *Rac1^{fl/fl}* and *Cx3cr1^{CreER+/+}; Rac1^{fl/fl}* mice (3 animals per genotype). Scale bar: 10 μ m. **(B, D, E)** Immunofluorescence of Rac1 or GDNF in HMC3 microglia expressing different plasmids (scrambled-mCherry, Rac1-shRNA_mCherry, FKBP-YFP+FRBLYn, or Rac1 YF+FRBLYn). Violin plots display medians with quartiles (n=36-58 cells pooled across 3 different cultures). Pseudocolor ramps display representative fluorescence ratio values. **p<0.01; ****p<0.0001 (Mann-Whitney test). Scale bars: 20 μ m **(B)**; 10 μ m **(D, E)**. **(C)** Flow cytometry analysis of GDNF expression in microglia from *Rac1^{fl/fl}* and *Cx3cr1^{CreER+/+}; Rac1^{fl/fl}* mice (n=5 animals per genotype). Violin plots (median and quartiles) depict GDNF⁺ microglia. *p<0.05 (Mann-Whitney test). **(F)** mTORC1 activity measurement in control (*Rac1^{WT}*) or Rac1 dominant-negative (*Rac1^{T17N}*) HMC3 microglia expressing a mTORC1 FRET biosensor. Whisker plots (median and min/max) show FRET/donor ratio (n = 126 cells per group pooled across 3 different experiments). ***p < 0.001 (Mann-Whitney).

The mammalian Rab family comprises more than 60 genes, and the proteins of the Rab27/3 branch regulate vesicular trafficking and exocytosis. Rab27a is present in brain microglia, while neurons highly express Rab3a. Using immunofluorescence, we observed that Rab27a colocalized with Rac1 in microglia (**Fig. 24A**). In addition, microglia KD for Rac1 had significantly less Rab27a⁺ puncta than control microglia, indicating that Rac1 modulates the microglial Rab27a content (**Fig. 24B**). Furthermore, we confirmed that GDNF colocalized with Rab27a in brain microglia (**Fig. 24C**) and microglia in culture (**Fig. 24D**). Like Rac1 KD, knocking down Rab27a decreased the GDNF content in microglia in culture (**Fig. 24E**). Thus, these results further support our previous transcriptomic data related to protein sorting (Socodato, Portugal, et al., 2020) and suggest that Rac1 controls Rab27a-mediated GDNF secretion by microglia.

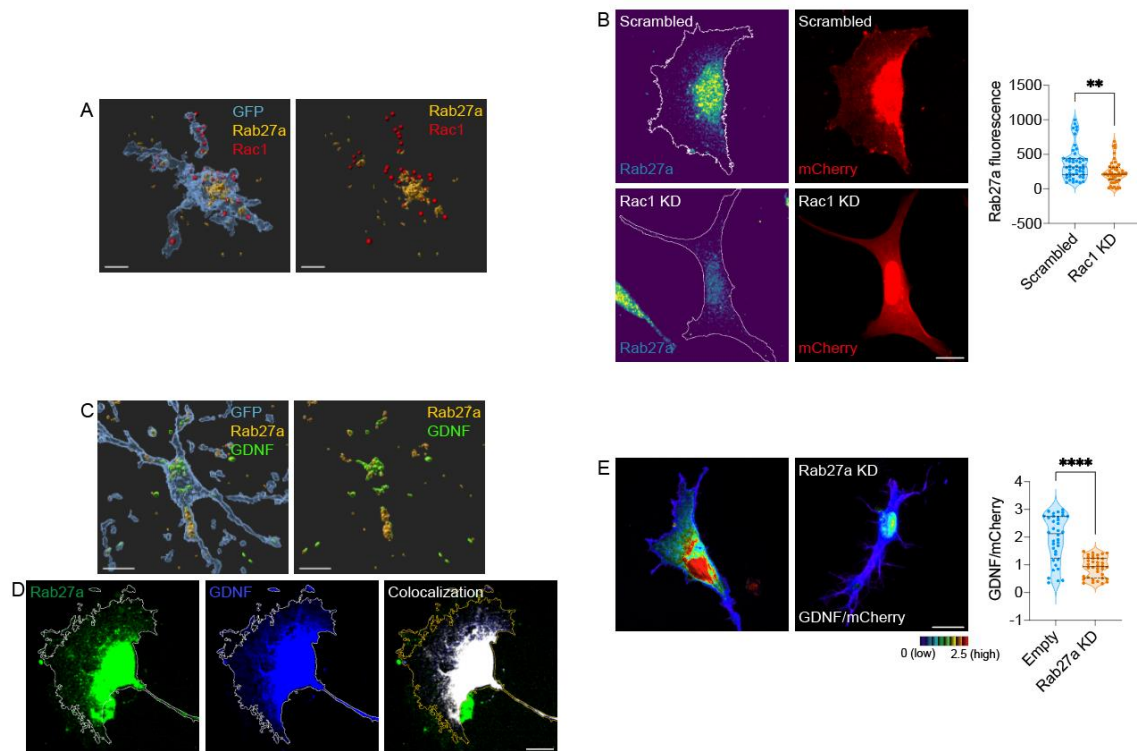


Figure 24 – *Rac1* controls a *Rab27a*/*GDNF* signaling axis in microglia. (A, C) Imapis rendering of confocal images from neocortical tissue sections of *Rac1^{fl/fl}* and *Cx3cr1^{CreER+/+}:Rac1^{fl/fl}* mice (3 animals per genotype). Scale bars: 10 μm. (B, D, E) Immunofluorescence of *GDNF* or *Rab27a* in HMC3 microglia expressing different plasmids (scrambled-mCherry, pLKO-empty, *Rac1*-shRNA_mCherry, pLKO-*Rab27a* shRNA). Violin plots display medians with quartiles (n=36-58 cells pooled across 3 different cultures). Pseudocolor ramps display representative fluorescence ratio values. **p<0.01; ****p<0.0001 (Mann-Whitney test). Scale bars: 10 μm.

Microglial *Rac1* is required for synaptic homeostasis, learning, and memory

To show that microglial *Rac1* directly impacts microglia-to-synapse communication, we looked for global effects in synaptic structure and density. We found that the brains of *Cx3cr1^{CreER+/+}:Rac1^{fl/fl}* mice had reduced spine density (Fig. 25A) and decreased excitatory (PDS-95⁺/vGlut1⁺) synapse number (Fig. 25B). The decrease in the numbers of spines and excitatory synaptic puncta caused by disrupting microglia-to-synapse communication should alter cognitive function, including learning and memory. Thus, we used the Morris Water Maze test to evaluate learning and memory. Indeed, *Cx3cr1^{CreER+/+}:Rac1^{fl/fl}* mice had a significant delay in finding the hidden platform in the learning phase (Fig. 25C) and spent significantly less time in the target quadrant in the probe trial (Fig. 25D). We concluded that *Rac1*-dependent disruption of homeostatic microglia-to-synapse communication leads to synaptic structural changes that correlate with deficits in learning and memory.

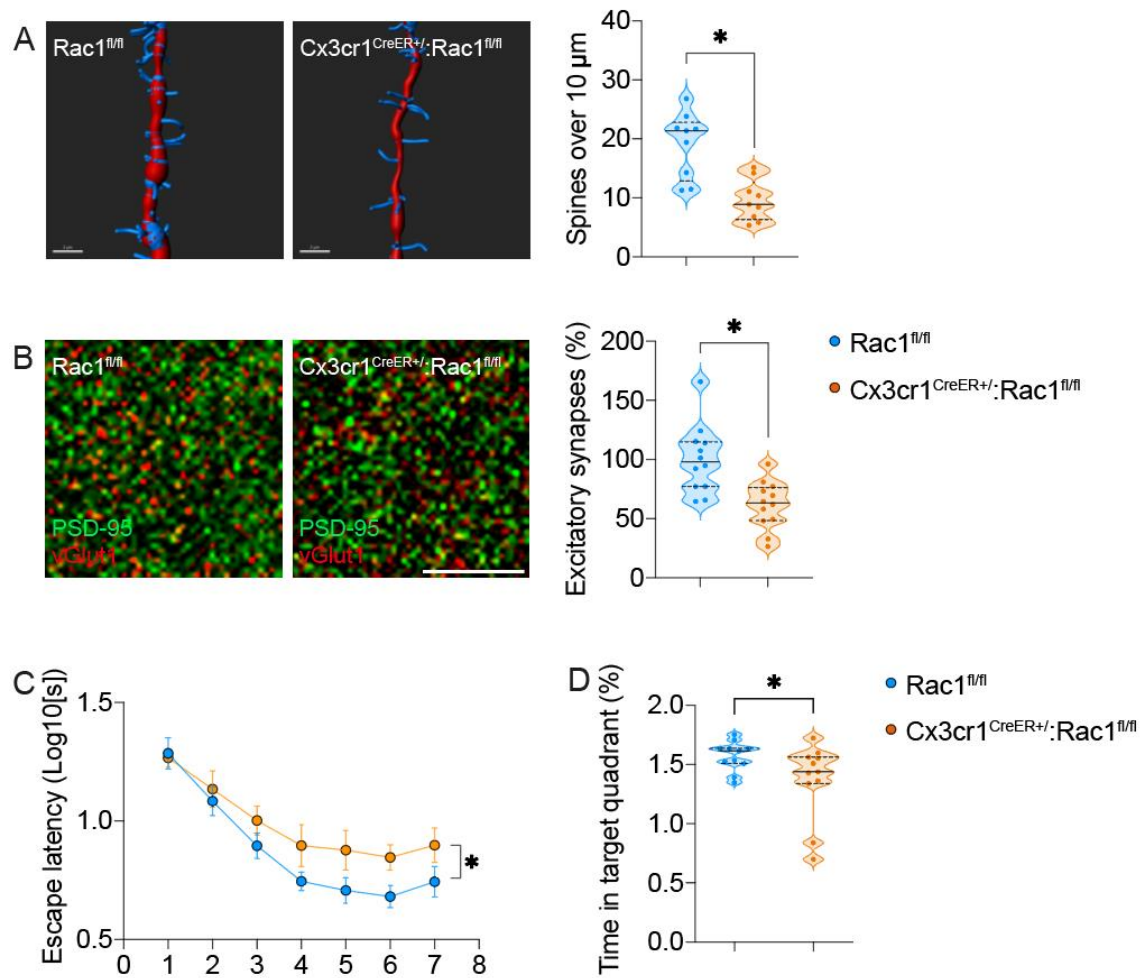


Figure 25 – Microglial Rac1 is critical for synaptic homeostasis. (A, B) Imaris rendering of dendritic spines and histological confocal colocalization of PSD-95 and vGlut1 on tissue sections from neocortices of Rac1^{fl/fl} and Cx3cr1^{CreER+/+}; Rac1^{fl/fl} mice (n=9 dendrites stacked across 3 animals per genotype (A) or n=12 slices pooled across 4 animals per genotype (B)). Violin plots (median and quartiles) depict spine density or PSD-95/vGlut1 double-positive puncta. *P<0.05 (nested t-test (A); Mann-Whitney test (B)). Scale bars: A: 2 μm ; B: 5 μm . (C, D) Performance of Rac1^{fl/fl} and Cx3cr1^{CreER+/+}; Rac1^{fl/fl} mice in the Morris Water Maze (n=11 mice per genotype). Histogram (mean and SEM) shows the learning curves, and violin plots (median and quartiles) depict the probe tests. *p<0.05 (two-way ANOVA (C); unpaired t-test (D)).

