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Final reports of the research projects of young researchers, supported by the Queen Elisabeth Medical Fondation in collaboration with the following professors and doctors (2025)

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Interuniversitaire onderzoeksprojecten
2023-2025 gefinancierd door de G.S.K.E.

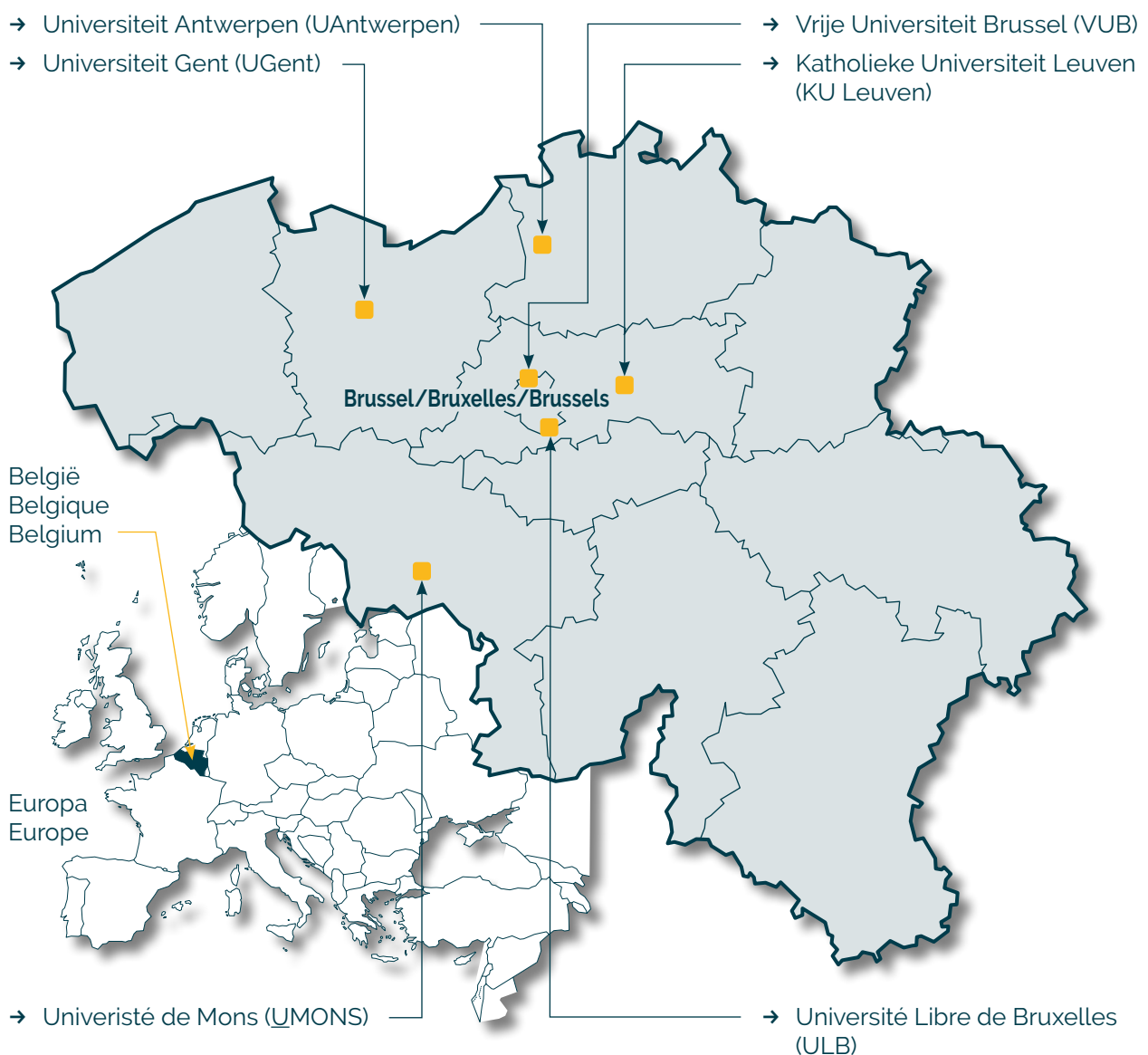
Projets de recherche interuniversitaire
2023-2025 subventionnés par la F.M.R.E.

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Prof. dr. Geert Van Loo (UGent)

Prof. Kiavash Movahedi (VUB)

OTULIN in neuroinflammation and Alzheimer pathology

Prof. dr. Sarah Weckhuysen (UAntwerpen)

Prof. dr. Bjorn Menten (UGent)

Detection of somatic mutations and disease-defining methylation patterns in brain tissue and cerebrospinal fluid of patients with non-acquired focal epilepsy

Prof. dr. Renzo Manusco (UAntwerpen)

Prof. dr. Joris De Wit (KU Leuven)

Dissecting the molecular basis of microglia-synapse communication in AD

Prof. dr. Ann Massie (VUB)

Prof. dr. Lutgarde Arckens (KU Leuven)

The xCT-/- killifish to validate the potential of system xc- as therapeutic target in Parkinson's disease

Prof. dr. Karelle Leroy (ULB)

Prof. dr. Laurence Ris (UMONS)

Prof. dr. Kristel Slegers (UAntwerpen)

Involvement of diabetes and antidiabetic treatment on tau pathology propagation



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Final report of the interuniversity research project of

Prof. dr. Geert Van Loo (UGent)
Prof. Kiavash Movahedi (VUB)

Prof. dr. Geert van Loo (UGent)

VIB-UGent Inflammation
Technologiepark 71
9052 Gent-Zwijnaarde
Belgium
T +32-(0)9 3313761
Geert.vanloo@irc.vib-ugent.be

Prof. dr. Kiavash Movahedi (VUB)

Research Center Vrije Universiteit Brussel
Laarbeeklaan 103 building E, room E234
1090 Jette-Brussels
Belgium
T +32-(0)2 4774569
kiavash.movahedi@vub.be

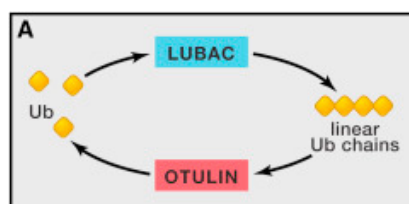
OTULIN in neuroinflammation and Alzheimer pathology

1. Summary of the research project:

Inflammatory signaling pathways are subjected to tight regulation to avoid chronic inflammation and the development of inflammatory pathology. One of the proteins involved in such regulation is the deubiquitinating enzyme OTULIN. In this project, we investigate the role of OTULIN in neuroinflammation and Alzheimer pathology.

2. Introduction

Inflammation is a protective response that induces repair in conditions of cellular damage and stress. However, strict control of inflammatory signaling pathways is essential to prevent chronic inflammation and the development of inflammatory pathologies. This control is exercised at various levels, including the reversible modification of signaling proteins. Ubiquitination is a posttranslational protein modification in which ubiquitin chains are covalently attached to target proteins. This process earmarks these proteins for proteasomal degradation or allows them to function as scaffolds to recruit other proteins and mediate downstream signaling [1,2]. Linear ubiquitination involves the linkage of ubiquitin via its amino-terminal methionine residue (Met1 or M1) to another ubiquitin molecule, a process mediated by the linear ubiquitin chain assembly complex (LUBAC) (Figure 1). **LUBAC is the sole E3 ubiquitin ligase complex known to generate linear chains**, and is essential for i) NF- κ B-mediated inflammatory gene expression in response to various stimuli, including TNF, IL-1 β , and ligands activating TLRs, NOD2 and NLRP3; and ii) for preventing TNF-induced cell death [3–12]. Genetic loss of LUBAC proteins causes immunodeficiency and auto-inflammation in humans [13,14], and embryonic lethality or inflammatory phenotypes in mice [4,10,11,15–17], establishing the importance of LUBAC-mediated M1 signaling for normal mammalian physiology.



Ubiquitination is reversed by **deubiquitinating enzymes (DUBs)** that cleave ubiquitin chains from their substrates [2]. About a hundred different DUBs have been identified so far, but mainly A20, Cyldromatosis (CYLD) and **OTULIN have been characterized as key DUBs involved in the negative regulation of NF- κ B activation and cell death** [18] (Figure 1). OTULIN (OTU Deubiquitinase with Linear Linkage Specificity), also known as Fam105b or Gumby, was only recently discovered in mice and human [19,20]. Homozygous hypomorphic mutations in the human *OTULIN* gene have been identified as causing an early-onset, potentially fatal, autoinflammatory disease [21–24]. Mice harboring a point mutation in *OTULIN*, abolishing its activity to cleave M1 chains, are not viable and die during development [20,25], while mice with inducible (postnatal) deletion of *OTULIN* develop acute systemic inflammation due to dysregulated NF- κ B activation and cytokine production [22]. **Together, these data identify OTULIN as a crucial regulatory mechanism in inflammatory signaling.**

Very little is known about the role of OTULIN in the central nervous system (CNS) and in CNS inflammation. A recent study showed that OTULIN controls microglia activation and suppresses neuroinflammation in a rat model of stroke [26]. Microglia, the resident tissue macrophages of the CNS, are important for maintaining CNS homeostasis, but also critically contribute to CNS pathology [27]. Interestingly, **a large meta-genome-wide association study (GWAS) by the European Alzheimer and Dementia Biobank reaffirmed the implication of microglia in AD and provided genetic evidence linking LUBAC and OTULIN to AD** [28].

The aim of the project is to characterize the role of OTULIN in neuroinflammation and Alzheimer disease (AD), the most common progressive form of dementia, affecting over 25 million people worldwide [29,30]. Neuroinflammation is increasingly regarded as a key component that actively contributes to AD pathology. NF- κ B signaling, activated by amyloid- β (A β) and/or by the microtubule-associated protein Tau, has been shown to drive microglia-mediated A β and Tau toxicity leading to AD-associated learning and memory deficits [31-33]. However, **the specific immune mechanisms through which microglia contribute to AD-associated neuroinflammation, as well as the eventual role of OTULIN in this process, remain largely elusive and require further exploration.** In our project, using a CNS myeloid-specific OTULIN knockout model in combination with AD disease models, **we aim to investigate the specific role of OTULIN in the regulation of microglia activation, neuroinflammation and AD pathology.**

3. Results so far

3.1. Characterization of unchallenged OTULIN^{Cx3Cr1-KO} mice

To specifically address the role of OTULIN in microglia in CNS homeostasis and pathology, the van Loo lab generated mice with a 'floxed' OTULIN allele [34] and crossed these mice with Cx3Cr1-CreErt2 transgenic mice, allowing Cre-mediated gene deletion in myeloid cells following tamoxifen treatment [35] (Figure 2A). CNS-resident macrophage populations including microglia can be specifically targeted using this approach which relies on microglial longevity and its self-renewing capacity [36].

Successful OTULIN deletion in microglia was confirmed by Western blot analysis of fluorescence-activated cell sorted (FACS) microglia *ex vivo* and of primary cultured microglia *in vitro* (Figure 2B). Tamoxifen-induced ablation of OTULIN in mice did not induce any gross abnormalities, as could be observed by histological analysis of the brain using H&E staining (Figure 2C). However, immunohistochemical analysis using the microglial marker Iba-1 revealed a spatially restricted increase in microglia number in the hippocampi, but not the cortex, of OTULIN^{Cx3Cr1-KO} mice compared to control littermates (Figure 2D). The enhanced presence of microglia in OTULIN^{Cx3Cr1-KO} CNS was confirmed by manual counting of Iba-1⁺ microglia (Figure 2E). This regional difference in the number of Iba-1⁺ cells suggest that the lack of OTULIN induces proliferation of adult microglia. We next analyzed how OTULIN deficiency in CNS resident myeloid cells has an impact on T-cell recruitment to the spinal cord in steady state conditions. Interestingly, the number of both CD4⁺ T-cells, CD8⁺ T-cells and Foxp3⁺ Tregs were significantly increased in the spinal cord of OTULIN^{Cx3Cr1-KO} mice (Figure 2F). This suggests that deficiency of OTULIN in microglia induces strong recruitment of different types of leukocytes to the spinal cord.

To reveal how microglial OTULIN deficiency is associated with CNS-wide inflammatory dys-homeostasis, we expression profiled by single-cell RNA sequencing CD45^{int}CD11b⁺ microglia sorted from brains of 10-12 week old OTULIN^{Cx3Cr1-KO} and control littermate mice. We performed clustering analysis, which resulted in the identification of 7 major subpopulations of microglia

(Figure 3A). Three of these clusters express high levels of so-called homeostatic microglia (HoM, H1M and H2M) markers (*Ptprc*, *Itgam*, *P2ry12*, *Slc2a5*, *Tmem119*, *Tgfb1*, *Siglech*, *Sall1*, *Fcrls*, *Mertk*, *Gpr34*, *Rhob*, *Olfml3*). This clustering analysis also identifies a distinct non-microglial CNS macrophage population, the 'Border-associated macrophages' ('BAM' cluster) (Figure 3A). Of particular interest are the clusters that show downregulated expression of homeostatic microglial genes in OTULIN^{Cx3Cr1-KO} and instead upregulate specific expression profiles associated with distinct functionalities of the activated microglia. These clusters, designated as 'interferon response microglia' (IRM), 'activated response microglia' (ARM), and 'cycling and proliferating microglia' (CPM), show upregulated expression of genes associated with interferon signalling (*Ifit2*, *Ifit3*, *Oasl2*, *Ifitm3*, *Irf7*), with a 'disease-associated microglia' (DAM) phenotype involved in inflammatory processes (*Cst7*, *Itgax*, *Cd74*, *Lgals3*, *Clec7a*, *Lpl*, *Csf1*, *Lilbr4a*), and with cell cycle regulation and proliferation (*Mki67*, *Cdk1*, *Top2a*, *Mcm2*), respectively, and are represented more strongly in conditions of OTULIN deficiency (Figure 3B-C) [37-39]. The ARM cluster also displays genes linked to inflammatory signaling (*Nfkb1*, *Nfkb2*, *Relb*, *Il1b*, *Tnf*, *Ccl4*) as being upregulated in OTULIN^{Cx3Cr1-KO} microglia (Figure 3B).

Together, these data demonstrate the importance of OTULIN in microglia activation and suggest that defects in OTULIN function may contribute to CNS pathology and neurodegeneration.

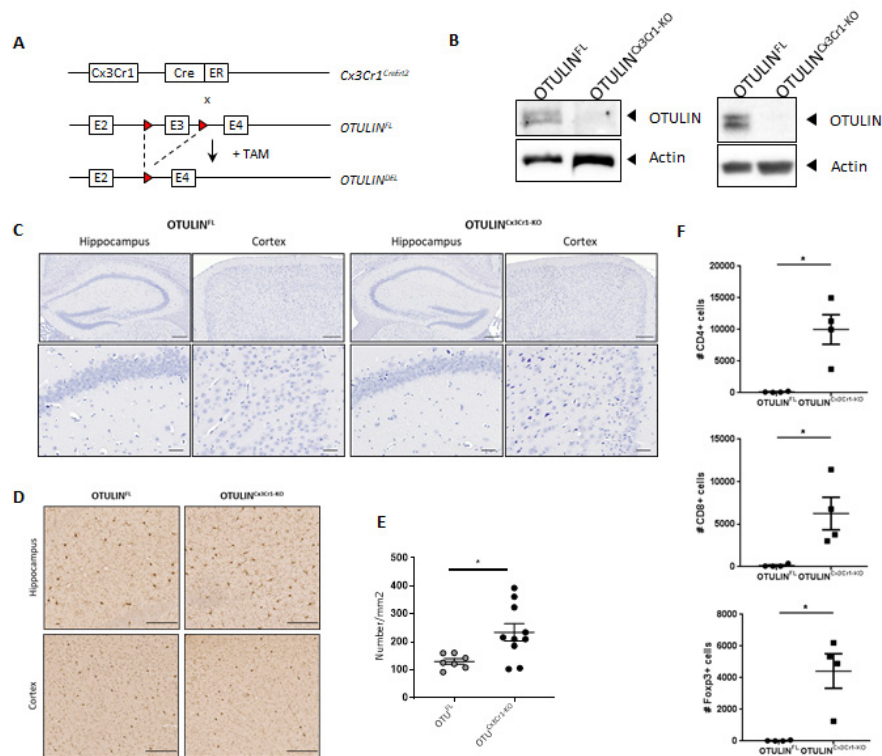


Figure 2. OTULIN deletion in CNS-resident myeloid cells induces a spontaneous neuroinflammatory phenotype. (A) Scheme for the Tam-inducible induction of Cre recombination and OTULIN deletion in CNS-resident myeloid cells. (B) Immunoblot for OTULIN expression in *ex vivo* FACS-sorted microglia from control (OTULIN^{FL}) and OTULIN^{Cx3Cr1-KO} mice 5 weeks after TAM injection (left), and in lysates from primary microglia from control (OTULIN^{FL}) and OTULIN^{Cx3Cr1-KO} mice after stimulation with 4-OH-TAM (right). Actin is shown as loading control. (C) H&E staining on section of cortex and hippocampus of 10-12 week old OTULIN^{FL} and OTULIN^{Cx3Cr1-KO} mice (5 weeks after TAM injection). Scale bars represent 200 μ m (overview, upper) and 50 μ m (zoom, lower). (D) Immunohistochemistry for Iba-1+ microglia in the hippocampus and cerebral cortex of control (OTULIN^{FL}) and OTULIN^{Cx3Cr1-KO} mice. Representative images are displayed and scale bars represent 100 μ m. (E) Number of Iba-1+ ramified parenchymal microglia. Each symbol represents one mouse, with 7 and 10 mice per group. Data presented as mean $\pm$ SEM. Significant differences determined by a Mann-Whitney U-statistical test (* $p < 0.05$). (F) Flow cytometric quantification of the number of CD3+CD4+ T cells, CD3+CD8+ T cells and CD3+CD4+Foxp3+ Tregs in the spinal cord of control (OTULIN^{FL}) and OTULIN^{Cx3Cr1-KO} mice 4 weeks post TAM injection. Each symbol represents one mouse and data are presented as mean $\pm$ SEM. Significant differences were determined by Mann-Whitney U-statistical test (* $p < 0.05$).

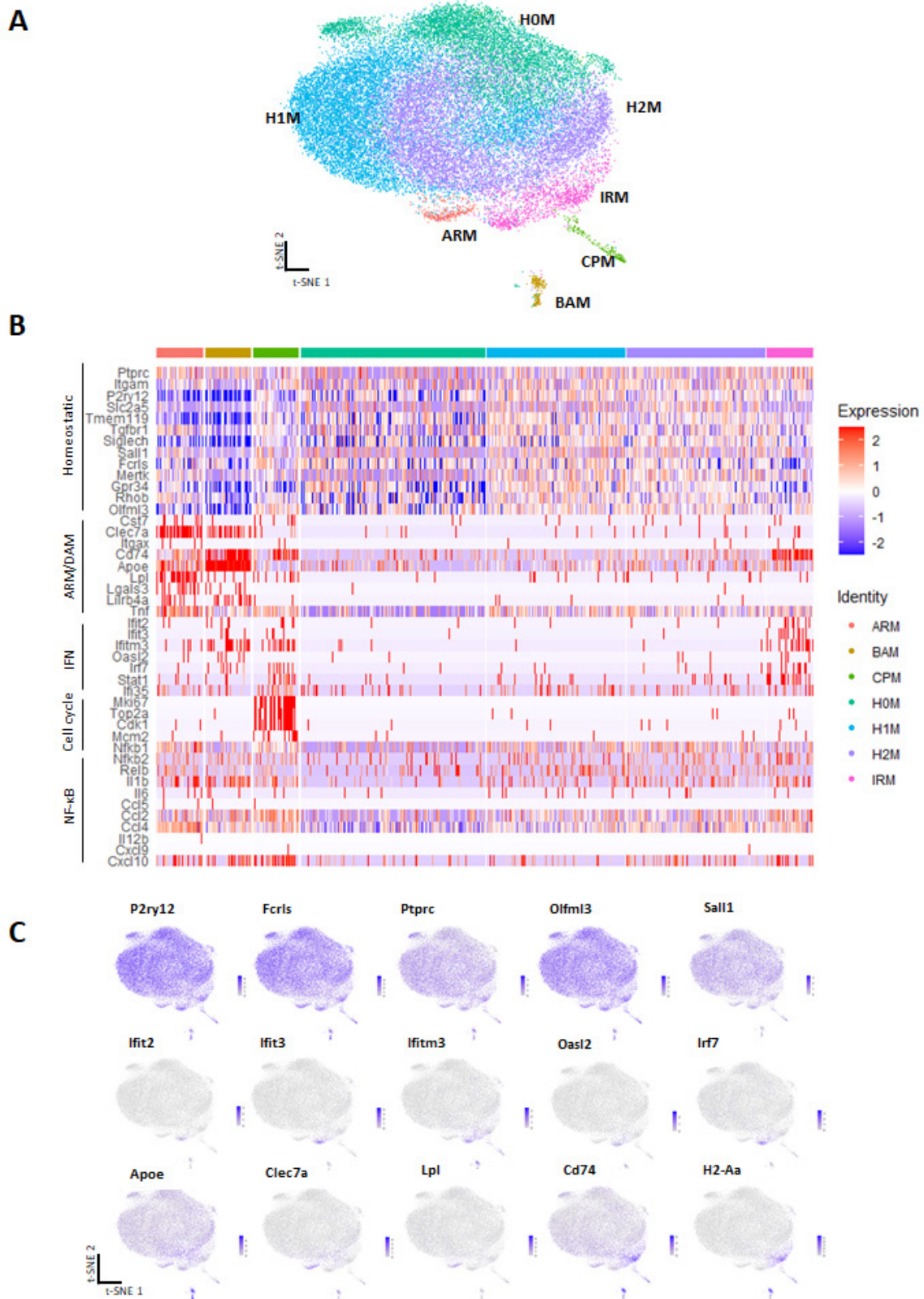


Figure 3. Single cell RNA sequencing reveals distinct subpopulations of activated microglia in conditions of OTULIN deficiency (A) t-distributed stochastic neighbour embedding (t-SNE) plot visualizing 13500 single microglial cells. Cells are coloured according to clusters identified with Seurat's k-nearest neighbours approach (H0M, H1M and H2M, homeostatic microglia; ARM, activated response microglia; IRM, interferon response microglia; CPM, cycling and proliferating microglia). (B) Heatmap showing clustering analysis of single cells, featuring 40 variable genes subdivided by cluster. (C) t-SNE plot as in (A), coloured by the level of normalized expression of selected genes listed in text. The ARM cluster shows increased expression of *Apoe* and inflammation markers (*Cst7*, *Itgax*, *Clec7a*), while the IRM cluster is enriched for interferon genes (*Ifit2*, *Ifit3*, *Ifitm3*, *Oasl2*, and *Irf7*). Both ARM and IRM clusters display a reduction in expression of homeostatic genes.

3.2. Study of OTULIN in MS/EAE pathology

To address the role of microglial OTULIN in the context of CNS pathology, OTULIN^{Cx3Cr1-KO} and control littermate mice were immunized with a myelin antigen (MOG35-55) and multiple sclerosis (MS)-like disease (so called experimental autoimmune encephalomyelitis, EAE) progression was monitored by assessing clinical disease symptoms and body weight (Figure 4A-B). Both OTULIN^{Cx3Cr1-KO} and control littermate mice developed EAE, however, OTULIN^{Cx3Cr1-KO} mice developed earlier disease onset as well as a more severe disease course as compared to control mice (Figure 4A-B), demonstrating a crucial role for microglial OTULIN activity in protecting against EAE. Clinical pathology in OTULIN^{Cx3Cr1-KO} was confirmed by histology on spinal cord sections at day 14 post immunization, the start of clinical manifestations, demonstrating extensive demyelination and immune cell infiltration of both T-cells and macrophages in the spinal cord of OTULIN^{Cx3Cr1-KO}, while nearly no signs of demyelination or infiltration could be detected in the spinal cord of control littermates at that time point (Figure 4C). The expression of TH1-, TH17-, and Treg-linked factors, as well as the expression of inflammatory cytokines and chemokines confirmed the hypersensitivity of OTULIN^{Cx3Cr1-KO} mice to EAE (Figure 4D-E). **Together, these results demonstrate the role of OTULIN in the regulation of inflammatory responses locally in microglia and indicate the importance of this microglial OTULIN control to prevent neuroinflammation.**

We're currently investigating the mechanisms by which OTULIN controls microglia activation to prevent neuroinflammation.

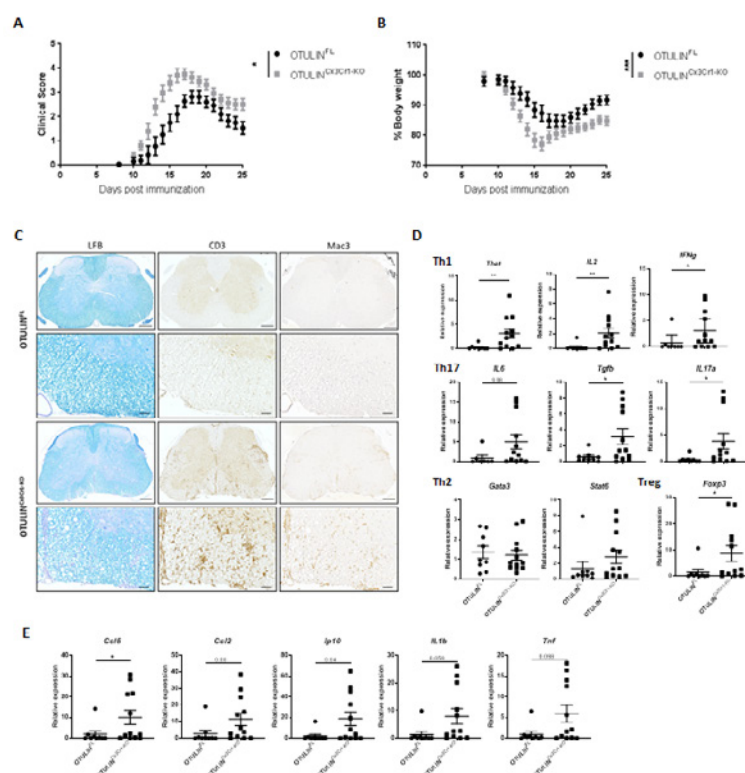


Figure 4. OTULIN deletion in CNS-resident myeloid cells exacerbates EAE pathology. (A-B) EAE was induced by active immunization of control OTULIN^{FL} (n = 14) and OTULIN^{Cx3Cr1-KO} mice (n = 18) with MOG35-55 peptide, and clinical disease development (A) and body weight (B) was followed over time. Each data point represents the mean $\pm$ SEM as estimated by the REML analysis, and changes in clinical score and relative body weight differ significantly (* p < 0.05, *** p < 0.001; F-test) between genotypes across the time span. Data are the combined results of two independent experiments. (C) Representative images of the spinal cord of control (OTULIN^{FL}) and OTULIN^{Cx3Cr1-KO} mice 14 days post immunization using Luxol Fast Blue (LFB) or using CD3 and MAC-3 antibodies. Scale bars represent 200 μ m (overview, upper) and 50 μ m (zoom, lower). Representative images from at least three mice per group are displayed. (D-E) Quantitative measurements of the indicated TH1, TH17, TH2 and Treg marker gene (D) and cytokine and chemokine (E) mRNA expression in spinal cord from OTULIN^{FL} (n=9) and OTULIN^{Cx3Cr1-KO} (n=12) littermate mice 12 d after immunization. Results are displayed as mean $\pm$ SEM and are representative of two independent experiments. *p < 0.05, **p < 0.01.

3.3. Study of OTULIN in AD pathology

A recent genome-wide association study (GWAS) identified *OTULIN* as a new risk locus for Alzheimer disease AD [28]. To experimentally validate the importance of OTULIN for microglia activation and AD pathology, OTULIN^{Cx3Cr1-KO} mice and control littermates have been crossed with three genetic models of AD : the *AppNL-G-F* knock-in mouse model of β -amyloid-induced neuropathology, the *APP/PS1* knock-in mouse model of β -amyloid-induced neuropathology, and the P301S Tau transgenic mouse model. *APP/PS1* mice ectopically overexpress the KM670/671NL 'Swedish' mutation in the amyloid precursor protein (APP) concomitant with mutant human presenilin-1 (PS1) in CNS neurons, and develop an early and robust AD pathology [40]. In contrast, *AppNL-G-F* mice express the human A β sequence which harbors the Swedish (KM670/671NL), Beyreuther/Iberian (I716F) and Arctic (E693G) mutations in the *App* gene [41]. These mice develop typical A β pathology, neuroinflammation, synaptic defects and memory impairment in an age-dependent manner, most similar to the defects which develop in human AD patients [41]. P301S Tau transgenic mice develop filamentous Tau lesions in association with neuroinflammation, prominent microgliosis and neuron loss [42].

Disease development in control (OTULIN-expressing) and microglia OTULIN-deficient (OTULIN^{Cx3Cr1-KO}) AD mice (*AppNL-G-F*, *APP/PS1* or P301S Tau) is being evaluated at different ages of the mice (20 weeks, 40 weeks and 60 weeks of age). At endpoint, brain sections are being evaluated for degree of A β deposition, Tau hyperphosphorylation, astro- and microgliosis, presence of inflammatory markers and neuronal cell death. These studies are still ongoing, but our preliminary results on *APP/PS1* mice demonstrate that OTULIN^{Cx3Cr1-KO} hippocampus shows more microglia and a higher number of A β plaques, suggest that OTULIN deletion in CNS-resident myeloid cells exacerbates AD pathology in *APP/PS1* mice. (Figure 5). Similar results were observed in cortex, demonstrating a higher number of A β plaques in OTULIN^{Cx3Cr1-KO} samples (data not shown).

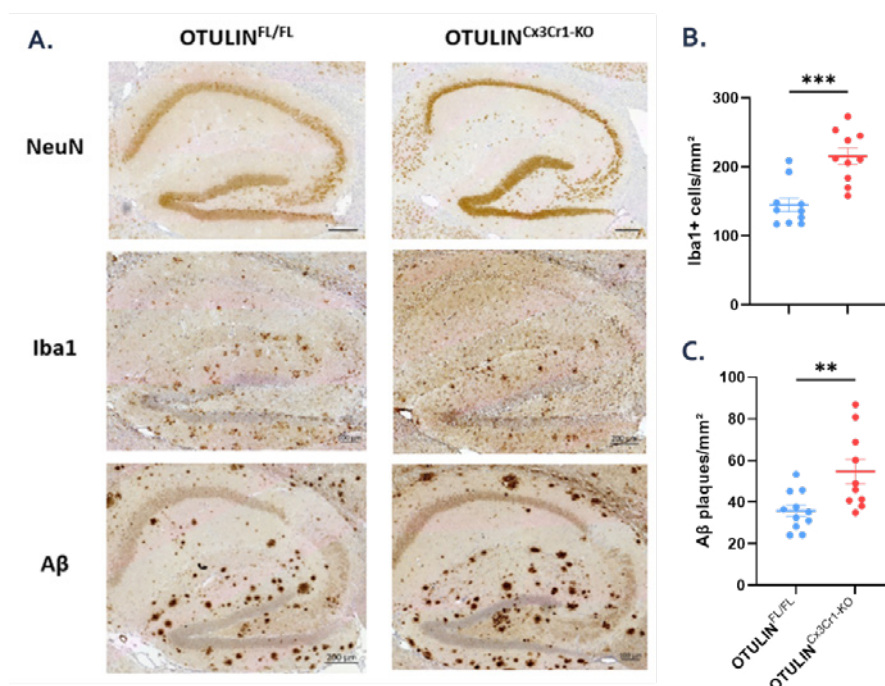


Figure 5. OTULIN deletion in CNS-resident myeloid cells exacerbates AD pathology in *APP/PS1* mice. (A) Immunohistochemistry for NeuN+ neurons, Iba1+ microglia and 6E10+ amyloid plaque load in the hippocampus of 50 week-old OTULIN^{FL} and OTULIN^{Cx3Cr1-KO} *APP/PS1* mice. Representative images are displayed. Scale bar = 200 μ m. (B) Quantification of the number of Iba1+ microglia in hippocampus of 50 week-old OTULIN^{FL} and OTULIN^{Cx3Cr1-KO} *APP/PS1* mice. (C) Quantification of the area covered by 6E10+ amyloid plaque deposits in hippocampus of 50 week-old OTULIN^{FL} and OTULIN^{Cx3Cr1-KO} *APP/PS1* mice. Each symbol represents one mouse. Data are represented as mean $\pm$ SEM.

These observations were confirmed in *AppNL-G-F* mice showing more microglia and a higher number of A β plaques in OTULIN^{Cx3Cr1-KO} hippocampus (Figure 6) and cortex (data not shown), again suggesting that OTULIN in CNS-resident myeloid cells suppresses AD pathology in this model of AD.

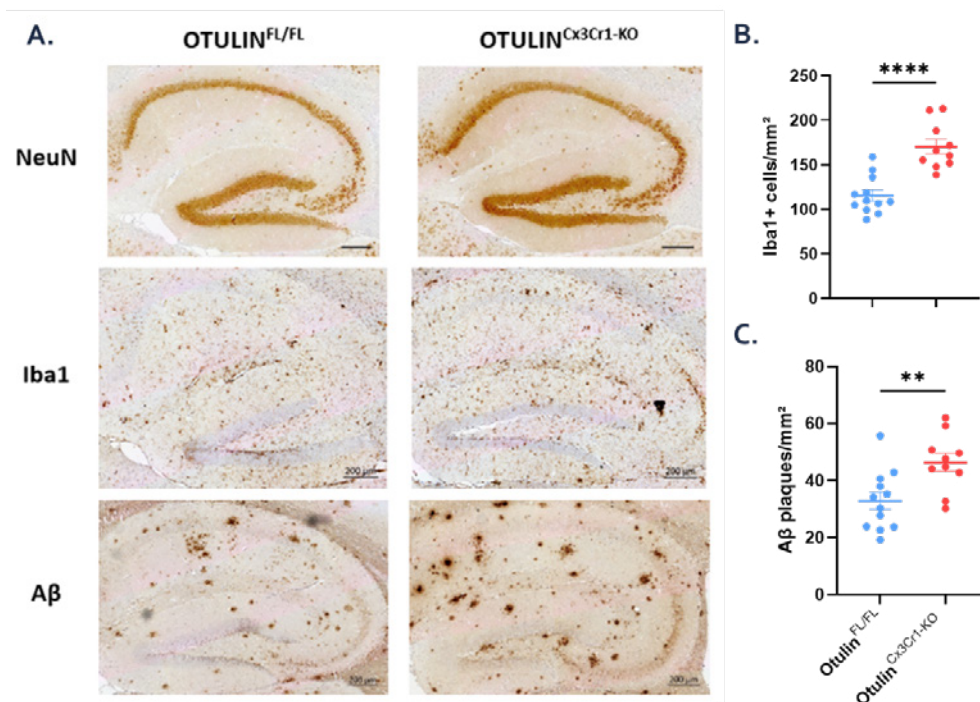


Figure 6. OTULIN deletion in CNS-resident myeloid cells exacerbates AD pathology in *AppNL-G-F* mice. (A) Immunohistochemistry for NeuN+ neurons, Iba1+microglia and 6E10+ amyloid plaque load in the hippocampus of 30 week-old OTULIN^{FL} and OTULIN^{Cx3Cr1-KO} *AppNL-G-F* mice. Representative images are displayed. Scale bar = 200 μ m. (B) Quantification of the number of Iba1+ microglia in hippocampus of 30 week-old OTULIN^{FL} and OTULIN^{Cx3Cr1-KO} *AppNL-G-F* mice. (C) Quantification of the area covered by 6E10+ amyloid plaque deposits in hippocampus of 30 week-old OTULIN^{FL} and OTULIN^{Cx3Cr1-KO} *AppNL-G-F* mice. Each symbol represents one mouse. Data are represented as mean $\pm$ SEM.

Similar analysis is ongoing for the P301S Tau transgenic mouse model of AD.

Finally, and in parallel with the studies described above, we have performed scRNA-seq analysis of immune cells obtained from control and OTULIN^{Cx3Cr1-KO} *APP/PS1* and P301S Tau transgenic brains at 50 and 18 weeks of age, respectively. We FACS-sorted CD45⁺ immune cells from the brains of individual *APP/PS1* : OTULIN^{FL} (n=3), *APP/PS1* : OTULIN^{Cx3Cr1-KO} (n=3), P301S Tau OTULIN^{FL} (n=3) and P301S Tau : OTULIN^{Cx3Cr1-KO} (n=3) mice. To enable efficient sample multiplexing to investigate the brain immune compartment of individual mice, we successfully implemented the 10x genomics FLEX assay. We captured > 100K cells from a total of 12 mice, that were all processed individually. The advantage of the 10x genomics FLEX approach is that we now have an n=3 for each genotype, allowing us to draw more robust conclusions with regard to immune cell dynamics for the various genotypes. The data underwent unsupervised analysis, identifying various cell types and cell states (Figure 7A). While this extensive dataset is still being analyzed, the first preliminary assessments on *APP/PS1* mice show a remarkable increased infiltration of peripheral immune cells in OTULIN^{Cx3Cr1-KO} during AD pathology, including monocyte-derived macrophages that are normally not or hardly observed (Figure 7 B,C). These results highlight how OTULIN deficiency has a significant effect on immune dynamics during progression of AD-related pathology in the *APP/PS1* mouse model of disease. This will be further assessed in-depth using our established analysis pipeline.

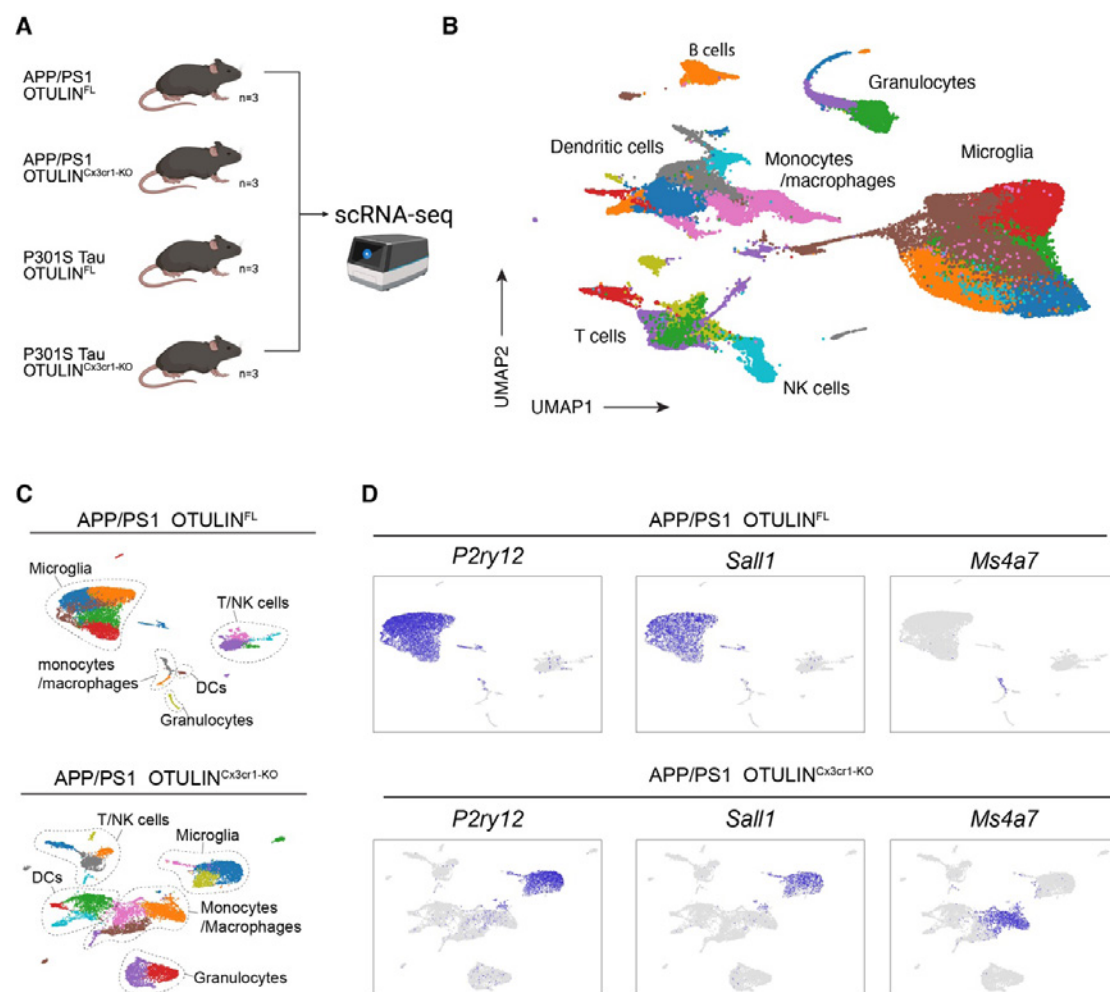


Figure 7. scRNA-seq analysis of the brain immune compartment during AD pathology in WT and OTULIN deficient mice. (A) Experimental layout. A total of n=12 individual mice were analyzed using the 10x Genomics FLEX assay. **(B)** All datasets were merged followed by unsupervised analysis and UMAP projection. The indicated cell populations were identified based on previously established gene expression signatures. **(C)** Representative examples of the brain immune compartment of APP/PS1 OTULIN^{FL} (upper panel) and APP/PS1 OTULIN^{Cx3cr1-KO} (bottom panel) mice. OTULIN deficiency resulted in strongly increased percentages of peripheral immune cells, including granulocytes, monocytes, monocyte-derived macrophages and DCs. **(D)** UMAP plots showing the expression of the indicated genes. *P2ry12* and *Sall1* are microglial signature genes, while *Ms4a7* is expressed monocyte-derived brain macrophages.

The scRNA-seq data will be further complemented with single-cell spatial profiling using the Akoya PhenoCycler-Fusion platform. We have now optimized this platform for multiplexed protein profiling of brain tissues and we developed a powerful analysis pipeline. In short, the PhenoCycler-Fusion system is an automated fluidics control unit coupled to a microscope that allows automatic successive rounds of staining on the same tissue slide. To do so, Akoya uses barcoded antibodies and complementary oligonucleotides labeled with fluorophores. In each cycle, up to three oligonucleotides specifically interacts with their corresponding tissue-bound barcoded antibodies, thus allowing imaging of the corresponding antibodies. Afterwards, the imaged reporters are washed away and new reporters are added in a new cycle, altogether resulting in the visualization of up to 100 markers in the same tissue slide. The Movahedi lab successfully custom-conjugated several markers relevant for studying neurodegenerative disease and DAMs: GFAP (astrocytes), Iba1 (microglia), Clec7a (DAMs), Lyve-1 (border associated macrophages), ERTR7 (dural fibroblasts), and Nestin (neuronal stem cells). Together with commercially available antibodies, we compiled a 30-marker panel that allows in-depth characterization of different immune populations in the mouse brain, which are being used for disease profiling in APP/PS1 and P301S mice. Analysis is still ongoing.

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5. Publications van Loo-Movahedi groups 2024 (acknowledging GSKE support)

- Srinivasan, S*, Kancheva, D*, De Ren, S*, Saito, T*, Jans, M., Boone, F., Vandendriessche, C., Paesmans, I., Maurin, H., Vandenbroucke, R.E., Hoste, E., Voet, S., Scheyltjens, I., Pavie, B., Lippens, S., Schwabenland, M., Prinz, M., Saido, T., Bottelbergs, A+, Movahedi, K+, Lamkanfi, M.+ and van Loo, G+. Inflammasome signaling is dispensable for β-amyloid-induced neuropathology in preclinical models of Alzheimer's disease. *Frontiers Immunol.* 15:1323409.
*shared first author
+shared last author
- Paesmans, I., Van Kolen, K., Vandermeeren, M., Shih, P-Y., Wuyts, D., Boone, F., Garcia Sanchez, S., Grauven, K., Van Hauwermeiren, F., Van Opdenbosch, N., Lamkanfi, M., van Loo, G.+ and Bottelbergs, B.+ (2024) NLRP3 Inflammasome Activation and Pyroptosis Are Dispensable for Tau Pathology. *Front Aging Neurosci.* 16:1459134
+shared last author



Geneeskundige Stichting Koningin Elisabeth
Fondation Médicale Reine Elisabeth
Königin-Elisabeth-Stiftung für Medizin
Queen Elisabeth Medical Foundation

Final report of the interuniversity research project of

Prof. dr. Renzo Manusco (UAntwerpen)
Prof. dr. Joris De Wit (KU Leuven)

Dr. Renzo Mancuso (he/him/his) (UAntwerpen)

Group Leader

Laboratory of Microglia and Inflammation in Neurological Disorders (MIND)

VIB-Center for Molecular Neurology (CMN), VIB.

Universiteit Antwerpen-CDE, Parking P4, Gebouw V 0.10

Universiteitsplein 1, B-2610 Antwerpen

T +32 (0) 3 265 25 28

Prof. Joris De Wit (KU Leuven)

Group Leader at VIB-KU Leuven

Center for Brain & Disease Research

Department of Neurosciences

ON5 Herestraat 49 – bus 602

3000 Leuven

Microglia-synapse molecular interactions in neurodegenerative disease (MicroSyn-MIND)

The goal of this project is to identify proteins that play a role in microglia-synapse interactions, and specifically those proteins whose expression is affected in Alzheimer's Disease (AD).

We are completing aim 1 and have started writing the manuscript, and are progressing with aim #2. We provide a detailed update on our findings and progress below.

First, a short summary of our projects' original aim and strategy. It is unclear what drives AD progression. Genetic risk¹, Amyloid- β (A β) plaques, neurofibrillary tau tangles, and vascular alterations lead to a loss of neurons and synapses²⁻⁴, followed by the patient's memory, cognition, and grasp on reality. However, A β builds up years before cognitive symptoms set-in⁵, and is not always followed by neurodegeneration³. Microglia prune synapses during development and could be key players in mediating synaptic loss in AD⁶. However, how microglia interact with neurons and synapses, and how AD affects these interactions, is poorly understood. Here, we combine the expertise of two labs to transplant human microglia in a mouse model of AD, perform a deep characterization of human microglia-synapse interactions at the molecular level using split-TurboID proximity labeling, and determine how these interactions are impacted by AD pathology. To address this overall goal, we formulated two main aims:

1. Identify AD-related changes to the microglial-synaptic cell surface interactome.

Following our update of January 2025, we are finishing up aim 1. The strategic adjustments we made involving the generation of "stable" iPSC lines, allowed us to generate the needed Mass Spectrometry (LC-MS/MS) data of *in vitro* differentiated iPSC-derived human microglia, both in a control state as well as following stimulation with soluble A β fibrils. We have processed a large number of biotinylated protein samples, and analyzed the generated (LC-MS/MS) data. During analysis, we normalized the data using quantile normalization, and combined the data of various different TurboID constructs (Membrane, cytoplasm, ER), to exclude potential noise. After filtering out potential background proteins, we proceeded to compare the proteins detected in the MARCKS-TurboID construct (used to identify proteins located at the inner-leaflet of the cellular outer membrane) with those identified in the CYTO-TurboID construct (used to identify proteins located throughout the cytoplasm). This allows us to estimate which proteins are enriched at the microglial outer membrane; including proteins that are expressed in- or on the outside of- the cellular membrane, and that have transmembrane tails/elements that receive a biotin tag. Specifically, this allowed us to identify over 2859 proteins that are enriched at the cellular outer membrane, including 1310 that are uniquely identified in the membrane TurboID construct (and not at all in the cytoplasmic TurboID construct). These enriched proteins include previously identified microglial membrane proteins (ITGAV, CD44/88, P2RY2, TMEM268, TYROBP), confirming we have extracted and identified the human microglial membrane proteome.

In order to link the entire protein set to specific functions, we perform a Gene Ontology analysis (GO). This analysis uses an online database (GO) that links specific genes to their previously identified cellular compartment, biological function, and molecular pathway. These include key

membrane biological functions ("cell-cell adhesion", "monoatomic ion transport", etc.), cellular compartments ("cell surface", "external side of the plasma membrane", etc.), and molecular functions ("transporter activity", "signaling receptor activity", "transmembrane signaling", etc.). These findings further confirmed our confidence in this human microglial membrane proteome dataset. As we stated before, this will serve as a valuable resource to the field in general.

In addition to the proteomes map, we have stimulated *in vitro* iPSC-derived human microglia with soluble Amyloid-beta fibrils, to determine whether this induces specific compartmental protein changes in the human microglia. We compared the A β -stimulated MARCKS-TurboID samples with unstimulated MARCKS-TurboID samples, to identify specifically those proteins affected by the A β . This allowed us to identify a large number of proteins that are enriched in the different cellular compartments in response to A β , including 265 unique proteins that are enriched in the MARCKS-TurboID group following A β stimulation. These include proteins that play a role in cell/cell-interaction (ADAM12/28), secreted proteins (IL34, SPP1) and known AD-risk genes (APOE, APP). In order to link all of these proteins to specific functions, we performed another Gene Ontology analysis (GO). The GO analysis identified several interesting processes; some of which could be linked to a clear response to a pro-inflammatory stimulus such as "pro-inflammatory response", "interferon-induced response", as well as "secreted factors", and "membrane- and signal-reception remodeling". As stated, these could be interpreted as a specific protein changes directed at dealing with the A β stimulus. Interestingly, a large number of proteins were involved in chromatin- and transcriptional remodeling, RNA processing and -splicing. Which role these proteins play in the exact response to A β is not yet clear. Further analysis is needed to identify the exact mechanism by which the microglial membrane responds to the A β fibrils. This could provide novel treatment targets for future drug development.

A scientific manuscript is being prepared at the moment for publication in Q1 2026, which will address specifically the *in vitro* response of human iPSC-derived microglia to A β .

In parallel, we have transplanted these "stable" iPSC-derived microglia that express these various constructs, *in vivo* in immunosuppressed WT mice as well as AD model mice. When these transplanted mice matured to an age where sufficient cortical A β build-up occurred (6 months of age), we activated the TurboID through biotin injections, and extracted protein for LC-MS/MS analysis. We are awaiting the LC-MS/MS data, once these data are in, we can proceed with the analysis as intended, to identify *in vivo* cell-type specific cell surface proteins, and infer potential ligand-receptor pairs of interest. From the differential expression of these proteins, comparing microglia from healthy WT mice compared to our AD model mice, we should be able to infer potential targets to study in aim 2.

2. Identify microglial cell surface proteins that mediate excessive synaptic loss in AD.

The original strategy for aim 2 still stands. As we discussed in our previous updates, the strategic adjustments have worked, and we are either finishing up data analysis (*in vitro* data) or are awaiting the LC-MS/MS data (*in vivo* data). Subsequently, we still aim to validate a sub-section of these first through an ELISA-based interaction assay, whereby we test whether these microglial proteins can interact with a broad spectrum of synaptic proteins, in collaboration with prof. Davide Comoletti (University of New Zealand). Once we have reduced the number of potential targets, we will directly knock these out using CRISPR/Cas9, in iPSCs. With these lines, we will generate iPSC-derived microglia, which we will use in an *in vitro* synaptosome phagocytosis assay, to test

the effects of the knockouts on the microglial capacity to phagocytose synapses. Following this, we will transplant one or several of these lines *in vivo*, to validate whether they affect synapse phagocytosis. We remain confident that we will provide an answer to our original question, of which key receptors-ligands affect the communication between microglia and synapses.

We hope this final report provides sufficient information, and would love to answer any additional questions you might have regarding the progress of our project. Even though our original timeline required adjustment to account for strategic shifts, we aim to have two scientific manuscripts based on this study; including one that will be submitted in early 2026.

3. Publications 2023-2025 with Q.E.M.F. acknowledgement

- Verpoort B, Amado L, Vandensteen J, Leysen E, Dascenco D, Vandenbempt J, Lemmens I, Wauman J, Vennekens K, Escamilla-Ayala A, Freitas A, Voets T, Munck S, Tavernier J, Wierda K, **de Wit J** (2025). A postsynaptic GPR158-PLCXD2 complex controls spine apparatus abundance and dendritic spine maturation. **Developmental Cell** 16:S1534-5807(25)00263-1. 10.1016/j.devcel.2025.04.025
IF: 8.7; Q factor: Q1 in Cell Biology

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6. Werneburg, S. *et al.* Targeted Complement Inhibition at Synapses Prevents Microglial Synaptic Engulfment and Synapse Loss in Demyelinating Disease. *Immunity* 52, 167-182.e7 (2020).
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9. Pluvinaige, J. V. *et al.* CD22 blockade restores homeostatic microglial phagocytosis in ageing brains. *Nature* 568, 187–192 (2019).



Geneeskundige Stichting Koningin Elisabeth
Fondation Médicale Reine Elisabeth
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Final report of the interuniversity research project of

Prof. dr. Karelle Leroy (ULB)
Prof. dr. Laurence Ris (UMONS)
Prof. dr. Kristel Slegers (UAntwerpen)

Prof. dr. Karelle Leroy (ULB)

Assistant professor, ULB

Head of the Alzheimer

research group in the

laboratory of Histology

<https://alzheimer-research.medecine.ulb.be>

Prof. dr. Laurence Ris (UMONS)

Department of

Neurosciences

Prof. dr. Kristel Sleegers (UAntwerpen)

Dr Sleegers Kristel

Professor, UA

Head of the Complex Genetics of

Alzheimer's disease group at

VIB – UA Center for Molecular

Neurology

<https://uantwerpen.vib.be/group/kristelSleegers>

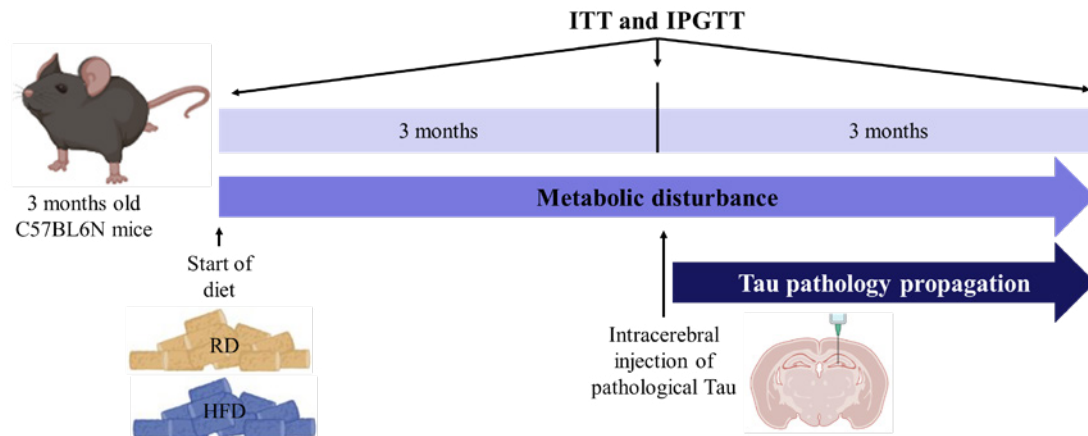
Involvement of diabetes and antidiabetic treatment on tau pathology propagation

1. Introduction:

The etiopathogenesis of Alzheimer's disease (AD) is relatively unknown as only 5 % of AD cases can be explained by familial mutations. However, several risk factors have been described in AD. Among them, epidemiological studies showed a clear link between AD and type 2 diabetes mellitus (T2D), a medical condition frequent in industrial but also in developing countries. Indeed, T2D increases the risk of AD by at least twice. Moreover, a study has shown that T2D in elderly population with mild cognitive impairment influences the progression to dementia. The features of T2D are high levels of blood glucose (hyperglycemia) and insulin resistance. Most insulin is produced by beta-pancreatic cells and is transported to the brain by crossing the blood brain barrier. Insulin binds to the insulin receptors that are present in neurons triggering the brain insulin signaling pathway by activating AKT resulting in the inhibition of GSK3beta. Interestingly, a recent study has shown that beta-pancreatic cells and neurons shared common mechanisms such as the ability to express tau proteins or shared causal genes or pathways. Under conditions of insulin resistance, GSK3beta is converted to its active form by the decrease of the inhibitory serine 9 phosphorylation therefore conducting to the degradation of IRS1 (Insulin receptor 1) leading to insulin pathway disturbances. As the activity of GSK3beta is increased in AD and is involved in tau phosphorylation in this disease, this kinase could be the possible link between these two pathologies. Indeed, an abnormal tau phosphorylation appears in beta-pancreatic cells of T2D patients and in the brain of T2D mouse models (High fat diet model) in which the brain insulin signaling pathway is impaired. The possibility that targeting AD and T2D through antidiabetic agents may constitute an approach to treat defective brain insulin signaling, cognitive impairment and neurodegeneration.

2. Aim of the study:

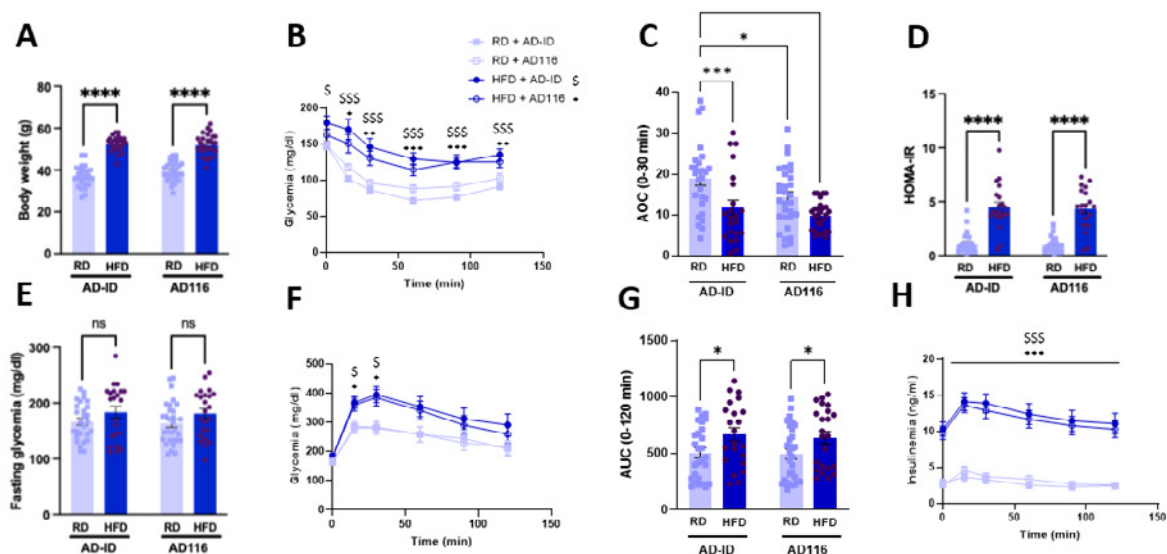
This study is be devoted to the analysis of the role played by **metabolic disturbances observed in type 2 diabetes mellitus (T2D)** but also the **effect of an anti-diabetic treatment** on tau seeding and on the **propagation of tau pathology in Alzheimer's models** mice fed with high fat diet (HFD) to mimic T2D. These experiments will be conducted in mice models in which tau seeding will be induced by intracerebral injection of PHF-tau proteins from AD brain.



3. Results:

3.1. Analysis of metabolic disturbances after the intracerebral injection of pathological Tau.

3 months after the intracerebral injection of AD sarkosyl fraction containing pathological tau or ADID sarkosyl fraction, we analyzed the metabolic parameters in RD and HFD fed mice. HFD fed mice showed insulin resistance and hyperinsulinemia but, surprisingly, mice fed with RD and injected with AD sarkosyl fraction developed insulin resistance.

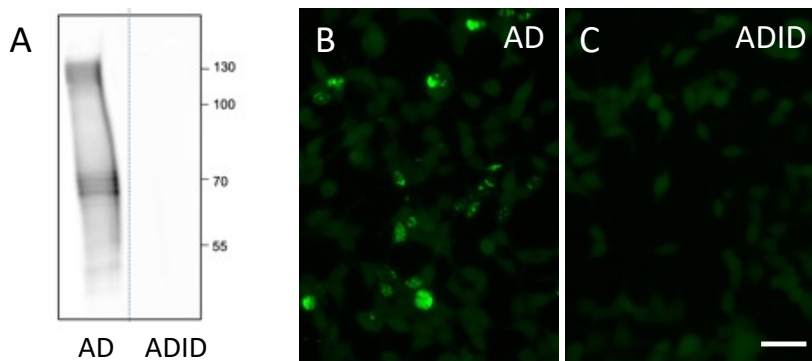


High-fat diet (HFD) induces metabolic disorders that mimic type 2 pre-diabetes by inducing insulin resistance, hyperglycemia, glucose intolerance and hyperinsulinemia. Tau pathology induces insulin resistance in C57BL6N mice fed with a normal diet.

3-month-old C57BL6N mice were fed with a normal diet (RD) or a high-fat diet (HFD) for 3 months and then randomized to receive stereotaxic injection of AD or AD-ID sarkosyl fractions. 3 months post-injection metabolic parameters were measured in these mice (RD AD-ID n= 27; RD AD n= 30; HFD AD-ID n= 22; HFD AD n= 16). The following assessments were made at the end of the study: (A) body weight; (B) blood glucose during the insulin tolerance test (ITT). Insulin sensitivity was quantified by the area on the curve (AOC) during (C) the insulin tolerance test and (D) the homeostatic model assessment of insulin resistance (HOMA-IR). (E) Fasting blood glucose (after a 6-hour fast). (F) Blood glucose, (G) AUC for glucose, and (H) insulin levels during intraperitoneal glucose tolerance test (IPGTT). Data points correspond to individual mice, mean is shown in column, and SEM is represented by bar. Two Way ANOVA Tukey's multiple comparisons test * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ RD + AD-ID vs HFD + AD-ID and § $p < 0.05$; §§ $p < 0.01$; §§§ $p < 0.001$ RD + AD-ID vs HFD + AD.

3.2. Characterization of sarkosyl fractions.

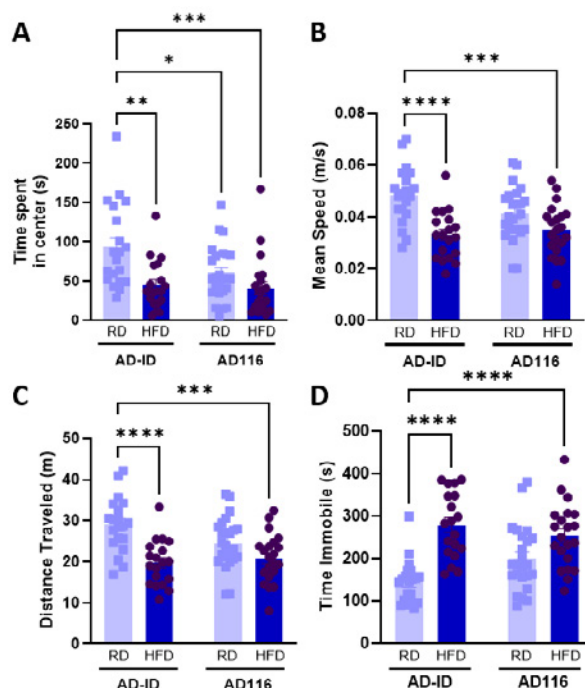
The seeding ability of AD sarkosyl fractions have been evaluated in a bioassay based on the formation of a FRET signal when tau is seeded. We observed a FRET signal when cells were treated with AD sarkosyl fraction but not with ADID sarkosyl fraction (AD sarkosyl fraction immunodepleted for tau). These results confirmed that AD sarkosyl fraction can be used in vivo to induce tau pathology propagation and ADID sarkosyl fraction can be used as a control.



A: Western blotting of sarkosyl fractions with anti-tau antibody. B-C: Cells treated with AD sarkosyl fractions showed a FRET signal (B) but not when they are treated with immunodepleted fractions.

3.3. Analysis of anxiety and exploratory behavior after the intracerebral injection of pathological Tau.

To assess the anxiety-like behavior, the exploration, and the locomotion of the mice, an Open Field test was used after 5 months of diet and 2 months after the stereotaxic injection. The mice were placed into the middle part of a box, and the recording started immediately after placement and lasted for 10 min. Time spent in the periphery, mean speed, distance and immobility time were measured. Regarding graph A, we can tell that HFD mice and pathological tau induce a change the anxiety behavior of our mice. Indeed, those mice spent more time in the periphery of the Open Field. But interestingly, we can see that HFD mice injected with AD-ID and AD have exploration and locomotor problems (B-D).



High-fat diet (HFD) mice have an abnormal exploratory behavior.

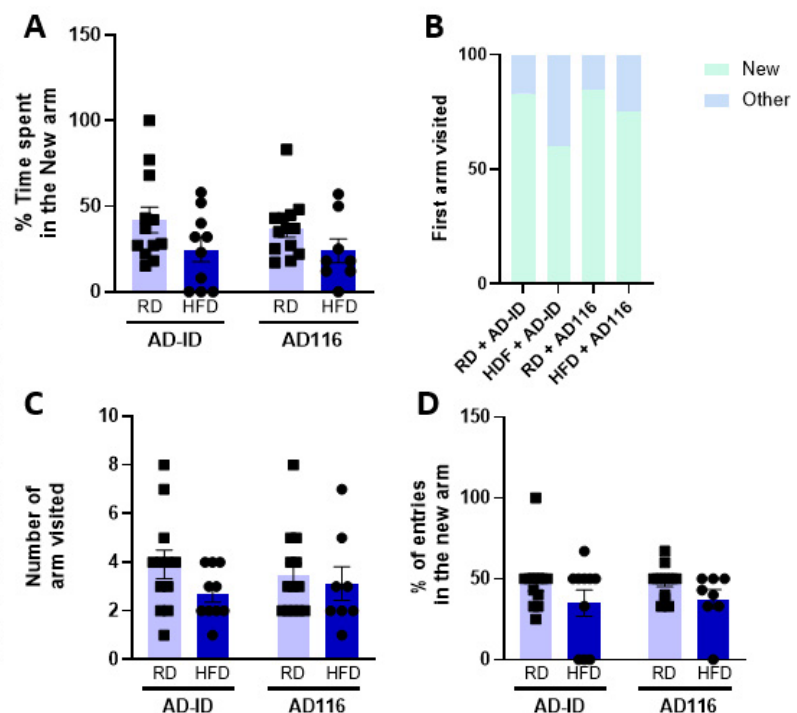
3-month-old C57BL/6N mice were fed with a normal diet (RD) or a high-fat diet (HFD) for 3 months and then randomized to receive stereotaxic injection of AD or AD-ID sarkosyl fractions and dissected 3 months post-injection (RD AD-ID n= 27; RD AD n= 30; HFD AD-ID n= 22; HFD AD n= 16). The exploratory behavior was assessed in these mice 1 month before the dissection. The mice were placed during 10 minutes in an Open Field, and the following assessments were measured: (A) the percentage of time spent in the periphery; (B) the mean speed; (C) the total distance travelled by the mice and (D) the time that the mice spent immobile. Data points correspond to individual mice, mean is shown in column, and SEM is represented by bar. Two Way ANOVA Sidak's multiple comparisons test * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ by.

3.4. Analysis of short-term spatial memory after the intracerebral injection of pathological Tau.

Short-term spatial memory was tested by a Y-maze behavioral test. 5 months after the start of diet and 2 months after the stereotaxic injection, the mice were placed in the start arm of the Y-maze and were free to explore for 10 minutes the start arm and the other arm (the new arm being blocked by a door). After this phase of habituation, the mice returned to their home cage for one hour. Then the mice were put back in the start arm and the following parameters were measured during the first minute after the mice exited the start arm: time spent in the new arm, first arm visited, number of arms visited and percentage of entries in the new arm. HFD mice tend to spend less time in the new arm and to enter less in this arm (A and C). The time spent in the new arm is not significantly different between the groups.

HFD mice seems to lead to an abnormal short-term memory independently of the injections.

6 months after the beginning of the diet and 2 months after the stereotaxic injection, the mice ($n = 8-13$ per group) were placed during 10 minutes in the start arm of which Y-maze in which the new arm was blocked by a door. Then the mice were back in their home cage for 1 hour before being replaced in the start arm of the Y-maze with the new arm now accessible. The following assessments were measured during the first minute after the exit of the mice from the start arm: (A) the percentage of time spent in the new arm; (B) the first arm visited; (C) the number of arms visited and (D) the percentage of entries in the new arm. Data points correspond to individual mice, mean is shown in column, and SEM is represented by bar. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ by Two Way ANOVA Sidak's multiple comparisons test.



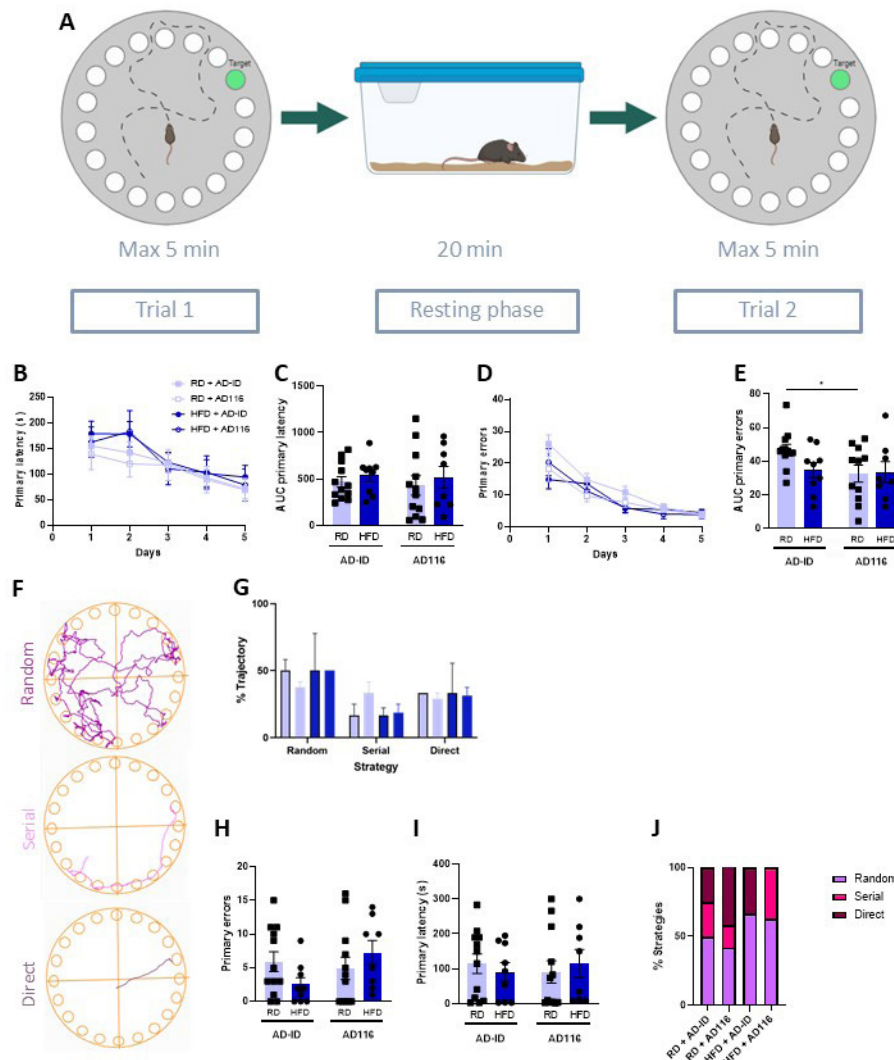
3.5. Analysis of the learning after the intracerebral injection of pathological Tau.

Barnes maze test is used to study hippocampus-dependent spatial learning. Briefly, the Barnes maze is a circular platform (1 m diameter) with 20 holes equally spaced and located close to the edge of the platform. A black escape box is placed under one of the holes (target) and spatial cues are located at the 4-cardinal points above the platform. The mice were placed in the middle of the maze and allowed to explore. The session ended when the mice found the box or after 5 min had elapsed. The mice performed 2 trials per day with a 20 min inter-trial interval for 5 consecutive days. The mice were tested 2 months after injection of AD-ID or AD fraction ($n=10$). To evaluate the spatial learning of the mice, several parameters were analyzed for each trial. These include the time spent trying to find the correct hole (latency), the number of incorrect holes checked prior to find the target (number of errors) and the search strategy used by the mice to find the escape box. The search strategies are defined as direct, serial or random (F). When the mice move directly to the target hole or they make 2 errors that are adjacent to the target, the strategy is direct. Trials in which the mice investigate 3 or more adjacent holes in a sequential order that start at a minimum of 3 holes from the target and follow a clockwise or anticlockwise direction are serial searches. All other trials are counted as random strategies, including those in which the mice failed to find the target.

Our results showed that HFD or AD do not have any effect on the latency of the mice to find the target, on the number of errors or on the strategies used during the 5 days of learning. Moreover, learning performance was measured by calculating the area under the learning curve (AUC) for latency and errors. RD-AD had a reduced AUC compared to RD-ADID but this result is probably due to the bigger number of errors made by RD-ADID mice the first day of the learning phase. We have observed no significant differences between HFD and RD conditions or between RD-AD and HFD-AD.

