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Born on 23 April 1994 in København (Denmark)

MICROGLIA PROGRAMS UNDER *PARK7*/DJ-1 DEFICIENCY, A GENETIC CAUSE OF PARKINSON'S DISEASE

A dissertation by

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Contents

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Abbreviations

AD	Alzheimer's disease
AP1	Activator protein 1
α-syn	α-synuclein
ATP	Adenosine triphosphate
BAM	Border-associated macrophages
BBB	Blood-brain barrier
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
CP	Choroid plexus
CSF	Cerebrospinal fluid
CSF1	Colony-stimulating factor 1
DAMP	Damage-associated molecular pattern
DAVID	Database for Annotation, Visualization, and Integrated Discovery
DJ-1	Deglycase 1
DMEM	Dulbecco's modified Eagle Medium
DAPI	4',6-diamidino-2-phenylindole
EMP	Erythroid-myeloid progenitor
FBS	Fetal bovine serum
FACS	Fluorescence-activated cell sorting
GO	Gene ontology
GW	Gestational week
GWAS	Genome-wide association studies
HSC	Hematopoietic stem cell
IBA1	Ionized calcium-binding adaptor protein 1
IGF1	Insulin-like growth factor 1
IL-6	Interleukin 6
IFN-γ	Interferon-gamma

iPSC	Induced pluripotent stem cell
IRF8	Interferon regulatory factor 8
KEGG	Kyoto Encyclopedia of Genes and Genomes
KI	Knock-in
KO	Knock-out
LB	Lewy body
LPS	Lipopolysaccharide
MACS	Magnetic-activated cell sorting
MIRO1	Mitochondrial Rho GTPase 1
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NO	Nitric oxide
PAMP	Pathogen-associated molecular pattern
PARK7	Parkinsonism-associated deglycase
PCA	Principal component analysis
PET	Positron emission tomography
PD	Parkinson´s disease
REM	Rapid eye movement
RBD	REM sleep behavior disorder
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RUNX	Runt-related transcription factor 1
scRNA	Single-cell RNA sequencing
SNpc	Substantia nigra pars compacta
TGF-β	Transforming growth factor-beta
TH	Tyrosine hydroxylase
TNFα	Tumor necrosis factor-alpha
TLR	Toll-like receptor
UMAP	Uniform manifold approximation and projection
WT	Wildtype

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Frida

Affidavit

I hereby confirm that the PhD thesis entitled “Microglia programs under *PARK7*/DJ-1 deficiency, a genetic cause of Parkinson’s disease” has been written independently and without any other sources than cited. All necessary ethical approvals have been obtained in accordance with the animal welfare law in Luxembourg (Règlement Grand-Ducal adopted on January 11th, 2013).

Luxembourg, 12th of February, 2025 Frida Lind-Holm Mogensen

List of publications

List of publications included in the thesis:

1. **PARK7/DJ-1 in microglia: Implications in Parkinson's disease and relevance as a therapeutic target**

Lind-Holm Mogensen F., Scafidi A., Poli A., Michelucci A. *Journal of Neuroinflammation* 2023 20:95
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2. **Protocol for immunofluorescence staining and large-scale analysis to quantify microglial cell morphology at single-cell resolution in mice**

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3. ***PARK7*/DJ-1 deficiency impairs microglial activation in response to LPS-induced inflammation**

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4. **Microglial dynamics and neuroinflammation in prodromal and early Parkinson's disease**

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List of publications not included in the thesis:

1. **Metformin impacts the differentiation of mouse bone marrow cells into macrophages affecting tumor immunity**

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DOI: 10.1016/j.heliyon.2024.e37792

2. **Protocol for the generation and assessment of functional macrophages from mouse bone marrow cells**

Scafidi A., **Lind-Holm Mogensen F.**, Michelucci A. *Manuscript under review in STAR Protocols*

3. **AllergoOncology: Biomarkers and refined classification for research in the allergy and glioma nexus – A joint EAACI-EANO position paper**

Turner C.M., Radzikowska U., Ferastraoaru E.D., Pascal M., McCraw A., Backes C., Bax H.J., Bergmann C., Bianchini R., Cari L., De las Vecillas L., Izquierdo E., **Lind-Holm Mogensen F**., Michelucci A., Nazarov P.V., S.P., Nocentini G., Ollert M., Preusser M., Rohr-Udilova N., Scafidi A., Toth R., Van Hemelrijck M., Weller M., Jappe U., Escribese M.M., Jensen-Jarolim E., Karagiannis S.N., Poli A. *Allergy* 2024 79(6):1419-1439
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4. **Targeting the ACOD1-itaconate axis stabilizes atherosclerotic plaques**

Harber K.J., Neele A.E., van Roomen C.P.A.A., Gijbels M.J.J., Beckers L., den Toom M., Schomakers B.V., Heister D.A.F., Willemsen L., Griffith G.R., de Goede K.E., van Dierendonck X.A.M.H., Reiche M.E., Poli A., **Lind-Holm Mogensen F.,** Michelucci A., Verberk S.G.S., de Vries H., van Weeghel M., Van den Bossche J., de Winther M.P.J.
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5. **Parkinson's disease-related mutant Miro1 causes mitochondrial dysfunction and loss of dopaminergic neurons**

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DOI: 10.1093/brain/awaf051

Abstract

Microglia, the resident immune cells of the central nervous system, play a critical role in neuroinflammatory responses associated with Parkinson's disease (PD). Their activation and the resulting inflammatory cascades are significant contributors for the progression of the pathology. Approximately 10-15% of PD cases are linked to genetic factors, including mutations in the *PARK7* gene that lead to a deficiency in DJ-1, a protein with vital antioxidant functions. DJ-1 is involved in transcriptional regulation, it has chaperone and protease functions and protects cells against reactive oxygen species. However, the specific mechanisms by which DJ-1 deficiency contributes to the development of early-onset PD remain unclear. This PhD project explores microglial transcriptional, functional and morphological changes during DJ-1 deficiency both at baseline and under inflammatory conditions. By combining single-cell and bulk RNA sequencing with multicolor flow cytometry and immunofluorescence analyses, we identified distinct activation profiles in DJ-1 deficient microglia when compared with corresponding wildtype (WT) cells. Furthermore, we detected morphological changes of microglia in *PARK7*/DJ-1 knock-out (KO) mouse brains both at baseline and under inflammatory conditions as well as in an additional mouse model of PD, the Miro1 p.R285Q knock-in mouse.

Our findings indicate that microglia in *PARK7*/DJ-1 KO mice treated with lipopolysaccharide (LPS) exhibit a unique transcriptional profile, specifically evidenced by changes associated with type II interferon and DNA damage response signaling, when compared with WT mice. Human *PARK7*/DJ-1 mutant iPSC-derived microglia showed comparable transcriptional signatures, highlighting the translational relevance of our findings. These genetic alterations were also reflected at the morphological level, with microglia in LPS-treated *PARK7*/DJ-1 KO mice displaying a less amoeboid cell shape compared with WT mice. These observations suggest that the underlying oxidative stress under DJ-1 deficiency impacts both microglial homeostatic and activated states. As balanced immune responses are crucial for the CNS homeostasis, compromised microglial responses under DJ-1 deficiency could contribute to PD development and progression. Hence, the identification of targets, which may restore microglial functions and immune responses, could pave the way to the development of novel therapeutic approaches, not only for PD patients carrying *PARK7* mutations, but also for a broader spectrum of idiopathic PD patients where DJ-1 functions are lost as a result of its oxidation.

CHAPTER Ⅰ - OBJECTIVES & RESULTS OUTLINE

Parkinson's disease (PD) is a highly complex, systemic and multifactorial neurodegenerative disease with no available cure. It is the fastest growing neurological disease worldwide, significantly impacting patients' wellbeing and imposing substantial burdens on their families and society, as many patients require assistance for self-care. PD is caused by a complex interplay of genetic and environmental factors, making the modelling of PD highly challenging. Various cell types contribute to the disease, with microglia, the brain's resident immune cells, being among the first to respond to brain perturbations that can occur decades before the disease onset. Dysregulation of microglial functions are implicated in PD pathogenesis both at early and late disease stages, although not yet clearly elucidated. However, brain tissue from PD patients is rare and often obtained post-mortem, limiting the investigation of early disease mechanisms.

By deeply collecting and analyzing past and current literature on the role of DJ-1 in microglia, we found that the vast majority of studies were conducted using murine *in vitro* models. Hence, we sought to conduct further studies *in vivo*, both to compare our findings with *in vitro* outcomes and to further investigate morphological adaptations and immunological functions of microglia in a living organism enabling to study relevant functions, including cell-cell interactions and compensatory mechanisms, which are essential for understanding PD and cannot be fully recapitulated in the existing *in vitro* models. Furthermore, for translational perspectives, we conducted similar analyses in microglia derived from human DJ-1 mutant induced pluripotent stem cells (iPSCs).

Taken together, the aim of my PhD project was to characterize the transcriptional, morphological and functional changes of microglia during DJ-1 deficiency under homeostatic and inflammatory conditions, both in a mouse model and human iPSC-derived microglia. In addition to genotype differences, studying age-related changes in microglia is critical as aging is a major risk factor for many neurological diseases. Therefore, we took advantage of our animal studies to additionally investigate the impact of age, sex and treatment on the transcriptomic signatures of microglia as well as an additional PD mouse model for studying their morphological changes.

The results of the current project are presented across chapter **Ⅳ**, **Ⅴ**, **Ⅵ** and **Ⅶ**. The articles presented in chapter **Ⅳ** and **Ⅵ** were published in the *Journal of Neuroinflammation* (IF=9.3), while the article presented in chapter **Ⅴ** was published in *STAR Protocols* (IF=1.3). Lastly, the article presented in chapter **Ⅶ** is currently under review in the *Journal of Neuroinflammation.*

Chapter Ⅳ - This chapter presents the manuscript entitled *"PARK7/DJ-1 in microglia: Implications in Parkinson's disease and relevance as a therapeutic target"*, where we reviewed the functions of DJ-1 and their role in microglia. In addition, we discussed the usage of DJ-1 enhancers to restore microglial functions and as potential neuroprotective agents to treat PD patients.

Chapter Ⅴ - Here, we present the manuscript titled "*Protocol for immunofluorescence staining and large-scale analysis to quantify microglial cell morphology at single-cell resolution in mice"*. This detailed protocol outlines the entire process from handling the animals to extracting and analyzing morphological features and creating 3D reconstructions of microglia in the mouse brain.

Chapter Ⅵ - This chapter presents the manuscript titled *"PARK7/DJ-1 deficiency impairs microglial activation in response to LPS-induced inflammation"* in which the main findings of the characterization of microglia under DJ-1 deficiency at the transcriptional and morphological level, both in a mouse model and in human iPSC-derived microglia, are described. Further data not included in the original publication, such as additional functional studies, preliminary transcriptional analyses from the 13-month-old cohort as well as from male and female mice at baseline and in response to LPS, are added to this chapter. Additional data also show a summary of the characterization of morphological features of microglia in another PD mouse model, the *Miro1* knock-in mouse model.

Chapter Ⅶ - In this chapter, we present the article "*Microglial dynamics and neuroinflammation in prodromal and early Parkinson's disease*", which is a review article currently under review in the *Journal of Neuroinflammation*. In this manuscript, we describe the changes occurring in microglia before and immediately after the diagnosis of PD, emphasizing the significance of studying early microglial alterations in disease progression.

CHAPTER Ⅱ - MATERIALS & METHODS

Methods for molecular biology

- RNA extraction, reverse transcription, primer design and qPCR in Chapter Ⅵ.
- RNA sequencing (bulk and single cell) described in Chapter Ⅵ.

In vitro

- iPSC differentiation into microglia and quality control described in Chapter Ⅵ.

In vivo mouse work

- DJ-1 KO mouse model description, housing and genotyping in Chapter Ⅵ.
- The Miro1 knock-in (KI) mouse model was created using CRISPR/Cas9 gene editing as described in manuscript Ⅵ (manuscripts not included in the thesis).

Primary cell culture and functional assays

- Adult microglia isolation by CD11b enrichment described in Chapter Ⅵ.
- Bone marrow cell isolation and differentiation into macrophages described in Chapter Ⅵ.
- ROS assay described in Chapter Ⅵ.

Tissue preparation and immunofluorescence staining

- Perfusion, fixation, embedding, immunofluorescence staining, confocal imaging described in Chapter Ⅵ and specifically detailed in chapter Ⅴ.
- For the staining of the Miro1 mouse brain tissue, the slices were treated as free floating tissue as described in Chapter Ⅵ. However, the microscopy settings were slightly different. Pixel dwell time were faster (0.34 µs per pixel), to reduce the acquisition time. The images were required with a 10% overlap between tiles and therefore stitched using Imaris Stitcher without background subtraction, with a smooth filter width of 0.5 µm. The downstream analyses were conducted using Imaris following the protocol described in Chapter Ⅴ, except for a manual removal of cells at the border of the images.

Other

- Protein extraction and western blotting described in Chapter Ⅵ.

Additional methods not described in the chapters:

Blood isolation and analysis

A total of 100-300 µL of blood were isolated from the right ventricle of mice under deep ketamine/medetomidine anesthesia. Subsequent analysis was done using a hemocytometer (Ms4se). Immediately after analysis (10-50 µL used for hemocytometer analysis), the plasma was isolated by centrifugation for 30 minutes at 13000 rpm at 4°C in EDTA-coated tubes (**Table 1**) to sediment the blood cells.

Spleen and thymus isolation and analysis

Immediately after cardiac perfusion of DJ-1 KO and WT littermates, the spleen and thymus were dissected out and kept in DMEM at 4°C for a maximum of 24 hours (**Table 1**). Fat surrounding the organs was removed after keeping the organs in a petri dish for 2 minutes. The organs were measured using a ruler. The organs were placed along the ruler and width 1 (W1), width 2 (W2) and length were measured. Subsequently, the organs were measured in a weighing boat on an analytical scale and organ-to-bodyweight ratio were calculated.

Ex vivo phagocytosis assay

After 6 hours of LPS treatment *in vivo*, mice were anesthetized and perfused as described in Chapter Ⅴ. Microglia were isolated via magnetic activated cell sorting (MACS) with CD11b enrichment, followed by incubation with 1 µg/mL of pHrodo™ Red *E. Coli* bioparticles™ conjugates for phagocytosis (Invitrogen, P35361) in FACS tubes in suspension for 1 hour. Subsequently, cells were washed two times with FACS buffer. Fc block was added followed by incubation with antibodies (**Table 1**) for 30 minutes and L/D staining for another 30 minutes. Data were acquired on a Novocyte Quanteon Flow cytometer (Agilent) using the 405 nm violet laser, a 488 nm blue laser, a 561 nm yellow/green laser and a 633 nm red laser. Data were analyzed using FlowJo™ software (version 10.8.1) and each biological replicate (microglia from N=3 mice) represents the mean of two technical replicates.

Sex determination of cells from the single-cell RNA sequencing dataset

Single-cell RNA sequencing was performed after FACS sorting CD45int, CD11b+ cells and sequencing was performed as previously described (Sousa, Golebiewska et al. 2018) and in Chapter Ⅵ. CellXY and *Xist* expression were used to determine the sex of each cell from the single-cell RNA sequencing data GitHub - phipsonlab/cellXY: R package to predict sex of single cells and identify Male/Female

doublets using machine learning approaches. DOI: 10.5281/zenodo.14065058. CellXY is based on the expression of genes located on the chromosome X and Y.

Table 1. List of reagents and materials.