Discussion

By integrating our previous data on Rac1 ablation in microglia with further experimental procedures, which included immunofluorescence, flow cytometry, stereotaxic administration of viral particles, and *in vitro* strategies, we aimed at identifying and specifying part of the signaling mechanisms that could explain the microglia-synapse crosstalk modulation observed in Cx3cr1^{CreER+/+}:Rac1^{fl/fl} mice. Our data revealed that Rac1 plays a crucial role in regulating several key pathways that not only modulate microglial functionality but also influence their interaction with neuronal elements, particularly at the synapse.

GDNF expression in the healthy brain is mainly attributed to neurons, with the contribution of glial cells being only around 6% of the total GDNF-expressing cells in this context (Duarte Azevedo et al., 2020). Microglia/macrophage expression of GDNF mainly occurs during disease or pathological conditions such as LPS-induced inflammation (Hashimoto et al., 2005) or mechanical injuries (Batchelor et al., 1999; Batchelor et al., 2002). Given the alterations in critical elements of the GDNF signaling cascade on the Cx3cr1^{CreER+/+}:Rac1^{fl/fl} microglia, we evaluated GDNF in microglia from Rac1^{fl/fl} and Cx3cr1^{CreER+/+}:Rac1^{fl/fl}. Firstly, we confirmed that microglia expresses GDNF, both by immunofluorescence and flow cytometry, even in the Rac1^{fl/fl} mice. Additionally, our results showed that GDNF and Rac1 colocalized intracellularly and that the cellular GDNF content was dependent on Rac1 activity. The protein complex mTORC1 is involved in several signaling pathways, including the response to growth factors, autophagy, and cell growth (Zou et al., 2020). Moreover, several studies suggest that mTORC1 signaling regulates vesicle sub-cellular localization (Chatterjee et al., 2020) and trafficking (Zou et al., 2019). Rac1 binds directly to mTOR, one of the components of both mTORC1 and mTORC2 complexes, and its ablation leads to a decrease in the activation of these two complexes (Adler, 2011; Sacci et al., 2011). Therefore, we evaluated mTORC1 activation in microglia overexpressing WT Rac1 or T17N Rac1 and observed a significant decrease in mTORC1 activity, suggesting that GDNF expression can be mediated via mTORC1.

Given mTORC1's known function as a negative regulator of exocytosis in various cellular systems (Zou et al., 2019), the relationship between Rac1 and mTORC1 offers an interesting area of study in microglia, particularly in understanding how Rac1 may mediate GDNF expression indirectly through mTORC1 signaling pathways. However, considering Rac1's diverse roles in cellular signaling, we decided to explore additional pathways that could, independently or in conjunction with mTORC1, regulate GDNF availability in the microglia.

IQGAP1 is an effector of Rac1 and Cdc42 involved in vesicle trafficking and tethering (Grohmanova et al., 2004). IQGAP1 specifically binds to the GTP-bound forms of Rac1, facilitating its interaction with various signaling molecules and vesicle transport systems (Mosaddeghzadeh et al., 2021). In the context of GDNF regulation by Rac1 (Kimura et al., 2013), the interaction of IQGAP1 with Rab27a—a key regulator in the endo- and exocytic processes—becomes particularly relevant in microglia-dependent secretion of growth factors (Fukuda, 2013). Thus, we started by confirming that Rac1 colocalized with Rab27a *in vivo* and *in vitro*. Following the knockdown of Rac1 in HMC3 microglial cells, we observed a corresponding decrease in Rab27a expression, suggesting a pathway through which Rac1 can affect the trafficking and availability of vesicles containing GDNF in microglia. Moreover, the colocalization of GDNF with Rab27a further supports the hypothesis that Rac1's regulation of GDNF availability is mediated through the Rab27a pathway. Indeed, the knockdown of Rab27a in microglia resulted in a significant decrease in GDNF expression levels, indicating that Rab27a is crucial for GDNF sorting and secretion in microglial cells. This finding not only highlights the importance of Rac1 and its downstream effectors in the regulation of neurotrophic factors but also sets the stage for exploring therapeutic strategies that could enhance GDNF availability through the modulation of these signaling pathways.

Functionally, the Rab27a-mediated pathway's integrity is crucial for maintaining optimal GDNF levels, which support neuronal survival and function. In pathological conditions, such as inflammation or mechanical injury, where the demand for neurotrophic factors increases, the efficient functioning of Rab27a becomes even more critical. Disruptions in Rab27a activity, as influenced by Rac1 signaling perturbations, could, therefore, lead to insufficient GDNF release, exacerbating neuronal damage and impeding recovery.

Given these dynamics, targeting Rab27a or its regulatory pathways might be a promising therapeutic avenue. Enhancing Rab27a function could potentially elevate GDNF secretion, supporting neuroprotection and regeneration in various neurodegenerative diseases or injury models. Such strategies would need to consider Rac1's fine-tuned regulation, aiming to optimize rather than simply increase Rab27a activity, ensuring that GDNF is delivered in a controlled and efficacious manner.

Impairments in microglia-synapse crosstalk lead to substantial modifications in the synaptic architecture and plasticity, ultimately resulting in cognitive impairments. Therefore, the changes in spine density, excitatory synapse numbers, and learning and memory deficits observed in *Cx3cr1^{CreER+/+}·Rac1^{fl/fl}* mice agree with this view, suggesting that the lack of microglial GDNF in these mice could decrease the neuronal protection provided by this growth factor.

While currently, there is no information on the specific mechanisms of GDNF production and export in microglia and on the importance of microglial GDNF in the physiological brain, there are studies on other cell types that highlight the relevance of GDNF in neuron-glia interactions. Transduction of hippocampal dopaminergic neurons, using an adeno-associated virus to promote the expression of constitutively active Rheb, led to mTORC1 activation in these neurons and increased GDNF and BDNF production. Furthermore, specific inhibition of mTORC1 using rapamycin attenuated the AAV-induced upregulation of GDNF. This study also reported that neuronal GDNF could be mediating the upregulation of the GDNF receptor GFR α -1 in astrocytes, incrementing GDNF production and ultimately contributing to neuronal protection (Yun et al., 2020). Interestingly, a recent study showed that GDNF delivery via microglia/macrophages derived from hematopoietic stem cells in a Parkinson's disease mouse model led to an amelioration of the motor symptoms and to neuronal protection (Ge et al., 2024). The data summarized in this chapter, in agreement with the literature, can, therefore, constitute the first steps in the identification of a similar GDNF communication mechanism between microglia and synapses, leading to control of learning and memory mechanisms in homeostatic conditions.

The findings detailed in this chapter, combined with corroborating evidence from the literature, collectively underscore the potential of GDNF as a pivotal mediator in the communication between microglia and synapses. The co-localization of GDNF with Rac1 in microglia and the dependency of GDNF levels on Rac1 activity highlight a direct pathway through which Rac1 could modulate microglial ability to support neuronal health and synaptic plasticity (**Fig. 26**). Our data allow speculating that this Rac1-driven modulation of GDNF should be particularly important in contexts where microglia are required to respond to neuronal damage or dysfunction, suggesting that Rac1's role extends beyond mere intracellular trafficking to actively shaping the microglial contribution to synaptic plasticity.

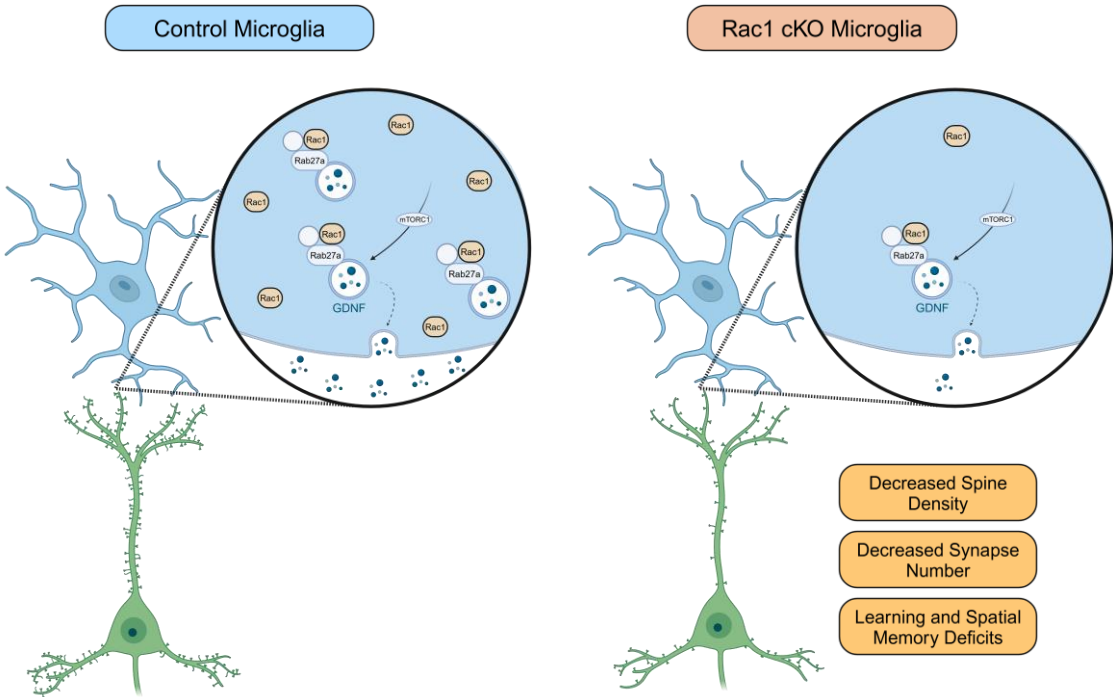


Figure 26 – A Rac1-dependent Rab27a-to-GDNF signaling axis critical for microglia-synapse crosstalk.