3.6. Analysis of long-term spatial memory after the intracerebral injection of pathological Tau.

3 days after the last training trial on the Barnes maze, the mice were put again on the Barnes maze to assess the long-term memory. The same parameters were analyzed during the learning phase. We do not see any differences between the different conditions meaning that HFD or tau pathology do not lead to long-term memory deficits.

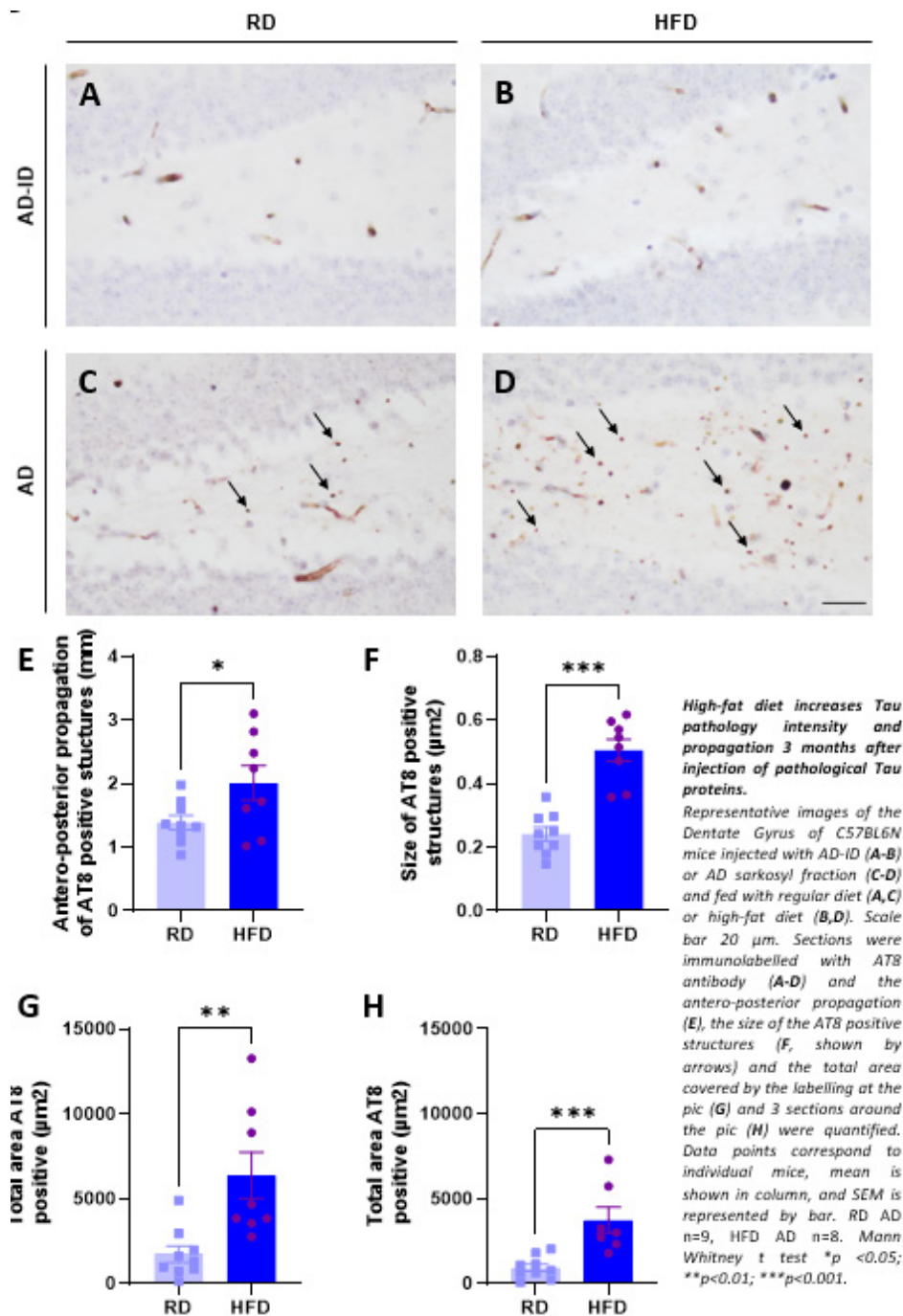


No difference between the treatments in the learning or in the long-term spatial memory.

Learning and long-term memory parameters were measured at the end of the study. For this the mice were placed twice a day on a Barnes Maze during 5 consecutive days (A), this represent the learning phase. During the tests, different parameters were measured: (B) primary latency; (C) AUC of the primary latency; (D) primary errors; (E) AUC of the primary error and (F-G) strategies. On day 8, the mice were replaced on the Barnes to assess the long-term memory by measuring (H) the primary latency; (I) primary error and (J) the kind of strategy used. Data points correspond to individual mice, mean is shown in column, and SEM is represented by bar. Two Way ANOVA Sidak's or Tukey's multiple comparisons test * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

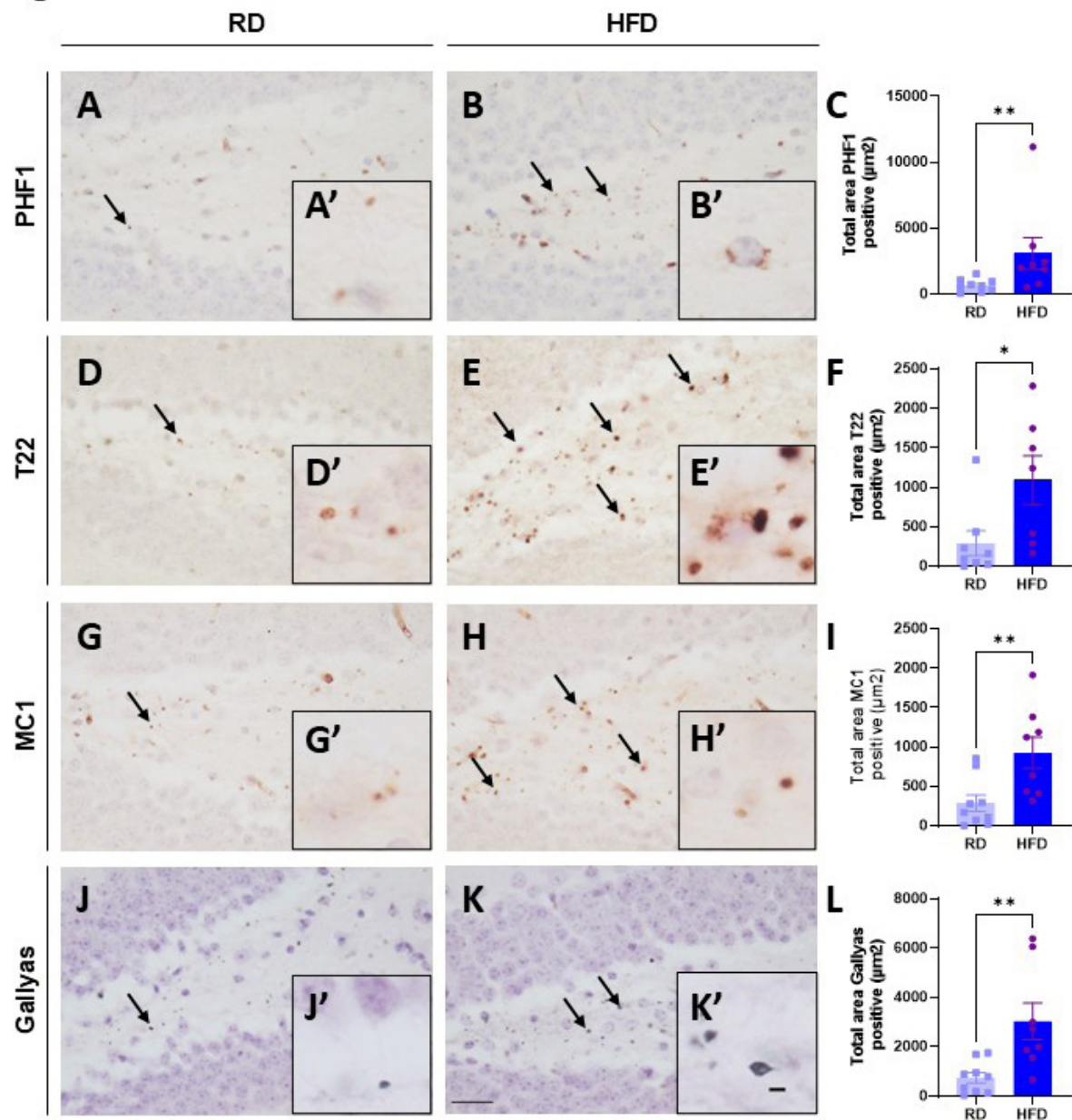
3.7. Analysis of Tau pathology propagation.

As expected, we didn't observe tau pathology in mice intracerebrally injected with ADID sarkosyl fractions and fed with RD or HFD. In AD injected mice, HFD induces a significant increased tau pathology detected with AT8 antibody recognizing phosphorylated tau on Ser 202/Thr 205. Moreover, the tau propagation evaluated by the presence of tau pathology in the anteroposterior axis of the brain is significantly increased in HFD fed mice compared to RD fed mice.



The results are similar with PHF1 antibody which recognizes tau proteins phosphorylated on Ser 396/404 to those observed with AT8 antibody: increase of tau pathology propagation. We also performed immunolabellings with T22 antibody which recognizes oligomers of tau proteins, with MC1 antibody which recognizes tau proteins in an abnormal conformation. A Gallyas staining was also performed to detect tau aggregates. In our injected model, we observe a significant increase of tau pathology maturation with T22, MC1 and Gallyas.

Figure 3:

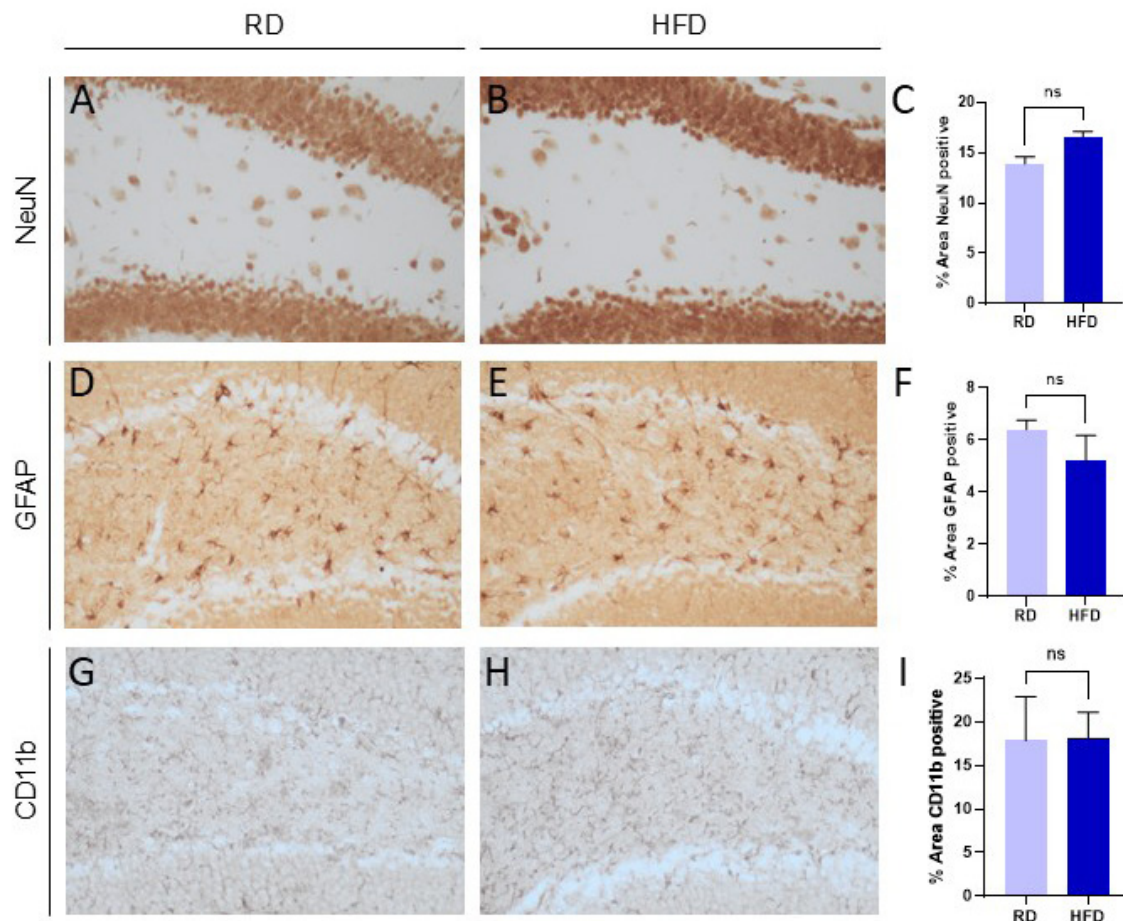


High-fat diet increases Tau pathology maturation 3 months after injection of pathological Tau proteins.

Representative pictures of immunolabeling with PHF1 (A-B), T22 (D-E), MC1 (G-H) antibodies or staining with Gallyas silver staining method (J-K). Quantifications of the total area covered by the labelling are shown in C, F, I and L. Data points correspond to individual mice, mean is shown in column, and SEM is represented by bar. RD AD n=9, HFD AD n=8. Mann Whitney t test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Scale bar 20 μm in K and 2 μm in K'

3.8. Analysis of the neuroinflammation

Because inflammation is a common risk factor for AD and T2D, we wanted to analyze the neuroinflammation and the neuronal loss after 3 months of diet and before the stereotaxic injection. We did not observe significant astrocytic (GFAP) or microglial (CD11b) activation or neuronal (NeuN) loss after 3 months of diet.



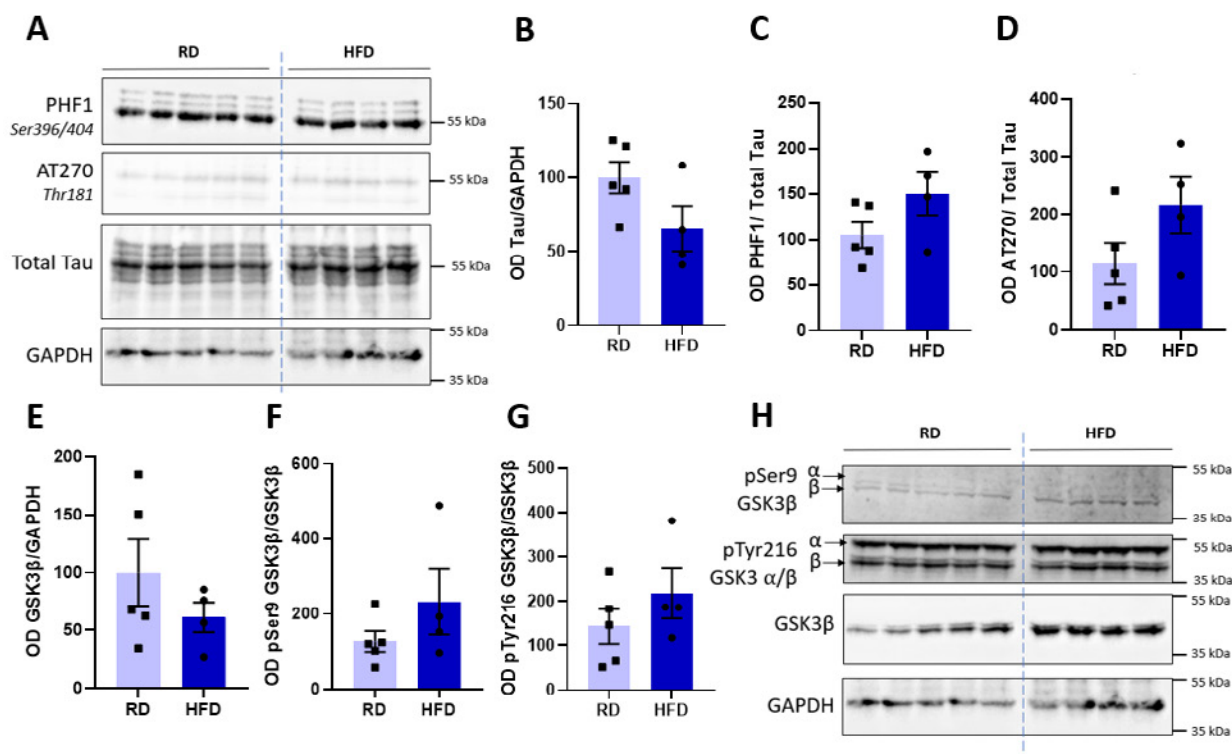
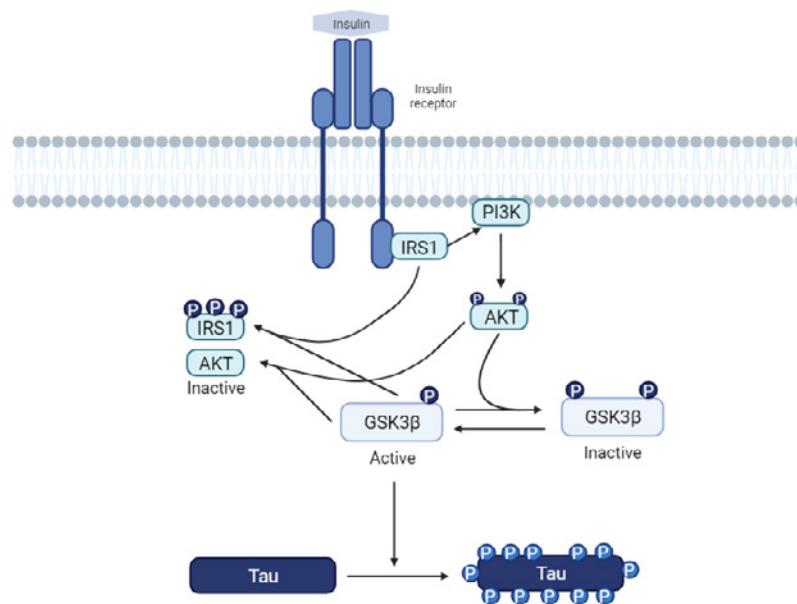
High-fat diet (HFD) do not lead to neuronal loss or astrocyte and microglial activation.

3-month-old C57BL6N mice were fed a normal diet (RD) or a high-fat diet (HFD) for 3 months ($n = 4-5$ per group). Sections were immunolabelled with NeuN (A-B), GFAP (D-E) or CD11b antibodies (G-H). Quantifications of the total area covered by the labelling are shown in C, F and I. Mean is shown in column, and SEM is represented by bar. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ by Mann Whitney t test.

3.9. Analysis of the insulin pathway in the brain.

As explained in the introduction, GSK3 β could be the possible link between diabetes and Alzheimer's disease. In physiological conditions, insulin binds to the insulin receptors that are present in neurons triggering the brain insulin signaling pathway by activating AKT resulting in the inhibition of GSK3 β (see figure below). Under conditions of insulin resistance, GSK3 β is converted to its active form by the decrease of the inhibitory serine 9 phosphorylation therefore conducting to the phosphorylation of Tau.

Therefore, we analyzed by western blot the effect of insulin resistance on tau phosphorylation and on GSK3 β activity after 3 months of diet (RD $n = 5$; HFD $n = 4$). As shown with the PHF1 and AT270 antibodies (recognizing respectively tau phosphorylated on Ser 396/404 and Thr181) in C and D, HFD doesn't lead to a change of tau phosphorylation or GSK3 β activity. Indeed, HFD doesn't change GSK3 β phosphorylation on Ser9, an inactivator site of GSK3 and on Tyr216, an activator site of GSK3 (F and G).

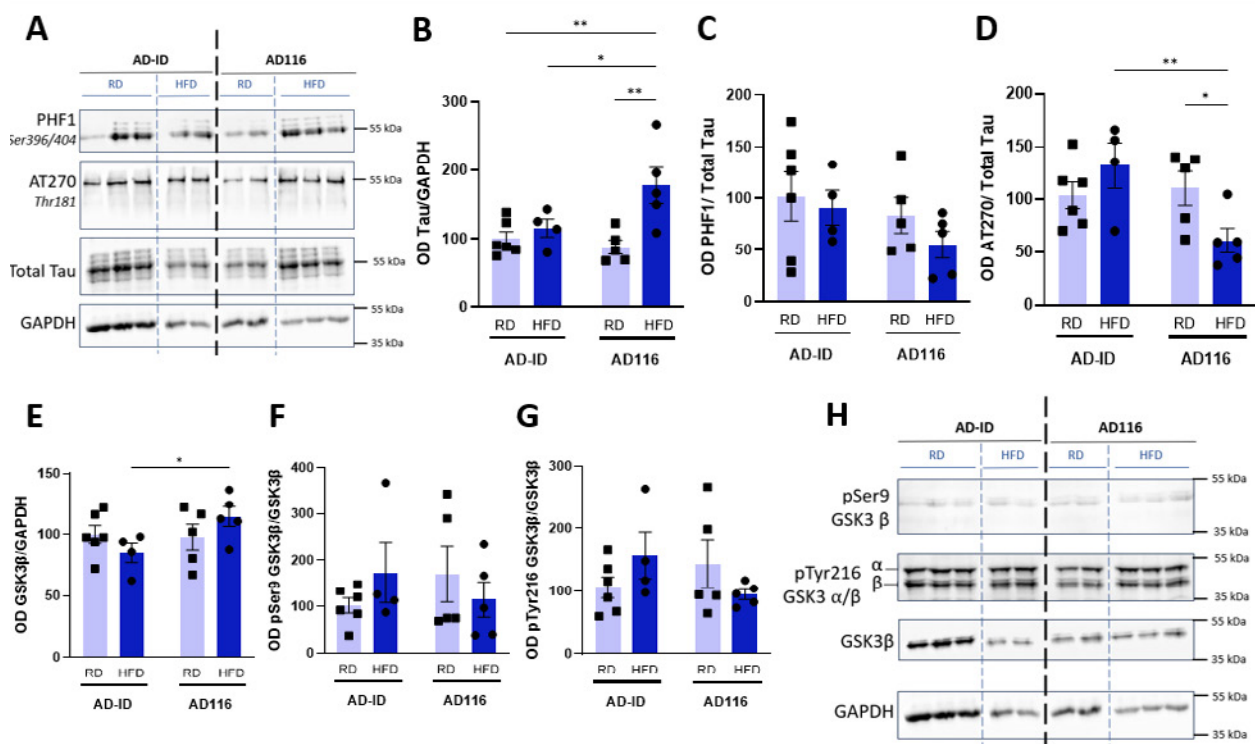


High-fat diet (HFD) does not change Tau phosphorylation or GSK3β activity after 3 months of diet.

3-month-old C57BL6N mice were fed with a normal diet (RD) or a high-fat diet (HFD) for 3 months (n= 4-5 per group). (A-D) Western blotting analysis with anti-phosphoTau antibodies (PHF1, Ser396/404 and AT270, Thr181) of mouse brain homogenates. (E-H) Western blotting analysis with anti-phosphoGSK3β antibodies (pSer9, Inactive GSK3 and pTyr216, active GSK3) of mouse brain homogenates. Data points correspond to individual mice, mean is shown in column, and SEM is represented by bar. Mann Whitney T test: *p < 0.05; **p < 0.01; ***p < 0.001.

3 months after the intracerebral injection of AD sarkosyl fraction containing pathological tau or ADID sarkosyl fraction, we analyzed tau expression/phosphorylation and GSK3beta expression/activity through phosphorylation in RD and HFD fed mice. Here we see an effect of HFD only in the mice injected with AD and fed with HFD. In those mice we observe an increase in tau content

(B) and an increase in GSK3 β content (E). These increased expressions of these two proteins could be explained by two mechanisms: an increase in the transcription or a decrease in the degradation of these proteins. The first hypothesis is going to be tested by checking the level of mRNA by sequencing. Moreover, we observe a decreased Tau phosphorylation on Thr181 (AT270 antibody) in HFD mice injected with AD compared to HFD-ADID and RD-AD. This decrease is probably due to the higher expression of tau (B) in this condition as the result for AT270 is expressed as a ratio between AT270 and total tau OD signals.



High-fat diet (HFD) does not change Tau phosphorylation or GSK3 β activity after 3 months of diet but increases tau expression when tau pathology developed.

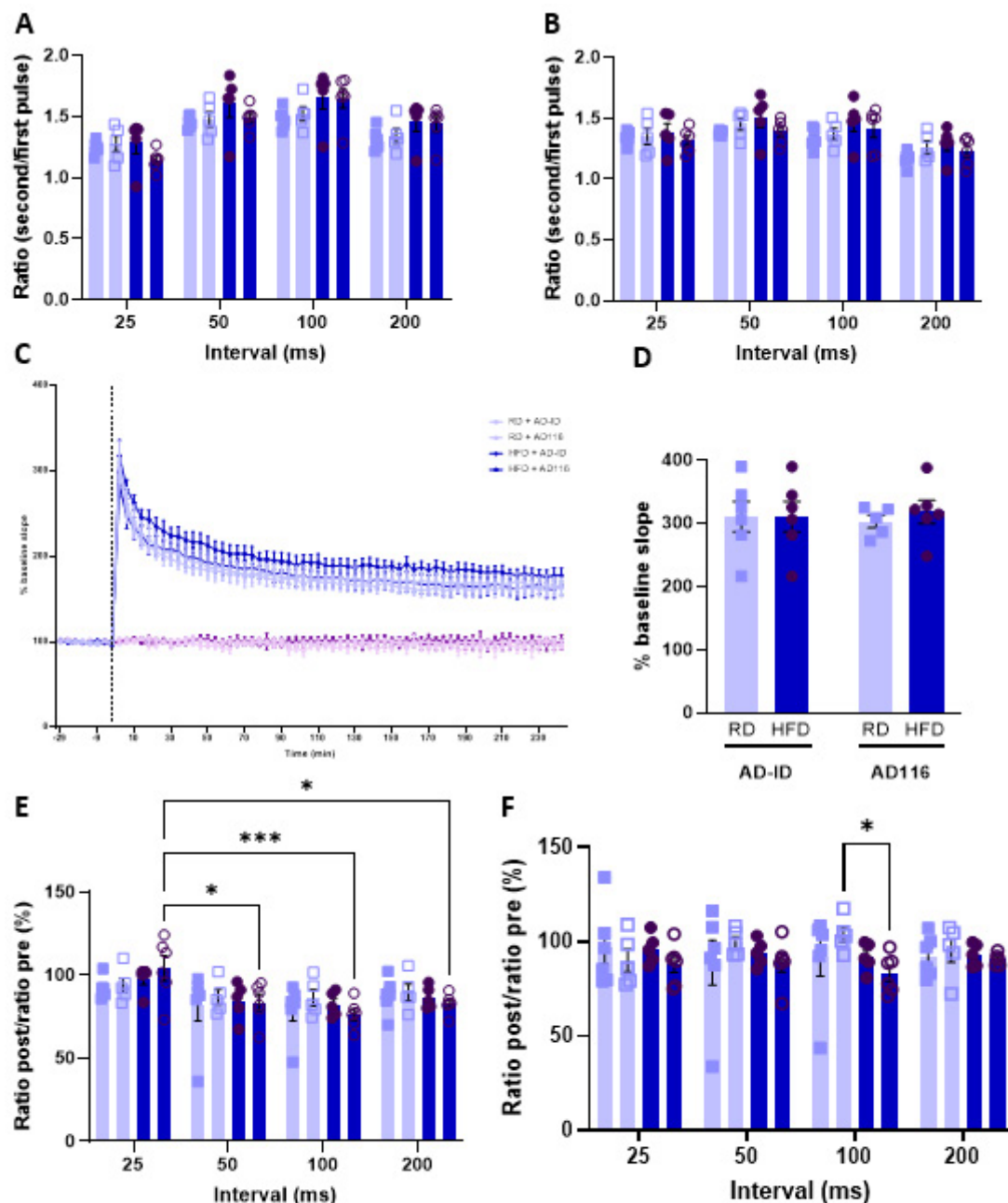
3-month-old C57BL/6N mice were fed with a normal diet (RD) or a high-fat diet (HFD) for 3 months and then randomized to receive stereotaxic injection of AD-or AD-ID sarkosyl fractions and protein expression was analyzed 3 months post-injection (n= 4-6 per group). (A-D) Western blotting analysis with anti-phosphoTau antibodies (PHF1, Ser396/404 and AT270, Thr181) of mouse hippocampus homogenates. (E-H) Western blotting analysis with anti-phosphoGSK3 β antibodies (pSer9, Inactive GSK3 and pTyr216, active GSK3) of mouse brain homogenates. Data points correspond to individual mice, mean is shown in column, and SEM is represented by bar. Two Way ANOVA Uncorrected Fisher's test *p < 0.05; **p < 0.01; ***p < 0.001.

3.10. Analysis of synaptic function after the intracerebral injection of pathological Tau.

The mouse is first sedated with isoflurane before being sacrificed by decapitation and dissected. The brain is maintained in an artificial cerebrospinal fluid (aCSF) bath at room temperature. The bath is oxygenated with carbogen (95% O₂/5% CO₂). The hippocampus of the left hemisphere is dissected and cut into 400 μ m-thick slices using a chopper. The slices are then placed in both installations (3 slices per installation) containing an oxygenated aCSF bath at 28°C. After 90 minutes' rest, the stimulation electrodes are placed on a slice in the CA1 region, stimulating Schaffer's collaterals. The recording electrode will be placed in the stratum radiatum to measure field excitatory post-synaptic potentials. Stimulation intensity is determined by the stimulation that will give 40% of the maximum amplitude on this slice. Paired pulses are recorded before and after LTP. They consist of two stimulations sent at 25, 50, 100 and 200 ms intervals. Two stimulations are averaged per interval and per electrode. After a stable baseline for 30 minutes,

LTP is induced by 2 TBSs separated by 20 seconds on one of the stimulation electrodes, with the other electrode acting as a control channel. These TBS comprise 10 packs of 4 stimulations sent at 200 ms intervals. Recording is maintained for 4 hours after LTP induction. Each point corresponds to 4 averaged stimulations separated by 60 seconds. Slope is measured and expressed as a percentage of baseline.

LTP was maintained at 170% after 4 hours in all groups. There were therefore no differences between groups. And no differences were found for the paired-pulses either.



No difference between the treatments on the synaptic plasticity or on the neuronal function .

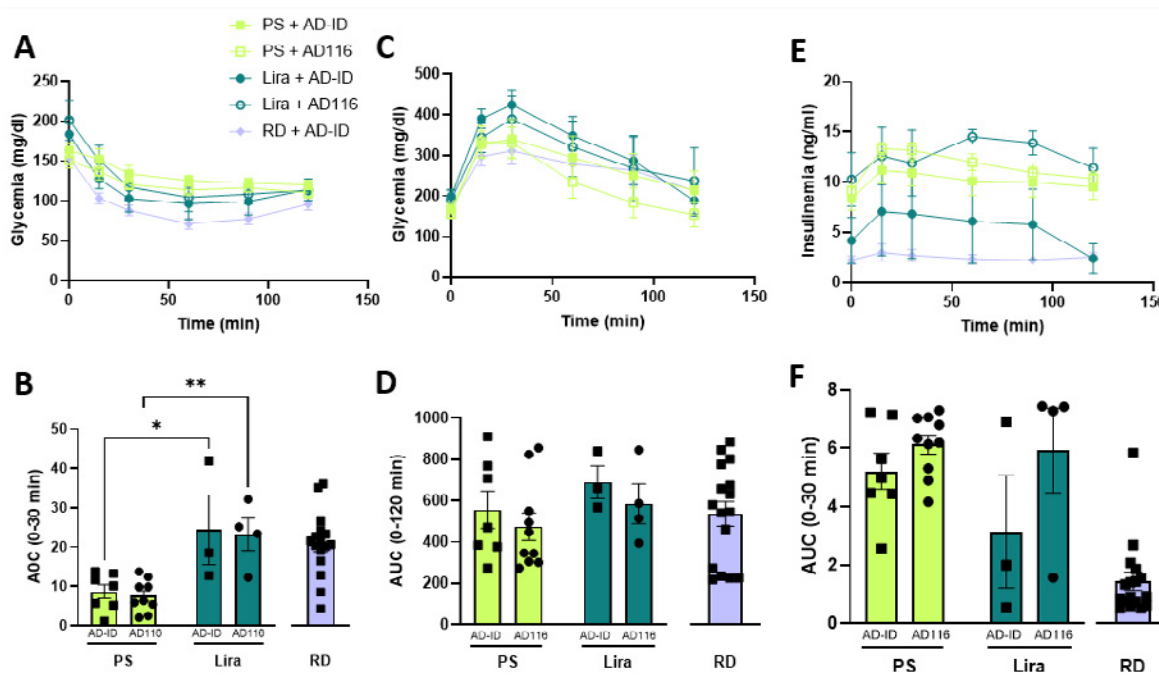
Synaptic plasticity and neuronal functions were measured at the end of the study by electrophysiology. Different parameters were measured: amplitude (A) and slope (B) during paired-pulse; LTP (C); LTP induction(D); paired-pulse post- vs pre-LTP amplitude (E) and slope (F). Data points correspond to individual mice, mean is shown in column, and SEM is represented by bar. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ by Tukey's multiple comparisons test or uncorrected Fisher's LSD.

3.11. Single cell RNA expression analysis

Hippocampal tissue was sent to the VIB-UAntwerp Center for Molecular Neurology (PI Sleepers) for single cell RNA sequencing in Q3 2025. To maximize the number of samples to be sequenced within the budget constraints, we opted for the 10x Genomics Gene Expression Flex protocol. We first optimized the nuclei extraction protocol to optimize the yield, using contralateral hippocampus samples. We tested a small gradient with and without sorting using BD FACSaria Fusion, and the large gradient without sorting, adapted from the protocol of Habib et al. From these experiments, the small gradient without sorting resulted in the largest number of nuclei for library preparation. We will perform nuclei extraction on all samples according to this optimized protocol, followed by library preparation using the 10x Flex protocol (ready by mid February 2026), and sequencing of the libraries on the NovaSeq platform. Data delivery is foreseen for mid March 2026.

3.12. Analysis of Liraglutide on metabolism.

The effect of an antidiabetic treatment (liraglutide) on the metabolic disturbances shown in our HFD mice was analyzed by ITT and IPGTT as explained before. After 3 months of HFD, mice were treated with physiological serum (PS) or with liraglutide (Lira; 300µg/kg/day) via an osmotic pump placed subcutaneously in the back of the mice. Liraglutide can rescue the insulin resistance in HFD mice to reach the same level as the RD mice but is not able to rescue the glucose sensitivity in those mice. Interestingly, Liraglutide tends to decrease the insulin level in AD-ID injected mice but not in AD injected mice.

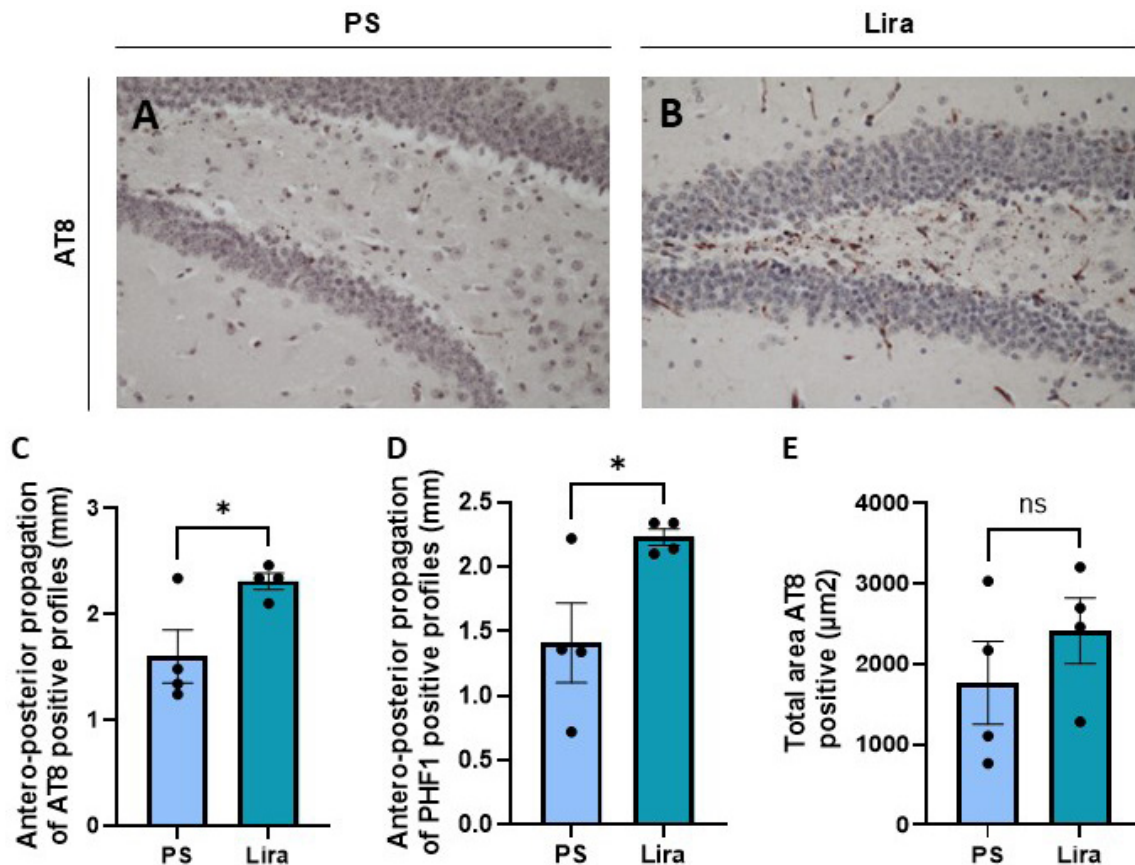


Liraglutide (Lira) rescues insulin sensitivity in high-fat (HFD) mice.

3-month-old C57BL6N mice were fed with a normal diet (RD) or a high-fat diet (HFD) for 3 months and then randomized to receive stereotaxic injection of AD or AD-ID sarkosyl fraction and treated with Liraglutide (Lira; 300µg/kg/day) or with physiological liquid (PS) as a control delivered by an osmotic pump. 3 months after the surgery metabolic parameters were measured in these mice. The following assessments were made at the end of the study: (A) blood glucose during the insulin tolerance test (ITT). Insulin sensitivity was quantified by the area under the curve (AOC) during (B) the insulin tolerance test. (C) Blood glucose, (D) AUC for glucose, and (E-F) insulin levels during intraperitoneal glucose tolerance test (IPGTT). Data points correspond to individual mice, mean is shown in column, and SEM is represented by bar. Two Way ANOVA Tukey's multiple comparisons test * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.13. Analysis of Tau pathology propagation with Liraglutide.

Interestingly, after Liraglutide treatment Tau pathology propagation is significantly increased in HFD mice, and we see a tendence to an increase for the intensity of the pathology with AT8 antibody.



Liraglutide (Lira) worsens Tau pathology in high-fat (HFD) mice.

3-month-old C57BL6N mice were fed a normal diet (RD) or a high-fat diet (HFD) for 3 months and then randomized to receive stereotaxic injection of AD116 or AD-ID and an osmotic pump placement containing physiological serum (PS) or Liraglutide (Lira; 300µg/kg/day; n= 4 per group). Sections were immunolabelled with AT8 (A-B) or PHF1 and the antero-posterior propagation (C-D) and the total area covered by the labelling at the pic (E) were quantified. Data points correspond to individual mice, mean is shown in column, and SEM is represented by bar. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ by Unpaired t test.

4. Conclusions and perspectives:

Metabolic tests showed that WT mice are a good model **to study the effect of metabolic** disturbances induced by high-fat diet on tau pathology propagation.

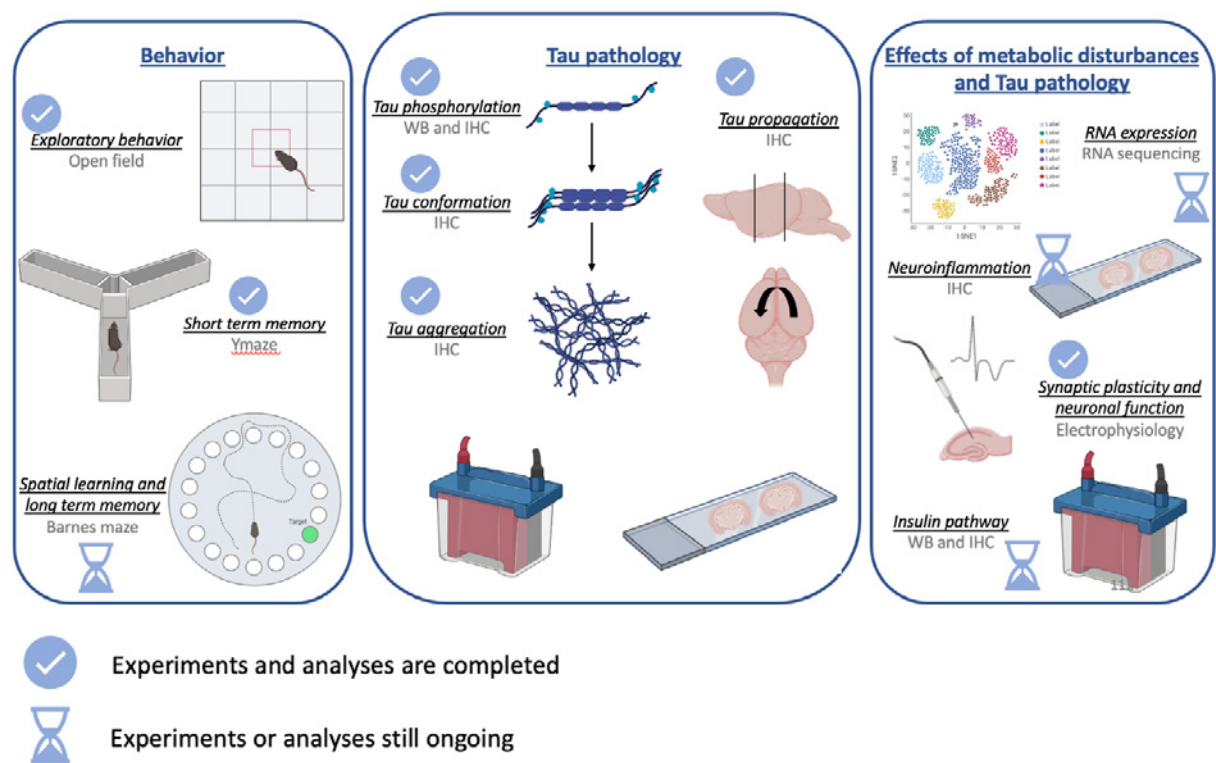
AD sarkosyl fractions induced a tau pathology that increases when the mice are fed with HFD compared to RD.

AD sarkosyl fractions induced an increase in Tau and GSK3 β proteins expression level in HFD mice. Those results would be furthermore investigated by sequencing.

Regarding the behavior test, we conclude that AD sarkosyl fraction does not have any effects, but HFD tends to lead to a change of exploratory behavior but not on short-term or long-term memory.

Liraglutide can rescue some metabolism parameters in HFD mice but worsens Tau pathology. Insulin pathway in the hippocampus will be further investigated.

More experiments or analyses still need to be completed as represented on the picture below.



More mice are under treatment to complete the groups for histology and western blot as planned in the table below.

	Group 17	Group 18
PS + AD-ID	3	4
PS + AD	5	5
Lira + AD-ID	6	5
Lira + AD	6	6
	N = 20	N = 20
Next availabilities	04/26	05/26



Geneeskundige Stichting Koningin Elisabeth
Fondation Médicale Reine Elisabeth
Königin-Elisabeth-Stiftung für Medizin
Queen Elisabeth Medical Foundation

Final report of the interuniversity research project of

Prof. dr. Sarah Weckhuysen (UAntwerpen)
Prof. dr. Bjorn Menten (UGent)

Prof. dr. Sarah Weckhuysen, MD, PhD (UAntwerpen)
Applied&Translational Neurogenomics Group VIB-UAntwerp
Center for Molecular Neurology UAntwerp CDE
Parking P4, Building V Universiteitsplein 1, 2610 Antwerpen
Belgium
uantwerpen.vib.be

Neurology Department
Antwerp University Hospital
Drie Eikenstraat 655
2650 Edegem
www.UZA.be/neurologie

Prof. dr. ir. Björn Menten (UGent)
Lab for Genome Research, Center for Medical Genetics, Department of biomolecular Medicine (UGent)
Associate professor (Faculty of Medicine and Health Sciences, UGent)
Lab supervisor Cytogenomics Laboratory Center for Medical Genetics, UZGent
Lab address: UZ Gent, Medical Research Building 1 (entrance 34),
Corneel Heymanslaan 10
9000 Gent

Detection of somatic mutations and disease-defining methylation patterns in brain tissue and cerebrospinal fluid of patients with non-acquired focal epilepsy

1. Background

The majority of non-acquired focal epilepsies (NAFE) have a presumed genetic etiology. However, the current yield of diagnostic genetic testing in affected patients is very low. This diagnostic gap is an important barrier to the broader use of gene-targeted therapies. Increasing evidence from resected brain tissue of individuals with NAFE points towards an important role of pathogenic somatic variants and methylation abnormalities. Most NAFE patients, however, do not undergo brain surgery, and the lack of brain tissue precludes a genetic diagnosis. In this project, we aim to prove that cell-free DNA (cfDNA) circulating in cerebrospinal fluid (CSF) and serum of patients with NAFE can be used to bridge this diagnostic gap. 1) We will apply a deep-sequencing protocol to detect novel somatic variants and 2) pathognomonic methylation patterns in cfDNA from CSF. 3) Simultaneously, we will use nanopore sequencing on native cfDNA to identify somatic copy number changes. We prioritize patients who undergo epilepsy surgery for our study cohort to validate the detected somatic and epigenetic changes in paired brain tissue. By identifying the genetic etiology and disease methylation profiles from cfDNA, we can improve the diagnostic yield and clinical decision-making in this severely affected population.

The previous report outlines significant in-roads in sample collection, sample preparation, and library preparation. In our new report, we would like to highlight important developments in library data analysis and critical results, in addition to ongoing sample acquisition.

2. Summary of achievements

2.1. Sample Acquisition

In the past 6 months, we have added 5 additional paired brain-CSF-blood samples from focal epilepsy patients. This expands our cohort from 42 to 47 individuals. In compensation for the slower acquisition rate, we now collaborate with Baulac laboratory in Paris to analyze their paired brain-CSF samples, which included 90 individuals with FCD-I, FCD-II, Rasmussen Syndrome, HME, PMG, and TSC. This access provides us with twice the number of samples we targeted for the whole project. This collaboration has since grown further to include 60 patients with sEEG-derived tissue and 32 with paired resected tissue, alongside an additional cohort of over 250 patients spanning major epilepsy-associated malformation subtypes (MOGHE, FCD I/II, HME, PMG) who have already undergone deep sequencing, over half of whom carry a known pathogenic somatic mutation.

2.2. Data Analysis

Targeted Methylation Sequencing

In our previous reports, we highlighted our baseline pretrained neural network machine model, which was able to **discern and classify methylation differences between these FCD, TLE, and control samples**. The classification scoring is derived by calculating the relative proximity of the model output encodings to known target encodings or combinations of target encodings. The model consistently gave higher focal epilepsy scores (EPI) for the FCD and TLE samples compared to controls ($p < 1e-3$) and higher FCD scores for FCD samples compared to controls ($p < 1e-3$).

This baseline model gave higher FCD scores for FCD samples compared to TLE, and higher TLE scores for TLE samples compared to controls, though these comparisons were not yet significant. Since our previous report, we have upgraded from this original supervised neural-network classifier to a mixture-of-experts (MoE) architecture, which improves discrimination between closely related focal epilepsy subtypes and enhances sensitivity to subtle epigenomic differences. Applying this new MoE model to the same cfDNA samples, the FCD-versus-TLE comparison now approaches statistical significance and we observe meaningful separation between FCD subtypes that was not achievable with the original baseline classifier. We will continue to improve our MoE model by mining the cfDNA methylation data to simulate true background noise better, which is expected to further widen the score differences between focal epilepsy subtypes. Nonetheless, this analysis demonstrates that our methylation sequencing protocol and analysis pipeline, now enhanced by the MoE architecture, can distinguish major subtypes based on cfDNA with improved sensitivity compared to our previous report.

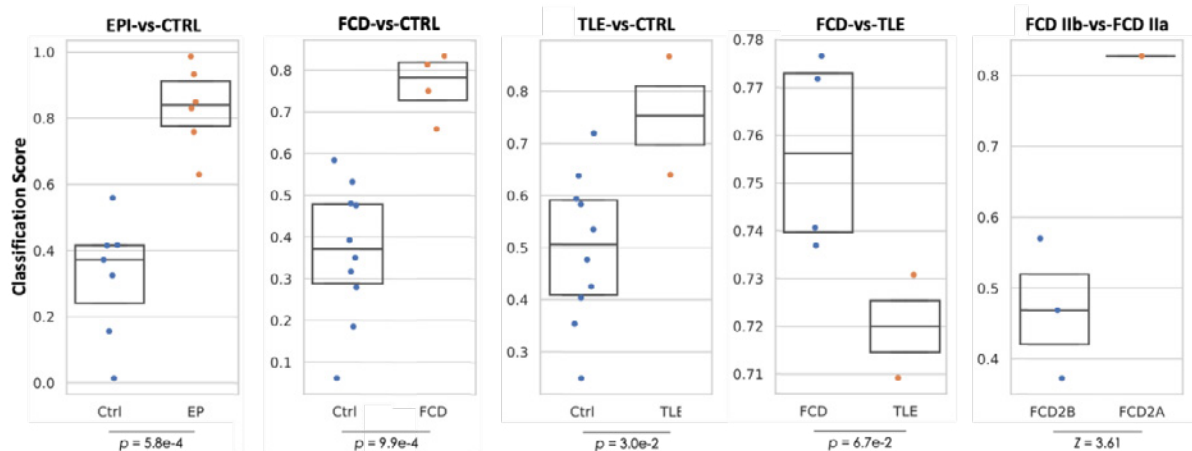


Figure. Classification scoring of cfDNA methylation using a pretrained neural network machine model. Our pre-trained machine models can, with statistical significance, discern between epileptic vs. non-epileptic, FCD vs. non-FCD, and TLE vs. non-TLE samples, and near statistical significance for FCD vs. TLE samples. Meaningful separation is achieved between FCD subtypes (statistical testing limited by sample size). Significance calculated using Mann-Whitney U.

Targeted Deep Mosaicism Sequencing

Since our last report, we have made substantial improvement to our pipeline for detection of pathogenic somatic variants. We have identified two individuals harboring somatic mutations in established epilepsy genes *RELN* and *CLN5*. Most importantly, *CLN5* variant was also detectable in the paired CSF-derived cfDNA (<1 ng), demonstrating that our lowinput optimizations remain effective at recovering meaningful low-frequency variants even from this extremely limited starting material. The mutation yield shall continue to increase as we sequence more samples.

Table Predicted Pathogenicity

ID	Gene	Protein Change		Epileptogenic Zone VAF	CSF cfDNA VAF
EP3387	<i>RELN</i>	F1743L	Probably Damaging (0.961, PolyPhen)	5.6%	not detected
EP3303	<i>CLN5</i>	Exon 4 Hypermutation	Destabilizing)	7.6%	2.3%

3. Library Preparation protocol optimization

A major methodological objective of the project was to establish a robust library preparation workflow for the ultra-low amounts of cfDNA obtained from CSF. This optimization has now been completed. We systematically refined the workflow to minimise DNA loss, improve reaction efficiency, and reduce PCR-associated duplication and bias. The resulting protocol allows us to reproducibly generate high-quality sequencing libraries from as little as 0.5 ng of input DNA, while maintaining sufficient library complexity for downstream detection of low-frequency somatic variants and methylation-based signatures.

We have also evaluated alternative amplification strategies, including linear amplification approaches, to further minimise amplification bias. The optimized workflow is now being applied to the larger sample set and provides the methodological foundation for the ongoing sequencing and analysis of paired brain tissue and CSF-derived cfDNA samples.

4. Further Planning

Following the extension of the project timeline until the end of 2026, we have continued to advance the experimental work. In particular, we have completed sequencing of an additional batch of brain tissue samples, both to identify somatic variants that can subsequently be compared with the paired cfDNA samples, and to obtain more comprehensive DNA methylation profiles. Analysis of these data is currently ongoing and will provide an important basis for assessing the extent to which brain-derived molecular signals can be detected in cfDNA.

In the coming months, we will focus on analysing the data generated from the first batch of samples from UZ Leuven and integrating the somatic variant and methylation data with the corresponding cfDNA analyses. In parallel, we will start sequencing and analysing samples from the other participating centres. This will allow us to validate the findings obtained in the initial cohort and increase the sample size and statistical power for detecting brain-derived signals in cfDNA.

For the methylation analyses, we are also continuing to explore the potential added value of generating data at a much higher CpG density than that available in the commercial methylation array on which our current neural network models were trained. By generating methylation data covering approximately 30 million CpG sites, we aim to develop and refine models that incorporate a broader set of brain-specific epigenetic signals, potentially improving the signal-to-noise ratio of cfDNA-based detection. Ultimately, these efforts will help determine the feasibility and robustness of cfDNA analysis as a minimally invasive approach for detecting brain-derived molecular signatures in patients with focal epilepsy.



Geneeskundige Stichting Koningin Elisabeth
Fondation Médicale Reine Elisabeth
Königin-Elisabeth-Stiftung für Medizin
Queen Elisabeth Medical Foundation

Progress report of the interuniversity research project of

Prof. dr. Ann Massie (VUB)
Prof. dr. Lutgarde Arckens (KU Leuven)

Prof. dr. Ann Massie (VUB)

Vrije Universiteit Brussel
Center for Neurosciences
Laarbeeklaan 103
1090 Brussels
ann.massie@vub.be
T 02 477 45 02

Prof. dr. Lutgarde Arckens (KU Leuven)

Katholieke Universiteit Leuven
Department of Biology
Naamsestraat 59
3000 Leuven
lut.arckens@kuleuven.be
T 016 32 39 51

The xCT^{-/-} killifish to validate the potential of system x_c⁻ as therapeutic target in Parkinson's disease

1. Project summary

Targeting the cystine/glutamate antiporter system x_c⁻ has great potential in the treatment of age-related neurological diseases. We recently showed lifespan extension with reduced age-related *inflammaging*, and preservation of hippocampal function and memory in aged mice lacking the specific xCT subunit of this antiporter. xCT deletion in mice also results in protection of the nigrostriatal dopaminergic pathway against toxin-induced degeneration (as a model for Parkinson's disease; PD). This protection, however, seems to be age- and toxin-dependent. Access to a spontaneous -and therefore toxin free- PD animal model is of paramount importance to create indisputable evidence about the power of system x_c⁻ inhibitors for the treatment of PD patients.

The African turquoise killifish *Nothobranchius furzeri* is the shortest-living vertebrate laboratory species showing all cellular and molecular hallmarks of human aging, including spontaneous α -synuclein accumulation with degeneration of dopaminergic neurons. These features make the killifish an excellent model to study PD, as there is no need for toxins to induce dopaminergic neurodegeneration. In this project, we therefore aimed at generating a transgenic killifish with a deletion of xCT, at characterizing the aging process of this fish -with focus on glutamate transporters, neuroinflammation, metabolic changes and the ubiquitin-proteasome system (UPS)- and at studying its susceptibility to spontaneous age-related neurodegeneration. This study will not only allow us to confirm or refute the therapeutic potential of targeting system x_c⁻ in PD and generate a valuable tool to further investigate the function of system x_c⁻ in aging and age-related (neurological) diseases, but it will also provide a detailed characterization of the aging brain of the shorter-lived GRZ-AD *N. furzeri* strain (until now most studies were done on the longer-lived MZM strain) that will be of interest for studying other potential neuroprotective targets in Parkinson's disease.

2. Project outline

State-of-the-art: The **cystine/glutamate antiporter system x_c⁻**, with xCT (Slc7a11) as specific subunit, imports cystine in exchange for glutamate and is the major source of extracellular glutamate in several mouse brain regions, including hippocampus and striatum^{1,2}. In the central nervous system (CNS), system x_c⁻ is mainly expressed on astrocytes^{3,4}. Glutamate released by system x_c⁻ is believed to be confined to the extrasynaptic space. This glutamate can act on metabotropic glutamate receptors to modulate glutamatergic transmission, and on ionotropic NMDA receptors thereby decreasing the threshold for glutamate toxicity (excitotoxicity). System x_c⁻ also regulates (neuro)inflammation: genetic xCT deletion (xCT^{-/-}) in mice⁵ results in a faster normalization of peripheral cytokine levels after i.p. injection of a sublethal dose of bacterial lipopolysaccharide (LPS) -thereby reducing LPS-induced sickness and neuroinflammation⁶- and favors the anti- over the pro-inflammatory microglial phenotype in pathological conditions^{7,8}. Accordingly, absence of system x_c⁻ is protective in neurological disorders that are characterized by excitotoxicity and (neuro)inflammation^{1,2,9-11}. The therapeutic potential of targeting system x_c⁻ in age-related neurological disorders has however been questioned, as the oxidative shift in the plasma cystine/cysteine ratio of xCT^{-/-} mice was suggested to accelerate the aging process⁵. We therefore studied the effect of genetic deletion of xCT on aging and indeed unveiled system x_c⁻ as

a regulator of life- and healthspan¹². Contrary to the expectations, however, mice with a genetic deletion of xCT showed a significant lifespan extension, while preserving their hippocampal function and spatial memory¹². Possible underlying mechanisms include attenuated *inflammaging* as well as metabolic changes at the level of the aged xCT^{-/-} brain, including reduced levels of N6-carboxymethyllysine¹², an advanced glycation end product (AGE) that has been linked to cognitive decline and neurodegenerative disorders¹³.

Glutamate toxicity and neuroinflammation are central to Parkinson's disease (PD) pathogenesis, and an accumulation of AGE levels has been linked to oxidative stress and α -synuclein (α -syn) aggregation in PD^{14,15}. Given the effect of system x_c⁻ deficiency on these pathogenic mechanisms, we validated the therapeutic potential of targeting system x_c⁻ in PD, by comparing the impact of three different toxin-based PD models between young-adult and aged xCT^{-/-} and xCT^{+/-} mice^{2,11}. Although we obtained very promising results, we remain somehow inconclusive. Whereas both young-adult and aged xCT^{-/-} mice were protected against neurodegeneration induced by intrastriatal 6-hydroxydopamine injection², the dopaminergic neurons of only aged (and not adult) xCT^{-/-} mice were protected against lactacystin (inhibitor of the ubiquitin proteasome system (UPS); intranigral injection)-induced degeneration, and neither young-adult nor aged xCT^{-/-} mice showed reduced susceptibility in the chronic, progressive 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model¹¹.

Objectives: With this project we aimed at developing a novel short-lived vertebrate model with spontaneous age-related degeneration of dopaminergic neurons that will allow us to unambiguously -and independent of PD-inducing toxins- reinforce or refute the potential of system x_c⁻ as therapeutic target in PD and to study the mechanisms underlying the anticipated neuroprotection.

To reach our **overarching goal**, i.e. reinforcing the validity of system x_c⁻ inhibition as a therapeutic target in PD, we initially defined four objectives:

The **first objective** was to create and validate an xCT^{-/-} African turquoise killifish.

The **second objective** was to investigate whether the beneficial effects on lifespan and general health, that we observed in aged xCT^{-/-} mice, can be replicated in aged xCT^{-/-} killifish.

The **third objective** was to study the effect of xCT deletion on the spontaneous age-related α -syn accumulation and dopaminergic neurodegeneration in the killifish brain.

The **fourth objective** was to investigate how xCT deletion affects the age-related pathogenic mechanisms that underlie neurodegenerative disorders, in the fast-aging killifish.

One additional objective has been added to our study. As most of the available data on the aging killifish brain have been collected from the longer-lived MZM strain and we here use the shorter-lived GRZ-AD strain to fully exploit the advantages of a short-lived model, our **fifth objective** was to map age-related neurodegeneration, protein accumulation and proteasome function in the GRZ-AD strain. *Moreover, given the recently discovered link between xCT and advanced glycation end products (AGEs) in the mouse brain, we decided to also analyze changes in AGE biology in the killifish brain. Until now, nothing is known about (age-related changes in) AGEs and their regulation mechanisms in any of the killifish strains.*

3. Workplan

(results obtained since the previous report are in italic)

3.1. Work package 1 (WP1): Generation and validation of an xCT^{-/-} killifish

xCT is expressed in the killifish¹⁶, showing a 73-75% DNA sequence homology with mouse and human xCT, and we had preliminary findings supporting the presence of system x_c⁻ activity in synaptosomes that were prepared from adult and aged killifish brains. In this WP we tried to generate an xCT^{-/-} killifish that allows time-efficient and in-depth investigation of the molecular mechanisms of aging and spontaneous age-related neurodegeneration that are affected by system x_c⁻ and could underlie our promising observations - both in the context of aging and in PD - in mice ^{2,11,12}.

To induce **xCT deletion** by a CRISPR-Cas9 strategy, proven to be efficient in *N. furzeri*^{17,18}, the Cas9-sgRNA complex targeting *slc7a11* was injected into the one-cell stage of killifish embryos, that were grown to adulthood. The knockout (KO) was confirmed on genomic DNA extracted from the caudal fin, followed by sequencing of the targeted region. After extensive training of our new PhD student for the injection of fish embryos, she obtained a heterozygous male fish. However, we lost this fish due to breeding issues (not related to the genotype) before he could fertilize eggs.

Given the low success rate when using the approach described above, we adopted a new approach to create the xCT KO killifish by replacing the Cas9 mRNA by the Cas9 protein, to facilitate CRISPR-Cas9 expression within the cell. The Cas9 protein was co-injected with a sgRNA targeting the *slc7a11* gene and a plasmid encoding the GFP protein. This construct enables KO of the xCT gene by inserting the GFP gene into the *slc7a11* locus, thereby disrupting the *slc7a11* gene and allowing GFP expression instead. This strategy has the potential to simultaneously generate a reporter killifish as GFP will be expressed in those cells that would express xCT in case of a wildtype (WT) fish. Using this construct, we confirmed the successful insertion of the GFP gene into the killifish genome. However, until now, we have been unable to confirm GFP gene insertion in the *slc7a11* gene.

We are currently exploring alternatives to delete xCT in the killifish. One option is to directly inject viral vectors in the killifish brain. However, due to limited knowledge about xCT expression in the killifish brain, it is hard to identify the ideal location for injection of these viral vectors.

In addition, besides the creation of the xCT KO killifish we further investigated (age-related changes in) xCT expression in the killifish brain. Single-nuclei RNA sequencing data from the telencephalon of young and old killifish brains, both before and after brain injury, revealed almost no xCT expression in young killifish telencephalon, with upregulation following brain injury and during aging. Interestingly, and in accordance with findings in mice, xCT was predominantly localized in an astrocyte-like radial glia subcluster. These results align with findings at protein and functional level. After successfully optimizing a Western blotting protocol to detect xCT in killifish brain lysates, we showed increased xCT protein expression in full brain lysates of old compared to young killifish brain, and a system x_c⁻ activity assay demonstrated enhanced system x_c⁻ activity in synaptosomes isolated from aged -compared to young- killifish brains.

We next used quantitative PCR (qPCR) to analyze brain mRNA levels of xCT over the lifespan of the killifish at 6w, 12w, 18w, and 24w, corresponding to young sexual mature fish, adult fish, old fish and very old fish. xCT expression was upregulated with aging, aligning with our previous results at the

protein and functional level. Together, these results clearly show an age-related upregulation of xCT in the killifish brain.

3.2. WP2: Characterization of the general health status and aging process of the xCT^{-/-} killifish

We previously observed that xCT deletion results in lifespan extension, without deterioration of general health status and with preservation of spatial memory in aged mice¹². In this WP, we aimed at evaluating whether these findings can be generalized to *N. furzeri* by comparing life- and health span of xCT^{+/+} and xCT^{-/-} killifish.

While trying to generate an xCT^{-/-} killifish, we have been optimizing the behavioral test battery and a strategy for detailed analysis of longitudinal age-dependent changes in locomotor function in WT killifish. We used the open field and the novel tank diving test to study killifish locomotion, exploration-related behavior, and behavioral changes over their adult lifespan. The characterization of this behavioral baseline is important for future experiments in our xCT^{-/-} fish. Fish were tested from the age of 6w to the age of 24w, and measurements were performed every 3 weeks. In the open field test, we found an increase in the time spent in the center zone from 18w onward, which indicates altered exploration behavior. Upon aging, the fish also showed an increased immobility frequency and duration. In addition, after the age of 15w, their locomotion decreased. In the novel tank diving test, we did not observe this aging effect on locomotion or exploration. Killifish spent around 80% of their time in the bottom half of the tank. We could not observe habituation effects, indicating slow habituation to novel environments. Moreover, we observed that killifish showed homebase behavior in both tests. These homebases are mostly located near the edges of the open field test and at the bottom of the novel tank diving test. Altogether, in the open field test, the largest impact of aging on locomotion and exploration was observed beyond the age of 15w. In the novel tank diving test, no effect of age was found. Therefore, to test the effects of xCT-related interventions on the aging process, the open field test is ideally suited. The novel tank test is better suited to test possible effect on anxiety as there is no confounding effect of aging in this readout. Whether the preference to spend time at the bottom of the tank truly reflects anxiety still needs to be confirmed with proven anxiolytic drugs though. These data have been published (Mariën et al, 2024, Frontiers in Behavioral Neuroscience¹⁹).

We validated spinal curvature as a longitudinal measure of aging in several batches of killifish, with and without drug treatment to alter aging. This has created optimized baseline data to compare the xCT^{-/-} killifish to once available.

3.3. WP3: Validation of the protective effect of xCT deletion on the spontaneous age-related α -synuclein accumulation and dopaminergic neurodegeneration in the killifish brain

The neuroprotective effects of xCT deletion in mouse models for PD were shown to be age- and toxin/model-dependent^{2,11}. We therefore aimed at using the xCT^{-/-} killifish to investigate how the absence of system x_c⁻ affects the occurrence and extent of spontaneous degeneration of dopaminergic neurons and the accumulation of α -syn, as was observed previously in WT killifish brain²⁰.

We have been optimizing a set of protocols to detect the presence of A β plaques and tauopathy in the aged WT killifish brain, mainly using histological and immunofluorescent stainings as well as a staining protocol to identify protein aggregates in the killifish brain. We also collaborated with the lab of Prof. Lies De Groef to validate the potential of new PD models in killifish.

3.4. **WP4: Identification of the mechanisms underlying the anticipated neuroprotective effect of xCT deletion in the aged killifish brain**

Multiple mechanisms that can underlie the neuroprotective potential of genetic xCT deletion have been identified in the mouse brain. Using the xCT^{-/-} killifish we wanted to investigate how xCT deletion affects the (age-related) pathogenic mechanisms described below and correlate them to the degree of neurodegeneration throughout the lifespan.

(WP4a) Glutamate transporters: We identified the presence of different excitatory amino acid transporters (EAAT) in our single-nuclei RNA sequencing data from the telencephalon of young and old killifish brains. Specifically, we detected EAAT1 (GLAST) and EAAT2 (GLT-1), which are known to be present on murine astrocytes. Our single nuclei data revealed that GLAST is expressed across different cell types in the killifish telencephalon, whereas GLT-1 expression is restricted to astrocyte-like radial glia (mainly RG1; Ayana, Zandecki et al.²¹).

Due to the limited sequence similarity between mouse and killifish, performing immunohistochemistry on killifish brain slices using mouse antibodies is challenging. To overcome this, we plan to conduct a glutamate uptake assay specific for the GLAST and GLT-1 in the coming months, to evaluate the effects of aging on the activity of these transporters. This assay has already been optimized by our collaborator Prof. E. Hermans (UCLouvain).

(WP4b) (Neuro)Inflammation: For this WP all methods are already optimized on WT fish (immunocytochemistry for L-Plastin; Hybridization Chain Reaction for APOEB; RT-qPCR for inflammatory marker genes *il6*, *il8*, *il10*, *sirt*, *il1β*, *tnf*, *csfr1a*).

*We quantified the expression levels of *il6*, *il8*, *csfr1a* and *tnf-α* over the lifespan of male killifish (6w, 12w, 18w and 24w). Aging was associated with an upregulation of *csfr1a*, a cytokine linked with microglial activation, and *tnf-α*. In contrast, *il6* and *il8* remained unchanged across the different age groups. These data indicate that aging in the killifish brain is accompanied by a selective increase in pro-inflammatory signaling, reflecting an age-associated inflammatory state. In the coming month, a staining using a custom-made (Eurogentec) and in-house validated antibody against a killifish-specific peptide of the microglial antigen LCP1 will be performed on brain slices originating of the different age groups to assess microglial activation during aging in the short-lived killifish.*

(WP4c) Metabolic changes: we began optimizing a protocol to cultivate killifish brain cells to perform Seahorse metabolic flux analyses on these cells.

We continued optimizing this protocol and tested different immunohistochemical stainings to identify the cultivated cells. Yet, till now, we have not been able to identify the exact cell-types we cultivated with our current protocol. Therefore, we are adapting the protocol with the aim to cultivate more specifically astrocyte-like radial and microglia-like cells.

(WP4d) The ubiquitin-proteasome system: While generating the xCT^{-/-} killifish, we developed different assays to study the UPS in the killifish. Dysfunction of the UPS has been implicated in several neurodegenerative diseases including PD. However, the UPS remains unexplored in the killifish and nothing is known about the inducible subunits of the immunoproteasome (IP) -a proteasome type which is only present in inflammatory conditions- in the killifish.

By analyzing our single nuclei sequencing data from the telencephalon of young and aged killifish, we identified four of the six proteasome subunits - responsible for the enzymatic activity of the UPS - described in human and mouse. Notably, we observed that some subunits of the

constitutive proteasome (CP; PSMB6-7) were broadly expressed across various cell subtypes, while the inducible subunits (PSMB8-10) were restricted to microglia. PSMB8 and 10 - but not PSMB6 and 5- are upregulated upon aging (the PSMB5 subunit is also upregulated in post-mortem brain tissue of PD patients²²), and after traumatic brain injury, the inducible subunits (PSMB8 and 10) are also upregulated.

In the single-cell sequencing of the young killifish telencephalon, we could identify all catalytic proteasome subunits that are expressed in human and mouse, i.e. PSMB5, PSMB6, PSMB7, PSMB8a, PSMB9 and PSMB10²¹.

We also optimized several tools to investigate the function of the UPS in the killifish brain: an activity assay using different fluorogenic substrates as well as qPCR protocols to quantify the expression of the different UPS subunits in the killifish brain.

qPCR analysis of the different catalytic subunits in whole brain lysates revealed that the expression of the constitutive subunits $\beta 1$ (PSMB6) and $\beta 5$ (PSMB5) was not altered with aging, whereas $\beta 2$ (PSMB7) expression was increased. A similar age-associated pattern was observed for the IP subunits: $\beta 2i$ (PSMB10) expression was increased in 18w and 24w compared to 6w brains, while $\beta 5i$ (PSMB8) mRNA levels were elevated at 12w compared to 6w. These data are in line with our single-cell and single-nuclei RNA sequencing data. We are currently optimizing primers to measure the expression of $\beta 1i$ (PSMB9).

To determine whether these transcriptional changes translate to the activity level, we measured the chymotrypsin and caspase-like activity, corresponding to the activity of the $\beta 5$ and $\beta 1$ subunit, respectively, in brain lysates. In accordance with the observations at mRNA level, an increased chymotrypsin activity was measured in 18w brain lysates compared to 6w, while a trend toward a decrease in activity was seen at 24w. Similar findings were found for the caspase-like activity, with an increase in activity at 18w compared to 6w and a decrease in activity in 24w compared to 6w.

Together with the single-cell, single-nuclei, and qPCR findings, these data demonstrate an age-associated upregulation of IP subunit expression in the killifish brain, particularly in microglia, while the expression of the CP subunits is only partially affected by age. Importantly, increased transcript levels do not consistently translate into sustained proteasome activity at advanced age, indicating a partial transcript-protein decoupling of the UPS in old killifish brains.

Currently, we are still working on other RNA-level techniques such as hybridization chain reaction, to visualize the localization of the different UPS subunits in the untreated killifish brain.

Since the mRNA expression of the UPS subunits and the UPS activity are increased in the aging killifish brain, we investigated whether induction of a neuroinflammatory response in young killifish could similarly upregulate IP subunits. Young fish were injected with a moderate (5mg/kg) or high dose of LPS (20mg/kg), and brains were collected after 4h and 24h post-injection to assess the induction and resolution of the inflammatory response.