Name	Description	Reference n°	Brand
FBS	Fetal Bovine Serum	10270-106	Gibco
p/S	Penicillin-Streptomycin	DE-17062^{E} Lot no : 6MB057	Lonza
DMEM	Dulbecco's Modified Eagle Medium	12604F	Lonza
HEPES	HEPES Buffer	15630056	Gibco
DMEM/F12	Dulbecco's Modified Eagle Medium + F12		Gibco
PBS	Phosphate Buffered Saline	BE17-517Q	Lonza
BSA	Bovine Serum Albumin		Sigma
Trypan blue	Trypan blue		Invitrogen
pHrodo™ beads	pHrodo™ Red E. coli BioParticles™ Conjugate for Phagocytosis	2539804P lot no : 35361	Invitrogen
EDTA 0,5M	Ethylene diamine tetraacetic acid		Sigma
LPS	*E.Coli* 0111:B4 Source:0000128736	L4391 Lot.no : 0000130083	Sigma
96 well plate	96 well TC standard, flat bottom	655180	Greiner
48 well plate	48-well cell culture CT standard	677180	Greiner
Poly-l-lysine	Poly-L-lysine hydrobromide CAS no. P6282-5MG	P6282-5MG	Merck
CD80	CD80 FACS antibody BV510		Biolegend
MHC-class I, H2kb/H2Db antibody	MHC-class I, H2kb/H2Db FACS antibody Clone 28-8-6	114616 Lot. No : B290883	Biolegend
I-AB	I-Ab antibody against MHC class II, APC	116418	Biolegend

Antibody	Clone AF6-120,1	
Hematology cell counter		MS4SE
Blood collection tubes	EDTA K3E coated blood collection tubes	KABE Labortechnik

CHAPTER Ⅲ - SYNOPSIS INTRODUCTION

1. Microglia: from "nerve glue" to a defined glial cell type

The term *neuroglia* was first introduced by Rudolf Virchow (1821-1902) in 1856 to describe "the thing between neurons", meaning glue or plaster (Virchow 1856). By the late 19th century, scientists recognized that the *neuroglia* mass represented defined cell types with nuclei. One notable scientist, Ramon y Cajal, developed a microscopy method by which he could label star-shaped cells with gold chloride sublimate and became famous for labeling astrocytes. While conducting these analyses, he also observed poorly stained apolar cells that he termed the "third element". Subsequent research, built upon Cajal's observations, led to the discovery of microglia by Pío del Río Hortega in 1919, when he recognized the "third element" as microglia and oligodendrocytes (Sierra, de Castro et al. 2016) (**Fig. 1**). Río Hortega claimed that microglia were present in all the parts of the brain and that they were able to transform into an amoeboid shape, a morphological change that was previously detected in the brain tissue under pathological conditions and heavily debated at the time. He proposed that microglia originate from the mesoderm and described microglia as cells with unique abilities, such as high phagocytic and migratory abilities in the brain parenchyma (Sierra, de Castro et al. 2016). Notably, all his pioneer microscopic observations were later confirmed using more sophisticated technologies.

Microglia are the main immune cells and represent the professional phagocytes of the central nervous system (CNS), which constitute 5-12% of the brain parenchymal cells (Lawson, Perry et al. 1990). Microglia occupy the CNS including brain parenchyma, spinal cord as well as the eye and optic nerve, though their distribution varies. Microglial density is higher in the gray matter than in the white matter, with higher densities in the hippocampus, olfactory bulb, basal ganglia and *substantia nigra* (SN) (Mittelbronn, Dietz et al. 2001). Microglial cells can serve as crucial indicators of changes in the brain, such as during infections or neurodegeneration, as they are the first to respond to changes in the CNS environment.

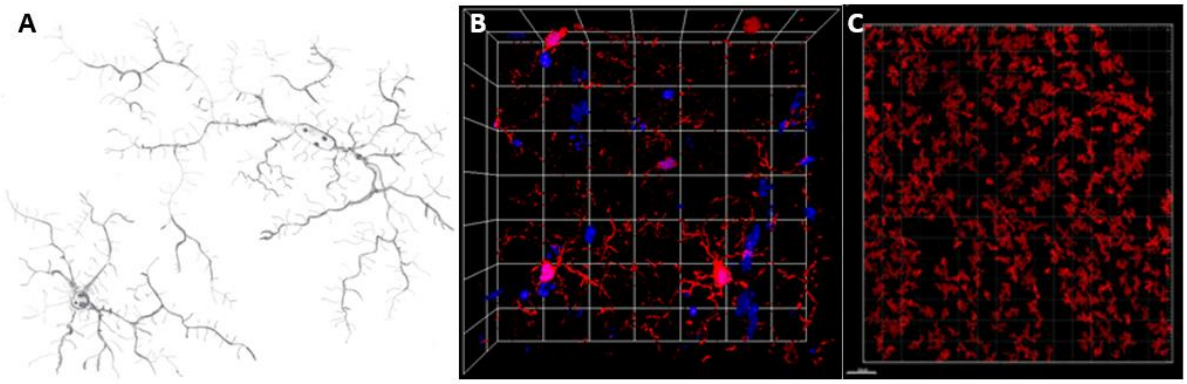

Figure 1. Microglia: From their discovery over a century ago to modern insights. (**A**) Via staining with ammoniacal silver carbonate, Pío del Río Hortega could separate within the "third element" microglia and oligodendrocytes. Drawing of how microglia looked under the light microscopy. Today we use fluorescent antibodies. (**B**) 3D confocal image of cortical mouse microglia stained with nuclei marker, 4'6- diamidino-2-phenylindol (DAPI), and antibody against the microglial marker ionized calcium-binding adaptor protein 1 (IBA1) (red). Scale bar: 50 µm. (**C**) 3D reconstruction of tile scan of mouse cortex with IBA1+ cells. Scale bar: 150 µm. Drawing by Ms Amandine Bernard and own microscopic images.

2. Microglia ontogeny

2.1 In mice

Traditionally, it was believed that hematopoietic stem cells found in the bone marrow were the only source of immune cells throughout life and, because of the resemblance with dendritic cells and macrophages, microglia were believed to be of hematopoietic origin. However, more recent studies have now established that various waves of macrophage migration to different tissues occur during early development in the fetus. In mice, cells with properties of microglial progenitors develop in the yolk sac blood islands at embryonic day 7.5-8 (E7.5-8) (Alliot, Godin and Pessac 1999). Around embryonic day 8.5 (E8.5), runt-related transcription factor 1 (Runx1)-positive microglia progenitors migrate into the brain via blood vessels and sparsely colonize it (Ginhoux, Greter et al. 2010). When the blood-brain barrier (BBB) is formed, microglia proliferate *in situ* and colonize all the areas of the brain (Ginhoux, Greter et al. 2010) (**Fig. 2A**). Microglia originate from erythroid myeloid progenitors (EMPs) in the yolk sac (Nayak, Roth and McGavern 2014) and their differentiation relies on specific transcription factors, including interferon regulatory factor 8 (IRF8) and PU.1 (Kierdorf, Erny et al. 2013), without the contribution of cells from the bone marrow. In

the first postnatal weeks, microglia start to occupy specific areas in a mosaic-like pattern, coined tiling, and develop an increasingly complex morphology over time. Microglia rely on cues from the environment, mainly the colony stimulating factor 1 (CSF1), which binds the CSF1 receptor on microglia and an alternative ligand, IL-34, mainly binding microglia in the grey matter (Easley-Neal, Foreman et al. 2019). CSF1 expression facilitates a spatiotemporal control of colonization pattern of microglia during development and induces microglial proliferation during development. Both CSF1 and IL-34 regulate microglial survival during adulthood (Easley-Neal, Foreman et al. 2019). Throughout life under homeostatic conditions, microglia can self-renew, but through a low rate of proliferation combined with apoptosis. Recent findings indicate that there is a daily turnover of 0.69% of microglia in the mouse brain retaining a remarkable stable density over lifetime (Askew, Li et al. 2017). Outside of the brain, a fraction of macrophages have pre-natal origin from EMPs, which give rise to tissue resident macrophages in e.g., skin, lung and liver. A percentage of macrophages in tissue is derived from hematopoietic stem cells from the bone marrow, which give rise to short-lived circulating monocytes in the blood, which replenishes a fraction of tissue-resident macrophages in the steady state (Mass, Nimmerjahn et al. 2023) (**Fig. 2B**). During inflammation, monocytes infiltrate damaged tissue and mediate antimicrobial responses at these sites (Shi and Pamer 2011).

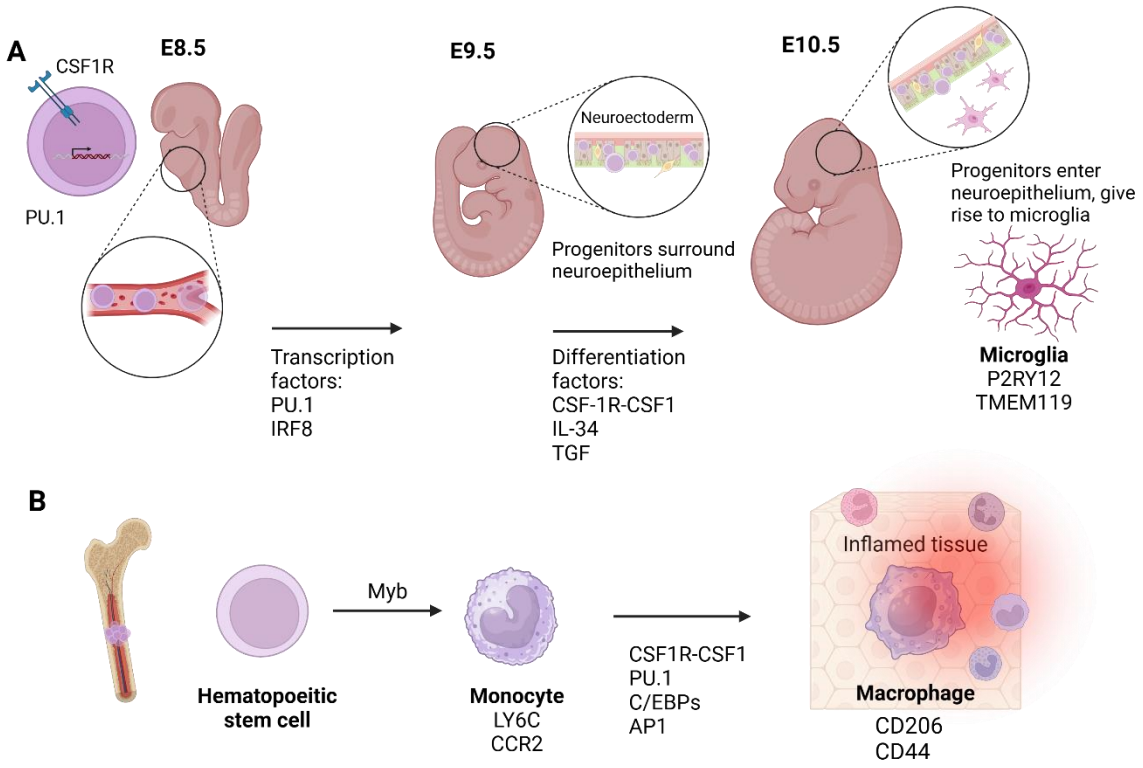

Figure 2. Microglia and macrophage ontogeny. (**A**) Microglia originate from EMPs in the yolk sac and colonize the brain via blood vessels around embryonic day 8.5 in mice. They colonize and proliferate in the brain and mature gradually during development. (**B**) Peripheral monocytes and bone marrow-derived macrophages arise from HSCs in the bone marrow and mature in tissue, such as liver or dura of the brain. The image is inspired by (Nayak, Roth and McGavern 2014) and created using Biorender. CSF-1: Colony stimulating factor 1, PU.1: transcription factor PU.1, IRF8: Interferon regulatory factor 8, TGF: transforming growth factor, AP1: Activator protein 1, C/EBPs: CCAAT-enhancer binding proteins.

2.2 In humans

Initially, addressing the question of microglia ontogeny in humans was more challenging due to few available samples and the absence of fate mapping approaches as were used in rodents. Single-cell sequencing of human fetal tissue recently confirmed the similarities with murine development. It seems to be an evolutionary conserved hematopoietic development and program of individual cell types (Popescu, Botting et al. 2019, Bian, Gong et al. 2020), underlining that EMPs generate long-lived immune cells, including microglia, without the addition of HSCs in healthy conditions, and that they are self-maintained throughout life. In human embryos, microglia expressing ionized calcium-binding adaptor protein 1 (IBA1) are first observed at gestational week (GW) 4.5 within the leptomeninges and choroid plexus of the brain. From these regions, they colonize the telencephalon and diencephalon where they become ramified as early as GW12 (Monier, Adle-Biassette et al. 2007). A recent study transcriptionally characterized human fetal microglia from the 9th until the 18th gestational week and found that microglia were heterogeneous and resembled adult microglia along the developmental stages. Already during the gestational weeks, microglia started to express immune-sensing genes, pointing towards an already mature and CNS-surveilling cell population in the fetus (Kracht, Borggrewe et al. 2020). The authors claimed that this could explain why the developing human CNS is vulnerable to environmental perturbations in early pregnancy (Kracht, Borggrewe et al. 2020). The expansion of microglia in humans follows a less linear, wave-like pattern that corresponds to distinct neurodevelopmental stages. This pattern is more complex compared to the microglial development observed in mouse brains (Menassa and Gomez-Nicola 2018). In the human brain, microglia renew at a median rate of 28% per year (0.08% per day) and are on average 4.2 years old, which means that the majority of the microglia population is renewed throughout life and at a much slower rate in humans than in mice (Reu, Khosravi et al. 2017). The self-autonomous nature of microglia makes it vulnerable to local disturbances. Therefore, it is key for brain homeostasis, both in development and throughout life,

that the number of microglia are maintained, as reduced numbers can result in behavioral and learning deficits (Parkhurst, Yang et al. 2013).

3. Microglial functions in the healthy brain

3.1 Functions of microglia during development

The developing CNS has a unique immune status, characterized by the presence of long-lived resident phagocytes that originate during early embryogenesis before the BBB closure and before neurogenesis takes place in the brain. This suggests that the microglial population has a lifelong history, potential memory of past interactions, and a unique symbiotic relationship with the developing brain (Thion, Ginhoux and Garel 2018).

Microglia serve functions essential for neuronal maturation and wiring during development (Squarzoni, Oller et al. 2014). During embryogenesis, microglia facilitate the removal of apoptotic cells and thereby maintain homeostasis and well-functioning neuronal circuits (Ginhoux, Greter et al. 2010). Microglia carry out what is termed synaptic pruning, which is an essential mechanism to eliminate excess synapses (Stevens, Allen et al. 2007, Paolicelli, Bolasco et al. 2011) (**Fig. 3A**). Microglia recognize tagged synapses via the classical complement system, and remove the excess synapses via phagocytosis (Stevens, Allen et al. 2007). Since microglia are equipped with both immune and neurotransmitter receptors, they are able to sense neuronal activity and other molecular features of their microenvironment (**Fig. 3A**). Via the fractalkine receptor CX3CR1, microglia can bind the fractalkine (CX3CL1) expressed by neurons and sense them (Cardona, Pioro et al. 2006, Paolicelli, Bisht and Tremblay 2014). Additionally, nucleotides like ATP and ADP released by damaged neurons can interact with purinergic receptors expressed by microglia e.g., P2RY2 and P2RY12 (Haynes, Hollopeter et al. 2006, Umpierre and Wu 2021). These interactions are essential for brain homeostasis, therefore microglia can be seen as the housekeeper of the brain, expressing high levels of genes important for these functions (e.g., *Olfml3, Fcrls, Siglech, Gpr34, Cx3cr1, Hexb* and *P2ry12*) (Hickman, Kingery et al. 2013).

By the release of cytokines and growth factors, microglia also influence gliogenesis and neurogenesis, which represent key processes contributing to brain plasticity, function and memory formation (Parkhurst, Yang et al. 2013) (**Fig. 3A**). Microglia are especially important for supporting the survival of cortical layer V neurons during postnatal development where they secrete insulin

like growth factor 1 (IGF1), which is an important element for neuronal survival (Ueno, Fujita et al. 2013). Microglia has also been recently showed to be important for myelogenesis in the developing neonatal brain (Santos and Fields 2021) by the release of IGF1 (Wlodarczyk, Holtman et al. 2017).

A series of studies has demonstrated that dysregulated synaptic pruning by microglia during critical windows of development, such as those caused by pre-natal maternal immune activation, can lead to neurodevelopmental disorders, including autism spectrum disorders (Morgan, Chana et al. 2010, Liao, Yang et al. 2020) or schizophrenia (Ruzzo and Geschwind 2016, Sekar, Bialas et al. 2016).

3.2 Microglial functions during adulthood – immune and synaptic surveillance

During adulthood, microglia continue to maintain the CNS homeostasis by removing debris and by modulating synaptic strength by contributing to synapse formation and elimination in response to neuronal activity (Tremblay, Lowery and Majewska 2010, Zhao, Umpierre and Wu 2024). By removing debris and dead cells, they prevent inflammatory responses that could disrupt the neuronal circuits (Chan, Magnus and Gold 2001, Magnus, Chan et al. 2001, Wang, Ulland et al. 2016). Additionally, ATP-dependent negative feedback control by microglia is essential for protecting the brain from excessive activation (Badimon, Strasburger et al. 2020).

Immune surveillance is one of the main functions of microglia during adulthood. Microglia are equipped with various receptors to sense changes in the environment and endogenously. Genes encoding these receptors are often referred to as homeostatic microglia genes (**Fig. 3B**). They can sense chemokines, cytokines, purinergic molecules, changes in pH, lipids, inorganic substances and amino acids (Hickman, Kingery et al. 2013).