Chapter 3 – Rac1 drives microglial signaling remodeling during neuroinflammation

Introduction

Neuroinflammation, defined as the inflammation of the nervous tissue, arises in the brain as a multifaceted response to various threats (DiSabato et al., 2016). While the process is predominantly recognized as the brain's reaction to bacterial pathogens, other triggers, such as viral infections, traumatic brain injuries, and neurodegenerative diseases, may also play significant roles (DiSabato et al., 2016). Among the bacterial components that incite neuroinflammation, lipopolysaccharide (LPS) is paramount. Present in Gram (-) bacteria, LPS serves as a prominent pathogen-associated molecular pattern, eliciting reactions from the brain's immune system.

Microglia, the brain's primary immune cells, act as the first line of defense against these threats. They detect and initiate various defensive mechanisms tailored to counteract the perceived tissue threats (Hickman et al., 2018). One widely recognized mechanism involves their response to systemic LPS administration, a paradigm frequently employed in experimental organisms such as mice. A primary reaction of microglia to LPS is through the activation of the Toll-like receptor (TLR) 4, which further prompts various inflammation-associated signaling pathways, such as the NF- κ B and MAPK/AP-1 (Kraft & Harry, 2011).

Transcriptomic data have illuminated the intricate mechanisms of this microglial response (Das et al., 2016; Jordão et al., 2019; Sousa et al., 2018). The interaction between LPS and TLR4 catalyzes a cascade, with the NF- κ B and MAPK/AP-1 pathways standing out as primary drivers of inflammation (Das et al., 2016; Jordão et al., 2019; Sousa et al., 2018). This eventually leads to the production of reactive oxygen species (ROS) and proinflammatory cytokines such as IL-1 β , IL-6, and Tnf (Das et al., 2016; Jordão et al., 2019; Sousa et al., 2018). Furthermore, interferon regulatory factors refine and amplify the overall neuroinflammatory reaction (Das et al., 2016; Jordão et al., 2019; Sousa et al., 2018). It is crucial to understand that while short-lived neuroinflammatory responses can aid tissue defense and repair, the implications of sustained inflammation remain a topic of debate (Sochocka et al., 2017). While traditionally seen as potentially harmful, there is growing evidence to suggest that certain prolonged inflammatory states might also have protective or reparative facets.

Rac1, a member of the Rho GTPase family, has gained considerable attention among various molecules implicated in neuroinflammation. Although the Rho GTPase family is primarily recognized for its role in the reorganization and dynamics of the actin cytoskeleton, Rac1 has a broader spectrum of functions, including its significant influence on phagocytosis (Cox & Greenberg, 2001) and the modulation of Nrf2 and NF- κ B pathways

(Cuadrado et al., 2014) in myeloid cells. Its role as a component of the NADPH oxidase complex also highlights its importance in ROS production (Abo et al., 1991; Zhao et al., 2003). However, while Rac1's impact on myeloid cells is evident, its direct involvement in neuroinflammation needs to be clarified.

Given the myeloid lineage of microglia and Rac1's apparent influence on myeloid cells, its role in neuroinflammation becomes intriguing. Recognizing the potential regulatory effect of Rac1 on microglial functionality, particularly during experience-dependent brain plasticity (Socodato, Almeida, et al., 2023), in this study, we seek to pinpoint Rac1's precise role in microglial responses during neuroinflammation.

Results

LPS modulates Rho GTPase signaling in microglia

To understand the signaling pathways altered by LPS administration in microglia *in vivo*, we injected 23-week-old Rac1^{fl/fl} mice with LPS 4mg/kg intraperitoneally (**Fig. 27A**). Microglia was isolated by MACS 24 hours after LPS administration to perform quantitative label-free phosphoproteomics. Bioinformatic analysis of the microglial phosphoproteome modulated by LPS revealed statistically significant differences in 377 phosphopeptides, corresponding to 255 altered phosphoproteins (LPS-dependent microglia phosphoproteome). Amidst these alterations, the expression of 240 phosphopeptides was increased, and 137 were decreased in microglia from LPS-injected Rac1^{fl/fl} mice compared with microglia from saline-injected Rac1^{fl/fl} mice (**Fig. 27B**). To explore the molecular signaling mechanisms regulated by LPS in this context we performed STRING functional enrichment analysis focused on the Reactome pathways database with the 255 altered proteins. Cluster categorization revealed that LPS administration profoundly impacted microglia phosphoproteome in 10 signaling modules: Rho GTPase signaling, intracellular signaling, cell death, development, cell cycle, molecule transport, response to stimuli, immune system, synaptic transmission, and hemostasis (**Fig. 27C**). Notably, Rho GTPase related pathways were among the most enriched pathways in this functional enrichment analysis.

Focusing on the Rho GTPase signaling cluster and considering the three main Rho GTPases (RhoA, Rac1, and Cdc42), the Rac1 GTPase cycle was the most significantly represented pathway in this dataset (FDR = 5.72×10^{-6}), followed by RhoA GTPase cycle (FDR = 6.3×10^{-4}), while Cdc42 GTPase cycle was not significantly modulated in this context (**Fig. 27D**). RhoA, Rac1 and Cdc42 can cycle between an active GTP-bound state and an inactive GDP-bound state. Guanine exchange factors (GEFs) and GTPase-activating proteins (GAPs) promote this interchange between states. In agreement with the functional enrichment analysis, we identified 12 GEFs, GAPs and effectors associated with Rac1, 9 with RhoA and 8 with Cdc42 (**Fig. 27E**). Arhgap1 (p-S49, p-S50 and p-S51), Arhgef7 (p-S497), Bcr (p-S122) and Rasgrf2 (p-S924) were shared between RhoA, Rac1 and Cdc42. Abi1 (p-S183), Ncf2 (p-T233), Sos1 (p-S1147), and Tiam2 (p-S1590) were specific to Rac1, Cavin1 (p-S42), Diaph1 (p-S22) and Tjp2 (p-S1135) were specific to RhoA and Cdc42bpb (p-S1692) to Cdc42 (**Fig. 27F**). These data suggests that Rho GTPase signaling might be critical for the microglial response to LPS and that there is a hierarchical organization of Rho GTPases in microglia, led by Rac1 signaling, in this process.

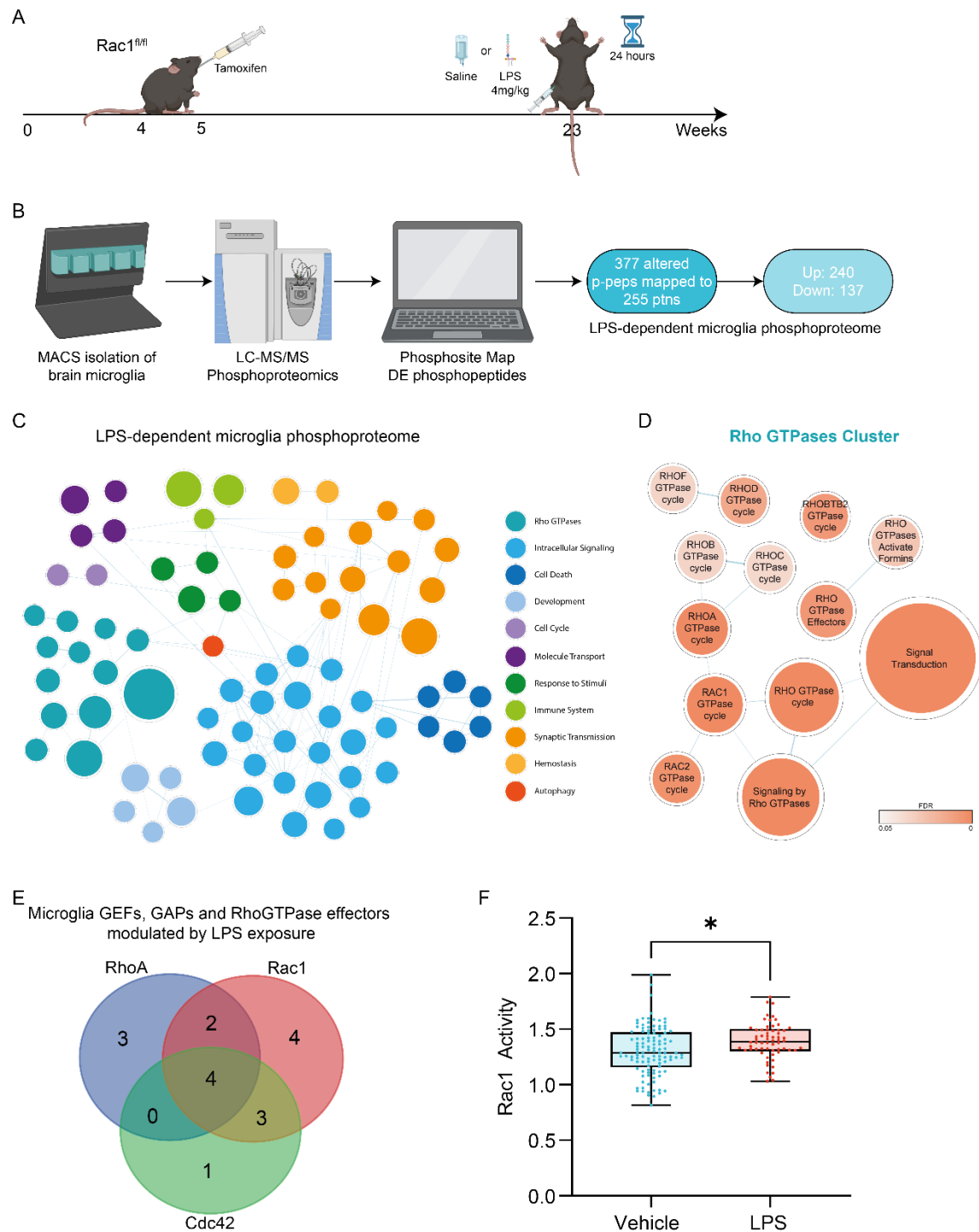


Figure 27 - LPS rewires the microglial phosphoproteomic signaling. (A) Experimental timeline. (B) Workflow of phosphoproteomics profiling in microglia from Rac1^{f/f} mice after LPS administration (FDR < 0.05). (C) Microglial pathways modulated by neuroinflammation (LPS vs Sal) revealed by phosphoproteomic analysis (n = 5 mice per group). Networks display the enriched pathways (nodes) modulated by LPS. Node size is scaled according to the number of phosphoproteins within. (D) Focus on the Rho GTPases cluster network. Nodes are colored according to the FDR value retrieved from the functional enrichment analysis. (E) Venn diagram representing the differentially expressed GEFs, GAPs and Rho GTPase effectors associated with Rac1, RhoA and Cdc42. (F) HMC3 microglia expressing a Rac1 activity FRET biosensor was stimulated with 100ng/mL LPS or vehicle for 15 minutes. Whisker plots (mean and min/max) represent the FRET/donor ratio (n = 63-113 pooled across 3 independent experiments). *p < 0.05 (Mann-Whitney).