*Increased *il8* and *csf1ra* mRNA levels were seen 4h after injection of 5mg/kg LPS, confirming the induction of an inflammatory response in the central nervous system. At this timepoint, the IP subunits $\beta 2i$ and $\beta 5i$ were upregulated, while among the CP subunits, only $\beta 5$ was significantly increased. At 24h after LPS injection, all cytokine levels returned to baseline, as did the different UPS subunits. Correlation analyses revealed significant correlations between most UPS subunits and the different cytokine levels over time and across the different LPS concentrations.*

A higher dose of LPS (20mg/kg) induced the expression of all cytokines- $\text{tnf-}\alpha$, csf1ra , il6 and il8 . The $\beta 2i$ and $\beta 5i$ subunits were mainly upregulated 24h post LPS injection as were the constitutive subunits $\beta 1$ and $\beta 2$, whereas the expression of the $\beta 5$ subunit remained unchanged. These results show that both a moderate and high dose of LPS trigger an increase in cytokine expression in young killifish, with a return to baseline 24h post moderate LPS dose. In contrast, high-dose LPS induced a more persistent inflammatory response, mirrored by sustained upregulation of both CP and IP subunits at 24h.

We next investigated whether aged killifish were able to upregulate their UPS in a similar way. Surprisingly, only $\text{tnf-}\alpha$ and il8 expression were affected by 20mg/kg LPS exposure. No effects were seen on csf1ra and il6 expression levels, or any of the UPS subunits, suggesting a reduced adaptive capacity of the UPS.

(WP4e) AGEs are harmful compounds that are formed as a side product during glycolysis and accumulate in the brain with aging. The products formed during glycolysis can undergo further chemical reactions to form highly reactive dicarbonyl compounds known as AGE-precursors, with glyoxal (GO) and methylglyoxal (MGO) being the best known. Further reactions of these reactive compounds with amino acid residues generate a wide range of AGEs, among which N-carboxy-methyl-lysine (CML) and N-carboxy-ethyl-lysine (CEL) are the most abundant in tissues and blood plasma^{23,24}.

AGEs can be cleared through the UPS. On the other hand, the UPS can be inhibited when there is an accumulation of AGEs. AGE-precursors are cleared through the glyoxalase system, which consist of the enzymes glyoxalase 1 (Glo1) and glyoxalase 2 (Glo2).

The levels of different AGE-precursors and AGEs were measured in the killifish brain of different age groups using ultra-high-performance-liquid chromatography coupled to mass-spectrophotometry (UPLC-MS-MS; collaboration with Prof. C. Schalkwijk, Maastricht University; The Netherlands). Brain GO levels increased with aging, while there were no differences in MGO levels across the different age groups. An even more pronounced age-related increase was seen for the CML and CEL levels. Next, we investigated whether the enzymes responsible for the detoxification of AGE-precursors were also altered with aging. While whole brain Glo1 mRNA expression was upregulated in aged killifish brain lysates, no corresponding increase in enzymatic activity was detected, indicating that aging does not enhance the glyoxalase detoxification capacity in the killifish brain, and once again the presence of transcript-protein decoupling in the aging brain.

Consistent with increased AGE burden, brain mRNA expression of the receptor for AGE -RAGE- was increased with aging.

Altogether, these data indicate that, like in rodents and humans, AGE-precursors and protein-bound AGEs accumulate in the aging killifish brain. This accumulation occurs in the absence of a compensatory increase in Glo1 enzymatic activity -suggesting an impaired capacity to detoxify reactive dicarbonyls with aging- and is accompanied by increased RAGE expression, consistent with enhanced AGE-RAGE signaling in the aged brain.

In conclusion, although a confirmed and validated $\text{xCT}^{-/-}$ killifish line is not yet available, we continued to use the assays optimized last year to further characterize the UPS and AGE biology across the lifespan of the African turquoise killifish. These efforts resulted in novel findings that are currently being prepared for publication.

4. References

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Queen Elisabeth Medical Foundation

Progress report of the interuniversity research project of

Prof. dr. Sebastiaan Engelborghs (VUB)
Prof. dr. Chris Baeken (UGent)

Prof. dr. Sebastiaan Engelborghs

Vrije Universiteit Brussel (VUB)
Center for Neurosciences (C4N)
Laarbeeklaan 103, 1090 Brussel

Prof. dr. Chris Baeken

Universiteit Gent
Department of Psychiatry & Neuropsychology
C. Heymanslaan 10, 9000 Gent

Unraveling the link between depression and dementia to improve diagnostic and treatment options

1. Publications 2025

- Amyloid Biomarker Study Group; Wiels, W. A., Oomens, J. E., Engelborghs, S., Baeken, C., von Arnim, C. A. F., Boada, M., Didic, M., Dubois, B., Fladby, T., van der Flier, W. M., Frisoni, G. B., Fröhlich, L., Gill, K. D., Grimmer, T., Hildebrandt, H., Hort, J., Itoh, Y., Iwatsubo, T., Klimkiewicz-Mrowiec, A., Lee, D. Y., Lleó, A., Martínez-Lage, P., de Mendonça, A., Meyer, P. T., Kapaki, E. N., Parchi, P., Pardini, M., Parnetti, L., Popp, J., Rami, L., Reiman, E. M., Rinne, J. O., Rodrigue, K. M., Sánchez-Juan, P., Santana, I., Sarazin, M., Scarmeas, N., Skoog, I., Snyder, P. J., Sperling, R. A., Villeneuve, S., Wallin, A., Wiltfang, J., Zetterberg, H., Ossenkoppele, R., Verhey, F. R. J., Vos, S. J. B., Visser, P. J., Jansen, W. J., & Alcolea, D. (2025). Depressive symptoms and amyloid pathology. *JAMA Psychiatry*, 82(3), 296–310. <https://doi.org/10.1001/jamapsychiatry.2024>

Background:

Depressive symptoms in later life are frequently associated with cognitive decline and are considered a potential risk factor or prodromal feature of dementia. However, the underlying neurobiological mechanisms remain unclear. In particular, it is uncertain to what extent late-life depressive symptoms are related to Alzheimer's disease (AD) pathology, such as cerebral amyloid accumulation. This uncertainty complicates diagnostic differentiation and the development of targeted treatment strategies.

Hypothesis and objectives:

The primary objective of this study was to investigate the association between depressive symptoms and amyloid pathology in older individuals without dementia. The authors further aimed to examine whether this association is modified by age, sex, educational level, or APOE 4 genotype, and whether differences exist between individuals with normal cognition (NC) and mild cognitive impairment (MCI).

Materials and methods:

This cross-sectional study used harmonized data from the Amyloid Biomarker Study, a large international data pooling initiative including research cohorts, population-based studies, and memory clinic samples. Data from 49 studies across 95 centers were included. The sample comprised 9,746 individuals with normal cognition and 3,023 individuals with MCI, aged 34 to 100 years, all without dementia. Amyloid pathology was determined using cerebrospinal fluid amyloid- β 1–42 levels or amyloid PET imaging. Depressive symptoms were defined based on validated depression rating scales, a current clinical diagnosis of depression, or self-reported depressive symptoms. Statistical analyses were performed between December 2022 and February 2024.

Results:

In cognitively normal individuals, the presence of depressive symptoms was not significantly associated with amyloid pathology. In contrast, among individuals with MCI, depressive symptoms were associated with a significantly lower likelihood of amyloid pathology. Subgroup analyses revealed that in cognitively normal participants, depressive symptoms were associated with a higher frequency of amyloid pathology only in APOE 4 noncarriers, but not in 4 carriers. No such subgroup effect was observed in the MCI group. The observed associations were not influenced by age, sex, or educational level.

Conclusions:

Depressive symptoms in older individuals were not consistently associated with increased amyloid pathology and were linked to a lower probability of amyloid positivity in those with MCI. These findings suggest that mechanisms other than amyloid accumulation—such as vascular, inflammatory, or stress-related processes—may commonly underlie late-life depressive symptoms. The results underscore the importance of avoiding a simplistic interpretation of depressive symptoms as an early marker of Alzheimer's disease and highlight the need for broader pathophysiological models linking depression and cognitive decline.

- De Witte, S., De Raedt, R., Wu, G. R., De Smet, S., Dedoncker, J., Klooster, D., Tandt, H., Van De Velde, N., De Schrijver, N., Herremans, S., Debruyckere, I., Nobels, A., Koster, E. H. W., Lemmens, G., Vanderhasselt, M. A., & Baeken, C. Assessing accelerated iTBS in treatment-resistant depression: Doubling the number of pulses per session does not double the response rates. *Brain Stimul.* 2025 Mar-Apr;18(2):195-197. doi: 10.1016/j.brs.2025.02.004.

Background:

Accelerated intermittent theta burst stimulation (aiTBS) offers rapid antidepressant effects for treatment-resistant depression (TRD). Yet, dose response relationships (number of sessions and number of pulses per session) are not fully understood. Objectives: This first phase of our Stepped-Care aiTBS 2 Depression double-blind randomized controlled trial (NCT03288675) aims to assess whether the increased pulse count enhances reductions in depressive symptoms, suicidal ideation, anxiety, psychomotor disturbances, and hopelessness among patients with TRD.

Methods:

We extended our previous aiTBS research by increasing the pulse count per session from 1,620 to 3,000 pulses—nearly doubling it—while keeping all other clinical and stimulation parameters unchanged from our earlier trial (NCT01832805). Sixty-eight TRD patients, all of the melancholic subtype, received 20 either active or sham aiTBS sessions over four days, targeting the left dorsolateral prefrontal cortex. Primary outcomes were assessed through changes in clinician-rated depressive symptoms.

Results:

Although active treatment demonstrated higher response (12.50%) and remission rates (6.25%) than sham (8.82% response; 0.00% remission), these rates were unexpectedly lower than those observed in our previous comparable studies using nearly half the number of pulses per session. We found no significant differential effect of active or sham stimulation on suicidal ideation, hopelessness, psychomotor disturbances, or anxiety.

Conclusion:

Increasing the pulse count per session in aiTBS treatment does not significantly improve clinical outcomes in melancholic TRD, indicating that there is no linear association with increasing the number of pulses per session and clinical outcome rates.

- Van Rossem, D., Allemeersch, G.-J., Barros, N., Temmerman, J., Desmet, M., Ringoot, I., Ribbens, A., Michiels, V., De Mey, J., Baeken, C., Engelborghs, S., & De Witte, S. (2025). Assessing the link between cerebellar volume and cognitive function in Alzheimer's disease: a pilot study. *Scientific Reports - Nature*, 15(1), 1-12. Artikel 32943. <https://doi.org/10.1038/s41598-025-12975-8>, <https://doi.org/10.1038/s41598-025-12975-8>, <https://doi.org/10.1038/s41598-025-12975-8>

Background:

Neurodegenerative diseases, such as Alzheimer's disease (AD), are characterized by pathological changes in the brain including specific patterns of brain atrophy. These atrophy patterns often mirror intrinsic connectivity networks linked to cognition, such as the default mode network. While research has focused on the changes in the cortex, emerging evidence highlights similar alterations in the cerebellum. Traditionally linked to motor functions, the cerebellum is now recognized for its role in (social) cognition.

Hypothesis and objectives:

We hypothesize that individuals within the AD continuum will exhibit significant reductions in grey matter volume, particularly in the posterior cerebellar lobule VII especially Crus I/II, with more pronounced reductions in the later stages of AD. We also expect that cerebellar atrophy will be linked to cognitive performance. This study investigates the relationship between differences in cerebellar volume and cognitive function across the AD continuum.

Materials and methods:

Subjects with subjective cognitive decline (SCD, n = 45), mild cognitive impairment due to AD (MCI, n = 23), and dementia due to AD (ADD, n = 62) were selected from an existing memory-clinic based research database. A comprehensive neuropsychological assessment conducted as part of the diagnostic process was available for all participants. The Mini-Mental State Examination (MMSE) and the Addenbrooke's Cognitive Examination-Revised (ACE-R) were selected for this study as an indication of cognitive performance. Brain magnetic resonance imaging (MRI) scans were processed using the automated brain volumetry software icobrain dm. For this pilot study, the cerebellar segmentations from icobrain were combined with an atlas-based approach to obtain a finer anatomical parcellation of the cerebellum. Finally, the volume for different cerebellar regions was determined, including the entire cerebellum (both grey and white matter), as well as grey matter of the left and right anterior cerebellum (lobules I-IV), posterior cerebellum (lobules VI-IX, with lobule VII being subdivided into VIIa with Crus I/II, VIIb), and the flocculonodular lobe (lobule X).

Results:

Our results identified significant volumetric differences in 14 cerebellar regions between the SCD and ADD groups, including lobule VII/Crus II. Notably, no differences in volume were found in the MCI group as compared to the ADD group. Further analysis demonstrated that cognitive performance (measured by the MMSE and ACE-R) positively correlated with grey matter volume in various regions of the posterior cerebellum for both the MCI and ADD groups. However, no correlations between brain volume and cognitive performance were observed in the SCD group.

Conclusions:

These findings support the hypothesis that cerebellar atrophy is associated with cognitive symptoms of neurodegenerative diseases like AD. Understanding this association could provide new insights into the pathophysiology of AD symptoms and possibly pave the way to targeted neuromodulatory therapeutic strategies.

- Zhang, Y., Wu, G., De Witte, S., & Baeken, C. (2025). Microstructural alterations in superficial white matter associated with anhedonia and suicidal ideation in major depressive disorder. *Biological Psychiatry: Cognitive Neuroscience and Neuroimaging*, 10(–), 1–13. <https://doi.org/10.1016/j.bpsc.2025.02.010>

Background:

Major depressive disorder (MDD) is characterized by disturbances in emotional and cognitive functioning, including anhedonia and suicidal ideation. Neuroimaging research has traditionally focused on deep white matter (DWM) tracts connecting distant brain regions. However, superficial white matter (SWM)—consisting of short-range fibers connecting adjacent cortical areas—accounts for the majority of cortico-cortical connections and may play a critical role in integrating gray and white matter alterations. Despite its relevance, SWM abnormalities in MDD remain understudied.

Hypothesis and objectives:

The authors hypothesized that patients with MDD would show significant microstructural abnormalities in SWM compared with healthy controls (HCs). They further expected that these SWM alterations would be associated with core depressive symptoms, particularly anhedonia and suicidal ideation. Finally, the study aimed to determine whether neurite orientation dispersion and density imaging (NODDI) metrics would provide more specific information on SWM pathology than conventional diffusion tensor imaging (DTI) metrics.

Materials and methods:

The study included 62 antidepressant-free patients with melancholic, treatment-resistant MDD and 77 healthy controls aged 18–65 years. All participants underwent diffusion-weighted MRI on a 3T scanner. SWM bundles were reconstructed using probabilistic tractography seeded from the gray–white matter interface. Microstructural properties were assessed using DTI metrics (fractional anisotropy [FA], mean diffusivity [MD]) and NODDI metrics (neurite density index [NDI], orientation dispersion index [ODI]). Voxelwise tract-based spatial statistics restricted to SWM were used for group comparisons, controlling for age and sex. Correlations between diffusion metrics and clinical measures—including anhedonia (SHAPS) and suicidal ideation (SSI)—were examined with correction for multiple comparisons.

Results:

Compared with healthy controls, patients with MDD showed widespread SWM abnormalities. DTI analyses revealed reduced FA and increased MD in SWM bundles connecting frontal, temporal, parietal, and occipital cortices. NODDI analyses demonstrated reduced NDI in SWM bundles connecting frontal–parietal–temporal regions and increased ODI in parietal, occipital, and posterior cingulate connections. Crucially, reduced neurite density in SWM bundles connecting prefrontal cortex and insula was significantly negatively correlated with anhedonia and suicidal ideation in the MDD group, but not in healthy controls.

Conclusions:

This study provides evidence that superficial white matter microstructure is significantly altered in major depressive disorder and is closely linked to clinically relevant symptoms. Reduced neurite density in prefrontal–insula SWM connections may constitute a structural substrate for anhedonia and suicidal ideation. Furthermore, NODDI metrics appear more specific than conventional DTI measures in detecting SWM abnormalities. These findings highlight SWM as a critical but previously underrecognized component of the neurobiology of MDD and suggest potential targets for neuromodulatory interventions.

2. Presentation during Conferences and Symposia in 2025

Besides the PI's, several researchers have contributed to the GKSE research project, each bringing unique expertise and insights.

- Dr. Dieter Zeeuws (MD, Staff Psychiatrist), a PhD candidate at Universitair Ziekenhuis Brussel (UZ Brussel), is involved in psychiatric research and clinical applications.
- Dr. Sara De Witte (PhD, Psychologist), a postdoctoral researcher and research manager, is affiliated with the Neuroprotection and Neuromodulation (NEUR) Research Group at the Center for Neurosciences (C4N), Vrije Universiteit Brussel (VUB), and the Departments of Neurology, Psychiatry and Bru-BRAIN at UZ Brussel. She is also associated with the Ghent Experimental Psychiatry (GHEP) Lab at Ghent University.
- Deborah Van Rossem (PhD student, Psychologist and Ma in Neurosciences) is also connected to the NEUR Research Group, C4N, VUB, and the Departments of Neurology, Psychiatry and Bru-BRAIN at UZ Brussel.

All participants have attended various symposia and conferences related to the topic of this project. In the following section we give an overview of presentations at conferences:

Dr. Sara De Witte:

- 19th International Conference on Alzheimer's & Parkinson's Diseases (AD/PD), April 2025, Vienna, Austria.

Oral presentation.

Abstract: Brain Stimulation for Depression: More Pulses, Same Blues?

(Randomized controlled trial on accelerated intermittent theta burst stimulation in treatment-resistant melancholic depression, examining whether increased pulse dose improves clinical outcomes.)

- C4N Spring Day, 2025, Brussels, Belgium.

Oral presentation.

Presentation linked to ongoing neuromodulation research within C4N.

Deborah Van Rossem:

- 19th International Conference on Alzheimer's & Parkinson's Diseases (AD/PD), April 2025, Vienna, Austria.

Oral presentation.

Abstract: Association between Cognitive Function and Cerebellar Atrophy in Alzheimer's Disease.

(Structural MRI study investigating associations between cerebellar subregional volumes and cognitive performance across stages of Alzheimer's disease.)

- C4N Spring Day, 2025, Brussels, Belgium.

Oral presentation.

Presentation based on ongoing MRI and cognitive biomarker research in Alzheimer's disease.

- International Exchange Trajectory – University of Siena, June 30 – August 1, 2025.

Selection by the Resident and Research Fellow Section (RRFS) of European Alzheimer Disease Consortium (EADC) to participate in an international clinical and research exchange program.

Focus on MRI methodologies and cognitive biomarkers in Alzheimer's disease.

3. Research Progress

3.1. WP1: Development of a dementia risk score amongst depressed elderly

Data collection and analyzing data finished. A research paper '**Depressive Symptoms and Amyloid Pathology**.' was published in *JAMA Psychiatry* with Wietse Wiels as first author and Sebastiaan Engelborghs and Chris Baeken as co-authors:

Wiels WA, Oomens JE, Engelborghs S, Baeken C, et al. Depressive Symptoms and Amyloid Pathology. *JAMA Psychiatry*. 2025 Mar 1;82(3):296-310. doi: 10.1001/jamapsychiatry.2024.4305.

3.2. WP2: Validate the dementia risk score in a prospective longitudinal clinical study

WP2 aims to examine the prognostic relevance of depressive symptoms and amyloid status in the elderly, through a prospective evaluation of cognitively healthy controls, depressed patients, and cognitively impaired patients (all groups >65 years of age). Data collection is finished.

Following publications have resulted from this WP:

- Wiels WA, Wittens MMJ, Zeeuws D, Baeken C, Engelborghs S. Neuropsychiatric Symptoms in Mild Cognitive Impairment and Dementia Due to AD: Relation With Disease Stage and Cognitive Deficits. *Front Psychiatry*. 2021 Aug 17;12:707580. doi: 10.3389/fpsyt.2021.707580. eCollection 2021.
- Wiels W, Baeken C, Engelborghs S. Depressive Symptoms in the Elderly-An Early Symptom of Dementia? A Systematic Review. *Front Pharmacol*. 2020 Feb 7;11:34. doi: 10.3389/fphar.2020.00034. eCollection 2020.

3.3. WP3: Evaluate the predictive and clinical effects of non-invasive brain stimulation in the elderly depressed with and without prodromal AD

WP3 will evaluate non-invasive brain stimulation techniques on the elderly depressed, and the impact of this treatment on imaging parameters. Recruitment and testing of depressive patients are ongoing. An amendment of the protocol for the use of rTMS/tDCS in patients with Alzheimer's disease was approved by the Ethical Committee of the UZ Brussel. Recruitment has started, and testing will be continued in 2026.

4. Budget 2025

There was a budget neutral prolongation of the study. No budget was used in 2025.

5. Future directions for 2026

- **WP1:** Data collection and data analysis finished. Research paper published.
- **WP2:** Data collection and data analysis finished. Research papers published.
- **WP3:** Continue recruitment and data collection.

6. Prolongation of the project

We would like to kindly request a one-year budget-neutral extension of the interuniversity research project "Unraveling the link between depression and dementia to improve diagnostic and treatment options." The project is currently scheduled to run until the end of 2025, and we would like to extend it until the end of 2026.



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Universitaire onderzoeksprojecten
2023-2025 gefinancierd door de G.S.K.E.

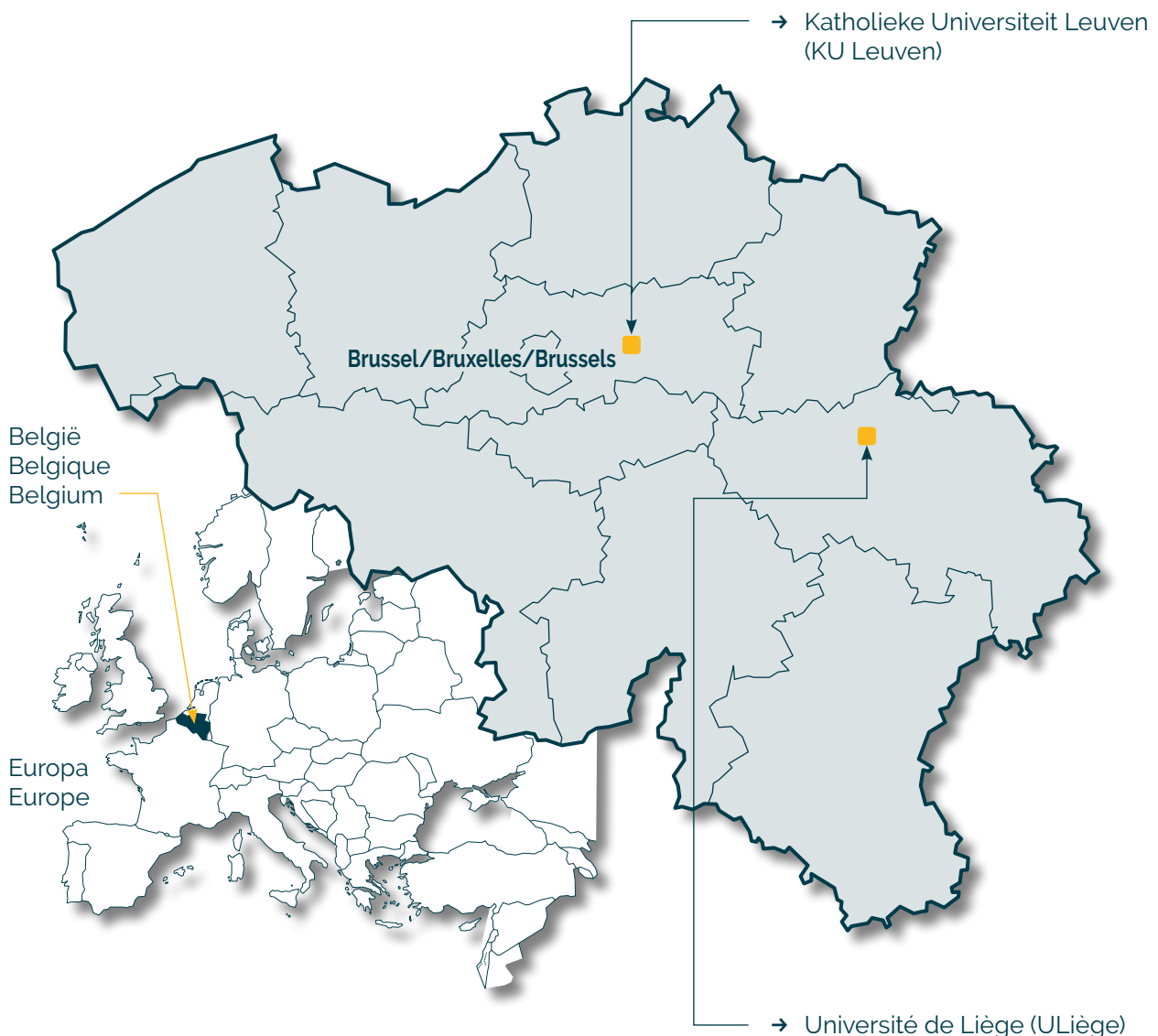
Projets de recherche universitaire
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Universiteiten met onderzoeksprogramma's die gesteund worden door de G.S.K.E.

Universités ayant des programmes de recherche subventionnés par la F.M.R.E.

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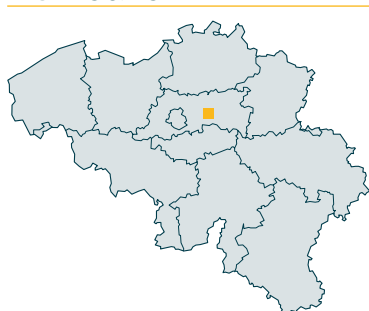


Universitaire onderzoeksprojecten 2023-2025 gefinancierd door de G.S.K.E.

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KU Leuven



Prof. dr. Bart De Strooper (VIB)

A vicious A β oligomers-dependent neuron-microglia cycle fuels Alzheimer's Disease

Prof. dr. Pierre Vanderhaeghen (VIB)

Deciphering the mechanisms underlying neurogenesis defects in mitochondrial diseases

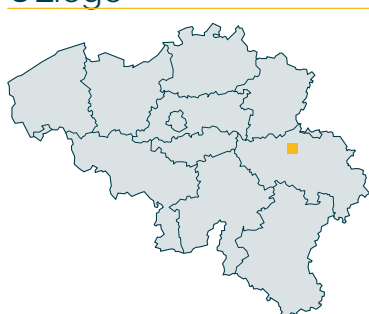
Prof. dr. Veerle Baekelandt

The role of LRRK2 in the peripheral immune system and gut-to-brain spreading of alpha-synuclein pathology in Parkinson's disease

Prof. dr. Thomas Voets

Unraveling the etiology of TRPM3-dependent neurodevelopmental disorders

ULiège



Prof. dr. Pierre Maquet

Quantitative MRI at 7 Tesla addresses ten questions about brain small vessel diseases



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Final report of the university research project of

Prof. dr. Bart De Strooper
Katholieke Universiteit Leuven
(KU Leuven-VIB)

Prof. dr. Bart De Strooper (KU Leuven-VIB)
VIB Center for Brain and Disease Research
Herestraat 49
3000 Leuven
Belgium
T +32 495 77 10 44
Bart.Destrooper@vib.be

A vicious A β oligomers-dependent neuron-microglia cycle fuels Alzheimer's Disease

1. Project overview

The overall aim of this project is to understand how interactions between neurons and microglia contribute to Alzheimer's disease (AD) pathology, with a particular focus on the role of soluble A β oligomers and the mechanisms through which anti A β immunotherapies modify neuroimmune crosstalk. Increasing evidence indicates that soluble A β species perturb neuronal activity and initiate a pathological dialogue between neurons and microglia that contributes to synaptic dysfunction, neuroinflammation, and ultimately neurodegeneration.

During the funding period 2023–2025, our work has focused on three major questions:

1. How soluble A β species influence communication between neurons and microglia in early stages of AD.
2. Whether microglia are required for the neurotoxic effects of soluble A β on neurons and synapses.
3. How anti A β immunotherapies modify microglial responses and neuronal pathology in a humanized experimental context.

In parallel, the project contributed to broader discoveries regarding glial contributions to AD pathology, including the identification of disease associated microglial states, the role of astrocytic APOE isoforms in amyloid deposition, and the identification of necroptosis as a mechanism of neuronal death in AD.

2. Progress during the reporting period

2.1. Neuron to microglia communication in early Alzheimer's disease

A central hypothesis of the project is that soluble A β species perturb neuronal activity and thereby influence microglial behavior. Increased neuronal activity is one of the earliest functional abnormalities observed in AD patients and experimental models. We therefore investigated whether neuronal hyperactivity could influence microglial motility and transcriptional activation. Initial experiments aimed to monitor human microglial dynamics using two photon imaging in xenotransplanted mice.

We have begun manipulating neuronal activity and neuron to glia signaling pathways and subsequently analyzing microglial transcriptional states using single cell transcriptomics. These experiments aim to determine whether neuronal perturbations are sufficient to induce the cytokine and chemokine response microglial (CRM) state that we previously identified as a response to soluble A β oligomers.

2.2. Microglia as mediators of A β induced neuronal dysfunction

A key unresolved question in AD research is whether soluble A β oligomers directly damage neurons or whether their neurotoxicity is mediated through microglial activation. To address this question we combined pharmacological microglial depletion with functional analyses of neuronal activity.

Using mouse models in which microglia were pharmacologically depleted, we examined the effects of soluble A β aggregates injected into the medial prefrontal cortex. Removal of microglia significantly attenuates neuronal damage as measured by NeuN staining, suggesting that microglia are necessary mediators of A β induced neurotoxicity.

Analysis of synaptic density revealed that soluble A β causes significant synaptic loss in control animals. In contrast, mice depleted of microglia did not show a comparable synaptic reduction, indicating that microglial responses contribute substantially to A β induced synaptic pathology.

2.3. Mechanisms of anti A β immunotherapy

Another important goal of the project was to understand how therapeutic antibodies targeting A β modify microglial responses and neuronal pathology.

Using sequences derived from patent literature we generated recombinant forms of four antibodies that have been tested in clinical trials: Lecanemab, Aducanumab, Gantenerumab, and Donanemab. Biochemical analysis using ELISA and dot blot assays demonstrated that Lecanemab and Gantenerumab show strong binding to aggregated A β species. Aducanumab displayed lower affinity under our experimental conditions, while Donanemab showed little binding to the synthetic A β aggregates used in our assays, consistent with its specificity for pyroglutamate modified A β found in dense core plaques.

Immunohistochemical analysis in AppNL G F mice confirmed that Lecanemab shows robust binding to amyloid deposits in vivo. Based on these results and the promising clinical data reported for this antibody, we focused subsequent mechanistic studies on Lecanemab.

We performed chronic Lecanemab treatment in mice transplanted with human induced pluripotent stem cell derived microglia. After eight weeks of treatment we observed a significant reduction in plaque load compared with animals receiving control antibodies.

Importantly, Lecanemab treatment not only reduced amyloid pathology but also improved neuronal health. Treated animals showed reduced neuritic dystrophy, improved synaptic density around plaques, and improved cognitive performance in behavioral assays.

Single cell RNA sequencing of human microglia isolated from treated animals revealed a strong enrichment of disease associated microglial states associated with phagocytosis and amyloid clearance. This activation was confirmed at the protein level. In vivo phagocytosis assays further demonstrated that microglia actively engulf A β in the presence of Lecanemab.

2.4. Identification of disease associated human microglial states

A major methodological advance during this project was the generation of a large single cell transcriptomic dataset of human microglia transplanted into mouse models of amyloid pathology. In total we analyzed more than 138,000 single cell transcriptomes from human stem cell derived microglia xenotransplanted into AppNL G F mice and control animals. These analyses demonstrated that human microglia adopt a disease associated activation state in response to amyloid plaques that resembles but is not identical to the states observed in mouse models.

A prominent feature of the human microglial response was strong expression of human leukocyte antigen genes, indicating an important role for antigen presentation in the human immune response to amyloid pathology. We also identified a cytokine and chemokine response microglial state induced by oligomeric A β .

Importantly, genetic variants associated with AD risk significantly altered these responses. Microglia carrying variants in APOE or TREM2 showed distinct activation patterns, highlighting the importance of genetic background in shaping microglial responses and influencing disease progression.

2.5. Microglia as regulators of amyloid plaque dynamics

Through genetic and pharmacological approaches we further examined the role of microglia in the formation and evolution of amyloid plaques.

Depletion of microglia prior to plaque formation significantly reduced plaque deposition and neuritic dystrophy. However, transplantation of human microglia restored plaque formation, demonstrating that microglia actively contribute to plaque initiation.

Interestingly, microglial depletion reduced insoluble A β levels but did not significantly alter soluble A β concentrations. This observation challenges the prevailing view that microglia primarily act as A β clearing cells and instead suggests that they influence the aggregation and compaction of amyloid.

At later disease stages microglial depletion resulted in less compact plaques and increased neuritic dystrophy, indicating that microglia also play a protective role by restricting plaque expansion and limiting neuronal damage.

Microglia carrying the AD risk variant TREM2 R47H exacerbated plaque pathology, further emphasizing the importance of microglial activation states in disease progression.

2.6. Astrocytic APOE as a driver of amyloid pathology

Another major discovery during the project concerns the role of astrocytes in regulating amyloid pathology through APOE expression.

Using an ApoE deficient AD mouse model we expressed human APOE isoforms selectively in astrocytes. Astrocytic expression of APOE was sufficient to restore amyloid plaque formation and to induce transcriptional changes in nearby microglia.

Interestingly, both APOE4 and the protective APOE2 isoform increased fibrillar plaque deposition, although they had distinct effects on soluble A β aggregates. These findings identify astrocytes as central regulators of APOE dependent amyloid pathology and demonstrate that astrocyte derived signals strongly influence microglial activation states.

2.7. Necroptosis as a mechanism of neuronal death in Alzheimer's disease

Beyond amyloid pathology, we investigated mechanisms of neuronal death associated with AD progression. Our work identified necroptosis as an important contributor to neuronal loss.

Markers of necroptosis accumulate in granulovacuolar degeneration vesicles and are closely associated with Tau pathology. We identified a neuron specific pathway in which activation of the necrosome appears linked to endolysosomal dysfunction and granulovacuolar degeneration.

Experiments in mouse models and neuronal cultures demonstrated that necroptosis related proteins are strongly upregulated in APP Tau transgenic mice and in neurons exposed to phosphorylated Tau seeds. In contrast, APP models with A β deposition alone did not show comparable activation of this pathway.

Importantly, pharmacological inhibition of necroptosis significantly reduced neuronal loss in experimental models. These findings suggest that necroptosis represents a delayed but therapeutically targetable mechanism of neurodegeneration in AD.

3. Conclusions

During the 2023–2025 funding period this project has significantly advanced our understanding of neuron glia interactions in Alzheimer's disease.

Major contributions include:

- Demonstration that microglia are essential mediators of soluble A β induced neuronal dysfunction.
- Identification of human microglial activation states triggered by amyloid pathology and modulated by AD risk genes.
- Evidence that microglia play dual roles in amyloid pathology by promoting plaque formation while also limiting plaque associated damage.
- Discovery that astrocyte derived APOE isoforms regulate amyloid deposition and influence microglial activation.
- Identification of necroptosis as a potential therapeutic target for preventing neuronal loss.

- Development of humanized experimental models that reveal how anti A β antibodies such as Lecanemab modify microglial responses and amyloid pathology.

Together these findings provide important mechanistic insights into the cellular processes that drive AD progression and identify new opportunities for therapeutic intervention targeting glial responses and neuronal survival.

4. Publications:

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2. Sanjuan-Ruiz I, Serneels L, Craessaerts K, Goate A, Annaert W, Ch vez-Guti rrez L, Shi Y, Sheikh-Bahaei N, Jen JC, Ramos EM, Campan M, Ward PM, Magaki S, Bartlone K, Vinters HV; Dominantly Inherited Alzheimer Network; Craig DW, Ringman JM, De Strooper B. The curious case of a heterozygous loss-of-function <i>PSEN1</i> variant associated with early-onset Alzheimer's disease. *Mol Neurodegener Adv*. 2025;1(1):4. IF and Q not known yet (new journal)
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9. De Strooper B, Haass C, Hardy J, Zetterberg H. Divisions over the regulation of lecanemab. *Lancet*. 2024 Nov 30;404(10468):2160-2161. IF: 98.4; Q1 (but is only a letter)
10. Preman P, Moechars D, Fertan E, Wolfs L, Serneels L, Shah D, Lamote J, Poovathingal S, Snellinx A, Mancuso R, Balusu S, Klenerman D, Arranz AM, Fiers M, De Strooper B. APOE from astrocytes restores Alzheimer's A β -pathology and DAM-like responses in APOE deficient microglia. *EMBO Mol Med*. 2024 Dec;16(12):3113-3141. IF 9.0 Q1
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Geneeskundige Stichting Koningin Elisabeth
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Prof. dr. Pierre Vanderhaeghen
Katholieke Universiteit Leuven
(KU Leuven-VIB)

Prof. dr. Pierre Vanderhaeghen (KU Leuven-VIB)
VIB Center for Brain & Disease Research
Department of Neurosciences, KU Leuven
Stem Cell and Developmental Neurobiology Lab
O&N5 Herestraat 49 box 602 | 3000 Leuven | Belgium
Email: pierre.vanderhaeghen@kuleuven.be
T +32 16 3 77038
F +32 16 3 30827
<https://pvdhlab.org>

Deciphering the mechanisms underlying neurogenesis defects in mitochondrial diseases.

1. State of the art and objectives

Mitochondrial dysfunction can result in various forms of neurodevelopmental diseases (NDD), from microcephaly to intellectual disability. This suggests important roles for mitochondria in neural development, but these have remained poorly known, as their links with diseases. We had previously shown that mitochondria dynamics play a key role in neurogenesis of mouse and human NSPCs of the cerebral cortex ^{1,2}. We also identified intriguing differences in this process between mouse and human cortical cells, which could be linked to increased cortical size and complexity in our species.

In this project we aimed to study the molecular mechanisms by which mitochondria dynamics and metabolic activity impact neuronal fate acquisition, and relate these to human corticogenesis in normal and pathological conditions.

This data obtained in this project, as detailed below, led to deep mechanistic insights on mitochondria function during neural development, and will enable to build a robust and diverse pipeline of analysis of mitochondria/metabolic defects in human neural development.

2. Results

In the frame of this project we have first shown that mitochondria metabolism exerts a direct effect on neuronal differentiation in a species-specific fashion, not only *in vitro* but also *in vivo* in xenotransplanted human cortical neurons ³. We are now focusing on the downstream mechanisms by implementing epigenetic and proteomic analyses to our systems in control conditions and following metabolic reprogramming ^{4,5}. We first found that protein turnover is two-fold lower human neurons than in mouse counterparts, and that manipulation of mitochondria does not seem to alter these differences. Next we obtained interesting evidence that ETC activity is higher in mouse than human cortical progenitors, while it also increases with developmental time in the mouse. As these differences could lead to distinct NAD⁺/NADH metabolism, we then implemented sonar imaging ⁶, to test this during neuronal fate acquisition, in relation to timing differences, species differences, or both. We first examined the dynamics of NAD⁺ and NADH levels during mouse neurogenesis using NAD⁺/NADH ratio sensor, SoNar, using time-lapse imaging of mouse cortical progenitors in *ex vivo* cultures (prepared from mouse embryonic cortex at E14). By implementing this approach, we found that there is a rapid pre-mitotic increase and post-mitotic decrease in NAD⁺/NADH ratios in self-renewing cortical progenitors. We next compared them in self-renewing and neurogenic divisions, but could not find significant differences.

We thus turned to other mitochondria-derived metabolites, and discovered that the generation of Acetyl-CoA has a dramatic effect on the timing of corticogenesis in human cortical organoid models ⁷⁻⁹. Given the association of Ac-CoA with protein acetylation, we next tested its impact on Histone acetylation, and found a direct relationship between fatty acid oxidation, Acetyl-coA production, and Histone H4K16 levels in cortical progenitors and neurons. Moreover we obtained similar results during neuronal differentiation, thus extending the impact of metabolism of all aspects of corticogenesis, from early to later stages, through the epigenetic regulation of cortical gene expression ⁸.

Finally we tested whether and how acceleration of neuronal developmental timing of human neurons actually impacts their functional properties. In this context we have recently obtained the important proof of principle that acceleration of synaptogenesis, obtained by loss of function of the SYNGAP1 synaptic protein, can lead to precocious responsiveness to visual stimulation and altered plasticity of human neurons^{10,11}, as we previously hypothesized. We are now pursuing these findings by testing the direct impact of metabolism on neuronal functional development and plasticity, and plan to explore the relationships between mitochondria metabolism and SYNGAP1.

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- Mitochondria Metabolism Sets the Species-Specific Tempo of Neuronal Development. Iwata R., Casimir P., Erkol E., Boubakar L., Planque M., Ditekowska M., Vints K., Gaspariunaite V., Bird M., Corthout N., Vermeersch P., Davie K., Gounko V., Aerts S., Ghesquière B., Fendt S-M, [Vanderhaeghen P](#). *Science* (2023) 379, eabn4705 DOI: 10.1126/science.abn4705. IF: 41
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Final report of the university research project of

Prof. dr. Veerle Baekelandt
Katholieke Universiteit Leuven (KU Leuven)

Prof. dr. Veerle Baekelandt (KU Leuven)
Laboratory for Neurobiology and Gene Therapy
Division of Molecular Medicine K.U.Leuven –
Faculty of Medicine Kapucijnenvoer 33 VCTB+5
B-3000 Leuven
Belgium
Tel.: +32 16 37 40 61
Fax: +32 16 33 63 36
E-mail: Veerle.Baekelandt@kuleuven.be

The role of LRRK2 in the peripheral immune system and gut-to-brain spreading of alpha-synuclein pathology in Parkinson's disease

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder. The neuropathological hallmarks consist of dopaminergic (DA) neuron loss in the *substantia nigra pars compacta* (SNpc) and cytoplasmic alpha-synuclein (α Syn) aggregates, termed Lewy bodies. **Cell-to-cell transmission** of misfolded α Syn has been proposed to account for the pathology stages described by Braak^{1,2}, suggesting that synucleinopathy might start in the periphery within the gastrointestinal tract (GI) and progress towards the brain via the vagus nerve. Mutations and variations in **LRRK2** (leucine-rich repeat kinase 2) are the most common cause of both familial and sporadic forms of PD³. Most pathogenic mutations are associated with enhanced kinase activity, thus making LRRK2 a very attractive therapeutic target⁴.

However, many questions remain, especially on how LRRK2 gain-of-function affects disease pathophysiology and how it correlates with PD.

We can summarize the findings of the project supported by GSKE in two main projects, which have been reported in two publications in *Acta Neuropathologica*^{5,6}.

Summary project 1 Mutant LRRK2 exacerbates immune response and neurodegeneration in a chronic model of experimental colitis.

The link between the gut and the brain in PD pathogenesis is currently a subject of intense research. Indeed, gastrointestinal dysfunction is known as an early symptom in PD and inflammatory bowel disease (IBD) has recently been recognised as a risk factor for PD. The leucine-rich repeat kinase 2 (LRRK2) is a PD- and IBD-related protein with highest expression in immune cells. We have completed a study to provide evidence for a central role of LRRK2 in gut inflammation and PD. The presence of the gain-of-function G2019S mutation significantly increased the disease phenotype and inflammatory response in a mouse model of experimental colitis based on chronic dextran sulphate sodium (DSS) administration (Fig.1, 2). Bone marrow transplantation of wild-type cells into G2019S knock-in mice fully rescued this exacerbated response, proving the key role of mutant LRRK2 in immune cells in this experimental colitis model (Fig. 3). Furthermore, partial pharmacological inhibition of LRRK2 kinase activity also reduced the colitis phenotype and inflammation (Fig.3). Moreover, chronic experimental colitis also induced neuroinflammation and infiltration of peripheral immune cells into the brain of G2019S knock-in mice. Finally, combination of experimental colitis with overexpression of α -synuclein in the substantia nigra aggravated motor deficits and dopaminergic neurodegeneration in G2019S knock-in mice (Fig.5). Taken together, our results link LRRK2 with the immune response in colitis and provide evidence that gut inflammation can impact brain homeostasis and contribute to neurodegeneration in PD.

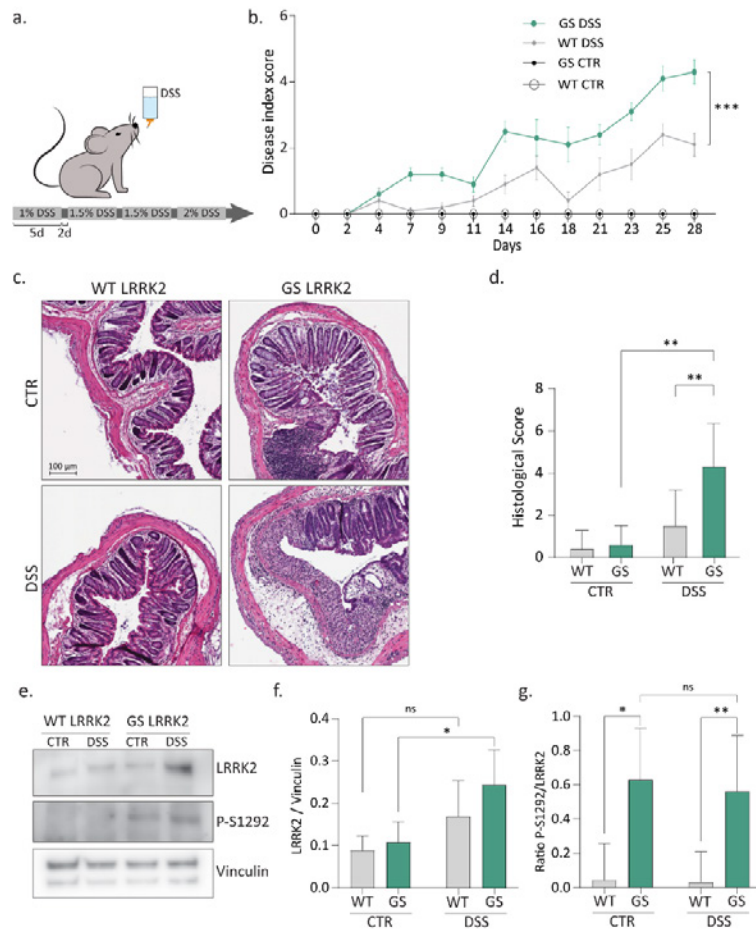


Figure 1 : The G2019S LRRK2 mutation increases the sensitivity to experimental colitis in mice. (a) DSS administration protocol. Mice received DSS in drinking water at increasing concentrations each time for 5 days with a pause of 2 days during one month. (b) Disease index score evaluation of stool consistency and haematochezia measured three times per week revealed a more severe phenotype in GS LRRK2 mice. (c) Representative image of Haematoxylin and Eosin (H&E) staining of the distal colon showing the damage to the epithelium and inflammation after DSS. Scale bar = 100 µm. (d) Histological scoring of the H&E images based on crypt architecture, infiltration of immune cells, presence of oedema and depletion of the goblet cells. Graphs represent mean $\pm$ SD. Statistical differences were assessed using two-way ANOVA and Tukey's post hoc test, $n = 10$ animals/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

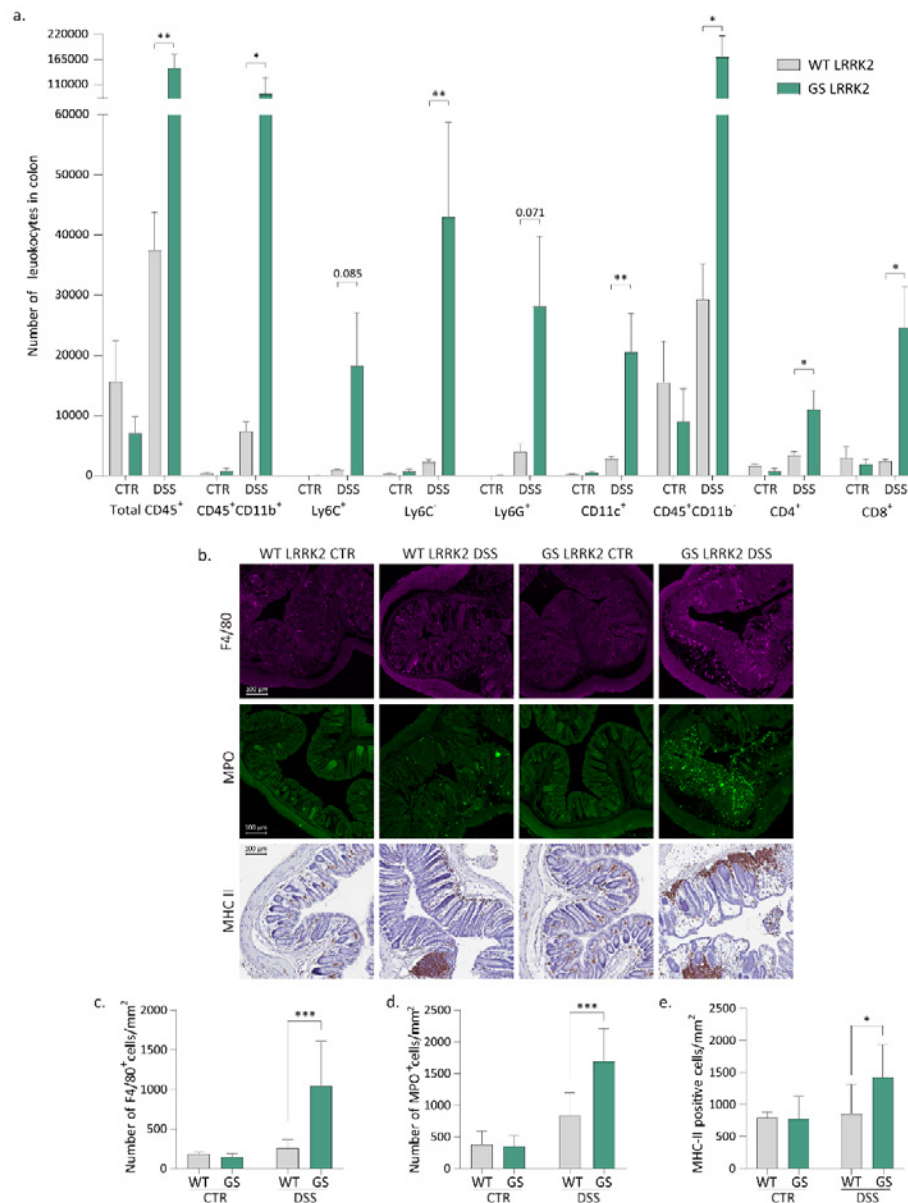


Figure 2. Increased inflammatory response in the colon of GS LRRK2 mice after DSS. (a) Flow cytometry immunophenotyping of the colon of WT and GS LRRK2 mice treated with DSS. Number of total leukocytes (CD45⁺), myeloid cells (CD45⁺ CD11b⁺), Ly6C⁺ and Ly6C⁻ monocytes, dendritic cells (CD11c⁺), neutrophils (Ly6G⁺), lymphocytes (CD45⁺ CD11b⁻) and T cells (CD8⁺ and CD4⁺) are shown. Graphs represent mean $\pm$ SD. Statistical differences were assessed using Student's t-test between WT and GS DSS groups, n = 5 (DSS), 2 (CTR) animals/group. *p < 0.05, **p < 0.01 (b) Immunostaining of paraffin-embedded distal colon sections. Representative images of macrophage (F4/80), neutrophil (MPO) and MHC II immunostaining in the distal colon of WT and GS LRRK2 mice. Quantification of macrophages (c), neutrophils (d) and MHC II expressing cells (e). Graphs represent mean $\pm$ SD. Statistical differences were assessed using two-way ANOVA and Tukey's post hoc test, n = 10 animals/group. *p < 0.05, ***p < 0.001.

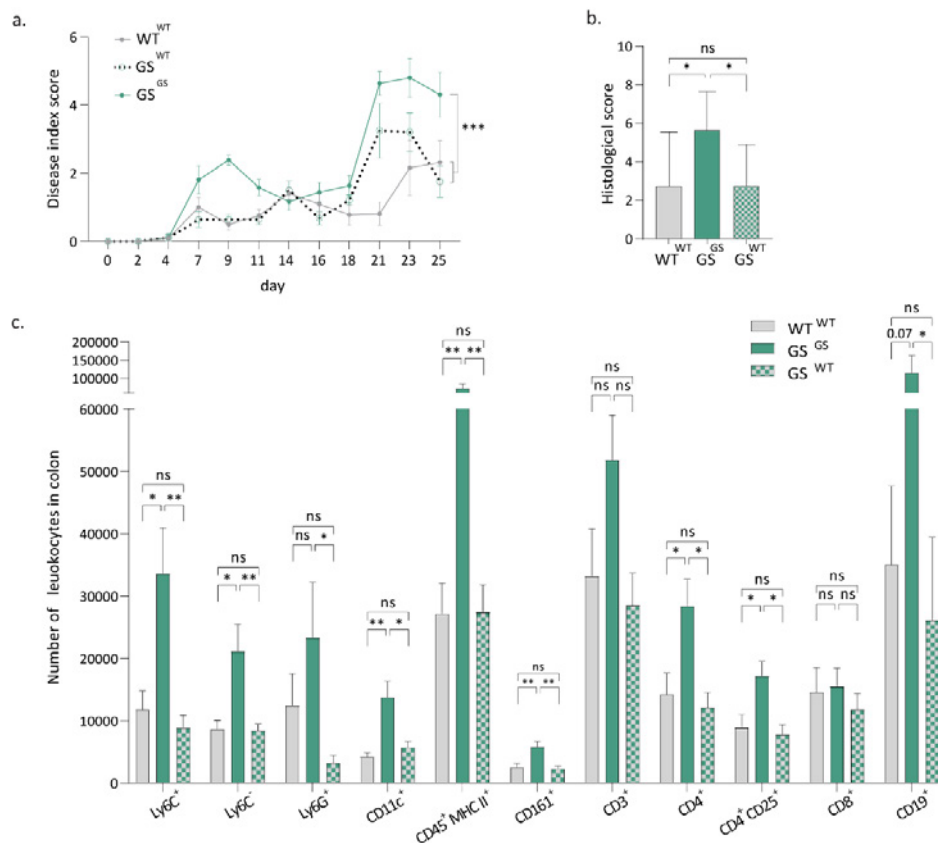


Figure 3. Bone marrow transplantation of wild type into GS LRRK2 mice reverts the susceptibility to DSS-induced colitis. (a) Disease index score of GS mice is normalised to WT levels after bone marrow transplantation from WT mice. Graph represents mean $\pm$ SD. Statistical differences were assessed using two-way ANOVA and Tukey's post hoc test, $n = 8-9$ animals/group. *** $p < 0.001$. (b) Histological score of GS mice is reduced to WT levels after bone marrow transplantation. Graph represents mean $\pm$ SD. Statistical differences were assessed using one-way ANOVA and Tukey's post hoc test, $n = 8-9$ animals/group. * $p < 0.05$. (c) Colonic immunophenotyping by flow cytometry of the GS^{WT} as well as the WT^{WT} and GS^{GS} mice treated with DSS. Quantification of total myeloid cells (CD45⁺ CD11b⁺), Ly6C⁺ and Ly6C⁻ monocytes, neutrophils (Ly6G⁺), dendritic cells (CD11c⁺), MHC II⁺ antigen presenting cells, natural killer cells (CD161⁺), lymphocytes (CD45⁺ CD11b⁻), T cells (CD3⁺), CD4 T cells, CD25⁺ CD4 T cells, CD8 T cells and B cells (CD19⁺). Graphs represent mean $\pm$ SD. Statistical differences were assessed using one-way ANOVA and Tukey's post hoc test, $n = 8-9$ animals/group. * $p < 0.05$, ** $p < 0.01$.

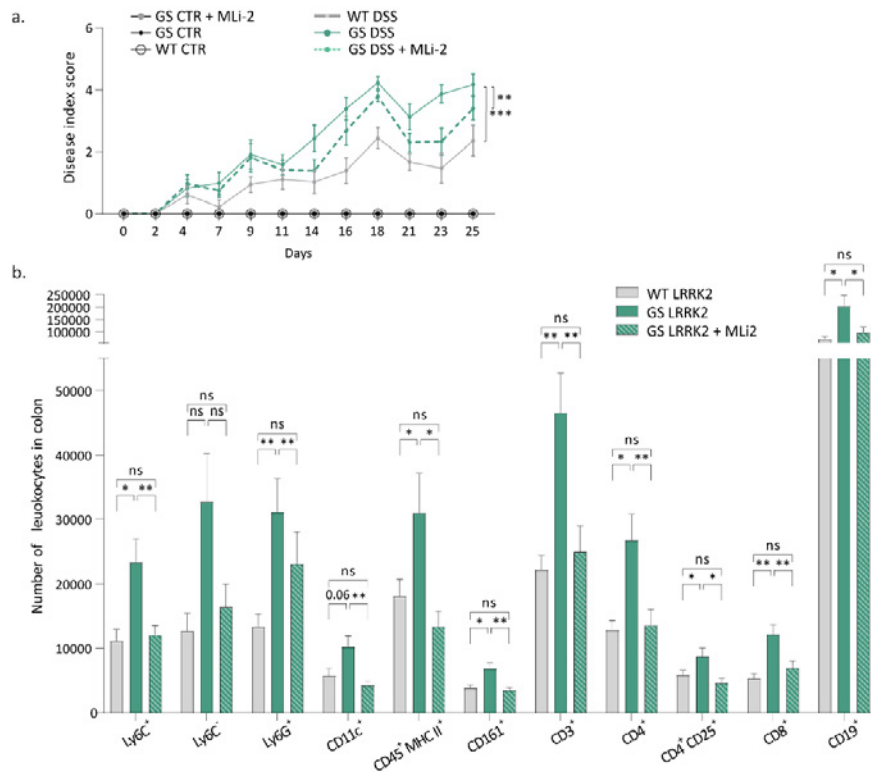


Figure 4. The LRRK2s kinase inhibitor MLI-2 partially rescues the enhanced susceptibility of GS LRRK2 mice to DSS. (a) Disease index score evaluation in WT and GS mice treated with placebo or MLI-2, as well as controls (CTR). Graph represents mean $\pm$ SD. Statistical differences were assessed using three-way ANOVA and Tukey's post hoc test, $n = 9-15$ animals/group. *** $p < 0.001$. (b) Colonic immunophenotyping by flow cytometry of GS mice treated with MLI-2 or placebo, as well as WT mice, after DSS administration. Quantification of total number of myeloid cells (CD45⁺ CD11b⁺), Ly6C⁺ and Ly6C⁻ monocytes, neutrophils (Ly6G⁺), dendritic cells (CD11c⁺), MHC II⁺ antigen presenting cells, natural killer cells (CD161⁺), lymphocytes (CD45⁺ CD11b⁺), T cells (CD3⁺), CD4 T cells, CD25⁺ CD4 T cells, CD8 T cells and B cells (CD19⁺). Graphs represent mean $\pm$ SD. Statistical differences were assessed using one-way ANOVA and Tukey's post hoc test, $n = 9-15$ animals/group. * $p < 0.05$, ** $p < 0.01$.

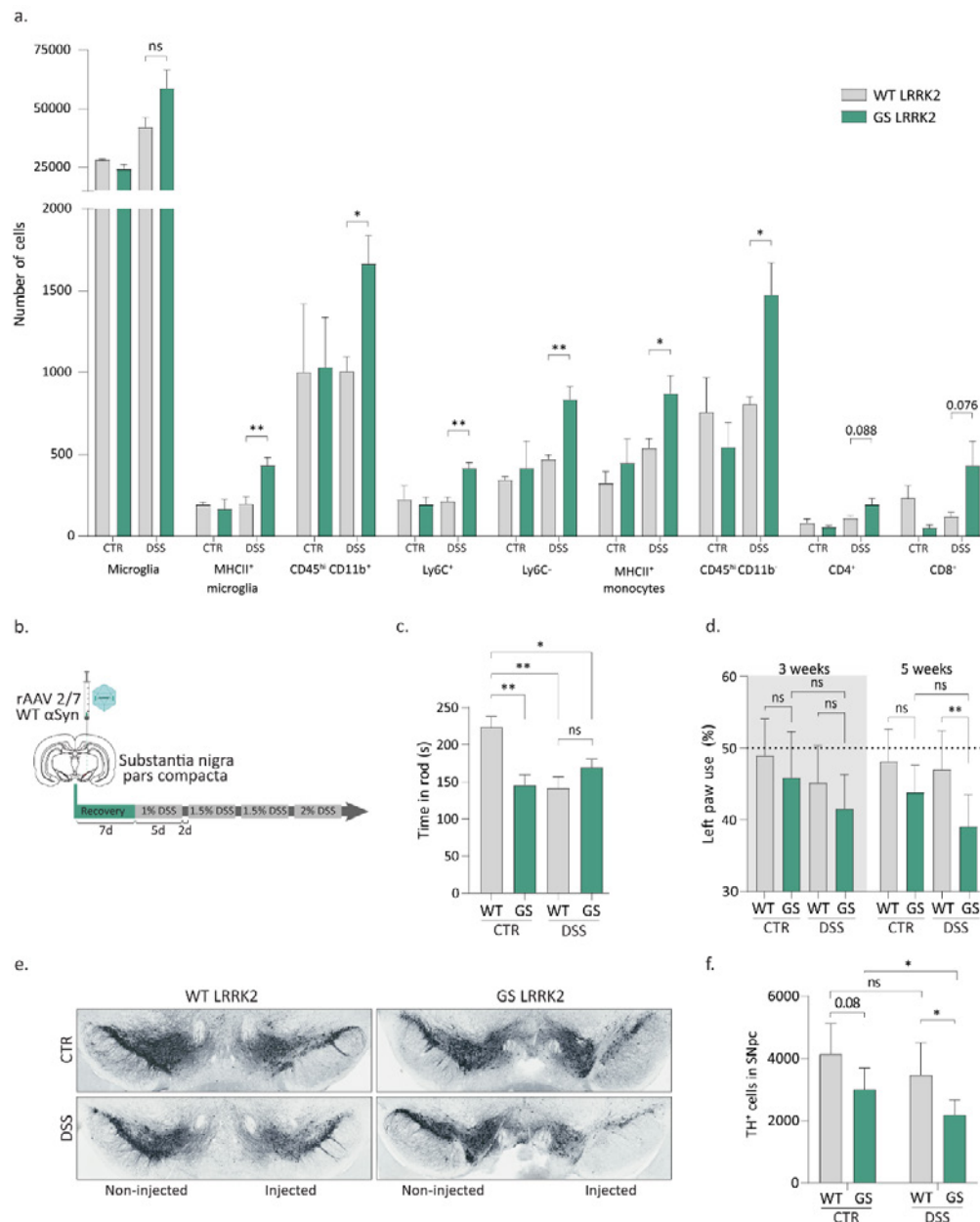


Figure 5. DSS-colitis aggravates α-synuclein-mediated dopaminergic neurodegeneration in GS LRRK2 mice. (a) DSS-colitis induces infiltration of immune cells in the brain of GS LRRK2 mice. Immunophenotyping of brain immune cells through flow cytometry. Graphs display number of microglia, MHC II expressing microglia, infiltrating myeloid cells (CD45^{hi} CD11b⁺), monocytes both Ly6C⁺ and Ly6C⁻, MHC II⁺ monocytes/macrophages and lymphocytes (CD45^{hi}, CD11b⁺), including CD4⁺ and CD8⁺ T cells. Graphs represent mean ± SD. Statistical differences were assessed using Student's t-test between WT and GS DSS groups, n = 5 (DSS), 2 (CTR) animals/group. *p < 0.05, **p < 0.01. (b) Experimental design. Mice were unilaterally injected with α-synuclein rAAV 2/7 in the substantia nigra. DSS was administered starting 7 days after surgery. (c) Rotarod test. Graph displays the time to fall from the rotarod 5 weeks post injection. (d) Cylinder test. Graph displays the % left forepaw use 3 and 5 weeks after α-synuclein rAAV injection in WT and GS mice with or without DSS (CTR). (e) TH immunostaining on coronal sections of the SN 5 weeks after α-synuclein rAAV injection in WT and GS mice with or without DSS (CTR). (f) Stereological quantification of TH⁺ cells in the right SN relative to the non-injected side 5 weeks p.i. Graphs represent mean ± SD. Statistical differences were assessed using two-way ANOVA and Tukey's post hoc test, n = 8-10 animals/group. *p < 0.05, **p < 0.01.

Summary project 2 The G2019S LRRK2 mutation exacerbates α-synuclein and tau neuropathology through divergent pathways in Parkinson's disease models

Aggregated α-synuclein (αSyn) is a pathological hallmark of Parkinson's disease (PD), yet other protein aggregates, including tau, are commonly observed in PD brains. This suggests that PD is not solely a synucleinopathy but may involve multiple, coexisting proteinopathies. Mutations in

LRRK2, particularly the G2019S (GS), are the most common cause of familial PD. LRRK2-PD has been associated with both α Syn and tau pathology, however the mechanistic links between LRRK2 dysfunction and protein aggregation remain incompletely defined. Here we opted to investigate whether LRRK2 contributes to α Syn and tau pathology through common molecular pathways or via distinct cellular mechanisms. Viral vector-mediated α Syn overexpression in GS LRRK2 knock-in mice led to enhanced dopaminergic neurodegeneration, increased phosphorylated α Syn levels (Fig.6), pronounced neuroinflammation (Fig.7,8), and accumulation of lysosomal proteins (Fig.9), suggesting impaired α Syn clearance and immune activation as key drivers. Human iPSC-derived dopaminergic neurons from GS LRRK2 PD patients mirrored these findings (Fig.10). In contrast viral vector-mediated overexpression of tau in GS LRRK2 knock-in mice promoted tau phosphorylation but did not significantly affect neuroinflammation, lysosomal markers, or neurodegeneration, indicating a primarily cell-autonomous mechanism (Fig. 11). Our results reveal a mechanistic divergence in how GS LRRK2 impacts α Syn and tau pathologies, supporting the notion that LRRK2 kinase activity contributes to PD pathogenesis through different pathways, thereby highlighting its potential as a therapeutic target in both familial and sporadic PD.

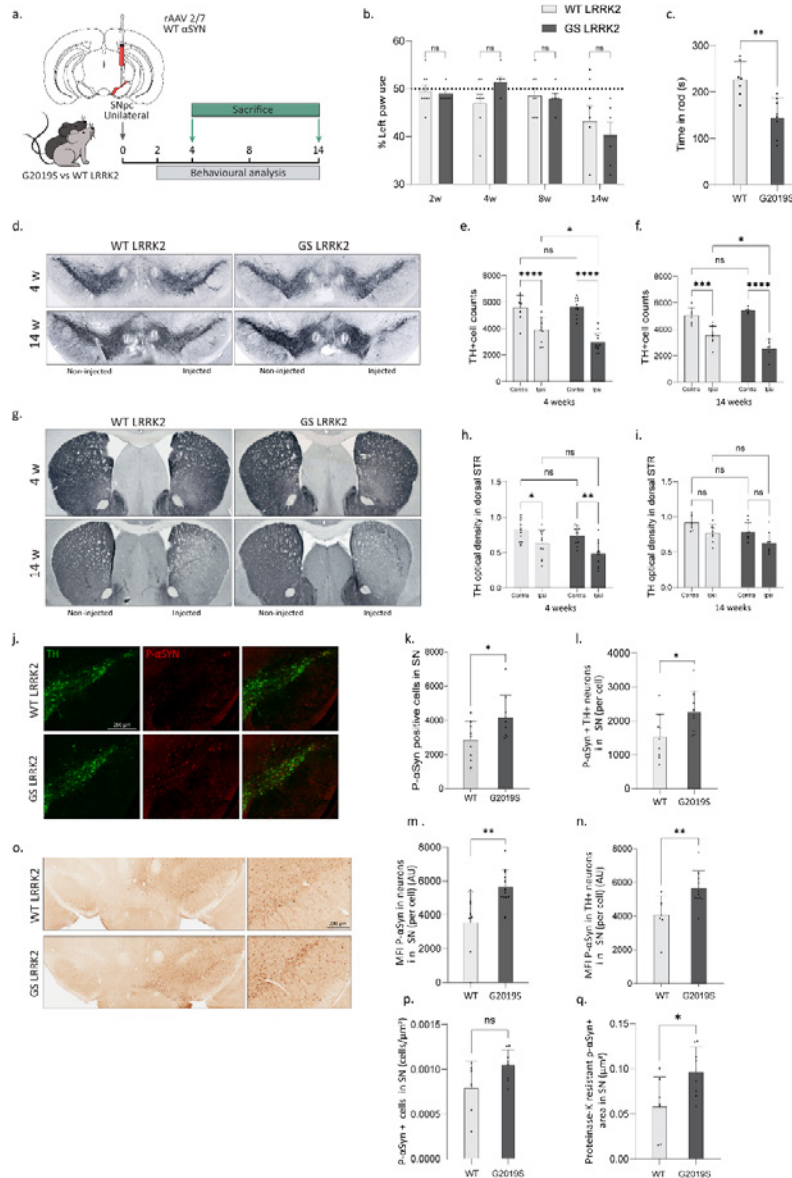


Figure 6. The G2019S LRRK2 mutation exacerbates α Syn neuropathology and neurodegeneration upon α Syn overexpression in the SN. (a) Schematic representation of the experimental set up. WT and GS LRRK2 mice were unilaterally injected with α Syn rAAV 2/7 in the SN. Mice were monitored behaviourally over time and sacrificed at 4 and 14 weeks post-injection. (b) Left forepaw use in the cylinder test for α Syn rAAV injected WT and GS LRRK2 mice. (c) Time to fall from the rotarod test 4 weeks post injection. (d) Representative images of TH immunostaining in the SN 4 and 14 weeks post-injection of α Syn rAAV 2/7 injected WT and GS LRRK2 mice. (e, f) Stereological quantification of the TH+ cells in the injected side and to the non-injected side 4 and 14 weeks post-injection. (g) Representative images of TH immunostaining in the STR 4 and 14 weeks post-injection of α Syn rAAV 2/7 in WT and GS LRRK2 mice. (h, i) Quantification of the optical density of the TH+ area in the dorsal striatum (STR) at 4 and 14 weeks post-injection in WT and GS LRRK2 mice. (j) Representative confocal images of TH+ (green) and pS129 α Syn+ (red) cells in the SN 4 weeks post-injection. Quantification of pS129 α Syn+ (k) and pS129 α Syn+ TH+ (l) cells in SN, as well as MFI of pS129 α Syn staining in neurons (m) and in TH+ neurons (n) of the SN. Representative images of proteinase K resistant pS129 α Syn immunostaining in the SN 4 weeks post-injection (o), and quantification of α Syn+ cells (p) and α Syn+ area (q) in the SN. N=11-13 at the 4 weeks time point from two independent experiments, and N=6-7 at the 14 weeks time point. Graphs represent mean $\pm$ SD. Statistical differences were assessed using unpaired Student's t-test or two-way ANOVA followed by Tukey's multiple comparison test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

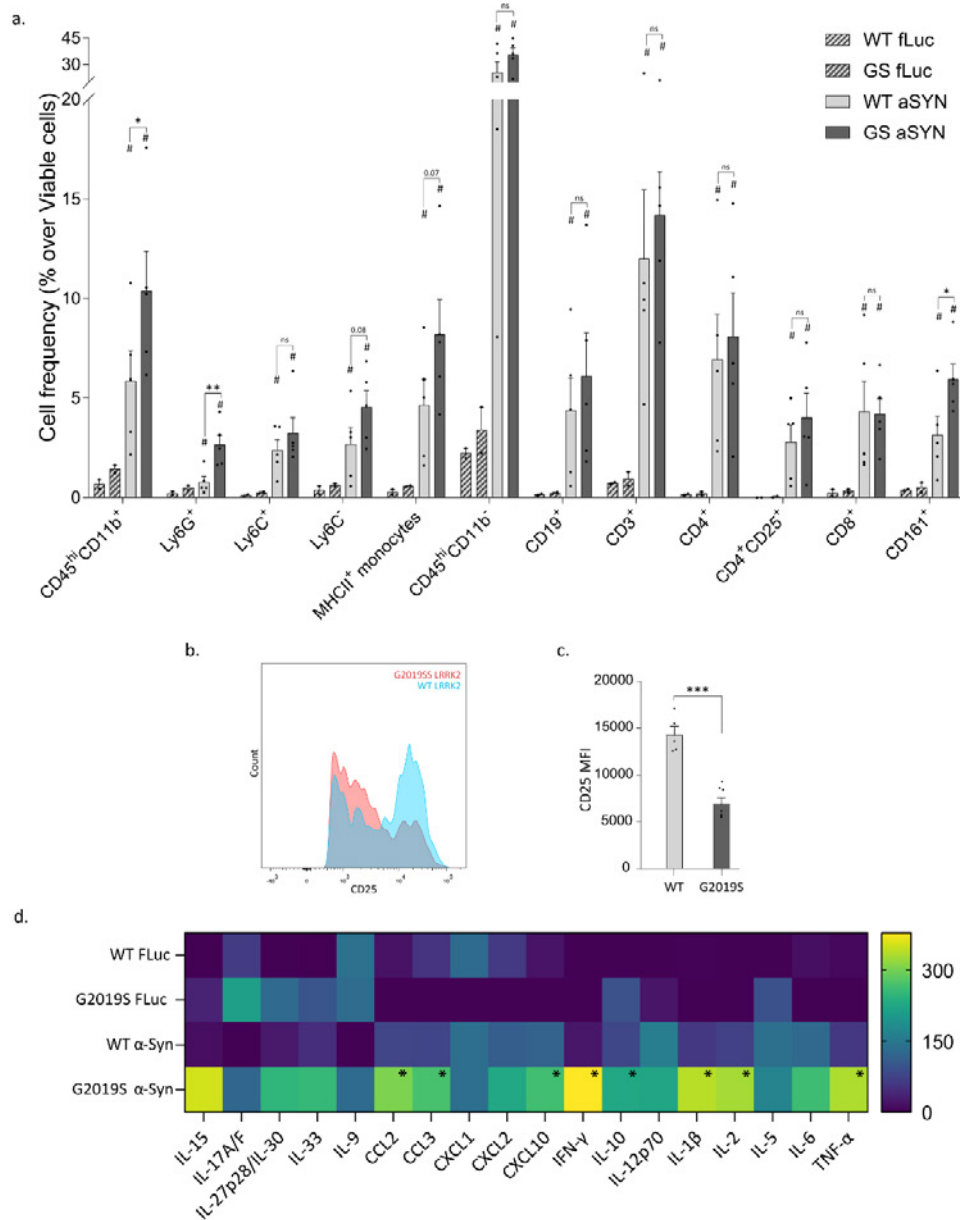


Figure 7. Increased inflammatory response in the SN of G2019S mice after αSyn rAAV 2/7. (a) Immunophenotyping by flow cytometry of WT and GS LRRK2 mice 4 weeks post injection of αSyn/fLuc rAAV 2/7. Number of myeloid cells (CD45⁺ CD11b⁺), neutrophils (Ly6G⁺), Ly6C⁺ and Ly6C⁻ monocytes, MHCII⁺ monocytes, lymphocytes (CD45⁺ CD11b⁺), B cells (CD19⁺), T cells (including CD3⁺ mature T cells, CD8⁺, CD4⁺ and CD4⁺ CD25⁺), and natural killer cells (CD161⁺). (b) Representative spectral emission profile of CD25 immunobinding and (c) quantification of CD25 immunoreactivity in CD4⁺ CD25⁺ cells in GS LRRK2 mice. (d) Quantification of pro-inflammatory cytokines in the injected SN 4 weeks post injection. Cytokines were measured in SN protein extracts from WT and GS LRRK2 mice after αSyn rAAV 2/7 injection and after control fLuc rAAV 2/7 injection. Increased levels of CCL2, CCL3, CXCL10, IFN-γ, IL-10, IL-1β, IL-2, and TNF-α were found in GS LRRK2 mice. N=5 and graphs represent mean ± SD. Statistical differences were assessed using unpaired Student's t-test between WT and GS LRRK2 αSyn rAAV 2/7 injected groups. *p < 0.05, **p < 0.01.