To simplify their functional heterogeneity, microglial activation states *in vitro* have been initially divided into two distinct states, with the M1 polarization representing the activated inflammatory state and the M2 phenotype standing for the resolving state (Michelucci, Heurtaux et al. 2009). Similarly to macrophages, the M1 state is termed classical activation, which can be induced by various pathogen-associated molecular patterns (PAMPs), structurally conserved molecules in various pathogens, and disease-associated molecular patterns (DAMPs), typically released by injured cells, such as ATP, DNA and RNA, attracting microglia to the inflamed site. The contact with PAMPs or DAMPs induces microglia to produce cytokines, nitric oxide (NO) and other reactive oxygen species (ROS). A typical inducer of inflammation in microglia is lipopolysaccharide (LPS), which is a part of the gram-negative bacterial cell wall. Briefly, LPS binds toll-like receptor 4 (TLR4) in the plasma membrane of microglia and starts a cascade of intracellular signaling, consequently

leading to the release of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNFα), IL-6 and IL-1β as well as ROS (Chow, Young et al. 1999). Various well-established protocols mimic infection and systemic inflammation depending on the route, e.g., intravascular injections can be used as models of sepsis and intraperitoneal injections can model systemic inflammation and neurodegeneration (Qin, Wu et al. 2007, Lind-Holm Mogensen, Sousa et al. 2024). A specific inflammatory signature is induced in microglia in response to LPS, often referred to as "microglia activated signature" and is characterized by the downregulation of homeostatic microglial genes, phagocytosis-related genes and upregulation of inflammatory genes (Sousa, Golebiewska et al. 2018). It is known that none to very little LPS crosses the BBB when injecting it in the periphery (Banks and Robinson 2010), where induced systemic inflammatory mediators, such as circulating cytokines, mainly mediate the transient neuroinflammatory processes and microglial activation (Shemer, Scheyltjens et al. 2020). More permeable barriers, such as the blood-cerebrospinal barrier in the choroid plexus (CP) or the circumventricular organs are believed to be the interfaces between the peripheral immune system and microglia (Rustenhoven and Kipnis 2022). Cytokines and chemokines secreted in response to peripheral LPS injection change the microglial signature (Sousa, Golebiewska et al. 2018, Shemer, Scheyltjens et al. 2020). Microglia drastically change their morphology, ability to phagocytose, proliferate and secrete cytokines when activated by LPS. In contrast to microglia under homeostatic conditions, microglia in disease models have the ability to clonally expand (Ajami, Bennett et al. 2007, Tay, Mai et al. 2017).

The M1 state has been extensively studied *in vitro* by treating microglial cells, for example with LPS or interferon gamma (IFNγ). At the opposite side of the continuum of activation states, microglia can also acquire an M2 or alternative activation state characterized by the promotion of genes involved in the resolution of inflammation, extracellular matrix reconstruction and tissue repair, such as *Arg1, Fizz1, Ym(1/2), and Chi3l3* (Colton 2009, Michelucci, Heurtaux et al. 2009). The M2 state can be induced *in vitro* by treating microglia with M2-inducing factors, such as interleukin-4 and interleukin-13. This state is also typically induced by tumor cells and other cells within the tumor microenvironment, which via cytokines, chemokines and other mediators, recruit monocytes to the neoplastic site, resulting in M2-like tumor-associated macrophages (Ghebremedhin, Athavale et al. 2024). Nevertheless, the classifications represent the extreme ends of a broader spectrum of polarization states and do not accurately reflect the complexity and heterogeneity of *in vivo* conditions, where individual microglia can express markers associated with both M1 and M2 phenotypes simultaneously, indicating a more nuanced and dynamic range of activation states, also depending on time, spatial location and disease context (Muller, Kohanbash et al. 2017).

3.3 Microglial phenotypes during aging

Very little is known about the general processes involved during aging, and most changes that occur are irreversible. One of the hallmarks of immune aging is an unresolved inflammation in the absence of a pathogen, a process called inflammaging (Mogilenko, Shchukina and Artyomov 2022, Li, Li et al. 2023).

In aging brains, several microglial phenotypes have been identified. The two main types described are 1) senescent and 2) dystrophic, which represent distinct states, both being dysfunctional compared to young microglia. The senescent microglia phenotype is characterized by an inability to self-renew, by specific gene expression patterns and age-related changes in their secretory phenotype, termed senescence-associated secretory phenotype. Senescence often occurs in non-dividing cells due to elevated ROS and DNA damage over time (Angelova and Brown 2019). On the other hand, dystrophic microglia are identified based on their morphological changes (**Fig. 3C**). The first evidence of aging-related changes in microglia was described by Streit, who observed morphological alterations, such as process deramification, shortening, formation of spheroids and cytoplasmic fragmentation (Streit, Sammons et al. 2004). In addition, homeostatic microglial genes and functions decrease with age (Olah, Patrick et al. 2018). The microglial sensome is altered during ageing and more than 80% of the downregulated sensome genes are involved in sensing endogenous ligands e.g., *P2ry12, P2ry13* and receptors recognizing apoptotic cells, such as *Siglech* (Hickman, Kingery et al. 2013). An important function for homeostasis is the removal of debris via phagocytosis or removal of dead cells by efferocytosis, functions described by many to be reduced in aged brains (**Fig. 3C**) (Pluvinage, Haney et al. 2019, Mogilenko, Shchukina and Artyomov 2022, Thomas, Lehn et al. 2022). Furthermore, it is known that the expression levels of homeostatic genes, such as TGFβ, are downregulated in microglia from old individuals (Olah, Patrick et al. 2018). Interestingly, age-related changes of microglia were shown to be brain region specific. The main source of heterogeneity is the difference in bioenergetics and immune-related pathways. With aging, the most significant changes to microglia occur in the cerebellum and hippocampus when compared to other brain regions (Grabert, Michoel et al. 2016). Recent data show that microglia protect against age-associated brain pathologies. In a mouse model lacking microglia, a significant acceleration and intensification of thalamic calcification occurred, which was prevented by populating the mouse brain with microglia (Munro, Bestard-Cuche et al. 2024).

Characterizing senescent microglia phenotypes is crucial for understanding how they influence the molecular environment of the brain during aging, particularly in the context of inflammation.

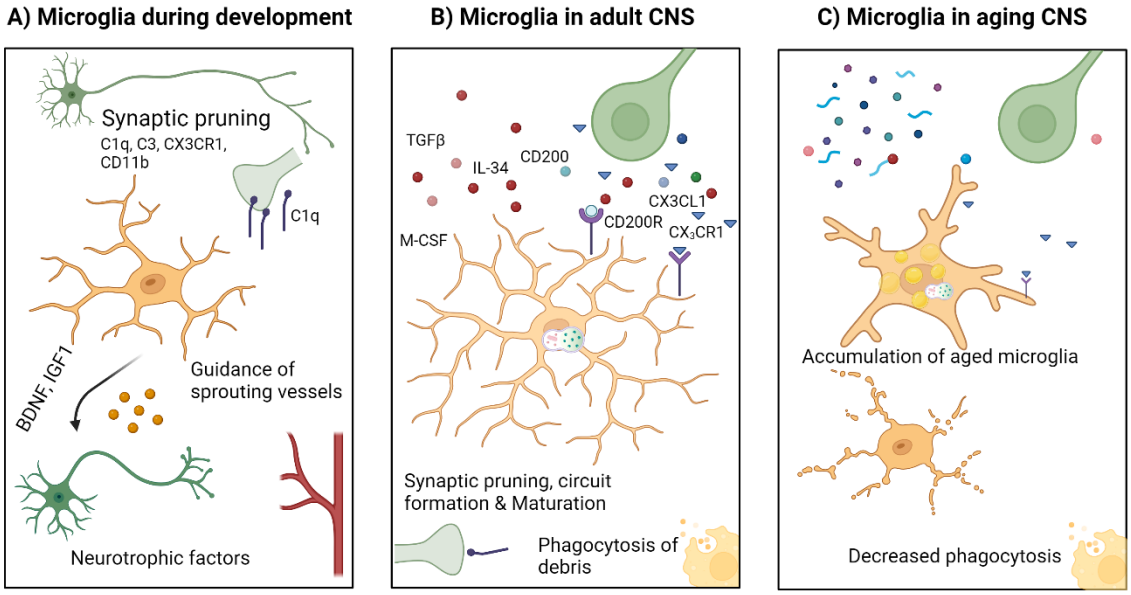

Figure 3. Main functions of microglia during development, adulthood and during aging. (**A**) In the developing brain, microglia are important for synaptic pruning, secretion of neurotrophic factors and neuronal wiring. (**B**) In the adult brain, microglia are important for immune surveillance and cell debris removal. (**C**) In aged microglia, there are changes in morphology and decrease in homeostatic functions, such as phagocytosis. The image is inspired by (Yoo and Kwon 2021) and created using Biorender.

4. Morphology of microglia

The recognition of microglia's role in the diseased CNS e.g., encephalitis or neurodegenerative diseases, was achieved through observing their morphological changes. In the healthy brain, microglia display processes branching off from the small cell soma followed by further branching, thus they exhibit a clear unique morphology compared to macrophages (Stence, Waite and Dailey 2001). Under threatening or pathological conditions, such as infections, the soma becomes larger and more rounded with fewer processes, a shape that is closer to macrophages. Microglial morphologies are regulated by changes in the actin and microtubule cytoskeleton. The dynamic reorganization of their cytoskeleton is important for changes in morphology and carrying out critical functions, such as brain surveillance and phagocytosis (Socodato and Relvas 2024). Early studies showed changes in microtubule organization and their stability when treating microglia with LPS to induce the "activated" state related to the transition to an amoeboid morphology (Ilschner and

Brandt 1996). Proteins controlling cytoskeletal reorganization, such as the transforming protein Rho GTPase Ras homolog family member A (RhoA), play an important role in the microglia response to inflammation, inducing morphological changes, metabolic reprogramming and reactivity to inflammation. A recent study found that a decrease in RhoA made microglia unresponsive to LPS-stimuli, whereas a complete ablation of RhoA triggered microglial apoptosis (Socodato, Rodrigues-Santos et al. 2023). A balanced RhoA activity is therefore essential not only for cytoskeletal reorganization in microglia in response to LPS, but also for metabolic rewiring and inflammatory responses.

In the healthy brain, the soma of microglia does not move, but stay relatively fixed. On the contrary, the processes of microglial cells are strikingly motile continuously forming and retracting (Davalos, Grutzendler et al. 2005). This leads to dynamic changes over time. During a 1-hour time-lapse series with 2-photon imaging, all branches except for a few, changed their morphology. The velocity of extensions and retractions were similar with about 1.47 μm/min and 0.08 μm/min, respectively (Nimmerjahn, Kirchhoff and Helmchen 2005). Laser ablation or mechanical damage to tissue surrounding microglia induces a protective response by microglia. Within the first minute's post injury, the tips of the microglial processes appear enlarged and bulbous and within the following minutes, microglial cells extend their processes toward the damaged area (Davalos, Grutzendler et al. 2005). Interestingly, 30 minutes following injury, the processes of neighboring microglia start to fuse to contain the injured area. It is believed that initial ATP release from the damaged tissue triggers further release of ATP and possibly other molecules, which activate purinergic receptors (e.g., P2RY12) on microglia and drive the dynamic process response of microglial cells (Davalos, Grutzendler et al. 2005). Furthermore, Cl^- channels are required for the rapid response of microglia to damage. Microglia move toward the injury and a 37% decrease in lesion volume is observed when microglia processes are in contact with the damaged area (Hines, Hines et al. 2009). In addition to the extension of large processes, hair-like filopodia further extend the surveillance area. Localized at the tip of larger processes, filopodia are actin-dependent structures. Contrary to the ATP-driven movement of large processes, filopodia are enhanced by increasing intracellular cyclic adenosine monophosphate (cAMP) concentration (Bernier, Bohlen et al. 2019). Thus, the resting surveillance behavior of microglia is performed both via large movement by their processes and on a nanoscale by filopodia.

The morphology of microglia are by many used as an identifying characteristic of their activation state. However, this approach is overly simplistic. Today, the field is shifting toward defining microglial states based on specific signatures (Paolicelli, Sierra et al. 2022). Consequently, for each

distinct molecular interaction, there is a corresponding microglial state and potentially a morphological characteristic (Bosch and Kierdorf 2022, Green and Rowe 2024).

5. Spatial heterogeneity and plasticity of microglia and border-associated macrophages in the brain

The majority of studies, which were able to distinguish microglia from other myeloid cells, raised after 2016. In 2016, the group of Ben Barres identified a stable and robustly expressed marker, transmembrane protein 119 (TMEM119), for mouse and human microglia (Bennett, Bennett et al. 2016). Previously, methods could distinguish microglia from other cells of the CNS based on relative marker expression, such as $CD45^{hi/lo}$, or based on morphology, which could not always distinguish microglia from macrophages. In addition, recent progressions in other methods, like cytometry by time-of-flight and single-cell RNA-sequencing (scRNA-seq), have revealed the diversity of microglia subsets or substates in the brain. Further, the heterogeneity of microglia and tissue-resident macrophages across different disorders showing the plasticity of macrophage phenotypes is currently emerging (Mass, Nimmerjahn et al. 2023).

Besides the changing functions of microglia during development and with aging, microglia also serve different roles depending on the brain region (de Haas, Boddeke and Biber 2008, Tan, Yuan and Tian 2020). The microenvironment throughout the brain varies across the specific brain regions as they consist of various neuronal subtypes, metabolic demands and neurotransmitter profiles. It remains unclear whether the brain microenvironment affects the local microglial phenotype and/or if the microglial phenotype itself influences the surrounding microenvironment. For example, microglia in the olfactory bulb, cerebellum and hippocampus were shown to be more immune vigilant compared to other brain regions (Grabert, Michoel et al. 2016). Additionally, microglia in these areas exhibit a higher rate of turnover compared to other brain regions (Tay, Mai et al. 2017). In the cerebellum, the brain area responsible for coordinating motor control and cognitive functions, microglia are less dense compared to SN (Lawson, Perry et al. 1990, Masuda, Sankowski et al. 2019). They display a less ramified morphology than in other brain regions and are described as physiologically unique microglia compared to cortical populations (Stowell, Wong et al. 2018).

Besides microglia, other anatomically distinct macrophages, called border-associated macrophages (BAMs), populate the CNS (Goldmann, Wieghofer et al. 2016). As the name infers, BAMs are not found in the brain parenchyma, but they are located at brain boundaries, such as meninges, dura

and choroid plexus (Goldmann, Wieghofer et al. 2016). The brain is a highly compartmentalized multi-layered organ, which contains diverse borders that form the interface with the periphery. The brain contains a heterogeneous immune compartment, with the majority of the diversity restricted to its border region. The brain parenchyma, wherein microglia reside, is covered by a three-layered meninges. The outermost layer, the dura mater layer, constitutes a more permissive border because of fenestrated blood vessels and meningeal lymphatic vessels. BAMs found in the dura exhibit high heterogeneity and express few microglial signature genes at a low level (Van Hove, Martens et al. 2019, Sankowski, Suss et al. 2024). In contrast, the vasculature in the layer below, the subdural meninges, contains tight junctions, restricting access and forming the blood-leptomeningeal barrier. The perivascular space surrounding the parenchymal vasculature forms a border region with low accessibility due to the presence of the BBB, which tightly regulates molecular and cellular entry to the brain parenchyma.

The choroid plexus (CP) is located in the ventricles of the brain, and its secretory epithelium produces the cerebrospinal fluid (CSF). The CP contains fenestrated blood vessels around the CP stroma and forms the blood-CSF barrier. Interestingly, BAMs found in the CP express microglial signature genes, although at lower levels compared to *bona fide* microglial cells. CP BAMs and microglia all have embryonic origin, whereas leptomeningeal macrophages and a fraction of CP BAMs are replenished by bone marrow-derived macrophages (Goldmann, Wieghofer et al. 2016, Van Hove, Martens et al. 2019). BAMs from different border regions contain separate transcriptional profiles, reflecting both their functional adaptations to the specific microenvironment and their ontogeny (Van Hove, Martens et al. 2019, Masuda, Amann and Prinz 2022).

6. Epidemiology of Parkinson's disease

PD is the globally fastest growing neurological disorder in terms of prevalence, disability and mortality (Collaborators, Feigin et al. 2021). PD is the most prevalent type of parkinsonism, which encompasses a range of movement disorders, including tremor, postural instability, muscular rigidity, freezing, flexed posture and bradykinesia (Poewe, Seppi et al. 2017).

Age is the primary risk factor for PD, with an incidence increasing 5-10-fold from the sixth to the ninth decade of life. Therefore, while it is relatively rare before 50 years of age, it rises to 1-3% in the population over 60 and it increases sharply up to 5% in individuals above 85 years of age (Ou, Pan et al. 2021). However, as the incidence rate of PD is more prominent than estimated, longer

life expectancy only partially explains the increased prevalence of PD. Therefore, additional factors, including environmental elements, such as exposure to pollution, nanoplastics and pesticides, are supposed to also contribute to the rise (Liu, Sokratian et al. 2023, Ben-Shlomo, Darweesh et al. 2024). Between 1990 and 2016, the number of PD cases doubled from 2.6 million to over 6 million worldwide (Dorsey 2018). If this trend persists, the number of cases could exceed 12 million by 2040, with some estimates suggesting up to 17 million patients (Todd Sherer 2020). The prevalence of PD is increasing so rapidly that it is considered a modern-day pandemic (albeit non-infectious), with the lifetime risk of developing PD being 1 in 15 (Dorsey, Sherer et al. 2018, Wanneveich, Moisan et al. 2018).