Thus, to functionally assess if LPS was modulating Rac1 activity and validate the bioinformatic observations, we evaluated membrane Rac1 activation *in vitro* by stimulating HMC3 cells transfected with Rac1 Raichu, a Rac1 activity FRET sensor, with 100ng/mL LPS for 15 minutes. Our FRET results confirmed that LPS stimulation increased Rac1 activation levels in microglia (**Fig. 27F**).

Moreover, our data revealed that systemic LPS administration profoundly altered the microglial phosphoproteomic landscape and that this effect was widespread to several signaling modules. Rac1 GTPase cycle was the most significantly altered pathway by LPS among the Rho GTPases, and this effect was further confirmed by *in vitro* FRET experiments, in which we observed an increase in Rac1 activity following LPS stimulation, which suggests that Rac1 signaling is a critical pathway for the microglial response to neuroinflammation.

Rac1 ablation reshapes the LPS-dependent microglia phosphoproteome.

Our previous data showed that Rac1 ablation in microglia altered the microglial transcriptome. Gene set enrichment analysis and string-ranked enrichment revealed changes in two main microglia signaling programs. The microglial environmental sensing program comprised a cluster of innate immune signaling pathways, including Toll-like receptor 4 and Fc receptor pathways (**Fig. 28**), already suggesting the potentially essential role of microglial Rac1 in the neuroinflammatory response (Socodato, Almeida, et al., 2023).

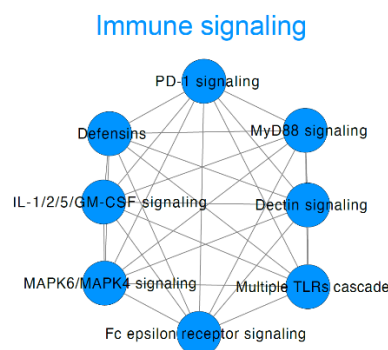


Figure 28 – Rac1-dependent immune signaling cluster. Rac1-dependent microglia transcriptomic signature revealed by analogy concatenation of GSEA and SRA (n = 3 mice per genotype). Interaction networks display the top enriched pathways.

Thus, to further dissect the role of Rac1 signaling in the LPS-dependent microglia phosphoproteome, we injected Cx3cr1^{CreER/+}:Rac1^{fl/fl} intraperitoneally with 4mg/kg LPS (**Fig. 29A**). Brain microglia were then isolated by MACS 24 hours after administration and protein

extracts were used for quantitative label-free phosphoproteomics. Bioinformatic analysis and data filtering identified statistically significant changes in 346 phosphopeptides mapped to 304 proteins. Among these modifications, the expression of 180 phosphopeptides was increased. In contrast, the expression of 166 was decreased (**Fig. 29B**). To understand the relevance of Rac1 for microglia LPS-dependent phosphoproteome reshaping, we combined the information obtained from *Rac1^{fl/fl}* and *Cx3cr1^{CreER+/+};*Rac1^{fl/fl}** mice to identify which changes were modulated by Rac1. Out of 432 unique proteins altered by LPS, 299 (69%) were dependent on microglial Rac1 upon LPS exposure (**Fig. 29C**). Functional enrichment analysis revealed that there were 11 signaling modules dependent on Rac1 upon LPS exposure: Rho GTPase signaling, intracellular signaling, cell death, development, cell cycle, molecule transport, response to stimuli, immune system, synaptic transmission, hemostasis, and autophagy (**Fig. 29D**). Signaling by Rho GTPases was the most represented pathway (FDR = 3.34×10^{-11}) of the entire network. As expected, the Rac1 GTPase cycle was the most significant pathway among the main Rho GTPases represented within the Rho GTPase signaling cluster (FDR = 1.8×10^{-4}), confirming the dependency on Rac1 for microglia to respond to LPS. While there seemed to be an overall similarity between the clusters in the LPS-dependent microglia phosphoproteome and the ones on the LPS-dependent microglia phosphoproteome modulated by Rac1, a deeper observation of these modules revealed that there were several differences in the pathways involved. Of note, the LPS-dependent microglia phosphoproteome modulated by Rac1 revealed that Rac1 takes part in more than one autophagy-related pathway in response to LPS. In contrast, LPS itself in *Rac1^{fl/fl}* mice only modulated a specific autophagy pathway (aggrephagy). Additionally, only part of the cell death signaling cluster modulated by LPS was shown to be dependent on microglial Rac1, as well as intracellular signaling, synaptic transmission, response to stimuli, and molecule transport, bringing forth the specific involvement of Rac1 within these modules.

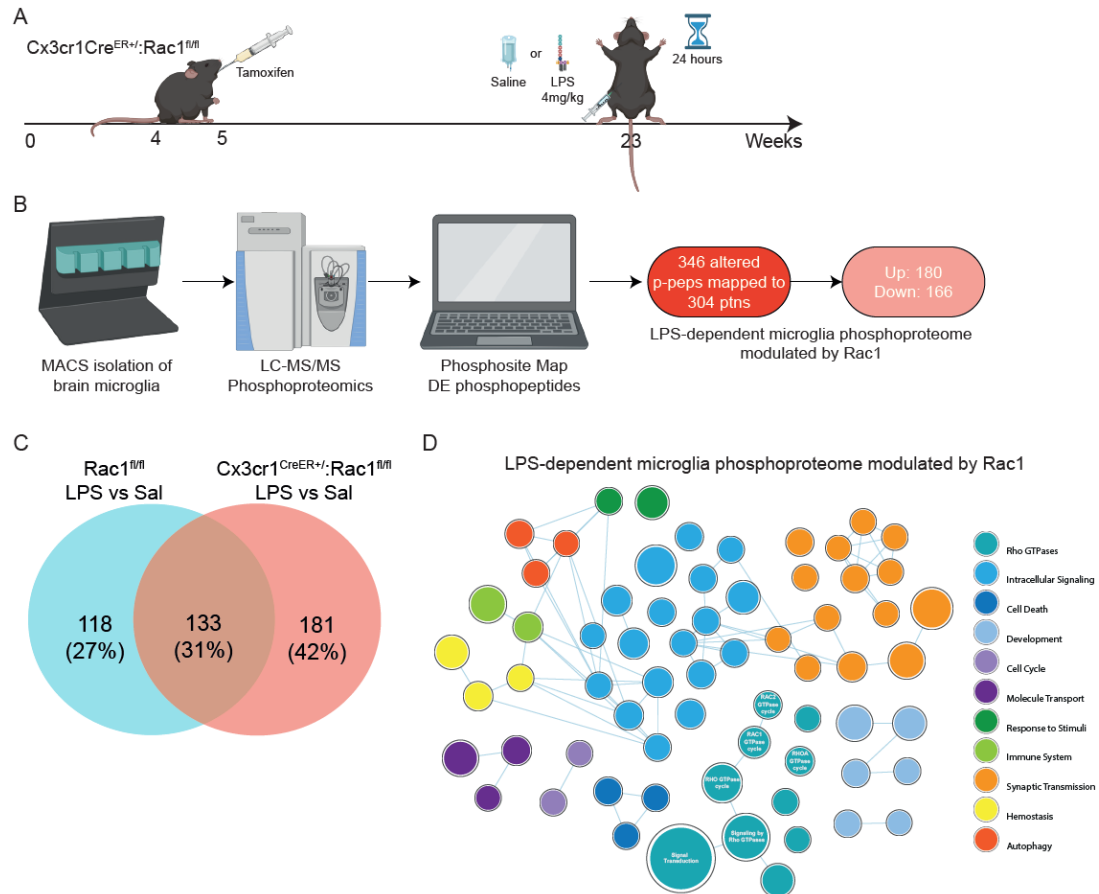


Figure 29 – Microglial Rac1 modulates the LPS-induced microglial phosphoproteome. (A) Experimental timeline. **(B)** Workflow of phosphoproteomics profiling in microglia after LPS administration (FDR < 0.05). **(C)** Venn diagram allocating the differentially expressed phosphoproteins across stimulation and genotype. **(D)** Segregation of microglial pathways modulated by Rac1 during neuroinflammation (n = 5 Rac1^{fl/fl} – Saline mice; n = 5 Rac1^{fl/fl} – LPS; n = 5 Cx3cr1^{CreER/+};Rac1^{fl/fl} – Saline; n = 4 Cx3cr1^{CreER/+};Rac1^{fl/fl} – LPS). Node size is scaled according to the number of phosphoproteins within.

Focusing on the Rho GTPases module, and in agreement with the pathway enrichment analysis, we identified 12 GEFs, GAPs, and effectors associated with Rac1, 8 with RhoA, and 7 with Cdc42 altered in the LPS-dependent microglia phosphoproteome modulated by Rac1. (**Fig. 30A**). Additionally, we observed that Rac1 ablation prevented the LPS-induced changes in several Rho GTPase GEFs, GAPs, and effectors phosphopeptides (**Fig. 30B**).