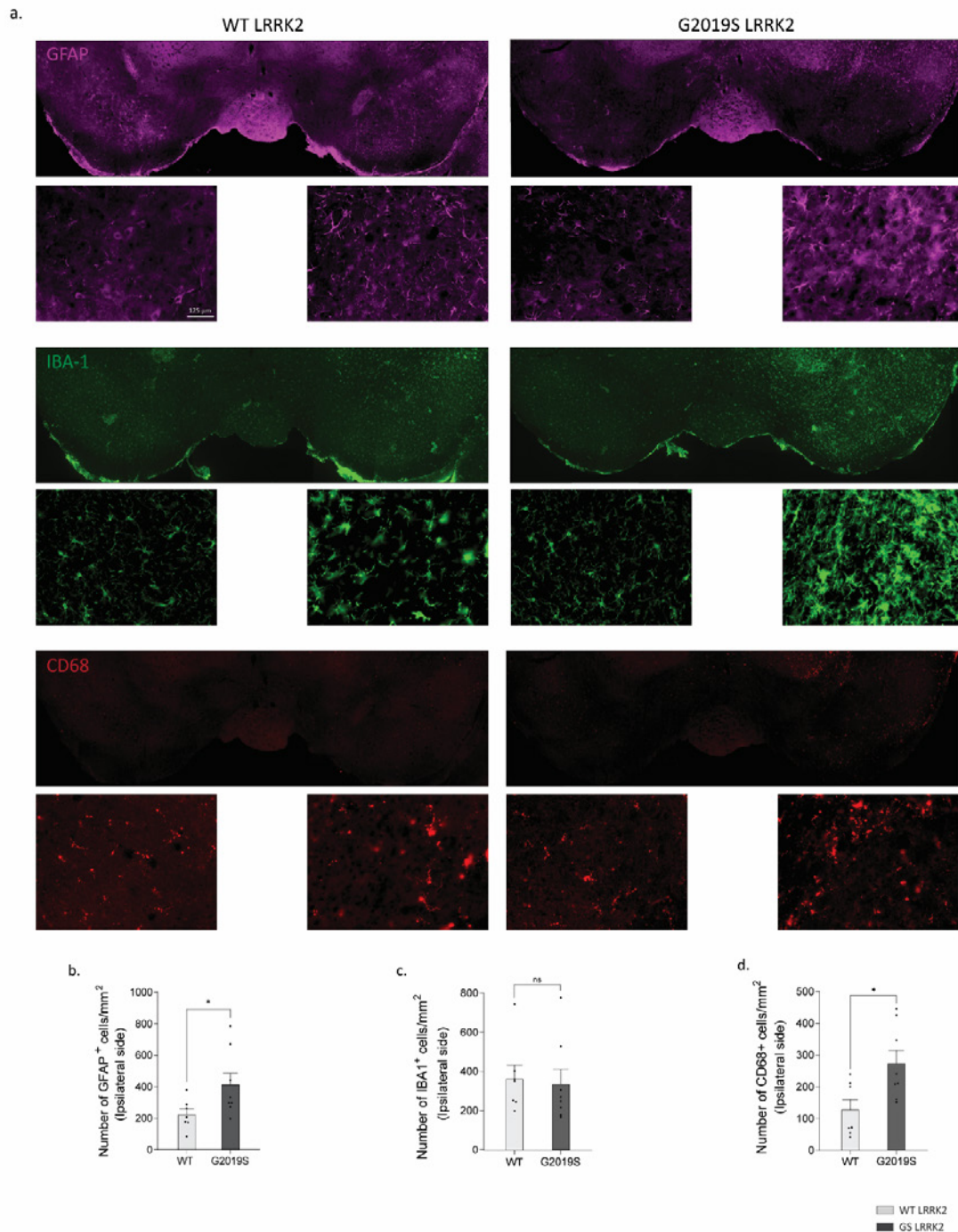


Figure 8. Immunohistochemical characterization of inflammatory response in the SN of G2019S mice after α Syn rAAV 2/7. Representative images (a) and quantification of astrocytes (GFAP) (b), microglia (IBA1) (c), and CD68 immunoreactivity (d) in the SN from WT and GS LRRK2 mice 4 weeks post injection. N=7-8 and graphs represent mean $\pm$ SD. Statistical differences were assessed using unpaired Student's t-test. * $p < 0.05$. Additional comparisons between animals of the same genotype injected with either α Syn rAAV2/7 or fLuc rAAV2/7 were performed using an unpaired Student's t-test. # $p < 0.05$ versus the respective fLuc group.

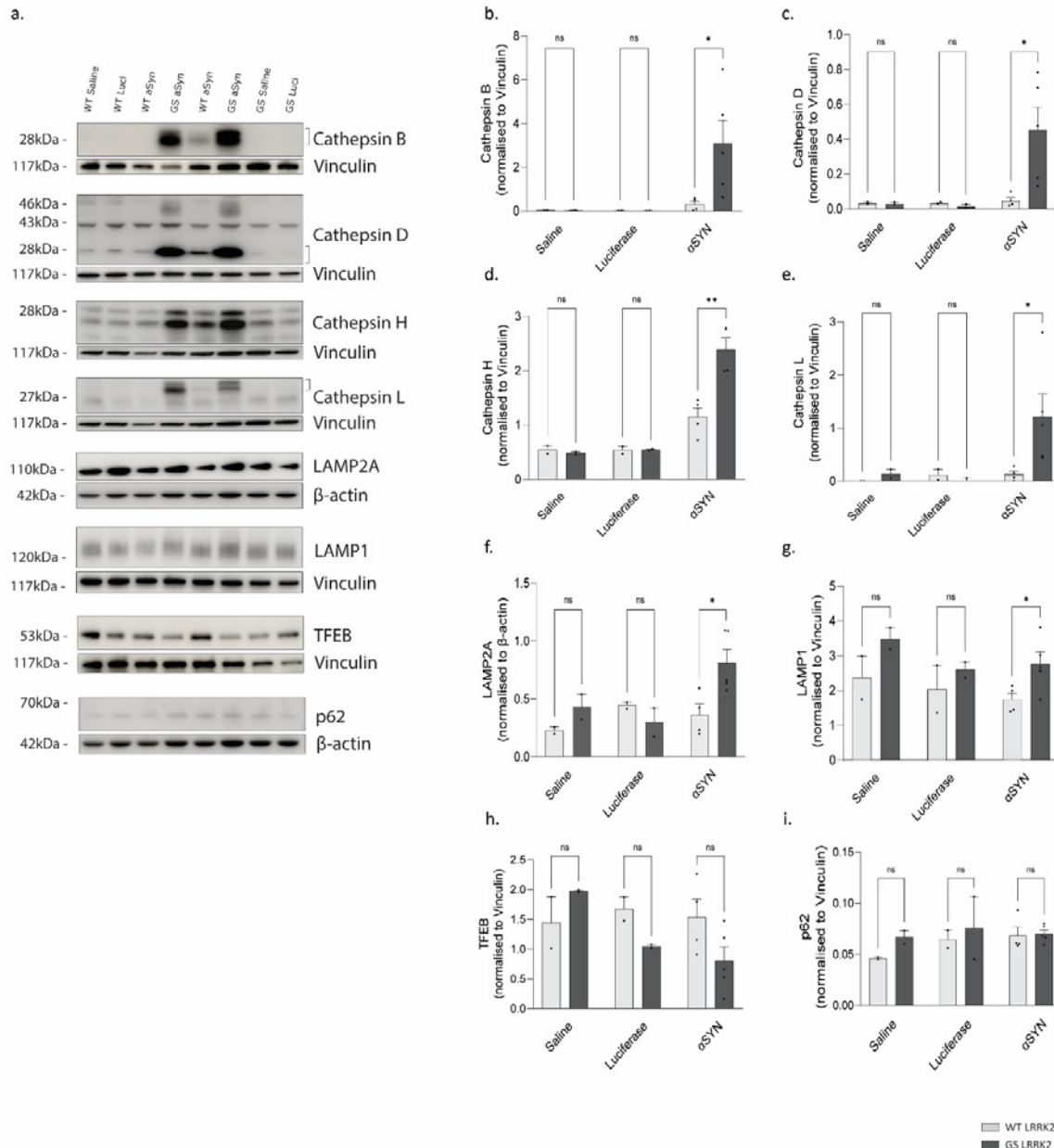


Figure 9. LRRK2 G2019S mice demonstrate lysosomal protein accumulation upon αSyn overexpression in SN. (a) Representative western blot images from whole SN protein extracts from WT and GS LRRK2 mice 4 weeks post injection with αSyn rAAV 2/7 and control groups (fLuc rAAV 2/7 or saline injected mice). Immunoblots for Cathepsin B,D,H,L and, Lamp2a, Lamp1, TFEB, and p62 are shown. (b-i) Quantification of immunoblots. Values were normalised to the endogenous protein (vinculin or β-actin) levels. N=4-5 for αSyn rAAV 2/7 groups and N=2 for the control groups. Graphs represent mean ± SD. Statistical differences were assessed using unpaired Student's t-test. *p < 0.05, *** p < 0.001

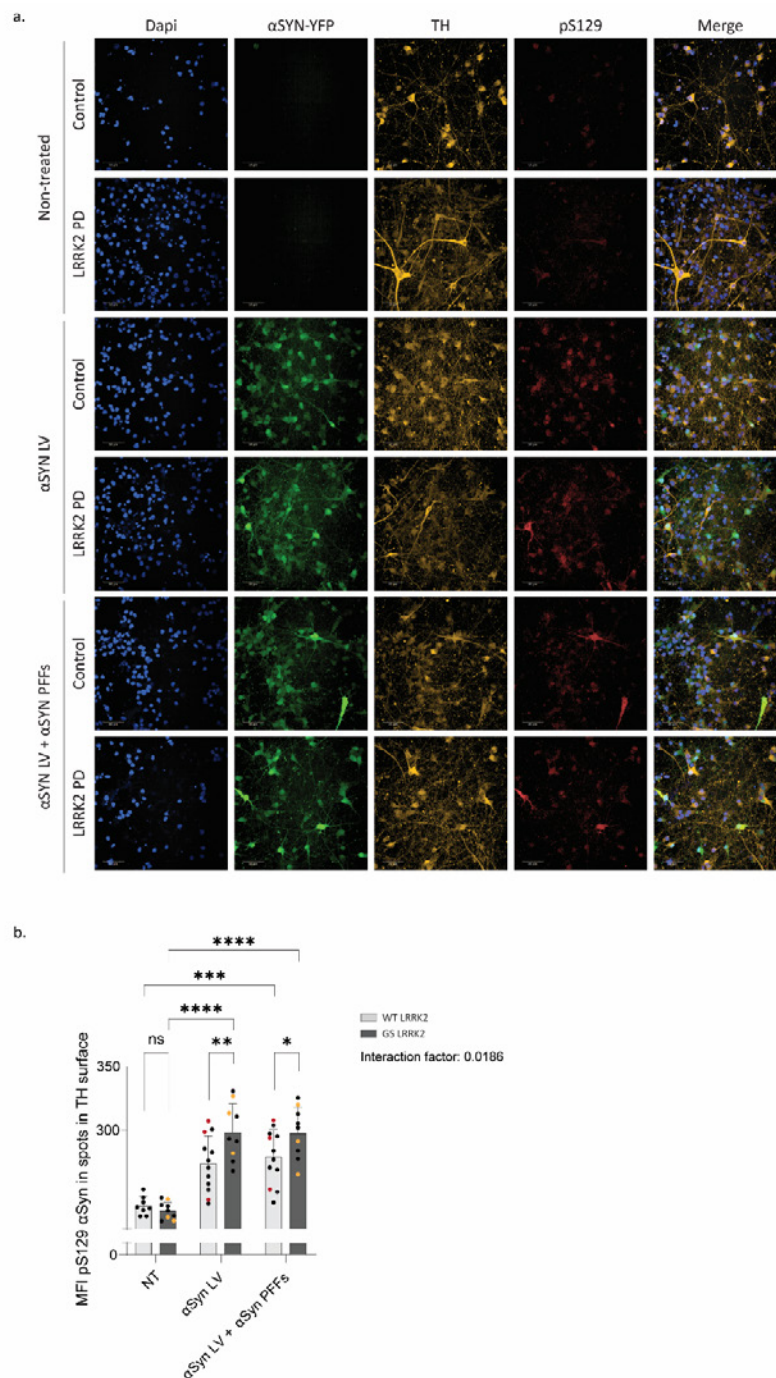


Figure 10. LRRK2 G2019S increases αSyn phosphorylation in human iPSC-derived dopaminergic neurons. (a) Representative fluorescence images of iPSC lines differentiated to dopaminergic neurons (DIV40) from patients carrying the GS LRRK2 mutation or controls (healthy control and corrected isogenic lines). Three experimental conditions are shown: non-treated cells (NT), cells transduced with αSyn-YFP LV, and cells transduced with αSyn-YFP LV followed by treatment with αSyn PFFs. Immunocytochemistry shown for TH, pS129 αSyn and αSyn-YFP in neurons transduced with LV to induced overexpression of αSyn-YFP. (b) Quantification of MFI of pS129 αSyn+ spots in TH+ neurons. Data points represent an iPSC line differentiated to dopaminergic neurons from an independent differentiation. N=3 iPSC-DaN lines, 3 differentiations. The orange-coloured spot indicates a GS LRRK2 PD line, while the red spot represents its isogenic control. Data are presented as mean ± SD. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparison test. A significant effect was observed for the treatment factor ($p = 0.0186$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

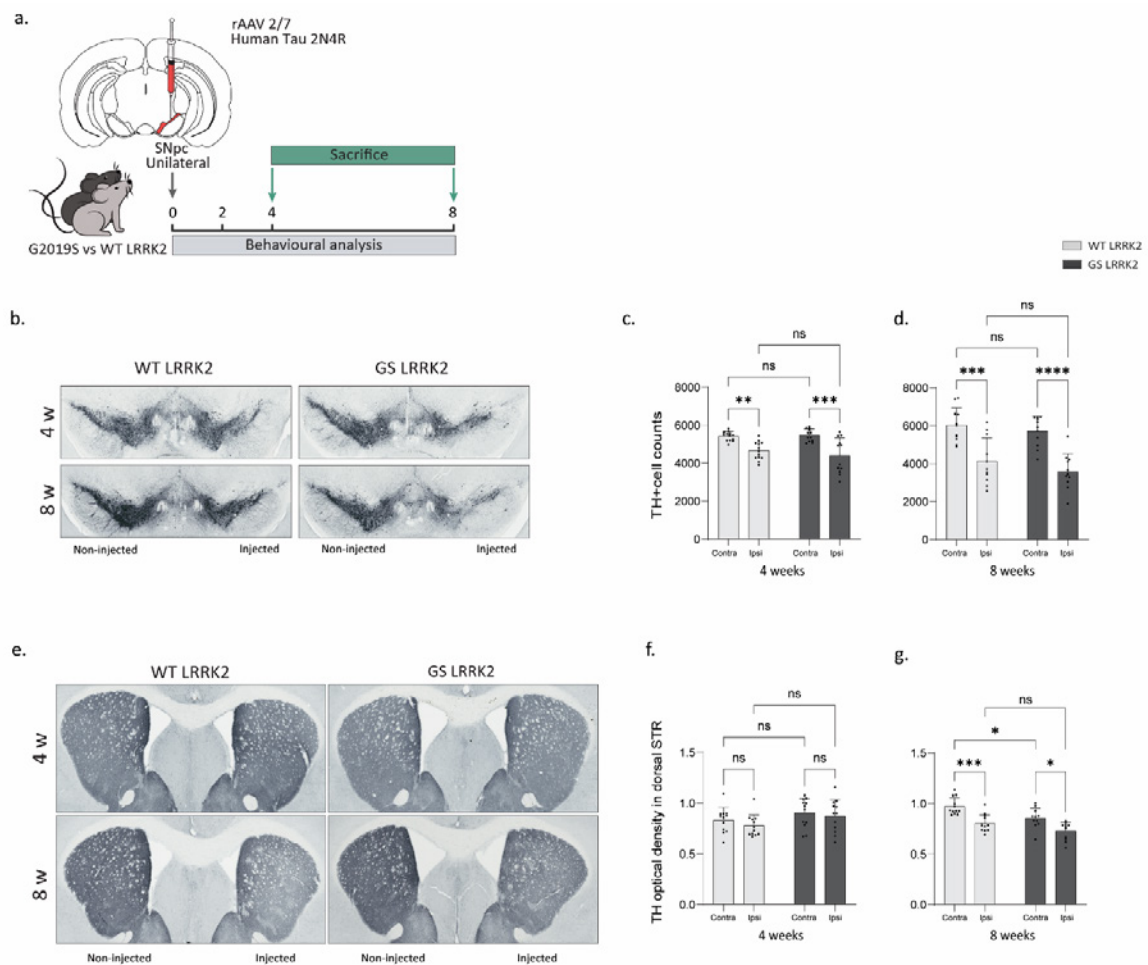


Figure 11. G2019S LRRK2 mice do not show exacerbated neurodegeneration upon tau overexpression in the SN. (a) Schematic representation of the experimental set up. Mice were unilaterally injected with tau rAAV 2/7 in the SN. (b) Representative images of TH immunostaining in the SN 4 and 8 weeks post-injection of tau rAAV 2/7 in WT and GS LRRK2 mice. (c) Stereological quantification of the TH⁺ cells in the ipsilateral and to the contralateral side 4 and 8 weeks post-injection. (d) Representative images of TH immunostaining in sections from the STR 4 and 8 weeks post-injection of tau rAAV 2/7 in WT and GS LRRK2 mice. (e) Quantification of the TH optical density in the STR at 4 and 8 weeks post-injection in WT and GS LRRK2 mice. N=12-13 at both the 4 and 8 weeks timepoint. Graphs represent mean $\pm$ SD. Statistical differences were assessed using two-way ANOVA followed by Tukey's multiple comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

2. Publications with Q.E.M.F. acknowledgement

- Cabezudo D, Tsafaras G, Van Acker E, Van den Haute C, Baekelandt V. (2023) Mutant LRRK2 exacerbates immune response and neurodegeneration in a chronic model of experimental colitis. *Acta Neuropathologica*. 146(2):245-261. (Q1 Neurosciences, IF 15.9, cit 22)
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Geneeskundige Stichting Koningin Elisabeth
Fondation Médicale Reine Elisabeth
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Queen Elisabeth Medical Foundation

Final report of the university research project of

Prof. dr. Thomas Voets
Katholieke Universiteit Leuven (KU Leuven)

Prof. dr. Thomas Voets

Laboratory of Ion Channel Research
VIB-KU Leuven Center for Brain & Disease Research
KU Leuven, Department of Cellular and Molecular Medicine
Herestraat 49 bus 801
3000 Leuven
Tel: +32 16 33 02 17
Thomas.voets@kuleuven.vib.be

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Unraveling the etiology of TRPM3-dependent neurodevelopmental disorders

1. Background and aim

TRPM3, a member of the transient receptor potential (TRP) superfamily of tetrameric ion channels, is a Ca^{2+} -permeable cation channel activated by increasing temperature and by ligands, including the endogenous neurosteroid pregnenolone sulphate (PS)³⁻⁵. Several studies, including pioneering work from our research group, have established the channel's role in peripheral somatosensory neurons, where it is involved in heat sensation and in the development of pathological pain^{3,6-12}. Surprisingly, TRPM3 is also highly expressed in several neuronal cell types of the developing and adult mammalian central nervous system (CNS). These include various neuronal types in the cortex, hippocampus and cerebellum, as well as non-neuronal cells such as astrocytes^{1,2,13}. This is in stark contrast to other highly temperature-sensitive TRP channels such as TRPV1 and TRPA1, and the cold- and menthol sensitive TRPM8, which are almost exclusively restricted to peripheral sensory neurons (Figure 1).

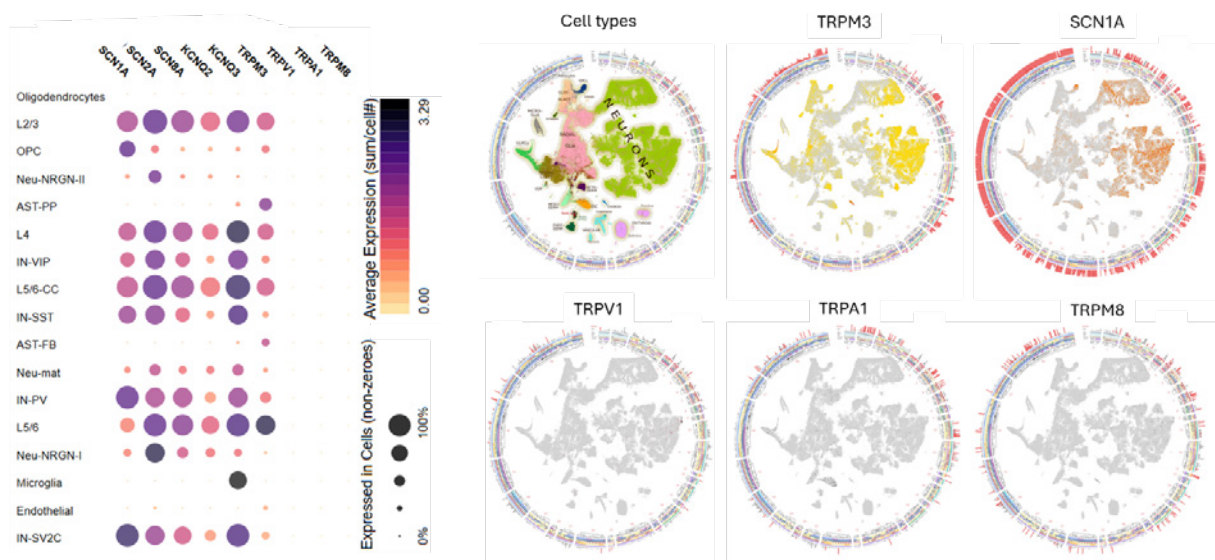


Figure 1: Unique expression profile of TRPM3 in the human and mouse brain (left) Expression of TRPM3 mRNA in the human cortex, compared to other temperature-sensitive sensory TRP channels (poorly visible due to negligible expression levels) and to voltage-gated Na^+ and K^+ channels implicated in neuronal excitability and epilepsy (Figure created at autism.cells.ucsc.edu with publicly available data¹). (right) Expression of TRPM3 mRNA in cells of the developing mouse brain, compared to other temperature-sensitive sensory TRP channels and the Nav1.1 (SCNA1) voltage-gated Na^+ channel. Yellow/orange colours indicate cells with high levels of gene expression; grey dots indicate cells that do not express the specific channel. (Figure created at mousebrain.org with publicly available data²)

Recently, rare heterozygous *de novo* variants in TRPM3 were identified in patients with neurodevelopmental disorders, consistently presenting with global developmental delay and mild to severe intellectual disability, in variable combination with other symptoms such as childhood-onset epilepsy, hypotonia, altered heat and/or pain sensitivity and cerebral palsy¹⁴⁻¹⁹. At the start of this project, our team had performed for the first time a detailed analysis of the electrophysiological and pharmacological properties of two disease-associated TRPM3 variants, following heterologous expression in the HEK293 cell line^{20,21}. We found that these variants show a dominant gain of function (GoF) when co-expressed with wild type TRPM3 (mimicking the situation in patients), seen as increased basal activity and enhanced responses to heat and PS. In particular, the recurrent TRPM3^{V1002M} variant, which to data has been described in 24 patients

worldwide, provokes a more than 10-fold increase in inward currents. Moreover, our team initiated an international consortium around TRPM3-related disease, via which we (1) facilitate rapid exchange of knowledge between geneticists, neurologists and basic scientists working on TRPM3¹⁸, (2) perform functional characterization of new variants, thereby allowing the diagnosis in new patients, and (3) organize information and Q&A sessions with parents of affected children. The aim of this project was to better understand how disease-associated TRPM3 variants lead to human neurodevelopmental disease, and use this knowledge as a basis for the development of TRPM3-based therapies to treat brain disease. In the past year, important progress was made regarding (1) structure-function of TRPM3-dependent disease and pharmacology, (2) pharmacological development of TRPM3-based therapies for brain disorders.

2. Results

2.1. New structure-function insights into TRPM3-dependent disease and pharmacology

As initiating team in the international consortium around TRPM3-related neurodevelopmental disease, we have been directly involved in the biophysical characterization of disease-associated TRPM3 variants, and in the systematic description of the phenotype of the patients. At this point, we have described the gain of function characteristics of 9 disease-associated variants in three publications^{13,20,23}, and summarized the current knowledge on the disease and disease-associated variants in a comprehensive review²⁴. However, the number of new patients carrying additional variants of unknown significance is steadily growing. In the last year, we optimized our plate reader-based analytical pipeline, which now allows us to provide a rapid assessment of the functionality of new variants, thus providing information to the patients and their caretakers whether the variants are the likely cause of neurodevelopmental disease. In this assay, we co-express equal amounts of cDNA of wild type and variant TRPM3, cloned into an IRES-GFP vector, together with the red calcium indicator jRCAMP1b. Then, in an automated fluorescence plate (96-well) reader system, we quantify changes in jRCAMP1b fluorescence to high doses of the antagonist primidone, and to a concentration-range of the neurosteroid PS. For the WT channel, primidone has little effect on the fluorescence, since the channel has low basal channel activity. However, in the case of gain-of-function variants, primidone leads to a quantifiable decrease in jRCAMP1b fluorescence, which can be used as a direct measure of the degree of basal channel activity.

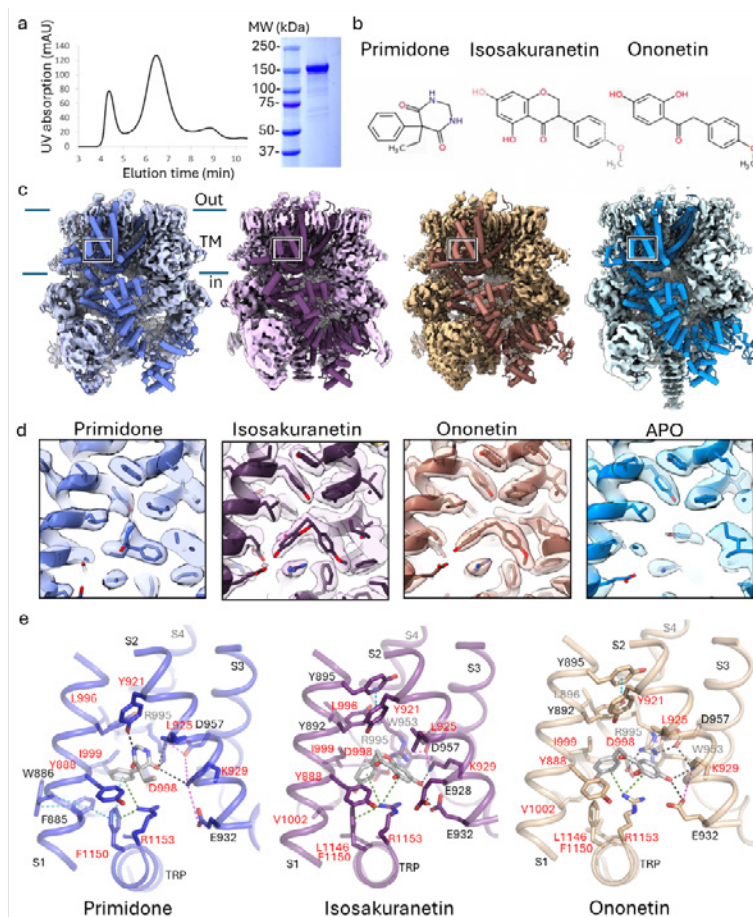


Figure 2: Structural insights into the interaction of TRPM3 with antagonists. (a) Representative chromatogram from a single size exclusion chromatography run of TRPM3 (left) and SDS-PAGE analysis. (b) Chemical drawings of primidone, isosakuranetin and ononetin. (c) Structures of TRPM3 bound to primidone, isosakuranetin, ononetin and in the apo-state displayed as surface representation with one subunit shown as cylinders. (d) Close-up view from the side on the VSLD ligand binding pocket with ligands and interacting residues lining S1-S4 shown as sticks. (e) Close-up views on the ligand binding sites are shown with important interactions highlighted as dashed lines; hydrogen bonds in black; salt bridges in pink; cation- π stacking in green and π - π stackings in cyan. Data from Bazeli et al.²²

In the last years, we have performed detailed analyses of newly identified channel variants from patients, thereby offering important diagnostic progress, as well as steering therapy. Moreover, by performing structural analysis of TRPM3 in complex with antagonists, including the clinically used drug primidone, we obtained important new insights into ligand action, further steering drug development (Figure 2). Importantly, thanks to these structural insights we also obtained crucial insights into the molecular basis of TRPM3-dependent neurodevelopmental disease²². In particular, we identified two patients with intractable epilepsy and neurodevelopmental abnormalities carrying the previously undescribed mutants I999S and F1150S. Based on our structural data, we found that these mutations directly affect the binding site for primidone, which is currently the only therapeutic antagonist with established therapeutic benefit for a large subset of patients with TRPM3-dependent neurodevelopmental disorder (Figure 3). Functional characterization revealed that both disease mutants abolish the inhibitory effect of primidone (Figure 3). These findings explain why these patients do not respond to primidone treatment, and urge research into other antagonists or other therapeutic approaches to restore normal TRPM3 activity in patients²².

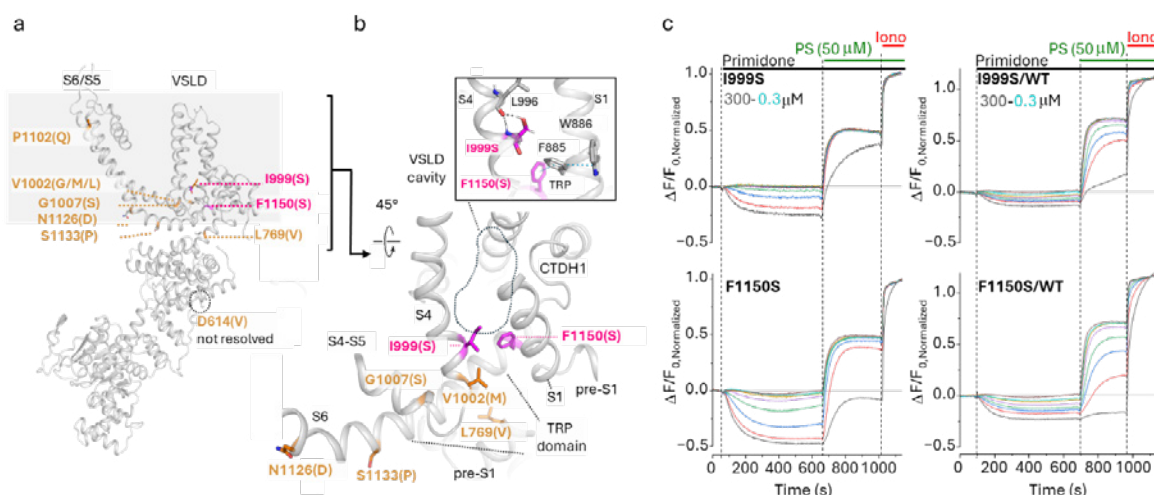


Figure 3: TRPM3-dependent disease mutations that affect antagonist binding (a,b) Indication of published (yellow) disease-associated mutations on the structure of TRPM3, as well as two newly discovered mutants (I999S and F1150S; pink) that line the ligand binding site. . Plasma membrane is indicated in grey. (c) Functional characterisation reveals that I999S and F1150S cause increase basal activity and strongly reduce the inhibitory effect of primidone. Data from Bazeli et al.²².

2.2. Towards the development of pharmacotherapy for TRPM3-dependent brain diseases

Based on our assessment of the sensitivity of disease-associated TRPM3 variants to primidone, a well-known medication to treat epilepsy and tremor, several children suffering from TRPM3-dependent neurodevelopmental disease have been treated with primidone. In a first case report, we were involved in the description of two children suffering from pharmacoresistant developmental and epileptic encephalopathy with spike-and-wave activation in sleep (DEE-SWAS) due to TRPM3 mutations. Following treatment with primidone, developmental regression was stopped, psychomotor development improved, and epileptic activity was fully suppressed²³. Currently, more than 50% of the known patients are being treated with primidone, often (but not always) with beneficial effects on developmental progress and the occurrence of seizures. One issue is that primidone is not very selective, and is metabolized to the barbiturate phenobarbital, leading to important central side effects occlude potential beneficial effects of the suppression of overactive TRPM3²⁵. A second issue, as outlined above, is that several disease mutations affect the inhibitory binding of primidone to the channel. Therefore, the development of more selective TRPM3 antagonists would likely allow a better treatment of these patients.

In this regard, our team is collaborating with the Center for Drug Design and Discovery (CD3; www.cd3.be), in partnership with the company Biohaven (www.biohaven.com), to develop highly potent and specific TRPM3 antagonists (described in the granted patent US 12,209,081 B2 (Thomas Voets is one of the inventors) and in related patent filings). In 2024-2025, an important milestone of this drug development was reached, with the successful finalization of Phase Ia and Phase Ib studies with of BHV-2100, an orally available, potent and selective TRPM3 antagonists developed in this collaboration (described in the granted patent US 12,209,081 B2; Thomas Voets is one of the inventors). Most importantly, these Phase 1 studies revealed that BHV-2100 did not cause any relevant adverse effects, indicating that TRPM3 inhibition can be safely achieved in human. Currently, BHV-2100 is being tested in phase 2 studies for migraine and neuropathic pain. However, BHV-2100 is largely peripherally restricted, and is therefore not optimally suited to treat the major CNS impact of disease-associated TRPM3 gain-of-function variants. Therefore, in the collaboration with CD3 and Biohaven LTD, and supported by the finding that TRPM3-deficient mice show reduced seizure susceptibility (Figure 4), we have recently achieved to develop potent, selective, brain-permeant TRPM3 antagonists. Key properties of one example

compound (CIM796) are shown in Figure 5: it inhibits wild type and mutant (V1002M) TRPM3 with single-digit nanomolar concentrations and, following oral dosing, shows potent antiseizure activity in established models of epilepsy, such as the 6Hz, maximal electroshock (MES) and PTZ models in mice. These tools put us in a unique position to evaluate the feasibility of targeted pharmacological therapies to treat TRPM3-dependent neurodevelopmental disorders, and also provide strong evidence that TRPM3 represents a druggable target for the treatment of other forms of epilepsy. Currently, in collaboration with our partners Biohaven and CD3, we are evaluating the efficacy of this and related brain-permeant TRPM3 antagonists in knockin mouse models of human TRPM3-dependent neurodevelopmental disorders and a battery of models of epilepsy. We are also evaluating their pre-clinical safety, with the aim of obtaining a clinical candidate for treatment of patients in the course of 2026.

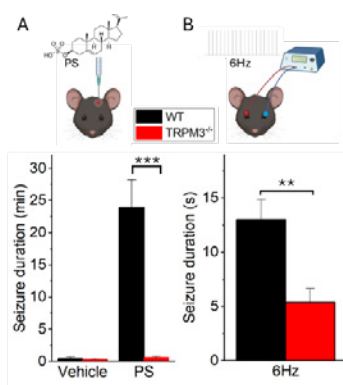


Figure 4: TRPM3 activity dictates seizure activity in mice (A) ICV injection of the neurosteroid TRPM3 agonist PS (250 nmole) causes seizures in WT but not in TRPM3^{-/-} mice. (B) TRPM3^{-/-} mice show reduced seizure duration in the 6Hz model of partial seizures.

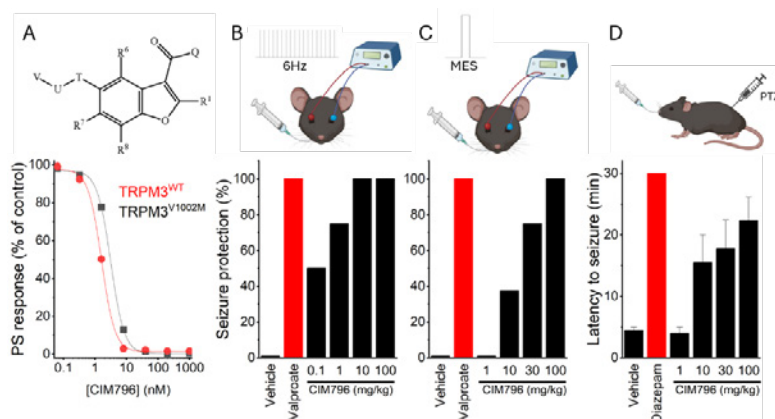


Figure 5: Seizure protection by a brain-permeant, specific TRPM3 antagonist (A) General structure of brain-permeant TRPM3 antagonists, and concentration-dependent inhibition of PS-evoked responses of TRPM3^{WT} and TRPM3^{V1002M} by CIM976. (B-D) CIM976 protects mice in the MES and 6Hz seizure models and delays seizure onset following PTZ injection. Positive controls were Valproate (250mg/kg; B,C) and Diazepam (5mg/kg; D).

2.3. Next steps and outlook

Thanks to the support of the GSKE, we have been able to make important progress in understanding TRPM3-dependent neurodevelopmental disease and the role of TRPM3 in the brain. By establishing a quantitative functional pipeline of novel TRPM3 variants, we were able to support the diagnosis of patients with neurodevelopmental disease by establishing gain of channel function as the underlying cause. Moreover, since we are making great progress in the development of brain-permeant TRPM3 antagonists, along with encouraging results using primidone, there is a realistic outlook that this research may lead to the development of an

effective treatment for patients with rare mutations in TRPM3. However, our recent structural insights indicate that in some patients the disease-associated mutations directly affect the binding site for ligands such as primidone and CIM796. These findings highlight the importance of personalized medicine approaches in the treatment of TRPM3-dependent neurodevelopmental disease, and indicate that other, non-pharmacological approaches (e.g. gene therapy or antisense oligonucleotides (ASOs)) may be needed as therapy for some patients.

Importantly, this research also revealed an important role for TRPM3 in non-genetic forms of epilepsy. Indeed, TRPM3 antagonists have strong antiseizure activity in models of electrically or chemically evoked epilepsy, whereas TRPM3 agonists are seizure evoking. These exciting findings indicate that TRPM3-targeted therapies may become a completely novel approach to treat patients with (refractory) epilepsy.

Overall, the project yielded important new insights into TRPM3-dependent brain disease, with a high translational value, both for the diagnosis of the cause of disease in patients with newly identified variants in the TRPM3 gene, and for the development of therapy. Thanks to our close interactions with geneticists, neurologists and parents of affected children, new findings and potential treatments can be directly communicated to the relevant stakeholders.

3. Publications in 2023-2025 with support from GSKE

(*: shared last and corresponding authors)

- Deseure, K., Dallali, I., Pinto, S., Luyten, K., Segal, A., Van Ranst, N., Roelens, R., Corbett, S., Murray, S., Persoons, E., Hans, G., Vriens, J. & **Voets, T.** TRPM3 mediates spontaneous pain and mechanical allodynia in a mouse model of chronic orofacial neuropathy. *Cell Reports Medicine* (in press). (IF: 10.6; Q1)
- Bazeli, B., Shkhumatov, A. V., Schenck, S., Vanherck, J. C., Janssens, A., Spieser, S. A., Marchand, D., Roelens, R., Chaltin, P., Marchand, A., Vriens, J., **Voets, T.*** & Brunner, J. D.* Stereoselectivity and functional plasticity of a common ligand-binding pocket in TRPM3. *Nature Communications* (in press). (IF: 15.7; Q1)
- Robben, S., Davidson, P., Rodrigues Ribeiro, R.S., **Voets, T.** A high-throughput assay for the measurement of Ca²⁺-oscillations and insulin release from uniformly sized β -cell spheroids eLife (in press). (IF: 6.4[#]; Q1)
- Held, K., Van Hoeymissen, E., Dallali, I., Persoons, E., Bazeli, B., Roelens, R., Robben, S., Wuyts, C., Mulier, M., Pinto, S., Janssens, A., **Voets, T.** & Vriens, J. A duo of redox-sensitive pore-loop cysteines controls the activity of the neural ion channel TRPM3. *Commun Chem* (in press)
- Toth, B. I., Bazeli, B., Janssens, A., Lisztes, E., Racsko, M., Kelemen, B., Herczeg, M., Nagy, T. M., Kover, K. E., Mitra, A., Borics, A., Biro, T. & **Voets, T.** Direct modulation of TRPM8 ion channels by rapamycin and analog macrolide immunosuppressants. *Elife* **13** (2025). <https://doi.org/10.7554/eLife.97341>. (IF: 6.4; Q1).
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- Daniluk, J. & **Voets, T.** pH-dependent modulation of TRPV1 by modality-selective antagonists. *Br J Pharmacol* **180**, 2750-2761 (2023). <https://doi.org/10.1111/bph.16173>. (IF: 7.7; Q1)
- Burglen, L. *et al.* **Voets, T.*** & Vriens, J.* Gain-of-function variants in the ion channel gene TRPM3 underlie a spectrum of neurodevelopmental disorders. *Elife* **12** (2023). <https://doi.org/10.7554/eLife.81032>. (IF:6.4; Q1).

(#: see <https://elifesciences.org/inside-elifesciences/c11c6101/the-elifesciences-model-an-update-on-progress-following-changes-in-web-of-science-indexing-status>).

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Final report of the university research project of

Prof. dr. Pierre Maquet
Université de Liège (ULiège)

Prof. dr. Pierre Maquet (ULiège)
Faculté de Médecine
Département des sciences cliniques
Neurologie
GIGA CRC In vivo Imaging – Sleep and chronobiology

Bât. B35 Neurologie
Quartier Hôpital
avenue de l'Hôpital 13
4000 Liège 1
Belgique
Téléphone de service: +32 4 366 23 67
Téléphone de service: +32 4 366 23 62

Quantitative MRI at 7 Tesla addresses ten questions about brain small vessel diseases

1. Project

Cerebral small vessel diseases (CSVD) are common and potentially severely disabling disorders. They are typically associated with stroke, both ischemic and hemorrhagic, cognitive impairment and dementia, gait disturbance and mood disorders. However, signs of CSVD are often detected in individuals with no apparent neurological history.

Diagnosis of CSVD is mainly based on the detection of typical neuroimaging features on brain MRI. These features include small subcortical infarcts, white matter hyperintensities of presumed vascular origin, enlarged perivascular spaces, lacunes of presumed vascular origin, cerebral microbleeds, cortical superficial siderosis, cortical cerebral microinfarcts and brain atrophy.

Most of the time, CSVD results from two main etiologies: arteriolosclerosis and cerebral amyloid angiopathy (CAA). These processes are not easily distinguishable using conventional MRI techniques.

This ongoing study aims to use ultra-high field (7 Tesla) quantitative MRI (qMRI) to measure microstructural abnormalities within CSVD macroscopic lesions and in normal-appearing brain tissue (NABT). The characterization of these CSVD features will be associated with the measurement of inflammation markers in the blood.

We contend that high-resolution quantitative MRI at very high field can reveal the spectrum of microstructure of brain SVD lesions. We expect to find a correlation between clinical data, advanced MRI data and blood biomarkers of inflammation to identify potential new biomarkers that (1) could enhance the distinction between arteriolosclerosis and CAA (2) improve evaluation of CSVD severity (in terms of clinical manifestation and MRI lesion load) and (3) potentially identifying new targets for future treatment.

2. Technical development

Before embarking on the acquisition of a large patient cohort, we developed new acquisition sequences and analytic tools, by running several pilot acquisitions.

We needed to improve the multi-parameter protocol, which grounds most of the project. The initial protocol was based on a single transmit coil and did not allow a proper investigation of the brainstem and cerebellum. It is well-known that ultra-high field MRI, as it is currently applied in neuroimaging, has substantial limitations in imaging the subtentorial regions. Due to the reduced wavelength of proton MRI at 7T, conventional MRI technology suffers from standing wave effects in whole-brain MRI, which often results in strongly reduced signal-to-noise ratio (SNR) in specific brain regions, mostly the lower part of the temporal lobes and the posterior fossa. The effect is also known as transmit field inhomogeneity, which is pronounced at 7 Tesla. This is inevitable if conventional excitation with a single radiofrequency (RF) transmit coil is performed. RF-coils specifically designed for the cerebellum would trade off other parts of the brain, which is not an option when critical measures must be extracted from the whole brain. Therefore, in the

framework of an international collaboration on spinocerebellar ataxias, we considered parallel transmission (pTx) with multiple RF channels. While it has been known for a long time that pTx can mitigate transmit field inhomogeneity, the technology became mature to be applicable in larger studies only recently. Our 7T imaging device (Siemens Terra) has a fully integrated pTx system, which can be used in the standard workflow. We also took advantage of novel pTx pulse design methods that have been developed and enable fast and calibration-free routine application. In a second step, we tweaked the sequence to reduce the acquisition time, allowing us to squeeze other signals in the MR run, e.g. TOF, which localizes the major arteries nearly up to their pial segment.

Currently, we have acquired five top-quality MRI pilot studies. Based on the acquired data, we developed an automatic detection of extravascular spaces, which constitute one of our main features of interest in this project. Building on the generalized segmentation available in SPM12, we designed a Bayesian framework including the extravascular spaces as a separate tissue type. The segmentations obtained on control participants are encouraging and are being generalized to data of SVD patients.

Finally, we have broadened the project and implemented the measurement of blood inflammatory biomarkers. The latter provide practical and innovative tools for linking advanced MRI data (such as inflammatory biomarkers like iron deposition), to plasma parameters which could be easily implemented in clinical practice.

3. State of the recruitment

We aim to recruit approximately 100 participants with a proportion of one healthy control for every 3 patients (~65 patients for ~35 healthy controls). We have been recruiting patients for 8 months during a dedicated medical consultation. Currently, approximately 30 patients have agreed to participate. We expect that all participants will be recruited by the end of 2026.

4. Time plan

As the preliminary results are encouraging, we included the first participants in September 2025. We now include between 2 and 4 participants each week. We hope to finish acquiring data by late 2026. We expect to finish the data analysis, which includes blood measurements, imaging segmentation, quantitative map extraction, and statistical analysis, by late 2027. We plan to publish our results in 2028.

5. Scientific communication

An abstract of the project has been accepted by the Belgium Stroke Council, and the project has been presented at the 8th Belgian Scientific Stroke Workshop on October 17th, 2025. We have been selected to also present our project at the Belgium Stroke Council symposium on December 13th, 2025.



Geneeskundige Stichting Koningin Elisabeth
Fondation Médicale Reine Elisabeth
Königin-Elisabeth-Stiftung für Medizin
Queen Elisabeth Medical Foundation

Projecten jonge onderzoekers
2023-2025 gefinancierd door de G.S.K.E.

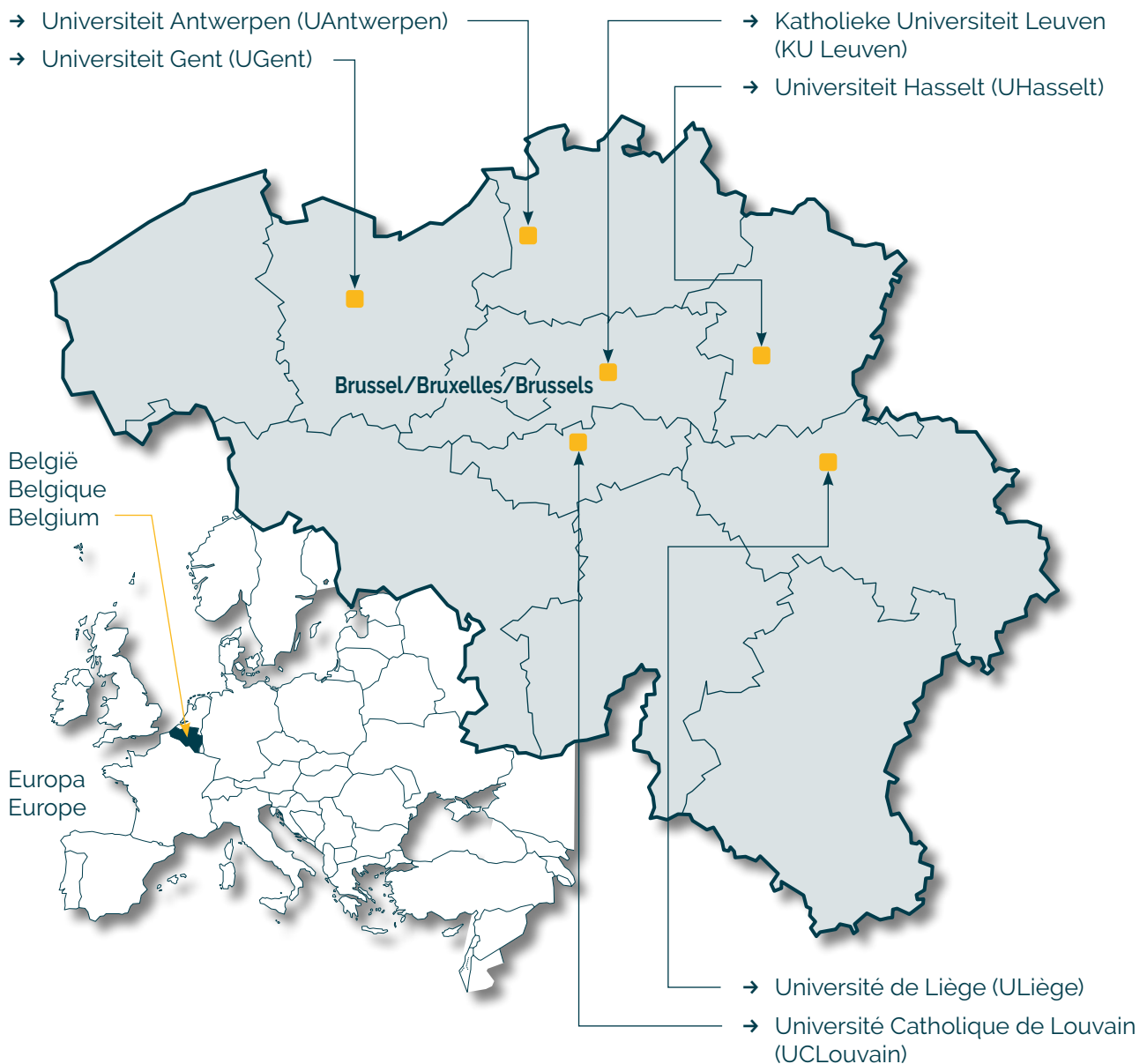
Projets de recherche de jeunes chercheurs
2023-2025 subventionnés par la F.M.R.E.

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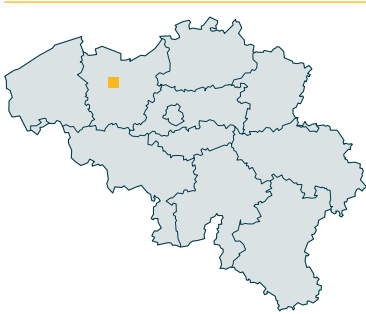


Projecten jonge onderzoekers 2023-2025 gefinancierd door de G.S.K.E.

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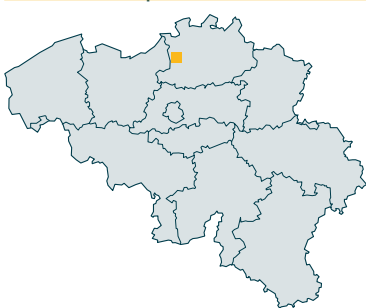
Sielke Caestecker (PhD student) & promotor prof. Robrecht Raedt

The role of the locus coeruleus noradrenergic system in seizures and epilepsy

Dr. Delfien Syx

Zebrafish as a model to study pain in Ehlers-Danlos syndromes

UAntwerpen



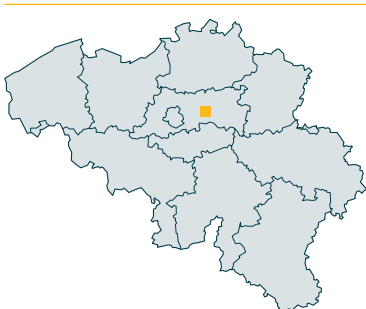
Dr. Marijne Vandebergh

World-wide systematic characterization of TMEM106B and ATXN2 genetic status Towards implementation of genetic testing of modifiers in clinical practice

Dr. Barbara M.P. Willekens

Unravelling the role of antigen-specific T cells in NMOSD and MOGAD

KU Leuven



Dr. Wouter Peelaerts

Peripheral infections as a trigger of multiple system atrophy

Dr. Sarah van Veen

The impact of ATP13A4-mediated polyamine transport in astrocytes on synaptogenesis and neurodevelopmental disorders

UCLouvain



Prof. Giulia Liberati

STIM-WAVES: Identifying pain biomarkers with invasive and non-invasive brain stimulation targeting ongoing neural oscillations

(UHasselt)



Prof. dr. Jeroen Bogie

Getting a grip on slippery protein modifications in multiple sclerosis

Prof. dr. Bieke Broux

High salt diet causes blood-brain barrier disturbances in multiple sclerosis: involvement of the renin-angiotensin-aldosterone system

ULiège



Dr. Sophie Laguesse

Unveiling the alcohol-dependent alterations in mRNA local translation and its consequences on adolescent prefrontal cortex maturation and function



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Königin-Elisabeth-Stiftung für Medizin
Queen Elisabeth Medical Foundation

Final report of the research project of the young researcher

Sielke Caestecker (PhD student) &
promotor prof. Robrecht Raedt
Universiteit Gent (UGent)

Prof. dr. Robrecht Raedt (UGent)

Principal investigator – 4BRAIN

Associate Professor (Faculty of Medicine and Health Sciences)

Director of the Animal Facility of the Faculty of Medicine and Health Sciences

Member of GATE steering committee

Secretary of the Belgian Society for Neuroscience

Lab address: 4BRAIN,

Campus UZ Gent, o Blok B (entrance 36),

Corneel Heymanslaan 10,

9000 Gent, Belgium

Robrecht.Raedt@UGent.be

Sielke Caestecker (UGent)

PhD student

Lab address: 4BRAIN,

Campus UZ Gent, o Blok B (entrance 36),

Corneel Heymanslaan 10,

9000 Gent, Belgium

sielke.caestecker@ugent.be

The role of the locus coeruleus noradrenergic system in seizures and epilepsy



Geneeskundige Stichting Koningin Elisabeth
Fondation Médicale Reine Elisabeth
Königin-Elisabeth-Stiftung für Medizin
Queen Elisabeth Medical Foundation

Progress report of the research project of the young researcher

Dr. Delfien Syx
Universiteit Gent (UGent)

Delfien Syx, PhD (UGent)

Department of Biomolecular Medicine

Center for Medical Genetics Ghent (CMGG)

Ghent University Hospital

Medical Research Building I (MRB I) – Ingang 34

C. Heymanslaan 10

B-9000 Ghent

Belgium

T +32 9 33 2 1346

Delfien.Syx@ugent.be

www.cmgg.be

www.ugent.be/ge/biomolecular-medicine

Zebrafish as a model to study pain in Ehlers-Danlos syndromes

1. Background

Chronic pain affects up to 20% of the global population and represents a socio-economical health challenge. [1,2] The etiology of chronic pain is complex, and the underlying mechanisms are poorly understood. Consequently, treatment options are limited and often associated with considerable side effects and serious health risks. [3–5]

Over the years, a role for the extracellular matrix (ECM) in the development and persistence of pain has emerged. The ECM is a complex three-dimensional network that supports and maintains tissue and organ structure. The ECM consists of collagens, glycoproteins, proteoglycans, and secreted factors, which, together with the cells that produce them (e.g., fibroblasts), forms the connective tissue that is found throughout the body (e.g., in skin, bone, tendon and ligaments). [6] An ECM network also surrounds and functionally supports neuronal and non-neuronal cells (including nociceptors) in the peripheral and central nervous systems (PNS/CNS). [7,8] Furthermore, painful peripheral injuries were shown to cause acute and chronic ECM remodeling and/or alter ECM modifying enzymes. [8–10]

The link between the ECM and pain is further reinforced by the presence of chronic widespread pain in several monogenic heritable connective tissue disorders, caused by genetic defects affecting individual ECM components. [11–14] One of these disorders are the Ehlers-Danlos syndromes (EDS), a heterogeneous group of conditions hallmarked by joint hypermobility, soft and hyperextensible skin, abnormal wound healing and generalized tissue fragility. [15] To date, 13 distinct EDS types are recognized, of which 12 are molecularly elucidated and caused by defects in 20 different genes, mainly affecting collagens, proteoglycans and glycoproteins. [15,16] All these EDS-related defects compromise ECM architecture, often visible as ultrastructural changes in the organization and/or structure of collagen fibrils. [17] Chronic widespread pain occurs in 90% of EDS patients and is often one of the main reasons patients seek medical care. [12,18] Despite its high prevalence, little is known about the precise origins and mechanisms contributing to EDS-related pain and the pain is usually inadequately controlled by currently used treatments. An important reason for this is the lack of studies investigating the existing knowledge gaps.

Zebrafish (*Danio rerio*) has emerged as a promising animal model to reliably study pain-related behavior and associated mechanisms since their nervous system shows great similarity to humans. [19–23] Over the years, our group has invested in the generation of several knockout zebrafish models of EDS and validated them as robust models for the respective human EDS types. [24–26] that are caused by mutations in the genes encoding type I collagen and include specific forms of osteogenesis imperfecta (OI) We created *b4galt7*^{-/-} and *b3galt6*^{-/-} zebrafish, models of spondylodysplastic EDS (spEDS) [25,26] encoded by B4GALT7, that plays a pivotal role in the proteoglycan linker region formation during proteoglycan biosynthesis. Defects in this enzyme give rise to a rare autosomal recessive form of Ehlers-Danlos syndrome (EDS and *chst14*^{-/-} zebrafish, a model of musculocontractural EDS (mcEDS) (unpublished). Both spEDS and mcEDS result from genetic defects in enzymes involved in proteoglycan biosynthesis. In addition, a zebrafish model with defective collagen biosynthesis was previously investigated: *col1a2*^{-/-} zebrafish, a model of cardiac-valvular EDS (cvEDS). [24]

My research aims to assess the contribution of the abnormal ECM and untangle the specific mechanisms that induce pain and peripheral nervous system changes using EDS zebrafish as a model.

2. Preliminary results

2.1. Evaluating pain-related behavior in EDS zebrafish

Potentially painful events lead to behavioral and physiological changes in zebrafish. [19] Studies have demonstrated that healthy zebrafish are usually swimming constantly, using mid-water and all areas of their tank. In contrast, zebrafish subjected to potentially painful treatment showed increased use of the bottom of the tank (diving behavior) and reduce swimming and activity. [23,27] an automated method, the Fish Behaviour Index (FBI

To analyze the general swimming behavior of young wild-type zebrafish of 5 days post fertilization (dpf), several experimental setups were tested, including the use of different container sizes (e.g., zebrafish individually placed in 6, 12, 24 or 48 well plates) and several setups with different duration in light and dark conditions. Following this initial exploratory phase in wild-type zebrafish, we opted to use 12-well plates to accommodate 5 dpf zebrafish for subsequent testing since this gave the most consistent results and still allows for a relatively high throughput during testing. The first 10 minutes of the experiment were considered an acclimatization period and were not used for analyses. During the experiment, the temperature was kept constant at 28°C using a Temperature Control Unit (Noldus, The Netherlands). Locomotor activity was analyzed using the DanioVision Observation Chamber (Noldus, The Netherlands) equipped with a Basler GenICam camera capturing 30 frames/second for image acquisition. Data was recorded and analyzed using the EthoVision XT 15 software (Noldus, The Netherlands). For each zebrafish, center-point detection was used. Movement was expressed as cumulative time spent moving (%), movement frequency and distance moved (mm). Following behavioral analysis, all zebrafish were genotyped. For each EDS zebrafish line, wild-type (+/+), heterozygotes (+/-), and knockout (-/-) animals were examined.

We confirmed decreased movement of 5 dpf wild-type zebrafish following addition of 0.25% acetic acid to the water (Figure 1A-C), which mimics published findings [23] 0.1% and 0.25%, and indicates that our experimental setup and conditions can pick up altered swimming behavior in response to a known stimulus.

Analysis of the behavior (movement, movement frequency and distance travelled) of 5 dpf *chst14*^{-/-}, *b3galt6*^{-/-} and *col1a2*^{-/-} zebrafish, models for mcEDS-*CHST14*, spEDS-*B3GALT6* and cvEDS, respectively, did not show differences in movement compared to wild-type siblings (Figure 1D-F, 1G-I and 1M-O). For these models no differences could be observed between the knockout zebrafish or wild-type and heterozygous zebrafish from the same clutch, except for the *chst14* line where wild-type siblings moved less compared to heterozygous siblings (Figure 1D), but with unaltered movement frequency and distance traveled.

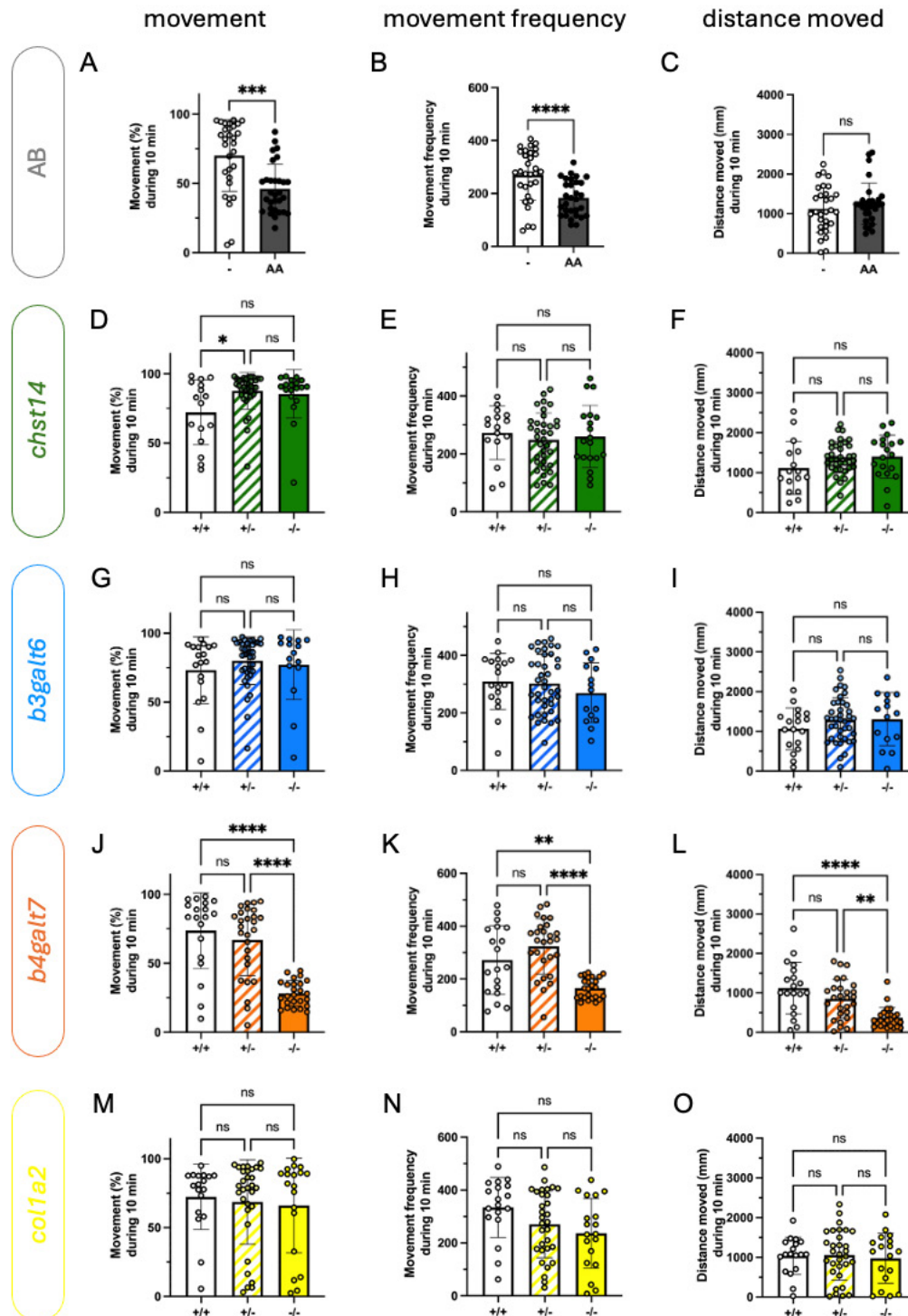


Figure 1 | Behavioral analysis of 5 dpf zebrafish. Movement (in %), movement frequency and distance moved (in mm) during 10 minutes tracking in light conditions. (A-C) Positive control without (-) or with (AA) addition of 0.25% acetic acid in the water of wild-type (AB) zebrafish. (D-F) *chst14*^{-/-}, (G-I) *b3galt6*^{-/-}, (J-L) *b4galt7*^{-/-} and (M-O) *col1a2*^{-/-} zebrafish and their wild-type and heterozygous siblings. Data is presented as mean ± standard deviation. Every dot represents an individual zebrafish. +/+ : wild-type, +/- : heterozygous, and -/- : homozygous zebrafish. * p<0.05; *** p<0.001, **** p<0.0001, t-test (A) or one-way ANOVA (B-E).

When studying locomotor activity of 5 dpf *b4galt7*^{-/-} zebrafish, a model for spEDS-*B4GALT7*, we found that *b4galt7*^{-/-} zebrafish moved significantly less compared to wild-type and *b4galt7*^{-/-} zebrafish (Figure 1J-L). The findings for the movement and movement frequency parameters is similar to what is seen when treating 5dpf zebrafish with a painful stimulus (Figure 1A-C). Given the observed variability in behavior between wild-type 5 dpf zebrafish and the fact that different

clutches (= offspring from one breeding) can show differences in severity of the phenotype, the behavior experiments were repeated at least three times starting from independent clutches, which resulted in comparable results.

To confirm that the altered behavior in *b4galt7*^{-/-} zebrafish, corresponds to a pain-related response, we tried to reverse the behavior by addition of analgesics. Several analgesics have been shown to lead to effective pain-relief in zebrafish. [28] Addition of the non-steroidal anti-inflammatory drugs aspirin (0.01 mg/ml) to the water of the *b4galt7* line did not convincingly show amelioration of the phenotype. Other analgesics will be administered to ameliorate the observed behavioral changes.

Since pain-related behavior can also develop at a later age, behavioral analysis was also performed in adult zebrafish (> 3 months). General swimming behavior of adult wild-type zebrafish was analyzed in a custom box (Noldus, The Netherlands) equipped with a Basler GenICam camera capturing 30 frames/second for image acquisition. Zebrafish were placed individually in a tank. Data was recorded and analyzed using the EthoVision XT 17 software (Noldus, The Netherlands). For each zebrafish, center-point detection was used. The first 10 minutes of the experiment were considered an acclimatization period and were not used for analyses. Movement was expressed as cumulative time spent moving (%), movement frequency and distance moved (cm). For each EDS zebrafish line that is viable at adult age, wild-type (+/+), and knockout (-/-) animals were examined. Of note, *b4galt7* zebrafish are lethal around 11 dpf, hence no adult *b4galt7*^{-/-} zebrafish could be tested.

Preliminary analysis of the behavior (movement, movement frequency and distance travelled) of adult *b3galt6*^{-/-} zebrafish, a model for spEDS-*B3GALT6*, did not show differences in movement compared to wild-type siblings (Figure 2D-F). In contrast to 5dpf zebrafish, adult *chst14*^{-/-} zebrafish, a model for mcEDS-*CHST14*, showed a decreased distance moved compared to wild-type siblings (Figure 2C), but unaltered movement and movement frequency (Figure 2A-B). Adult *col1a2*^{-/-} zebrafish show an increased movement frequency compared to wild-type siblings but unaltered distance moved, indicating that the zebrafish pause more, but in the end swim the same distance (Figure 2G-I). How the observed behavior relates to a potential pain phenotype requires further examination.

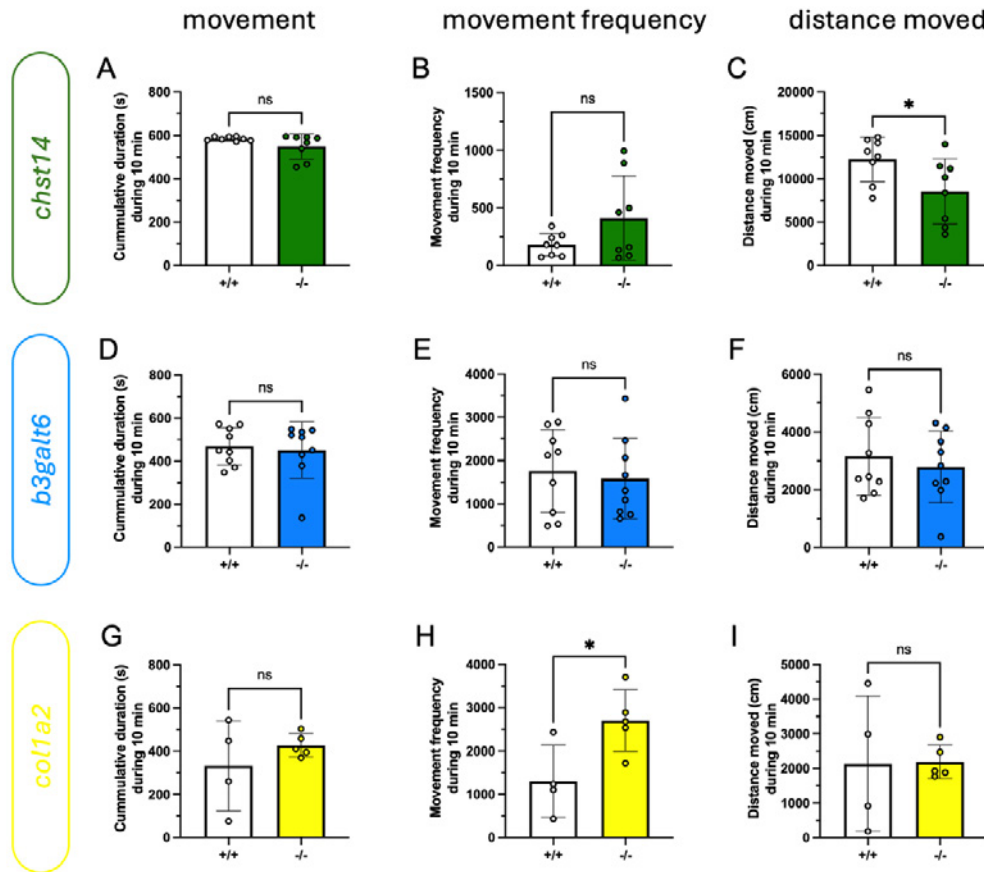


Figure 2 | Behavioral analysis of adult zebrafish. Movement, movement frequency and distance moved (in cm) during 10 minutes tracking in light conditions. (A-C) *chst14*^{-/-}, (D-F) *b3galt6*^{-/-} and (G-I) *col1a2*^{-/-} zebrafish and their wild-type siblings. Data is presented as mean ± standard deviation. Every dot represents an individual zebrafish. +/+ : wild-type, and -/- : homozygous zebrafish. * p<0.05; ns: not significant, unpaired t-test.