More than 200 years have passed since James Parkinson first described patients with Parkinson's symptoms (Parkinson 2002). Today, we know that PD is more common in men than in women by a ratio of approximately 1.4:1, and that only around 10-15% of cases have a genetic cause. Notable genes implicated in familial PD include *SNCA, LRRK2, PARK2*, *PINK1* and *PARK7*, among others (**Fig. 4**). Mutations in these genes can lead to mitochondrial dysfunction, abnormal protein folding and aggregation and these impaired cellular pathways contribute to the often early onset and progression of the disease in these patients. The large majority of PD cases (85-90%) are idiopathic (sporadic) and arise from a complex interplay of aging, environmental factors, genetic susceptibility without a familial pattern, making PD among the most heterogeneous known diseases. Ongoing scientific research is essential for understanding the mechanisms of the disease and developing therapies that not only alleviate symptoms, but also slow down or halt the disease progression.

7. Cellular hallmarks of Parkinson's disease

The majority of possible disease mechanisms known for PD comes from studies of genetic PD, which have revealed how various mutations can lead to protein misfolding, disruption of clearance mechanisms in cells of the brain and an array of other dysregulated cellular functions. It is clear that PD is a complex and systemic disease involving various cell types of the CNS, the peripheral nervous and the immune system. An approach to improve the understanding of PD is to focus on hallmarks of the disease, such as calcium homeostasis, protein misfolding, synaptic dysfunction, lysosome and proteasome, cell death, oxidative stress, neuroinflammation and mitochondrial dysfunction (Antony, Diederich et al. 2013). These hallmarks can provide a framework for understanding the complex biology and guide research and therapeutic development (**Fig. 4**). As my project focuses

on mitochondrial dysfunction, oxidative stress and neuroinflammation, these hallmarks are detailed in the next sections.

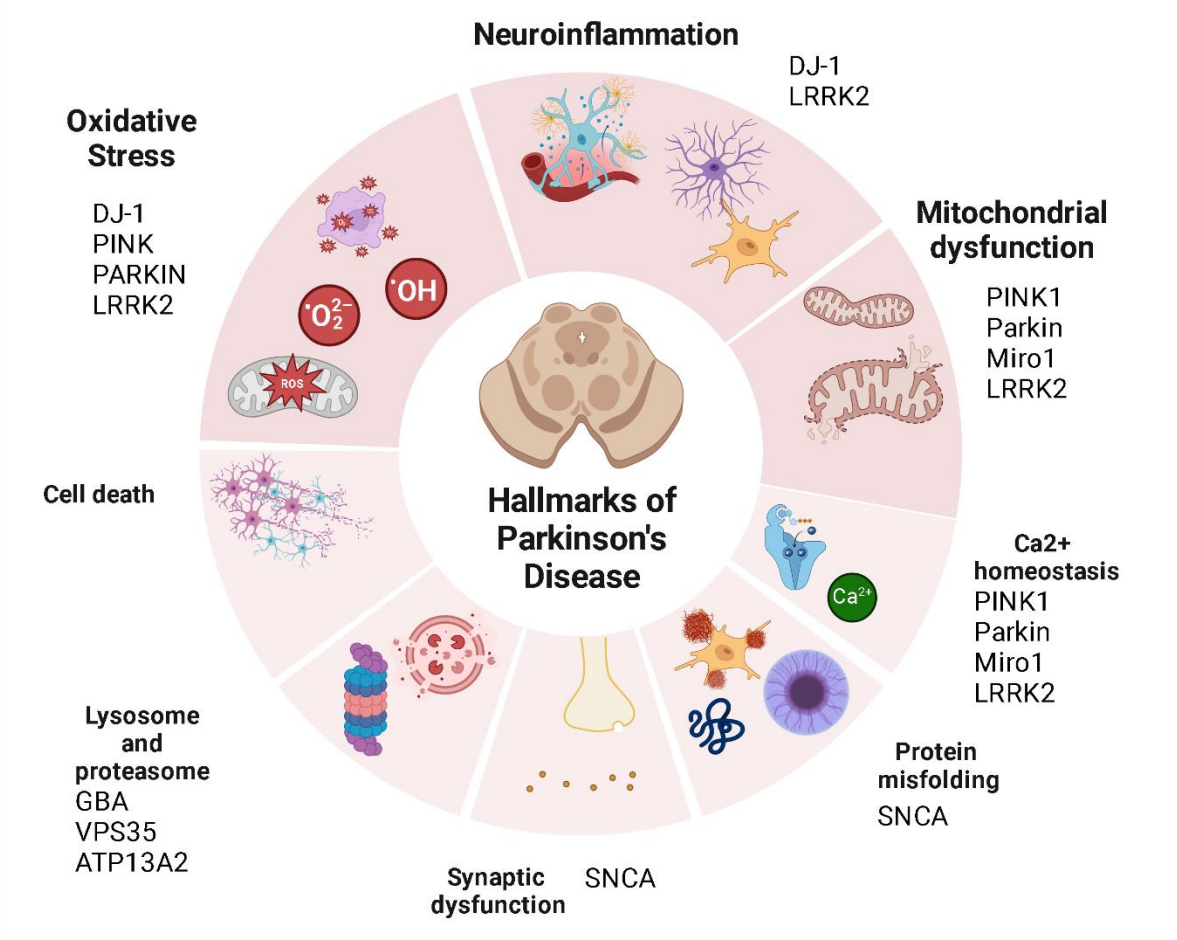

Figure 4. Cellular hallmarks partly shared across PD subtypes. It is believed that at least one of the eight cellular hallmarks occurs in neurons or glial cells of patients with PD. The figure is inspired by (Antony, Diederich et al. 2013) and created using Biorender.

7.1 α-synuclein misfolding and aggregation

At the cellular level, one of the main hallmarks of PD is the progressive loss of dopaminergic neurons in the SN *pars compacta* (SNpc) of the midbrain. Post-mortem studies exhibited Lewy bodies in the brains of the majority of PD cases. Lewy bodies and Lewy neurites are aggregates of α-synuclein (α-syn), which is an ubiquitous protein involved in the regulation of neurotransmitter release and synaptic functions (Burre, Sharma et al. 2010). Mutations in the *SNCA* gene, encoding α-syn, were the first evidences showing a genetic cause of PD (Polymeropoulos, Lavedan et al. 1997). Abnormal protein structure of α-syn can result from mutations or overexpression of physiological α-syn, which

can lead to oligomerization, fibrillization and aggregation, resulting in progressive motor deficits due to the loss of dopaminergic neurons (Calabresi, Mechelli et al. 2023).

Microglia can carry out an important protective function in response to α-syn. By forming tunneling nanotubes, which are F-actin dependent hollow tubes, they can transport organelles, Ca^{2+} or mRNA between cells. Microglial cells can share the burden of α-syn by exchanging it among them (Scheiblich, Dansokho et al. 2021). In addition, mitochondria can be supplied to stressed or α-syn-burdened microglia and neurons (Chakraborty, Nonaka et al. 2023). Interestingly, a recent follow-up study showed that microglia can rescue neurons by taking up α-syn from dying neurons and donate mitochondria, thereby decreasing mitochondrial stress and ROS in neurons (Scheiblich, Eikens et al. 2024). It is therefore now clear that microglia play dual roles in response to α-syn, not only inducing neuroinflammation and perpetuating neurodegeneration, but also protecting neurons from α-syn aggregation.

7.2 Mitochondrial dysfunction

In the eighties, it was noted that seven users of synthetic heroin were hospitalized with Parkinsonism. All patients had a loss of dopaminergic neurons and never recovered (Langston, Ballard et al. 1983, Ballard, Tetrud and Langston 1985). They later found out that a byproduct of the synthetic drug, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), caused neurodegeneration and PD symptoms. It was later discovered that MPTP is an electron transport chain complex I inhibitor leading to dopaminergic neuron cell death (Langston 2017). The blocking of the electron transport chain came to be a central element in PD research and later other drugs, such as the pesticides paraquat and rotenone, were discovered to have similar effects (Henrich, Oertel et al. 2023). Not only were mitochondrial dysfunction found in idiopathic PD patients, but also several mutations leading to non-functional proteins in genetic PD, such as Parkin, PINK, Miro1 and DJ-1, affect mitochondria (Abou-Sleiman, Muqit and Wood 2006). For example, PINK1 and Parkin are important for mitochondrial turnover dynamics and homeostasis.

7.3 Oxidative stress

DJ-1 plays a multifaceted role in various diseases and across different immune cell types. In cancer, DJ-1 acts as an oncogene by promoting cell survival (Kim, Kitaura et al. 2009), proliferation and

metastasis (He, Zheng et al. 2012, Jin, Wang et al. 2020). Elevated levels of DJ-1 have been linked to poor prognosis in lung, breast and pancreatic cancers (Ismail et al. 2014). Mutations in Parkinsonism associated deglycase (*PARK7*) gene (encoding DJ-1) account for approximately 1-2% of all recessively inherited early-onset PD cases (Abou-Sleiman, Healy et al. 2003). *PARK7,* located on chromosome 1p36, is an eight exon gene of 24kb. Of note, a total of 20 pathogenic mutations in the *PARK7* gene causing loss of function or complete loss of the DJ-1 protein leading to the early-onset PD have been described (Abou-Sleiman, Healy et al. 2003, Bonifati, Rizzu et al. 2003, Repici and Giorgini 2019). DJ-1 is ubiquitously expressed, especially in metabolically active tissues, such as the brain. Several functions have been ascribed to DJ-1, ranging from oxidative stress and transcriptional regulation, thus making the exact function unknown. However, its main function is to act as an antioxidant and oxidative stress sensor in several neuroprotective mechanisms (Skou, Johansen et al. 2024). DJ-1 is mainly found in the cytosol under homeostatic conditions, while it translocates to the mitochondria during high oxidative stress to protect the cell from mitochondrial damage. DJ-1 mutation carriers are rare, but demonstrate a high percentage of non-motor symptoms (57%), showing that in addition to being the cause of motor symptoms, DJ-1 is also linked to symptomology, such as cognitive decline and anxiety (Kasten, Hartmann et al. 2018). Consistently with symptoms in patients, the DJ-1 KO mouse model show cognitive dysfunctions and slight behavioral abnormalities. Despite having a normal number of dopaminergic neurons in the SN, *PARK7*/DJ-1 KO mice exhibit subtle neurochemical and behavioral abnormalities, including reduced dopamine overflow in the striatum, impaired long-term depression, and decreased activity in the open field (Wang, Chandran et al. 2008, Pham, Giesert et al. 2010).

We provided an overview of the role of DJ-1 in neuroinflammation, with a special focus on its functions in microglia genetic programs and immunological traits in the article entitled "*PARK7*/DJ-1 in microglia: implications in Parkinson's disease and relevance as a therapeutic target" included in Chapter Ⅳ (Lind-Holm Mogensen, Scafidi et al. 2023).

7.4 Neuroinflammation

Changes in the peripheral and central immune system happens in the majority of PD cases (Tansey and Romero-Ramos 2019). Neuroinflammation and microgliosis have been observed both with immunohistochemistry of post-mortem tissue of PD brain tissues and via positron emission tomography (PET) scans in early PD using tracers indicating microglial activation. Changes in diagnosed PD and especially related to changes in microglia in patients and animal models with

mutations in DJ-1 can be found in chapter Ⅳ and Ⅵ (Lind-Holm Mogensen, Scafidi et al. 2023, Lind-Holm Mogensen, Sousa et al. 2024). We addressed the importance of understanding microglial cell changes in the early phases of the disease in the opinion article entitled "Microglial dynamics and neuroinflammation in prodromal and early Parkinson's disease" (Chapter Ⅶ, Lind-Holm Mogensen et al. Under review).

8. Models and approaches to study microglia in Parkinson's disease

This chapter addresses human and murine models that are commonly used to study microglia biology. However, other species are available to investigate microglial immunological functions. For instance, zebrafish models have been crucial for studying, through live imaging, synaptic pruning during embryogenesis (Moller, Brambach et al. 2022) and phagocytosis (Peri and Nusslein-Volhard 2008, Villani, Benjaminsen et al. 2019). Still, the mouse represents the principal animal model for studying microglia due to its genetic accessibility, the availability of numerous transgenic and knockout strains, and its well-characterized immune system (Konnova and Swanberg 2018, Zhang and Cui 2021).

When isolated from mouse or human brain, microglia have a low proliferation rate and can survive in culture between 10 days and 4 weeks, depending on the added growth factors, making the study of these cells difficult and complex (Witting and Moller 2011). A way to overcome this drawback is the immortalization of isolated cells via c-myc or SV40, as carried out for various murine (e.g. N9 and BV-2) and human (e.g. CHME-5, HMC3 and C20) microglial cell lines (Dello Russo, Cappoli et al. 2018, Zhang and Cui 2021). A main advantage of immortalized cell lines is that they can be studied for months in culture without limitations in terms of quantity. However, immortalized microglia are proliferative cells, which do not recapitulate their terminally differentiated state with a low proliferative capacity by nature (Pesti, Legradi and Farkas 2024). Moreover, contamination by other cell types is more prevalent than commonly assumed. Recent findings revealed that the human microglial cell line CHME-5 actually originates from transformed rat microglia (Garcia-Mesa, Jay et al. 2017). However, further investigations are needed to determine whether this applies to all laboratories. Additionally, the karyotype of many transformed cell lines is abnormal with an incorrect gene dosage (Maslova, Plotnikov et al. 2023). This obviously represents a problem when studying neuroinflammatory processes. For example, it has been recently showed that an extra copy of the gene encoding α-syn results in PD pathology (Bido, Muggeo et al. 2021). Additionally, as microglia are essential in sensing their microenvironment, multiple studies showed specific

transcriptomic changes *in vitro,* where they lose or drastically downregulate their homeostatic signature genes and upregulate inflammatory genes (Bohlen, Bennett et al. 2017, Cadiz, Jensen et al. 2022). The microglia field therefore is moving away from studying microglia in 2D monocultures and towards studying them in co-culture with neurons or astrocytes, in brain organoids, directly *ex vivo* or in the mouse brain if possible (**Fig. 5**). However, there are multiple disadvantages of mouse studies as not all pathways are conserved. For example, polymorphisms found in human PD patients are not recapitulated in mice, which do not also reiterate the human disease (Kitada, Tong et al. 2009).

Most recent discoveries about microglia biology comes from bulk or scRNA-seq analyses of microglia isolated *ex vivo* from mouse or human brains. Therefore, it is critical that the best practice for isolation is chosen to obtain reliable measurements of *in vivo* transcriptional states. The most common method used to isolate microglia from the mouse brain is by fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS) with the main goal of investigating these cells either acutely *ex vivo* or for *in vitro* studies. However, various studies showed that the process of tissue extraction via mechanical and enzymatic tissue dissociation can induce activation signatures and that microglia are preferentially sensitive to *ex vivo* artefacts (Marsh, Walker et al. 2022). Interestingly, it was shown that even prolonged room temperature storage of post-mortem brain tissue or biopsies could induce an activated microglia signature, originally thought to be induced solely by enzymatic digestion (Marsh, Walker et al. 2022). Additionally, various studies showed that FACS can activate microglia (Haimon, Volaski et al. 2018), affecting their redox state and impacting their metabolism (Llufrio, Wang et al. 2018). Usage of FACS and MACS provides highly viable samples, but with a relatively low yield. By MACS CD11b enrichment, a small fraction of other CD11b+ myeloid cells cannot be avoided, whereas with FACS isolation by specifically gating $CD11b^{+}$ $CD45^{int}$ cells, the yield is lower, but the purity is higher (Pan and Wan 2020). Irrespective of the conditions, the yield of even an optimized cell isolation protocol is less than 30% of all microglia from an intact mouse brain (Lawson, Perry et al. 1990, Mass 2024). A way to avoid transcriptional changes when isolating microglia is, for example, to inhibit the transcriptome by transcription and translation inhibitors (Ocanas, Pham et al. 2022). These proved to be efficient and reversed the "activated signature" previously shown.