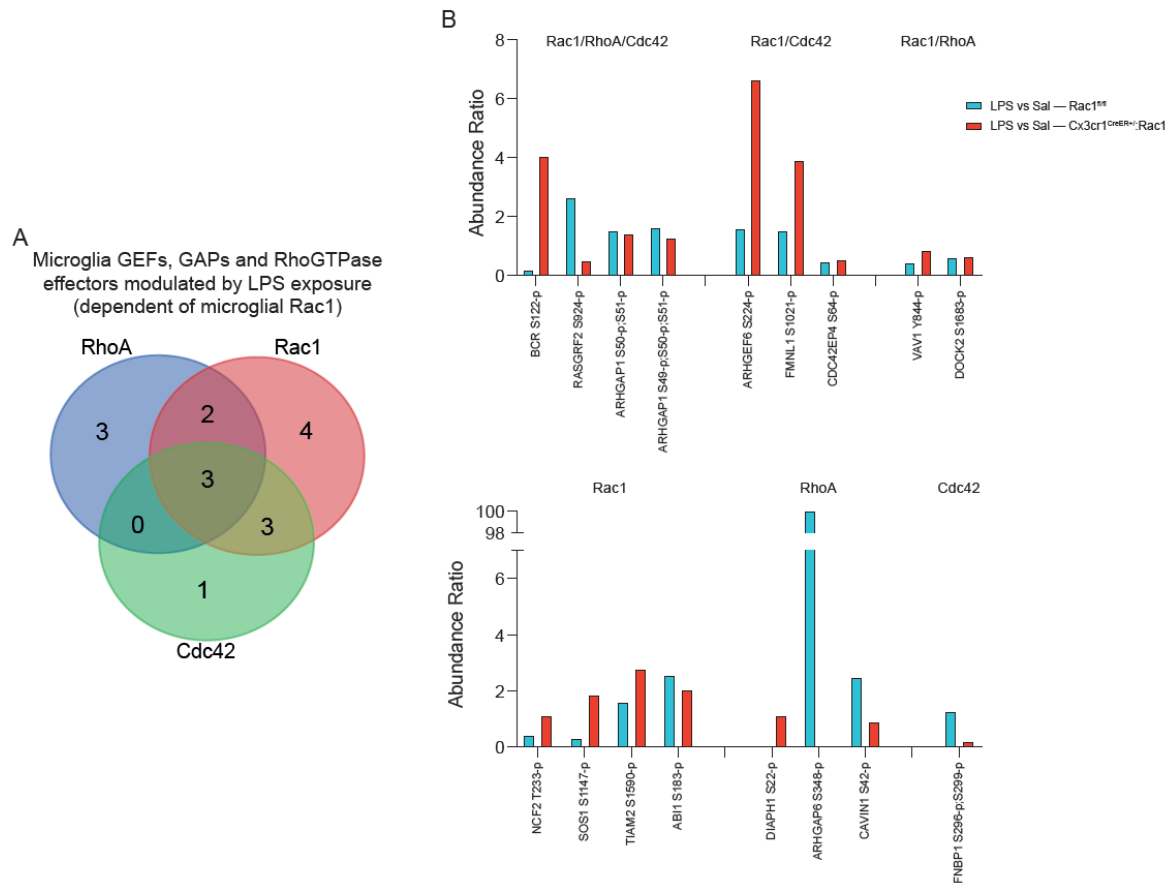


Figure 30 – Rac1 ablation impairs LPS-induced changes in Rho GTPase signaling. (A) Venn diagram representing the differentially expressed GEFs, GAPs and Rho GTPase effectors retrieved from the Rho GTPase signaling cluster in Fig. 27D. (B) Abundance ratio of Rac1, RhoA and Cdc42 GEFs, GAPs and Rho GTPase effector phosphopeptides retrieved from the Rho GTPase signaling cluster in Fig. 27D.

These observations suggest that Rac1 is a key regulator of the hierarchical organization of the microglial Rho GTPase signaling and coordinates a wide range of signaling modules in neuroinflammation.

Rac1 ablation impairs the microglial intracellular signaling machinery in response to inflammatory stimuli.

To further explore the functional relevance of Rac1 in the intracellular signaling mechanisms during neuroinflammation, we evaluated the production of ROS (Guzy et al., 2005) and NF- κ B pathway activation (Shcherbakova et al., 2016) by control and Rac1 knockdown microglia *in vitro* using fluorescent biosensors and live-cell imaging. Our results showed an increase in ROS production in control (Scrambled) cells upon LPS stimulation. Rac1 knockdown (Rac1 KD), however, prevented this increase in ROS production (**Fig. 31A**). To study the NF- κ B pathway activation, we evaluated the levels of I κ B. This inhibitor prevents

NF- κ B translocation to the nucleus. We observed that there was a reduction in I κ B expression in control cells stimulated with LPS and that this reduction was not present in Rac1 knockdown microglia (**Fig. 31B**). These results suggest that Rac1 is critical in two of the most prominent pathways (ROS and NF- κ B) related to the proinflammatory response in microglia, raising the hypothesis that microglia deficient in Rac1 signaling become largely non-responsive to proinflammatory stimulation.

Production and release of pro-inflammatory cytokines such as IL-1 β , IL-6, and TNF α is part of the microglial pro-inflammatory response (Pawate et al., 2004; Qin et al., 2004). Through its specific receptors, pro-inflammatory cytokine stimulation of microglia can induce changes in intracellular calcium (Ca²⁺) (Goghari et al., 2000). To further dissect the roles of Rac1 in a neuroinflammatory context, we overexpressed either wild-type (WT) or a dominant-negative mutant form (T17N) of Rac1 on HMC3 microglia *in vitro*, stimulated them with IL-1 β , IL-6, or TNF α and evaluated an overall time course of intracellular calcium mobilization with the GCaMP6f biosensor (Chen et al., 2013). Each pro-inflammatory cytokine led to a specific response in intracellular Ca²⁺ mobilization, which, in all cases, was largely dependent on Rac1 activity (**Fig. 31C**). Specifically, IL-1 β led to an increase in intracellular calcium levels 10 minutes after stimulation. However, while in Rac1 WT microglia, this increase in Ca²⁺ levels was only transient, Ca²⁺ levels in Rac1 T17N microglia were kept at high levels for the remaining time points (**Fig. 31C**). There were no apparent alterations in intracellular Ca²⁺ levels after IL-6 exposure in Rac1 WT at all time points. However, the intracellular Ca²⁺ levels in Rac1 T17N microglia increased 1 hour after stimulation. They were significantly different from the Rac1 WT microglia at the same time points (**Fig. 31D**). Finally, stimulation with TNF α reduced the intracellular calcium levels in Rac1 WT cells. Still, this reduction was not observed in Rac1 T17N cells (**Fig. 31E**).

LPS-induced inflammation can lead to metabolic reprogramming in microglia and potentially imbalance the intracellular AMP:ATP ratio (Hardie et al., 2012). AMP-activated protein kinase (AMPK), an intracellular sensor of the AMP:ATP ratio, is critical to promote the anti-inflammatory response and homeostasis reestablishment after neuroinflammation (Saito et al., 2019). Since our results suggested that Rac1 ablation/deficiency was abrogating the homeostatic microglial response to inflammation, we wondered if this effect was also translated into AMPK activation. To evaluate AMPK activation, we transfected Rac1 WT and Rac1 T17N microglia with a fluorescent biosensor to measure cytosolic AMPK activity (Miyamoto et al., 2015) and stimulated them with 100ng/ml LPS for 30 minutes. LPS induced an increase in cytosolic AMPK activity in Rac1 WT microglia. Overexpression of Rac1 T17N, however, prevented the LPS-induced increase in cytosolic AMPK activity (**Fig. 31F**).