2.2. Monitoring neuronal development in EDS zebrafish

To acquire information about the effect of an aberrant ECM on **neuronal development**, EDS zebrafish models will be crossed with the Tg(*neurog1:EGFP*) line (available in our lab), expressing the pro-neural transcription factor neurogenin1 which is a key regulator for dorsal root ganglion (DRG) neuron development. [29]

To introduce a less time-consuming method to evaluate neuronal development, we started optimizing the NeuroTracer Dil tissue-labeling paste (Thermo Fisher Scientific) that serves as an alternative staining to visualize the nervous system. Several experiments did not result in proper visualization of the nervous system.

Breeding of the EDS zebrafish models will require some time to obtain homozygous zebrafish in the Tg(*neurog1:EGFP*) background. We are currently exploring alternative imaging techniques to obtain proper visualization of the nervous system.

2.3. Generation and phenotypic characterization of *col5a1*^{-/-} zebrafish

Our group previously demonstrated the presence of pain in patients with classical EDS (cEDS), caused by genetic defects in the *COL5A1* and *COL5A2* genes. [30] In addition, we showed the presence of pain-related behavior and concomitant alterations in the dermal nociceptive innervation in *Col5a1*^{-/-} mice, a validated model of cEDS. [31] In view of these findings, we aimed to create a zebrafish counterpart that mimics the human cEDS phenotype to assess pain-related

behaviors as a proof-of-concept. Since young zebrafish are well-suited for large-scale drug/compound screenings, these EDS zebrafish would open opportunities to start exploring potential therapeutic options.

2.3.1 Generation of *col5a1* zebrafish models

CRISPR/Cas9-based genome editing was used to generate mutant *col5a1* zebrafish lines (Figure 3). The aim is to obtain zebrafish carrying a premature termination codon, predicted to result in *col5a1* haploinsufficiency, the most common molecular defect observed in cEDS patients and similar to the defect in *Col5a1*^{-/-} mice. Two different models were generated to account for possible off-target effects.

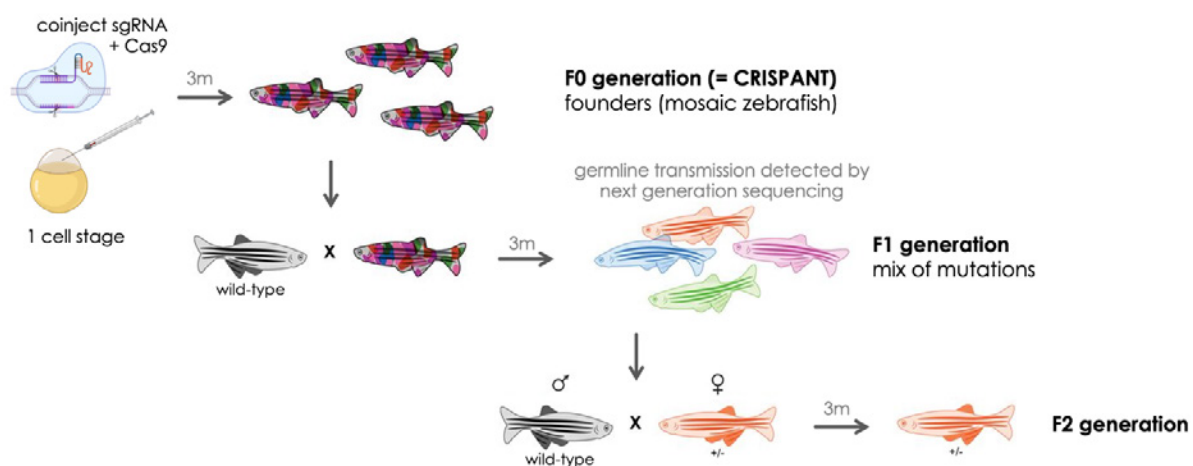


Figure 3 | Schematic overview of the generation of the *col5a1* zebrafish lines using CRISPR/Cas9 genome editing. Adapted from Varshney *et al.* Genome Research, 2015. [32] We present a high-throughput targeted mutagenesis pipeline using CRISPR/Cas9 technology in zebrafish that will make possible both saturation mutagenesis of the genome and large-scale phenotyping efforts. We describe a cloning-free single-guide RNA (sgRNA)

Two sgRNAs were designed that target either exon 8 or exon 10 of the zebrafish *col5a1* gene and were individually injected with Cas9 protein into one-cell stage zebrafish eggs. This resulted in an overall indel efficiency of 86.6% for exon 8 and 95.4% for exon 10. Overall, there was a high mortality of injected zebrafish. The surviving zebrafish represent the F0 generation, also called crispants, and are mosaic for different genetic alterations in the *col5a1* gene. These crispants were grown till 3 months of age, bred with wild-type zebrafish and their offspring was tested for germline transmission of the out-of-frame indel variants. Next, a founder crispant zebrafish which carries a high frequency of out-of-frame variants was selected for each of the targeted exons. These founders were subsequently crossed with a wild-type zebrafish and their offspring was allowed to grow till adulthood. This resulted in a mixture of zebrafish, each carrying a particular mutation. We selected zebrafish with a 5bp deletion in exon 8 (cmg109) and 1bp duplication in exon 10 (cmg111) for establishing stable mutant zebrafish lines. Both variants are predicted to introduce a premature termination codon.

To further validate the *col5a1* zebrafish lines, the consequences of the mutations were examined. However, the study of the effect of the generated defects on type V collagen quantity (and quality) was severely hampered by the lack of an antibody to detect type V collagen. This is a known problem that is frequently encountered when working with zebrafish proteins. Testing of different antibodies for Western blot analysis did unfortunately not result in visualization of the type V collagen protein in zebrafish.

To overcome the issue with detection of the (mutant) type V collagen protein, expression levels of the *col5a1* gene were evaluated using reverse transcription quantitative PCR (RT-qPCR). In line with the expected haploinsufficiency, caused by the introduction of a premature termination codon, a significant decrease of the *col5a1* expression levels were observed in both the heterozygous *col5a1* cmg109 and cmg111 lines (Figure 4). This suggests that the mutant allele is subject to nonsense-mediated mRNA decay, which mimics the most common molecular defect seen in cEDS patients.

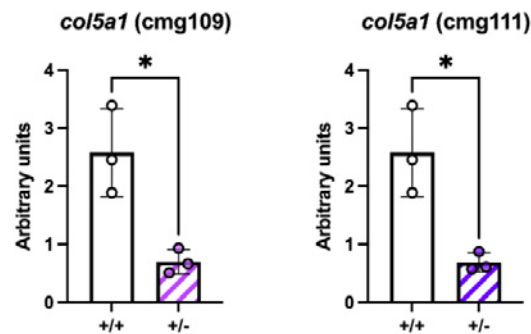


Figure 4 | Expression analysis. Decreased expression of *col5a1* in cmg109 and cmg111 lines. Every dot represents an individual zebrafish. +/+ : wild-type, and +/- : heterozygous zebrafish. * $p < 0.05$, t-test.

2.3.2 Phenotypic characterization of *col5a1* zebrafish models

Behavior testing of the *col5a1* cmg109 and cmg111 lines was performed. Behavior analysis of 5 dpf heterozygous *col5a1*^{-/-} zebrafish (the expected model of cEDS) did not show differences in movement compared to wild-type siblings (Figure 5). However, we found that homozygous *col5a1*^{-/-} zebrafish moved significantly less compared to wild-type and *col5a1*^{+/-} zebrafish. This finding resembles what is seen when treating 5 dpf zebrafish with a painful stimulus (Figure 1A-C) and the altered behavior in *b4galt7*^{-/-} zebrafish (Figure 1J-L).

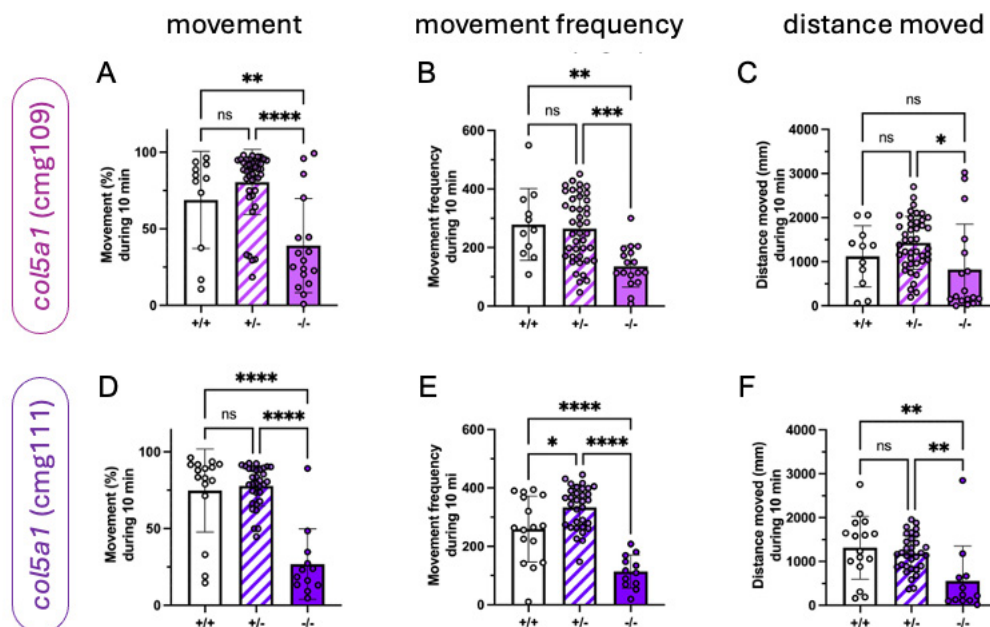


Figure 5 | Behavioral analysis in *col5a1* zebrafish. Movement (in %), movement frequency and distance moved (in mm) during 10 minutes tracking in light conditions. (A-C) *col5a1* cmg109 line and (D-E) *col5a1* cmg111 line. Data is presented as mean $\pm$ standard deviation. Every dot represents an individual zebrafish. +/+ : wild-type, +/- : heterozygous, and -/- : homozygous zebrafish. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$, one-way ANOVA.

Given the reported embryonic lethality of homozygous knockout (*Col5a1*^{-/-}) mice, it was initially surprising to notice that homozygous knockout (*col5a1*^{-/-}) zebrafish were found at the 5 dpf timepoint when performing genotyping following behavior analysis. [33]type I collagen accounts for the majority of the collagen mass, and collagen type V, the functions of which are poorly understood, is a minor component. Type V collagen has been implicated in the regulation of fibril diameter, and we reported recently preliminary evidence that type V collagen is required for collagen fibril nucleation (Wenstrup, R. J., Florer, J. B., Cole, W. G., Willing, M. C., and Birk, D. E. (2004 This prompted us to evaluate the survival of the *col5a1* zebrafish lines over time. This showed that at 1 dpf, the different genotypes approximate the expected Mendelian ratios. This ratio changes during the early stages of development with a clear decrease to absence of homozygous zebrafish at 14 dpf (Figure 6A-B). When following survival over time, a rapid drop in survival of homozygous animals is seen around 10 dpf with no homozygous animals left by 20 dpf for cmg109 and 28 dpf for cmg111 (Figure 6C-D). This indicates that, at least in early developmental stages in zebrafish, the lack of type V collagen is viable, but is associated with behavioral alterations.

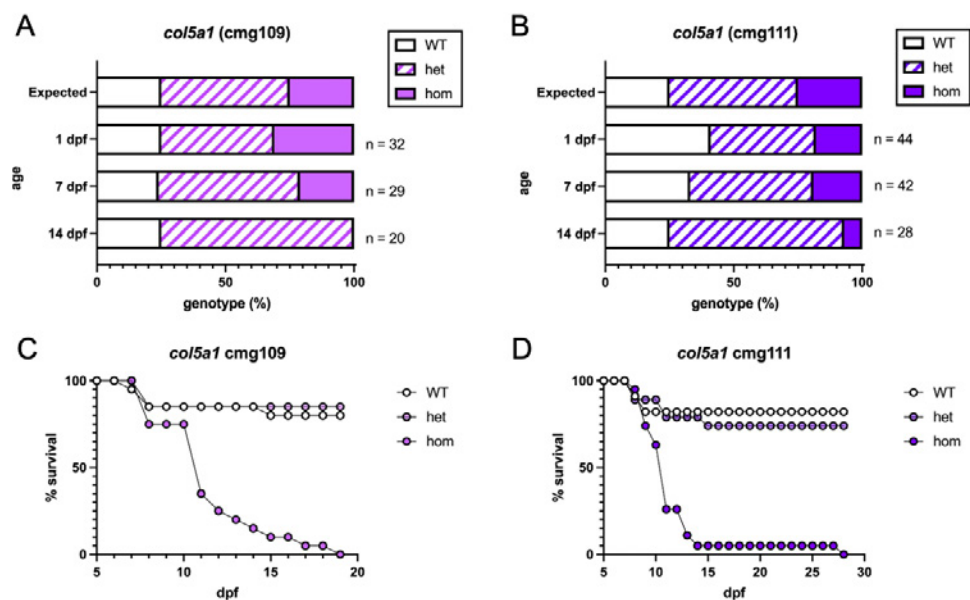
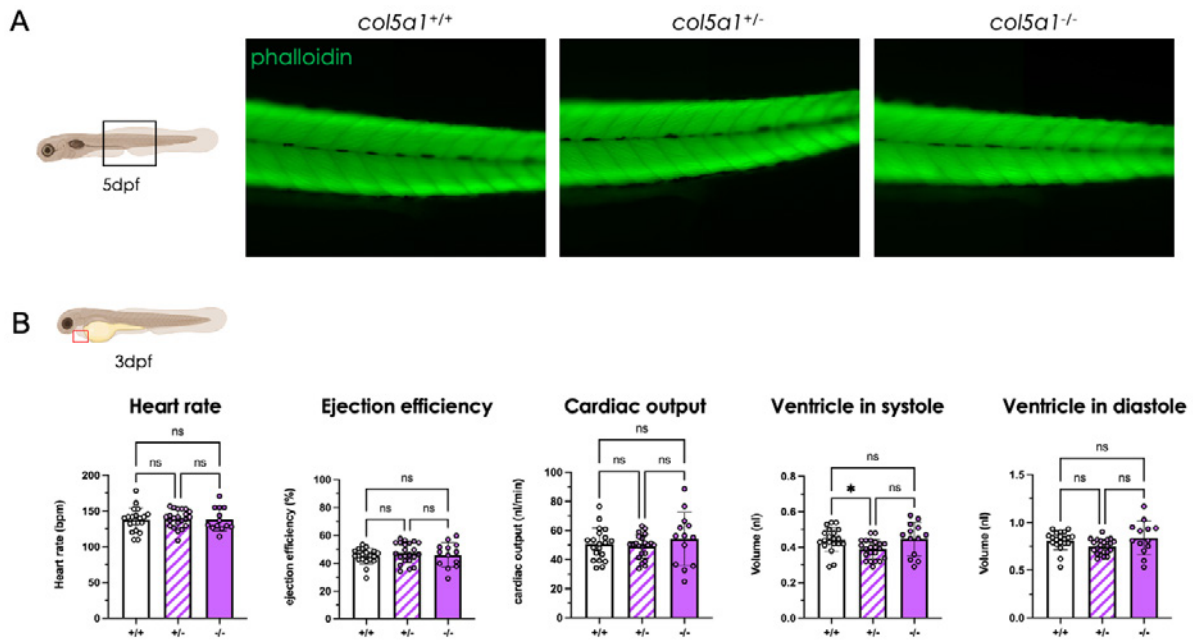
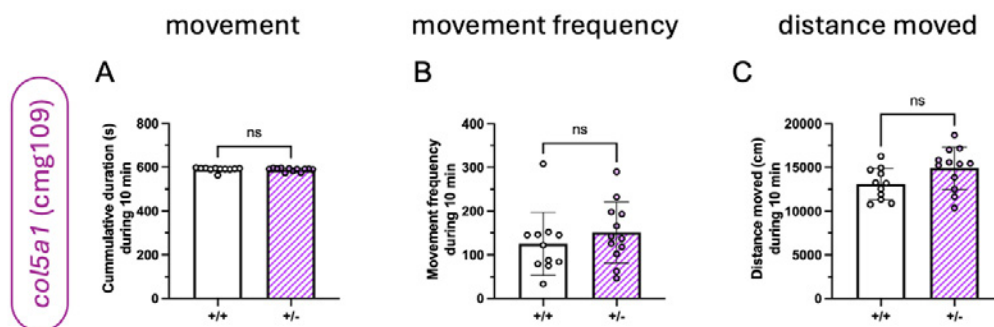


Figure 6 | Survival analysis. A clutch of zebrafish offspring from the *col5a1* cmg109 line (A) and the *col5a1* cmg111 line (B) were divided in three equal parts (initial number of zebrafish per part: n = 32 for cmg109 and n = 44 for cmg111) and genotypes were determined after 1, 7 and 14 dpf. The number of surviving animals at each age is indicated next to the bars. In addition, survival was followed over time for both the *col5a1* cmg109 line (C) and the *col5a1* cmg111 line (D).

Further phenotypic analysis included evaluation of the muscle structure by whole mount phalloidin staining of 5 dpf zebrafish, which did not show significant differences between *col5a1* genotypes (Figure 7A). In addition, cardiac assessment was performed at 3 dpf. In the *Col5a1*^{-/-} mouse model, embryonic lethality of the homozygous mice was attributed to cardiovascular failure. [33]type I collagen accounts for the majority of the collagen mass, and collagen type V, the functions of which are poorly understood, is a minor component. Type V collagen has been implicated in the regulation of fibril diameter, and we reported recently preliminary evidence that type V collagen is required for collagen fibril nucleation (Wenstrup, R. J., Florer, J. B., Cole, W. G., Willing, M. C., and Birk, D. E. (2004 Analysis of several cardiac parameters (heart rate, ejection efficiency, cardiac output, ventricle in systole and ventricle in diastole) did not show significant differences between *col5a1* genotypes (Figure 7B), indicating lack of cardiac involvement, at least at 3 dpf. Follow-up at an older age might provide more clarity about the involvement of cardiovascular insufficiency and whether it plays a role in the observed lethality.



Behavior analysis of adult *col5a1* zebrafish was performed as described above and did not show alterations between *col5a1*^{-/-} zebrafish and wild-type siblings (Figure 8).



Subsequent phenotypical and molecular characterization of *col5a1* zebrafish will include assessing mechanical properties of soft connective tissue specimens as well as ultrastructural analysis to assess collagen architecture in the ECM using transmission electron microscopy, since similar alterations are seen in the human (and murine) counterpart of cEDS. To pinpoint the molecular changes associated with the different genotypes, RNA was collected from wild-type, heterozygous and homozygous *col5a1* zebrafish and bulk RNA sequencing will be performed.

3. Project extension

Because of a parttime change in occupation, the proposed research has been delayed. Therefore, an extension of the project for one year was asked. This request was granted.

4. Output

Part of this work was presented as a **poster** at the Annual Symposium of the Belgian Society of Human Genetics in Leuven.

4.1. Abstract details:

Zoë Malfait, Filipa Oliveira, Fransiska Malfait, Delfien Syx, **Zebrafish as a model to study pain in Ehlers-Danlos syndromes**. Annual Symposium of the Belgian Society of Human Genetics, Leuven, Belgium, April 12, 2024.

Part of this work was presented as **oral presentations** at the Gordon Research Conference on Collagen in New London, NH, USA (invited presentation) and at the 2025 International Scientific Symposium on Ehlers-Danlos syndromes, Toronto, Canada (selected presentation).

4.2. Presentation details:

Delfien Syx: **Type V collagen: of man, mouse and zebrafish**, Gordon Research Conference on Collagen, New London, NH, USA, July 6-11, 2025.

Delfien Syx: **Classical Ehlers-Danlos syndrome as a model to unravel the role of defective extracellular matrix in chronic pain**, 2025 International Scientific Symposium on Ehlers-Danlos syndromes, Toronto, Canada, September 17-21, 2025.

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Queen Elisabeth Medical Foundation

Final report of the research project of the young researcher

Dr. Marijne Vandenberg
Universiteit Antwerpen (UAntwerpen)

Dr. Marijne Vandebergh (UAntwerpen)
Campus Drie Eiken
Center for Molecular Neurology
Universiteitsplein 1
2610 Wilrijk
marijne.vandebergh@uantwerpen.be
Tel. +3232653978

World-wide systematic characterization of TMEM106B and ATXN2 genetic status Towards implementation of genetic testing of modifiers in clinical practice

1. Research Summary

Frontotemporal lobar degeneration (FTLD) is a quickly progressing debilitating neurodegenerative disease and one of the leading causes of young-onset dementia. There are no treatments available that delay the onset or slow down disease progression. The most common form causes changes in behavior and personality, hence FTLD imposes a dramatic impact on social life. In about 30% of patients, a strong family history is present. Autosomal dominant mutations in the chromosome 9 open reading frame 72 (*C9orf72*) gene, the microtubule-associated protein tau (*MAPT*) gene and the progranulin (*GRN*) gene are the most common causes of genetic frontotemporal dementia (FTD). FTD is characterized by variability in age at onset and clinical presentation, even within families. This variability remains poorly understood. Previous studies suggest the transmembrane protein 106 B (*TMEM106B*) and ataxin 2 (*ATXN2*) loci as genetic modifiers of disease risk, especially in patients who already carry dominant disease mutations. The discovery of these loci is exciting; however, studies have been limited in sample size and detailed characterization of the patient cohorts.

In this project, the aims are to:

1. Characterize the *TMEM106B* protective haplotype and *ATXN2* intermediate repeat carrier status by systematic genotype screens in well-characterized large FTD cohorts and within families
2. Investigate whether the *TMEM106B* protective haplotype and *ATXN2* intermediate repeats correlate with progression of disease by linking genetic data with detailed longitudinally collected clinical, neurophysiological and imaging data

2. Final report

2.1. Characterization of the ATXN2 intermediate repeat carrier status in large FTD cohorts and investigation whether ATXN2 intermediate repeat carrier status correlates with progression of disease

2.1.1. ATXN2

The ARTFL/LEFFTDS Longitudinal Frontotemporal Lobar Degeneration study (ALLFTD, NCT04363684) is a consortium with 28 centers across the US and Canada. ALLFTD evaluates participants with FTLD spectrum disorders (bvFTD, svPPA, nvPPA, FTD-ALS, CBS, PSP), with strong family histories of FTLD, or known FTLD-associated genetic variants within the family. Study enrollment started in 2015 (till present).

As part of the Genetics Core of the ARTFL/LEFFTDS Longitudinal Frontotemporal Lobar Degeneration study (ALLFTD, NCT04363684), *ATXN2* intermediate repeat carrier status was determined in ALLFTD participants. We defined intermediate *ATXN2* repeat expansion carriers as those with at least 27 repeat units. In a cohort of 1926 individuals, we have demonstrated that there is an enrichment of intermediate *ATXN2* repeat expansion carriers in *C9orf72* mutation carriers (8.7%) compared with participants not carrying a pathogenic mutation in any of the known FTD genes (1.5% in *GRN* mutation carriers, 2.4% in *MAPT* mutation carriers and 2.8% in non-mutation carriers) (Fisher exact test $p < 0.05$, adjusted for multiple pairwise comparisons with the false-

discovery rate method). The distribution of *ATXN2* repeat expansions across the different genetic groups was further investigated through mixed effects logistic and mixed effects linear regression analyses, taking the relatedness between individuals into account through random effects. These analyses confirmed an enrichment of *ATXN2* intermediate repeat expansion carriers in *C9orf72* (**Table 1**). In addition, a mixed effects zero-inflated negative binomial model was performed as sensitivity analysis, which also showed a significant increase of *ATXN2* intermediate expansion carriers among individuals in the *C9orf72* group ($p < 0.001$).

Logistic regression			Linear regression		
Predictors	OR (95% CI)	p-value	Predictors	Estimates (95% CI)	p-value
<i>GRN</i> vs <i>C9orf72</i>	0.11 (0.02,0.67)	0.016	<i>GRN</i> vs <i>C9orf72</i>	-0.46 (-0.72,-0.21)	<0.001
<i>MAPT</i> vs <i>C9orf72</i>	0.18 (0.04,0.86)	0.032	<i>MAPT</i> vs <i>C9orf72</i>	-0.50 (-0.76,-0.24)	<0.001
NONE vs <i>C9orf72</i>	0.19 (0.08,0.44)	<0.001	NONE vs <i>C9orf72</i>	-0.45 (-0.61,-0.29)	<0.001

Table 1: the odd's ratios and estimates, corresponding 95% confidence interval (95% CI) and p-value for each predictor of the mixed effects logistic regression and linear regression model respectively

Besides the 27 repeat unit threshold, other cut-offs have been used as well in published literature. Therefore, additional analyses were conducted with a cut-off at 31 repeat units. This cut-off was applied by Henden et al.¹. In their control population, 0.66% of the individuals carried an intermediate expansion of 31 or more repeat units. In the ALLFTD cohort, 0.64% of the control individuals have 31 or more repeat units, a number very similar to published literature. Remarkably, in the ALLFTD cohort, a total of nine individuals have an intermediate expansion of at least 31 repeat units, of which two are in the control group (0.64%) and the other seven carry a pathogenic *C9orf72* expansion (2.65% of *C9orf72* carriers), leading to a significant difference between control individuals and *C9orf72* carriers ($p < 0.001$).

Interestingly, upon comparison of the clinical phenotypes of *C9orf72* carriers with *ATXN2* intermediate repeats, we observed that there are two *C9orf72* mutation carriers in ALLFTD with the progressive supranuclear palsy (PSP) phenotype, a phenotype only rarely associated with *C9orf72*, and both individuals carry an intermediate *ATXN2* repeat (**Figure 1**).

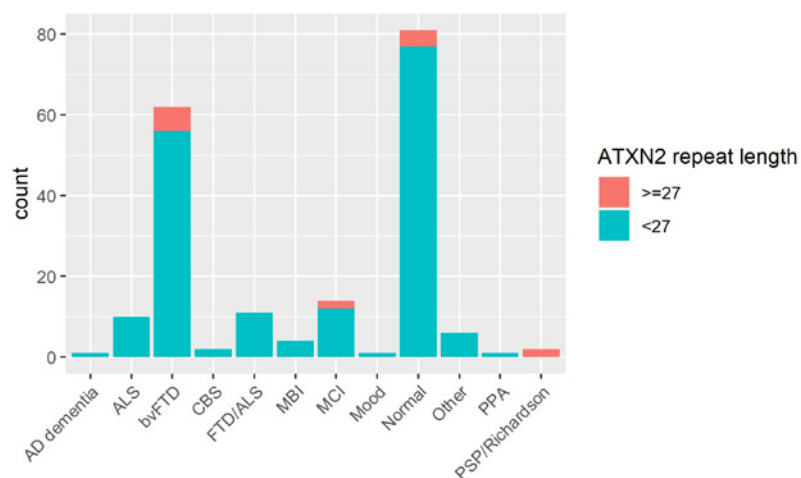


Figure 1. The distribution of *ATXN2* intermediate repeats across the clinical phenotypes of *C9orf72* mutation carriers in ALLFTD.

2.2.2. *TMEM106B*

Clinical genetic testing has been focused on autosomal dominant risk-increasing mutations rather than on genetic modifiers of disease. While *TMEM106B* has been shown to modify disease penetrance in *GRN* pathogenic variant carriers, genotyping of *TMEM106B* in *GRN* pathogenic variant carriers is currently not conducted in a clinical setting, not even in the event of a positive genetic test result for *GRN* in an asymptomatic individual. To further investigate the modifying effect of *TMEM106B*, we have conducted systematic genotype screens for *TMEM106B* in well-characterized cohorts, including cohorts from the ARTFL LEFFTDS Longitudinal Frontotemporal Lobar Degeneration (ALLFTD) consortium and the Genetic FTD Initiative (GENFI).

TMEM106B rs1990622 is a common variant, with a minor allele frequency of ~ 40 % in the European non-Finnish population (gnomAD v4.1). In the general population, ~20% of individuals are expected to be homozygous for the protective haplotype. In ALLFTD and GENFI, we have mapped the distribution of *TMEM106B* rs1990622 across the genetic groups (*GRN*, *C9orf72* and *MAPT*) and non-carrier control individuals. Both in ALLFTD and GENFI, there is a lower percentage of *TMEM106B* rs1990622*G/G carriers in the *GRN* group. The distribution of the rs1990622 genotype is significantly lower in *GRN* mutation carriers than in participants not carrying a pathogenic mutation in any of the known FTD genes and *C9orf72* mutation carriers (Fisher exact test $p < 0.05$, adjusted for multiple pairwise comparisons with the false-discovery rate method). The *TMEM106B* locus was identified as a genetic modifier of disease risk in individuals with *GRN* mutations, with carriers of the protective haplotype (rs1990622*G) having a 50% lower odds to develop disease symptoms compared to *TMEM106B* non-protective haplotype carriers with *GRN* mutations². Furthermore, our group has shown that *TMEM106B* alters the penetrance of *GRN* mutation. A patient in their 50s presented in the clinic with symptoms of behavioral variant FTD, and genetic testing revealed a pathogenic *GRN* mutation. The patient's asymptomatic parent who was in their 80s was also found to carry the *GRN* mutation, but also carried two copies of the protective *TMEM106B* allele³. Hence, we believe that that *GRN* mutation carriers with *TMEM106B* rs1990622*GG are seen less often in the clinic as they remain without symptoms, and are hence not enrolled in research studies. This would explain the lower proportion of *TMEM106B* rs1990622*GG carriers across *GRN* mutation carriers (Vandebergh et al. *Neurology*, 2025). Currently, we are collecting data of *GRN* carriers beyond ALLFTD and GENFI to further identify and investigate *GRN* mutation carriers with the *TMEM106B* protective haplotype to demonstrate its protective effects

Beyond disease susceptibility, we have further characterized the effect of *TMEM106B* on disease endophenotypes. In ALLFTD participants, I have investigated the effect of *TMEM106B* on MRI volumetric measures and cognitive phenotypes. Looking at changes in volumetric gray matter measures, *GRN* carriers homozygous for the protective allele (rs1990622*GG) had significantly greater total gray matter volumes than *GRN* mutation carriers without or with only one protective allele (linear mixed model with years of education, sex, age at visit and the CDR+NACC-FTLD sum of boxes as covariate and with pedigree as random effect, $\beta = 3.25$ [0.37,6.19], $p = 0.034$). Looking at individual (sub)cortical regions of interest, the left thalamic region was the top individual region of interest ($p = 0.006$). To further map the modifying effect of *TMEM106B*, we've investigated the association between *TMEM106B* and the National Alzheimer's Coordinating Center Uniform Data Set (v3.0) executive function composite score (UDS3-EF). This is an item response theory-based composite score derived from 7 total UDS3-EF test scores: category fluency (animals and vegetables, total correct), lexical fluency (F and L words, total correct), number span backward (total correct trials), Trail Making Test parts A and B (correct lines per minute). In *C9orf72* carriers, we identified an association between the protective *TMEM106B* allele and higher cognitive scores ($\beta = 0.36$ [0.05,0.66], $p = 0.021$). This was retained considering only presymptomatic *C9orf72*

mutation carriers ($\beta = 0.33$ [0.03,0.63], $p = 0.036$). In conclusion, we identified associations of *TMEM106B* with gray matter volume and cognition in the presence of *GRN* and *C9orf72* pathogenic variants. The association of *TMEM106B* with outcomes of interest in presymptomatic *GRN* and *C9orf72* pathogenic variant carriers could additionally reflect *TMEM106B*'s effect on divergent pathophysiologic changes before the appearance of clinical symptoms. This study has been published (Vandenberg et al., *Neurology*, 2024)⁴.

3. Presentation at international conferences (selection)

- National Society of Genetic Counselors (USA) – ALS/FTD working group meeting (online, December 10th, 2025)
- GENFI Winter Meeting 2025 (online, December 4th, 2025)
- American Academy of Neurology (AAN) Annual Meeting 2025 – San Diego, USA (April 5-9 2025)
- AFTD Holloway Summit 2025 – Miami, Florida, USA (January 22nd, 2025)
- 14th International Conference on Frontotemporal Dementias – Amsterdam, the Netherlands (September 19th, 2024)
- CReATe Consortium Annual Meeting – Miami Beach, Florida, USA (August 19th, 2024)

4. Publications with Q.E.M.F. acknowledgement

Gene-Specific Effects on Brain Volume and Cognition of TMEM106B in Frontotemporal Lobar Degeneration

Marijne Vandenberg, Eliana Marisa Ramos, Nick Corriveau-Lecavalier, Vijay K. Ramanan, John Kornak, Carly Mester, Tyler Kolander, Danielle E. Brushaber, Adam M. Staffaroni, Daniel H. Geschwind, Amy A. Wolf, Kejal Kantarci, Tania Gendron, Leonard Petrucelli, Marleen Van den Broeck, Sarah Wynants, Matthew Baker, Sergi Borrego-Écija, Brian Appleby, Sami Barmada, Andrea C. Bozoki, David Clark, R. Ryan Darby, Bradford C. Dickerson, Kimiko Domoto-Reilly, Julie A. Fields, Douglas Galasko, Nupur Ghoshal, Neill R. Graff-Radford, Ian M. Grant, Lawrence S. Honig, Ging-Yuek R. Hsiung, Edward D. Huey, David J. Irwin, David S. Knopman, Justin Y. Kwan, Gabriel C. Léger, Irene Litvan, Joseph C. Masdeu, Mario F. Mendez, Chiadi U. Onyike, Belen Pascual, Peter S. Pressman, Aaron Ritter, Erik D. Roberson, Allison Snyder, Anna Campbell Sullivan, Maria Carmela Tartaglia, Dylan Wint, Rosa Rademakers, ALLFTD Consortium.

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Association of Initial Side of Brain Atrophy With Clinical Features and Disease Progression in Patients With GRN Frontotemporal Dementia

Sergi Borrego-Ecija, Jordi Juncà-Parella, **Marijne Vandenberg**, Agnès Pérez Millan, Mircea Balasa, Albert Llado, Arabella Bouzigues, Lucy Louise Russell, Phoebe H Foster, Eve Ferry-Bolder, John C Van Swieten, Lize Corrine Jiskoot, Harro Seelaar, Robert Laforce Jr, Caroline Graff, Daniela Galimberti, Rik Vandenberghe, Alexandre de Mendonça, Pietro Tiraboschi, Isabel Santana, Alexander Gerhard, Johannes Levin, Sandro Sorbi, Markus Otto, Florence Pasquier, Simon Ducharme, Christopher Butler, Isabelle Le Ber, Elizabeth Finger, Maria Carmela Tartaglia, Mario Masellis, James B Rowe, Matthis Synofzik, Fermin Moreno, Barbara Borroni, Rosa Rademakers,

Jonathan Daniel Rohrer, Raquel Sánchez-Valle; for the Genetic Frontotemporal Initiative (GENFI)

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Q factor: Q1

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The road towards implementation of TMEM106B genetic testing in clinical practice in progranulin pathogenic variant carriers

Marijne Vandebergh, Eliana Marisa Ramos, Daniel Geschwind, Saira Mirza, Mario Masellis, Ekaterina Rogaeva, Lucy Chisman-Russell, Jonathan Rohrer, Hilary Heuer, Leah Forsberg, Adam Boxer, Howard Rosen, Bradley Boeve, and Rosa Rademakers

Published: April 7, 2025

Journal: Neurology (online)

Impact factor: 9

Q factor: Q1

doi: <https://doi.org/10.1212/WNL.0000000000210476>

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Fondation Médicale Reine Elisabeth
Königin-Elisabeth-Stiftung für Medizin
Queen Elisabeth Medical Foundation

Progress report of the research project of the young researcher

Dr. Barbara M.P. Willekens
Universiteit Antwerpen (UAntwerpen)

Prof. dr. Barbara Willekens (UAntwerpen)
Neurologist | MS & CNS Neuroimmune Disorders
T +32 3 821 34 23
Barbara.willekens@uza.be

Department of Neurology
UZA, Drie Eikenstraat 655, 2650 Edegem
T +32 3 821 30 00
www.uza.be/neurologie

University of Antwerp
Translational Neurosciences Research Group
Laboratory of Experimental Hematology, Vaccine & Infectious Disease Institute (VAXINFECTIO)
Faculty of Medicine and Health Sciences
Universiteitsplein 1, 2610 Antwerp, Belgium
Barbara.willekens@uantwerpen.be

Unravelling the role of antigen-specific T cells in NMOSD and MOGAD: first progress report

1. Project overview

Neuromyelitis Optica Spectrum Disorders (NMOSD) and Myelin Oligodendrocyte Glycoprotein Antibody Associated Disease (MOGAD) are rare autoimmune diseases of the central nervous system (CNS) that are distinct from multiple sclerosis (MS), a more prevalent CNS autoimmune disease [1, 2]. The key role of T cells in the pathogenesis of NMOSD and MOGAD remains underinvestigated. Improved understanding of antigen-specific T cell functions is relevant to improve understanding of disease pathogenesis and develop more targeted therapies, such as antigen-specific cell based treatments [3].

The importance of T cells in the pathogenesis of NMOSD and MOGAD is supported by (i) pathological features in human tissue, (ii) T cell help is needed for blood-brain-barrier breakdown and pathogenicity of auto-antibodies, (iii) Th1 and Th17 pro-inflammatory cytokine profile. However, detection of antigen-specific T cells in the peripheral blood of people with AQP4+ NMOSD and even more so in MOGAD, has been challenging so far [4].

Previous research of our group in the field of MS has demonstrated that longitudinal analysis of myelin-specific T cells with IFN- γ ELISPOT assay in a cohort of MS patients treated with natalizumab showed a high variability over time [5]. The low frequencies of MOG-specific T cells when measured in a cross-sectional setting could be a potential explanation for the previous failure of Hofer et al. to detect MOG-specific T cells in a small cohort of MOGAD patients [4].

Detection and functional characterisation of antigen-specific autoreactive T cells in the peripheral blood is a necessary step to demonstrate and strengthen the evidence for the pivotal role of T cells in the pathogenesis of NMOSD and MOGAD and may pave the way towards future development of antigen-specific T cell modulatory treatments [3, 6].

2. Research hypothesis and objectives

Unravelling the role of antigen-specific T cells in AQP4+ NMOSD and MOGAD will aid in paving the road towards development of antigen-specific tolerance inducing cell-based treatments for NMOSD and MOGAD [3]. The main hypothesis is that T cell responses are detectable in a substantial proportion of patients with MOGAD and AQP4+ NMOSD [4]. The second hypothesis is that antigen-specific T cells are functionally different between NMOSD and MOGAD patients versus healthy controls. Furthermore, antigen-specific T cell responses are likely variable over time and could be associated with disease activity and clinical disease phenotypes.

The objectives are:

1. To identify MOG specific and AQP4 specific T cells in patients with MOGAD and AQP4+ NMOSD.
2. To characterize the functional phenotype of antigen-specific T cells in patients with MOGAD and AQP4+ NMOSD

3. Achievements and ongoing work

Study protocol and informed consent documents have been written, submitted and approved by the ethical committee. Final approval by the ethics committee was obtained on July 24, 2023. The project was registered in the Antwerp Biobank, for which contracts between University Hospital Antwerp and the University of Antwerp have been put in place. All necessary documents and contracts were finalized in December 2023. Identification of potential study participants has been done and a workflow has been set up. Due to familial reasons occurring unexpectedly in 2024, but unfortunately also in 2025, additional delays in this project have occurred.

In MS patients, we have demonstrated that the number of circulating myelin specific T cells is variable over time between and within individuals [5]. Hence, longitudinal analysis, with multiple sampling points in individual patients, may increase the chance to detect MOG specific T cells in MOGAD patients. While others have used the same myelin peptides as have been implicated in multiple sclerosis, we will use intact MOG protein [4]. Our research group has established a unique protocol for enrichment of autoreactive T cells in MS using a broad antigen panel of myelin peptides [7, 8]. This protocol will be optimized (IFN- γ and IL-6 ELISPOT assay) to detect autoreactive T cells towards full-length MOG and AQP4+ peptides as described before [4]. Supernatant of cell cultures will be collected and frozen for later cytokine analysis using ELISA to measure IFN- γ , IL-6, IL-22 and GM-CSF [4, 9]. These results can provide information on the proinflammatory character of antigen-specific T cells. As pathogenic CD4+ T cells have been detected in brain lesions of MOGAD and AQP4+ NMOSD patients [10-12], it is important to clarify the mechanisms through which they may enter the CNS. Identifying expression of brain-homing markers on antigen-specific T cells could help our understanding of cell trafficking from the periphery to the CNS in these disorders [13]. The Flow Cytometry and Cell Sorting Core Facility of the University of Antwerp (FACSUA) recently acquired a new state-of-the-art Cytex Aurora which will be used to analyze surface markers of antigen-specific T cells and retain the remaining cells for downstream applications.

A request for extension of the grant period with 1 year (until 31.12.2026) was approved and an updated end-report will be provided shortly thereafter.

4. Research output with QEMF acknowledgement

A Review article has been submitted to the journal *Methods* (Q1, IF 4.3), entitled: "Novel methods for the discovery of disease-associated T cell epitopes in autoimmunity". The manuscript is currently being rewritten after a first round of revision, and planned for resubmission mid-January 2026. QEMF support is acknowledged in this manuscript.

4.1. Other relevant impact

No other impact in the past year

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Queen Elisabeth Medical Foundation

Final report of the research project of the young researcher

Dr. Wouter Peelaerts
Katholieke Universiteit Leuven (KU Leuven)

Prof. Dr. Wouter Peelaerts (KU Leuven)
Department of Neurosciences
Laboratory for Neurobiology and Gene Therapy
Department of Pharmacy and pharmaceutical
O&N5b-Herestraat 49 box 1023
3000 Leuven
Belgium

Peripheral infections as a trigger of multiple system atrophy

Multiple system atrophy (MSA) is a progressive and fatal neurodegenerative disease. The clinical presentation of MSA is heterogeneous as the disease affects a variety of central and peripheral autonomic functions. Although it is believed that MSA risk can be influenced by genetic or environmental factors, no risk factors have been identified to date. In the absence of crucial insights into the molecular etiopathogenesis there are no available biomarkers for the early detection of early disease-related changes, which limits efficient treatment options.

The hypothesis of this project is that MSA can be triggered by an infectious pathogen. We recently proposed that a trigger can occur before or during advanced disease stages and elicit a local or systemic host response¹. Infections are prominent in MSA also during the prodrome, and manifest months or years before MSA diagnosis²⁻⁴. Our overarching goal is to study both viral and bacterial infections in MSA and identify those pathogens that might have a significant impact in the development of neuropathology.

Infections manifest frequently in MSA but can go unnoticed because of autonomic failure, lower body temperature, desensitisation of the peripheral pain response or anhidrosis which can mask sudden changes, and which would otherwise warrant signs of infection. Peripheral symptoms can mask ongoing infection and worsen disease progression. During advanced stages, infections can lead to neuropsychiatric effects^{5,6} and sepsis⁷. Yet, and even though infections are common, it is not known if there is a causal link between infections and MSA.

This project focuses on the role of urinary tract infections in MSA. We discovered that urinary tract infections (UTIs) are strongly associated with MSA diagnosis when the infection occurs prior to disease onset. To the best of our knowledge, this is the first significant risk factor identified for MSA to date.

Our lab is currently investigating the contribution of several types of infections in MSA, including viral infections, but these studies are currently more exploratory whereas the focus of this project is on the role of UTIs in MSA. The link between UTIs and MSA is currently better established⁸. Our scientific goals build on these links with new methodological tools and animal models that recapitulate the complexity of genetic and environmental risk in MSA.

1. Scientific goals

Even though infections are common in MSA, it is not yet known if there is a direct or causal link between infections and MSA and/or if infections can influence disease progression. The neuroinflammatory response during UTIs has not been fully examined in MSA and it is also not known if infections in the lower urinary tract could influence central effects related to glial or neuronal health.

For our experimental approach, we use uropathogenic *E. coli* (UPEC) which is commonly used to experimentally model lower urinary tract infections, as this pathogen leads to robust inflammatory responses in mouse.

To examine how peripheral infections influence Syn pathology and trigger central changes, we have defined the following three aims:

1. establish a direct link between peripheral infections and Syn function (**report 2023**)
2. identify neuroinflammatory triggers in response to peripheral infection (**report 2023, 2024 and this report, 2025**)
3. examine the influence of peripheral infection on MSA disease progression (**report 2023, 2024 and this report, 2025**)

The main objective of this project is to identify pathogen-related triggers of MSA and investigate how disease triggers contribute to disease progression in MSA. We will focus on the specific mechanisms underlying UTI-related disease triggers.

2. Research results

Central to MSA is Syn that deposits throughout the central nervous system in oligodendroglial cells leading to demyelination and neurodegeneration⁹. The pathognomonic feature of MSA is the presence of deposits of the protein α -synuclein (Syn) in oligodendroglial cells. Accumulation of Syn results in progressive pathology in oligodendroglia with demyelination and cellular dysfunction. We and others have shown that aggregated Syn can spread between cells and convert endogenous Syn into its pathogenic form, which is reminiscent of prions¹⁰. Glial immune activation is often found in areas with high Syn burden^{11,12}. Considerable experimental and clinical evidence suggests that interactions between Syn and the immune system occur physiologically¹³⁻¹⁵.

2.1. Publication 1: Chronic UTIs cause persistent microglial changes in a humanized α -synuclein mouse model

Building on our previous epidemiological and experimental data linking UTIs to synucleinopathy⁸, we published a study examining the neuroinflammatory consequences of chronic recurrent UTIs in a humanized Syn mouse model (Mercado, Clabout et al., *Journal of Parkinson's Disease*, 2024)¹⁷. UTIs are more common in people with PD or MSA than in the general population, and their incidence is notably evenly distributed between men and women in these patient groups – contrasting with the female predominance seen in the general population. UTIs occur especially during disease, but also in the years prior to clinical diagnosis, making them a compelling candidate environmental trigger.

To model the chronic disease-relevant exposure, we used a humanized Syn mouse model (FVB;129S6-Snca^{tm1Nbm} Tg(SNCA)^{1Nbm}/J), which expresses wild-type human Syn at physiological levels while lacking endogenous mouse Syn. These mice do not normally develop motor deficits or detectable brain pathology within their lifespan, making them an ideal model to isolate the contribution of peripheral infection. Chronic recurrent UTIs were induced by repeated transurethral catheterization with UPEC (10^8 CFU), generating a non-resolving chronic UTI phenotype with persistent bacteriuria, confirmed by serial urine cultures. Bladder inflammation was confirmed by quantification of CD45⁺ leukocytes and MPO⁺ neutrophils.

Gait and behavioral motor analysis (CatWalk XT, Noldus) at 11 months of age revealed detectable motor changes in UTI-infected humanized Syn mice, nine months after the first superinfection. This is notable as these transgenic mice are not expected to develop a motor phenotype, suggesting that the chronic infectious burden contributes to functional neurological changes.

Brain-wide microglial analysis using AI-assisted deep learning (see Publication 2 below) demonstrated that recurrent UTIs lead to **persistent neuroinflammatory changes in multiple brain areas**. Critically, regions showing significantly increased microglial cell numbers were selectively those early affected in the cerebellar variant of MSA (MSA-C), including multiple lower brainstem regions, while regions associated with the parkinsonian subtype (MSA-P) did not show detectable changes. This regional specificity is a key finding, as it suggests that peripheral infectious triggers may engage distinct neuroanatomical circuits relevant to different MSA subtypes.

Focusing on the dorsal vagal complex – a brainstem hub integrating peripheral autonomic and immune signals and controlling sickness behavior – all three subregions (area postrema [AP], nucleus tractus solitarius [NTS], and dorsal motor nucleus of the vagal nerve [DMX]) showed significantly increased microglial cell numbers following recurrent UTIs. However, **microglial reactivity** (assessed by morphometric change in the area-to-perimeter ratio, reflecting the amoeboid shift of reactive microglia) was significant only in the **area postrema**, not in the NTS or DMX. This dissociation between microglial proliferation and morphological activation indicates region-specific microglial phenotypes within the same anatomical complex. The area postrema, which lacks a complete blood-brain barrier, is positioned as a key peripheral-to-central immune interface and may represent an entry point through which systemic UTI-driven inflammation reaches the brain. Microglial changes in the cingulate cortex were also observed.

Importantly, despite this persistent neuroinflammatory state, no significant changes in phosphorylated Syn (Pser129-Syn) pathology were detected in the brainstem or brain-wide in the UTI-exposed humanized Syn mice. This finding suggests that neuroinflammation, while a consequence of peripheral infection, may not be sufficient on its own to drive overt Syn aggregation in this model – and that additional factors, such as genetic susceptibility, the presence of a seeding competent Syn template, or prolonged unresolved inflammation, may be required.

2.2. Publication 2: Development of convolutional neural networks for automated brain-wide histopathological analysis in mouse models of synucleinopathies

A major methodological advance supporting our research program was published this year (Barber-Janer et al., *npj Parkinson's Disease*, 2025)¹⁸. Traditional histological quantification in preclinical studies is labor-intensive, time-consuming and susceptible to sampling bias. To address this, we developed and validated a pipeline of five convolutional neural network (CNN) models for high-throughput, unbiased, brain-wide analysis of key histopathological readouts in mouse synucleinopathy models.

The five models were trained on the Aiforia® cloud platform and validated across three synucleinopathy models: a PD model (rAAV-mediated neuronal Syn overexpression in the substantia nigra), an MSA model (rAAV-mediated oligodendrocyte-specific Syn overexpression via the MAG promoter in the striatum), and a prion-like seeding model (striatal injection of mouse preformed Syn fibrils). The five CNN models comprise:

- **DCD (Dopaminergic Cell Detector)**: detects and quantifies TH neurons in the substantia nigra, with validation against stereological counting (Pearson's $r = 0.96$, $p < 0.0001$ for ipsilateral cell counts; no significant difference from stereology for absolute counts or percent cell loss).
- **DAD (Dopaminergic Axon Detector)**: measures TH axonal staining density in the dorsal striatum (Spearman's $r = 0.86$ vs. ImageJ).
- **NCD (Neuronal Cell Detector)**: recognizes 28 brain regions and quantifies NeuN neuronal density brain-wide. In the MSA model, this detected a $30.4 \pm 9.7\%$ reduction of NeuN cells in

the globus pallidus and a $9.1 \pm 2.3\%$ reduction in the dorsal striatum ($p < 0.01$), validated against stereology ($r = 0.73-0.75$).

- **MCD (Microglial Cell Detector)**: identifies individual Iba1 microglia and extracts morphometric reactivity parameters (area/perimeter ratio) across 28 brain regions. In the MSA model, this revealed 10–20% increases in microglial density across regions including the cingulate cortex, corpus callosum, striatum, hypothalamus, and thalamus, with a partially overlapping pattern of morphological reactivity, indicating region-specific microglial phenotypes.
- **pSynD (Pser129-Syn Detector)**: maps Syn pathology brain-wide across 33 regions, classifying inclusions as neuritic or cellular. In the PFF seeding model, the highest cellular inclusion densities (~ 800 inclusions/ mm^3) were found in motor cortex and striatum, with significant involvement of the amygdala, nucleus accumbens, corpus callosum, substantia nigra, and thalamus.

All models delivered F1-scores predominantly above 80%, with analysis time reduced from weeks to hours for an entire cohort. Crucially, the CNN models showed **lower variability than stereological counting**, improving the statistical power of preclinical studies. The observation that microglial density and reactivity show only partially overlapping brain-wide patterns is an important conceptual contribution: it demonstrates that proliferation and morphological activation are dissociable, region-specific processes that must be assessed independently. Code and trained models are publicly available (github.com/DieterPlessers/Neuro_IHC_analysis).

This CNN pipeline was directly applied in Publication 1 to detect the subtle but persistent brain-wide microglial changes induced by chronic UTIs, illustrating the translational value of these methodological tools for the broader research program on infectious triggers of MSA.

3. Summary

With our epidemiological data and experimental models⁸, and the two publications resulting from this GSKE-supported project, we have substantially advanced the evidence base linking peripheral infections to synucleinopathy. Our key findings from the 2024–2025 reporting period are:

- Chronic recurrent UTIs in a humanized Syn mouse model induce **persistent microglial changes** in multiple brain regions, with regional selectivity for areas relevant to MSA-C, and are accompanied by detectable motor changes.
- The **area postrema** of the dorsal vagal complex, a circumventricular organ lacking a full blood-brain barrier, shows both increased microglial density and morphological reactivity, supporting its role as a peripheral-to-central immune communication hub in the context of UTI-driven neuroinflammation.
- Peripheral infection alone may not be sufficient to trigger overt Syn aggregation: neuroinflammation and Syn pathology appear dissociable, suggesting that genetic susceptibility or additional environmental co-factors may act in concert with infection to drive full synucleinopathy.
- We have developed and validated a suite of five **CNN-based histopathological analysis tools** for high-throughput, unbiased, brain-wide quantification of neurodegeneration, neuroinflammation, and Syn pathology in mouse synucleinopathy models, reducing analysis time from weeks to hours while improving reproducibility and statistical sensitivity.

These results have been disseminated through two peer-reviewed publications (see Publications section below) and presented to wider audiences including scientific conferences and patient organization meetings (Defeat MSA, MSA Coalition, de Vlaamse Parkinson Liga). This work provides the experimental and methodological foundation for ongoing and future studies

examining how UTIs and other peripheral infections contribute to the onset and progression of MSA.

4. Publications 2023–2025

4.1. Publication 1:

Mercado G, Clabout A-C, Howland V, Arkin E, Barber Janer A, Plessers D, Steiner JA, Smith WW, Hannan T, Brundin P, Peelaerts W. Chronic urinary tract infections cause persistent microglial changes in a humanized -synuclein mouse model. *Journal of Parkinson's Disease*. 2024;14(8):1559–1574. DOI: 10.1177/1877718X241289046.

Impact Factor: 5.0 – Q1

4.2. Publication 2:

Barber-Janer A, Van Acker E, Vonck E, Plessers D, Rosada F, Van den Haute C, Baekelandt V, Peelaerts W. Development of convolutional neural networks for automated brain-wide histopathological analysis in mouse models of synucleinopathies. *npj Parkinson's Disease*. 2025;11:317. DOI: 10.1038/s41531-025-01170-1.

Impact Factor: 8.2 – Q1

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Geneeskundige Stichting Koningin Elisabeth
Fondation Médicale Reine Elisabeth
Königin-Elisabeth-Stiftung für Medizin
Queen Elisabeth Medical Foundation

Final report of the research project of the young researcher

Dr. Sarah van Veen
Katholieke Universiteit Leuven (KU Leuven)

Dr. Sarah van Veen, PhD (KU Leuven)
FWO postdoctoral fellow

Laboratory of Cellular Transport Systems
KU Leuven, Department of Cellular and Molecular Medicine
ON Ibis Herestraat 49, box 802 – 3000 Leuven (Belgium)

T +32 (0)16 32 90 55
sarah.vanveen@kuleuven.be

The impact of ATP13A4-mediated polyamine transport in astrocytes on synaptogenesis and neurodevelopmental disorders

1. General aim of the project

The research supported by the Geneeskundige Stichting Koningin Elisabeth during the period 2023-2025 aimed to improve our understanding of molecular mechanisms that regulate brain development and function. Specifically, the project focused on the role of ATP13A4, a member of the P5B-type ATPase family, in astrocytes and its contribution to polyamine homeostasis and neuron-glia interactions in the central nervous system.

2. Scientific work performed and main results

During the funding period, a multidisciplinary experimental approach was employed, combining genetic mouse models, primary neural cell cultures, molecular and biochemical assays, targeted metabolomic analyses, quantitative imaging, and developmental phenotyping.

A central outcome of the project was the identification of ATP13A4 as a key determinant of polyamine compartmentalization in the brain. Targeted metabolomic analyses revealed that loss of ATP13A4 leads to pronounced and opposing changes in polyamine levels across compartments. Brain homogenates from ATP13A4-deficient mice showed reduced levels of putrescine and acetylated spermidine, whereas cerebrospinal fluid (CSF) displayed increased levels of these polyamines. This divergence indicates defective polyamine compartmentalization and supports a role for ATP13A4 in maintaining extracellular polyamine balance *in vivo*.

These alterations in polyamine distribution were associated with clear cellular consequences. ATP13A4 deficiency resulted in deficits in astrocyte morphology and was accompanied by increased excitatory synapse density, indicating that dysregulated extracellular polyamine levels affect astrocyte structure and synaptic development. These findings identify a metabolic mechanism through which astrocytes can influence neuronal circuit formation.

At the organismal level, ATP13A4 knockout mice exhibited delayed acquisition of early postnatal developmental milestones, consistent with disrupted neurodevelopment. In contrast, young adult animals showed only very mild behavioral alterations, predominantly in females, suggesting partial developmental compensation and/or sex-specific sensitivity.

Finally, building on prior genetic links between ATP13A4 and neurodevelopmental disorders, this project provided molecular and functional evidence that disease-associated ATP13A4 variants result in partial loss-of-function. This establishes a mechanistic framework linking ATP13A4 dysfunction, altered polyamine compartmentalization, and neurodevelopmental phenotypes.

3. Scientific and societal relevance

This project provides new insight into the role of ATP13A4 in the central nervous system and highlights polyamine handling as an important factor in brain development and astrocyte–neuron interactions. By establishing ATP13A4 as a polyamine transporter linked to cellular polyamine uptake and extracellular polyamine balance, this work contributes to a better understanding of neurodevelopmental disorders associated with ATP13A4 dysfunction and more broadly to the emerging field of glial metabolic regulation.

4. Publications (2023–2025)

The research conducted with support from the Geneeskundige Stichting Koningin Elisabeth during the 2023–2025 funding period has resulted in one preprint, which is currently under peer review. This manuscript acknowledges the support of the Queen Elisabeth Medical Foundation and represents the primary scientific output of the project to date.

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Geneeskundige Stichting Koningin Elisabeth
Fondation Médicale Reine Elisabeth
Königin-Elisabeth-Stiftung für Medizin
Queen Elisabeth Medical Foundation

Progress report of the research project of the young researcher

Prof. Giulia Liberati
Université Catholique de Louvain (UCLouvain)

Giulia Liberati (UCLouvain)

Université catholique de Louvain Institute of Neuroscience (IoNS)

Avenue Mounier 54

UCL 54.02 B-1200 Brussels

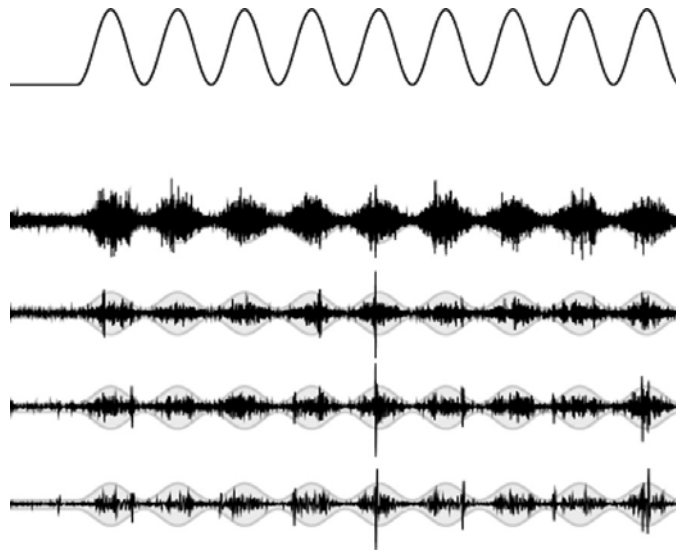
Belgium

T +32 (2) 7645467

giulia.liberati@uclouvain.be

STIM-WAVES: Identifying biomarkers with invasive and non-invasive brain stimulation targeting ongoing neural oscillations

*The research credit has been officially extended until the end of 2026, and the report will be updated accordingly



1. Introduction

Around 10% of adults worldwide suffer from chronic pain conditions¹. The identification of effective neural biomarkers for pain – which would allow improving the stratification of pain conditions, predicting responses to treatment, and offering personalized interventions – is of major importance². In recent years, works from our group^{3,4} and others^{5,6} have strongly suggested that pain perception may arise from the modulation of ongoing neural oscillations within different frequency bands. However, despite these encouraging findings, there is still disagreement on the degree to which these modulations of neural oscillations are actually related to pain, or whether they are merely an epiphenomenon.

My project, STIM-WAVES, remedies this gap by directly assessing the causal relationship between ongoing neural oscillations and pain perception, combining both scalp electroencephalography (EEG) and intracerebral electroencephalography (iEEG) with **invasive and non-invasive brain stimulation**, with the aim of (i) better characterizing pain-related modulations of ongoing neural oscillations, and (ii) understanding whether disrupting these modulations through brain stimulation would also affect pain perception.

Since the beginning of the project, **7 distinct studies** – detailed below – have either been completed or are currently underway to achieve these objectives. In addition, I published **1 literature review** within the project framework⁷.

1.1. The effect of stimulus saliency on the modulation of ongoing neural oscillations related to thermonociception (complete)

Team members participating in the study:

- Giulia Liberati: Principal Investigator (UCLouvain)
- Chiara Leu: PhD student (UCLouvain)
- Sebastien Forest: Master student and intern (UCLouvain)
- Valéry Legrain

To explore the relationship between pain perception, saliency, and ongoing oscillations, we employed a sustained periodic thermonociceptive stimulation paradigm with periodic oddball events and frequency-tagging analysis in a cohort of 35 healthy volunteers. Oddball stimuli were delivered at either higher or lower intensities compared to baseline stimuli, referred to as the “high oddball” and “low oddball” conditions, respectively. During the stimulation, participants provided continuous pain ratings to capture their perception in real-time. In the “high oddball” condition, continuous pain ratings closely mirrored the variations in stimulus intensity, indicating clear perception of intensity differences. In contrast, in the “low oddball” condition, participants’ ratings did not distinguish between different stimulus intensities. Consistently, oddball stimuli in the “high oddball” condition modulated ongoing oscillations, whereas no such modulation was observed in the “low oddball” condition. These findings suggest that the modulation of ongoing oscillations is associated with the perception of thermonociceptive stimuli that are both salient and intense.

This study, after receiving in principle acceptance as a Registered Report (<https://osf.io/s3879/overview>) was published in Cortex⁸.

1.2. The effect of attention on pain-related oscillations recorded from the human insula using intracerebral EEG (complete)

Team members participating in the study:

- Giulia Liberati: Principal Investigator (UCLouvain)
- Chiara Leu: PhD student (UCLouvain)
- Aïcha Boutachkourt: PhD student (UCLouvain)

If ongoing oscillations are related to the pain experience, then cognitive processes that are known to affect pain intensity (e.g. distraction) should also affect these oscillations. Using scalp EEG, Leu et al.⁸ recently investigated the effects of a distraction task on pain perception and on ongoing neural oscillations in the theta, alpha, and beta frequency bands. Whereas the intensity of pain perception was significantly reduced by the arithmetic task, no clear effect was observed on ongoing oscillations in any of the frequency bands. Hence, the fact that, unlike pain perception, these oscillations did not appear to be affected by the task suggests that they are dissociable from pain perception. However, two reasons for the lack of pain-related observable effects on neural oscillations could be (i) the lower signal-to-noise ratio of scalp EEG compared to other techniques, such as iEEG³, as well as (ii) the fact that scalp EEG might not be able to sufficiently detect activity in deeper pain-related brain regions such as the insular cortex. To this end, we conducted a study in which we used iEEG recorded using depth electrodes implanted in the insula of 8 hospitalized patients undergoing a presurgical evaluation of focal epilepsy. The patients had to perform the same task as in Leu et al.⁸, namely repeatedly subtracting 7 from a 3-digit number, while receiving a sustained periodic thermonociceptive stimulation or a frequency matched non-nociceptive vibrotactile stimulation contralaterally to the electrode implanted in the insula. A thermal cutaneous stimulator was used to deliver sustained periodic thermonociceptive stimuli (0.2 Hz). Each sustained periodic stimulation comprised 15 stimulation

cycles, lasting a total of 75 s per stimulus^{3,8,9}. Inter-stimulus intervals were variable and self-paced by the experimenter to allow participants to provide intensity ratings, as well as to give responses to the arithmetic task. Sustained periodic vibrotactile stimuli were delivered via a recoil-type vibrotactile transducer driven by a standard audio, positioned between the fingertips of the index and the thumb. The vibrations consisted in a 251 Hz sine wave whose amplitude was periodically modulated at a frequency of 0.2 Hz. We chose this kind of stimulation because, despite being non-nociceptive and non-painful, it is generally perceived as highly intense, which makes it a suitable comparison to investigate whether the observed modulations might be preferential for pain perception.

The arithmetic task reduced perceived intensity for both stimulus types, but had different effects on brain activity: it decreased oscillations in the posterior insula for thermonociceptive stimuli, while increasing oscillations for vibrotactile stimuli. The posterior insula showed consistently stronger responses than the anterior insula, with thermonociceptive stimuli producing notably stronger alpha-band activity, in agreement with previous findings from the lab³. This suggests the posterior insula may have a specialized role in processing thermonociceptive signals, particularly in the alpha frequency band, though findings in other frequency bands (like theta) were less conclusive.

The manuscript of this study is currently in preparation. The study was presented at the *10th Science Factory: TMS-EEG Summer School and Workshop* — Espoo, Finland, 2024. and at the *EFIC Congress*, Lyon, France, 2025, and the *Society for Neuroscience (SFN) Conference*, San Diego, USA, 2025.

The study was awarded Best Poster Presentation at the EFIC 2025 Congress in Lyon.

1.3. Individualized alpha-transcranial alternating current stimulation (tACS) for modulating pain perception (complete)

Team members participating in the study:

- Giulia Liberati: Principal Investigator (UCLouvain)
- Yaser Fathi Arateh: Postdoctoral researcher (UCLouvain)
- Françoise Dissassuca: Master student and intern (UCLouvain)
- Gloria Ricci: Erasmus master student and intern (Università di Padova)

Pain encompasses sensory, affective, and cognitive dimensions, with neural oscillations increasingly recognized as key mechanisms in their integration. However, the underlying processes remain inadequately understood. Transcranial alternating current stimulation (tACS) offers a promising tool for modulating these oscillations, but its efficacy in pain modulation remains inconsistent, possibly due to a lack of individualized stimulation protocols. In this study, we investigated the effects of tACS at the individual peak alpha frequency (IAF) on both pain perception and neural oscillations. Using a within-subject, sham-controlled design, we applied tACS over the primary motor cortex (M1) contralateral to the dominant arm in 38 healthy participants. Sustained and periodic 0.2 Hz thermonociceptive stimuli were applied to the dominant forearm before and after tACS. Pain perception and heat pain thresholds (HPT) were assessed before and after stimulation, while scalp electroencephalography (EEG) was recorded during the thermonociceptive stimulation. To calculate IAF, we used a discriminative approach based on independent component analysis (ICA) to isolate somatosensory IAF (SM-IAF). The results revealed an overall increase in pain perception and a decrease in HPT in both sham and active conditions, with no significant interactions between conditions. However, a trend toward reduced sensitization post-tACS was observed. Exploratory analyses suggested a

potential effect of tACS on HPT in women, but not in men. Furthermore, a significant correlation emerged between SM-IAF and HPT. These findings provide novel perspectives on advancing individualized neuromodulation approaches for pain.

This study has been presented at the *IASP World Congress on Pain*, Amsterdam, Netherlands, 2024, at the *Transcranial Brain Stimulation Neuroscience Workshop*, Rovereto, Italy, 2024, at the *6th International Brain Stimulation Conferences*, Kobe, Japan, 2025, at the *EFIC Congress*, Lyon, France, 2025, and at the *Neupsig Conference*, Berlin, 2025.

The study received the **Annual Award of the Belgian Pain Society (BPS)** <https://www.belgianpainsociety.org/bps-prize> and was recently published in the *European Journal of Pain*¹⁰.

1.4. Using periodic oscillations to analyze tACS effects on IAF (in progress)

Team members participating in the study:

- Giulia Liberati: Principal Investigator (UCLouvain)
- Gloria Ricci: Erasmus master student and intern (Università di Padova)
- Yaser Fathi Arateh: Postdoctoral researcher (UCLouvain)

We aimed to assess whether tACS can modulate neural oscillations, focusing on changes in peak frequency and amplitude within the alpha band. We retrospectively analyzed EEG data from 38 healthy volunteers who followed a protocol in which tACS was delivered over M1, contralateral to a thermonociceptive stimulus applied on the forearm (see Study 4 of this document). To personalize stimulation, the tACS stimulation frequency was set to the individual peak alpha frequency (IAF), with sham stimulation as a control. To allow for a more precise analysis of neural oscillations by isolating genuine oscillatory activity from background noise, we decomposed the alpha-band spectrum into aperiodic and periodic components using the FOOOF algorithm. We then fit the periodic component with a gaussian curve. Parameters of the gaussian curve - IAF, Power (PW) and Bandwidth (BW) - from the pre-stimulation condition were used to compute the Arnold tongue for each participant, namely the parameter space where synchronization between neural activity and stimulation frequency is believed to occur. A linear mixed model was used to compare the effects of "time" (pre vs post), and "condition" (active vs sham) on neural oscillations across three sessions: Eyes Open (EO), Eyes Closed (EC), and during the perception of thermonociceptive stimuli (i.e. somatosensory session). While a modulation of neural oscillations was observed in all sessions under the active condition compared to sham, none of these effects reached statistical significance. However, further analysis of the BW/PW ratio using a Wilcoxon rank-sum test with Bonferroni correction revealed that the somatosensory session had a significantly higher ratio, suggesting it provides the most effective condition for neuromodulation. This study emphasizes the importance of removing aperiodic components in analyzing neuromodulation. Importantly, it paves the way for a more personalized tACS approach, where not only the IAF is taken into account, but the stimulation frequency is tailored using each individual's Arnold tongue.

The study was recently presented at the *Transcranial Brain Stimulation Neuroscience Workshop*, Rovereto, Italy, 2024, and at *6th International Brain Stimulation Conferences*, Kobe, Japan, 2025.

1.5. Precision targeting of temporal interference stimulation (TIS) over the insula using computational modeling and cadaver investigations (complete)

Team members participating in the study:

- Giulia Liberati: Principal Investigator (UCLouvain)
- Yaser Fathi Arateh: Postdoctoral researcher (UCLouvain)
- Aïcha Boutachkout

Noninvasive brain stimulation for the treatment of chronic pain typically targeted the primary motor cortex (M1), premotor cortex, and primary sensory cortex (S1), with a significant proportion of patients not responding to these interventions^{11–13}. This focus is largely because these regions are easier to access due to their proximity to the brain's surface. However, deeper brain regions, particularly the insula, cannot be neglected when developing interventions¹⁴, especially considering that the dysregulation of insular oscillatory activity is often associated with chronic pain¹⁵. Crucially, because the insula is an essential hub that mediates interactions between different networks^{16,17}, it offers the potential to induce more widespread, global effects across interconnected neural systems. Unfortunately, precisely stimulating the insula without the need for invasive electrode implantation presents a significant challenge. Advances in transcranial magnetic stimulation (TMS), like cooled double-cone coils, enable deeper cortical stimulation¹⁴ but lack selectivity, possibly leading to unpredictable side effects like seizures^{18,19}. Transcranial focused ultrasound stimulation (tFUS) offers greater depth and focal resolution²⁰, but requires high energy to overcome skull attenuation, risking tissue overheating and rare yet severe side effects, such as hemorrhages, cell death, cavitation damage, and blood-brain barrier disruption²¹. Temporal interference stimulation (TIS) could potentially enable precise targeting of deep brain structures without affecting nearby areas²². Given its novelty and the complexity of its implementation, the application of TIS in humans has been very limited^{23–25}, despite some preliminary research performed mostly in animals and in isolated neurons²⁶, and using computational models and simulations²⁷.

This study assessed selectivity and focality of TIS in targeting different subregions of the insula while minimizing electric field (EF) exposure to adjacent or overlying cortical areas. To achieve this, we integrated computations modeling with human cadavers, guided by simulations of EF distributions. Using high-resolution anatomical data and finite element modeling (FEM)²⁸, we simulated EF intensity and focality across insular subregions, optimizing electrode configurations and current parameters. It is important to point out that whereas it is known that *pre-* and *post-mortem* induced EFs differ significantly in strength and frequency, *relative EF distributions* (scaled to individual minimum and maximum values) are largely comparable across conditions²⁹, meaning that the focality and spatial distribution of stimulation can be reliably assessed in human cadavers. This approach also allows exploring the *steerability* of TIS fields, by adjusting the amplitude ratios of stimulation currents while keeping the carrier frequencies constant. Additionally, we compared the EF generated by insular TIS with that produced by deep brain stimulation of the insula using depth electrodes. Data from 2 cadavers has been acquired, demonstrating the potential of TIS to target specific insular subregions with focal precision.