It is incredibly difficult and rare to get biopsies from healthy individuals or patients for the study of microglial cells. Additionally, the risk of infection or trauma to the CNS tissue represents a potential risk when taking a biopsy. Furthermore, biopsies are often sampled under suspicious or defined pathological settings, therefore they are often resected from patients affected by neurological disorders. In 2006, a revolutionizing method was coined by Yamanaka, who later won the Nobel

Prize in physiology and medicine for his discovery of reprogramming any cell type into pluripotent stem cells, called induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka 2006). When exposed to specific factors, pluripotent stem cells can differentiate into any cell of the three germ layers: the ectoderm (e.g., skin cells, neurons), the mesoderm (e.g., red blood cells) or the endoderm (e.g., hepatocytes). Yamanaka's discovery revolutionized the field of biomedicine as it made it possible to take an uncomplicated skin biopsy from patients and healthy controls and derive cells into any cell of interest if exposed to specific transcription factors, growth factors or cytokines. These elements are represented by four specific reprogramming factors, namely *Sox2, Oct4, Klf4* and *c-myc*, also dubbed Yamanaka factors (Liu, Huang et al. 2008). These iPSCs can renew themselves indefinitely if properly managed and researchers can, for example, derive fibroblasts from a patient into dopaminergic neurons or microglia to study PD (Mahajani, Raina et al. 2019, Badanjak, Mulica et al. 2021). The maintenance of iPSCs in culture requires appropriate combination of media, matrices for the cells to grow on and passaging methods to support their health and growth. Additionally, an enormous number of quality controls is important for ensuring pluripotency and to control downstream cell differentiation into the cell type of interest. Since their discovery, a considerable number of culturing and differentiation protocols have been published and precise guidelines for ensuring pluripotency and high-quality culturing of iPSCs should be followed (Sullivan, Stacey et al. 2018). Multiple protocols with the aim of differentiating iPSCs into macrophages and microglia were established (van Wilgenburg, Browne et al. 2013, Abud, Ramirez et al. 2017). Co-culturing of microglia induces specific anti-inflammatory and pro-remodeling responses, which is not seen in microglia in monocultures (Haenseler, Sansom et al. 2017). By co-culture, it is possible to study secreted and contact-mediated interactions. However, by culturing in 3D, more physiologically relevant interactions can be studied (Hedegaard, Stodolak et al. 2020) (**Fig. 5**). A novel approach induced transcription factors important for microglia differentiation, such as IRF8, via CRISPR technology and showed strong similarities of iPSC-derived microglia (iMGs) with primary human microglia, using fewer cytokines and growth factors to differentiate them and shortened the differentiation time from months to eight days. Furthermore, this approach enabled large-scale screens of knockdown of various disease genes in human iPSC-derived microglia (Drager, Sattler et al. 2022).

Another relatively novel method is the culturing of patient-derived cells into organoids, where microglia can integrate mimicking *in vivo* settings (Ormel, Vieira de Sa et al. 2018). However, reproducibility in iPSC organoids is a current issue (Sandoval, Cappuccio et al. 2024). Interestingly, recent studies reported the successful transplantation of human iPSC-derived microglia into mice. Xenografted human microglia derived from iPSCs in these chimeric mouse models retain their

human microglial identity underlined by high expression levels of homeostatic signature genes (Xu, Li et al. 2020). This study also shows a dynamic response of human microglia and an overexpression of neurological disease-risk genes in response to cuprizone-induced demyelination (Xu, Li et al. 2020). All of these studies serve novel and important tools for studying the role of human microglia both in brain development and in neurodegeneration.

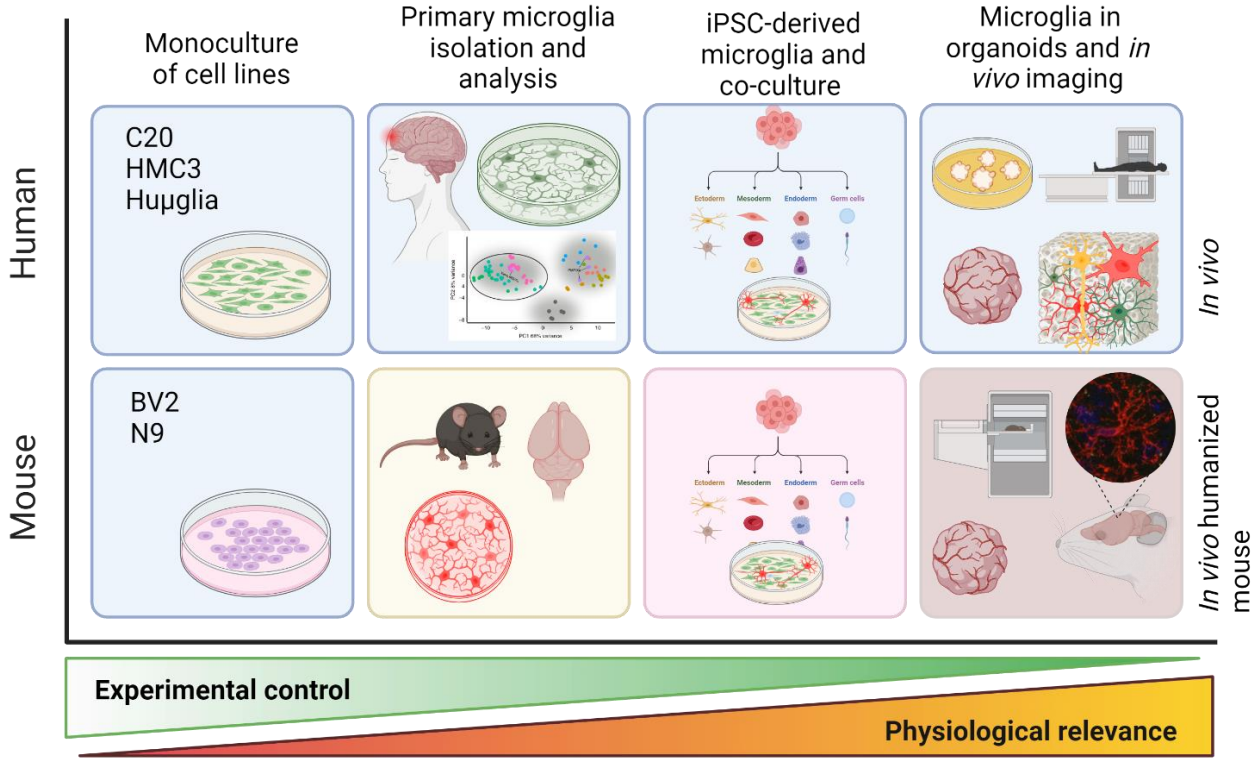

Figure 5. Methods to study microglia. Methods to investigate microglia in human and mouse with increasing complexity and physiological relevance and decreasing experimental control. Figure created using Biorender. Inspired by (Hedegaard, Stodolak et al. 2020).

CHAPTER Ⅳ - MANUSCRIPT Ⅰ: PARK7/DJ-1 in microglia: implications in Parkinson's disease and relevance as a therapeutic target

PARK7/DJ-1 in microglia: implications in Parkinson's disease and relevance as a therapeutic target

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MANUSCRIPT Ⅰ - PREFACE

Mutations in the gene *PARK7*, encoding for the DJ-1 protein, are rare and lead to early-onset autosomal recessive PD. DJ-1 has been extensively studied to understand its protective role in neurons, therefore the majority of studies have originally been conducted in those cells. However, as the role of neuroinflammation in PD has also been recognized, we were here interested in reviewing the studies that have been conducted to elucidate the role of DJ-1 in microglial cells. To this aim, we sought to summarize the relevant current literature addressing the role of DJ-1 briefly in neurons, but mainly in microglia, hoping that such overview would benefit the scientific community studying microglia in PD.

In this article entitled "*PARK7/DJ-1 in microglia: implications in Parkinson's disease and relevance as a therapeutic target"* published in the *Journal of Neuroinflammation,* we reviewed the main evidences showing that DJ-1 plays critical roles for microglial functions, such as regulating inflammatory responses and neuroprotective mechanisms. The loss of DJ-1 function in microglia exacerbates neuroinflammation and neuronal damage, suggesting that DJ-1 has a protective role in maintaining microglial homeostasis. The study summarizes various experimental models, including genetic manipulation and pharmacological interventions, to elucidate the specific mechanisms by which DJ-1 modulates microglial activity. It was observed that DJ-1 deficiency leads to an increased

release of pro-inflammatory cytokines and reactive oxygen species, contributing to a detrimental environment for neuronal survival. However, with our review we identified a knowledge gap, as the majority of studies investigated the role of DJ-1 in immortalized microglial cell lines and not *in vivo*, which may impact reliability and relevance of the findings due to the artificial nature of cell line studies. Additionally, we highlighted the potential of DJ-1 activators or enhancers in restoring microglial functions and providing neuroprotection. These findings suggest that therapies aimed at boosting the expression levels of DJ-1 or its activity in microglia could represent a novel approach for treating PD patients. Future research is encouraged to further explore the role of DJ-1 across different stages of PD and to develop targeted treatments that harness its neuroprotective capabilities.

I contributed to the conceptualization of the review and selected the relevant literature, written a first draft and edited the manuscript, created tables and figures.

CHAPTER Ⅴ - MANUSCRIPT Ⅱ: Protocol for immunofluorescence staining and large-scale analysis to quantify microglial cell morphology at single-cell resolution in mice

Protocol for immunofluorescence staining and large-scale analysis to quantify microglial cell morphology at single-cell resolution in mice

Lind-Holm Mogensen F, Ameli C., Skupin A., Michelucci A.

STAR Protocols (2024) 5(4):103467

DOI: 10.1016/j.xpro.2024.103467

MANUSCRIPT Ⅱ - PREFACE

Morphological changes in microglia are often linked to their functional states, such as surveillance or phagocytic ability, making their analysis essential for understanding brain health and disease.

Microglia morphology has been used as an important metric for their activation status in past research studies. The microglial field is going away from this view and working towards describing various morphological features and linking these specific morphometries to specific states and functions (Bosch and Kierdorf 2022, Paolicelli, Sierra et al. 2022, Green and Rowe 2024). The large majority of recent publications investigating microglia includes a summary of morphological changes along with transcriptomic and functional data. However, the microglial field now faces a big challenge finding links between specific morphological features, gene expression and cell function. The protocol presented in this chapter is an optimized way of extracting up to 16 morphological features from up to 350 microglial cells per field of view in a mouse brain. By scanning large tiles, it increases the number of cells per brain area of interest and it decreases the batch effect by taking several pictures in different brain areas. Additionally, it reduces the time

spent on the cumbersome procedure of finding small and precise neuroanatomical areas of interest while working on the confocal microscope. When interested in the volume and animal levels, the crucial advantage of this protocol is the large number of cells obtained in each volume, which allows for a better estimate of true parameters of the population in the brain area of interest. The protocol allows for the clustering of microglial cells based on their morphological features, such as compactness, reflecting their resting ramified or activated amoeboid phenotype. A similar approach was recently used to compare four different clusters of microglia based on the morphology in the brain of mice and post-mortem human samples. Interestingly, it was found that the morphology of mouse and human microglia were very similar across the two species (Torres-Platas, Comeau et al. 2014). This is in stark contrast to astrocytes, which are much larger and more diverse in humans compared to mice (Oberheim, Takano et al. 2009). Many similarities between microglia ontogeny, gene expression and functions are found between mammalian species, and therefore changes in microglia morphology in various mouse models are similar to human, thus serving as an indicator for how a response will take place in a human context.

We hope that this protocol will be useful for the community who wishes to quantify microglial morphology at the single cell level. We expect that a comprehensive protocol describing all the critical steps, from the mouse brain dissection to data analysis, will enhance reproducibility and transparency, ensuring that experiments can be replicated and validated by independent laboratories. The protocol serves as a valuable resource for training new researchers and encouraging broader expertise in microglial analysis techniques.

In the manuscript included in Chapter VI, the application of this method enabled to elucidate microglial morphological changes in the *PARK7*/DJ-1 KO mouse and in another PD mouse model, the Miro1 p.R285Q knock-in mouse, which is described in the additional data of Chapter VI.

CHAPTER Ⅵ - MANUSCRIPT Ⅲ: PARK7/DJ-1 deficiency impairs microglial activation in response to LPS-induced inflammation

PARK7/DJ-1 deficiency impairs microglial activation in response to LPS-induced inflammation

Lind-Holm Mogensen F, Sousa C., Ameli C., Badanjak K., Pereira S.L, Muller A., Kaoma T., Coowar D., Scafidi A., Poovathingal S.K, Tziortziou M., Antony P.M.A , Nicot N., Ginolhac A., Weisenhorn D.M.V, Wurst W., Poli A., Nazarov P.V., Skupin A., Grünewald A., Michelucci A.

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DOI: 10.1186/s12974-024-03164-x

MANUSCRIPT Ⅲ - PREFACE

This article represents an important outcome of the thesis project, which describes the results obtained in the *PARK7*/DJ-1 KO mouse model and human DJ-1 mutant iPSC-derived microglial cells at baseline and after activation by LPS. Our findings indicate that microglia from both mouse (*in vivo* and *ex vivo*) and human (*in vitro*) models show distinct activation profiles and an attenuated response to LPS-induced inflammation compared to WT counterparts. DJ-1 deficient microglia dampened their interferon-related pathways at the transcriptional level and had a distinct expression of genes related to actin and cell cytoskeleton. Furthermore, microglia and bone marrow-derived macrophages (BMDMs) from the *PARK7*/DJ-1 KO mouse model displayed enhanced compactness at baseline and a less amoeboid morphology following LPS exposure. According to the role of DJ-1 as an important regulator of oxidative stress conditions, we detected an upregulation of genes related to the oxidative response in microglia from *PARK7*/DJ-1 KO mice after LPS-induced inflammation. Hence, we investigated ROS production in the cytoplasm and in the mitochondria by live tracer imaging in microglia and BMDMs and found significantly elevated ROS levels in cells from *PARK7*/DJ-1 KO compared to WT mice. These results are in line with previous *in vitro* studies describing elevated ROS levels in DJ-1 deficient cells. As an appendix to this

manuscript, we added supplementary data of the quantification of tyrosine hydroxylase (TH) levels in *PARK7*/DJ-1 KO and WT SN after 24h of LPS treatment. Furthermore, we investigated the phagocytic ability as well as age, genotype and sex differences in organ to body weight ratios and whole blood parameters. Additionally, we investigated the transcriptional differences between microglia isolated from male and female mice under inflammatory conditions.

Lastly, we took advantage of the Miro1 R285Q/R285Q knock-in (KI) mouse to study morphological changes of microglia. The mouse model was created by inserting the orthologue of the human mutation (p.R272Q), R285Q, in the mouse, encoding for the mitochondrial Rho GTPase 1 (MIRO1) protein (Grossmann, Berenguer-Escuder et al. 2020). This mouse model is promising for studying PD, as it shows aggregation and accumulation of phosphorylated α-syn in 15-16-months old R285Q/R285Q KI mice and significant dopaminergic neuronal loss compared to WT mice (See manuscript Ⅵ in appendix) (Chemla, Arena et al. 2025). Within this model, we were interested in investigating changes of microglia morphology in the adult (3-8 months) and aged (15-16 months) mice. Furthermore, it was previously shown that knock out of Miro1 leads to reduced neuronal dendritic complexity and decreased mitochondrial distribution in dendrites (Lopez-Domenech, Higgs et al. 2016). We hypothesized that the loss of Miro1 function would lead to a decreased complexity in microglial morphology, therefore we aimed at quantifying and describe morphological feature changes of microglia in this mouse model.

Supplementary data related to manuscript Ⅲ

With our basic investigations of TH expression in midbrain regions of *PARK7*/DJ-1 KO and WT mice injected with LPS for 24 hours, we did not detect significant differences neither at baseline nor after LPS (**Fig. S1A-B**), therefore we concluded that 24 hours of LPS exposure did not lead to evident loss of dopaminergic neurons both in *PARK7*/DJ-1 KO and WT mice. It is known from previous studies that central administration of LPS or neurotoxin delivery in the *PARK7*/DJ-1 KO mice will lead to loss of dopaminergic neurons (Qin, Wu et al. 2007, Chien, Lee et al. 2016), hence longer time points are possibly needed for a single intraperitoneal dose of LPS injection to induce neurodegeneration (**Fig. S1A-B**).

To investigate if the morphological and transcriptional differences described in chapter Ⅵ (manuscript Ⅲ) were affecting key microglial immunological functionalities, we assessed their phagocytic ability. Although we detected a significant increase of the phagocytic ability of microglial cells when isolated from mice treated with LPS for 6 hours compared to baseline, we did not observe differences between *PARK7*/DJ-1 KO and WT mice (**Fig. S1C**).

Furthermore, we examined macroscopic differences in age (adult vs aged), sex and genotype across our cohort (**Fig. S1D-H**). As expected, aged mice and males exhibited increased body weight compared to females, with no significant differences between the genotypes (data not shown). Additionally, thymus-to-body weight significantly decreased with age, consistent with typical thymic atrophy observed in all mice, regardless of genotype (**Fig. S1D**) (Aspinall and Andrew 2001). In contrast, spleen-to-body weight increased with age, particularly in female mice, while no significant differences were observed between WT and *PARK7*/DJ-1 KO mice at 4 or 13 months of age (**Fig. S1E**). Notably, previous studies have reported a higher spleen-to-body weight ratio in female mice compared to males in both adult and aged populations (Menees, Earls et al. 2021).