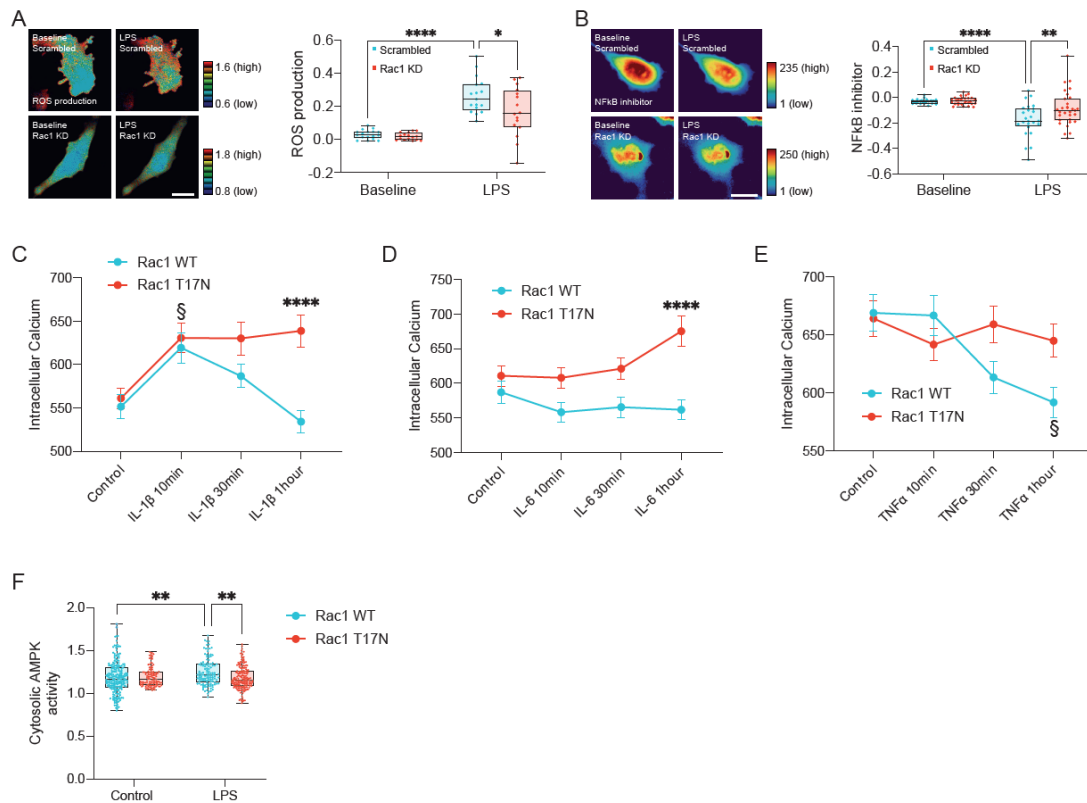


Figure 31 – Rac1 coordinates the microglial intracellular signaling during neuroinflammation. (A) Control (scrambled) or Rac1 knocked down (KD) HMC3 microglia expressing a ROS FRET biosensor were recorded in saline before (t=0 min; baseline) and after treatment with 100 ng/ml LPS. Whisker plots (median and min/max) show time-lapse Donor/FRET ratio changes (n=16-17 cells pooled across 3 different experiments). Pseudocolor ramps display representative ratio values. *p<0.05; ****p<0.0001 (Two-Way ANOVA). Scale bar: 10 μm. (B) Control (scrambled) or Rac1 knocked down (KD) HMC3 microglia expressing an NFkB inhibitor biosensor were recorded in saline before (t=0 min; baseline) and after treatment with 100 ng/ml LPS. Whisker plots (median and min/max) show time-lapse fluorescence changes (n=25-31 cells pooled across 3 different experiments). Pseudocolor ramps display representative fluorescence values. **p<0.01; ****p<0.0001 (Two-Way ANOVA). Scale bar: 5 μm. (C, D, E) Rac1 WT or Rac1 T17N HMC microglia expressing an intracellular Ca²⁺ biosensor, were treated with saline or 1ng/mL IL-1β (n = 244 – 35 cells pooled across 3 independent experiments) (C), 50pg/mL IL-6 (n = 235 – 297 cells pooled across 3 independent experiments) (D) or 50pg/mL TNF-α (n = 313 – 373 cells pooled across 3 independent experiments) (E) for 10 minutes, 30 minutes and 1 hour. Line graphs (mean and SEM) represent the changes in intracellular Ca²⁺ levels. ****p < 0.0001 (IL-1β or IL-6 1hour Rac1 WT vs Rac1 T17N – Two-way ANOVA); § p < 0.05 (comparison with control – Two-way ANOVA). (F) Rac1 WT or Rac1 T17N HMC microglia expressing a cytosolic AMPK activity FRET biosensor, were treated with saline or 100ng/mL LPS. Whisker plot (median and min/max) show changes in FRET/donor ratio (n = 122 – 213 cells pooled across 3 independent experiments). **p<0.01 (Two-way ANOVA).

These results suggest that Rac1 ablation or dysfunction perturbs the typical response of microglia to pro-inflammatory stimuli, placing Rac1 as a key element in the microglial response to neuroinflammation.

Rac1 ablation prevents microgliosis and phagolysosome formation upon LPS stimulation.

Ultimately, to understand the impact of Rac1 on the microglial response to LPS *in vivo*, we evaluated microgliosis and phagolysosome structures in microglia from Rac1^{fl/fl} and Cx3cr1^{CreER+/+}:Rac1^{fl/fl} mice. Firstly, we observed that the number of Iba1⁺ cells in the brains of Rac1^{fl/fl} mice injected with LPS was significantly higher than the number of Iba1⁺ cells in the brains of Rac1^{fl/fl} mice injected with a saline solution, indicative of microgliosis. LPS administration in Cx3cr1^{CreER+/+}:Rac1^{fl/fl} mice, however, did not trigger the same effect, with the number of Iba1⁺ cells being comparable with the ones from saline-injected mice (**Fig. 32A**). This result was also confirmed by evaluating the number of CD11b⁺CD45^{low}Ly6C⁻ cells by flow cytometry (**Fig. 32B**).

Basal CD68 expression in microglia is considered low in homeostatic conditions and increases significantly when microglia respond to stimuli. Thus, we evaluated if LPS increased CD68 expression in microglia and if this response was prevented by ablating Rac1. As expected, CD68 expression in microglia from Rac1^{fl/fl} mice injected with LPS was significantly increased compared to those injected with a saline solution. In Cx3cr1^{CreER+/+}:Rac1^{fl/fl} mice, however, this increase in CD68 expression was prevented (**Fig. 32C**).

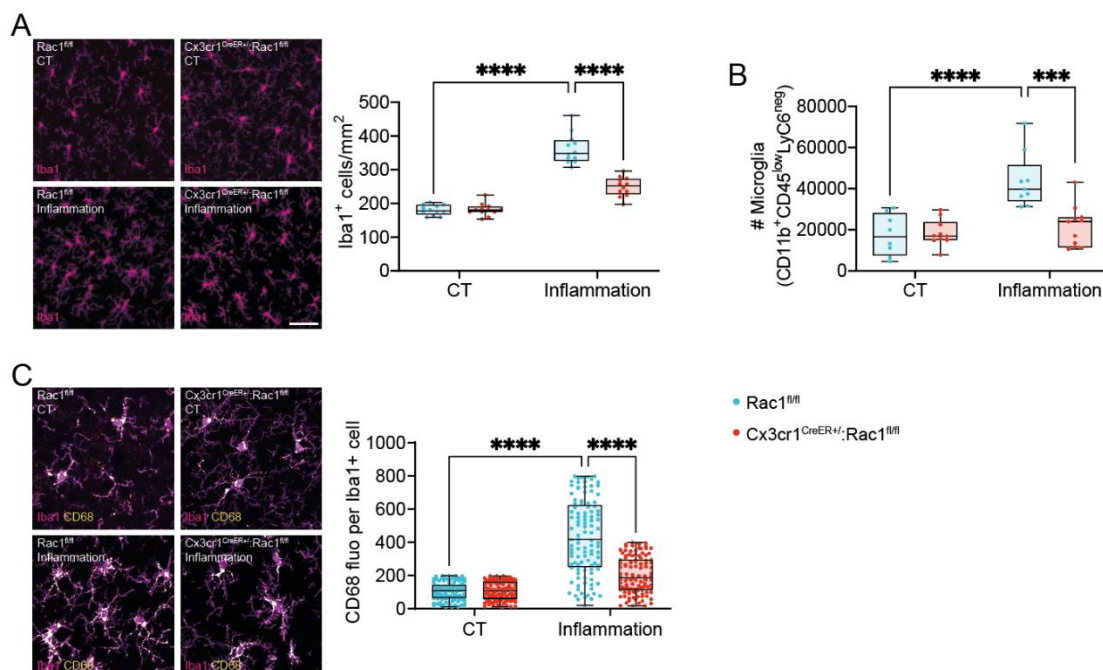


Figure 32 – Rac1 is critical for the microglial response to neuroinflammation *in vivo*. (A) Histological confocal analysis of Iba1 on tissue sections from neocortices of Rac1^{fl/fl} and Cx3cr1^{CreER+/+}:Rac1^{fl/fl} mice after treatment with saline (CT) or intraperitoneal injection of 4 mg/kg LPS (inflammation) for 24 h (n=3 slices from 4 animals per group). Whisker plots (median and min/max) depict Iba1⁺ cell counts. *p < 0.05 (Two-way ANOVA). Scale bar: 50 μ m. (B) Flow cytometry analysis of microglia from the brains of Rac1^{fl/fl} and Cx3cr1^{CreER+/+}:Rac1^{fl/fl} mice after treatment with saline (CT) or intraperitoneal injection of 4 mg/kg LPS

(inflammation) for 24 h (n=8-11 animals per group). Whisker plots (median and min/max) depict microglial cell numbers. * $p < 0.05$ (Two-way ANOVA). **(C)** Histological confocal analysis of CD68 and Iba1 on tissue sections from neocortices of $Rac1^{fl/fl}$ and $Cx3cr1^{CreER+};Rac1^{fl/fl}$ mice after treatment with saline (CT) or intraperitoneal injection of 4 mg/kg LPS (inflammation) for 24 h (n=108 cells pooled across 4 animals per group). Whisker plots (median and min/max) depict CD68 fluorescence per Iba1⁺ cell. **** $p < 0.0001$ (Two-way ANOVA). Scale bar: 20 μ m.

These results confirm the critical role of microglial Rac1 in microgliosis and the increase in CD68 expression during neuroinflammation, highlighting that the intracellular signaling impairments extend and impact the overall cellular response to LPS.

Discussion

Combining microglia-specific gene ablation with high-throughput phosphoproteomics, live-cell imaging, high-content screening strategies, and immunofluorescence, we showed that Rac1 is a critical regulator of the intracellular signaling machinery necessary to respond to neuroinflammatory stimuli with a specific focus on LPS (**Fig. 33**).

Phosphoproteomics of microglia isolated from Rac1^{fl/fl} injected with LPS revealed a general effect on several signaling networks, with robust Rho GTPase signaling network modulation. This bioinformatic analysis also highlighted the Rac1 GTPase cycle as the most significantly represented pathway from the Rho GTPases affected in this context, followed by RhoA, demonstrating the hierarchical organization of Rho GTPases in this context. While our previous data on environmental enrichment also revealed a robust modulation of the Rho GTPase signaling (with a preponderance of Rac1) (Socodato, Almeida, et al., 2023), it is worth mentioning that there were differences both in the hierarchical organization of the three main Rho GTPases and of the phosphoproteins involved in each paradigm. This emphasizes the adaptability of the Rho GTPase signaling and reinforces the position of these proteins as crucial signal integrators. Integration with the data from Cx3cr1^{CreER+/+}:Rac1^{fl/fl} injected with LPS pointed out specific signaling networks modulated specifically by Rac1 in this context, underscoring the intense dependency on microglial Rac1. *In vitro* experiments with HMC3 further confirmed the LPS-induced Rac1 activation, raising the hypothesis that Rac1 signaling drives the response to neuroinflammation.

To further investigate this hypothesis, we relied on our *in vitro* system to explore key signaling steps of the inflammatory process. In agreement with the classical functions of Rac1 in the NADPH oxidase complex (producer of ROS) (Abo et al., 1991; Zhao et al., 2003) and NF-κB pathway regulation (Cuadrado et al., 2014), we observed that removing Rac1 from microglia abrogated the LPS-induced ROS production and NF-κB pathway activation.

Pro-inflammatory cytokine release and sensing are vital parts of the neuroinflammatory process. These molecules can be produced by microglia but can also be released by peripheral myeloid cells and cross the blood-brain barrier (BBB) in paradigms of systemic inflammation. Given the indications provided by the phosphoproteomics on immune signaling and intracellular signaling, we explored if Rac1 was involved in the signaling integration of classical pro-inflammatory cytokines by measuring the global intracellular Ca²⁺ mobilization in microglia. IL1β, IL6, and TNFα stimulation are described to promote a transient increase of intracellular Ca²⁺ levels in microglia. However, this effect depends on

cytokine concentration, which can explain why it was only possible to observe this effect with IL1 β .