Preliminary findings were presented at the *6th International Brain Stimulation Conferences*, Kobe, Japan, 2025. An article is currently in preparation.

1.6. Exploring the link between ongoing oscillations and pain perception through high-frequency rTMS: a brain-state dependent TMS-EEG study (in progress)

Team members participating in the study:

- Giulia Liberati: Principal Investigator (UCLouvain)
- Chiara Leu: PhD student (UCLouvain)
- André Mouraux (UCLouvain)
- Paolo Belardinelli: external collaborator (University of Trento)

This study initiated a collaboration between my lab and the lab of Prof. Paolo Belardinelli (University of Trento).

Pain is a highly flexible phenomenon, which is likely mediated by ongoing neural oscillations. To fully understand the functional relationship between ongoing oscillations and pain perception, it is imperative to investigate a causal link between these phenomena. To this end, we used high-frequency brain-state dependent repetitive transcranial magnetic stimulation (rTMS) to selectively alter cortical excitability and inhibition, by stimulating either at the trough or peak of the μ -rhythm (measured online using EEG), using 200 triplets at 100 Hz at 80% of the resting motor threshold (RMT). Not unlike rTMS, pain has been shown to alter corticomotor excitability, as illustrated by a reduction in motor-evoked potentials (MEPs) following experimental pain interventions. Thus, changes in cortical excitability could potentially modulate pain perception. Using a pre-/ post-intervention paradigm, we will assess changes in the response to brief thermonociceptive stimuli (62°C, 200ms) in cortical activity measured by EEG as well as in the subjective perception of the individual. Following a high-frequency rTMS intervention targeting the trough of the μ -rhythm, we expect to induce a state of higher cortical excitability. We hypothesize that this will cause an increase in the modulation of ongoing oscillations as well as in the pain perception. Conversely, targeting the peak of the μ -rhythm would lead to an opposite effect. The obtained results will further inform us about the neurophysiology of pain and facilitate the development of novel therapeutic interventions for chronic pain patients.

The study protocol has been presented at the *Transcranial Brain Stimulation Neuroscience Workshop* and at the *6th International Brain Stimulation Conferences*, Kobe, Japan, 2025. Preliminary data will be presented at the *IASP* conference, Bangkok, 2026.

1.7. Exploring the effects of transcutaneous auricular vagus nerve stimulation (taVNS) on pain perception (in progress)

Team members participating in the study:

- Giulia Liberati: Principal Investigator (UCLouvain)
- Javier Chavez Cerda: postdoctoral researcher (UCLouvain)

Whereas non-invasive brain stimulation approaches have demonstrated analgesic potential¹⁵, their use is constrained by limited portability, high cost, operator dependency, and the need for controlled settings^{25,26}. Within this landscape, transcutaneous auricular vagus nerve stimulation taVNS has gained increasing attention due to its low cost, ease of use, safety, and high tolerability (dropout rate of 2.6%)²⁷⁻²⁹ its safety and tolerability profile is unclear. Objective: We performed a systematic review of treatment harms from tVNS in humans. Methods: A systematic published and grey literature search was carried out to identify studies which deployed tVNS in human subjects. Study authors were contacted for safety/tolerability data if these were not available in the publication. Databases were searched from 1966 to May 2017. We noted study type, population, stimulation parameters, type and prevalence of side effects and/or serious adverse events (SAE, as well as its reduced sensitivity to operator-dependent parameters³⁰ the persistent secondary

neuropsychiatric effects are still not fully understood. These “long COVID” symptoms, also referred to as post-acute sequelae of SARS-CoV-2 infection (PASC), taVNS targets the auricular branch of the vagus nerve (ABVN) via the external ear, activating key brainstem nuclei such as the nucleus tractus solitarius, raphe nuclei, and locus coeruleus^{31,32} mood, health, and even decision making in our everyday lives. In support of this interaction, the vagus nerve is a crucial pathway transmitting diverse gut-derived signals to the brain to monitor of metabolic status, digestive processes, or immune control to adapt behavioural and autonomic responses. Hence, neuromodulation methods targeting the vagus nerve are currently explored as a treatment option in a number of clinical disorders, including diabetes, chronic pain, and depression. The non-invasive variant of vagus nerve stimulation (VNS). This activation leads to the release of neurotransmitters involved in descending pain inhibitory pathways³³ and shares mechanistic similarities with invasive VNS³⁴. Clinical and experimental studies report reductions in pain intensity and duration across several pain conditions³⁵. Nevertheless, taVNS findings remain highly heterogeneous and difficult to replicate^{36,37} including randomized controlled trials published until October 2023, which assessed the effects of noninvasive tVNS. Cohen's d effect size and 95% confidence intervals (CIs – a variability reflecting fundamental methodological limitations. Current taVNS research is limited by two major methodological gaps: large variability in stimulation parameters and locations, and inadequate blinding. Anatomical variability in auricular innervation undermines both active and sham conditions, while suprasensory, physiologically timed stimulation increases expectancy biases. This study addresses these issues by introducing an individualized mapping device to optimize stimulation parameters and a novel blinding strategy that dissociates stimulation timing from perceptual cues, enabling unbiased assessment of timing-dependent taVNS effects. Preliminary data have been successfully acquired to validate the experimental setup and feasibility of the approach. In parallel, custom-made auricular electrodes for taVNS were designed and fabricated, and the individualized mapping procedure has been implemented and tested, laying the groundwork for systematic data collection

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Final report of the research project of the young researcher

Prof. dr. Jeroen Bogie
Universiteit Hasselt (UHasselt)

Prof. dr. Jeroen Bogie (UHasselt)

Assistant Professor

T +32(0)11 26 92 61

www.uhasselt.be

Universiteit Hasselt - Campus Diepenbeek

Agoralaan Gebouw D - B-3590 Diepenbeek

Kantoor BMO-C107a

Getting a grip on slippery protein modifications in multiple sclerosis

1. Acknowledgements

With this report, I would like to sincerely thank the GSKE for the financial support of the project entitled "Getting a Grip on Slippery Protein Modifications in Multiple Sclerosis." Over the past year, this funding has enabled us to make substantial progress toward the objectives outlined in the original proposal. Moreover, it has allowed me, as an early-stage tenure-track principal investigator and research group leader, to further establish myself as an expert in the fields of immunometabolism, neuroinflammation, and remyelination.

We have thoroughly investigated the impact of S-palmitoylation on microglial physiology and remyelination, and we are currently finalizing the revision of our manuscript in *Nature Neuroscience*. In parallel, we examined the role of S-palmitoylation in autoimmunity, with ongoing efforts focused on elucidating the cellular and molecular mechanisms underlying the complete absence of autoimmunity observed in preclinical models of multiple sclerosis following inhibition of S-palmitoylation. This research line is progressing well, and submission is anticipated in 2027.

Beyond S-palmitoylation, we have also explored the contribution of other post-translational modifications to microglial phenotype determination, with a particular focus on ubiquitination. These findings were published in *Nature Communications* in 2025. Although funding was not specifically requested for the exact experiments described in this manuscript, consumables and resources are often shared across projects within my research group. We strive to ensure appropriate acknowledgment of all contributing funding agencies in such cases.

In addition, we authored literature reviews discussing these findings and presenting our perspective on studying lipid biology in health and disease, which were published in *Nature Reviews Neuroscience* and *Molecular Neurodegeneration*. While review articles are not experimental in nature, they are strongly rooted in experimental discoveries that are only made possible through sustained funding. Accordingly, we consistently acknowledge the relevant funding bodies in these publications. Further details on these manuscripts and the associated data can be found in the sections below ("*Scientific Output*" and "*Acquired Data*").

The progress and achievements described here would not have been possible without the generous support of the GSKE. I am deeply grateful for this opportunity and sincerely hope to have the privilege of applying for continued funding in the coming year.

2. Scientific output

2.1. Published

- Kuipers K, Vanherle S, Poelmans K, Wolfs E, **Bogie J**, Vangansewinkel T. Lipid alterations in hereditary peripheral neuropathies: common mechanisms in disease heterogeneity? *Molecular Neurodegeneration*. 2026 Feb 4. doi: 10.1186/s13024-026-00932-6. (IF=17.5, Q1).
- Loix M, Vanherle S, Bolkaerts L, Verberk SGS, Punt M, Wouters F, Moonen B, Verhagen R, Van Wouw SAE, Jongejan A, Distel B, Elgersma Y, Haidar M, Zelcer N, Hendriks JJA, **Bogie JFJ**. UBE3A promotes foam cell formation and counters remyelination by targeting ABCA1

for proteasomal degradation. [Nature Communications](#). 2025 Aug 29;16(1):8077. doi: 10.1038/s41467-025-62053-w. (IF=15.7, Q1)

- Vanherle S, Loix M, Miron VE, Hendriks JJA, **Bogie JFJ**. Lipid metabolism, remodelling and intercellular transfer in the CNS. [Nature Reviews Neuroscience](#). 2025 Apr;26(4):214-231. doi: 10.1038/s41583-025-00908-3. (IF=26.7, Q1)

2.2. Under revision

- Jeroen Guns, Sam Vanherle, Samiksha Sardana, Kai Hong, Brecht Moonen, Lien van Otterdijk, Melanie Loix, Koen Kuipers, Flore Wouters, Kirsten Poelmans, Tess Dierckx, Leen Timmermans, Tim Vangansewinkel, Esther Wolfs, Bieke Broux, Bart Ghesquière, Luciana Berod, Monique T Mulder, Florian Hermans, Noam Zelcer, Erin M Gibson, Marc P Baggelaar, Miranda Nabben, Jerome JA Hendriks, Dante Neculai, Joost JFP Luiken, **Jeroen FJ Bogie**. zDHHC5-driven FABP5 palmitoylation impairs brain repair by disrupting foam cell physiology. [Nature Reviews Neuroscience](#) (under revision). (IF=21.1, Q1)

2.3. Ongoing

- Tentative title: zDHHC2 promotes dendritic cell maturation and autoimmunity. Expected submission Q2 2027.

Experimental details on these projects can be found in the section "*Acquired data*" for results that have not yet been published. For manuscripts that have been published, PDF copies have been attached to this progress report.

3. Financial summary

The final financial report can be found in addendum 1.

4. State-of-the-art of the submitted project

A major pathological hallmark of neuroinflammatory disorders such as multiple sclerosis (MS) is the accumulation of macrophage subsets and T cells within the CNS. Driving these immune cells towards an anti-inflammatory, repair-promoting phenotype is considered a promising therapeutic strategy to halt MS disease progression and promote CNS repair. Emerging evidence indicates that fatty acids control the fate of immune cells in inflammatory disorders such as MS. We and others demonstrated that changes in the desaturation, β -oxidation, and synthesis of fatty acids regulate the induction and maintenance of anti-inflammatory and reparative macrophage, microglia, and CD4⁺ T cell subsets in experimental models of MS. To date, however, the culprit fatty acids and molecular mechanisms that govern their influence on immune cell function and MS pathology remain ill-defined.

The saturated fatty acid palmitate is the most common fatty acid in the human body. It is situated at the heart of fatty acid metabolism, as it is the primary end-product of fatty acid synthesis and represents a major substrate for β -oxidation and desaturation. We recently demonstrated that subtle changes in cellular palmitate levels have a major impact on the inflammatory and reparative properties of immune cells *in vitro* and in animal models of MS. Most studies on fatty acids in general, and palmitate in particular, focused on their role in energy metabolism and their effect on membrane composition. Based on our preliminary findings, we propose that S-palmitoylation (S-palm), a posttranslational modification that consists of the reversible addition of palmitate to

cysteine residues on proteins, is an important determinant of palmitate function in immune cells and MS pathology. In line with the importance of this modification, palmitoyl-proteome screens indicate that over 25% of the proteome is susceptible to S-palm, and accordingly, a family of 24 zinc finger DHHC-domain containing proteins (zDHHCs) have been identified as palmitoyl-transferases. Ample evidence indicates that S-palm dynamically regulates protein membrane interactions, intracellular sorting, and stability, and in doing so, tightly controls cellular function in health and disease

Our understanding of the role of S-palm in immunity is limited. Importantly, no studies have thus far addressed how changes in the S-palm landscape and machinery impact the polarization of primary macrophage, microglia, and T cell subsets, and to what extent these alterations can be harnessed to reduce neuroinflammation and promote CNS repair. The short-term goal of this project is to address the role of S-palm in the induction of disease- and repair-associated immune cell subsets in MS. The long-term goal of this project is to identify S-palm as a novel therapeutic target to suppress neuroinflammation and promote CNS repair in MS.

5. Acquired data

The data we have acquired so far pertain to the impact of S-palmitoylation on T cell physiology and autoimmunity (1), as well as on microglia physiology and remyelination (2). In this section, we will briefly address the findings related to (1) and outline upcoming experiments. For (2), we have included a version of the manuscript that has passed initial revision rounds in *Nature Neuroscience*.

5.1. Inhibition of S-palmitoylation prevents autoimmunity in MS.

Autoimmunity is widely recognized as a key driver of disease initiation and progression in MS. In this context, autoreactive T cells have been extensively studied, and strategies that promote the formation of immunosuppressive immune cell subsets are considered highly promising therapeutic approaches to halt disease progression. With the support of the Geneeskundige Stichting Koningin Elisabeth, we identified S-palmitoylation (S-palm) as a pivotal regulator of autoimmune-mediated brain pathology in MS.

In the experimental autoimmune encephalomyelitis (EAE) model, which is commonly used to study T cell-mediated autoimmunity in MS, systemic inhibition of S-palmitoylation using 2-bromopalmitate (2BP) completely abrogated disease severity (**Fig. 1A**). Given this striking protection, we hypothesized that early immunological changes in secondary lymphoid organs underlie the beneficial effects of S-palm inhibition. Indeed, immunophenotyping revealed that reduced EAE severity was associated with diminished antigen-specific T cell proliferation and a relative increase in regulatory and anergic T cells (Treg and Tan) in secondary lymphoid tissues (data not shown). Notably, *in vitro* experiments demonstrated that S-palmitoylation inhibition does not directly affect T cell physiology (data not shown).

These findings prompted us to investigate the role of antigen-presenting cells. We found that inhibition of S-palmitoylation induces a tolerogenic dendritic cell (DC) phenotype *in vitro* (**Fig. 1B-C**). In co-culture experiments, these DCs failed to effectively activate autoreactive T cells (**Fig. 1D**). Importantly, these observations were validated in human cell cultures in collaboration with Prof. Nathalie Cools (data not shown). To directly assess the contribution of DCs *in vivo*, we performed adoptive transfer experiments in which EAE mice received DCs treated with either the S-palmitoylation inhibitor or vitamin D3, a well-established inducer of tolerogenic DCs.

Remarkably, DCs exposed to the palmitoylation inhibitor conferred superior protection compared to vitamin D3-treated DCs, as evidenced by a greater reduction in EAE severity (**Fig. 1E**). This protection was associated with increased Treg frequencies and reduced Th17 cell abundance in the spleen (**Fig. 1F**). Extending these findings to another autoimmune model, we treated collagen-induced arthritis (CIA) mice with 2BP and observed a reduction in disease severity, albeit less pronounced than in EAE (**Fig. 1G**).

As S-palmitoylation is catalyzed by 24 acyltransferases (zDHHC1–24), we next sought to identify which enzyme mediates the protective effects of pan-inhibition. DC maturation was associated with minimal changes in zDHHC expression, except for zDHHC2, which exhibited a striking 11-fold increase in mature compared to immature DCs (**Fig. 1H**). To validate its functional relevance, we obtained zDHHC2-deficient mice and generated bone marrow-derived DCs. Notably, zDHHC2 deficiency impaired DC maturation, closely phenocopying the effects of 2BP-mediated pan-inhibition (**Fig. 1I**).

Currently, we are: (1) assessing the impact of zDHHC2 deficiency on T cell activation and differentiation *in vitro*; (2) breeding zDHHC2 knockout mice for EAE experiments, including adoptive DC transfer studies; (3) performing palmitoyl-proteomics and RNA sequencing on wild-type and knockout DCs to elucidate underlying molecular mechanisms; (4) establishing access to lymph node tissue from MS patients to validate our findings in a clinical context; and (5) analyzing our single-cell RNA sequencing datasets from spleen and inguinal and cervical lymph nodes of EAE mice treated with vehicle or 2BP. These experiments, particularly the proteomic and transcriptomic analyses, will allow us to identify differentially S-palmitoylated target proteins regulated by zDHHC2 and to define the molecular pathways responsible for the induction of DC tolerance.

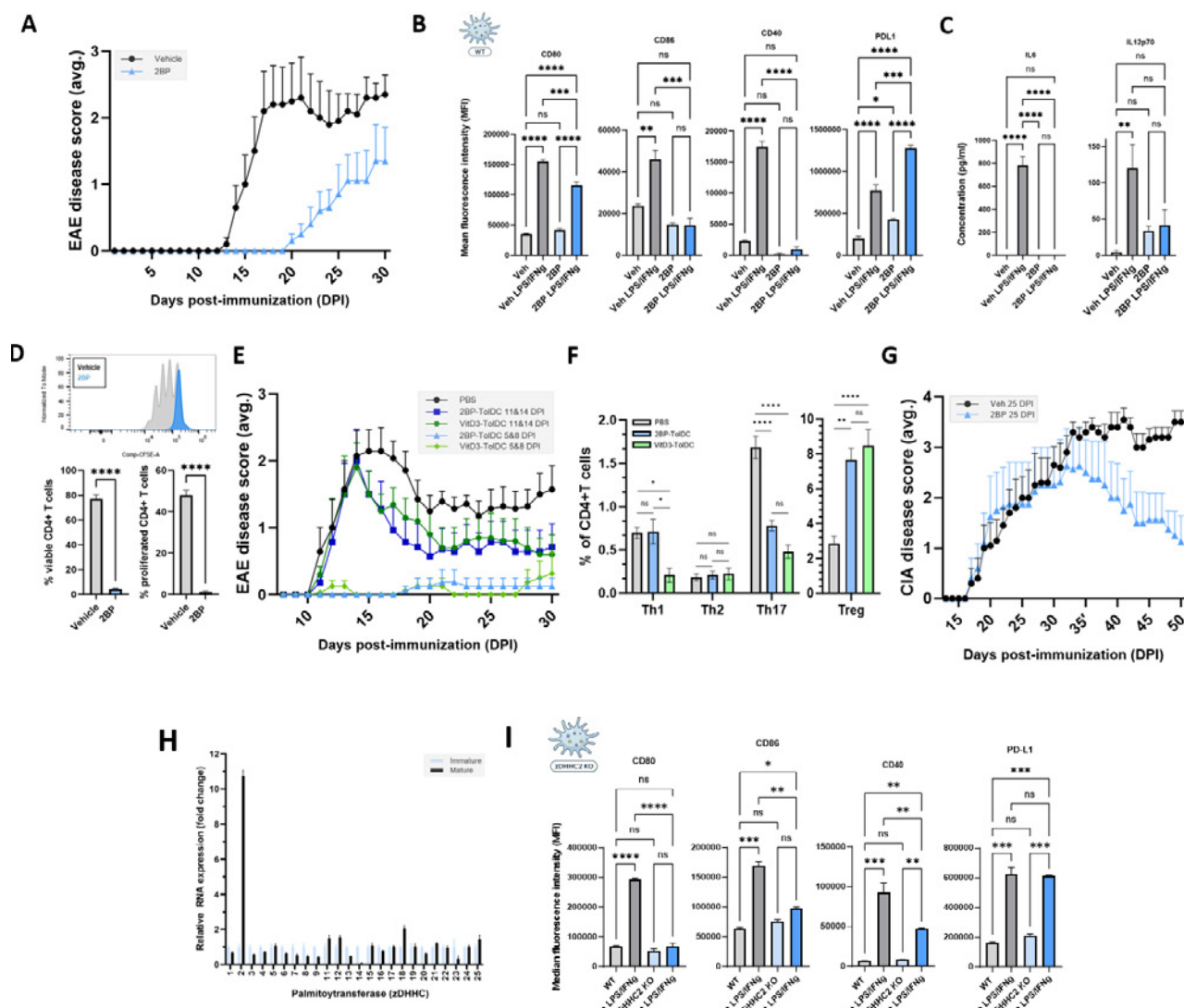


Figure 1. Inhibition of S-palm through ZDHHC2 suppresses autoimmunity by inducing tolerogenic dendritic cells. (A) Clinical disability scores of EAE mice treated daily with an intraperitoneal injection of 40mg/kg 2-bromopalmitate (2BP; n=5) or vehicle (n=5), starting on day 5 post immunization (DPI). (B) Bone marrow-derived DCs (BMDCs) were treated with vehicle (0.01% DMSO) or 20μM 2-BP on day 0 of culture. On day 7 the BMDCs were matured with 100 ng/mL lipopolysaccharide (LPS) and IFN-γ. After 24h, surface marker expression was analyzed by flowcytometry. Mean fluorescence intensity (MFI) is shown for (left to right) costimulatory molecules CD80, CD86, CD40 and inhibitory marker PD-L1 (n=3). (C) BMDCs were treated with vehicle (0.01% DMSO) or 20μM 2-BP on day 0 of culture. On day 7 the BMDCs were matured with 100 ng/mL lipopolysaccharide (LPS) and IFN-γ. After 24h, supernatant was collected and the cytokine expression of inflammatory cytokines IL6 and IL12p70 was determined by ELISA (n=6). (D) BMDCs were treated with vehicle (0.01% DMSO) or 20μM 2-BP on day 0 of culture. On day 7 the BMDCs were matured with 100 ng/mL lipopolysaccharide (LPS) and IFN-γ. After 24h, BMDCs were MOG₃₅₋₅₅-pulsed for 4h and brought into coculture with CFSE-labeled CD4+ T cells from 2D2 mice at a 1:5 ratio for 72h. Afterward, viability and proliferation of the CD4+ T cells was assessed with flowcytometry by zombieNIR staining and CFSE-dilution (top) respectively. (E) Clinical EAE disability scores of EAE mice treated with MOG₃₅₋₅₅-pulsed 2BP-tolDCs, MOG₃₅₋₅₅-pulsed VitD3-tolDCs or vehicle (PBS). Treatment administered by IV injection of 1*10⁶ Tol-DCs or 100μl vehicle either prophylactically on 5&8 DPI or therapeutically on 11&14 DPI. Prophylactically: 2-BP-tolDCs, VitD3-tolDCs (n = 7), or vehicle (PBS) (n = 5). Therapeutically: 2-BP-tolDCs (n = 7), VitD3-tolDCs (n = 5), or vehicle (PBS) (n = 7). (F) Flowcytometry staining determining the T cell subsets (Th1, Th2, Th17, Treg) within splenocytes isolated at 10 DPI from EAE mice treated with 1*10⁶ MOG₃₅₋₅₅-pulsed 2BP-tolDCs, 1*10⁶ MOG₃₅₋₅₅-pulsed VitD3-tolDCs or 100μl vehicle (PBS) on 5&8 DPI. (G) Clinical disability scores of CIA mice treated daily with an intraperitoneal injection of 40mg/kg 2BP (n=5) or vehicle (n=5), starting on day 25 post immunization. (H) Relative mRNA expression levels of 24 ZDHHC palmitoyltransferases in immature and mature BMDCs. On day 7, BMDCs were left unstimulated (immature) or stimulated with 100 ng/ml lipopolysaccharide (LPS) and IFN-γ (mature). (I) BMDCs generated from wildtype (WT) or ZDHHC2 KO mice. On day 7 the BMDCs were matured with 100 ng/mL lipopolysaccharide (LPS) and IFN-γ. After 24h, surface marker expression was analyzed by flowcytometry. Mean fluorescence intensity (MFI) is shown for (left to right) costimulatory molecules CD80, CD86, CD40 and inhibitory marker PD-L1 (n=3). All data are represented as mean± SEM. Statistical significance was determined by one-way ANOVA with Tukey's post hoc test (B,C,D,F). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns = not significant.

5.2. Inhibition of S-palmitoylation enhances remyelination by inducing reparative microglia.

Below is the submitted version of the manuscript. For brevity, we have omitted the corresponding figures in this document. A pdf-version of the complete manuscript including figures was attached as addendum (Guns et al 2026_Nature Neuroscience).

zDHHC5-driven FABP5 palmitoylation impairs brain repair by disrupting foam cell physiology

Jeroen Guns^{1,2,3,4}, Sam Vanherle^{1,2}, Samiksha Sardana^{5,6}, Kai Hong³, Brecht Moonen^{1,2}, Lien van Otterdijk^{1,2}, Melanie Loix^{1,2}, Koen Kuipers^{1,7}, Flore Wouters^{1,2}, Kirsten Poelmans^{1,2}, Tess Dierckx⁸, Leen Timmermans^{1,2}, Tim Vangansewinkel^{7,9}, Esther Wolfs⁷, Bieke Broux^{1,2}, Bart Ghesquière¹⁰, Luciana Berod^{11,12}, Monique T Mulder¹³, Florian Hermans⁷, Noam Zelcer^{14,15,16}, Erin M Gibson⁸, Marc P Baggelaar^{5,6}, Miranda Nabben^{4,17}, Jerome JA Hendriks^{1,2}, Dante Neculai³, Joost JFP Luiken^{4,17}, Jeroen FJ Bogie^{1,2}

1. Hasselt University, Department of Immunology and Infection, Biomedical Research Institute, Diepenbeek, Belgium.
2. University MS Center Hasselt, Pelt, Belgium.
3. International Institutes of Medicine, The Fourth Affiliated Hospital of Zhejiang University School of Medicine, Yiwu 322001, China.
4. Maastricht University, Department of Cardiology, Cardiovascular Research Institute Maastricht (CARIM), Maastricht, the Netherlands.
5. Utrecht University, Biomolecular Mass Spectrometry & Proteomics Group, Bijvoet Center for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, Utrecht, the Netherlands.
6. Netherlands Proteomics Center, Utrecht, the Netherlands.
7. Hasselt University, Department of Cardio and Organ Systems, Biomedical Research Institute, Diepenbeek, Belgium.
8. Stanford University School of Medicine, Department of Psychiatry and Behavioral Sciences, Palo Alto, CA 94305, USA.
9. Peripheral Neuropathy Research Group, Department of Biomedical Sciences, University of Antwerp, Antwerp, Belgium.
10. Laboratory of Applied Mass Spectrometry, Department of Cellular and Molecular Medicine, KU Leuven, Leuven, Belgium and Metabolomics Core Facility Leuven, Center for Cancer Biology, VIB, Leuven, Belgium.
11. Research Center for Immunotherapy (FZI), University Medical Center of the Johannes Gutenberg University Mainz, Mainz, Germany.
12. Institute of Molecular Medicine, University Medical Center of the Johannes Gutenberg University Mainz, Mainz, Germany.
13. Erasmus University Medical Center, Department of Internal Medicine, Division of Pharmacology, Vascular and Metabolic Diseases, Rotterdam, the Netherlands.
14. Department of Medical Biochemistry, Amsterdam UMC location AMC, University of Amsterdam, Amsterdam, the Netherlands.
15. Amsterdam Gastroenterology Endocrinology and Metabolism Institute, Amsterdam University Medical Center, the Netherlands.
16. Amsterdam Cardiovascular Sciences Institute, Amsterdam University Medical Center, the Netherlands.
17. Maastricht University, Department of Clinical Genetics, Maastricht University Medical Center, Maastricht, the Netherlands.

Corresponding author: Jeroen F.J. Bogie (Jeroen.bogie@uhasselt.be)

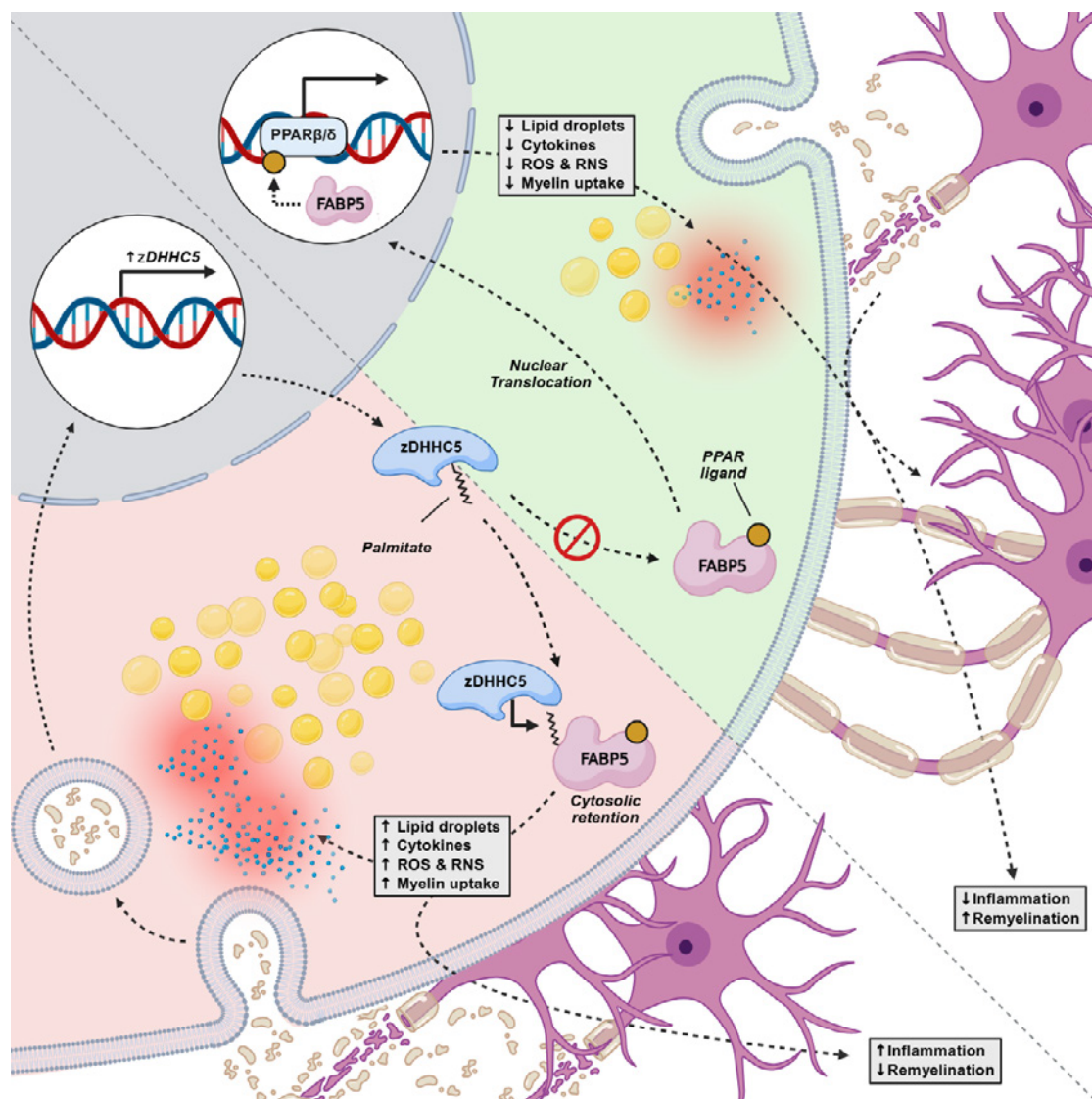
Abstract

Despite lipids being essential in controlling the phenotypic fate of macrophages and microglia in brain disorders, the exact lipid species and molecular mechanisms involved remain poorly understood. Here, we show that S-palmitoylation, a post-translational modification involving palmitate binding to proteins, is dysregulated in actively demyelinating brain lesions of multiple sclerosis patients. Mechanistically, we find that S-palmitoylation impedes remyelination by perturbing foamy macrophage and microglia physiology, and identify a key role for palmitoyl acyltransferase 5 (zDHHC5) in this process. zDHHC5-mediated S-palmitoylation was found to

hamper the nuclear translocation of fatty acid binding protein 5 (FABP5), consequently attenuating PPAR β/δ activation and the induction of a reparative foamy phagocyte phenotype. Finally, we demonstrate the efficacy of lomitapide, a recently repurposed zDHC5 inhibitor, as a promising therapeutic agent for promoting remyelination by enhancing the formation of pro-regenerative phagocytes. These findings position S-acylation, and particularly zDHC5-mediated FABP5 S-palmitoylation, as a promising target to enhance myelin regeneration.

Keywords: Protein S-palmitoylation; foamy macrophages and microglia; lipid metabolism; remyelination; central nervous system

Graphical abstract



Introduction

Failure of remyelination, characterized by deficient formation of myelin sheaths around demyelinated axons by oligodendrocytes, constitutes a key driver of disease progression in chronic demyelinating disorders such as multiple sclerosis (MS)¹⁻³. Recent insights underscore disruptions in the physiology of brain-resident microglia and infiltrated peripheral macrophage subsets as fundamental determinants of this failure⁴⁻⁷. Dysregulations in fatty acid (FA) metabolism, encompassing both their anabolism and catabolism, are linked to the compromised regenerative

potential of these phagocyte subsets within brain lesions⁸⁻¹⁰. Specifically, aberrations in key metabolic pathways, including FA desaturation^{11,12}, elongation¹³, and β -oxidation^{14,15}, are posited to influence the dualistic roles of macrophages and microglia in the progression and resolution of MS brain lesions. This concept mirrors the critical involvement of FA metabolism in modulating the bifunctional nature of myeloid cells in peripheral disorders^{16,17}. Yet, despite serving as a central substrate and product of these metabolic pathways, the precise impact of the saturated FA palmitate on macrophage and microglia physiology within demyelinating brain lesions remains elusive and warrants further investigation at the molecular level.

Most studies on FAs in general, and palmitate in particular, have focused on their integral function in energy metabolism and membrane composition, as well as the consequences of alterations in these metabolic and structural processes on cellular phenotypes^{18,19}. However, recent research spotlights S-palmitoylation, a post-translational modification involving the binding of palmitate to cysteine residues on proteins, as a crucial regulator of protein trafficking, stability, and interactions^{20,21}. Disruptions in S-palmitoylation significantly affect cellular function and metabolism across both healthy and diseased states²²⁻³¹, with ample evidence indicating a key role for S-palmitoylation in controlling immune cell activity³²⁻³⁴. Underscoring its significance and specificity, palmitoyl-proteome screens indicate that over 20% of the proteome is susceptible to S-palmitoylation, and accordingly, a family of 24 zinc finger and DHHC (aspartate-histidine-histidine-cysteine) motif-containing palmitoyl acyltransferases (zDHHCs) is identified to catalyze protein S-acylation by palmitate²⁰. Here, we sought to determine the impact of S-palmitoylation on MS lesion pathology, probing whether alterations in S-palmitoylation associated with demyelination affect remyelination and the regenerative capacity of macrophages and microglia in the brain.

Results

Active MS lesions display an altered S-acylproteome

Recent studies demonstrate marked alterations in the lipidome of human actively demyelinating MS lesions, particularly in palmitate levels^{11,35-37}. To assess changes in protein S-palmitoylation in these lesions, we applied acyl-biotin exchange (ABE), a cysteine-centric proteomic approach for assessing protein S-acylation (workflow depicted in **Figure S1A**)³⁸. Although ABE identifies the sparse attachment of FAs other than palmitate to protein cysteine residues, palmitate attachment is the dominant form of S-acylation, and ABE remains the main method to measure the S-acylproteome in metabolically inactive cryopreserved tissue³⁹. Actively demyelinating MS lesions were characterized by enrichment of HLA-DR⁺ phagocytes, loss of PLP⁺ myelin sheaths, and accumulation of lipid droplet (LD)-containing ORO⁺ foam cells (**Figures 1A** and **S1B**), as described previously¹¹.

Prior to assessing lesional differences in protein S-acylation, we defined the efficacy of ABE in defining the S-acylproteome in human post-mortem brain tissue of MS patients. Our findings highlight a notable absence of proteins in lysates of active MS lesions and adjacent normal-appearing white matter (NAWM) not subjected to hydroxylamine (HA; negative control; **Figure S1A**), whereas lysates exposed to HA showed an enrichment of established S-palmitoylated proteins, including GNAQ, GNA13, VAMP3, HRAS, SNAP25, and CAV1 (**Figure 1B**)^{20,21,40}. By consulting the SwissPalm database⁴¹, we further found that the majority of differentially S-acylated proteins identified in our ABE analysis had either been recognized (63%) or validated (18%) in earlier S-acylproteome studies (**Table S1**). Protein hits included several S-palmitoyl transferases (zDHHC2, 5, 9, 17, and 20), pointing towards their abundance and likely enhanced activity in brain tissue.

Having established the robustness of our ABE analysis in post-mortem human brain tissue, we next examined differences in protein S-acylation between MS lesions and adjacent NAWM. Our analysis revealed that 31 S-acylated proteins distinguished active demyelinating MS lesions from adjacent NAWM, with 12 exhibiting decreased levels and 19 exhibiting increased levels in active MS lesions (**Figure 1C**). Pathway analysis using Ingenuity Pathway Analysis (IPA) showed enrichment of these differentially S-acylated proteins in biological processes and molecular functions associated with FA and cholesterol metabolism, cell migration, and endocytosis (**Figure 1D**). Upstream regulator analysis further revealed that differentially S-acylated proteins were linked to inflammatory signaling molecules and pathways, including LPS, IFNG, IL1B, TNF, and STAT6 (**Figure 1E**). This aligns with inflammation being a driver of MS lesion pathology and supports the molecular relationship between S-palmitoylation and immunity^{2,32,34}.

To determine whether changes in the abundance of S-palmitoylated proteins are reflected in overall protein levels, proteomic analyses were conducted on MS lesion and NAWM lysates. The majority of the proteins clustered along the $x = y$ diagonal when plotting the fold changes in abundance between MS lesions and NAWM as observed in the S-acylproteome compared to the proteome (**Figure S1C**), indicating an equal change in S-palmitoylated protein and total protein abundance. However, several proteins that distinguished MS lesions and NAWM in the ABE analysis, including NQO1, HSP90AB2P, EFHD2, and TTYH2, deviated from this diagonal, indicating altered S-palmitoylation stoichiometry in MS lesions compared to the proteome. The validity of our proteomic analysis was confirmed by the increased lesional abundance of proteins previously associated with MS pathology, including HLA-DRB1, HLA-DRBA, C3, and STAT3 (**Figure S1D** and **Table S1**)⁴²⁻⁴⁴. Collectively, these findings highlight that active MS lesions have an altered S-acylproteome, and likely S-palmitoyl-proteome, compared to adjacent NAWM.

S-palmitoylation impairs remyelination by affecting phagocyte physiology

Aberrations in the FA lipidome and metabolism are tied to failure of remyelination in MS lesions^{11,13,45}. To elucidate whether S-palmitoylation impacts remyelination, the cerebellar brain slice culture (BSC) model was utilized. In this model, lysolecithin-induced demyelination is followed by spontaneous endogenous remyelination, enabling the study of remyelination at both cellular and molecular levels (workflow in **Figure S2A**)⁴¹. Our results reveal that exposure to 2-bromopalmitate (2BP), a broad-spectrum inhibitor of zDHHCs^{46,47}, enhanced the colocalization of myelin (myelin basic protein; MBP) and axons (neurofilament; NF) in demyelinated BSCs (**Figures 2A** and **2B**), indicative of increased remyelination. Three-dimensional reconstruction of these slices using Vaa3D confirmed more efficient axonal myelination upon exposure to 2BP (**Figure 2A**)⁴⁸.

Given the abundant presence of LD-containing foamy microglia in demyelinated BSCs and their importance in de- and remyelination¹¹, we proceeded to characterize their presence in control and 2BP-exposed BSCs. Although immunostaining analysis revealed a similar number of F4/80⁺ microglia containing BODIPY⁺ LDs (**Figures 2C** and **2D**), the intracellular LD load in microglia from 2BP-exposed BSCs was markedly reduced (**Figures 2C** and **2E**). Correspondingly, reduced ORO reactivity was observed in 2BP-exposed BSCs, indicating decreased neutral lipid content and LD load (**Figures S2B** and **S2C**). Building on our previous studies which revealed that sustained lipid accumulation and excessive formation of LDs in foamy microglia induces a repair-inhibitory phenotype^{11,13,45}, we postulated that 2BP enhances remyelination in BSCs by modulating the physiology of resident microglia. In support of this notion, microglia depletion using clodronate liposomes (CL), but not empty liposomes (EL), nullified the pro-regenerative impact of 2BP in BSCs (**Figures 2F** and **S2D**). Notably, vehicle-treated brain slices depleted of microglia exhibited a reduced remyelination efficacy compared to those sufficient in microglia (**Figure 2F**), consistent

with microglia being essential for remyelination^{11,13,49}. Efficient targeting and depletion of F4/80⁺ microglia using clodronate liposomes was confirmed using immunostainings (**Figure S2E**). Collectively, these findings show that pharmacological inhibition of S-palmitoylation enhances remyelination in brain slices in a microglia-dependent manner.

To evaluate the significance of these findings *in vivo*, the cuprizone-induced de- and remyelination model was applied. Cuprizone feeding leads to reproducible toxic demyelination in distinct brain regions, the corpus callosum (CC) in particular, while discontinuing its use prompts endogenous remyelination (workflow depicted in **Figure S2F**; efficient demyelination/remyelination is depicted in **Figures S2G-S2I**). We find that intraperitoneal administration of 2BP, starting after cessation of cuprizone feeding (5w), increased MBP abundance in the CC during remyelination (5+1w) (**Figures 2G and 2H**). Mirroring these findings, cuprizone mice treated with 2BP exhibited a reduced g-ratio (the ratio of the inner axonal diameter to the total outer fiber diameter) in the CC during remyelination (**Figures 2I and 2J**), indicative of thicker myelin sheaths and effective remyelination, along with an increase in myelinated axons (**Figure 2K**). In particular, small diameter axons showed thicker myelin sheaths in 2BP-treated cuprizone mice as compared with control mice (**Figure S2J**). Similar to BSCs, improved remyelination in 2BP-treated cuprizone mice was paralleled by a reduced abundance of ORO⁺ LDs in the CC (**Figures 2L and 2M**). These observations indicate that pharmacological inhibition of S-palmitoylation augments remyelination in the cuprizone model, potentially by modulating foamy microglia physiology.

Studies indicate that 2BP can impact cellular physiology in ways unrelated to S-palmitoylation⁵⁰. Hence, to confirm the importance of S-palmitoylation on remyelination and foam cell abundance, we next defined the impact of inhibition of acylprotein thioesterases 1 and 2 (APT1 and APT2) – the primary enzymes involved in depalmitoylation^{20,21} – on remyelination. Exposure to the APT1 inhibitor (ML348), but not the APT2 inhibitor (ML349), reduced the colocalization of MBP and NF in BSCs (**Figures S3A and S3B**), which was paralleled by an increased ORO load (**Figures S3C and S3D**). Similarly, intraperitoneal administration of ML348, starting after cessation of cuprizone feeding, reduced MBP abundance and increased LD load in the CC (**Figures S3E-S3H**). These findings corroborate the repair-inhibitory impact of S-palmitoylation in demyelinated brain lesions and argue for APT1 being essential in controlling S-palmitoylation dynamics during remyelination.

S-palmitoylation promotes LD formation and enhances myelin uptake by phagocytes

To examine the impact of S-palmitoylation on LD accumulation in more detail, we first used mouse bone marrow-derived macrophages (BMDMs) that were exposed to isolated myelin to induce foam cell formation. Analogous to the BSC and cuprizone models, 2BP exposure reduced the abundance of ORO⁺ and BODIPY⁺ LDs in foamy BMDMs *in vitro* (**Figures 3A-3C**). Conversely, inhibition of APT1, but not APT2, led to an increased LD load in these cells (**Figures S4A-S4C**). A similar increase in LD load was observed when using the dual APT1/APT2 inhibitor palmostatin B (**Figures S4A-S4C**)⁵¹. Cell viability was not affected in macrophage cultures exposed to the divergent S-palmitoylation modulators (**Figure S4D**). Noteworthy, alongside macrophages, 2BP also reduced the ORO⁺ and BODIPY⁺ LD load in mouse microglia and human monocyte-derived macrophages (MDMs) exposed to isolated mouse and human myelin, respectively (**Figures 3D, 3E; and S4E**). These experiments demonstrate that S-palmitoylation enhances the intracellular LD burden in foamy macrophages and microglia.

Cellular lipid metabolism and accumulation in foam cells is regulated through a complex interplay of myelin uptake, intracellular processing, and efflux¹⁰. Here, we explored the impact of S-palmitoylation on these different metabolic processes. By using pHrodo-labeled myelin, we found that 2BP exposure resulted in a marked reduction in the internalization of myelin by

BMDMs (**Figures 3F** and **3G**). Interestingly, 2BP did not impact the uptake of latex beads by BMDMs (**Figure S4F**), excluding an effect of 2BP on non-specific phagocytosis. Similar to BMDMs, 2BP reduced pHrodo-labeled myelin uptake by mouse microglia and human MDMs (**Figure 3G**). Despite affecting ORO load in a manner opposite to 2BP, blocking depalmitoylation with ML348 and palmostatin B did not enhance myelin uptake by BMDMs (**Figure S4G**). The latter finding may reflect the multifaceted impact of S-palmitoylation on the metabolic phenotype of foam cells, suggest a pre-existing reduction in APT activity in foam cells *in vitro*, or indicate regulation by S-palmitoylated proteins with inherently low or limited lipid turnover rates. Our data further indicate that 2BP did not affect cholesterol efflux via ATP binding cassette transporter A1 (ABCA1) and G1 (ABCG1) to their acceptors ApoA-I and HDL, respectively (**Figure S4H**), nor did it impact the breakdown of ORO⁺ LDs when BMDMs were pre-treated with myelin (**Figure S4I**). The latter finding suggests that changes in LD degradation via lipolysis and lipophagy – processes we previously demonstrated to be essential for controlling LD load in myelin-induced foam cells^{45,52} – are not influenced by S-palmitoylation. Overall, these findings demonstrate that S-palmitoylation drives the internalization of myelin by macrophages and microglia, without affecting the intracellular processing or efflux of myelin-derived lipids.

S-palmitoylation induces an inflammatory foamy macrophage and microglia phenotype

To further study the impact of S-palmitoylation on the phenotype of myelin-induced foam cells, bulk RNA-seq was performed on control and foamy BMDMs either left untreated or exposed to 2BP. Prior to sequencing, cultures were exposed to LPS/IFN to mimic the inflammatory activation seen in active MS lesions¹¹. Differential gene expression analysis revealed that 3,938 genes distinguished untreated and 2BP-exposed control BMDMs, with 1,724 upregulated and 2,214 downregulated in 2BP-exposed BMDMs (**Figure 3H** and **Table S2**). Foamy BMDMs treated with 2BP displayed a highly similar transcriptional profile, with 4,137 differentially expressed genes (DEGs), of which 2,221 were upregulated, 1,916 were downregulated, and 1,978 (~50%) overlapped between control and foamy BMDMs exposed to 2BP (**Figure 3H** and **Table S2**). Mirroring this overlap, upstream regulator analysis using IPA demonstrated that DEGs in 2BP-exposed control and foamy BMDMs were associated with a reduced activation of inflammatory signaling pathways (e.g., IRF3, IRF7, IRF9, IFNAR, STAT1, IFNA, IFNG, and LPS), increased anti-oxidative stress response (e.g., NFE2L2 and quercetin), and peroxisome proliferator-activated receptor (PPAR) ligation (e.g., rosiglitazone, pioglitazone, genistein, and troglitazone) (**Figure 3I**). Consistent with these findings, the transcriptional profile of 2BP-treated control and foamy BMDMs showcased heightened engagement in biological pathways associated with ROS detoxification and nuclear receptor activation (e.g., RAR, LXR, and PPAR), alongside a reduced activity of pathways linked to inflammation, endocytosis, and, most notably, MS pathology (**Figure 3J**). Taken together, the RNA-seq analysis indicates that pharmacological inhibition of S-palmitoylation leads to a less-inflammatory macrophage phenotype, characterized by enhanced activation of nuclear receptors such as PPARs. This phenotype resembles a macrophage profile that is considered to alleviate neuroinflammation and promote remyelination^{11,13}.

To confirm alterations in inflammatory and antioxidant pathways observed in the RNA-seq analyses, we assessed the expression and abundance of cytokines, along with the formation of reactive nitrogen and oxygen species (RNS and ROS) in foamy macrophages and microglia exposed to 2BP. We demonstrate that 2BP reduced the expression and abundance of IL1B, IL6, and CCL5 in myelin-treated BMDMs (**Figures 3K** and **3L**). Similarly, 2BP lowered the intracellular abundance and release of ROS and NO, respectively, in foamy BMDMs (**Figures 3M** and **3N**). While APT1 inhibition using ML348 increased ROS and NO formation by foamy BMDMs, it reduced *Il1b*, *Il6*, and *Ccl5* expression, albeit to a lesser extent than 2BP (**Figures S5A-S5C**). Alike mouse macrophages, 2BP decreased the formation of ROS/RNS and expression of *Il6* by foamy

microglia (**Figures S5D–5F**), while a trend toward reduced expression of *Il1b* and *Ccl5* was observed (**Figure S5D**). Human foamy MDMs exposed to 2BP showed a decreased expression of *CCL5* and generated less ROS (**Figures S5G and S5H**). No NO was detected in culture supernatants of foamy human MDM cultures (data not shown), which could point towards murine and human macrophages differing in their responses to inflammatory stimuli^{53,54}. Altogether, these findings indicate that S-palmitoylation promotes the formation of inflammatory foamy macrophages and microglia.

S-palmitoylation suppresses PPAR β / signaling by reducing FABP5 nuclear translocation

Alongside affecting endocytic and inflammatory features, RNA-seq analysis identified increased PPAR activation in macrophages upon 2BP exposure (**Figure 3I and 3J**). With PPARs being drivers of cellular metabolism and inflammation⁵⁵, we reasoned that their enhanced activation may underpin the metabolic and inflammatory phenotype of macrophages and microglia upon exposure to 2BP. First, using luciferase-based nuclear receptor reporter assays, we confirmed that 2BP increased the ligation of PPAR, PPAR β /, and PPAR α upon myelin exposure, with PPAR β / showing the most pronounced increase (**Figure 4A**). No ligation of PPAR isoforms was observed in control cultures exposed to 2BP, excluding a direct impact of the palmitate analogue 2BP on PPAR ligation (**Figure 4A**)⁵⁶. Consistent with enhanced PPAR ligation, 2BP increased the expression of canonical responsive genes of PPAR activation such as *Cpt1a*, *Scd1*, and *Cd36* in foamy BMDMs (**Figure 4B**). Given the pronounced ligation of PPAR β / upon 2BP exposure and our published findings highlighting the critical role of PPAR β / in regulating foamy phagocyte biology⁵⁷, we next investigated whether blocking PPAR β / could counteract the less-inflammatory phenotype of foamy macrophages exposed to 2BP. Our data indicate that antagonism of PPAR β / does not reverse the 2BP-driven reduction in *Il1b*, *Il6*, and *Ccl5* expression in foamy BMDMs (**Figure 4C**), whereas it effectively abolishes the 2BP-mediated decrease in ROS and NO production (**Figures 4D and 4E**). PPAR β / antagonism further reversed the reduced myelin uptake by BMDMs exposed to 2BP (**Figure 4F**), and simultaneously prevented the decline in BODIPY⁺ and ORO⁺ LDs in these cells (**Figures 4G–4I**). While the former finding does not match the reported positive correlation between PPAR β / activation and receptor-mediated endocytosis of FAs and apoptotic cells⁵⁸, few studies indicate that PPAR β / may not affect, or could even suppress, the uptake of (lipid-containing) macromolecules^{59,60}. In this respect, we found that PPAR β / agonism attenuated the uptake of myelin by macrophages (**Figure 4J**). Overall, these data highlight that enhanced PPAR β / activation mediates, at least partially, the effects of 2BP on the metabolic and inflammatory phenotype of foamy macrophages.

FA binding protein 5 (FABP5) is tuned to selectively stimulate PPAR β / transactivation through its capability to localize to the nucleus and distribute FAs to PPAR β / ^{61,62}. As 2BP enhanced PPAR β / ligation (**Figures 4A and 4B**) and MS lesions showed increased FABP5 acylation compared to NAWM (**Figure 1C**), we next sought to determine to what extent FABP5 S-palmitoylation controls its nuclear translocation and underpins PPAR β / ligation and LD formation in foam cells. Using an untargeted click-chemistry approach with 17-octadecynoic acid (17-ODYA) as a metabolic probe (workflow in **Figure S6**), we first confirmed the S-palmitoylation of FABP5 in macrophages, along with other proteins previously reported to be S-palmitoylated, including CD36^{23,29}, CANX⁶³, CALR⁶⁴, and ABL1⁶⁴ (**Figure 5A and Table S3**). In total, we identified 166 S-palmitoylated proteins in BMDMs (**Table S3**). Furthermore, by utilizing cysteine mutants predicted by SwissPalm to undergo S-palmitoylation on FABP5⁴¹, we identified Cys120 - rather than Cys43, Cys67, or Cys127 - as essential for its S-palmitoylation (**Figure 5B**). Having established FABP5 S-palmitoylation, we next defined its impact on the nuclear localization of FABP5. Immunostainings demonstrated that 2BP increased the nuclear localization of FABP5 in foamy BMDMs but not in control BMDMs (**Figures 5C and 5E**), consistent with 2BP enhancing PPAR ligation in BMDMs only after exposure

to myelin (**Figure 4A**). Similar to 2BP, palmitoylation-deficient FABP5^{C120S} COS7 mutants - but not Cys43, Cys67, or Cys127 mutants - exhibited increased nuclear localization of FABP5 upon myelin exposure (**Figures 5D and 5F**). Accordingly, luciferase-based nuclear receptor reporter assays showed enhanced PPARβ/ ligation in FABP5^{C120S} mutants, while FABP5 mutants lacking the nuclear localization signal (FABP5^{NLSm}) showed reduced PPARβ/ ligation (**Figure 5G**)⁶⁵. Finally, at a functional level, we show that, similar to 2BP treatment (**Figure 3**), FABP5^{C120S} mutants exhibited a reduced ORO⁺ LD burden following myelin exposure (**Figures 5H and 5I**), along with a diminished capacity to internalize myelin (**Figure 5J**). Collectively, these findings indicate that FABP5^{C120} S-palmitoylation suppresses FABP5 nuclear import and PPARβ/ ligation, and suggest that this signaling axis is key in controlling myelin uptake and LD formation in foam cells.

zDHHC5 induces an inflammatory macrophages and microglia phenotype

S-palmitoylation is catalyzed by the action of 24 zDHHCs that attach palmitate to cysteine residues on proteins^{20,21}. Hence, we next set out to identify zDHHCs whose inhibition underlies the impact of 2BP on phagocyte physiology and remyelination. To this end, we measured the expression of zDHHCs in actively demyelinating human post-mortem MS lesions, the corpus callosum of cuprizone-fed mice following demyelination, and inflammatory and myelin-treated macrophage subsets *in vitro*. An increase in the expression of both *zDHHC5* and *zDHHC18* was observed across all four models, suggesting that their inhibition could be a key target of 2BP (**Figure 6A**). Here, we opted to focus on *zDHHC5* due to its detection by ABE in active MS lesions (**Table S1**), supporting its presence and substantial expression.

Upon verifying enhanced *zDHHC5* abundance in macrophages and microglia in actively demyelinating MS lesions (**Figures 6B and 6C**), we next asked whether deficiency of *zDHHC5* induces an inflammatory and metabolic phagocyte phenotype akin to cells exposed to 2BP. To this end, BMDMs were isolated from *Zdhhc5*^{fl+/+} and *Zdhhc5*^{fl+/+}*LysM*^{Cre+/-} mice²⁴. Absence of *zDHHC5* in BMDMs isolated from *Zdhhc5*^{fl+/+}*LysM*^{Cre+/-} mice was previously confirmed using immunoblotting and immunohistochemistry²⁴, and further validated by us using qPCR (**Figure S7A**). Similar to foamy BMDMs exposed to 2BP, *Zdhhc5*^{-/-} foamy BMDMs showed a decreased intracellular LD load (**Figures 6D; S7B and S7C**), endocytosis of myelin (**Figure 6E**), and release of ROS and NO (**Figures 6F and 6G**), which was reversed in the presence of the PPARβ/ antagonist (**Figures 6D-6G; and S7B and S7C**). The absence of *Zdhhc5* further reduced the expression of *Il1b* and *Ccl5*, but not that of *Il6*, and PPARβ/ antagonism was able to reverse this reduction (**Figure 6H**). An untargeted click-chemistry proteomics approach further revealed reduced S-palmitoylation of FABP5 in *Zdhhc5*^{-/-} BMDMs compared to wild-type BMDMs (**Figure 6I and Table S3**), indicating that FABP5 is a *bona fide* target of *zDHHC5*. Alongside FABP5, palmitoyl-proteome analysis revealed that CD36, LGL1, and H2-Q4 showed a reduced S-palmitoylation status in *Zdhhc5*^{-/-} BMDMs. Notably, several proteins - including PRSS3B, PFN1, LYZ2, EIF5A, and S100A4 - showed increased S-palmitoylation levels in *Zdhhc5*^{-/-} BMDMs, suggesting compensatory activity of other acyltransferases. All in all, these findings show that the absence of *Zdhhc5* induces a favorable metabolic and inflammatory foamy macrophage phenotype in a PPARβ/-dependent manner, resembling the phenotype observed after exposure to 2BP.

zDHHC5 knockout boosts remyelination in a phagocyte-specific manner

With *zDHHC5* deficiency mimicking the impact of 2BP on foamy phagocyte physiology, we next determined if deficiency of *Zdhhc5* in macrophages and microglia enhances remyelination. To this end, we utilized *Zdhhc5*^{fl+/+} and *Zdhhc5*^{fl+/+}*LysM*^{Cre+/-} mice and the cuprizone model. Our findings indicate that *Zdhhc5*^{fl+/+}*LysM*^{Cre+/-} mice exhibited increased MBP abundance in the CC during remyelination (5+1w) as compared to *Zdhhc5*^{fl+/+} littermates (**Figures 6J and 6K**). Macrophage and microglia-specific depletion of *Zdhhc5* did not impact cuprizone-mediated demyelination

in the CC (5w) (**Figures S7D** and **S7E**), indicating that its absence in phagocytes does not protect against demyelination. Similar to cuprizone mice exposed to 2BP, enhanced remyelination in *Zdhhc5*^{fl/+}/*Lysm*^{cre/+} mice was associated with a reduced ORO load in the CC (**Figures 6L** and **6M**). Thus, these findings indicate that phagocyte-specific deficiency of *Zdhhc5* enhances remyelination, potentially by reducing the harmful accumulation of LDs in foamy phagocytes.

Lomitapide induces a benign foamy phagocyte phenotype and enhances remyelination

Lomitapide is an FDA-approved microsomal triglyceride transfer protein (MTTP) inhibitor utilized to reduce very low-density lipoprotein (VLDL) cholesterol levels⁶⁶. However, a recent study has introduced a compelling repositioning of lomitapide, identifying it as a potential inhibitor of zDHHC5⁶⁷. In this context, we explored the impact of lomitapide on foam cell physiology and remyelination. Our data indicate that BMDMs exposed to lomitapide exhibit a reduced capacity for myelin internalization (**Figure 7A**). Foam cells exposed to lomitapide further showed less LD accumulation (**Figures 7B**; and **S8A** and **S8B**), a decreased formation of ROS and NO (**Figures 7C** and **7D**), and a less-inflammatory phenotype (**Figure 7E**). PPAR β / antagonism reversed the reduction in myelin uptake, LD load, and ROS production upon exposure to lomitapide (**Figures 7A-7C**), while also showing a trend toward counteracting the decrease in NO (**Figure 7D**). However, except for *Ccl5*, PPAR β / antagonism did not reverse the reduced expression of *Il1b* and *Il6* in foamy BMDMs exposed to lomitapide (**Figure 7E**). Finally, by using luciferase-based nuclear receptor reporter assays, immunostainings, and a click-chemistry proteomic approach, we found that lomitapide enhances PPAR β / ligation in foam cells (**Figure 7F**), promotes FABP5 nuclear translocation (**Figures 7G** and **7H**), and counteracts FABP5 S-palmitoylation (**Figure 7I** and **Table S3**). In addition to FABP5, BMDMs treated with lomitapide exhibited reduced S-palmitoylation of CD36, FABP5, and LGL1, which together account for 60% of the proteins showing reduced S-palmitoylation in *Zdhhc5*/ BMDMs (**Figure 7I** and **7J**). Lomitapide treatment further significantly increased the S-palmitoylation of H2AC4, CD40, M6PR, and PRSS3B, with the latter also being elevated in *Zdhhc5*/ BMDMs (**Figure 7I** and **7J**). In contrast, lomitapide exposure reduced S-palmitoylation of several proteins not affected in *Zdhhc5*/ BMDMs, including LAMP1, C1QBP, HMGA1, and PSMB1 (**Figure 7J**). Collectively, these findings show that lomitapide induces a benign foamy phagocyte phenotype and acts as an inhibitor of zDHHC5.

To assess the potential of lomitapide to enhance remyelination, we next used the BSC and cuprizone models. Akin to 2BP and *Zdhhc5* deficiency, lomitapide exposure enhanced axonal myelination and reduced ORO abundance in the BSC model (**Figures 7K-7M**). Importantly, the pro-myelinating effect of lomitapide was lost upon elimination of microglia using clodronate liposomes (**Figures S8C** and **S8D**). Similarly, in the cuprizone model, intraperitoneal administration of lomitapide enhanced remyelination, evidenced by an increased MBP abundance (**Figures 7N** and **7O**), decreased g-ratio (**Figures 7P** and **7Q**), elevated percentage of myelinated axons (**Figures 7P** and **7R**), and reduced LD load in the CC (**Figures 7P** and **7S**). Further, in line with general S-palmitoylation inhibition, smaller diameter axons showed thicker myelin sheaths in mice treated with lomitapide compared to vehicle-treated mice (**Figure 7R**). As expected, lomitapide administration reduced serum VLDL, triglyceride, and cholesterol levels, while not affecting HDL levels (**Figure S8E**), confirming its efficacy in inhibiting hepatic MTTP but complicating the isolation of its direct regenerative effects on remyelination through S-palmitoylation. All in all, these experiments position lomitapide as a promising therapeutic tool to enhance remyelination in the CNS.

Discussion

In this study, we demonstrate that active human MS lesions exhibit significant alterations in their S-palmitoyl-proteome and that pharmacological inhibition of S-palmitoylation enhances

remyelination. Foamy macrophages and microglia were found to serve as a critical cellular conduit for this enhanced remyelination. Specifically, we show that abrogation of zDHHC5-mediated S-palmitoylation enhances nuclear translocation of FABP5, leading to heightened activation of PPAR β / and the formation of disease-resolving foam cells. Our findings further highlight that the VLDL-lowering drug lomitapide promotes remyelination, at least partially, through the suppression of the zDHHC5-FABP5-PPAR β / signaling axis in macrophages and microglia. Together, we show for the first time that S-palmitoylation by zDHHC5 impairs remyelination by inducing the formation of repair-inhibitory foam cells, having significant mechanistic and therapeutic implications for MS and other demyelinating disorders.

Within the CNS, palmitate plays a multifaceted role, supporting both energy homeostasis and membrane structure⁶⁸. Alongside these canonical functions, S-palmitoylation has emerged as a key mechanism of palmitate action in the CNS, particularly in fine-tuning neuronal morphology and physiology, synaptic plasticity, and oligodendrogenesis^{69,70}. Accordingly, alterations in S-palmitoylation dynamics contribute to neuropathological, locomotor, and cognitive deficits observed in conditions like Alzheimer's disease (AD), Parkinson's disease, psychiatric disorders, and intellectual disabilities⁷¹⁻⁸². In these studies, loss-of-function mutations in acyltransferases, particularly zDHHC8, 9, 13, 15, and 17, drive brain pathology, leading to locomotor and cognitive impairments, whereas inhibition of depalmitoylation reverses these deficits. While these studies argue for attenuated S-palmitoylation driving various brain pathologies, our findings indicate that active S-palmitoylation can have detrimental effects in the CNS as well. We show that zBP (pan-zDHHC inhibition), phagocyte-specific deficiency of *Zdhhc5*, and lomitapide (zDHHC5 inhibition) enhance remyelination following toxin-induced demyelination by promoting the formation of pro-regenerative foam cells. Supporting these findings, we demonstrate that inhibition of APT1-mediated depalmitoylation perturbs myelin regeneration and the pro-regenerative features of foam cells. This discrepancy may reflect disease- and cell type-specific expression patterns of zDHHCs, the varying involvement of zDHHCs in brain disorders, or alternatively, point towards the high abundance of lipid-loaded foam cells in MS lesions compared to other CNS disorders¹⁰. Though, lipid-laden microglia accumulate in AD brains, where they contribute to Tau phosphorylation and neurotoxicity⁸³. It is worth noting that we did not conduct an in-depth assessment of dendritic and axonal structures or evaluate potential behavioral and locomotor deficits. Thus, while we provide strong evidence that palmitate promotes MS lesion progression via S-palmitoylation, specifically identifying increased zDHHC5-mediated S-palmitoylation in foam cells as a key factor in remyelination failure, further research is necessary to investigate deficits in neuronal physiology and cognition in our models.

Recent studies demonstrate that foamy macrophages and microglia exhibit dual phenotypes in demyelinating lesions¹⁰. Early foam cells display a less inflammatory, pro-regenerative phenotype marked by the formation of protective lipid species and intermediates, such as oxysterols, desmosterol, and FAs, which activate PPAR β / and LXRs^{57,84-88}. Conversely, disruptions in FA desaturation and elongation, lipophagy, and lysosomal processing, and the resulting accumulation of LDs and inflammatory lipid species, are associated with late-stage, lipid-overloaded, disease-promoting foam cells^{4,11,13,45,52,89}. With zDHHC5-mediated S-palmitoylation impacting functional parameters characteristic of both early- and late-stage foam cells, including PPAR β / activation, myelin internalization, LD accumulation, and the release of ROS, RNS, and inflammatory mediators, our findings indicate that S-palmitoylation serves as a critical molecular regulator of early- and late-stage foam cell physiology. Of note, the importance of zDHHC5 in driving the harmful phenotype switch of foam cells in our study aligns with other studies showing that zDHHC5 activity correlates with inflammatory myeloid cell formation²⁴, NLRP3 inflammasome activation and pyroptosis^{90,91}, and heightened lipid endocytosis^{23,29}.

Similarly, the interplay between zDHHc5-mediated FABP5 S-palmitoylation and PPAR β / activation fits with reports showing that FABP5 is a FA chaperone that promotes nuclear PPAR activation^{62,92}. However, given that FABP5 is susceptible to S-glutathionylation, a process that relies on C127 and also controls FABP5 nuclear translocation and PPAR β / activation⁹³, it would be valuable to investigate the interaction and necessity of both C127 S-glutathionylation and C120 S-palmitoylation in nuclear trafficking of FABP5 in future studies. Collectively, we present a deeper layer of molecular complexity regarding foam cell physiology in the CNS, and identify several therapeutically targetable regulators that mitigate the harmful impact of S-palmitoylation on foamy phagocyte physiology and remyelination, including zDHHc5, PPAR β /, and FABP5.

With macrophages and microglia being the most abundant cell types in early demyelinating brain lesions¹⁰, ample evidence links changes in their functional phenotypes to the fate of these lesions. In this context, several functional properties of foamy phagocytes are associated with efficient remyelination, including the release of neurotrophic and anti-inflammatory mediators^{94,95}, the clearance of inhibitory myelin debris⁹⁶⁻⁹⁸, and the intercellular recycling of lipids, including cholesterol, via lipoproteins to oligodendrocytes^{4,11,13,45,89,99}. Our findings highlight that changes in foam cells underpin enhanced remyelination upon exposure to 2BP and lomitapide, as well as following genetic deficiency of *Zdhhc5*. However, this pro-myelinating effect did not seem to rely on increased ABCA1- and ABCG1-mediated horizontal transfer of lipids from foamy phagocytes to oligodendrocytes, nor did it depend on increased clearance of myelin debris. In fact, *Zdhhc5* deficiency and exposure to 2BP and lomitapide attenuated endocytosis of myelin debris by macrophages and microglia *in vitro*. Given the pronounced anti-inflammatory effects and antioxidant response, it is tempting to speculate that reduced zDHHc5-mediated S-palmitoylation enhances oligodendrogenesis and remyelination by alleviating the differentiation block induced by overt inflammation and oxidative stress¹⁰⁰⁻¹⁰². Yet, more research is needed to identify changes in the secretome and microenvironment involved, and assess to what extent and how these outweigh the anticipated accumulation of myelin debris, or, alternatively, drive compensatory clearance of myelin debris by other cell types such as astrocytes¹⁰³⁻¹⁰⁵. Likewise, given that S-palmitoylation controls various aspects of extracellular vesicle (EV) production and release¹⁰⁶, it would be of interest to assess the impact of EVs released by macrophages and microglia in the regenerative impact of 2BP, lomitapide and *zDhhc5* deficiency. In this regard, we recently demonstrated that macrophages enhance remyelination by recycling cholesterol via EVs to oligodendrocytes⁴⁹. Thus, while our findings indicate that S-palmitoylation impairs remyelination by modulating foam cell biology, further research is necessary to elucidate the molecular mechanisms and cellular interactions involved.

Several other unresolved questions persist concerning our findings. We cannot entirely rule out that S-palmitoylation of targets beyond FABP5, such as CD36, beclin-1, or NOD1^{23,24,29,107}, impact foam cell function and remyelination in our experiments^{45,108,109}. Additionally, we did not clarify the mechanisms behind reduced myelin uptake downstream of PPAR β /, which based on our RNA-seq analysis could involve reduced caveolar endocytosis. Finally, with palmitate levels being increased in MS brain¹¹⁰, it would be of interest to study a substrate-dependent harmful impact of S-palmitoylation on phagocyte physiology and remyelination. Despite the remaining gaps, our findings highlight the zDHHc5–FABP5–PPAR β / axis in foam cells as a promising therapeutic target for remyelination, and identify lomitapide as a potential agent for inhibiting this signaling pathway.

Material and methods

Antibodies and chemical reagents

The following antibodies were used for immunofluorescence and immunohistochemical stains or immunoblotting: antiMBP (1:250, cat. no. MCA409S, Millipore, ex vivo cerebellar brain slices), anti-MBP (1:250, cat. no. MAB386, Millipore, brain cryosections), antiNF (1:1000, cat. no. ab8135, SigmaAldrich), anti-CD68 (1:100, cat. no. 14-0688-82, Invitrogen), anti-zDHHC5 (1:100, cat. no. 21324-1-AP, ProteinTech), anti-F4/80 (1:100, cat. no. MCA487G, Bio-Rad), anti-FABP5 (1:1000; ICC, 1:500; WB, cat. no. 12348-1-AP, ProteinTech), and anti-F-actin (iFluor 647 Phalloidin; 1:1000, cat. no. 40762ES75, Yeasen). The following secondary Alexa Fluor antibodies were used: Alexa Fluor488 (1:500, Invitrogen) and Alexa Fluor555 (1:500, Invitrogen). BODIPY® 493/503 (2µM, cat. no. D3922, Thermo Fisher Scientific) was used to stain the lipid droplets. To assess cellular viability, cells were incubated with 7-aminoactinomycin D (7-AAD; cat. no. A1310, Thermo Fisher Scientific) for 10 min at 4°C and analyzed with the FACS Fortessa (BD Biosciences). 2-Bromopalmitate (2BP; 20 µM, cat. no. 18263-25-7, Sigma-Aldrich) was used to inhibit general S-palmitoylation. Palmostatin B (50 µM, cat. no. 5.08738, Sigma-Aldrich), ML348 (20 µM, cat. no. 899713-86-1, Sigma-Aldrich), and ML349 (20 µM, cat. no. 890819-86-0, Sigma-Aldrich) were used to inhibit depalmitoylation via APT1 and/or APT2. Lomitapide (1 µM, cat. no. 182431-12-5, Simson Pharma Limited) was used to inhibit zDHHC5. Wy14643 (10 µM, cat. no. 50892-23-4, Sigma-Aldrich), GW501516 (1 µM, cat. no. 317318-70-0, Sigma-Aldrich), and rosiglitazone (1 µM, cat. no. 122320-73-4, Sigma-Aldrich) were used as agonists of peroxisome proliferator-activated receptor (PPAR), PPARβ/γ, and PPAR, respectively. GSK3787 (1 µM, cat. no. 516567, Sigma-Aldrich) was used as antagonist of PPARβ/γ.