Lastly, in the 13 months old mice, the percentage of monocytes significantly increased in the whole blood after 6 hours of LPS treatment (**Fig. S1F**), a trait that we detected also 24 hours after LPS injection in adult mice when checking the percentage of monocytes in $CD45^+$ cells isolated from the brain (see manuscript Ⅲ). Interestingly, we observed a significantly stronger increase in the percentage of monocytes in the blood of WT mice compared to *PARK7*/DJ-1 KO mice. Furthermore, we observed a significantly decrease in the percentage of lymphocytes 6 hours after LPS treatment in WT mice (**Fig. S1G**). Additionally, there was a tendency to a higher percentage of granulocytes in the blood of WT mice treated with LPS for 6 hours compared to *PARK7*/DJ-1 KO mice (**Fig. S1H**). Except for a higher percentage of monocytes in WT mice after LPS, our preliminary results of immune organs and immune cells in the peripheral blood showed no differences between WT and

PARK7/DJ-1 KO mice, suggesting that systemic immune changes, if any, are not detectable using our macroscopic and cell blood counting approaches. However, further and deeper examinations of the changes of the immune cell proportions in aged *PARK7*/DJ-1 KO mice would be required to conclude on the changes taking place in this model.

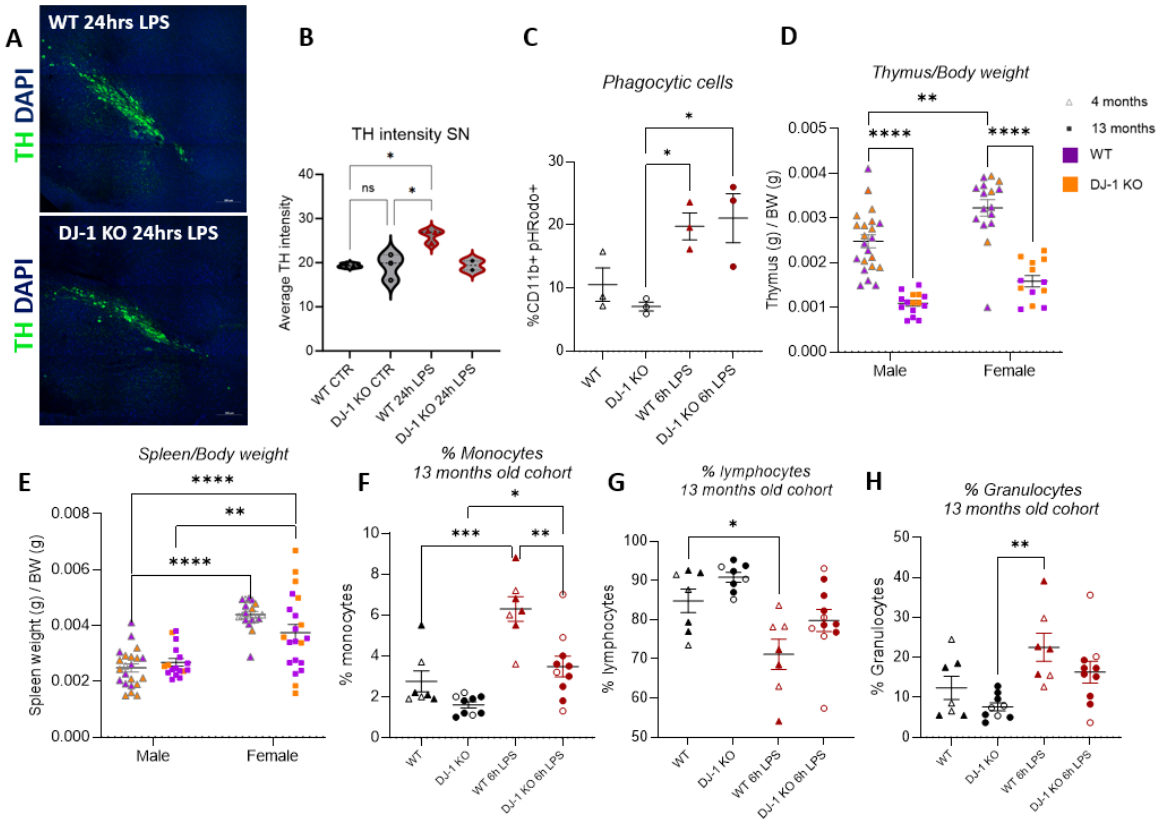

Figure S1. Supplementary information on genotype, sex and age differences in tyrosine hydroxylase expression, phagocytosis, organ and bodyweight. (**A**) Representative images of ventro-lateral SNpc of WT and DJ-1 KO mouse brain 24 hours after LPS injection. (**B**) Quantification of tyrosine hydroxylase (TH) intensity between WT and DJ-1 KO mice, both at baseline and after 24 hours of LPS treatment. (**C**) Percentage of $CD11b^{+}$ phagocytic cells isolated from WT and DJ-1 KO mice. (**D**) Thymus (g) to bodyweight (g) across age and sex (N=5-15 per group). 2-way ANOVA, **=p<0.01, ****=p<0.0001. (**E**) Spleen (g) to bodyweight (g) across age and sex (N=5-20 per group). 2-way ANOVA, **=p<0.01, ****=p<0.0001. (**F-H**) Percentage of (**F**) monocytes, (**G**) lymphocytes and (**H**) neutrophils in whole blood in 13-months old mice. N=7-8 mice per group. Samples from males (filled circles), female (empty circles). 2-way ANOVA with multiple comparisons *=p<0.05, **=p<0.01, ***=p<0.0005.

Additional data: transcriptional differences of microglial cells between male and female mice at baseline and after LPS-induced neuroinflammation

Understanding sex differences in microglia and their responses under inflammatory conditions is essential for several reasons. First, numerous neurological and neurodegenerative disorders, including multiple sclerosis, Alzheimer's disease (AD), and PD, exhibit gender disparities in prevalence and progression. For example, multiple sclerosis is more common in women, whereas PD is more prevalent in men (Mazure and Swendsen 2016, Ullah, Ahmad et al. 2019). A comprehensive study examining sex-specific transcriptional differences across all immune cells of the unstimulated immune system identified the most significant differences in macrophages, including brain-resident microglia (Gal-Oz, Maier et al. 2019). Microglia in male mice are characterized by a more primed transcriptomic signature during early life, whereas microglia from females display a more immune-reactive state later in life, suggesting that male and females may experience different periods of vulnerability to immune challenges throughout their lifespan (Hanamsagar, Alter et al. 2017, Han, Fan et al. 2021, Bobotis, Braniff et al. 2023). These sex-specific differences extend to responses to infections, for example the response to an inflammatory inducer, such as LPS, differ between male and female mice (Murtaj, Belloli et al. 2019). Male microglia often exhibit a heightened inflammatory response to pathogens, while female microglia may show a more regulated response (Guneykaya, Ivanov et al. 2018). The sexual dimorphism was later shown to be conserved after targeting sex hormones, ovariosectomy and after grafting male microglia into female mice, suggesting a long-lasting programming in early life (Villa, Gelosa et al. 2018). Another study showed that early postnatal microglia in rats contribute to masculinization of the preoptic area by releasing prostaglandins (Lenz, Nugent et al. 2013). A recent study uncovered age-dependent microglia genes and found sex differences during the aging process. Microglia from female mice gradually aged in a stepwise manner, whereas microglia from male mice rapidly aged (Hanamsagar, Alter et al. 2017). Others found an attenuated response to LPS in microglia from 12 and 24 months old mice, which was more pronounced in females (Li, Li et al. 2023). These differences suggest that underlying biological mechanisms, including microglial functions and inflammatory responses, may vary between male and females. Investigating these differences can reveal sex-specific pathways and targets for therapeutic interventions, potentially leading to more effective treatments tailored to men or women.

Therefore, another aspect of my project, was to investigate the differences between microglia from male and female mice taking advantage of the generated transcriptomic data of mice treated with LPS or saline vehicle for 6 hours (**Fig. S2**) or 24 hours (**Fig. S3**).

At baseline, applying a strict threshold (adjusted p value <0.05, log2FC ± 1), we only found transcriptomic differences in X- and Y-linked genes in the two datasets (*Xist*, *Ddx3y, Uty*, *Eif2s3y, Kdm5d*, *Gm18665*, *Kdm6a*, *Gm4017*, *Kdm5c*, *Gm29650, Tsix, Rps4y2)* (**Fig. S2C, Fig. S3E**). Therefore, we focused on the transcriptomic differences in response to LPS.

After 6 hours of LPS or saline vehicle injection, C57BL/6NCrl mice were perfused under deep anesthesia and microglia were isolated using CD11b enrichment (**Fig. S2A**), as previously described. Bulk RNA sequencing data revealed few but prominent differences in gene expression between microglia from male and female mice.

After 6 hours of LPS exposure, immune-related genes were significantly upregulated in microglia from male mice (e.g., *Arhgef10l, Batf2, Serpina3g, Ciita*) (**Fig. S2C**), which could indicate, as previously observed in other studies, an enhanced response to LPS in microglia from males compared to female mice. By applying a less strict threshold (p value < 0.05 and log2FC > 0.5), we found 604 DEGs upregulated in male vs female microglia after LPS. By submitting this gene list to Database for Annotation, Visualization, and Integrated Discovery (DAVID), the top Gene Ontology Biological Processes (GOBPs) enriched in males were "Defense response to protozoan", "Oligodendrocyte differentiation" and "Positive regulation of T cell activation" **(Fig. S2B)**. Top GOBPs enriched microglia from female mice were "Cell adhesion", "Chaperone mediated protein folding requiting co-factor" and "Positive regulation of IL-10 production" (**Fig. S2B**).

Interestingly, an observation across both WT and *PARK7*/DJ-1 KO C57BL/6NCrl mice was that males injected with LPS scored higher on severity measurements, a score from 0 to 3, based on mobility and behavior, body posture and grooming (**Fig. S2D**). Male mice showed almost complete immobility, piloerection and very often eye infection (score 3), whereas female mice in general reacted more to the opening of their cage, had less ruffled coat and no eye infection in response to 5-6 hours of LPS treatment (score 0-2). This was significantly different between male and female mice in 4-7 months old mice, whereas the severity of sickness behavior increased in both male and female mice at 13 months at age and there were no differences in the sickness behavior score (**Fig. S2D**). Previous studies showed an increased sickness score with age (Cai, van Mil et al. 2016). The stronger LPS-induced sickness behavior and inflammatory cytokine expression, such as IL-6 in male mice, were previously shown in C57BL/6N and BALB/c mice, but not CD1 mice (Everhardt Queen, Moerdyk-Schauwecker et al. 2016). This could partly be explained by elevated levels of TNFα and lower IL-10 levels in males compared to females (Cai, van Mil et al. 2016). Interestingly, one of the main biological processes upregulated in females compared to males were "Positive regulation of IL-10 production", which could indicate a higher IL-10 in circulation. Our results indicate a stronger

inflammatory response in microglia from male mice, but further experiments are needed to investigate cytokine release, morphological and functional consequences of these differential transcriptional phenotypes.

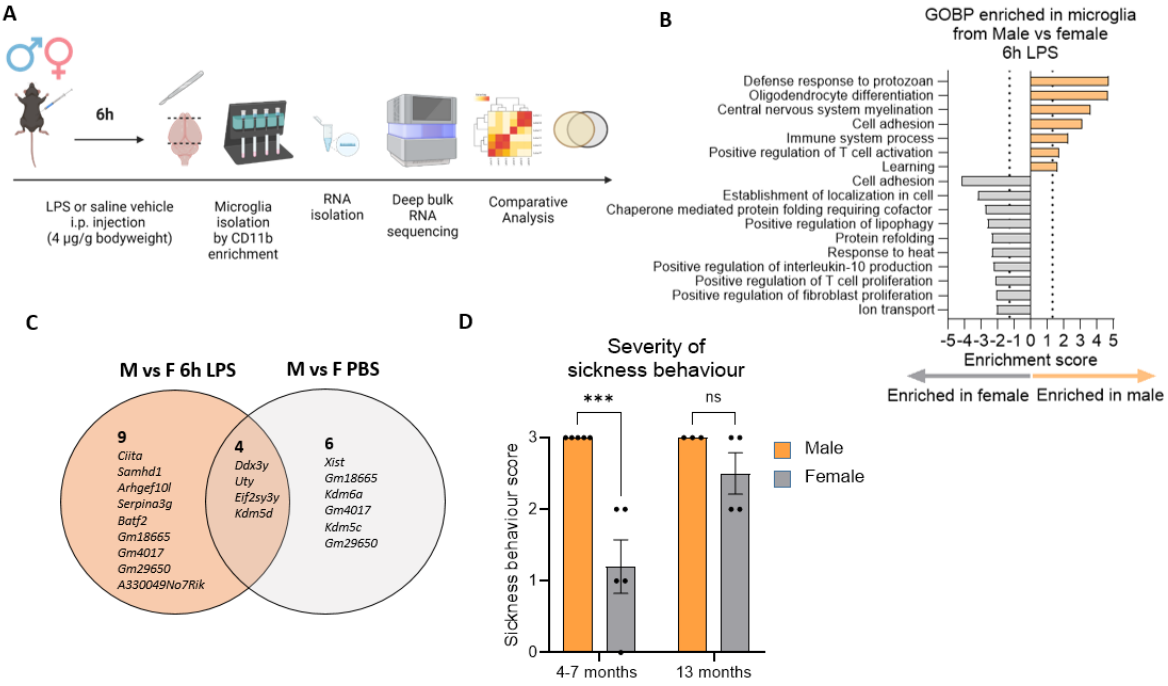

Figure S2. Transcriptional differences of microglia between male and female mice at baseline and after 6 hours of LPS-induced neuroinflammation. (**A**) Experimental design. (**B**) GOBPs from DAVID based on up- or down-regulated genes (p-value < 0.05, log2FC ± 0.5) in microglia from male 4 months old mice. Orange bars show GOBPs enriched in males and grey bars GOBPs enriched in microglia from female mice. The enrichment score equals –log10 (adj. p-value). (**C**) Venn diagram showing differences of DEGs between male and female mice w/wo 6h LPS treatment (log2FC ± 1, adj. p-val < 0.05). (**D**) Severity of sickness behavior in male and female mice at 4-7 and 13 months of age. 2-way ANOVA multiple comparisons. ***=p<0.0005 *=p<0.05.

Furthermore, we investigated the differences in the response to 24 hours-LPS treatment in microglia from male and female mice. Briefly, the brain from 3-4 mice per treatment were dissociated and $CD45^{int}$ $CD11b^{+}$ cells from pooled male and female mice were sorted by FACS and sequenced by Drop-Seq Illumina NextSeq 500 and a comparative analysis were performed (**Fig. S3A**). The single cell RNA sequencing of $CD11b^{+}CD45^{int}$ sorted cells resulted in four clusters based on gene expression (**Fig. S3B**). Cluster 1 and 3 were composed of cells from mice treated with LPS and cluster 2 and 4 from mice treated with saline vehicle. Using the cellXY package, the sex of the

single cells were predicted and categorized into either male or female. The partition of cells in the "CellXY male" and "CellXY female" enabled us to discriminate which cells and the proportion of cells in each of the 4 clusters were coming from female or male mice. Cluster 1 (502 cells from female, 727 cells from male), cluster 2 (258 female, 521 male), cluster 3 (211 female, 203 male) and cluster 4 (103 female, 148 male) (**Fig. S3C-D**).

Based on the similar composition of cells from male and female mice in the four transcriptomic groups, it was clear that the largest difference in expression came from the treatment and the differences coming from the sex of the mice were subtle (**Fig. S3D**).

Out of the top 13 DEGs comparing microglia from male and female mice after 24 hours of LPS treatment, we found 9 DEGs upregulated in males (*Msr1, Dab2, Rnf169, Sdc4, Lcn2, Ccl2, Mt2, Gas5* and *Gbp7*) to be pro-inflammatory (**Fig. S3E**). To further investigate the differences in expression between microglia from male and female mice, GOBPs were investigated by submitting a list of 1361 genes (adjusted p-value <0.05, Log2FC ± 0.5) to DAVID. The GOBP enriched in males were associated to "Immune system process", "Chromatin remodeling", "Synapse pruning" and "DNA damage response", whereas in females "(Positive) Regulation of transcription by RNA polymerase II", "Apoptotic process" and "mRNA processing", which could indicate, as previously observed in other studies and from our own studies after 6 hours of LPS treatment, an enhanced response to LPS in microglia from males compared to female mice. In summary, we found at the transcriptional level a higher inflammatory response towards LPS both at 6 and 24 hours in microglia from males compared to female mice. These findings were in line with a higher sickness behavior score observed in adult male mice compared to female mice 6 hours after LPS-induced inflammation. Further studies are needed to confirm this inflammatory phenotype both at the protein and functional level.