Nevertheless, there was a clear differential response to the three pro-inflammatory cytokines of microglia overexpressing wild-type Rac1 or the defective dominant-negative form. Interestingly, Rac1 T17N microglia responded to IL1 but failed to reestablish the Ca²⁺ up to 1 hour after stimulation, confirming a disruption of the typical response of microglia to these pro-inflammatory stimuli. Whether this increase in Ca²⁺ levels would lead to sustained activation of microglia or if it would lead to a complete disruption of the intracellular signaling remains elusive and requires additional experiments.

Several studies in microglia point out that LPS stimulation modulates AMPK phosphorylation on its catalytic domain (Thr172) by reducing its phosphorylation (Fan et al., 2018; Li et al., 2018). This, however, can be context-specific, meaning that depending on the cell line, stimulation time, and concentration, it is possible to observe an increase in AMPK phosphorylation (Wang et al., 2015). This can be an indirect effect due to an imbalance in the intracellular AMP:ATP ratio caused by the LPS-induced energy stress. Besides, activating AMPK promotes an anti-inflammatory response and homeostasis reestablishment (Chen et al., 2014). This can explain the rather unexpected observation in our experimental setup, where AMPK activity was increased after LPS exposure in Rac1 WT microglia. The lack of response by Rac1 T17N microglia further supports the idea that Rac1 is critical for microglia to sense or transduce the information from extracellular neuroinflammatory cues, which is firmly supported by the phosphoproteomics data.

Robust expansion of the microglial population characterizes the response to acute, deleterious neuroinflammatory triggers. This is accompanied by increased CD68 expression, a protein in the phagolysosomes (Jung et al., 2023; Ye et al., 2020). Its expression is kept at low levels in homeostatic conditions and dramatically increases when microglia are triggered. We decided to explore if Rac1 was exerting a role in these processes *in vivo*. LPS administration in Rac1^{fl/fl} mice reflected the expected outcomes, with an increase in the number of microglia and CD68 expression. Rac1 ablation prevented the LPS-induced changes in the microglial population.

While this data raises several questions and leaves some points unsolved, it seems clear that microglia without Rac1 either become unresponsive or exert an inefficient response to neuroinflammatory triggers. As previously mentioned, a normal neuroinflammatory reaction suppresses harmful triggers and contributes to CNS protection (Sochocka et al., 2017). Additionally, several neurodegenerative diseases are characterized by an exacerbated, uncontrolled, and sometimes perpetual pro-inflammatory response of microglia, which

ultimately becomes detrimental. Thus, precise regulation of the neuroinflammatory process is critical to minimizing damage to the brain and regaining tissue homeostasis. Rac1 works as a binary switch that can cycle between an active and an inactive form, a process mainly regulated by GEFs and GAPs. Considering its capacity to interchange between “on” and “off” states, our results suggest that Rac1 activity can be potentially targeted to tightly control and modulate the neuroinflammatory response of microglia.

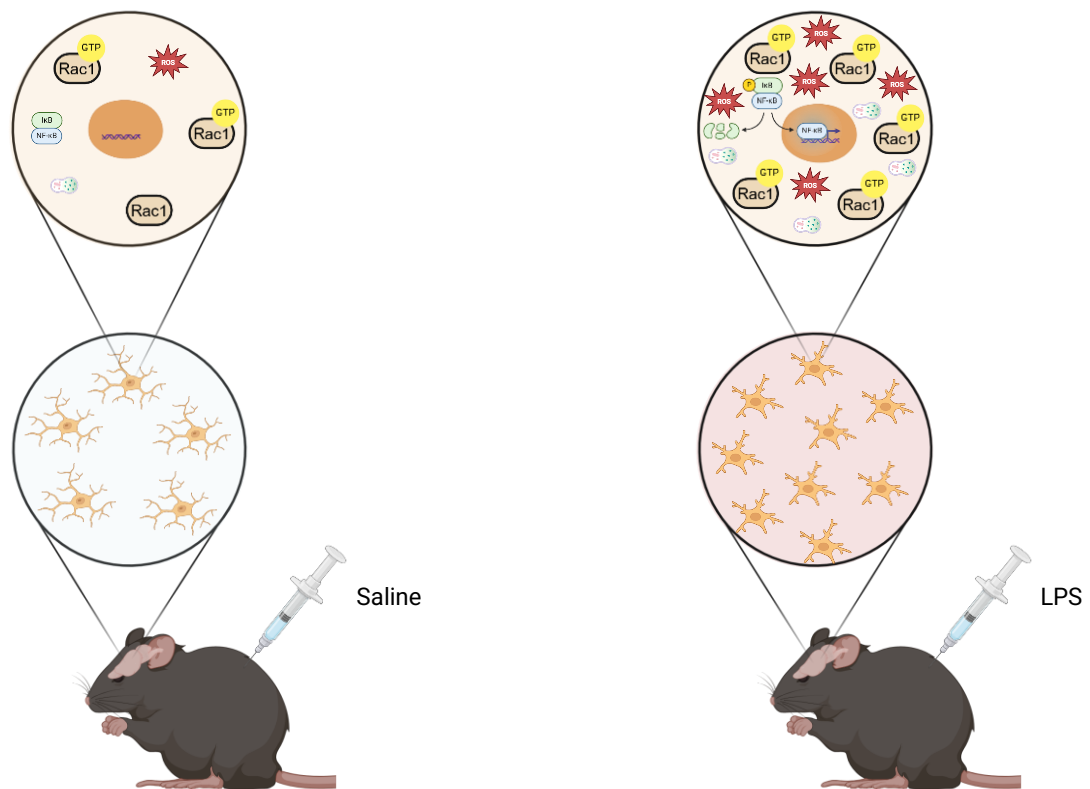


Figure 33 – Rac1 is a critical regulator of the intracellular signaling machinery during neuroinflammation.

General Discussion

General Discussion

The number of microglia-related publications has been constantly rising every year, demonstrating the interest and relevance of microglia biology (Dashwood & Liston, 2024). The present thesis aims at further contributing to our understanding of microglia function, by exploring the specific regulatory effects of Rac1, a protein associated both with cytoskeleton dynamics and regulation of the inflammatory response. While one would expect that Rac1 regulation of microglia is similar to that of other myeloid cells, the reality is that the functions regulated by Rho GTPase signaling are cell-type and context-specific. In this work, to study the functions of Rac1 in microglia, I employed complementary *in vivo* and *in vitro* models.

In Chapter 1, I started by demonstrating the requirement of Rac1 for microglia morphology *in vivo*. To complement and overcome the caveat of using a static approach in this evaluation, we performed FRET live-cell imaging in HMC3 microglia. This highly sensitive imaging strategy allowed real-time visualization of cellular processes with spatial resolution. In other cell types, Rac1 regulates lamellar protrusion dynamics, involved in processes such as cell motility (Fiedler, 2009; Fort et al., 2018; Stephanie & Timothy, 2006; Wittmann et al., 2003). Thus, we hypothesized that Rac1 could have a regulatory effect on microglia protrusions and studied protrusion dynamics on microglia in which Rac1 signaling was impaired by the expression of a dominant negative form of Rac1 (T17N). These cells presented reduced protrusion dynamics compared with microglia overexpressing wild-type Rac1. Several studies have already revealed the importance of microglia contact with other cells, including neurons, oligodendrocytes and astrocytes during homeostasis and in disease (Miyamoto et al., 2016; Rostami et al., 2021; Uranova et al., 2018). Decreased protrusion dynamics in Rac1 T17N microglia led us to start wondering if the impairment in protrusion dynamics could perturb these communications. Nevertheless, we understand that microglia morphology *in vitro* does not fully recapitulate the morphology of microglia in the brain. Therefore, it remains elusive the specific impact of Rac1 ablation in homeostatic microglia dynamics *in vivo*, as well as in response to stimuli, that could be best addressed using two-photon intravital microscopy as in (Davalos et al., 2005; Nimmerjahn et al., 2005).

Further exploration of Rac1 genetic ablation in homeostatic microglia revealed that Rac1 is not necessary for microglia engulfment of synaptic elements. Furthermore, the loss of Rac1 did not elicit changes in the microglia oxidative stress under homeostatic conditions. Given the classical participation of Rac1 in phagocytosis (Caron & Hall, 1998) and NADPH oxidase complex activation (Abo et al., 1991; Zhao et al., 2003), these observations were

quite unexpected. Caron & Hall described that Rac1 activity was not required for complement-mediated phagocytosis (Caron & Hall, 1998). Synaptic engulfment by microglia, also known as synaptic pruning, is regulated by the complement cascade (Stevens et al., 2007). While this could be a possible explanation for the absence of a significant impact in synaptic engulfment, it does not explain the lack of phagocytosis-related transcriptomic changes upon Rac1 ablation. Thus, in homeostasis, Rac1 does not seem to impact microglia in these two critical aspects of Rac1 signaling.

RNA sequencing, however, revealed that Rac1 ablation was indeed regulating microglial functions at the transcriptomic level. We complemented our transcriptomic data by generating a microglia phosphoproteomic dataset. Driven by the information extracted from both the transcriptomic and phosphoproteomic datasets, we identified a potential modulatory role of Rac1 in microglia-synapse crosstalk and environmental sensing capacity. Regulation of intracellular trafficking, Ca^{2+} and MAPK signaling, as well as synaptic transmission in Rac1-ablated microglia, further suggested that the communication between microglia and synapses could be impaired. It wasn't until we challenged our *in vivo* system, using a protocol of environmental enrichment, that we fully appreciated the intricate influence of microglial Rac1 signaling in the brains of $\text{Cx3cr1}^{\text{CreER+/-}};\text{Rac1}^{\text{fl/fl}}$ mice. Microglia are sensitive to neuronal activity and respond to these changes by modulating their Ca^{2+} signaling (Liu et al., 2021; Umpierre et al., 2020) and by sending protrusions in response to ATP released by neurons (Badimon et al., 2020; Cserép et al., 2020). We decided to use an environmental enrichment paradigm to potentiate synaptic activity and transmission and improve cognition (Abraham et al., 2019). Environmental enrichment provides a stimulating context where cognitive, sensory and social interactions can be promoted by the addition of elements that increase the complexity of the mice home cage. Since this can be modulated at several levels, the available protocols for environmental enrichment are quite diverse. One of the key aspects is the duration of enrichment. While short-term stimulation can produce transient effects or just reflect acute modifications in synaptic plasticity, such as increased excitability, long-term enrichment induces robust and long lasting structural consequences, that likely require microglia, in neuronal circuitry and behaviour (Ohline & Abraham, 2019). Therefore, this long-term and demanding experimental paradigm in terms of microglia-synapse crosstalk was chosen to evaluate the requirements of microglial Rac1 signaling in this bidirectional communication. Firstly, we observed that environmental enrichment strongly modulated the microglial phosphoproteomic signaling network, having a predominant effect on Rho GTPase signaling and synapse crosstalk, and that these changes were dependent on microglial Rac1. Synaptic regulation by microglia is achieved through a combination of direct physical contacts between both compartments and secreted

factors. Direct contacts allow precise monitoring and refinement of the brain circuitry, whereas microglia-released factors can have a widespread effect on synaptic function. In agreement with an impairment in microglial protrusion dynamics and protein secretion, we demonstrated that Rac1 is critical for microglia-synapse crosstalk by modulating both the physical contacts of microglia with synapses and the secretion of signaling factors necessary for synaptic activity. Although our data strongly suggest that synaptic remodeling during enrichment results from a combination of these two regulatory strategies by microglia, we did not evaluate the impact of secreted factors in this particular context. Therefore, the specific effects of microglia-secreted factors in environmental enrichment remain elusive. Impairments in microglia-synapse crosstalk ultimately led to an abrogation of the enrichment-induced cognitive improvements observed in control mice (Socodato, Almeida, et al., 2023). Taken together, the work presented in Chapter 1 allowed us to identify novel roles for Rac1 in microglia and CNS biology. Moreover, it opened the possibility of further dissecting the potential microglia secretory deficits elicited by Rac1 ablation.