Animals

Wild-type C57BL/6J mice were purchased from Envigo and were housed in a 12h light/dark cycle with *ad libitum* access to water and a standard chow diet or specific formulations as indicated. All procedures were conducted in accordance with the institutional guidelines and approved by the Ethical Committee for Animal Experiments of Hasselt University (protocol numbers 202402K, 202404). *Zdhhc5*^{fl/+} and *Zdhhc5*^{fl/+}*LysM*^{Cre/+} mice were generated at Zhejiang University. All experiments involving these mice were performed in compliance with institutional animal welfare regulations and approved by the Animal Care and Use Committee of Zhejiang University (protocol ZJU20170473)²⁴.

Macrophage and microglia differentiation and treatments

Mouse bone marrow-derived macrophages (BMDMs) were obtained as described previously¹¹¹. Briefly, femoral and tibial bone marrow was isolated from 11-week-old female mice and cultured in 14.5 cm petri dishes (cat. no. 639161, Greiner) at a concentration of 10×10^6 cells/Petri dish in RPMI1640 medium (cat. no. 724000-21, Gibco) supplemented with 10% fetal bovine serum (FBS; cat. no. S181B-500, Biowest), 50 U/mL penicillin (cat. no. 15070063, Gibco), 50 U/mL streptomycin (cat. no. 15070063, Gibco), and 15% L929-conditioned medium (LCM; in house production¹¹²) for 7 days. Next, cells were cultured at a density of 0.5×10^6 cells/mL in RPMI1640 supplemented with 10% FBS, 50 U/mL penicillin, 50 U/mL streptomycin, and 5% LCM. Microglia were obtained from the cortices of postnatal day 1–3 wild-type C57BL/6J mice and cultured in poly-L-lysine (PLL, 50 µg/mL, cat. no. P3513, Sigma-Aldrich)-coated T75 flasks (cat. no. 658175, Greiner) in high-glucose Dulbecco's Modified Eagle Medium (DMEM) medium (cat. no. 41966-029, Gibco), supplemented with 10% FBS, 50 U/mL penicillin, and 50 U/mL streptomycin. Three days after isolation, cultures were supplemented with 33% LCM. After 14 days, microglia were collected by means of the orbital shake-off method (230 rpm, 3h, 37°C), after which the cells were cultured at a density of 0.5×10^6 cells/mL in high-glucose DMEM supplemented with 10% FBS, 50 U/mL penicillin, 50 U/mL streptomycin, and 15% LCM. Murine BMDMs and microglia were treated with vehicle, 2BP (20 µM), palmostatin B (50 µM), ML348 (20 µM), ML349 (20 µM),

lomitapide (1 μ M), GW501516 (1 μ M), GSK3787 (1 μ M), and/or 100 μ g/mL mouse myelin for 24h, unless stated otherwise. For phenotypic analysis, BMDMs and microglia were stimulated with LPS (100 ng/mL, cat. no. 437627, Sigma-Aldrich) and IFN- (20 ng/mL, cat. no. 315-05, Thermo Fisher Scientific) for the indicated durations.

Human monocyte-derived macrophages (MDMs) of healthy controls were isolated as described previously¹¹¹. Isolated CD14⁺ cells were differentiated towards macrophages in DMEM supplemented with 10% human serum, 50 U/mL penicillin, 50 U/mL streptomycin, and 1% L-glutamine. After 6 days, adherent MDMs were harvested and plated at 0.3×10^6 cells/mL to be used for *in vitro* experiments. MDMs were treated with vehicle, 2BP (20 μ M) and/or 100 μ g/mL human myelin for 24h. For phenotyping, MDMs were stimulated with LPS (100 ng/mL) and IFN- (20 ng/mL, cat. no. 300-02, Thermo Fisher Scientific) for 6h to assess gene expression. All experimental protocols using human cells were conducted in accordance with institutional guidelines and approved by the Medical Ethical Committee of Hasselt University. Written informed consents were obtained from all individual participants included in the study.

Myelin isolation

Myelin was purified from postmortem mouse and human brain tissue by means of density gradient centrifugation, as described previously¹¹¹. Myelin protein concentration was determined by using the BCA protein assay kit (cat. no. A55864, Thermo Fisher Scientific), per the manufacturer's guidelines. By using the Chromogenic Limulus Amebocyte Lysate assay kit (cat. no. L00350, Genscript), endotoxin content of isolated myelin was determined to be negligible.

Cuprizone model

To induce acute demyelination, 9weekold male wildtype, *Zdhhc5*^{fl/+}, and *Zdhhc5*^{fl/+}*LysM*^{Cre/+} C57BL/6J mice were fed a diet containing 0.3% (w/w) cuprizone (bis(cyclohexanone) oxaldihydrazone, cat. no. 370-81-0, SigmaAldrich) mixed in powdered standard chow ad libitum for 5 weeks. After these 5 weeks, cuprizone diet was withdrawn to allow spontaneous remyelination for 1 week. During this week, wild-type mice were subjected to a daily intraperitoneal injection of either vehicle, 2BP (20 mg/kg), ML348 (25 mg/kg), or lomitapide (10 mg/kg) for 7 days. Mice were sacrificed either 5 weeks (5 w) or 6 weeks (5 + 1 w; including 1 week of recovery) after initiation of the cuprizone diet, and tissues and blood were collected for histological and biochemical analyses.

Cerebellar brain slice cultures

Cerebellar brain slices were obtained from wildtype C57BL/6J mouse pups at postnatal day 9 or 10 (P9-10), as described previously^{113,114}. Brain slices were cultured in MEM medium (cat. no. 32360-026, Thermo Fisher Scientific) supplemented with 25% horse serum (cat. no. 26050088, Thermo Fisher Scientific), 25% Hank's balanced salt solution (cat. no. H4641, SigmaAldrich), 50 U/mL penicillin, 50 U/mL streptomycin, 1% glutamax (cat. no. 35050038, Thermo Fisher Scientific), 12.5 mM HEPES (cat. no. 15630080, Thermo Fisher Scientific) and 1.45 g/L glucose (cat. no. G8644, SigmaAldrich). For microglia depletion, slices were treated with empty or clodronate liposomes (0.5 mg/mL, cat. no. CP-005-005, LIPOSOMA) immediately after isolation for 24h. Slices were allowed to recover for three days after isolation, after which demyelination was induced by treating them with 0.5 mg/mL lysolecithin (cat. no. L4129, SigmaAldrich) for 16h. Next, brain slices were allowed to recover in culture medium for 1 day and subsequently treated daily with vehicle, 2BP (20 μ M), palmostatin B (50 μ M), ML348 (20 μ M), ML349 (20 μ M), and lomitapide (1 μ M) for 1 week, followed by histological and biochemical analysis.

Transmission electron microscopy

Mouse brain samples were fixed in 2% glutaraldehyde (cat. no. 16320, Electron Microscopy Sciences), followed by post-fixation in 2% osmium tetroxide (cat. no. 201030, Sigma-Aldrich) prepared in 0.05 M sodium cacodylate buffer (cat. no. 6131-99-3, Merck) for 1h at 4 °C. Samples were then dehydrated through a graded acetone series. Dehydrated tissues were infiltrated overnight (O/N) in a 1:1 mixture of acetone and Araldite epoxy resin, followed by embedding in pure Araldite resin at 60 °C. Ultrathin sections (70 nm) were cut perpendicular to the corpus callosum using a Leica EM UC6 ultramicrotome. Sections were mounted on copper grids (Veco B.V.) coated with 0.7% formvar. Transmission electron microscopy was performed using a JEOL JEM1400 Flash operating at 80 kV, equipped with an Emsis 20 NP XAROSA camera system. For each brain sample, 5–12 images were acquired. The g-ratio (inner axonal diameter/total outer diameter) and number of myelinated axons were quantified using ImageJ. All analyses were performed by investigators blinded to the experimental groups.

BODIPY and ORO stains

BMDMs, microglia, and MDMs were stained for intracellular lipid load by 15 min incubation with BODIPY (493/503) at 37°C, and analyzed with the FACS Fortessa (gating strategy can be found in (Figure S4J)). For ORO stains, cells, brain slices, MS lesions, and cuprizone sections were fixed with paraformaldehyde (PFA, Sigma) for 15 min. Next, cells were stained with 0.3 % Oil Red O (ORO; cat. no. 1320-06-5, Merck) for 10 min and counterstained with nuclei stain haematoxylin (cat. no. 51275, Merck) for 1 min. Analysis was carried out using a Leica DM 2000 LED microscope and ImageJ software.

Immunofluorescence

Cells were cultured on glass cover slides and fixed in 4% paraformaldehyde (PFA) for 20 min at room temperature (RT). To stain BMDMs, samples were first permeabilized with 0.1% Triton X-100 for 20 minutes and blocked with 4% goat serum and 1% BSA in PBS-T for 1h. Next, cells are incubated with the relevant primary antibodies diluted in blocking buffer (1x PBS-T + 1% BSA + 4% goat serum). Nuclei were stained using 4,6-diamidino-2-phenylindole (DAPI; cat. no. 62248, Invitrogen). To assess phagocytosis, fixed cells (see phagocytosis assay section) were permeabilized for 15 min in 0.1% Triton X-100 and then stained with F-actin in 1x PBS for 1h at RT, and nuclei were stained using DAPI. Imaging of cells was performed using the LeicaDM2000 LED fluorescence microscope (Leica) or LSM900 confocal microscope (Zeiss) and ImageJ was used for quantification.

Cerebellar brain slices were fixed in 4% PFA for 15 min at RT. To stain cerebellar brain slices, samples were incubated with relevant primary antibodies diluted in blocking buffer (1x PBS + 5% horse serum + 0.3% Triton X100). Analysis of cerebellar brain slices was performed using the LSM880 confocal microscope (Zeiss) and ImageJ was used for analysis. Threedimensional analysis of cerebellar brain slices was performed using the zstack feature, and images were 3D rendered using the 3D rendering software Vaa3D⁴⁸.

Brain tissue of cuprizone mice was isolated, snapfrozen, and sectioned with a Leica CM1900UV cryostat (Leica Microsystems) to obtain 10 µm slices. Immunostaining and analysis of cryosections were performed as described previously¹¹¹. ImageJ was used to align the corpus callosum, followed by determination of the MBP or ORO-positive signal in this area.

Snap-frozen brain material containing active MS lesions was obtained from the Netherlands Brain Bank (NBB, Amsterdam, Netherlands) and sectioned with a Leica CM1900UV cryostat to obtain 10 µm slices. Cryosections were fixed in icecold acetone for 10 min. To determine the lesion and the adjacent normal appearing white matter (NAWM)¹¹¹, cells were stained with anti-human

HLA-DR (cat. no. 14-9956-82, Ebioscience) or PLP (cat. no. MCA839G, Biorad) O/N. After washing, HRP-conjugated rabbit-anti-goat (cat. no. 12-348, Sigma-Aldrich) was added. Subsequently, DAB substrate (cat. no. GV825, Dako) was used to stain slides. Lesional foamy myelin-containing phagocytes were visualized with ORO, which stains neutral lipids.

Representative images shown in the figures are digitally enhanced to increase the readability. All analyses were conducted by observers blinded to the experimental arm of the study.

Cholesterol efflux

Total and free cholesterol were measured in cell lysates and supernatant using the Amplex™ Red Cholesterol Assay Kit (cat. no. A12216, Invitrogen), according to the manufacturer's instructions. Cholesteryl ester concentration was calculated by subtracting free cholesterol concentration from total cholesterol concentration. To measure cholesterol efflux, cells were exposed to 10% FBS, ApoA-I (50 µg/mL; generous gift from Dr. Remaley, National Institute of Health, USA⁸⁹), or high-density lipoprotein (HDL; 50 µg/mL, cat. no. PRO-559, Biovalley) in phenol- and serum-free RPMI1640 medium (cat. no. 11835030, Gibco) for 4h prior to measuring intra- and extracellular total cholesterol. Cholesterol efflux was calculated by dividing the fluorescence in the supernatants by the total fluorescence in supernatants and cells. Fluorescence was measured at an excitation wavelength of 540 nm and an emission wavelength of 590 nm using the CLARIOstar PLUS optima microplate reader (BMG Labtech).

RNA extraction and real-time quantitative PCR

Total RNA from cells was isolated using QIAzol (cat. no. 79306, Qiagen) and the RNeasy mini kit (cat. no. 74106, Qiagen), according to the manufacturer's guidelines. Complementary DNA (cDNA) was synthesized using qScript™ cDNA SuperMix (cat. no. 95048-500, Quantabio) according to the manufacturer's instructions. Real-time quantitative PCR was conducted on a Quantstudio 3 detection system (Applied biosystems). Cycle conditions were 95 °C for 1 s, followed by 40 cycles of 95 °C for 3 s, and 60 °C for 20 s. The PCR reaction mixture contained Fast SYBR™ Green Master Mix (cat. no. 4385612, Thermo Fisher Scientific), 0.3 µM forward and reverse primer (IDT technologies), RNase free water and 12.5 ng cDNA template. Data were analyzed using the comparative $\Delta\Delta C_t$ method and normalized to the most stable reference genes. Primer sequences are available upon request.

Bulk RNA sequencing

Total RNA from BMDMs was isolated using QIAzol and the RNeasy mini kit, according to the manufacturer's guidelines. Preparation of RNA libraries and sequencing was outsourced to Novogene, specifying Eukaryotic RNA-Seq as the library type, followed by sequencing on a Novaseq X Plus (Illumina) with a PE 150 strategy. Following quality control, reads were aligned to the mouse reference genome (Mus musculus, GRCm38/mm10 release M21) and transcript abundancies were quantified using Salmon (1.4.0) using the --gcBias and --seqBias flags¹¹⁵. Gene-level count matrices were created using the tximport package (v1.18.0) and used as input for DEG analysis with DESeq2 (v1.30.1) as previously reported^{116,117}. PCA analysis was performed using the plotPCA function from the DESeq2 package after rlog transformation. Based on PCA analysis, two samples (one vehicle control, one 2BP-treated) were considered outliers and omitted from further downstream analyses. For visualization and gene ranking, log fold change (LFC) estimates were shrunk using the apegglm (v1.12.0) method for effect size shrinkage as described by^{116,118}. Pathway analysis of differentially expressed genes was conducted using Ingenuity Pathway Analysis. Data are accessible from ArrayExpress database (accession number E-MTAB-15390).

ELISA

IL1B and IL6 concentrations were determined in the supernatant of BMDM cultures, which were stimulated with LPS (100 ng/mL) and IFN- (20 ng/mL) for 18h, using the IL1B (cat. no. 88-7013-22, Thermo Fisher Scientific) and IL6 mouse ELISA kit (cat. no. 88-7064-22, Thermo Fisher Scientific) according to the manufacturer's guidelines.

Measurement of nitric oxide and reactive oxygen species

Cells were stimulated with LPS (100 ng/mL) and IFN- (20 ng/mL) for 18h, after which cell culture supernatants were collected. Nitric oxide (NO) was monitored by mixing supernatants with a 1:1 mixture of sulphanilamide (cat. no. S9251, Sigma-Aldrich) and N-(1-naphthyl)ethylenediamine dihydrochloride (cat. no. N9125, Sigma-Aldrich), followed by incubation at RT for 10 min. Absorbance of the azo derivative was measured at 540 nm using the CLARIOstar PLUS optima microplate reader. For determination of reactive oxygen species (ROS), cells were stimulated with LPS (100 ng/mL) and IFN- (20 ng/mL) for 15 min, and reactive oxygen species (ROS) production was measured using the fluorescent probe 2,7-dichlorodihydrofluorescein diacetate (DCFH; cat. no. D6883, Sigma-Aldrich) at 10 μ M in PBS for 30 min. Fluorescence was measured using the CLARIOstar PLUS optima microplate reader at 495 nm excitation and 529 nm emission.

Transfection of COS7 cells

COS7 cells were cultured in DMEM medium supplemented with 10% FBS, 50 U/mL penicillin, and 50 U/mL streptomycin (complete medium) at 37°C, 5% CO₂. One day before transfection, cells were plated at 0.3×10^6 cells/mL. For FABP5 constructs, 6 μ g (for one 10 cm dish), 0.5 μ g (for 1 well of a 24-well plate), or 0.125 μ g (for 1 well of a 96-well plate) DNA was diluted in respectively, 200 μ L, 67 μ L, or 17 μ L empty medium per well in the according plate. Polyethylenimine (PEI; cat. no. 24765, Polysciences) was diluted 1:16 in empty medium and added to the mix in a DNA:PEI ratio of 1:3. Suspension was briefly agitated and left to incubate at RT for 15 min before addition to the cells. 600 μ L mix was added to one 10 cm plate, 200 μ L mix to a well of a 24-well plate, and 50 μ L mix to a well of a 96-well plate. The medium was refreshed after 6-8h to complete medium. 24 or 48h post-transfection, cells were collected for acyl-biotin exchange assay, stimulated with 100 μ g/mL myelin for 24h and fixed in 4% PFA for stainings, or used for phagocytosis assays.

Phagocytosis assay

To evaluate the ability and extent of myelin phagocytosis, myelin was fluorescently labeled with pHrodo succinimidyl ester (cat. no. P36600, Invitrogen). Cells were exposed to 100 μ g/mL pHrodo-labeled myelin for 1.5h at 37°C and analyzed for fluorescence intensity using the CLARIOstar PLUS optima microplate reader or fixed with 4% PFA for immunofluorescence staining. To define the uptake of latex beads, cells were exposed to fluorescent red latex beads for 1.5h at 37°C (1:100, cat. no. L3030, Sigma-Aldrich) and analyzed for fluorescence intensity using the FACS Fortessa.

Luciferase-based nuclear receptor reporter assay

To determine the activation of PPAR, PPAR β /, and PPAR, ligand-binding luciferasebased reporter assays were performed using the ONEGlo™ Luciferase Assay System kit (cat. no. E6110, Promega). COS7 cells were transfected with bacterial plasmid constructs expressing luciferase under the control of the ligandbinding domain for PPAR, PPAR β /, and PPAR, which were kindly provided by Prof. dr. Bart Staels (National Institute of Health and Medical Research, University of Lille, Lille, France). Cells were grown to 60% confluency in 60 mm petri dishes and subsequently transfected with 1.8 μ g plasmid DNA, including 0.2 μ g pGAL4hLXR or pGAL4hLXR β , 1 μ g pG5 TKGL3, and 0.6 μ g pCMV β galactosidase. In case of luciferase-based nuclear receptor reporter assay with FABP5 constructs, 2 μ g of FABP5 construct was added to the mix. PEI was used as

transfection reagent. After O/N incubation, transfected cells were seeded at a density of 10,000 cells/well in a 96well plate in DMEM medium enriched with 50 U/mL penicillin and 50 U/mL streptomycin, and treated with modulators for 18h. Following treatment, cells were lysed in lysis buffer (25 mM GlycylGlycine, 15 mM MgSO₄, 4 mM EGTA, and 1x Triton, all from SigmaAldrich). To correct for transfection efficacy, β galactosidase activity was measured using cell lysate (10%) in β gal buffer, consisting of 20% 2Nitrophenyl β Dgalactopyranoside (ONPG; SigmaAldrich) and 80% BufferZ (0.1 M Na₂HPO₄, 10 mM KCl, 1 mM MgSO₄, and 3.4 μ L/mL 2mercaptoethanol; all from SigmaAldrich). Luminescence and absorbance (410 nm) were measured using the CLARIOstar PLUS microplate reader.

Acyl-biotin exchange (brain tissue)

Frozen brain tissues were lysed on ice using a Dounce homogeniser in a lysis buffer containing 50 mM TEA pH 7.5, 10 mM NaCl, 2 mM MgCl₂. The tissue homogenates were centrifuged at 2,500 g and 4°C for 5 min. The supernatant was carefully transferred to new tubes, and protein concentrations were measured using a BCA protein assay (Thermo Fisher Scientific). The following was added (to a final concentration): 0.5% (v/v) NP-40, 0.1% (w/v) SDS, 62.5 U/mL Benzonase nuclease, 1x complete EDTA-free protease inhibitor cocktail (Roche), 500 nM Palmostatin B.

For the S-acylproteomics analysis, the acyl-biotin exchange (ABE) method was performed as previously described³⁸ with minor modifications. The lysates (560 μ g, 0.72 μ g/ μ L) were reduced with 50 mM TCEP pH 7.5 for 30 min at RT, followed by alkylation with 30 mM N-ethyl maleimide (NEM; cat. no. 23030, Thermo Fisher Scientific) for 1h at RT. Additional NEM was added to reach a total concentration of 60 mM, along with 8 M urea, and the samples were incubated for 3h at RT. Samples were precipitated once with methanol and chloroform and resuspended in 50 mM TEA pH 7.5, 150 mM NaCl, 5 mM EDTA, 2% (w/v) SDS. They were reduced again with 25 mM TCEP pH 7.5 at RT for 30 min and alkylated with 50 mM NEM for 1h. Following four rounds of precipitation, the samples were split in two for +/- hydroxylamine (HA) treatment. The samples were incubated with 1 M hydroxylamine (cat. no. 8.14441, Sigma-Aldrich) pH 7.5 and biotinylated with 1.25 mM HPDP-biotin (in DMF/MQ = 2:1; cat. no. 21341, Thermo Fisher Scientific) for 2h at RT. For the negative control, the samples were treated with MQ replacing HA (+/- HA). After three rounds of precipitation, the samples were resuspended in 50 mM TEA pH 7.5, 150 mM NaCl, 5 mM EDTA, 2-4% (w/v) SDS. Samples were further diluted with 50 mM TEA pH 7.5, 150 mM NaCl, 5 mM EDTA to a final SDS concentration of 0.1% before enrichment. Each sample was incubated with 10 μ L NeutrAvidin beads (cat. no. 29201, Thermo Fisher Scientific) for 2h at RT with end-to-end rotation. The beads were washed and the proteins were eluted using 50 mM TEA pH 7.5, 50 mM TCEP, 5 mM EDTA, and 0.1% (w/v) Rapigest (cat. no. 186008090 Waters) and were collected in 50 mM HEPES pH 8. Enriched samples were alkylated with 25 mM chloroacetamide for 20 min at RT. They were digested sequentially with LysC (0.028 μ g) at 37°C for 1h, followed by O/N digestion with trypsin (0.028 μ g) at 37°C. Digestion was quenched with TFA, and samples were centrifuged before desalting on a C18 stage tip (Empore C18 SPE disk, Supelco). Finally, dried peptides were reconstituted in 2% (v/v) formic acid and stored at -20°C until LC-MS/MS analysis.

For the proteomics analysis of the brain samples, the S-trap (PROTIFI) method was applied to 20 μ g lysates (0.72 μ g/ μ L) as we previously described³⁸. After drying, peptides were resuspended in 2% (v/v) formic acid and stored at -20°C until LC-MS/MS analysis.

Samples were analyzed on a nanospray UHPLC system (Ultimate 3000, Thermo Fisher Scientific) coupled to an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific) operating in data-dependent acquisition mode. Peptides were loaded onto a Pepmap Neo 100 C18 trap column (5 mm x 0.3 mm, 5 μ m, Thermo Fisher Scientific) using solvent A (0.1% formic acid) and

separated on a homemade analytical column (ReproSil-Pur 120 C18-AQ, 50 cm x 75 µm, 2.4 µm, Dr. Maisch) with a flowrate of 300 nL/min. For the ABE experiment, a 120 min gradient was applied: 9% solvent B (0.1% formic acid in 80% acetonitrile) for 1 min, 9-13% for 1 min, 13-44% in 95 min, 44-55% in 5 min, 55-99% in 1 min, 99% for 7 min and 9% B for 10 min. For the total proteome analysis, a gradient of 180 min was used: 9% solvent B for 1 min, 9-13% for 1 min, 13-44% in 155 min, 44-55% in 5 min, 55-99% in 5 min, 99% for 3 min and 9% for 10 min. MS1 scans were acquired with a resolution of 60,000 between 375 and 1600 m/z after reaching the normalized automated gain control (AGC) target with automatic injection time. HCD fragmentation was performed with a normalized collision energy of 28% and 16-24s dynamic exclusion time, and scans were collected at a resolution of 30,000. The data analysis was done as previously described³⁸ using the human Uniprot database (version July 2023, UP000005640). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE¹¹⁹ partner repository with the dataset identifier PXD068689.

Acyl-biotin exchange (cells)

The ABE assay was performed as described by Wan et al.¹²⁰. Cells were lysed in a buffer containing 150 mM NaCl, 50 mM Tris, 5 mM EDTA, pH 7.4, 0.1 % SDS, and 1.7 % Triton X-100, with 1 × protease inhibitor cocktail (cat. no. 04693116001, Sigma-Aldrich) and 10 mM NEM, which protects free cysteines from capture by thiol-reactive resin. After three chloroform-methanol precipitations, pellets were resuspended in a buffer containing 8% SDS, and the sample was divided into two equal fractions. One fraction was incubated with 0.7 M HA a thioester cleavage agent for S-S-palmitoylation sites, while the rest of the proteins were incubated with Tris (-HA). The omission of hydroxylamine serves as a negative control. Since the thioester bonds at S-palmitoylation sites in the HA(-) fraction were not cleaved, proteins captured by the thiol-reactive resin represented non-specific binding. Samples were rotated for 1h at RT. After a chloroform-methanol precipitation, the pellets were resuspended in a buffer containing EZ-Link HPDP-Biotin and incubated for 1h at RT. Unreacted HPDP-biotin was removed by chloroform-methanol precipitation and pellets were resuspended in lysis buffer. Samples were diluted to 0.1% SDS and biotinylated proteins were affinity-purified using streptavidin-agarose beads (cat. no. 20359, Thermo Fisher Scientific). 1% Beta-mercaptoethanol (cat. no. BP176, Fisher Scientific) was used to cleave HPDP-biotin and release biotinylated proteins from the beads. Finally, proteins were denatured in sample buffer and Western blot was performed.

Immunoblotting

After acyl-biotin exchange, denatured proteins (input and pool-down) in sample buffer were separated by electrophoresis on a 10% Tris-Tricine gel and were transferred onto a PVDF membrane. Blots were blocked using 5% dried skimmed milk powder (Marvel) in 1x TBS0.1% Tween20 (TBST), incubated O/N with FABP5 primary antibody (1:500), followed by incubation with the appropriate HRPconjugated secondary antibody. Chemiluminescent signals were detected with the Amersham Imager 680 (GE Healthcare Life Sciences), using the enhanced chemiluminescence (ECL) FEMTO detection kit (cat. no. 34094, Thermo Fisher Scientific).

Click chemistry for detection of S-palmitoylated proteins

To determine the S-palmitoylation landscape of BMDMs, cells were pretreated with vehicle or lomitapide (1 µM) for 24h. Next, these cells or *Zdhhc5*^{-/-} BMDMs were incubated in RPMI1640 medium enriched with 50 U/mL penicillin, 50 U/mL streptomycin, 5% fatty acid-free BSA (cat. no. A8806, Sigma Aldrich), and 17-Octadecynoic acid (17-ODYA; 100 µM, cat. no. 34450-18-5, Cayman Chemical) for 16h. To lyse the cells, cells were resuspended in 400 µL lysis buffer (1X PBS, 1% Triton X-100, 0.1% SDS, and 1 × Complete EDTA-free protease inhibitor cocktail (cat. no. 4693159001, Sigma-Aldrich) and kept for 30 min on ice. Prior to proceeding to the pull-down

procedure, the protein concentration was determined using a BCA kit (cat. no. A55864, Thermo Fisher Scientific). Protein lysate (1 mg) was incubated with 100 μ M biotin-azide (cat. no. HY-129832, MedChemExpress), 1 mM CuSO₄ (cat. no. 451657, Sigma-Aldrich), 1 mM Tris(2-carboxyethyl) phosphine hydrochloride (TCEP; cat. no. C4706, Sigma-Aldrich), and 100 μ M Tris((1-benzyl-1H-1,2,3-triazol-4-yl)methyl)amine (TBTA; cat. no. 678937, Sigma-Aldrich) for 1h at RT while shaking at 750 rpm. 20 mM EDTA (cat. no. 324506, Millipore) was added to stop the click chemistry reaction. The samples were equally divided into two fractions, and three rounds of chloroform-methanol precipitations were performed. Next, the protein pellet was dissolved in 250 μ L lysis buffer, and 25 μ L of pre-washed neutravidin beads were added, followed by 2h end-over-end rotation at RT. After centrifugation for 2 min at 2,500g, samples were either treated with or without 0.7 M HA for 1h at RT while rotating. Finally, samples were centrifuged for 2 min at 2,500g and supernatants were prepared for mass-spectrometry analysis. Briefly, 20 mM chloroacetamide (CAA; cat. no. C0267, Sigma-Aldrich) was added to the samples for 20 min at RT and 750 rpm to label thiol groups. Next, samples were digested with 0.1 μ g LysC (cat. no. 324796, Sigma-Aldrich) and trypsin O/N at 37°C and 750 rpm. Finally, samples were diluted to 1 mL with MQ and quenched with 0.1% trifluoroacetic acid (TFA; cat. no. 302031, Sigma-Aldrich) for 30 min at 37°C and 750 rpm before proceeding with mass spectrometry analysis. The evoSEP-ZenoTOF platform was used, employing the 60SPD method with a 21-minute gradient in SWATH/DIA mode. For data analysis, Spectronaut was used referencing to the Mouse Proteome available at <https://www.uniprot.org/proteomes/UP000000589>. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE¹¹⁹ partner repository with the dataset identifier PXD068746.

Plasmids

FABP5 wild-type and mutant plasmids were kindly donated by Prof. dr. Ke and Prof. dr. Zhang (Zhejiang University, Hangzhou, China). Briefly, wild-type 3xFlagFABP5 was constructed by cloning cDNA into the pXJ403xFlag vector containing the aminoterminal Flag tag using HindIII and KpnI sites. Other FABP5-related mutants were generated with the Fast Mutagenesis System kit (Transgen, FM111) on the basis of their respective wild-type constructs. Wild-type and FABP5 mutants lacking the nuclear localization signal (FABP5^{NLSm}: K24A, K34A, and R33A) were kindly donated by Prof. dr. Ortlund (Emory University School of Medicine, Atlanta, USA). Briefly, full-length, codon-optimized wild-type human FABP5 (residues 1–135) was subcloned into pMCSG7-His, pCMV-Tag2B, and pEGFP-N3 expression vectors. FABP5^{NLSm} was generated in the pMCSG7-His pCMV-Tag2B. The mutagenesis was performed using QuikChange II XL (cat. no. 200521, Integrated Sciences).

Serum cholesterol measurements

Blood was collected by cardiac puncture prior to perfusion of sacrificed cuprizone-treated mice and collected with Vacutainer® Ethylene Diamine Tetra Acetic acid (EDTA) tubes that were promptly centrifuged at 4°C. Serum was aliquoted and stored at -80°C until batch-wise analyses. Hydragel analysis of lipoprotein profiles of serum samples was performed using Hydragel 7 Lipoprotein(e) (Sebia Inc., Georgia, USA) using a HYDRASYS System (Sebia Inc., Georgia, USA). In short, samples were loaded into wells of ready-to-use agarose gels (0.8 g/dL) and electrophoresis was performed using buffered strips. Samples were run at 160V for 20–25 min. Dried gels were stained with a Sudan Black solution provided by the manufacturer, washed with 75% ethanol and the migration pattern and lane density of the samples were calculated.

Statistical analysis

Data were statistically analyzed using GraphPad Prism v10 and are reported as mean $\pm$ SEM. The D'Agostino and Pearson omnibus normality test was used to test for normal distribution. When

datasets were normally distributed, an ANOVA (Tukey's post hoc analysis) or two-tailed unpaired Student's *t*-test (with Welch's correction if necessary) was used to determine statistical significance between groups. If datasets did not pass normality, the Kruskal-Wallis or Mann-Whitney analysis was applied. For click chemistry analysis, statistical analyses were performed using an unpaired *t*-test assuming a single pooled variance. To correct for multiple comparisons and control the false discovery rate (FDR), the two-stage step-up method of Benjamini, Krieger, and Yekutieli was applied. This procedure was implemented with a desired FDR (*Q*) set at 1.00%. Statistical significance was determined based on adjusted *p*-values, and only comparisons meeting the FDR threshold were considered statistically significant. No statistical method was used to predetermine the sample size for *in vitro* experiments. The sample size for *in vivo* experiments was determined with the G Power sample size calculation. *Ex vivo* and *in vivo* experiments were performed in a blinded fashion. The investigators were not blinded to allocation during experiments and outcome assessment of *in vitro* experiments. All statistical parameters, tests used, and reproducibility are reported in figure subscriptions. *P* values < 0.05 were considered to indicate a significant difference (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001).

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Declaration of generative AI and AI-assisted technologies in the writing process.

During the preparation of this work the author(s) used ChatGPT in order to assist with language polishing, grammar, and formatting. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

Author contributions

Conceptualization, J.G., S.V., and J.B.; **Formal analysis**, J.G., S.V., S.S., B.M., L.v.O., M.L., K.K., T.D., L.T., M.M., and F.H.; **Investigation**, J.G., S.V., S.S., K.H., B.M., L.v.O., M.L., K.K., F.W., K.P., T.D., L.T., T.V., E.W., B.B., B.G., L.B., M.M., F.H., N.Z., E.G., M.B., M.N., J.H., D.N., J.L., and J.B.; **Methodology**, J.G., S.V., S.S., K.H., B.M., L.v.O., M.L., K.K., F.W., K.P., T.D., L.T., T.V., E.W., B.B., B.G., L.B., M.M., F.H., N.Z., E.G., M.B., M.N., J.H., D.N., J.L., and J.B.; **Resources**, E.W., B.G., M.M., D.N., M.B., and J.B.; **Writing – original draft**, J.G., S.V., and J.B.; **Writing – review & editing**, J.G., S.V., S.S., K.H., B.M., L.v.O., M.L., K.K., F.W., K.P., T.D., L.T., T.V., E.W., B.B., B.G., L.B., M.M., F.H., N.Z., E.G., M.B., M.N., J.H., D.N., J.L., and J.B.; **Data curation**,

J.B.; **Funding acquisition**, M.N., D.N., J.L., and J.B. **Supervision**, M.N., J.H., D.N., J.L., and J.B.
Declaration of interests

The authors declare no competing interests.

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Geneeskundige Stichting Koningin Elisabeth
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Final report of the research group of

Prof. dr. Bieke Broux
Universiteit Hasselt (UHasselt)

Bieke Broux, PhD (she, her) (UHasselt)

Assistant Professor Immunology

Chronic inflammation and Blood brain barrier disruption in Neurodegeneration (CBN)

Neuroimmune Connections & Repair (NIC&R) lab

Hasselt University – Biomedical Research Institute

Agoralaan, building C – B-3590 Diepenbeek

T +32 11 26 92 54

T +32 468 14 30 54

High salt diet causes blood-brain barrier disturbances in multiple sclerosis: involvement of the renin-angiotensin-aldosterone system

1. Research summary

Multiple sclerosis (MS) is a chronic neurodegenerative disease affecting millions globally, characterized by progressive demyelination and neuronal damage, leading to significant physical and cognitive impairments. A hallmark of MS pathogenesis is the disruption of the blood-brain barrier (BBB), a critical structure that regulates the passage of molecules and immune cells between the bloodstream and the brain. The BBB is primarily composed of specialized endothelial cells (ECs), supported by astrocytes, which maintain the neuronal microenvironment. When the BBB is compromised, immune cells infiltrate the brain, exacerbating neuronal damage and driving disease progression. Despite extensive research into genetic and environmental factors, the precise etiology of MS remains unclear, limiting the development of effective therapies.

Among environmental factors, dietary influences, particularly high-salt diets (HSD), have emerged as significant contributors to MS progression. Studies using experimental autoimmune encephalomyelitis (EAE), a preclinical model for MS, demonstrate that HSD exacerbates disease severity and compromises BBB integrity. High salt intake is thought to disrupt the delicate balance of the neurovascular unit (NVU), which includes endothelial cells, astrocytes, and other supporting cells. However, the specific effects of HSD on the human BBB, particularly in the context of neuroinflammation and endothelial cell-astrocyte interactions, remain poorly understood. Additionally, recent research suggests that salt sensitivity and the renin-angiotensin-aldosterone system (RAAS) play a role in MS. RAAS activation, triggered by high salt levels, is associated with increased BBB permeability, potentially worsening neuroinflammation. Yet, the mechanisms by which HSD influences RAAS and subsequently affects BBB function in the human NVU are not well elucidated.

This study aims to investigate the effects of high salt concentrations on BBB activation and integrity in human endothelial cells and astrocytes, both in isolation and under inflammatory conditions mimicking MS. By generating detailed protein expression profiles and RAAS fingerprint datasets, the research will identify salt-induced, RAAS-mediated molecular changes that alter BBB function. The study will also explore how high salt affects interactions between endothelial cells and astrocytes, critical components of the NVU, and how these interactions influence BBB integrity in the context of MS. Through these investigations, the project seeks to establish a clear link between salt sensitivity, RAAS dysregulation, and BBB impairment in MS. By elucidating these mechanisms, the study aims to provide novel insights into how dietary factors, particularly high salt intake, contribute to MS progression. Ultimately, the findings could identify new therapeutic targets to mitigate BBB disruption and slow disease progression, offering potential avenues for personalized interventions in MS management.

The goal of this study is to elucidate the effects of high salt concentrations on BBB activation and integrity in human endothelial cells and astrocytes, both in isolation and under inflammatory conditions. By generating detailed protein expression profiles and RAAS fingerprint datasets, this project aims to uncover salt-induced, RAAS-mediated alterations in BBB integrity. Additionally, the research will explore the interactions between BBB endothelial cells and astrocytes under high-salt conditions and their impact on BBB function in MS. Ultimately, this project seeks to

establish the link between salt sensitivity, RAAS dysregulation, and BBB impairment in MS, providing new insights into the role of dietary factors in disease progression and offering potential therapeutic targets for intervention.

2. Results:

2.1. Evaluation of cell viability in astrocytes (ASTs) and endothelial cells (ECs) under varying sodium chloride (NaCl) Concentrations

As key cellular components of the blood-brain barrier (BBB), endothelial cells (ECs) HCMEC/D3 human cerebral microvascular endothelial cell line and primary astrocytes (ASTs) were selected for cellular experiments. To better determine the appropriate cellular stimulation concentration that corresponds to a high-salt diet, we reevaluated the experimental NaCl stimulation concentrations. The cytotoxic effects of these concentrations on ECs and ASTs were meticulously examined using the LDH assay (Figure 1 and Figure 2). The results demonstrated that in both cell type, a 40 mM NaCl concentration was manageable and corresponded well to high-salt conditions (with or without inflammatory) observed in vivo, making it suitable for subsequent experiments. Additionally, a 10 mM NaCl concentration was used as a control in certain experiments to provide comparative insights.

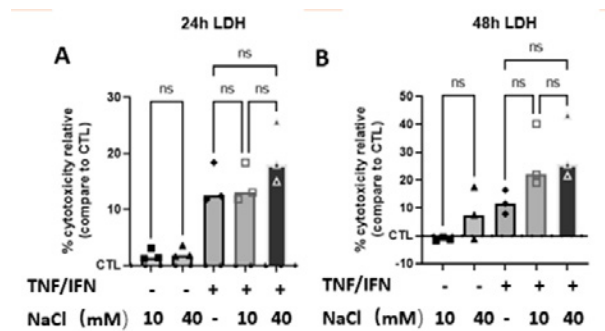


Figure 1 A/B: LDH kit analysis of cytotoxicity in hCMEC/D3 cells (n=3) after treated 24 and 48h with 10mM or 40mM NaCl in the presence/absence of 10 ng/ ml TNF- α /IFN- γ . Results were analyzed by one-way analysis of variance (ANOVA) with Friedman test, and correction for multiple comparisons performed with Dunn's test.

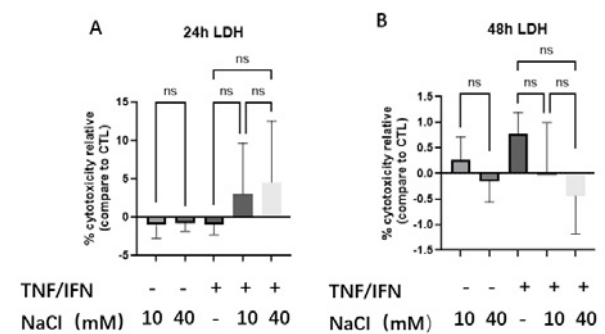


Figure 2 A/B: LDH kit analysis of cytotoxicity in astrocytes (n=3) after treated 24 and 48h with 10mM or 40mM NaCl in the presence/absence of 10 ng/ ml TNF- α /IFN- γ . Results were analyzed by one-way analysis of variance (ANOVA) with Friedman test, and correction for multiple comparisons performed with Dunn's test.

2.2. Tight junction gene and protein expression in ECs

To investigate the effects of high sodium concentration on the tight junctions of EC cells, we examined the gene and protein expression levels of Claudin-5. The results indicated that there was no significant difference in the mRNA and protein expression of Claudin-5 between the non-inflammatory and inflammatory control groups and the high-salt groups. (Figure 3)

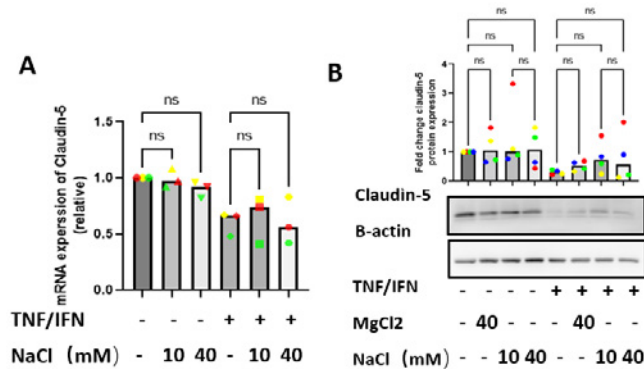


Figure 3 A. qPCR analysis of Claudin-5 mRNA in hCMEC/D3 cells (n=3) treated 24h. B. Western blot analysis of Claudin-5 in hCMEC/D3 cells (n=3) treated 48h. Here, MgCl₂ be taken as Anion control. Results were analyzed by one-way analysis of variance (ANOVA) with Friedman test, and correction for multiple comparisons performed with Dunn's test.

2.3. Adhesion molecule expression in ECs

Adhesion molecules such as ICAM-1, VCAM-1, and VE-Cadherin are critical for the maintenance of endothelial cell function and integrity. Therefore, we also investigated the expression of these adhesion molecules in ECs at the transcriptional level. The results showed no significant difference in the expression of ICAM-1, VCAM-1, and VE-Cadherin between the non-inflammatory/inflammatory control groups and the high-salt groups. (Figure 4)

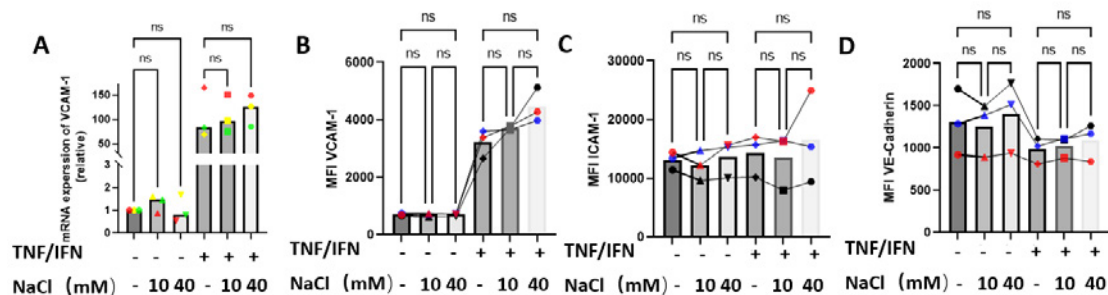


Figure 4 A: qPCR analysis of VCAM-1 mRNA in hCMEC/D3 cells (n=3), treated 24h. B/C/D: Flow cytometry analysis of VCAM-1, ICAM-1 and VE-Cadherin in hCMEC/D3 cells (n=3) treated 48h. Results were analyzed by one-way analysis of variance (ANOVA) with Friedman test, and correction for multiple comparisons performed with Dunn's test.

2.4. Differential gene expression analysis in ECs and ASTs under high-salt conditions

For the ECs, a total of 45 differentially expressed genes (DEGs) were identified in the comparison between the CTL and 40 mM NaCl groups. Of these, 34 genes were relatively upregulated in the NaCl group, while 11 were upregulated in the CTL group ($p < 0.05$). Genes with log₂ fold change > 1 were selected to create the volcano plot shown in the Figure 5A. In the comparison between inflammation without or with high salt concentration, a total of 57 differentially expressed genes (DEGs) were identified. Of these, 41 genes were relatively upregulated in the inflammation with NaCl group, while 16 were upregulated in the inflammation group ($p < 0.05$). Genes with log₂ fold change > 1 were selected to create the volcano plot shown in the Figure 5B.

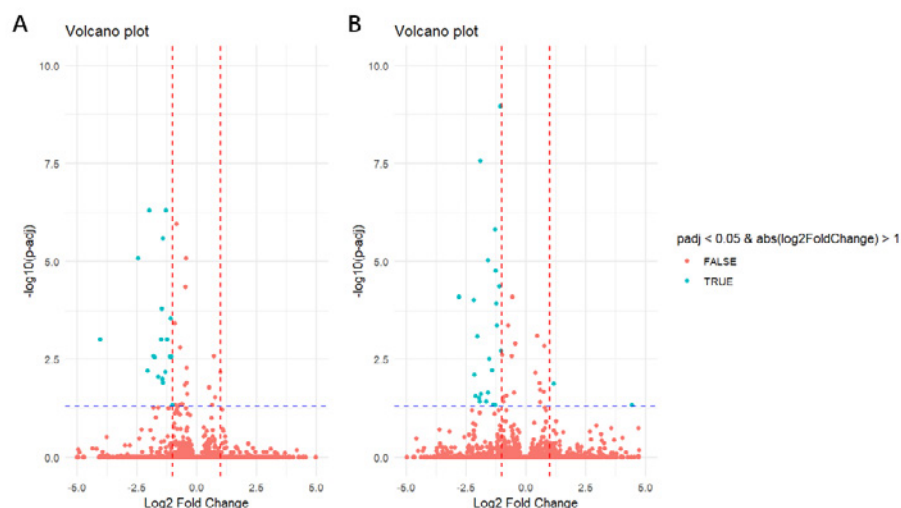


Figure 5. Overview of the gene expression profile in ECs under high-salt conditions. (A) Volcano plot of differential gene expression patterns from the control group ($n=4$) versus the NaCl(40mM) treated group ($n=4$). The blue plots indicate the significantly dysregulated genes. ($\log_2$ fold change > 1 and adjust p value < 0.05). (B) Volcano plot of differential gene expression patterns from the inflammation group (treated with 10 ng/ ml TNF- α /IFN- γ) ($n=4$) versus the group treated with both NaCl (40mM) and inflammation treated($n=4$). The blue plots indicate the significantly dysregulated genes. ($\log_2$ fold change > 1 and adjust p value < 0.05).

For the ASTs, 8 differentially expressed genes (DEGs) were identified in the comparison between the CTL and 40 mM NaCl groups. Genes with $\log_2$ fold change > 1 were selected to create the volcano plot shown in the Figure 6A (left). A total of 14 differentially expressed genes (DEGs) were identified in the comparison between inflammation group versus the inflammation with high salt concentration group. Genes with $\log_2$ fold change > 1 were selected to create the volcano plot shown in the Figure 6B (left). To further investigate the biological processes affected by high salt under inflammatory conditions, Gene Set Enrichment Analysis (GSEA) was performed using Hallmark gene sets. Pathways related to inflammatory responses and ion transport were significantly enriched, as shown in Figure 6A and 6B (right).

Through STRING database, protein-protein interaction analysis was performed based on differentially expressed genes (adjust $p < 0.05$). The results are shown in Figure 7. Interestingly, we found that under the CTL_versus_NaCl DEGs, ARG2 in EC cells interacts with ODC1 in AST cells, and under the inflammation versus inflammation with NaCl condition, PGM2L1 in EC cells interacts with IMPA2 in AST cells.

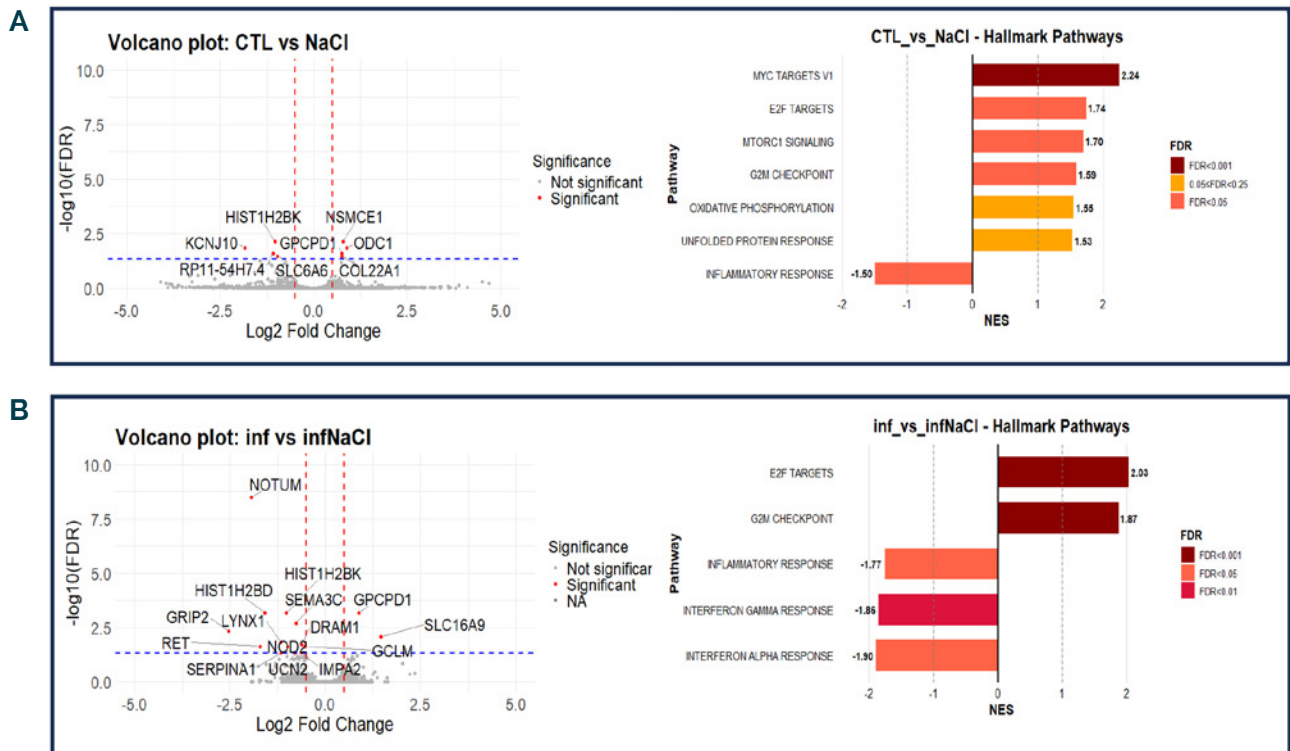


Figure 6. Overview of the gene expression profile in ASTs under high-salt conditions. (A) Volcano plot of differential gene expression patterns from the control group ($n=4$) versus the NaCl(40mM) treated group ($n=4$). The blue plots indicate the significantly dysregulated genes. ($\log_2$ fold change > 1 and adjust p value < 0.05). (B) Volcano plot of differential gene expression patterns from the inflammation group (treated with 10 ng/ ml TNF- α /IFN- γ) ($n=4$) versus the group treated with both NaCl (40mM) and inflammation treated($n=4$). The blue plots indicate the significantly dysregulated genes. ($\log_2$ fold change > 1 and adjust p value < 0.05). Figure 6 A/B: Bulk RNA-seq analysis ($n=4$) identified 8 differentially expressed genes (DEGs) between the CTL and high-salt groups (A left), and 14 DEGs between the inflammation and inflammation + NaCl groups(B, left). ($\log_2FC>0.5$, $FDR<0.05$), with pathways related to inflammatory response, ion transport being significantly enriched.(A,B right)

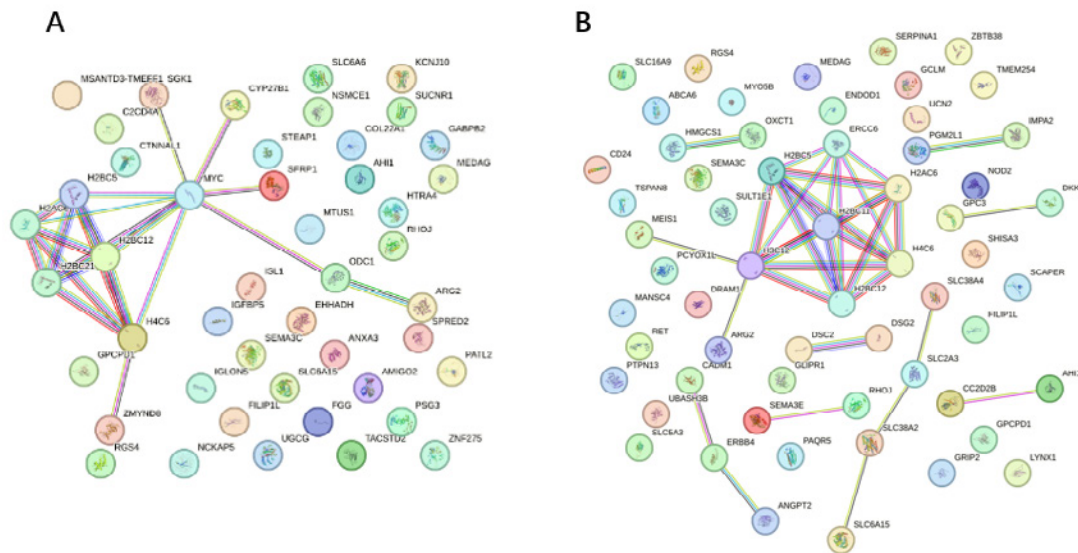


Figure 7 Protein–protein interaction network analysis under the same treatment conditions, a joint analysis of differentially expressed genes (DEGs) from ECs and ASTs. (A) DEGs between the CTL and 40 mM NaCl groups, while (B) DEGs between inflammation (treated with 10 ng/ ml TNF- α /IFN- γ) versus inflammation with 40 mM NaCl groups. The analysis was conducted on the STRING database official website (https://string-db.org/cgi/about?footer_active_subpage=references), each gene is represented as a node, and the minimum required interaction score is set to medium confidence (score = 0.400).

2.5. Protein expression in high-salt treated groups in astrocytes

By analyzing the culture supernatants of primary astrocytes exposed to high-salt stimulation, we identified significant alterations in protein expression profiles. Notably, PDGF-AA exhibited relatively elevated expression in the high-salt group across both comparative conditions, while IL-8 was consistently upregulated under high-salt treatment (Figure 8).

To further validate these findings, enzyme-linked immunosorbent assay (ELISA) kits were used to quantify IL-8 and PDGF-AA levels. The ELISA results showed that the difference in IL-8 expression was not statistically significant, while the PDGF-AA expression pattern was consistent with the results obtained from the cytokine array analysis (Figure 9 A, B).

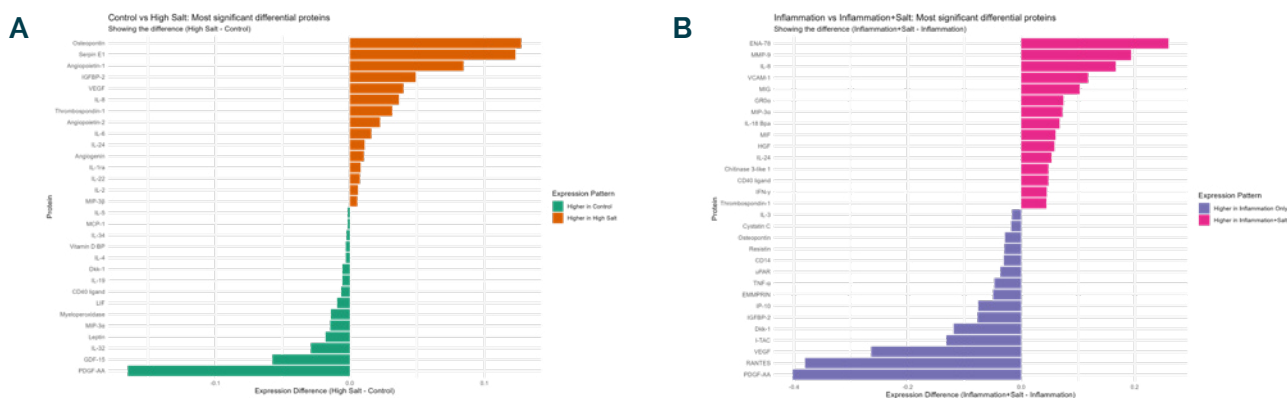


Figure 8 Detection of cytokine expression in astrocyte supernatants using the Human XL Cytokine Array Kit. (A) Comparison between the control (CTL) and 40 mM NaCl-treated groups. (B) Comparison between the inflammation group (treated with 10 ng/mL TNF- α and IFN- γ) and the inflammation + 40 mM NaCl group. Supernatants from four independent astrocyte batches were pooled for analysis. Pixel intensities were quantified using ImageJ, and data were analyzed and visualized in R.

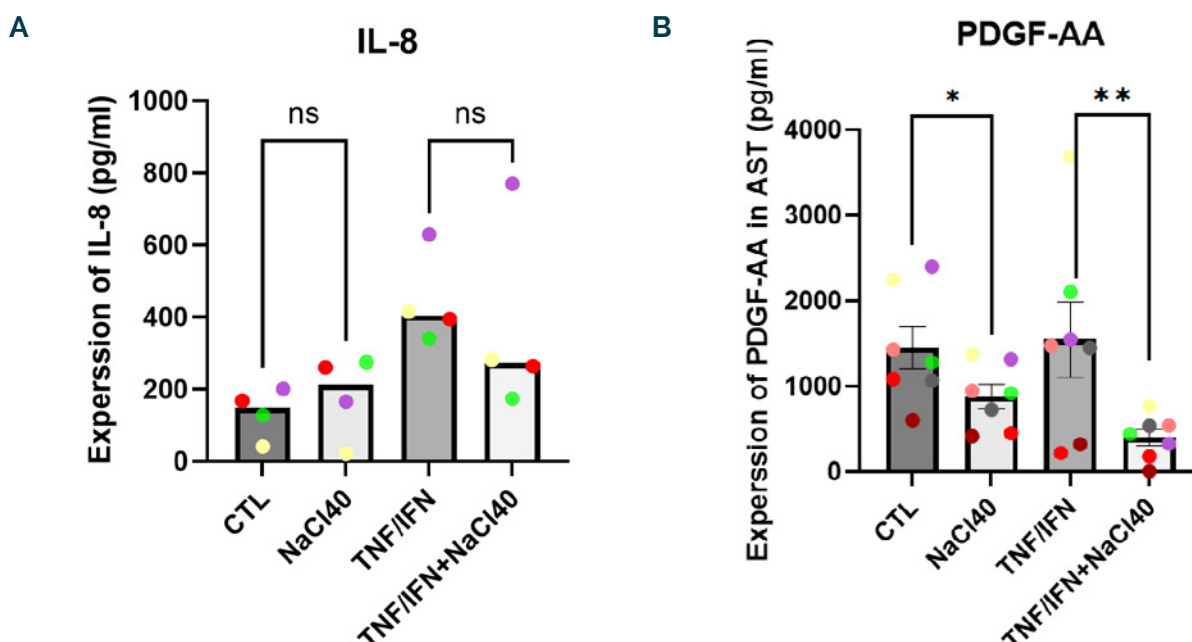


Figure 9 Comparisons between the control (CTL) group and the 40 mM NaCl-treated group, as well as between the inflammation group treated with 10 ng/mL TNF- α and IFN- γ and the inflammation plus 40 mM NaCl group, were performed using the nonparametric Wilcoxon test. The left panel (A) shows IL-8 levels ($n = 4$), whereas the right panel (B) shows PDGF-AA levels ($n = 7$). Pairwise comparisons are indicated by lines above the plots, with statistical significance denoted by p values.

2.6. Transendothelial/epithelial Electrical Resistance TEER

TEER measurements revealed that high-salt stimulation led to a significant decrease in transendothelial electrical resistance ($p < 0.05$). When endothelial cells were cultured in astrocyte-conditioned medium (ACM), TEER values showed a downward trend compared to monocultured endothelial cells, although the difference was not statistically significant ($n = 6$). (Figure 10

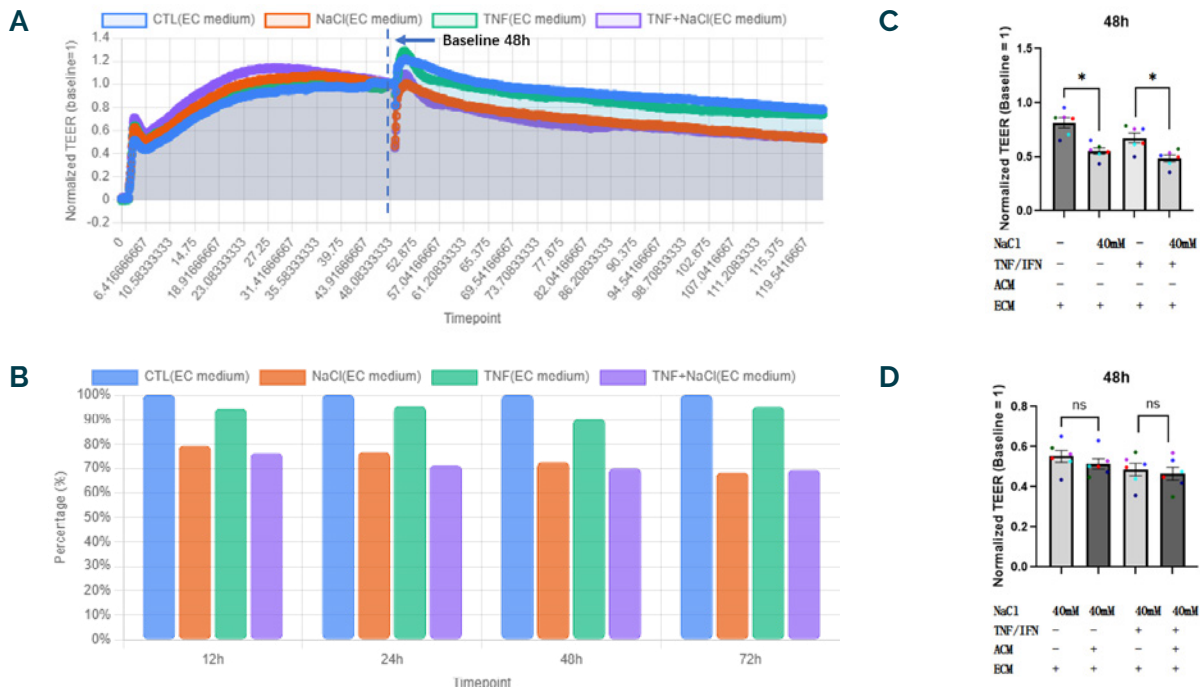


Figure 10. Transendothelial electrical resistance (TEER) measurements of hCMEC/D3 endothelial cell monolayers (A) Continuous TEER measurements of endothelial cells cultured in endothelial media under different stimulation conditions. Stimulation was initiated when cells reached ~90% confluence at 48 hours, and TEER values were normalized to this time point and continuously monitored until 72 hours. (B) Bar graphs comparing TEER values at selected time points. Panels A and B represent the same batch of cells (one of six independent batches), with similar trends observed across all batches (data not shown). (C) Statistical analysis of TEER values at 48 hours across six independent batches. (D) TEER measurements of endothelial cells cultured in 60% endothelial medium supplemented with 40% astrocyte-conditioned medium (ACM, supernatant collected from astrocytes stimulated for 48 hours). Statistical comparisons were performed using the nonparametric Wilcoxon test. Each condition included three technical replicates.

2.7. Bioinformatics analysis

Beyond experimental validation, this study employed bioinformatic approaches to systematically analyze data relevant to dietary salt intake and MS, providing computational support for the experimental findings. Differential gene screening and functional annotation highlighted signaling pathways related to inflammation and ion transport. An integrated strategy combining Mendelian randomization (MR) analysis with a systematic review was applied. MR analyses used genome-wide significant single-nucleotide polymorphisms (SNPs) associated with urinary sodium levels, dietary salt consumption, MS susceptibility, and MS severity as instrumental variables, selected with stringent criteria ($P < 5 \times 10^{-8}$, linkage disequilibrium $r^2 < 0.001$, F-statistic > 10). The primary method was inverse-variance weighted (IVW) estimation, complemented by sensitivity and directionality analyses using MR-Egger, weighted median, MR-PRESSO, and reverse MR.

In parallel, a PROSPERO-registered systematic review was conducted across PubMed, Embase, and the Cochrane Library, and the quality of evidence was assessed using the GRADE framework. Multiple data sources were integrated, including genome-wide association study (GWAS) summary statistics for urinary sodium, dietary salt intake, and MS phenotypes; expression quantitative trait locus (eQTL) data from the GTEx project and European cohorts; and linkage

disequilibrium reference data from the European population of the 1000 Genomes Project. The analytical pipeline consisted of three phases: (1) a transcriptome-wide association study (TWAS) using the FUSION framework, with Bonferroni-corrected significance thresholds to identify candidate genes; (2) summary data-based Mendelian randomization (SMR) to validate TWAS-identified genes, requiring $P_{\text{SMR}} < 0.05$ and HEIDI $P > 0.05$; and (3) three-way colocalization analysis (coloc.abf) within ± 500 kb loci to evaluate shared causal variants among salt-related traits, MS outcomes, and gene expression. Genes with posterior probability for hypothesis 4 (PP_{H4}) > 0.75 across all three pairwise comparisons were considered triangulated causal candidates, ultimately identifying RANBP10, PDE4C, and C16orf86 as the final targets. (Full text manuscript attached; will be submitted to MS Journal in February).

3. Conclusion

In conclusion, our work demonstrates that high-salt conditions affect both the molecular and functional properties of the blood–brain barrier (BBB). High salt altered gene expression in astrocytes and endothelial cells and significantly impacted protein expression in astrocytes, including differentially expressed proteins. Although no statistically significant changes were observed in the expression of the tight junction proteins examined, TEER measurements confirmed an overall impairment of barrier function under high-salt treatment. Additionally, exposure of endothelial cells to astrocyte-conditioned medium (ACM) suggested a trend toward further barrier disruption, although this effect did not reach statistical significance.

Separately, integrative bioinformatic analyses combining TWAS, SMR, and colocalization approaches identified RANBP10, PDE4C, and C16orf86 as high-confidence tissue-specific genetic regulators linking sodium exposure to multiple sclerosis (MS). These genes may affect MS risk through brain-intrinsic mechanisms, such as cellular signaling or epigenetic regulation. Overall, these findings reveal new pathways by which sodium could contribute to MS and suggest potential targets for future functional and therapeutic studies.



Geneeskundige Stichting Koningin Elisabeth
Fondation Médicale Reine Elisabeth
Königin-Elisabeth-Stiftung für Medizin
Queen Elisabeth Medical Foundation

Final report of the research group of

Dr. Sophie Laguesse
Université de Liège (ULiège)

Dr. Sophie Laguesse (ULiège)

Liège University

F.R.S-FNRS Research Associate

Giga-Stem Cells, Giga-Neurosciences

Laboratory of molecular regulation of Neurogenesis – Addiction development

Quartier Hôpital

15, Avenue Hippocrate

B-4000 Liège

Belgium

T +32 4 366.59.51

slaguesse@uliege.be

ORCID 0000-0002-9217-0087

Unveiling the alcohol-dependent alterations in mRNA local translation and its consequences on adolescent prefrontal cortex maturation and function

"AL(CO)TERATION"

1. Part A: Scientific Impact of the FMRE Young Researcher grant

1.1. Goals of the research

Alcohol use disorder (AUD) is characterized by an impaired ability to control alcohol use and ranks among the most prevalent mental disorders globally. The neural mechanisms of AUD have remained uncertain, and multiple genetic, psychological, and environmental factors are thought to be involved. For decades, AUD has been considered as a pathological condition that develops in adults, but growing evidence suggests that its roots may emerge during adolescence. Indeed, studies have shown that adolescent alcohol exposure (AAE) may interfere with the maturation of some frontal brain regions, including the prefrontal cortex (PFC), thereby leading to heightened vulnerability to develop AUD and comorbid psychopathology later in life. However, **the precise mechanisms by which alcohol perturbs the maturation of the PFC are not fully understood.** Addiction has been conceptualized as a maladaptive form of memory involving persistent drug-associated memories. In this view, alcohol is thought to usurp the molecular mechanisms underlying memory, including synaptic plasticity, which depends on the local translation of mRNAs at synaptic sites. Here, **we hypothesize that AAE interferes with local translation of mRNAs, thereby impairing prefrontal cortex maturation.** The main goals of this research are (i) to unveil whether AAE leads to PFC malfunction in mice, (ii) to decipher whether AAE modulates the activity of specific local translation regulators in the PFC, (iii) to identify the pool of mRNAs whose local translation is modulated by AAE in specific neuronal populations and (iv) to unveil their involvement in AAE-induced PFC defects, and finally (v) to decipher whether AAE differentially impacts male and female PFC maturation.

1.2. Research project

Task 1: Characterizing PFC malfunction induced by adolescent alcohol binge-drinking

Task 1.1 & 1.2 Mouse model and PFC-related behaviors induced by AAE

Using a modified version of the Drinking in the Dark paradigm, we previously reported that voluntary alcohol binge drinking in adolescent mice significantly increases anxiety-like and depressive-like behaviors and alcohol consumption, while impairing recognition memory and behavioral flexibility in adulthood, suggesting that AAE compromises long-term PFC function (Van Hees, Didone *et al.* 2022). Building on this work, the FMRE grant enabled mechanistic analyses of synaptic, structural, and circuit-level alterations in the PFC following AAE.

Task 1.3 Uncovering the structural and transmission defects induced by AAE in the PFC

We quantified glutamatergic and GABAergic neuron number and survival in the PFC at P43 and P80 and found no difference between AAE and water controls (data not shown). Microglial activation (Iba1, CD68) was also assessed, and we reported no increase in apoptosis or major inflammation in adolescent PFC after AAE (Van Hees *et al.*, 2022).

Task 1.3.1 Transmission defects

We performed whole cell patch-clamp recordings in layer V glutamatergic neurons of the prelimbic subregion of the PFC in adolescent (P43) and adult (P80) male mice. In late adolescent

mice, we observed a significant increase in the frequency of spontaneous excitatory postsynaptic currents in AAE animals as compared to control littermates, without significant change in amplitude (**Figure 1**).

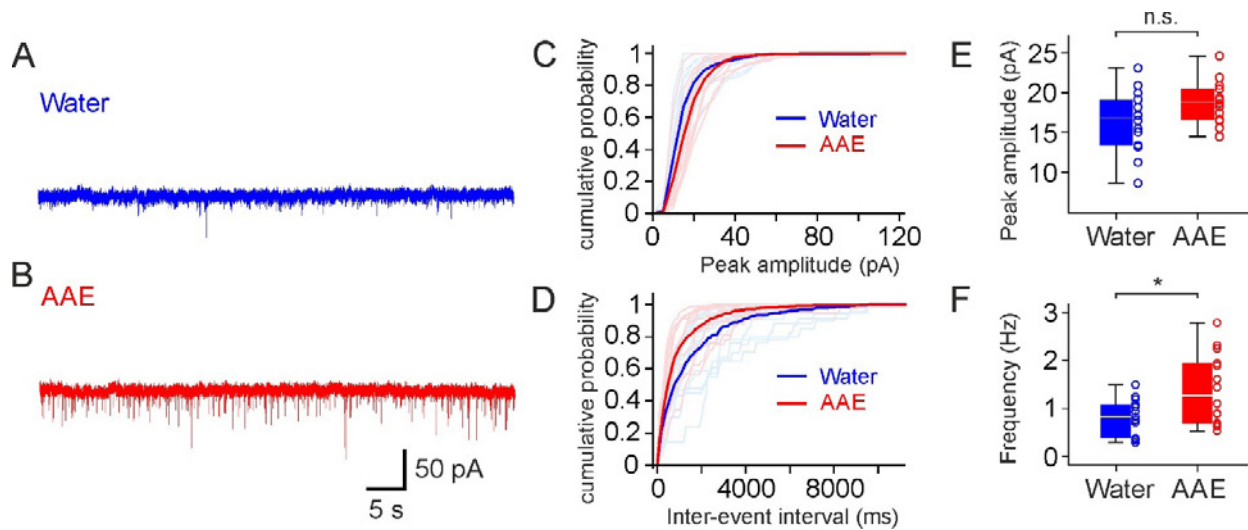


Figure 1. Alcohol consumption increases sEPSC frequency in layer 5 pyramidal neurons of the prelimbic cortex at P43. (A, B) Current traces showing spontaneous EPSCs at -60 mV in slices from water-consuming mice (top, blue traces) and from alcohol-consuming mice (bottom, red traces). External solution contained 50 μ M picrotoxin to block inhibitory synaptic currents. (C, D) Cumulative distributions of spontaneous EPSC amplitude (C) and inter-event intervals (D). Thick lines are average cumulative distributions from pyramidal neurons in water- (blue) and alcohol-consuming (red) mice, respectively. Thin lines are from individual cells. Data are from 14 and 14 individual neurons, respectively. (E, F) Histograms of EPSC peak amplitude (E) and frequency (F) for water-consuming mice and alcohol-consuming mice. Data are from 14 pyramidal neurons (water) and 14 pyramidal neurons (AAE). * $p < 0.05$.