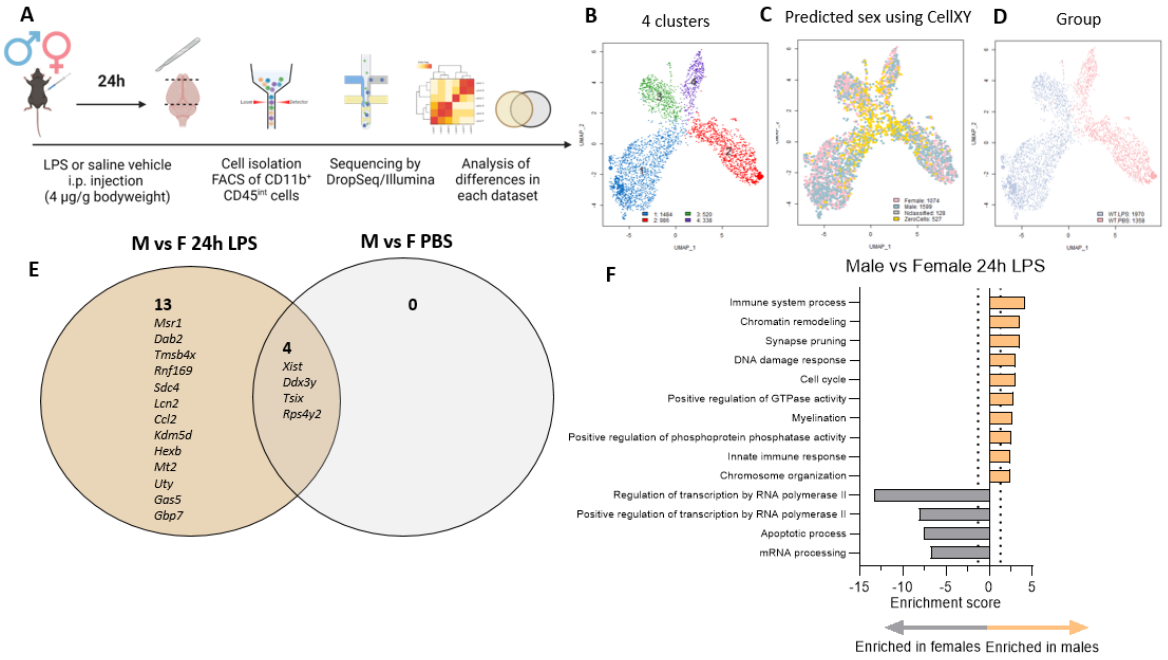

Figure S3. Transcriptional differences of microglia between male and female mice at baseline and after 24 hours of LPS-induced neuroinflammation. (**A**) Experimental design. (**B**) UMAP clustering cells in 4 clusters; cluster 1: 1484 cluster 2: 986 3: 520 cells 4: 338 cells. (**C**) Sex determination based on cellXY resulted in 4 clusters; 1074 female cells (light pink), 1599 male cells (light blue), 128 not classified (grey), 527 ZeroCells (yellow). (**D**) UMAP showing cells clustering based on treatment LPS (1970 cells, blue) PBS (1358 cells, rose-pink). The size of the dots shows counts per cells. (**E**) Venn diagram of DEGs between male vs female mice in cluster 1 and 3 = PBS and male vs female in cluster 2 and 4 (LPS), adj. p-value < 0.05, FDR < 0.05 & abs_logFC > 0.3. (**F**) GOBPs based on DEGs in microglia between male (orange) and female (grey) mice 24 hours after LPS treatment. The enrichment score equals –log10 (adj. p-value).

Additional data: morphological characterization of microglia in the Miro1 knock-in mouse model

To investigate the morphological differences between microglia in WT and homozygous R285Q/R285Q knock-in (KI) mice, we applied the protocol described in Chapter V. Four selected morphological features (compactness, polarity, 1 over 2 and S-metric) were extracted from microglia in the SN and cortex and were compared between 3-7 months old female R285Q/R285Q KI and wildtype (WT) mice and at 15-16 months of age in SN (**Fig. S4A**). In both the cortex and SN, the majority of microglia from R285Q/R285Q KI mice were less compact compared to the majority of microglia in WT mice (**Fig. S3B-D**). Compactness score is a measure of the thickness of the soma

and processes of microglia and can be seen as a measure of amoeboid characteristics. Thus, microglia in R285Q/R285Q KI mice were less amoeboid compared to microglia in WT cortex and SN. Surprisingly, microglia from aged mice had a less significant difference in compactness values between R285Q/R285Q KI mice and WT counterparts (**Fig. S3E**).

The S-metric, which is a measure of ramification complexity, was significantly higher in R285Q/R285Q KI mice, both in the cortex and SN compared to WT mice at the age of 3-7 months (**Fig. S3B-D**). However, there were no genotype differences in microglial ramification complexity in 15-16 months old mice. Based on these morphological features, we can refute the hypothesis that microglia without a functional Miro1 protein would have a decreased complexity. On the contrary, and different from what was described in neurons, we saw a less amoeboid and more ramified morphology in the microglia in R285Q/R285Q KI mouse brain both at 3-7 months and, although less significantly, at 15-16 months of age. In addition, we observed a distinct morphology of several microglial cells only in the SN of R285Q/R285Q KI mice, which was characterized by an elongated aspect with one or two long processes with several smaller processes (**Fig. S3C-D**). The ½ measurement is the ratio between the largest process and the second largest process, representing the elongation of the cell. This morphometrical type was mainly present in the SN at 3-7 months old R285Q/R285Q KI mice, but also significantly in 15-16 months old mice (**Fig. S3E**). This typical rod-shaped microglia was previously described in patients with brain injury and in AD. It is believed to be an adaptive response of microglia surrounding neuronal injury (Holloway, Canty et al. 2019). Further studies of microglial morphological changes would be interesting in even older mice, as 20-21 months old R285Q/R285Q KI mice showed motor symptoms and cognitive dysfunction (See appendix of original publications not included in the thesis). It would be interesting to see if the morphological changes found in microglia at 3-7 months and 15-16 months R285Q/R285Q KI mice would become increasingly evident with older age and whether other changes in microglia could be detected earlier than dopaminergic neuronal loss. Furthermore, changes of microglial morphology in additional brain areas, such as the striatum or olfactory bulb, would warrant further investigations. These preliminary morphological analyses have various limitations as different mouse cohorts were checked at different ages. Future studies should investigate changes of microglia over time combined with morphological analyses from the same mice. This could for example be strengthened by *in vivo* brain imaging of a marker of microglial activation, such as binding of the translocator protein (TSPO), in mice over time. Furthermore, at the functional level, it would be interesting to investigate if microglia would also show impaired mitochondrial function and a higher level of ROS release as seen in dopaminergic neurons in the R285Q/R285Q KI mice and patient-derived iPSC differentiated midbrain organoids.

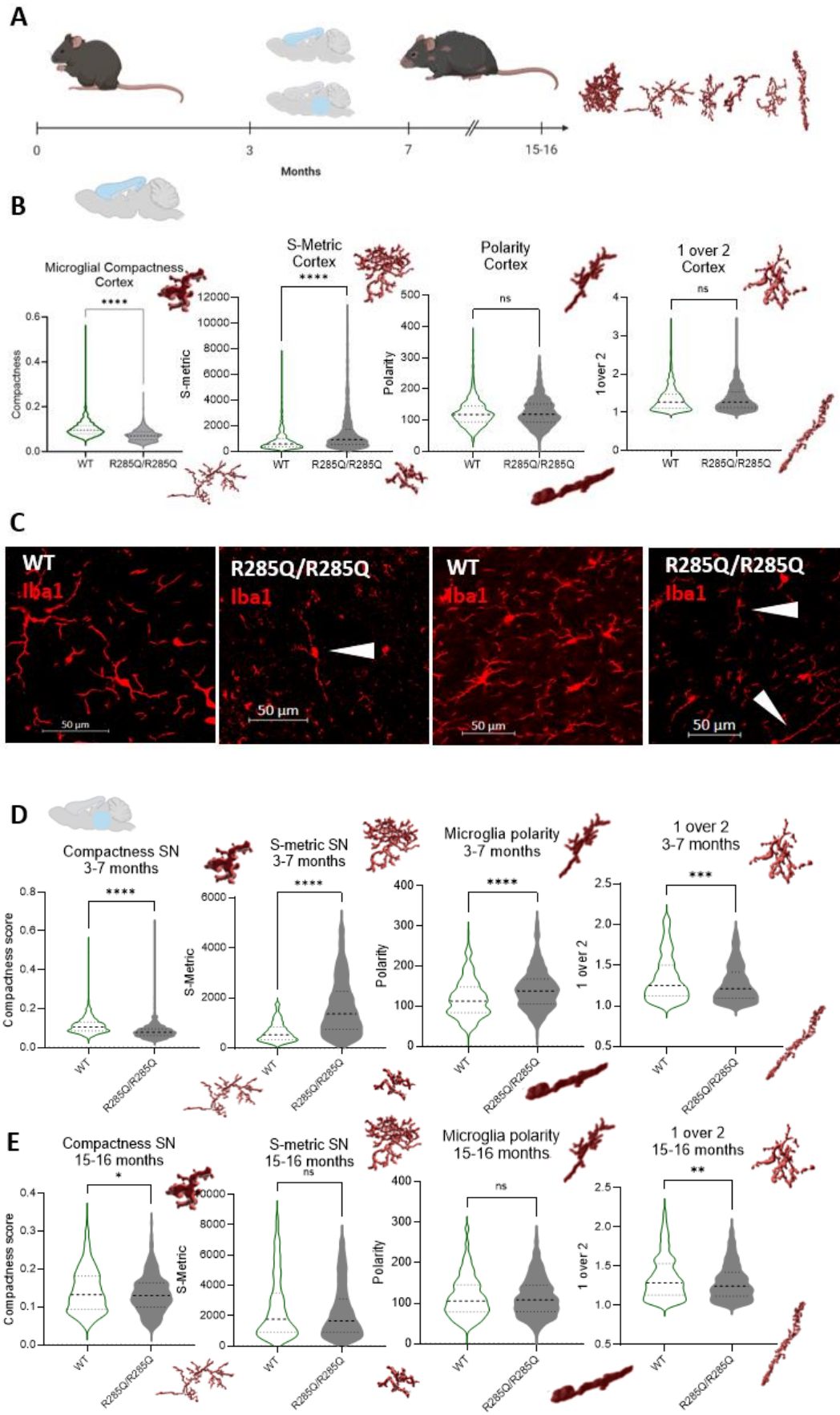
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Figure S4. Differences of microglial morphological features in the cortex and midbrain of Miro KI/R285Q/R285Q and WT mice. (**A**) Brains from 3-7 and 15-16 months old R285Q/R285Q and WT female mice were analyzed for microglial morphological features in cortex and SN. (**B**) Microglial features in cortex; compactness, S-metric (ramification complexity), polarity, 1 over 2 (ratio between longest and 2nd largest process). (**C**) Representative images of microglia in SN of 3-7 months old (left) and 15-16 months old (right). Scale bar: 50 µm. (**D**) Microglial features in the SN in 3-7 months old WT and R285Q/R285Q mice. An average of 220 cells ± 62 per field of view, each from 3-5 mice. (**E**) Microglial features in SN in 15-16 months old WT and R285Q/R285Q mice. An average of 202 cells ± 57 per field of view from 3-5 mice per group. Mann-Whitney test, *=p<0.05, **=p<0.01, ***=p<0.0005, *****=p<0.0001*, ns=not significant.

CHAPTER Ⅶ - MANUSCRIPT Ⅳ: Microglial dynamics and neuroinflammation in prodromal and early Parkinson's disease

Microglial dynamics and neuroinflammation in prodromal and early Parkinson's disease

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MANUSCRIPT Ⅳ - PREFACE

PD is characterized by the loss of dopaminergic neurons in the SN leading to motor symptoms such as resting tremor and bradykinesia. The human midbrain is estimated to contain around 400.000-600.000 dopaminergic neurons, which constitute only about 0.0007% of all the neurons (of a total of ~86 billion) in our brain (Pakkenberg, Moller et al. 1991, Azevedo, Carvalho et al. 2009). However, the connectivity of the dopaminergic neurons is extensive due to the large and complex human brain and each dopaminergic neuron can form around 10.000 to 100.000 synapses with other neurons in the striatum, prefrontal cortex and limbic areas (Haber and Knutson 2010). These connections are essential for regulating functions, such as cognition, movement and reward. At diagnosis, up to 60% of the dopaminergic neurons are lost and, at this stage, disease-modifying interventions have been unsuccessful. Focusing on the prodromal stage is therefore key to understanding etiology, pathophysiology and for finding therapeutic targets in PD. Relatively recently, researchers observed a changed morphology and a different expression profile in microglial cells in the brain of PD patients. Furthermore, PET tracer studies revealed microglia activation in prodromal and recently diagnosed PD patients together with a reduced dopaminergic function in the putamen (Gerhard, Pavese et al. 2006, Stokholm, Iranzo et al. 2017). Besides changing from a neuron-centered view into microglia focused research in PD, researchers also recently changed into focusing more on the prodromal stage of the disease. The prodromal stage

refers to the early stage in the development of PD, during which an individual exhibits symptoms or signs that suggest an increased risk of PD, but does not live up to the clinical diagnostic criteria. The prodromal stage is characterized by many physiological changes, which only recently have been collected and are now used to stratify patients in the hope for a better and earlier diagnosis and treatment. The early signs of PD are, amongst others, hyposmia, constipation, depression, fatigue and worsened sleep (Mahlknecht, Marini et al. 2022). A recent longitudinal study of patients suffering from REM sleep behavior disorder (RBD) showed that 80% of these patients develop PD.

In this review, we describe the different disease stages of PD and the contribution of microglia dysregulation in the prodromal stage. We discuss microglial activation in the brain, traced by TSPO-binding, brain-immune interfaces, the brain-first gut-first theories, animal models of prodromal PD and peripheral immune changes in prodromal PD patients.

CHAPTER Ⅷ - CONCLUSIONS AND PERSPECTIVES

In this cumulative thesis, we have investigated different aspects of the role of microglia in PD, with a specific focus on their phenotypic changes under *PARK7*/DJ-1 deficiency. In our main original article, we comprehensively addressed microglial transcriptional, functional and morphological changes at baseline and upon inflammatory conditions in the absence of *PARK7*/DJ-1, using both murine and human models. Additionally, we reviewed the known functions of DJ-1 in microglia, we optimized a protocol for analyzing the morphological features of microglia in the mouse brain and addressed the importance of detecting microglial changes in prodromal and early stages of PD. These four endeavors are briefly summarized here.

In **Chapter Ⅳ**, we highlighted the various functions of DJ-1 specifically focusing on its role in microglia. We underscored the need for *in vivo* and *ex vivo* studies in the investigation of the role of DJ-1 in microglia, as previous research showing significant effects on microglial neuro-immune responses was mainly carried out using immortalized cell lines. Interestingly, as DJ-1 is oxidized in idiopathic PD patients and thereby it results to be dysfunctional, non-genetic PD patients may show an immune phenocopy of DJ-1 deficient patients. Hence, we designated DJ-1 as a biomarker and as a relevant therapeutic target for a broader spectrum of PD patients.

In **Chapter Ⅴ**, we described the optimization of a protocol used for quantifying the density and the morphological features of microglia in mouse brain tissues using immunofluorescence staining and confocal imaging. This protocol is useful for quantifying microglial morphological features *in situ* at one time point, which is currently one of the best ways to describe microglial morphological changes. To reach another level of information, live imaging by two-photon or three-photon imaging takes also into account the dynamic movement of microglial processes and phagocytosis in real-time (Beccari, Diaz-Aparicio and Sierra 2018). However, even with the newest three-photon imaging, only 1.12 millimeters below the cortical surface can be imaged (Cheng, Tong et al. 2020) and deeper brain regions more relevant for PD, such as the striatum or SN, are inaccessible. Advancing non-invasive live imaging techniques will be crucial to accurately determine the relationship between microglial morphology and their functional states.