Chapter 2 focused on exploring potential molecules participating in this process. Our bioinformatic analyses suggested a dependency of the GDNF signaling on microglial Rac1. Thus, we dissected the GDNF availability and proteins involved in its secretion. We confirmed that microglial GDNF levels were dependent on microglial Rac1, possibly via mTORC1 activity, and explored the participation of an interactor of Rac1 in this process. Rab27a, a protein that decorates vesicles and coordinates its transport inside the cell, was decreased in Rac1 knocked-down microglia. Besides, Rab27a also regulated GDNF levels in microglia. In this chapter, we further explored the consequences of Rac1 ablation in the neuronal compartment and confirmed the essential role of microglial Rac1 in spine formation, synapse numbers and learning memory. Our observations strongly suggest that microglial GDNF, through Rac1 and Rab27a is critical for these processes.

Although our bioinformatics data supports Rac1 modulation of GDNF signaling and does not suggest a regulatory effect of Rac1 in BDNF signaling, at present we cannot formally exclude it. BDNF is a key regulator of synaptic development and plasticity (Chao, 2003). However, while microglia-derived BDNF is classically described to contribute for motor learning-dependent synapse formation (Parkhurst et al., 2013), GDNF signaling has been associated with neuronal survival and axonal regeneration (Lin et al., 1993; Sauer et al., 1995). Depending on the targeting approaches, mice with BDNF signaling impairments can present changes in risk assessment (Olsen et al., 2013), aggression (Ito et al., 2011), or depressive-like behaviors (Kojima et al., 2020). In contrast, disruption of GDNF signaling leads to learning and spatial memory deficits (Bonafina et al., 2019; Gerlai et al., 2001). Given the similarity between these observations and the behavioral phenotypes observed

in Rac1 mutant mice, this further strengthens our hypothesis that the synaptic and behavioural phenotypes observed in Rac1 mutants are caused by decrease of microglial GDNF secretion. In the future, we will also assess BDNF expression and secretion levels in Rac1 mutant microglia. It would also be interesting to clarify the specific contribution of microglial BDNF in the context of environmental enrichment, since this paradigm has been shown to increase BDNF levels in several brain regions (even though the BDNF origin was not addressed) (Harb et al., 2021). Overall, Chapter 2 complemented the previous observations in chapter 1 by identifying GDNF as a candidate to modulate growth-factor-dependent microglia-synapse crosstalk.

In Chapter 3, we explored the contribution of microglial Rac1 signaling in inflammation using an experimental paradigm of neuroinflammation. Oppositely to environmental enrichment, LPS-induced neuroinflammation is a deleterious paradigm in which a strong immune proinflammatory response is triggered in microglia. LPS profoundly affected the microglia phosphoproteomic landscape in a Rac1-dependent manner. Surprisingly, a simple comparison between the environmental enrichment and LPS-induced effects in microglia, reveals a common set of regulated pathways in both contexts. Among the classical Rho GTPases, Rac1 signaling was the most affected by both stimuli. This raises the hypothesis that there might be a conserved signaling machinery that is activated when microglia respond to both stimuli, with Rac1 occupying a central role in the coordination of these processes. A deeper look into both datasets, at the phosphorylation level, might highlight both common and specific signaling patterns for responsive microglia. Rho GTPases can be activated by several membrane receptors to integrate signals. Dynamic orchestration of the complex signaling mechanisms of Rho GTPase signaling (GEFs, GAPs, GDIs and effectors) can produce downstream signaling events that are specific but at the same time use common signaling pathways.

The microglial response to pro-inflammatory stimuli relies on multistep signaling steps, including ROS production, NF- κ B pathway activation, Ca²⁺ mobilization and AMPK activation to reestablish tissue homeostasis. Our *in vitro* results support a critical role of Rac1 in coordinating the intracellular signaling machinery required for the microglial response to inflammatory cues. While Rac1 was dispensable for microglial oxidative stress under homeostatic conditions, challenging the system with an inflammatory stimulus brought to light the contribution of Rac1 for microglial ROS production, in agreement with its classical role in the NADPH oxidase complex (Qin et al., 2004; Wu et al., 2006). Microglial response to LPS *in vitro* is known to activate PI3K/Akt/mTORC signaling (Ye et al., 2020). Moreover, mTORC1 is a master regulator of autophagy (Kim et al., 2011). We previously mentioned that mTORC1 activity was reduced in Rac1-defective microglia. Since

bioinformatic analyses also revealed Rac1-dependent regulation of autophagy in the context of LPS-induced inflammation, we can speculate that Rac1 signaling, via mTORC1, might be an additional contribution for the microglial response to neuroinflammation. Ultimately, we demonstrated that Rac1 was necessary for microgliosis and increased CD68 expression, features from neuroinflammation (Jung et al., 2023; Ye et al., 2020). Altogether, the work described in Chapter 3 supports an essential role of Rac1 in the microglial response to neuroinflammatory cues.

While more work is needed to understand the specific roles of Rho GTPases and the actin cytoskeleton in microglia, the data presented in this thesis proposing Rac1 as a critical protein for microglia biology, complement other studies recently performed in our lab on RhoA and type-II myosins (Melo et al., 2021; Socodato, Portugal, et al., 2020; Socodato, Rodrigues-Santos, et al., 2023). Our results identify Rac1 signaling as a novel regulator of microglia-synapse crosstalk, brain plasticity and cognitive performance and for microglia response to environmental cues. Rac1 signaling becomes then a potentially relevant investigation target to modulate cognitive performance and control microglia-mediated neuroinflammation.

General Conclusions

General Conclusions

Taken together, the work developed and presented in this thesis leads to the following conclusions:

1. Microglia morphology and protrusion dynamics are modulated by Rac1.
2. Rac1 is dispensable for synaptic engulfment and microglia redox state at steady state.
3. Ablation of Rac1 in microglia impairs microglia-synapse crosstalk, both via direct microglia-synapse contacts and microglia secretion of factors.
4. Microglial Rac1 is critical for brain plasticity and cognitive improvements induced by environmental enrichment.
5. GDNF availability in microglia is modulated by Rac1 activity, potentially via mTORC1 and Rab27a signaling pathways.
6. Rac1 signaling in microglia is necessary for synaptic homeostasis and cognitive functions.
7. Environmental enrichment and LPS profoundly and hierarchically remodel the microglial Rho GTPase signaling, preferentially affecting Rac1 GTPase cycle over RhoA and Cdc42 GTPase cycle.
8. Rac1 is necessary for the microglial response to neuroinflammation, by promoting ROS production, NF- κ B pathway activation, intracellular Ca^{2+} mobilization, microgliosis and CD68 expression.

Future Work

Future Work

While the work presented in this thesis provides novel insights on the roles of Rac1 in microglia biology, it also opens new research perspectives to be explored in the future. Thus, in this section, I propose a set of follow-up studies that would substantially add to the observations reported in this thesis:

- Evaluate the Rac1 effect on Fc-mediated phagocytosis of pathogens, apoptotic cells or cell debris in microglia *in vivo* and *in vitro*. To achieve this, one possibility could be injecting fluorescently labeled myelin into the brain of control and mutant Rac1 mice and then evaluate phagocytosis by immunofluorescence or flow cytometry. This would clarify the contribution of Rac1 for phagocytosis in microglia.
- Further dissect the signaling steps that regulate GDNF availability and secretion in microglia to establish the full signaling mechanism, using our streamlined pipeline for *in vitro* high-throughput screening and FRET-based biosensor imaging. Validation *in vivo* could be performed thereafter in our Rac1 mutant mice.
- Verify if Rac1 contributes for BDNF expression levels and availability in microglia. This could be performed with the same approaches used for GDNF (flow cytometry and immunofluorescence).
- Overexpress GDNF *in vivo* and evaluate recovery of synaptic homeostasis and behaviour in Rac1 mutant mice. This could be carried out using adeno-associated viral (Lin et al., 2022) or lentiviral delivery (Åkerblom et al., 2013) to transduce microglia. Phenotype recovery could be evaluated by counting the number of excitatory synapses and checking learning memory. If proved, it could also become interesting to overexpress GDNF in the context of environmental enrichment and understand if this would be enough to reestablish cognitive improvements in enriched Rac1 mutant mice.
- Compare the environmental enrichment and LPS phosphoproteomes. While a formal comparison was not yet performed between both datasets, it is possible to identify similarities on the pathways modulated in these two different contexts. Since the specific phosphoproteins involved in each situation can be different, it would be interesting to map the common and non-common phosphoproteins and respective phosphorylation residues for both stimuli. This could reveal a common phosphoproteomic signature of responsive microglia. The addition and integration of a third dataset on a different paradigm, such as tissue lesion or neurodegenerative disease, would also strengthen these bioinformatic analyses.

We believe that these experiments would reinforce and validate some of the observations described in this work. Moreover, they would be a valuable addition to our understanding of Rac1 regulation of microglial cell functions in different brain environments, ultimately contributing towards a better understanding of microglia biology.

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