Paired-pulse ratio analysis in layer V projection neurons of the prelimbic cortex of males P43 did not reveal any difference, arguing against altered presynaptic release probability (**Figure 2**).

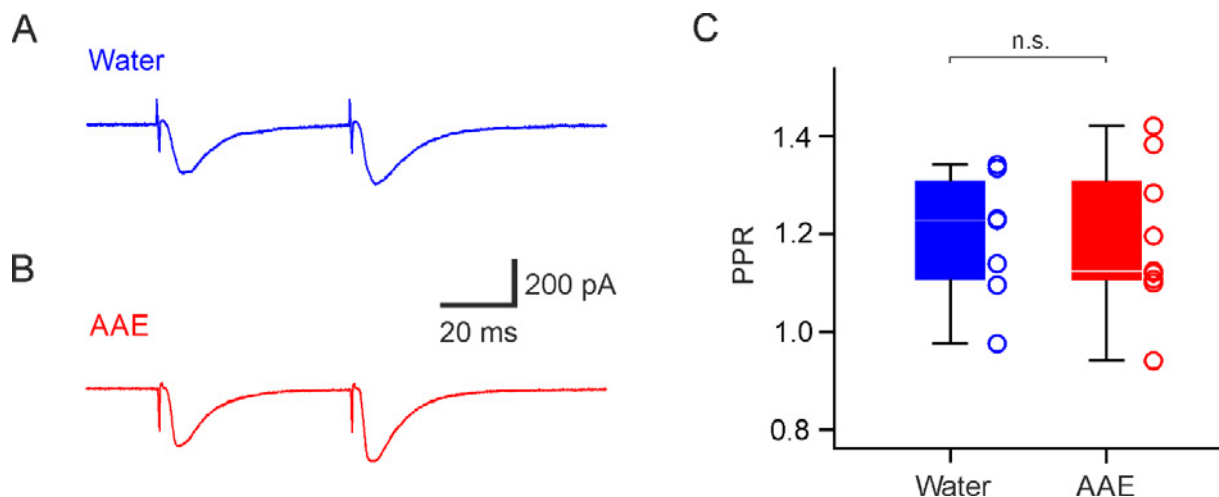


Figure 2. Alcohol consumption does not modify paired-pulse ratio in layer 5 pyramidal neurons of the prelimbic cortex at P43. (A, B) Current traces in response to a paired-pulse protocol (50 Hz) from water-consuming mice (top, blue traces) and from alcohol-consuming mice (bottom, red traces). (C) Histograms of PPR from 8 water neurons and 9 AAE neurons.

We then observed an increased NMDA/AMPA ratio in AAE condition, driven by larger NMDAR currents (**Figure 3**).

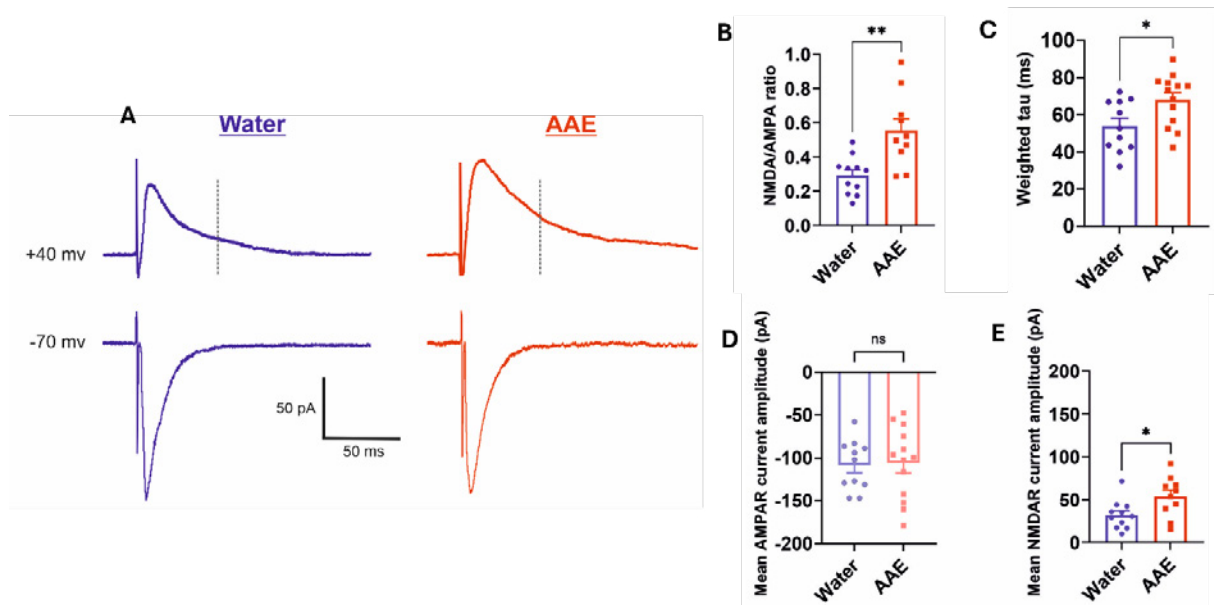


Figure 3. Adolescent alcohol exposure impacts NMDAR currents in layer 5 pyramidal neurons of the prelimbic cortex at P43. (A) Averaged traces of evoked excitatory postsynaptic currents at +40 mV (upper traces) and -70 mV (lower traces) from water-consuming mice (blue traces) and from alcohol-consuming mice (red traces). (B) Bar graph representing the NMDA/AMPA ratio. (C) Bar graph representing the weighted tau, illustrating the decay time. (D) Bar graph representing the mean AMPAR current amplitude calculated at -70 mV. (E) bar graph showing the mean NMDAR current amplitude calculated at 40 ms after the onset of the event at +40 mV. * $p < 0.05$, ** $p < 0.01$.

We next investigated potential modifications in NMDAR subunit contribution following AAE. Sensitivity to Ro25-6981 (a GluN2B antagonist) is consistent with a shift in NMDAR subunit composition at P43 (Figure 4). In contrast, GluN2A-related pharmacology did not reveal an effect of AAE at P43 (Figure 5).

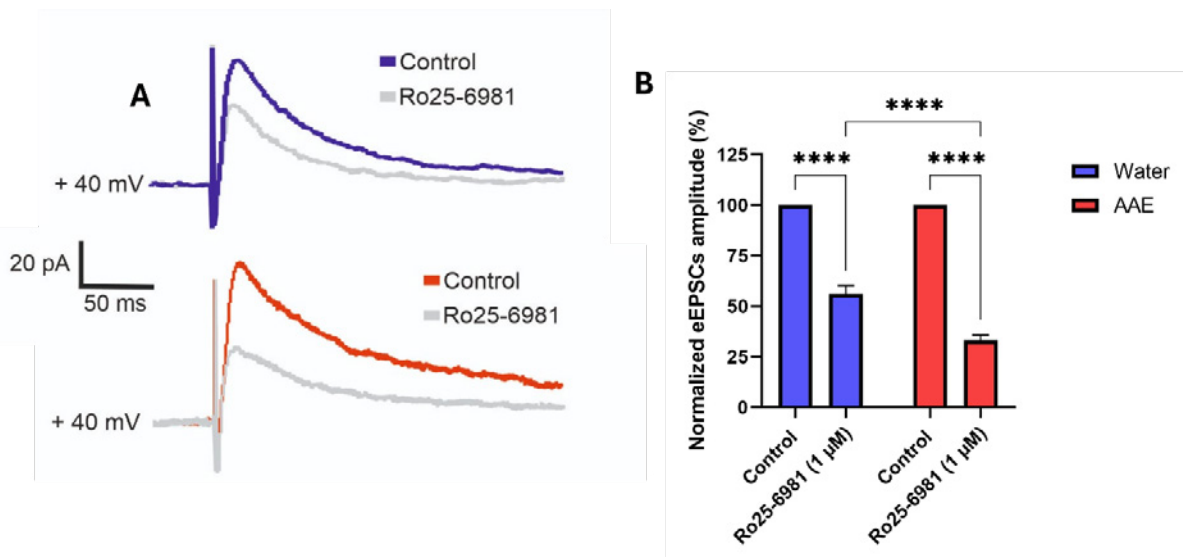


Figure 4 Adolescent alcohol exposure impacts the GluN2B subunits composition of NMDAR in layer 5 pyramidal neurons of the prelimbic cortex at P43. (A) Averaged traces of EPSCs +40mV, without and with Ro25-6981 (specific antagonist of GluN2B) application (grey lines). (B) Bar graph showing the effect of 1 μ M of Ro25-6981 on normalized NMDAR current in from water-consuming mice (blue) and from alcohol-consuming mice (red).

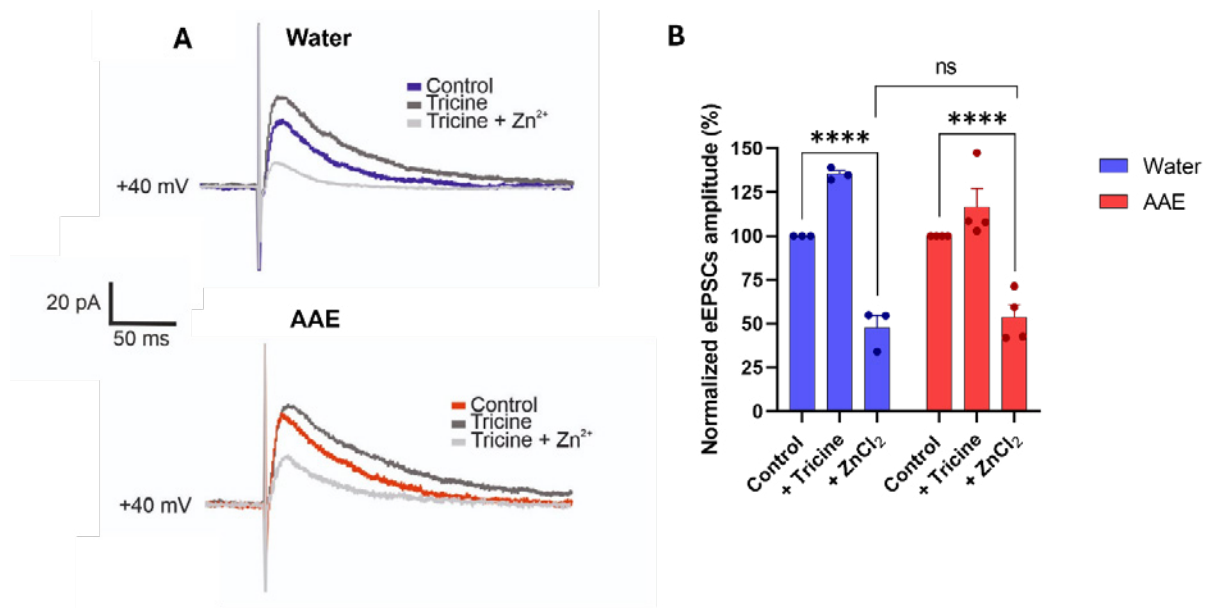


Figure 5. Adolescent alcohol exposure does not impact the GLUN2A subunits composition of NMDAR in layer 5 pyramidal neurons of the prelimbic cortex at P43. (A) Averaged traces of EPSCs +40mV, without and with Tricine only, or Tricine and zinc. (B) Bar graph showing the effect of Tricine ± Zinc on normalized NMDAR current from water-consuming mice (blue) and from alcohol-consuming mice (red).

In **adulthood** (P80), no difference was observed in the frequency or amplitude of sEPSCs between AAE and water animals (**Figure 6**). However, the AMPA/NMDA ratio remained elevated due to larger NMDA currents (**Figure 7**), indicating a persistent NMDAR phenotype.

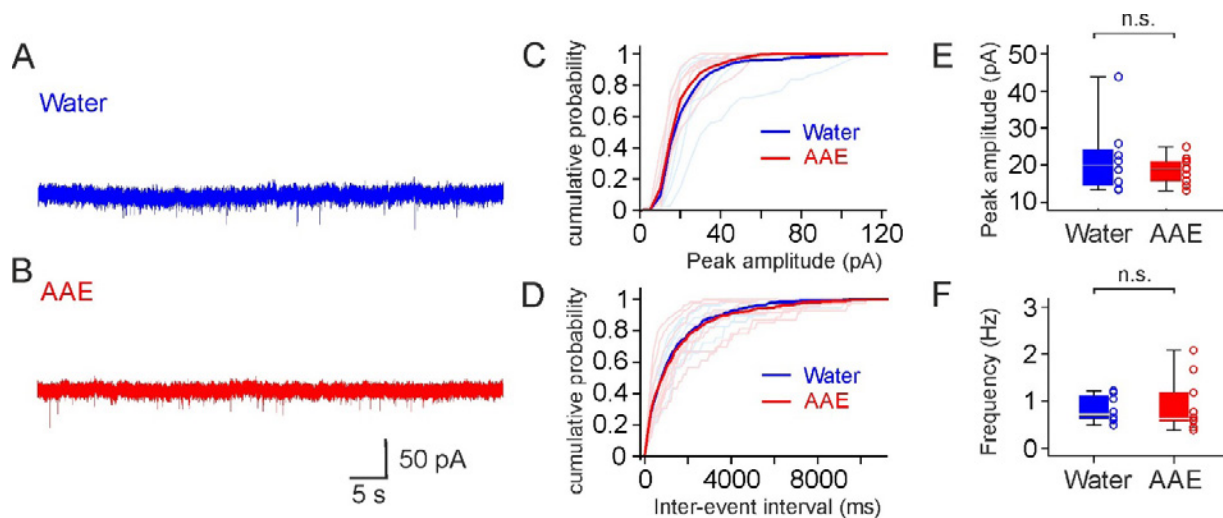


Figure 6. Alcohol consumption does not modify sEPSC frequency in layer 5 pyramidal neurons of the prelimbic cortex at P80. (A, B) Current traces showing spontaneous EPSCs at -60 mV in slices from water-consuming mice (top, blue traces) and from alcohol-consuming mice (bottom, red traces). External solution contained 50 μ M picrotoxin to block inhibitory synaptic currents. (C, D) Cumulative distributions of spontaneous EPSC amplitude (C) and inter-event intervals (D). Thick lines are average cumulative distributions from pyramidal neurons in water- (blue) and alcohol-consuming (red) mice, respectively. Thin lines are from individual cells. Data are from 8 and 10 individual neurons, respectively. (E, F) Histograms of EPSC peak amplitude (E) and frequency (F) for water-consuming mice and alcohol-consuming mice. Bars represent mean $\pm$ SEM; open circles indicate data from individual recordings. Data are from 8 pyramidal neurons (water) and 10 pyramidal neurons (AAE).

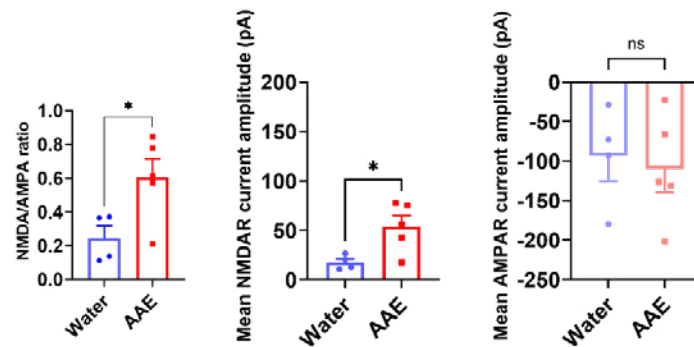


Figure 7. Adolescent alcohol exposure impacts NMDAR current in layer 5 pyramidal neurons of the prelimbic cortex at P80 (A) Bar graph representing the NMDA/AMPA ratio. **(B)** bar graph showing the mean NMDAR current amplitude calculated at 40 ms after the onset of the event at +40 mV. **(C)** Bar graph representing the mean AMPAR current amplitude calculated at -70 mV. (preliminary data)

Task 1.3.2 Structural defects

Post-hoc biocytin fills with 3D reconstructions showed no changes in dendritic arborization (apical/basal length, branching, Sholl) in layer V pyramidal neurons of the prelimbic PFC at P43 or P80 (**Figure 8**).

However, at P43 we observed spine remodeling, with increased mean spine head size/volume and a higher proportion of mushroom spines with reduced thin spines (**Figure 9**). By P80, spine density and morphology were no longer different (**Figure 10**).

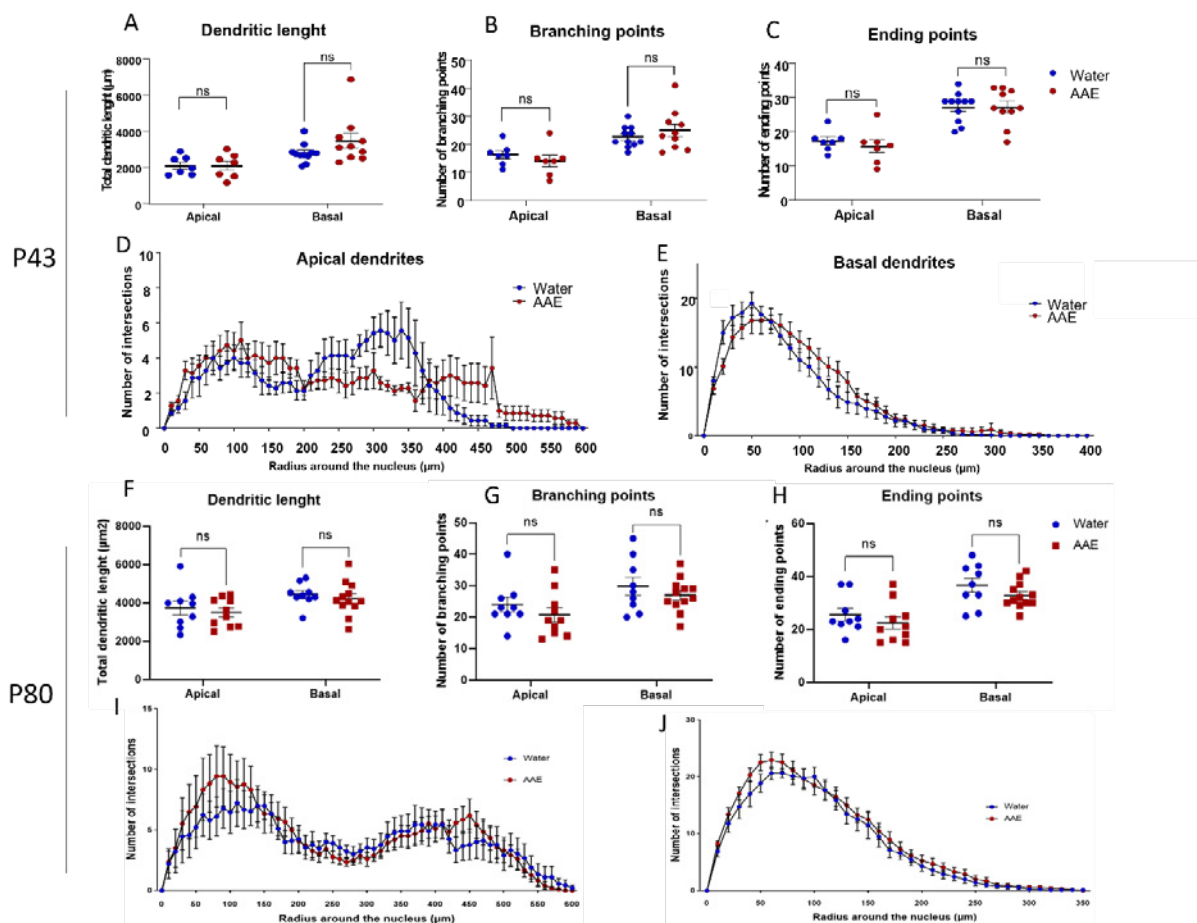


Figure 8: No change in apical and basal dendrites morphometric measurements after AAE in prelimbic layer V projection neurons at P43 or P80. (A) Total length of apical (left) and basal (right) dendritic arborizations. Unpaired t test showed no effect of alcohol on apical dendritic length ($p=0.9854$) nor on basal dendritic length ($p=0.1361$). **(B-C)** Number of branching and ending points per apical and basal dendritic arborization per neuron. Unpaired t test showed no effect of alcohol on the number of apical ($p=0.4114$)

and basal ($p=0.8279$) branching points nor on the number of apical ($p=0.50$) and ($p=0.9731$) basal ending points. (D-E) Sholl analysis of apical (D) and basal (E) dendrites. Two-way ANOVA showed no effect of AAE (apical: $p=0.715$, $F_{(1,12)}=0.1403$; basal $p=0.5116$, $F_{(1,19)}=0.4474$). Data are presented as mean $\pm$ SEM; for apical dendrites: $n=7$ water, $n=7$ AAE; for basal dendrites: $n=11$ water, $n=10$ AAE. (F) Total length of apical (left) and basal (right) dendritic arborizations, expressed in μm . Unpaired t test did not show any significant difference in apical ($p=0.5773$) and basal dendritic length ($p=0.5274$). (G-H) Number of branching and ending points per apical (left) and basal (right) dendritic arborization per neuron. Unpaired t test showed a similar number of apical ($p=0.3406$) and basal ($p=0.3518$) branching points and a constant number of apical ($p=0.3627$) and ($p=0.1907$) basal ending points between water and AAE mice. (I-J) Sholl analysis of apical (I) and basal (J) dendrites. Two-way ANOVA showed no effect of AAE (apical: $p=0.5814$, $F_{(1,19)}=0.3146$; basal: $p=0.341$, $F_{(1,19)}=0.9539$). Data are presented as mean $\pm$ SEM; for apical dendrites: $n=9$ water, $n=10$ AAE; for basal dendrites: $n=9$ water, $n=12$ AAE.

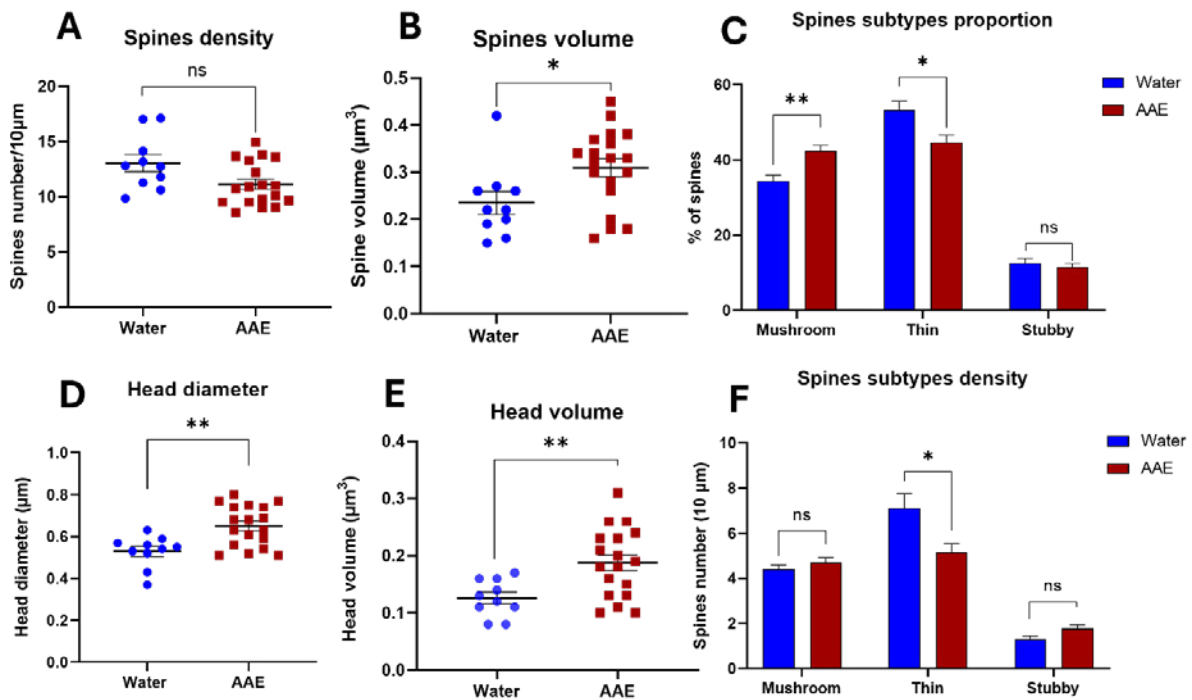


Figure 9: AAE modifies dendritic spine morphology in PFC layer V projection neurons at P43. (A) Spine density defined as the number of spines per 10 μm of dendrite. (B) Spine volume. (C) Percentage of mushroom, thin and stubby-type spines. Percentage is measured as the number of each type of spines on the total number of spines per dendrite $\times 100$. Two-way ANOVA showed a significant effect of AAE on the percentage of mushroom-type spines ($p=0.0427$), and thin- ($p=0.035$) but not stubby-type ($p=0.3597$) percentage. (D,E) Average head max diameter and head volume of water and AAE mice. (F) Spine subtypes density. Two-ways ANOVA showed a significant effect of AAE on thin spines density ($p=0.035$) but not stubby-type or mushroom-type. Data are presented as mean $\pm$ SEM; $n=14$ water, $n=16$ AAE; * $p<0.05$; ** $p<0.01$.

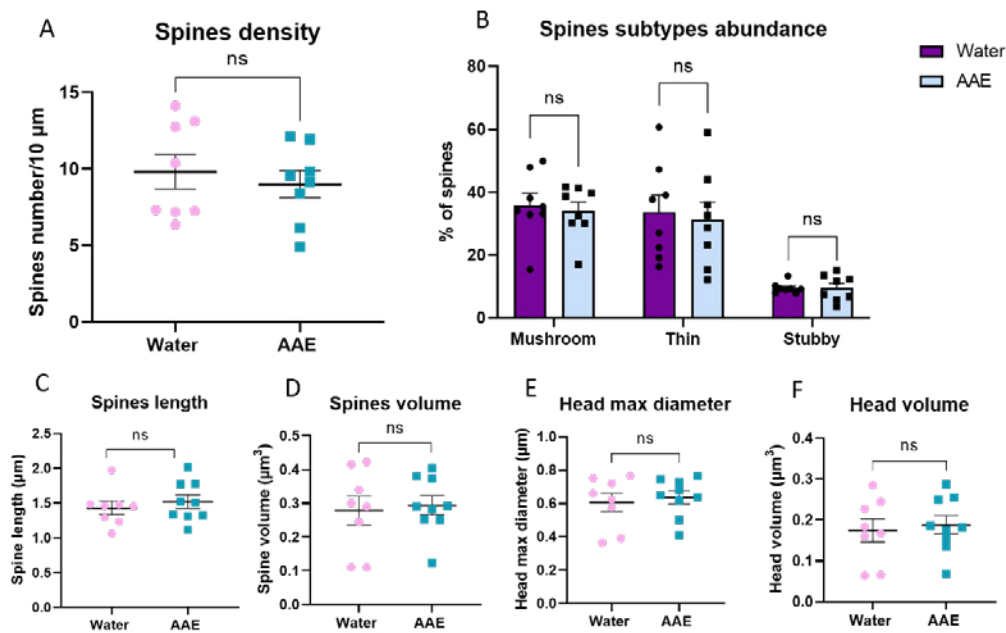


Figure 10: No defect in dendritic spines number or morphology in PFC layer V projection neurons at P80 (A) Spine density defined as the number of spines per 10 μm of dendrite. (B) Percentage of mushroom, thin and stubby-type spines. Percentage is measured as the number of each type of spines on the total number of spines per dendrite*100. (C-F) Average spine length, volume, head max diameter and head volume of water and AAE mice. Data are presented as mean $\pm$ SEM; n=8 water neurons, n=9 AAE neurons

Task 1.3.3. System-level readouts

The PFC is an integration center receiving inputs from several brain regions, including the Nucleus accumbens, the dorso-medial striatum (DMS), the basolateral amygdala (BLA) and the dorsal raphe nucleus (DRN). It also sends outputs to several brain regions including the DMS, BLA, and DRN. Altered maturation of the PFC during adolescence may disrupt top-down circuitry in adulthood, contributing to the behavioral defects observed following AAE. We initiated FDG micro-PET CT and c-Fos mapping (basal vs alcohol priming) with IMMI-CMMI (ULB, G. Doumont). Preliminary analyses suggest PFC dysfunction in adult AAE mice, with additional midbrain glucose uptake changes (**Figure 11**). C-fos analysis are currently ongoing.

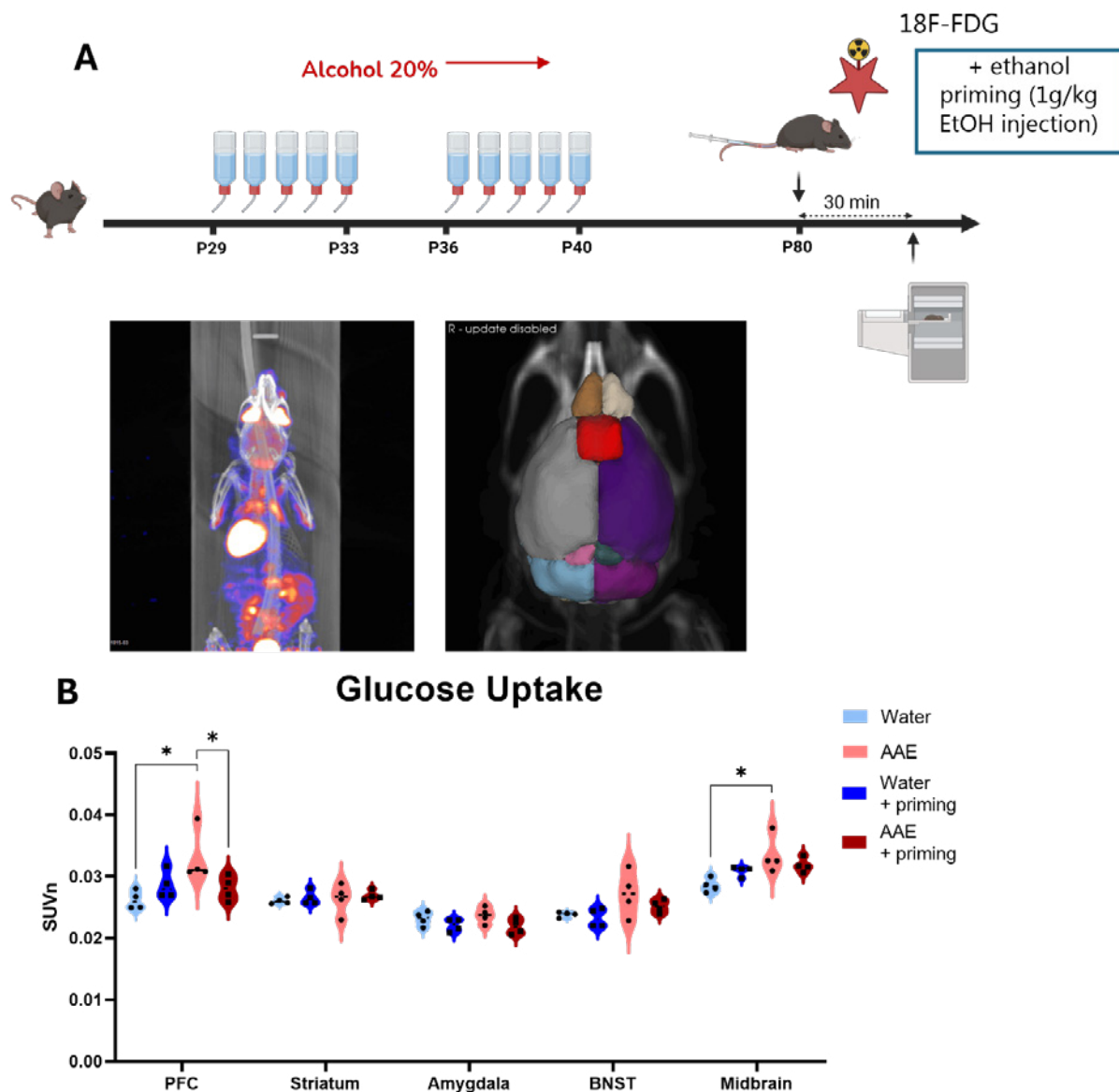


Figure 11. Micro-PET CT experiment shows PFC and midbrain dysfunction in AAE mice in adulthood. **(A)** Scheme and illustration of the micro-PET CT experiment.

WP1 conclusion

AAE produces adolescent-onset synaptic and spine phenotypes in prelimbic layer V neurons (increased sEPSC frequency, increased NMDAR currents, spine remodeling), without gross structural loss or inflammation. Most synaptic frequency and morphology changes normalize by adulthood, yet a sustained NMDAR elevation persists at P80. Ongoing E/I balance mapping across PFC subregions (PSD95/VGlut1 for excitation; Gephyrin/VGAT for inhibition, **Figure 12**), additional synaptic plasticity experiments (LTP/LTD) and expansion microscopy of GluN2A/GluN2B will anchor the subunit phenotype anatomically. Finally, optogenetic experiments are ongoing to investigate synaptic transmission changes within bidirectional PFC-VTA circuits and PFC-BNST circuits.

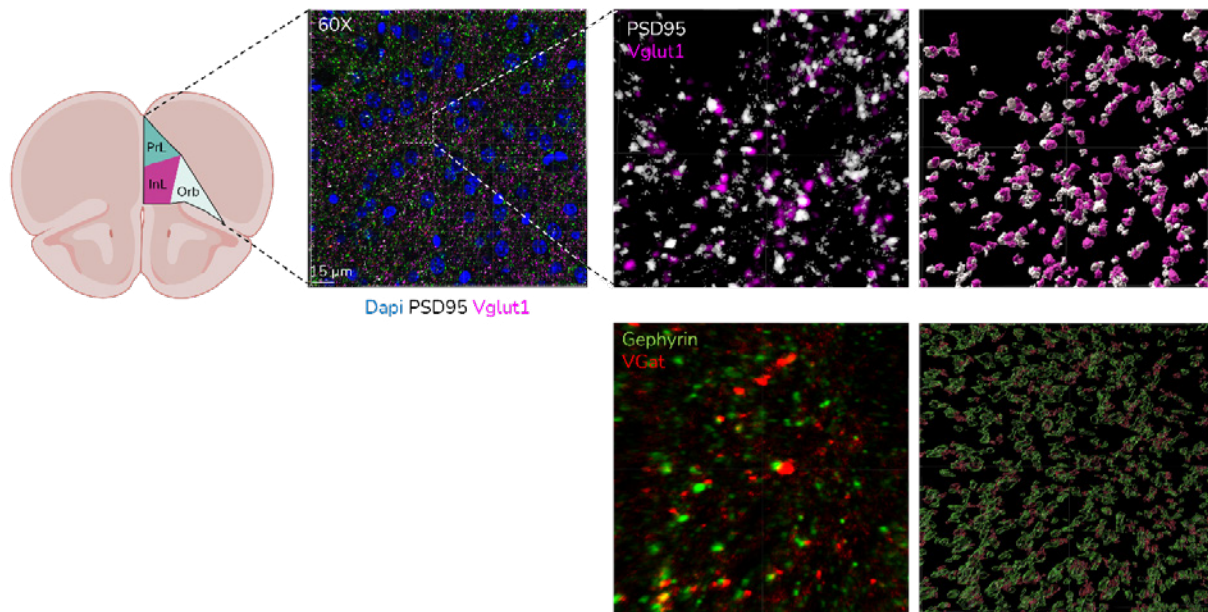


Figure 12. Excitatory and inhibitory synapses analysis in PFC.

Task 2: Deciphering whether AAE modulates local translation in the adolescent PFC

Alcohol is thought to hijack plasticity mechanisms, including local dendritic translation. We tested two master regulators of translational control: mTORC1 and eIF2. Synaptoneurosomes and total PFC homogenates were probed by Western blot at P40, P43, and P80 in males and females; spatial mapping across PFC subregions and neuronal types was performed using immunofluorescence.

Task 2.1. mTORC1 is activated in the PFC following AAE

We report that AAE increases the phosphorylation levels of S6 Kinase and S6 in the PFC in total homogenate and synaptic fraction in both sexes (**Figure 13**). Phosphorylation levels of S6 were not increased in the motor cortex or nucleus accumbens of alcohol-exposed adolescent mice (data not shown), suggesting regional specificity and local translation.

At P43, the increased S6 phosphorylation persisted in the PFC of both males and females, suggesting long-lasting activation of mTORC1 following AAE (**Figure 14**). Finally, when assessed at P80, no change in S6 phosphorylation levels was detected (**Figure 15**).

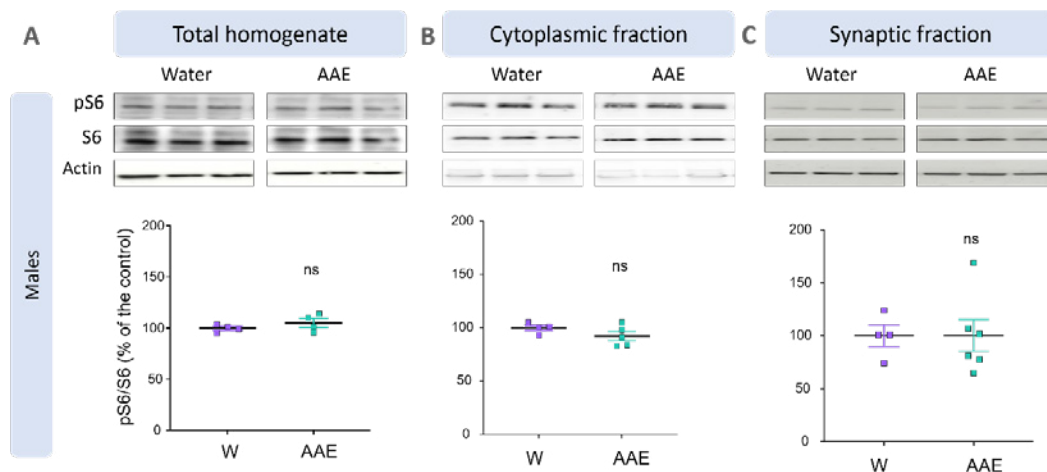


Figure 15. AAE-induced mTORC1 activation in the PFC is no longer present in adulthood. Western blot analysis of phosphorylation levels of ribosomal protein S6 in the PFC of males adult mice. Forty days after the last drinking session, PFC samples were dissected and analyzed by western blot. No significant change in S6 phosphorylation were observed in the total homogenate or synaptic/cytoplasmic fractions of PFC extracts.

We also assessed mTORC1 activation following AAE by immunofluorescence. As shown in **Figure 16**, we observed a significantly increased phosphorylation of S6 in AAE animals at P40, both in males and females, which may specifically localize in the Ctip2+ layer V projection neurons.

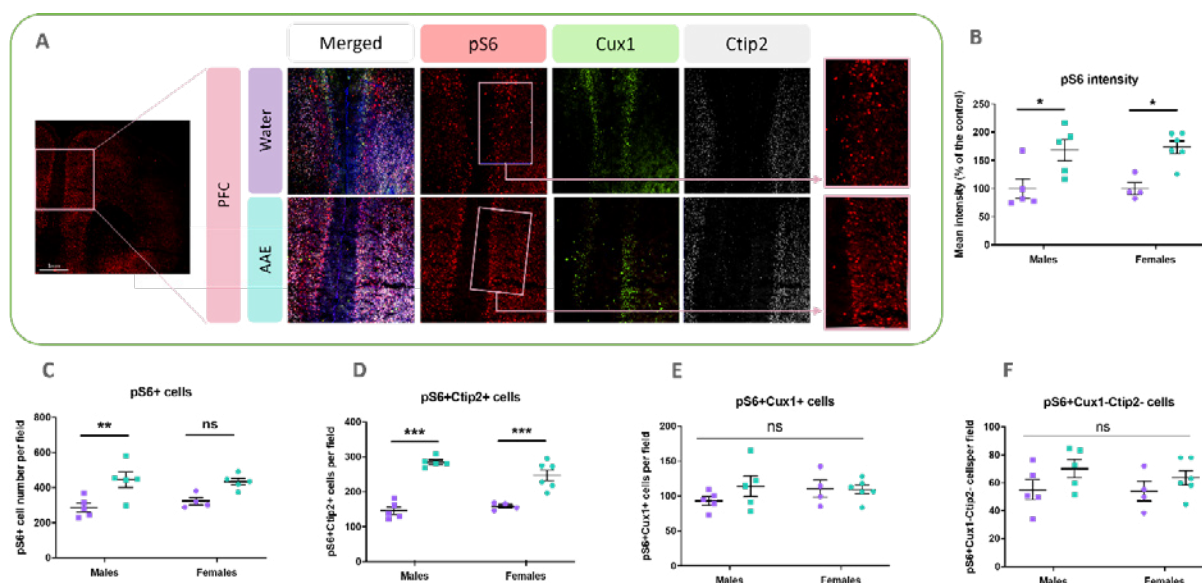


Figure 16. AAE increases S6 phosphorylation levels in PFC layer V neurons. Immunolabeling of the phosphorylated form of the ribosomal protein S6 in the PFC of males and females P40 adolescent mice. Immediately after the last drinking session, brains were dissected, fixed and used for immunofluorescence. (A) pS6 in red, Cux1 in green, Ctip2 in gray. (B) Significantly increased S6 phosphorylation intensity was observed in AAE animals compared to water control extracts in both sexes. (C) Significantly increased number of pS6-positive cells in AAE animals as compared to water controls. (D-F) The increased phosphorylation levels of S6 are localized mostly in layer V Ctip2-positive cells. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Additional experiments were conducted using additional neuronal identity markers (SatB2, Tbr1,...), and at later time-points (P43, P80), in both sexes. An immunofluorescence pipeline for automated analysis using the slide scanner Axioscan 7 has been implemented. **Figure 17** shows an example of a coronal brain slice labeled for phospho-S6, Cux1, Satb2, Ctip2 and DAPI. All images have been acquired and are currently being analyzed using automated workflow in Imaris.

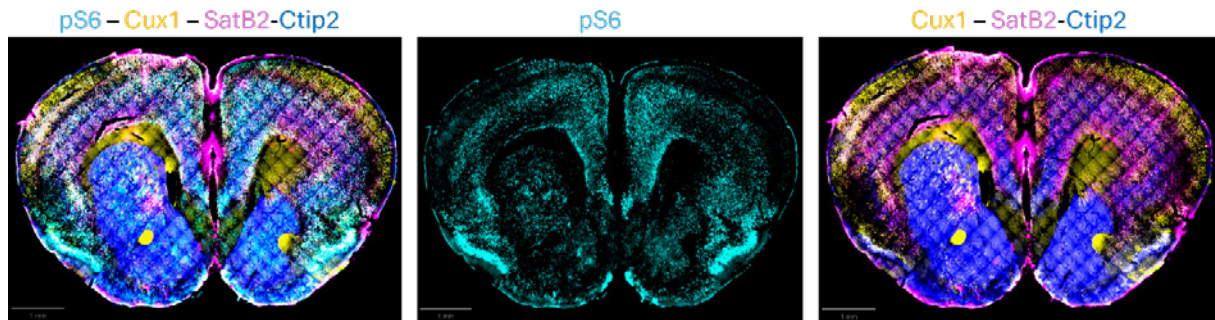


Figure 17. Representative picture of P40 coronal brain slice stained with phospho-S6 and neuronal identity markers.

Interestingly, eIF2 phosphorylation did not differ at P40, P43, or P80 in male or female PFC (data not shown). Finally, eEF2 activity is currently being assessed in the PFC of males and females by Western blot and immunolabeling.

Task 2.2 Deciphering whether mTORC1 mediates the PFC defects induced by AAE in the PFC

Task 2.2.1. Behavioral defects:

mTORC1 activity was inhibited using systemic rapamycin injections administered at the end of session 5 and session 10 of the AAE paradigm (**Figure 18**). This injection schedule was selected to minimize potential reduction in alcohol consumption during adolescence induced by rapamycin (Beckley, Laguesse *et al.* 2016). As shown in **Figure 18**, rapamycin administration during adolescence significantly reduced alcohol consumption in AAE adult animals tested in the intermittent access to alcohol 20% - two bottle choice paradigm. This effect is supported by a significant AAE × treatment interaction and post hoc comparisons indicating lower intake in the AAE/Rapamycin group compared with the AAE/Vehicle group.

Anxiety-like behavior was also reduced in adult AAE animals treated with rapamycin (**Figure 19**). These data confirm that AAE induces anxiety-like behavior in adulthood and suggest that adolescent rapamycin treatment attenuates this phenotype.

Together, these results support a role for mTORC1 signaling in the emergence of long-term behavioral outcomes following AAE.

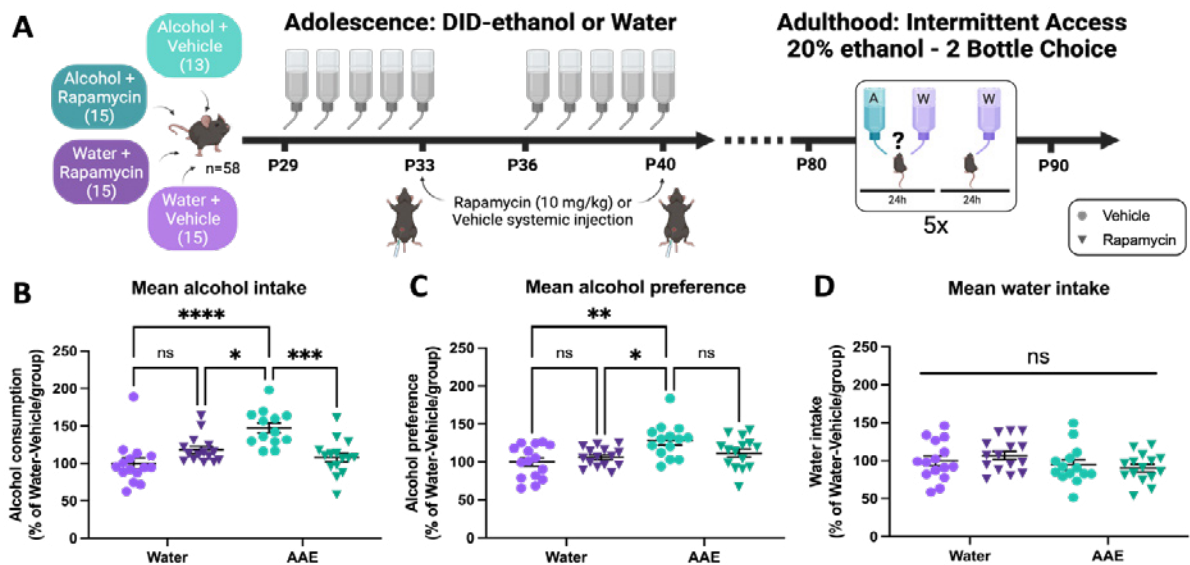


Figure 18. mTORC1 systemic inhibition during adolescent alcohol exposure reduces alcohol consumption and preference in adulthood. 4 groups of mice underwent the DID paradigm between P29 and P40. Rapamycin and Vehicle injections are shown in the scheme. After 40 days of abstinence, adult female mice underwent the Intermittent access to alcohol 20%-two-bottle choice (IA-20%-BC) paradigm for five sessions. **(B)** Mean alcohol consumption. Two-way ANOVA revealed a main effect of AAE ($p < 0.0001$) and an interaction ($p < 0.01$) but no significant main effect of rapamycin ($p = 0.10$). Post hoc Tukey test showed a significant difference between AAE/Vehicle and Water/Vehicle ($p < 0.0001$) as well as between AAE/Vehicle and AAE/Rapamycin ($p < 0.001$). **(C)** Alcohol preference. Two-way ANOVA showed a main effect of AAE ($p < 0.01$) and an interaction ($p < 0.05$) but no significant main effect of rapamycin. **(D)** Water consumption. Two-way ANOVA showed no significant main effect of AAE or rapamycin and no interaction. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

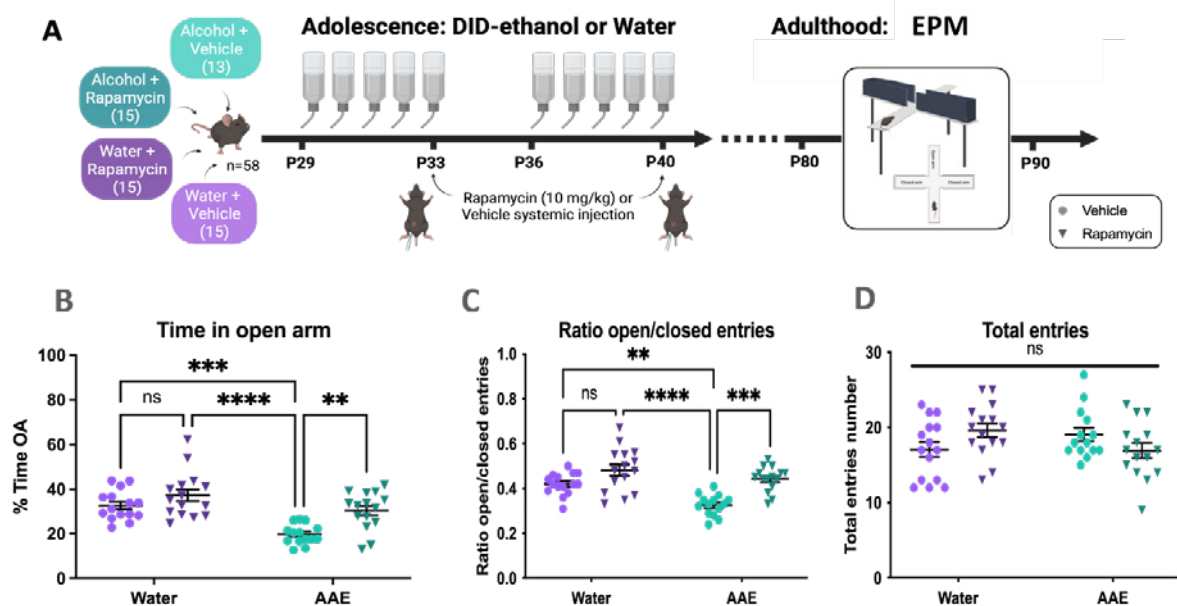


Figure 19. mTORC1 systemic inhibition during adolescent alcohol exposure reduces anxiety-like disorder in adulthood. 4 groups of mice underwent the DID paradigm between P29 and P40. Rapamycin and Vehicle injections are shown in the scheme. After 40 days of abstinence, adult female mice underwent the EPM test. **(B)** Time in open arms. Two-way ANOVA revealed a main effect of AAE ($p < 0.0001$), of rapamycin ($p < 0.001$) but no interaction. Post hoc Tukey test showed a significant difference between AAE/Vehicle and Water/Vehicle ($p < 0.001$) as well as between AAE/Vehicle and AAE/Rapamycin ($p < 0.01$). **(C)** Ratio Open/closed arm entries. Two-way ANOVA showed a main effect of AAE ($p < 0.001$) and rapamycin ($p < 0.001$) an interaction ($p < 0.05$). **(D)** Total entries. Two-way ANOVA showed no significant main effect of AAE or rapamycin and no interaction. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Because systemic rapamycin lacks brain-region specificity, we are now implementing PFC-targeted genetic approaches to directly test PFC mTORC1 involvement. For the next steps, we will use intra-PFC AAV-delivery of shRNA targeting Raptor, to achieve brain-region specificity. DNA

constructs have been generated, and stereotaxic coordinates have been established for targeting the PFC at several time-points between P21 and P40 (**Figure 20**). Following intra-PFC infusion of shRNA against Raptor, we will test anxiety-like behavior (EPM), alcohol consumption (IA20%-2BC), and flexibility (Barnes maze) in adulthood.

Sexe – Age	PFC coordinates	
Male P21	From Lambda (Bregma invisible)	AP: +3.2
		ML: +0.2
		DV: -2.1
Male P26	From Lambda (Bregma invisible)	AP: +4
		ML: +0.4
		DV: -2.3
Male P33	From Bregma	AP: +3.2
		ML: +0.2
		DV: -1.2
Male P40	From Bregma	AP: +3.0
		ML: +0.2
		DV: -1.7



Figure 20. Set up of stereotaxic coordinates for PFC targeting between P21 and P40.

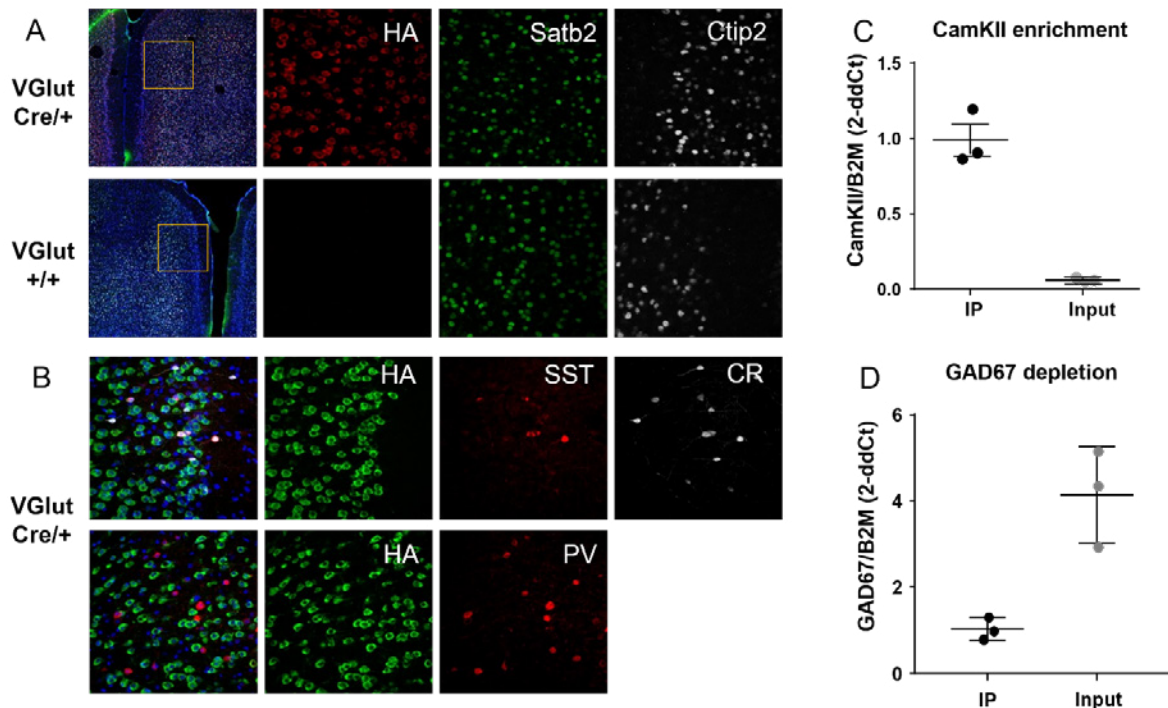
Task 2.2.2. Transmission defects:

Experiments are currently ongoing to determine whether mTORC1 activity contributes to the synaptic transmission phenotype identified in Task1. We are combining pharmacological inhibition (rapamycin) with projection neuron-targeted viral delivery of shRaptor in the PFC.

Task 3: Global approach: identify the mRNAs whose local translation is modulated by AAE in specific neuronal populations.

Task 3.1 Strategy and validation

We deployed a conditional RiboTag model (HA-Rpl22) crossed with VGlut1-Cre to purify ribosome-bound mRNAs from PFC projection neurons at P43 and P80. Specificity was verified by immunofluorescence: HA colocalized with Satb2/Ctip2-positive glutamatergic neurons but not with interneuron markers SST, CR, or PV. Moreover, the immunoprecipitate showed enrichment for CamKII and depletion for GAD67 (**Figure 21**).



Principal component analysis (PCA) of the RNA-seq dataset nicely separated the neuronal translatoome (IP) from the PFC transcriptome (input), but did not reveal major global separation between water and AAE conditions at either age (**Figure 22**).

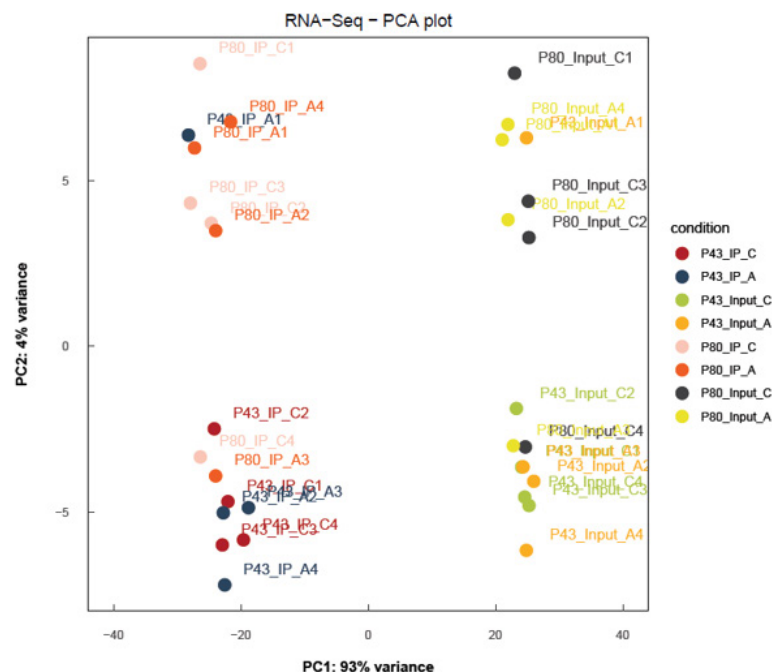


Figure 22. PCA plots of Ribotag-RNA seq experiment on P43 and P80 IP and input samples.

Task 3.2 P43 findings

We identified several ribosome-bound mRNA candidates upregulated by AAE in the projection neurons, notably the lysine demethylase 6B (Kdm6b), the tribbles pseudokinase 1 (Trib1) and the nuclear receptor Nr4a1 (**Figure 23**).

Kdm6B and Nr4A1 are of particular interest given their reported role in synaptic plasticity and alcohol dependence. Our preliminary data suggests that Kdm6b may contribute to a loss of H3K27me3 at the GluN2B locus, potentially driving the de-repression and impairing the developmental switch from GluN2B to GluN2A. The contribution of Kdm6b to the synaptic transmission phenotypes observed after AAE is currently under investigation.

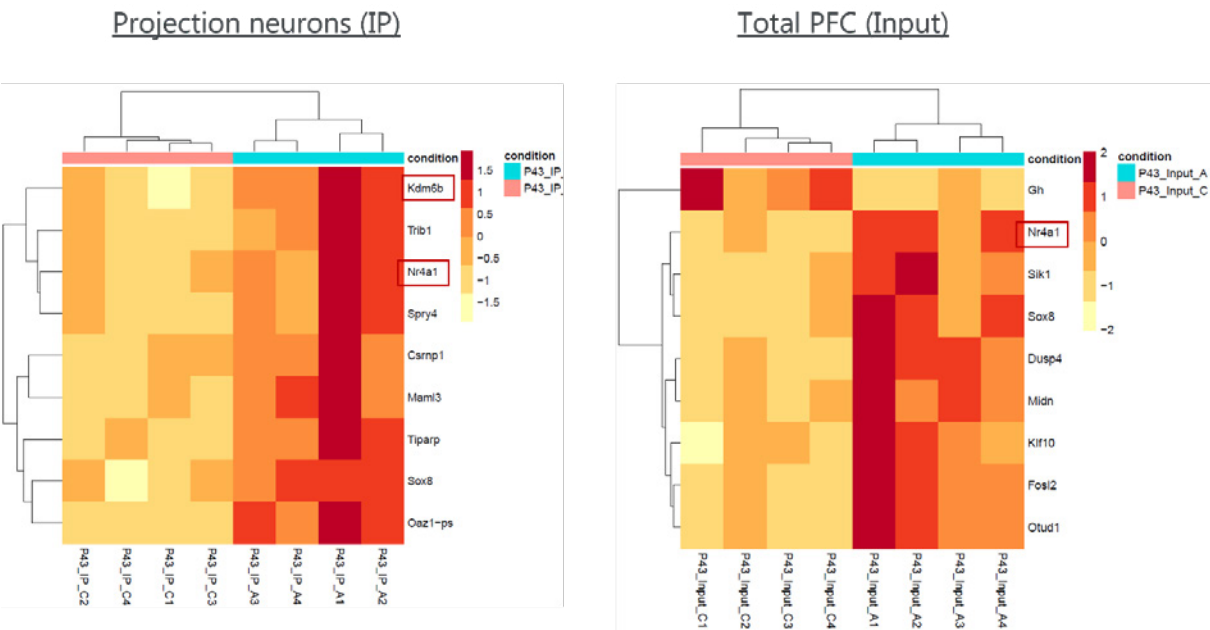


Figure 23. Differential expression of candidate genes at P43 in projection neurons (IP) or total PFC (input).

Conversely, Acetyl-Coenzyme A acetyltransferase (ACAT3) and Neuropeptide FF (NPFF) showed reduced expression in AAE animals compared with water controls. We validated a subset of profiling results by ribosome-bound mRNA purification from PFC projection neurons followed by qRT-PCR. These data support AAE-dependent reductions of ACAT3 and NPFF in the PFC at P43 (but not in motor cortex ,data not shown) (**Figure 24**).

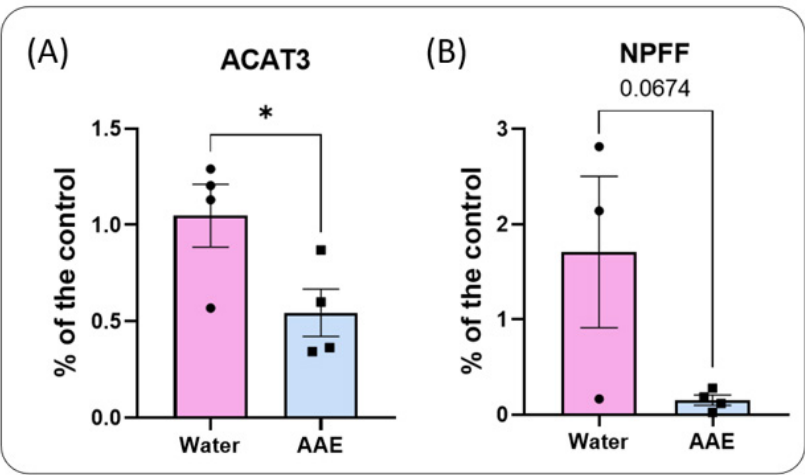


Figure 24: qRT-PCR analysis of ACAT3 and NPFF mRNAs in the IP and fraction after Ribotag IP in PFC samples at P43. (A) Unpaired t test showed ACAT3 mRNAs expression is decreased in alcohol-exposed mice as compared to water-exposed animals in IP fraction (p=0.0488). (B) NPFF mRNAs expression shows a tendency but is not significantly decreased in alcohol-exposed mice as compared

to water-exposed mice in the IP fraction ($p=0.0674$). Data are presented as the average ratio of ACAT3 and NPFF to the mean of B2M and HPRT1 $\pm$ SEM and expressed as the percentage of water control; * $p<0.05$.

Task 3.3 P80 findings

At P80, RNA-seq analysis has revealed 144 differentially expressed genes (DEG) in the IP fraction (Threshold >1.2 fold-up or <0.8 fold-down) and 236 DEG in the input fraction (**Figure 25**).

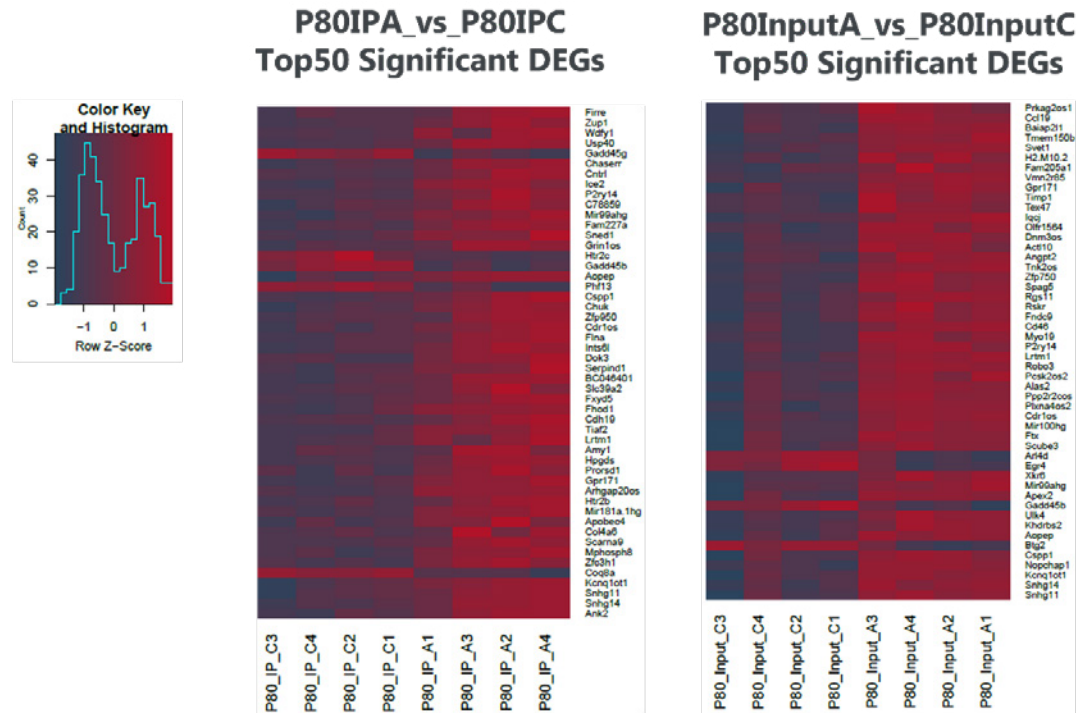


Figure 25. Differentially expressed genes in the IP (left) and input (right) fractions at P80.

We prioritized candidates based on functional relevance to synaptic structure and signaling, including the actin-binding protein Filamin A (Flna), the glutamate kainate receptor subunit Grik1, and the microtubule scaffolding protein IQ motif containing GTPase-activating protein 1 (Iqgap1). qRT-PCR analysis confirmed significantly increased levels of ribosome-bound Flna and Grik1 mRNAs in the IP fraction from PFC extracts of AAE animals compared with water controls (**Figure 26**). In motor cortex (control region), no significant differences in Flna or Grik1 translation were detected in the IP fraction (data not shown). In contrast, we did not observe significant changes in ribosome-bound Iqgap1 transcripts in the PFC IP fraction (Figure 26), and therefore did not confirm the RNA-seq result for this candidate.

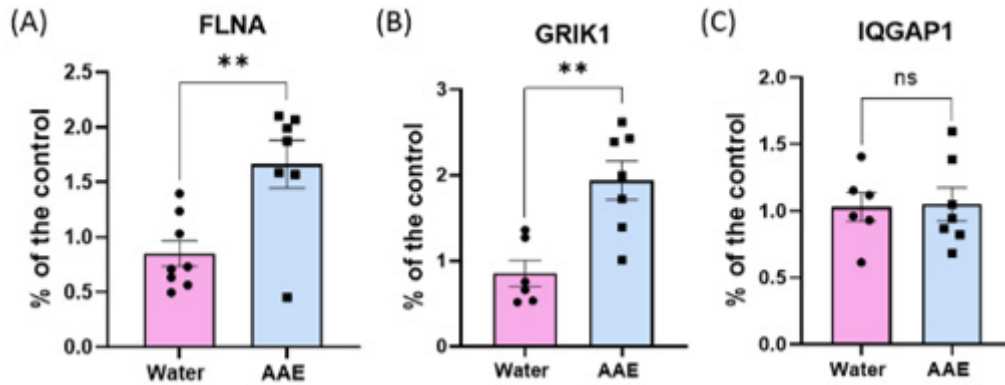


Figure 26. qRT-PCR analysis of FLNA, GRIK1 and IQGAP1 candidates at P80. Unpaired t test showed that (A) FLNA mRNAs expression is increased in the PFC IP fraction of alcohol-exposed mice as compared to water-exposed mice ($p=0.0046$). (B) GRIK1 mRNAs expression is also increased in the PFC IP fraction of alcohol-exposed mice as compared to water-exposed mice ($p=0.0026$). (C) No difference in the level of IQGAP1 mRNAs in the PFC IP fraction between the AAE and water groups was reported ($p=0.9133$). ** $p<0.01$.

Additional candidates are currently being validated; follow-up includes analysis of FACS-sorted PFC projection neurons for immunofluorescence and Western blot confirmation.

Overall Scientific impact

The grant enabled a comprehensive, multi-level analysis showing that adolescent alcohol binge-drinking reprograms prefrontal synaptic function via local translational control (mTORC1), producing adolescent-specific synaptic/spine phenotypes and persistent NMDAR abnormalities into adulthood that align with anxiety, elevated intake, and reduced flexibility. Cell-type resolved transcriptome profiling nominates KDM6B as potential mechanistic link linking AAE to epigenetic control of NMDAR subunits and plasticity. A manuscript grouping most of the data presented in this report is currently in preparation and will be submitted in a high-impact journal at the end of 2026.

2. Part B : Broader Impact of the FMRE grant

2.1. Career Development and scientific Independence

The FMRE Young Researcher Grant played a central role in consolidating my scientific independence and shaping my long-term research vision. It allowed me to reinforce an independent research line at the University of Liège, centered on how alcohol exposure disrupts prefrontal cortex maturation during adolescence. Importantly, the grant provided the means to recruit team members, initiate key experimental pipelines, and establish the infrastructure and collaborations required to deliver the project objectives. Collectively, these developments have strengthened the foundation for a sustainable, long-term program at the interface of developmental neuroscience and addiction biology.

2.2. International Visibility and Recognition

The visibility and credibility conferred by the FMRE Grant substantially strengthened my international profile. I was invited to present our findings at major international conferences, contributing to the recognition of our work in developmental and addiction neuroscience:

- **European winter congress on brain research 2025**, Les Diablerets, Switzerland – Invited symposium on alcohol exposure and cerebral cortex development.

- **International Society for Biomedical Research on Alcoholism (ISBRA) Meeting 2024**, Melbourne, Australia – Participation in two symposia presenting our results on prenatal and adolescent alcohol exposure and cortical development.
- **Mediterranean Society for Neurosciences (MSN) Meeting 2023**, Tunis, Tunisia – Invited talk on adolescent alcohol exposure and prefrontal cortex maturation.
- **European Society for Biomedical Research on Alcoholism (ESBRA) Meeting 2023** – Symposium presentations highlighting leadership in cortical development and alcohol research.
- **Volterra “Alcohol & Stress” Conference 2023**, Italy – Presentation of our work on adolescent alcohol exposure and brain maturation.
- **European Conference on FASD 2022**, Arendal, Norway – Invited talk linking prenatal alcohol exposure to cortical developmental trajectories.

This increased visibility also supported the acquisition of additional competitive funding, including:

- *FRS-FNRS Research Credit* (2024, €60 000)
- *Credits Sectoriels* (2022 – 2024, €155 000 total)

2.3. Collaborations and Network Building

The project catalyzed new national and international collaborations, broadening both technical expertise and conceptual scope. In particular, partnerships were established with **Dr. Dominique Engel (ULiège)** for electrophysiology, **Dr. Gilles Doumont (ULB)** for PET imaging, and **Dr. Jérôme Jeanblanc (France)** for behavioral neuroscience. These collaborations have already generated shared datasets, and contributed to joint funding applications, reinforcing our integration within the European neuroscience community and strengthening the long-term sustainability and competitiveness of the research program.

2.4. Research Dissemination and Societal Engagement

FMRE support also enhanced my capacity to disseminate our research beyond academia. In collaboration with **Réjouissiences (University of Liège)**, I participated in outreach activities targeting the general public and secondary-school students, contributing to prevention-oriented education on adolescent alcohol exposure. In parallel, I was invited to discuss the risks associated with adolescent alcohol consumption on television and radio programs, supporting science-based public health communication.

In recognition of these dissemination and engagement efforts, I received the **“Citoyen d'Honneur de la Ville de Liège”** award in 2022, an uncommon distinction for a fundamental researcher, underscoring the societal relevance and local impact of this work.



Geneeskundige Stichting Koningin Elisabeth
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Fondation Médicale Reine Elisabeth – F.M.R.E.
Queen Elisabeth Medical Foundation – Q.E.M.F.**

Mailing address:

The scientifique director:

Prof. dr. Jean-Marie Maloteaux
3, avenue J.J. Crocq laan
1020 Bruxelles – Brussel
Belgium
Tel.: +32 2 478 35 56
E-mail: jean-marie.maloteaux@uclouvain.be

Secretary:

Mr. Erik Dhondt
3, avenue J.J. Crocq laan
1020 Bruxelles – Brussel
Belgium
Tel.: +32 2 478 35 56
E-mail: fmre.gske@skynet.be
erik.dhondt@skynet.be
e.l.dhondt@skynet.be

www.fmre-gske.be
www.fmre-gske.eu
www.fmre-gske.com