In **Chapter Ⅵ**, we investigated microglial gene expression signatures under homeostatic conditions and systemic inflammatory conditions in the absence of *PARK7*/DJ-1. Neuroinflammation is a common feature of PD, where microglia activation and dysfunctions have been linked to both the initiation and progression of the disease. We found that microglia in the *PARK7*/DJ-1 KO mouse and human iPSC-derived microglia lacking DJ-1 downregulated inflammatory response genes both at

baseline and under inflammatory conditions. The inflammatory response to fight off bacterial or viral infections, which contributes to the resolution of inflammation, represents a key function of microglial cells (Waltl, Kaufer et al. 2018). It would be interesting to further investigate the long-term consequences of the hyporesponsive phenotype towards inflammation observed in the DJ-1 deficient models. Associated to this distinct transcriptional phenotype, we found a discrete morphological adaptation in microglia and BMDMs both at baseline and after LPS treatment in *PARK7*/DJ-1 KO mice, characterized by a less amoeboid morphology and hyper ramification compared to WT mice (**Fig. S5**). Furthermore, we conducted morphological analyses in another PD mouse model carrying a knock-in mutation of Miro1, a protein like DJ-1, involved in mitochondrial homeostasis and cellular protection. We found a higher prevalence of microglia having an elongated polar cell shape in the SN in both adult and aged Miro1 KI compared to WT mice. This shape of microglia was previously described in AD and further investigations of microglial changes in expression and function in this mouse model are warranted.

Microglia in both the *PARK7*/DJ-1 KO murine and DJ-1 mutant human models showed an upregulation of genes related to extracellular matrix organization, focal adhesion and cytoskeleton. Interestingly, a recent paper found those identical gene ontology pathways to be modulated in sporadic PD and various monogenic iPSC-derived neurons (Stern, Lau et al. 2022). In addition, we found an upregulation of DNA repair response genes and elevated ROS, which could be an early sign of DNA damage-induced senescence or oxidative stress-induced senescence (**Fig. S5**). However, we found no differences in the percentages of dead microglial cells between WT and *PARK7*/DJ-1 KO mice. The long life of microglia from the yolk sac throughout large parts of life might have significant implications when considering their susceptibility to ROS, DNA damage, replicative stress and protein aggregates. It is possible that DJ-1 deficient microglia could be more prone to senescence due to enhanced ROS levels, an impaired response to LPS and an elevated DNA damage response compared to microglia with a functional DJ-1. Currently, the contribution of senescent microglia in AD is relatively clear, but data is sparse in the context of PD (Angelova and Brown 2019, Yoon, You et al. 2022, Rim, You et al. 2024). Investigating how various challenges, such as chronic LPS stimuli, viral infections or oxidative stress, in order to assess susceptibility of microglia to enter cell death pathways, would be an interesting perspective of this study.

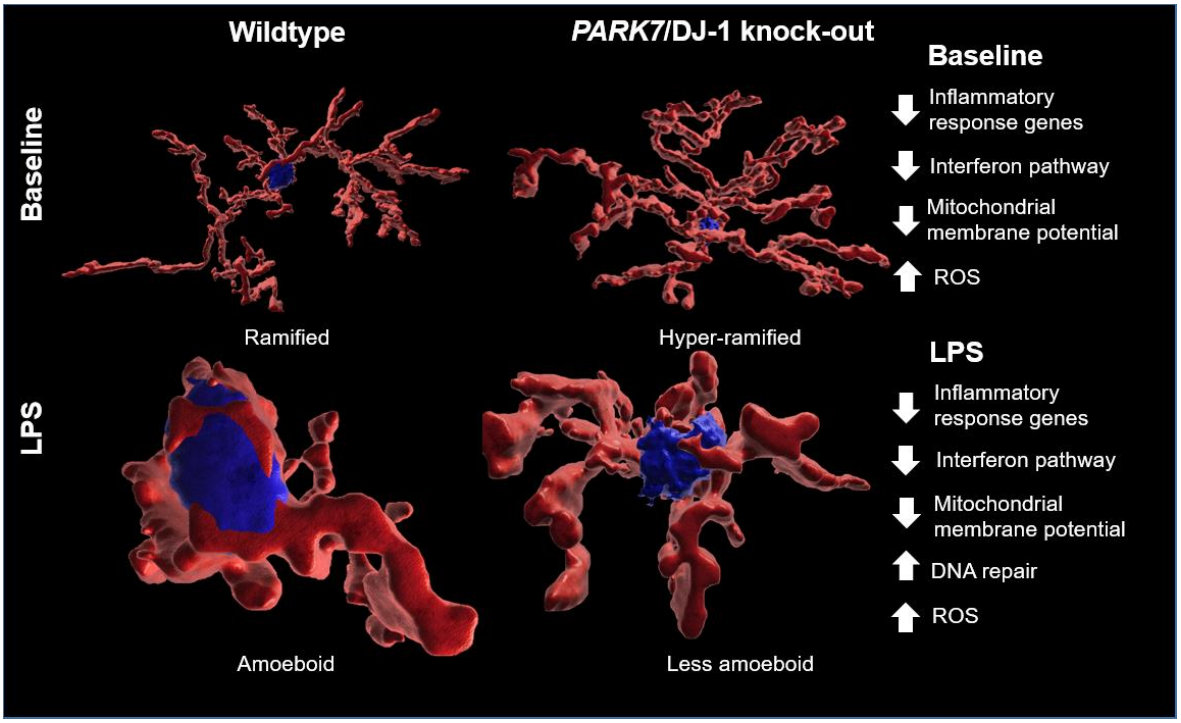

Figure S5. Graphical summary of the main results obtained in microglia from the *PARK7*/DJ-1 KO mouse model.

Various other functions of microglia are facilitated by changes in cytoskeleton dynamics, such as in actin filaments or microtubules. In this regard, it is important to mention that various post-translational modifications of microtubule-associated proteins or local changes in adenosine tri phosphate (ATP) or cAMP (Davalos, Grutzendler et al. 2005, Bernier, Bohlen et al. 2019), which are known to direct microglia surveillance movements and morphology, would not be discernable using RNA sequencing analyses as applied in our study. To investigate whether the observed morphological changes of microglia in *PARK7*/DJ-1 KO mice impact their surveillance ability, it would be interesting to measure their activities using live *in vivo* imaging directly within the brain or in organotypic cortical slices. Of note, our results are based on studies *in vivo* and *ex vivo* of a full knock out of DJ-1. As DJ-1 is ubiquitously expressed, we believe it is the most physiological relevant model for studying the consequences of DJ-1 deficiency. However, it would be interesting to confirm our results in a conditional KO of DJ-1 in microglia. This approach could also be interesting to investigate the consequences of DJ-1 loss in microglia at different stages, for example during fetal development, in adult and in aged mice.

Lastly, as our results obtained in adult mice were in contrast with the primed phenotype mainly described in microglial cell lines, we sought to investigate the effect of aging during DJ-1 deficiency. Surprisingly, we did not observe differences in bodyweight, size or weight of spleen and thymus between WT and *PARK7*/DJ-1 KO mice neither at 4- nor at 13-months of age. We observed a higher

percentage of monocytes and a lower percentage of lymphocytes in whole blood in 13-months old WT mice after 6 hours of LPS treatment compared to *PARK7*/DJ-1 KO mice, but no significant differences were found between other immune cells. These preliminary data could indicate that there are no significant macroscopic systemic and peripheral immune changes at the analyzed ages in this mouse model and that our focus on brain microglia is indeed more promising. Transcriptional differences between microglia from male and female mice at baseline were subtle, hence we focused on changes to LPS-treatment. We found a stronger inflammatory response to LPS in male mice, especially at the 6 hour time point, which was further supported by a higher sickness behavior score. Further investigations are necessary to confirm these signatures at a functional level.

In **Chapter VII**, we drew the attention of the role of microglia and neuroinflammation in prodromal and early-stage PD and discussed why and how future studies should focus on early changes of microglia in PD. We propose that longitudinal studies of infections and PD risk would be highly valuable, as currently all patients with infections as well as anti-viral or anti-inflammatory medications are excluded from the clinical trials. Multiple studies showed that infections could potentiate neurodegeneration, however the data available correlating infection and risk of PD is limited. Our study on DJ-1 in microglia shows that focusing on one immune cell in PD gave valuable insights, although the interplay with brain resident cell types and the whole immune system is essential to fully understand the disease. Similarly, for understanding what the immune system encounters throughout life and how this could potentially alter the risk of a later PD diagnosis would be an interesting study. The concept of establishing an "immunobiography" of each individual (Franceschi, Salvioli et al. 2017), could be a thrilling framework to study how and which exposure of various antigens could affect the risk of developing PD. Combining these aspects with societal and genetic background of healthy individuals and PD patients, would be an exciting new avenue.

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Xu, R., X. Li, A. J. Boreland, A. Posyton, K. Kwan, R. P. Hart and P. Jiang (2020). "Human iPSC-derived mature microglia retain their identity and functionally integrate in the chimeric mouse brain." Nat Commun **11**(1): 1577.
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ANNEX I: PUBLICATIONS INCLUDED IN THE THESIS

Chapter Ⅳ

PARK7/DJ-1 in microglia: implications in Parkinson's disease and relevance as a therapeutic target

Lind-Holm Mogensen F., Scafidi A., Poli A., Michelucci A.

Journal of Neuroinflammation (2023) 20:95

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Author contributions: I contributed to the conceptualization of the review and selected the relevant literature, written a first draft and edited the manuscript, created tables and figures.

Chapter V

Protocol for immunofluorescence staining and large-scale analysis to quantify microglial cell morphology at single-cell resolution in mice

Lind-Holm Mogensen F, Ameli C., Skupin A., Michelucci A. *STAR Protocol*. (2024) 5(4):103467

DOI: 10.1016/j.xpro.2024.103467

Author contributions: I contributed by optimizing the protocol and I carried out all animal work, tissue preparation, staining and microscopy acquisitions. I made the graphical abstract and wrote the manuscript draft, expect for points 44-50, which were written by Dr Corrado Ameli. Expected outcomes, limitations and troubleshooting were written together with Dr Corrado Ameli. All authors reviewed and edited the manuscript.

Chapter VI

PARK7/DJ-1 deficiency impairs microglial activation in response to LPS-induced inflammation

Lind-Holm Mogensen F, Sousa C., Ameli C., Badanjak K., Pereira S.L, Muller A., Kaoma T., Coowar D., Scafidi A., Poovathingal S.K, Tziortziou M., Antony P.M.A , Nicot N., Ginolhac A., Weisenhorn D.M.V, Wurst W., Poli A., Nazarov P.V., Skupin A., Grünewald A., Michelucci A. *J Neuroinflammation* 2024 21(1):174

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Author contributions: I contributed to this paper by carrying out the majority of the experiments (except figure 1 and 3) as well as all analysis and data visualization. I designed the figures and tables. The first manuscript drafts were written by me, but the final manuscript was written with the support of my supervisor Dr Alessandro Michelucci.

Chapter Ⅶ

Microglial dynamics and neuroinflammation in prodromal and early Parkinson's disease

Lind-Holm Mogensen F, Seibler P, Grünewald A, Michelucci A.

Under review in *Journal of Neuroinflammation*

Author contributions: I prepared the figures, tables, selected the literature and wrote the draft of the manuscript.

ANNEX II: PUBLICATIONS NOT INCLUDED IN THE THESIS

The appendix includes additional publications to which I have contributed, but they do not relate to the main project. It comprises the article titled *"Metformin impacts the differentiation of bone marrow cells into macrophages affecting tumour immunity"* published in *Heliyon* (IF = 3.4). Furthermore, it consists of a protocol article entitled "*Protocol for the generation and assessment of functional macrophages from mouse bone marrow cells*" under review in STAR Protocols (IF = 1.3) that we used to generate results included in Chapter Ⅵ (manuscript Ⅲ). Additionally, it comprises the article entitled "*AllergoOncology: Biomarkers and refined classification for research in the allergy and glioma nexus – A joint EAACI-EANO position paper",* which was published in *Allergy* (IF = 12.6). In addition, it includes the article titled *"Targeting the ACOD1-itaconate axis stabilizes atherosclerotic plaques***",** which was published in *Redox Biology* (IF = 10.7). Lastly, it comprises the article entitled "*Parkinson's disease-related mutant Miro1 causes mitochondrial dysfunction and loss of dopaminergic neurons*", which was published in *Brain* (IF = 11.9) and describes the establishment and characterization of the Miro1 KI mouse model further analysed using the protocol described in manuscript Ⅱ (see additional data manuscript Ⅲ).

1. **Metformin impacts the differentiation of mouse bone marrow cells into macrophages affecting tumour immunity.**

   Scafidi A., **Lind-Holm Mogensen F**., Campus E., Pailas A., Neumann K., Legrave N., Bernardin F., Pereira S.L., Antony P.M.A., Nicot N., Mittelbronn M., Grünewald A., Nazarov P.V., Poli A., Van Dyck E., Michelucci A.

   Heliyon, 2024 10(18):e37792.

   Co-author contributions: Figure 1b, Figure 4e, (H&E staining, Iba1 and other staining of metformin-treated mouse brain tissue, which were not included in the manuscript). Additionally, literature search and paragraph writing related to BMDM cell and mitochondrial morphology, manuscript review and editing.

I hereby declare that this article, which forms part of this cumulative PhD thesis, has also been included in the PhD thesis of Mr. Andrea Scafidi. This article represents collaborative work between multiple researchers, and its inclusion in more than one doctoral thesis has been approved by all the co-authors and relevant academic authorities. The specific contributions of each researcher to this work are clearly delineated in the article in the section “CRediT authorship contribution statement” and in the respective theses.

2. **Protocol for the generation and assessment of functional macrophages from mouse bone marrow cells.**

   Scafidi A., **Lind-Holm Mogensen F.**, Michelucci A.

   Under review in *STAR Protocols* (*Cell Press*).

   Co-author contributions: Optimization of protocol for BMDM differentiation, mainly addressing phalloidin staining and morphological analyses using CellProfiler™ (points 61-79 and graphical abstract). Manuscript review and editing.

I hereby declare that this article has also been incorporated in the PhD thesis of Mr. Andrea Scafidi. This article represents collaborative work between the group members of the Neuro-Immunology Group and its inclusion in more than one doctoral thesis has been approved by the co-authors. The specific contributions of each researcher to this work are clearly delineated in the article in the section “Author contributions” and in the respective theses.

3. **AllergoOncology: Biomarkers and refined classification for research in the allergy and glioma nexus – A joint EAACI-EANO position paper.**

   Turner C.M., Radzikowska U., Ferastraoaru E.D., Pascal M., McCraw A., Backes C., Bax H.J., Bergmann C., Bianchini R., Cari L., De las Vecillas L., Izquierdo E., **Lind-Holm Mogensen F**., Michelucci A., Nazarov P.V., S.P., Nocentini G., Ollert M., Preusser M., Rohr-Udilova N., Scafidi A., Toth R., Van Hemelrijck M., Weller M., Jappe U., Escribese M.M., Jensen-Jarolim E., Karagiannis S.N., Poli A.

   Allergy, 2024 79(6):1419-1439.

   Co-author contributions: Figure 4, box 1 with Aurélie Poli, manuscript review and editing.

I hereby confirm that this article has also been incorporated in the PhD thesis of Mr. Andrea Scafidi. This work is the result of a collaborative effort among multiple researchers, and its inclusion in multiple doctoral thesis has been approved by all the co-authors and relevant academic authorities. The specific contributions of each researcher are explicitly detailed within the article and the respective theses.

4. Targeting the ACOD1-itaconate axis stabilizes atherosclerotic plaques.

Harber K.J., Neele A.E., van Roomen C.P.A.A., Gijbels M.J.J., Beckers L., den Toom M., Schomakers B.V., Heister D.A.F., Willemsen L., Griffith G.R., de Goede K.E., van Dierendonck X.A.M.H., Reiche M.E., Poli A., **Lind-Holm Mogensen F.,** Michelucci A., Verberk S.G.S., de Vries H., van Weeghel M., Van den Bossche J., de Winther M.P.J. *Redox Biology*, 2024 70:103054.

Co-author contributions: Mouse takedown, dissections, and mouse cohort maintenance/genotyping, review and editing of final manuscript.

I affirm that this article has also been included in the thesis of Dr Karl Harber. This article was published in Redox Biology in 2024 and is a collaborative effort among numerous researchers, with its inclusion in multiple doctoral theses receiving formal approval by the first and last author. The article and corresponding theses clearly outline the specific contributions of each researcher involved.

5. **Parkinson's disease-related mutant Miro1 causes mitochondrial dysfunction and loss of dopaminergic neurons**

   Chemla A., Arena G., Sacripanti G., Barmpa K., Zagare A., Garcia P., Gorgogietas V., Antony P., Ohnmacht J., Baron A., Jung J., **Lind-Holm Mogensen F**., Michelucci A., Marzesco A.-M., Buttini M., Schmidt T., Grünewald A., Schwamborn J.C., Krüger R., Saraiva C.

   Brain, 2025 In Press.

   Co-author contributions: Staining of mitochondrial marker Tom20 in TH^+ neurons, image acquisition and analysis (Supplementary figure S9), Review and editing of manuscript and figures.

   By this means, I declare that this article has also been included in the PhD thesis of Dr Axel Chemla. This article represents collaborative work between multiple researchers, and its inclusion in more than one doctoral thesis has been approved by all the co-authors and relevant academic authorities. The specific contributions of each researcher to this work are clearly described in the article and in the respective theses.